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**REGULATION OF GLYCOGEN
METABOLISM BY PROTEIN
PHOSPHORYLATION DURING
ENVIRONMENTAL
STRESS**

By

HOSSEIN MEHRANI, B.Sc., M.Sc.

A thesis submitted to the faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

**OTTAWA CARLETON CHEMISTRY INSTITUTE AND INSTITUTE OF
BIOCHEMISTRY, CARLETON UNIVERSITY
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**"REGULATION OF GLYCOGEN METABOLISM BY
PROTEIN PHOSPHORYLATION DURING
ENVIRONMENTAL
STRESS "**

Submitted by HOSSEIN MEHRANI, B.Sc., M.Sc.

In Partial Fulfillment Of The Requirements
For The Degree Of Doctor Of Philosophy

Chairman, Department Of Chemistry

Thesis Supervisor

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Carleton University

1994

ABSTRACT

The enzymes involved in the phosphorylation controlled glycogen catabolic pathway were studied in two different model systems involving anoxia: functional anoxia in exercised fish and environmental anoxia in turtle. Glycogen phosphorylase **b** from rainbow trout, *Oncorhynchus mykiss*, white skeletal muscle was purified to near homogeneity. Glucose and ATP inhibited the enzyme; glucose inhibition decreased at lower pH values. Michaelis constants for glycogen, phosphate, and AMP were 128 μ M, 31 mM, and 142 μ M respectively, at pH 7.2; maximum enzyme activity was obtained at pH 7.5 and 25°C. Exhaustive swimming exercise altered tissue glycogen phosphorylase kinase (GPK) and protein kinase A (PKA), GPK activity increasing by 60% in liver and 40% in white muscle of exercised fish. The amount of active PKA rose from 12% to 21% in liver and from 32% to 57% in white muscle after exhaustive swimming coupled with 50 % and 70 % increases in cellular cyclic AMP levels, respectively. Three forms of α -glucosidase were identified in trout liver. Two forms showed acid pH optima, hydrolyzed glycogen, maltose and 4-methylumbelliferyl α -glucoside (MU α G), and were associated with lysosomes whereas the third was microsomal, had a neutral pH optimum and did not hydrolyze glycogen. Properties of acid α -glucosidase type I changed significantly during exercise; maximal activity increased by 80% and K_m values for glycogen and maltose dropped by 50% in exercised, versus control, fish.

Exposure of turtles, *Trachemys scripta elegans*, to submergence anoxia at 7°C, elevated activities of phosphorolytic and glucosidic enzymes in some organs. Phosphorylase **a** in liver and heart increased significantly after 5 h of anoxia. PKA activity increased 2.3-fold in liver within 1 h of anoxia accompanied by a 60% increase in cAMP levels; however, with longer anoxia active PKA was suppressed to 2.1-3.7% of the total. Protein phosphatase-1 (PP-1) activity in liver decreased to 63% of controls within 1 h and remained suppressed over the subsequent 20 h of anoxia. PP-1 activity

also fell in anoxic red muscle and decreased transiently in brain. Within one hour of anoxia, 40% of protein kinase C β isomer (PKC β) and over 80% of PKC α were translocated from cytosol to the membrane fraction. Activity of acid α -glucosidase also increased in liver of anoxic turtles. PKA, PP-1, PKC α , and PKC β from control turtle liver were purified to homogeneity; physical and kinetic properties of these are presented.

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LIST OF ABBREVIATIONS

% a - percent glycogen phosphorylase in active a form

a + b - total glycogen phosphorylase activity

ADP - adenosine diphosphate

AMP - adenosine monophosphate

ATP - adenosine triphosphate

BSA - bovine serum albumin

cAMP - cyclic adenosine monophosphate

DAG - dioleoylglycerol

EDTA - ethylenediamine tetraacetate

EGTA - ethylen glycol bis(β -aminoethyl ether) N, N'-tetraacetic acid

GPK - glycogen phosphorylase kinase

H₇ - 1-(5-isoquinolinesulfonyl)-2-methyl piperazine

HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HPLC - high performance liquid chromatography

IBMX - isobutylmethylxanthine

IEF - isoelectric focusing

IMP - inosine monophosphate

K_m - Michaelis-Menten constant

LPI - lysophosphatidylinositol

MARKS - protein, myristoylated alanine rich C kinase substrate

MS22 - 3-aminobenzoate ethyl ester methanesulfonate

MU α G - 4-methylumbelliferyl α -glucoside

PEG - poly (ethylene glycol)

P_i - inorganic phosphate

pI - isoelectric point

PI - phosphatidylinositol

PKA - cAMP-dependent protein kinase

PKAc - catalytic subunit of PKA

PKA-I - PKA inhibitor peptide (5-24)

PKC - phospholipid/calcium-dependent protein kinase

PKC α - PKC α isomer

PKC β - PKC β isomer

PKC-I - PKC inhibitor peptide (19-36)

PMSF - phenylmethylsulfonyl fluoride

PNPP - para-nitrophenyl phosphatase

PP α - phosphorylase a phosphatase

PP-1 - protein phosphatase type-1

PP-2 - protein phosphatase type-2

PS - phosphatidylserine

SDS-PAGE - polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate

TCA - trichloroacetic acid

TPA - 12-O-tetradecanoylphorbol-13-acetate

V_{max} - maximal enzyme velocity

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CHAPTER 1

GENERAL INTRODUCTION

Biochemical signal transduction pathways transmit information arriving at the plasma membrane to the interior of the cell. Signal transduction pathways are also responsible for coordinating cellular activity during complex functions. Reversible covalent modification of proteins by phosphorylation and dephosphorylation plays a dominant role in controlling the activities of proteins involved in signaling (Krebs 1986; Walsh et al. 1991). The net activity of a phosphoprotein at any given time depends on the proportion of molecules in the phosphorylated versus dephosphorylated states, which in turn depends on the relative activities of protein kinases and protein phosphatases. An important generality that has emerged is that enzymes in biodegradative pathways are typically activated, whereas those in biosynthetic pathways are typically inactivated by protein phosphorylation (Cohen 1980). This, in turn, implies that different metabolic pathways may be controlled by identical protein kinases and protein phosphatases, or by identical effector molecules that regulate different protein kinases and protein phosphatases.

Glucose is a major metabolic fuel source and is degraded via glycolysis to produce biosynthetic precursors, reducing equivalents and ATP. Storage of glucose and other monosaccharides would disturb the osmotic balance of cells, but storage of glucose in the form of polysaccharides, which have a small osmotic effect, entails minimal disturbance. Living organisms protect themselves from potential fuel shortage by polymerizing excess glucose for storage as high molecular mass glucans that may be readily mobilized in times of metabolic demand. In animals and some bacteria the storage glucan is glycogen. Glycogen was discovered and named by Bernard in 1870, who noted its ability to provide blood glucose (Bernard 1877). It occurs mainly in liver and muscle in 100 to 400 Å diameter cytoplasmic granules. These granules are complexed with the enzymes responsible for glycogen degradation and synthesis. Since the metabolism of glucose is central to energy production in the cell, maintenance of a

sufficient concentration of glucose is crucial. This is accomplished in part through regulation of the synthesis and degradation of glycogen.

Glycogen degradation begins with the action of the enzyme glycogen phosphorylase (EC.2.4.1.1). Cori and Green (1943) and later Cori and Cori (1945) showed that two interconvertible forms of glycogen phosphorylase exist, termed glycogen phosphorylase a (phosphorylated form) and glycogen phosphorylase b, each with differing requirements for the allosteric activator AMP for activity. Glycogen phosphorylase a is active independent of the presence of AMP whereas, glycogen phosphorylase b is fully dependent upon AMP activation to express activity. Glycogen phosphorylase has been the subject of intense biochemical and structural analysis and the basis for the control of enzyme activity, both by allosteric effectors (AMP and glucose) and by phosphorylation, is now well understood (Johnson et al. 1989; Palm et al. 1990; Madsen 1986). The enzyme catalyzes the phosphorolytic degradation of glycogen into glucose-1-phosphate and, as the rate limiting step of glycogenolysis, controls the rate of flux of storage carbohydrate into the glycolytic pathway. The activity of the enzyme is subject to regulation by metabolic, hormonal and neuronal signals, with metabolic effects being exerted by allosteric effectors and hormonal and neuronal effects being exerted by phosphorylation through the action of phosphorylase kinase and protein phosphatases.

Phosphorylase kinase (EC 2.7.1.38) is a key enzyme in the interconvertible enzyme cascade that regulates glycogenolysis. The physiological benefits of the complexity of this bicyclic cascade for the regulation of the rate of glycogenolysis remain to be fully established. Phosphorylase kinase is an oligomeric enzyme, a hexadecamer, composed of four kinds of subunit, named α , β , γ and δ (Cohen et al. 1978), which can express variable degrees of activity depending on pH, metal ions, allosteric effectors and covalent modification (Ludwig and Heilmeyer 1991). It is well known that the enzyme can be activated by phosphorylation of both the α and the β

subunits (Pickett-Gies and Walsh 1986). More extensive activation of phosphorylase kinase can occur during phosphorylation by cAMP-dependent protein kinase in the presence of high Mg^{2+} concentrations (Singh and Wang, 1977).

Phosphorylase kinase can be converted to activated states by an ATP dependent phosphorylation reaction which is catalyzed by either cAMP or cGMP dependent protein kinase, or phosphorylase kinase itself (Tam et al. 1982). Among the various modes of the enzyme activation, the activation by the PKA catalyzed has been best characterized. PKA is most ubiquitous in eukaryotic cells and is known to be involved in the regulation of a wide variety of metabolic processes (Nairn et al. 1985; Edelman et al. 1987). The holoenzyme consists of two catalytic subunits and a dimeric regulatory subunit. The enzyme is activated by cAMP according to Equation 1,

via an intermediate ternary complex of holoenzyme and cAMP, resulting in the release of the catalytically active free C subunit.

Regulation of glycogen metabolism is achieved by a balance between the activities of glycogen synthase and glycogen phosphorylase, which are under substrate control (through allosteric regulation) as well as hormonal control. Not only is phosphorylase activated by a rise in concentration of cAMP (via phosphorylase kinase), but glycogen synthase is, at the same time, converted to its inactive form. Both effects are mediated via PKA. Thus, inhibition of glycogenolysis enhances net glycogenesis, and inhibition of glycogenesis enhances net glycogenolysis. Of further significance in the regulation of glycogen metabolism is the finding that the dephosphorylation of phosphorylase α , phosphorylase kinase, and glycogen synthase β is all accomplished by a single enzyme of wide specificity; protein phosphatase type-1. In turn, protein phosphatase type-1 is inhibited by cAMP-dependent protein kinase via inhibitor-1 (Fig.

1.1). Thus, glycogenolysis can be terminated and glycogenesis can be stimulated synchronously, or vice versa, because both processes are keyed to the activity of PKA.

Cellular regulation between signalin pathways may also be mediated by protein kinase C; recent studies suggest that PKC may potentiate cAMP and cGMP mediated responses (Ho et al. 1988; Aasheim et al. 1989; Houslay 1991). PKC activity is regulated by changes in cellular free Ca^{2+} and lipid concentrations which cause a translocation of PKC from cytosol (where it is inactive) to the membrane where it becomes activated through binding to membrane lipids (Ho et al. 1988).

Compared with mammalian systems, information on the regulation of carbohydrate metabolism via protein phosphorylation and dephosphorylation in lower vertebrates and plants is weak or incomplete. This is particularly true with respect to the molecular controls expressed as responses to environmental stresses. Fish are by far the most numerous vertebrate. Physiological and metabolic responses of fish skeletal muscle to either endurance or burst swimming exercise have been characterized by a number of authors (e.g. Johnson, 1981; Wood and Perry, 1985; Parkhouse et al. 1987; Brooks and Storey, 1988). At relatively low swimming speeds, power is primarily provided by red muscle fibers utilizing aerobic, oxidative pathways of ATP generation. As swimming speed increases there is a progressive recruitment of white muscle fibers, and high speed or burst swimming derives most of its power needs from the white musculature. When high speed swimming is prolonged over many minutes, glycogen is fully depleted and the skeletal muscle may then switch to the use of blood glucose as a fuel supply (Dobson et al. 1987; Storey 1991). Whereas there is considerable information about skeletal muscle metabolism during swimming, less is known about the simultaneous control of metabolism in the liver and other organs. Many questions remain about the control of carbohydrate metabolism during exhaustive swimming. Is liver glycogenolysis in exercising fish regulated via reversible protein phosphorylation or via allosteric regulation of glycogen phosphorylase, or both? During burst

Fig. 1.1 Control of glycogenolysis and glycogenesis by cAMP-dependent protein kinase

The reactions that lead to glycogenolysis as a result of an increase in cAMP concentrations are shown with enzymes in their active a forms, and those that are inhibited are shown in their inactive b forms. The reverse occurs when cAMP concentrations decrease as a result of phosphodiesterase activity, leading to glycogenesis.

* PP-1 = Protein phosphatase type-1

swimming, how does liver glycogenolysis regulate the export and supply of glucose for use in skeletal muscle contraction and for the functioning of brain and other organs? Is glycogen phosphorylase, in fact, the only enzyme regulating glycogenolysis, and thereby glucose export, in liver? or other glycogenolysis pathway is operating in these animals.

This last question addresses the issue of whether other enzymatic activities, apart from glycogen phosphorylase, are also involved in supplying glucose from glycogen. The splitting of polysaccharide α -1,4 linkages by glycogen phosphorylase to release glucose-1-phosphate is certainly the main, if not only, process for endocellular glycogenolysis in mammalian tissues. However, this may not be true of lower vertebrates and indeed, several studies with fish give evidence that glycogen breakdown is only partially due to phosphorylase activity and that other enzymes must also be involved (Rush and Umminger, 1978; Umminger et al. 1975; Murat 1976). Alternative pathways for glycogen breakdown could involve several hydrolases which have been shown to be present in the liver, and in some other tissues and body fluids, such as α -amylase (Takeuchi et al. 1975; Das and Hems 1979), lysosomal acid α -glucosidase (Hers, 1963), neutral α -glucosidase (Rosenfeld, 1975), and amylo-1,6-glucosidase (Vaillant, 1975). The biological significance of these enzymes in animal cells is still poorly documented, but a role in the regulation of metabolism has been strongly suggested in some cases (Hers, 1972; Murat, 1976; Das and Hems, 1979).

Many freshwater turtles (e.g. genera such as *Trionyx*, *Stemotherus*, *Chelydra*, *Trachemys* and *Chrysemys*) can survive underwater without breathing air for days or even months, particularly during winter hibernation at low environmental temperatures. Although some species have the ability to extract dissolved O_2 from water by nonpulmonary gas exchange, all also possess an extremely well-developed anoxia tolerance (Belkin, 1968; Gatten, 1985; Herbert and Jackson, 1985; Ultsch and Jackson, 1982). Indeed, various species of *Trachemys* and *Chrysemys* can endure weeks of

submergence in nitrogen-gassed water, relying entirely upon anaerobic pathways for energy generation (Ultsch, 1985). This ability is dependent on the following adaptations: 1) the possession of large glycogen stores in the liver, the primary fuel for anaerobic metabolism (Clark and Miller 1973; Daw et al. 1967); 2) the ability to minimize and tolerate acidosis (Jackson and Heisler, 1982); 3) the adjustment of blood flow such that the brain is served at the expense of other organs (Davies, 1989); and a large reduction in metabolic rate (Herbert and Jackson, 1985; Jackson, 1968; Kelly and Storey, 1988).

This last mechanism, metabolic rate depression, is indeed the overriding factor and anoxic metabolic rate in turtles is reduced to only 10-20% of the corresponding aerobic metabolic rate at the same body temperature (Herbert and Jackson, 1985). Studies of glycolytic regulation during environmental anoxia, hibernation and estivation in a wide variety of animals have served to illustrate the molecular mechanisms which mediate metabolic depression (Storey and Storey, 1990). In the turtle *Trachemys scripta elegans*, these mechanisms include; 1) control of carbohydrate utilization via fructose 2,6-bisphosphate regulation of phosphofructokinase (Brooks and Storey, 1988, 1989); 2) changes in the percentage of enzymes associated with subcellular structures to directly alter enzyme activity (Duncan and Storey 1991); and 3) covalent modification of regulatory enzymes via reversible protein phosphorylation (Brooks and Storey, 1988, 1989). Cellular control of enzyme covalent modification, such as phosphorylation and dephosphorylation, is mediated by second messenger-dependent protein kinases such as protein kinase C (PKC), PKA and cGMP-dependent protein kinase.

The objectives of the present studies are to provide a clear picture of the *in vivo* regulation of glycogen metabolism, particularly the control of glycogen phosphorylase through protein kinases and protein phosphatases. The study focuses on regulation in response to two different stresses, exercise and anoxia, using appropriate vertebrate model system for each. These are summarized as follows.

Part 1: Exercise metabolism in fish (Rainbow trout, *Oncorhynchus mykiss*)

1). Glycogen phosphorylase *b* from trout skeletal muscle was purified to electrophoretic homogeneity and its physical and chemical properties were characterized. Kinetic analysis of the enzyme was carried out under physiological conditions.

2) Activities of glycogen phosphorylase, phosphorylase kinase and PKA, as well as levels of cAMP in organs of control and exercised fish were investigated.

3) The involvement of the glucosidic pathway of glycogen degradation was examined by analysis of α -glucosidase in liver and the effect of exercise on the activities and kinetic properties of different isozymes.

Part 2: Anoxic metabolism in turtles (Red-eared sliders, *Trachemys scripta elegans*)

1) The effect of anoxia exposure on the behavior of enzymes of both the phosphorolytic (glycogen phosphorylase, phosphorylase kinase, cAMP-dependent protein kinase) and glucosidic (α -glucosidase) pathways were assessed in five organs of control and anoxic animals.

2) The catalytic subunit of PKA of *T. scripta* was purified to homogeneity and physical and kinetic properties of the enzyme were studied.

3) The effect of anoxia on protein phosphatase activity was investigated in turtle organs and protein phosphatase type-1 from turtle liver was purified and physical and kinetic properties of the enzyme were assessed

4) The effect of anoxia on protein kinase C activities as well as enzyme translocation during anoxia in turtle liver were studied. PKC type II (β) and type III (α) from turtle liver were purified and physical and chemical properties of both isozymes were investigated.

PART 1

CHAPTER 2

CONTROL OF GLYCOGENOLYSIS DURING EXERCISE IN RAINBOW TROUT ORGANS

INTRODUCTION

Glycogen catabolism in vertebrates is regulated through a cascade of enzymes, each interconvertible between active a and inactive b forms. Glycogen phosphorylase (1,4- α -D-glucan:orthophosphate glucosyl transferase EC 2.4.1.1) catalyzes the phosphorolytic cleavage of glucosyl units from glycogen to form glucose-1-phosphate. The enzyme has been purified and crystallized from various animal species and its structure and properties have been extensively reviewed (Busby and Radda 1976; Dombradi 1981; Steele 1982; Madsen 1986). Glycogen phosphorylase from rabbit muscle was first shown to exist in two interconvertible forms by Krebs and Fischer (1956): the unphosphorylated inactive b form and the phosphorylated active a form. The conversion of b to a is catalyzed by phosphorylase kinase, which adds phosphate groups to specific serine residues on each subunit. This is reversed by phosphorylase phosphatase. The two forms are discriminated kinetically because phosphorylase b requires AMP as an allosteric activator to express its activity, whereas phosphorylase a is active in the absence of AMP. Glycogen phosphorylase kinase (GPK) which phosphorylates and activate glycogen phosphorylase, in turn, is activated through phosphorylation by cyclic AMP-dependent protein kinase (PKA) (Cohen 1980). The system has been extensively analyzed in mammalian systems, particularly skeletal muscle, but questions still remain about the regulation of glycogen metabolism in lower vertebrates. Although it is well known that glycogen is one of the major energetic fuels for fish skeletal muscle during exercise, many aspects of the regulation of glycogenolysis are still controversial. For example, Dobson et al. (1987) have recently questioned how glycolysis can continue to operate in fish muscle when interacellular pH of the muscle drops to very low, and presumably inhibitory, values under conditions of burst exercise. Thus, pH of trout white muscle dropped by 0.4-0.6 units to approximately pH 6.6 during exhaustive exercise (Milligan and Wood 1986b; Parkhouse et al. 1987). During high speed swimming in trout, glycogenolysis is rapidly

activated in white muscle and glycogen reserves are virtually fully depleted when trout are exercised to exhaustion (Parkhouse et al. 1987). Despite this, the percentage of glycogen phosphorylase in the a form rose to only 16 % (from 3.6%) in working muscle (Storey 1991). This low percent a seemed unusual for the support of maximal rates of glycogenolysis in trout muscle and lead to questions about the regulation of phosphorylase in fish muscle. We proposed two possible reasons for this behavior: 1) that muscle phosphorylase b might contribute to glycogenolysis in working muscle, and 2) that fish muscle might exhibit changes or limitations to the enzyme phosphorylation cascade that controls the activation of glycogen phosphorylase. The first possibility could be examined by a study of the kinetic and regulatory properties of purified trout white muscle glycogen phosphorylase b. This study, presented in this chapter, revealed that although phosphorylase b would be virtually inactive at the physiological concentrations of substrates and activators found in resting trout white muscle, the metabolic conditions (low pH, low ATP, and high levels of AMP, IMP and P_i) that prevail in exercising fish muscle could allow phosphorylase b to significantly complement phosphorylase a activity in working muscle.

The second possible limiting factor on phosphorylase activation in fish muscle could be examined with an analysis of the effects of exercise on the activation of GPK and PKA in rainbow trout organs. The structure and function of these enzymes in mammalian tissues have been extensively analyzed (Reimann et al. 1971; Hayakawa et al. 1973; Carlson et al. 1979; Zetterqvist et al. 1990) but limited information is available on the corresponding fish enzymes although GPK from elasmobranch (dogfish) muscle has also been purified and characterized (Pocinwong et al. 1981). However, the responses of these enzymes to swimming exercise has not previously been studied. In the present study we demonstrate that GPK and PKA are activated, and cAMP levels rise, in both skeletal muscle and liver of rainbow trout during

exhaustive swimming. These changes support the rapid mobilization of glycogen reserves in both muscle and liver to support skeletal muscle work.

MATERIALS AND METHODS

CHEMICALS AND ANIMALS

All chemicals, coupling enzymes and rabbit muscle PKA catalytic subunit were obtained from Sigma Chemical Co (St. Louis, MO) or Boehringer Mannheim (Montreal, PQ) and were of highest quality available. [γ - ^{32}P] ATP (3000 Ci.mmol⁻¹) was obtained from New England Nuclear (Montreal, PQ). Phosphocellulose paper (P81) was obtained from Whatman (Canlab Corp, Mississauga, Ont). Assay kits for cAMP determinations were obtained from Amersham (Mississauga, Ont.).

Rainbow trout were housed in the aquatic facility of the University of Ottawa in aerated circulating water at 12°C with a photoperiod 12L/12D and were fed ad libitum with commercial trout pellets. To exercise fish, individual animals were gently netted and transferred to another tank. There they were chased by repeatedly grasping them by the tail, thus forcing them to swim vigorously; this was continued for either 1 min or until fish were exhausted (approximately 10 min). Control fish were obtained by carefully netting fish from the holding tank so as to minimize any struggle. To study the effect of anaesthesia, fish were gently netted and placed in a container of buffered water (NaHCO₃, 2 g/l) containing MS-222 (3-aminobenzoate ethyl ester methanesulfonate) at a concentration of 1 part per 6000; fish were exposed to MS-222 for several seconds until they were unable to right themselves. All fish were killed quickly by a single sharp blow to the head. Organs were rapidly dissected out (within 60 sec) and immediately frozen in liquid nitrogen. Tissue samples were transferred to -80°C for storage.

PREPARATION OF TISSUE EXTRACTS AND ENZYME ASSAY

A). *Glycogen phosphorylase.*

Samples of frozen tissue were homogenized with four volumes of extraction buffer (30 mM imidazole (pH 7.2), 30 mM 2-mercaptoethanol, 2 mM EDTA, 2 mM EGTA, 100 mM sodium fluoride, and 0.1 mM phenylmethylsulfonylfluoride [PMSF]) and centrifuged at 15,000 x g for 15 min at 4°C. Supernatants were removed and desalted by passage through small columns (1 x 5 cm, 0.5 ml supernatant per column) of Sephadex G-25 (medium) equilibrated in extraction buffer (Helmerhorst and Stokes 1980). The columns were centrifuged in a IEC benchtop centrifuge for 1 min and the enzyme filtrate was collected and stored at 4°C for assay.

The activity of glycogen phosphorylase was assayed using a Gilford-240 recording spectrophotometer at 21°C in the direction of glycogen breakdown, using a coupled assay system, with the production of NADPH monitored at 340 nm. Optimum assay conditions were 10 mM MgCl₂, 0.25 mM EDTA, 1 mM glucose-1,6-bisphosphate, 2 mg/ml glycogen, 0.4 mM NADP, 50 mM KPi (pH 7.2), 1.4 U phosphoglucomutase, 0.5 U glucose-6-phosphate dehydrogenase and with or without 1.6 mM AMP in a total volume of 1 ml. One unit of phosphorylase activity is defined as the amount of enzyme that produces one micromole of glucose-1-phosphate per minute at 21°C.

B). *Phosphorylase kinase*

Frozen tissue samples were quickly weighed and then immediately homogenized in 4 vol (w/v) of extraction buffer (50 mM imidazole pH 7.2, 30 mM 2-mercaptoethanol, 2.5 mM EDTA, 2.5 mM EGTA and 50 mM NaF with 0.1 mM PMSF added immediately prior to homogenization) using a Polytron PT-10 homogenizer. Homogenates were then centrifuged and desalted as mentioned above. Glycogen phosphorylase b, the substrate for phosphorylase kinase, was purified from

trout white muscle as described in the following section. When needed, glycogen phosphorylase a was prepared from the b form using the conditions outlined below for the conversion of b to a in the GPK assay.

The GPK assay of Uyttenhove et al. (1991) was modified for use on a spectrofluorometer. The incubation mixture for phosphorylation of purified trout phosphorylase b contained 2.5 mM ATP, 10 mM magnesium acetate, 100 mM NaF, 0.1 mM CaCl₂, 25 mM Tris, 25 mM sodium glycerophosphate, pH 8.2 and 0.1 mg (8 units) purified trout glycogen phosphorylase b in a final volume of 200 μ l. Each sample was compared with a corresponding blank that contained 2.5 mM ATP and 20 mM EDTA but no Mg²⁺; this allowed the reaction to be controlled for trace levels of phosphorylase a in the crude GPK extracts. The reaction was started by adding an appropriate amount of enzyme preparation and the mixture was incubated at 22°C for 30 min. This optimal incubation time was determined from a preliminary experiment with incubation times ranging from 5 to 50 min in 5 min intervals. The phosphorylation reaction was stopped by the addition of 1 ml of glycogen phosphorylase assay mixture containing 50 mM potassium phosphate, pH 7.0, 10 mM MgCl₂, 0.25 mM EDTA, 50 μ M glucose-1,6-bisphosphate, 0.4 mM NADP, 2 mg/ml oyster glycogen, 5 mM caffeine, 1.4 U phosphoglucomutase, and 0.5 U glucose-6-phosphate dehydrogenase. NADPH production was monitored fluorometrically with an excitation wavelength of 360 nm and an emission wavelength of 460 nm; fluorescence standards used known amounts of NADPH. One unit of activity is defined as the number of μ moles of phosphorylase b converted to phosphorylase a per min under the above conditions at 22°C.

C). Activation of phosphorylase kinase by endogenous protein kinase

The reaction mixture for testing GPK activation by endogenous protein kinases contained the following in a total volume of 400 μ l: 21 mM imidazole buffer, 5 mM

KPi, pH 7.2, 5 mM 2-mercaptoethanol, 10 mM ATP, 50 mM NaF, 15 mM MgCl_2 , and a suitable amount of enzyme preparation (G-25 filtrate). The following additions were made to different tubes (all samples were run in triplicate): 2.5 μM cAMP, 2.5 μM cGMP, 1.5 μM CaCl_2 + 6.7 $\mu\text{g/ml}$ 12-O-tetradecanoylphorbol-13-acetate (TPA), or 2 mg/ml purified PKA catalytic subunit from rabbit muscle. Control samples substituted 20 mM EDTA instead of Mg^{2+} . Samples were incubated at 22°C for 45 min, then desalted by centrifugation through Sephadex G-25 columns (as previously), and assayed for GPK activity.

D). *cAMP-Dependent protein kinase activity*

For analysis of PKA activity, samples of frozen tissues were rapidly weighed and then immediately homogenized 1:10 (w/v) in ice cold PEM buffer (10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 2 mM 2-mercaptoethanol, 0.1 % v/v Triton X-100) in the presence of 1 mM PMSF and 1 $\mu\text{g/ml}$ aprotinin. Extracts were centrifuged for 1 min in an I.E.C. microfuge at 13,000 g and the supernatants were removed for PKA assay. This procedure took approximately 2 min to complete and then samples were immediately assayed. Activity was assayed essentially as described by (Corbin and Reimann 1974). Unless otherwise indicated, the reaction mixture contained in a 60 μl volume: 20 mM potassium phosphate (pH 6.8), 0.15 mM kemptide, 1 mM EDTA, 3.47 mM magnesium acetate, 0.231 mM unlabeled ATP, 10 $\mu\text{Ci/ml}$ radioactive ATP (0.6 μCi per assay), and 10 μl of enzyme extract with the absence versus presence of 10 μM cAMP for assays of active versus total protein kinase activity, respectively. The total Mg^{2+} and ATP concentration gave 0.2 mM $\text{Mg}\cdot\text{ATP}$ and 0.8 mM free Mg^{2+} in the assay mixture (Brooks and Storey 1992). Higher concentrations of free Mg^{2+} were inhibitory to the enzyme (data not shown).

E). *Determination of tissue cAMP levels*

For the determination of cAMP levels perchloric acid extracts of frozen trout tissues were prepared according to Storey and Storey (1984). cAMP concentrations were determined from competitive protein binding assays using the commercial assay kit from Amersham.

PROTEIN DETERMINATION

Protein concentration was determined using the Bio-Rad assay kit based on the Coomassie blue G-250 dye-binding method with bovine gamma globulin as the standard (Bradford 1976).

GEL ELECTROPHORESIS AND MOLECULAR WEIGHT DETERMINATION

The subunit molecular weight of glycogen phosphorylase was determined by SDS-PAGE performed according to Laemmli (1970) using 7.5% acrylamide gels and 5% acrylamide in the stacking gel. The proteins were visualized with silver stain (Wray et al. 1981).

The molecular weight of the native enzyme was determined by gel filtration on Sephacryl S-300 (Andrew 1964). The column (1.5 x 150 cm) was equilibrated with 50 mM phosphate buffer containing 10% (v/v) glycerol (pH 7.2), with or without 1 mM AMP. Calibration used cytochrome c, hemoglobin, fructose-1,6-bisphosphatase, aldolase, and phosphofructokinase as standards. The flow rate was 5 ml/h.

KINETIC DATA ANALYSIS

Throughout this study all enzymes presented were linear with respect to time of the assays and all kinetic constants (K_m , K_a , I_{50} , etc) were determined from the Hill equation, Job plot and activator plot from the statistical computer analysis program (Brooks 1992).

PURIFICATION OF GLYCOGEN PHOSPHORYLASE β

All steps of purification were performed at 4°C unless otherwise stated and used white skeletal muscle from resting rainbow trout.

A). Crude homogenate

Approximately 10 to 12 g of muscle was homogenized with 10 volumes of glycogen phosphorylase extraction buffer (see above). The homogenate was then centrifuged at 20,000 g for 30 min at 4°C (Sorvall RC-5B, Dupont Instruments). The high speed supernatant was removed and used in subsequent steps.

B). Glycogen-ConA chromatography

An affinity resin consisting of glycogen bound to ConA-Sepharose was prepared as outlined by Gurnack and Edstrom (1990). A column (2.5 x 20 cm) was poured using freshly prepared glycogen-Sepharose beads and equilibrated with buffer A (67 mM HEPES, 0.2 mM CaCl_2 , 10 mM MgCl_2 , pH 6.8). An aliquot of the high speed supernatant was applied to the column. The column was washed with 20 ml of buffer A and then phosphorylase activity was eluted by a linear gradient of 0-2 M glucose (60 ml) in buffer B (50 mM HEPES, pH 7.2, 1 mM EDTA). Fractions of 1 ml were collected and assayed for phosphorylase activity. The active fractions were pooled and dialyzed overnight against dialysis buffer (10 mM imidazole, pH 7.2, 2% v/v glycerol, 15 mM 2-mercaptoethanol).

C). DEAE-Sephacel chromatography

The pooled dialyzed enzyme solution was applied to a DEAE-Sephacel column (2.5 x 20 cm) that had been equilibrated with the extraction buffer. The enzyme was eluted using a linear gradient of α -glycerophosphate (0-200 mM) formed by two 50-ml

vessels. Fractions of 1 ml were collected and assayed, and peak fractions containing phosphorylase **b** activity were pooled and dialyzed overnight against dialysis buffer.

D). AMP-Sepharose chromatography

The dialyzed enzyme was applied to a column of 3',5'-cAMP-Sepharose 4B (2.5 x 5 cm) equilibrated with extraction buffer. The column was washed with the same buffer (20 ml) and then the enzyme was eluted by a linear gradient of AMP (0-20 mM). The eluant was collected in 1 ml fractions and assayed for enzyme activity. The active fractions were pooled and dialyzed as above for kinetic studies.

RESULTS

GLYCOGEN PHOSPHORYLASE

A). Enzyme purification

Table 2.1 shows the purification scheme for trout muscle phosphorylase **b**. The enzyme bound tightly to the glycogen-ConA column and was eluted in a sharp peak at approximately 0.7 M glucose. Enzyme elution from DEAE-Sepharose was also in a single peak at 50 mM α -glycerophosphate. The enzyme eluted from the AMP-Sepharose 4B column in a single peak at 8 mM AMP and was highly pure with an overall yield of 7%. The final specific activity of the enzyme was 135 $\mu\text{mol glucose-1-phosphate formed} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, which represents a 204-fold purification. The purification scheme combined techniques that had previously been used for single-step phosphorylase purification from other systems: e.g. rabbit liver phosphorylase by glycogen-ConA affinity chromatography (Kamp 1986; Gurnack and Edstrom 1990). With trout muscle phosphorylase, however, the combination of multiple techniques was needed to achieve a high purification factor. The enzyme preparation was examined by

TABLE 2.1
Purification of phosphorylase b from trout white muscle

Steps	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (fold)	Yield %
Supernatant	78.4	49.28	0.66	1.0	100
Glycogen-ConA	0.99	11.67	12.10	18.4	23
DEAE-Sephacel	0.53	8.59	16.20	24.5	17
AMP-Sepharose	0.025	3.37	134.7	204.0	7

Fig. 2.1 SDS-Polyacrylamide gel electrophoresis of purified phosphorylase b

Lane A shows molecular weight standards from top to bottom: mammalian glycogen phosphorylase b (97,000), bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000) and bovine lactalbumin (14,200). Lane B shows partially purified trout glycogen phosphorylase b after the DEAE-Sephacel column and lane C shows purified glycogen phosphorylase b after the AMP-Sepharose column.

SDS-PAGE (Fig. 2.1). The purified sample showed a single major protein band on the gel (silver stain) with very minor contaminating bands. The subunit molecular weight of trout white muscle glycogen phosphorylase b was estimated to be 87,000 daltons (Fig. 2.1). The molecular weight of the native form of glycogen phosphorylase b was determined to be $193,000 \pm 4,000$ daltons by gel filtration on Sephacryl S-300. When 1 mM AMP was added to the elution buffer, the apparent molecular weight increased to $335,000 \pm 6,000$ daltons, indicating a change from a dimeric to tetrameric form of the enzyme.

B). Kinetic properties of phosphorylase b from trout muscle

Table 2.2 shows the affinity of phosphorylase b for its substrates and for the activator AMP at two pH values and at optimal versus suboptimal cosubstrate concentrations. The two pH values were chosen because they represent the pH values *in vivo* in trout white muscle at rest (pH 7.2) versus after exhaustive exercise (pH 6.6)(Milligan and Wood 1986a, 1986b). When assayed at optimal substrate concentrations, the K_m for glycogen increased significantly by two-fold when the pH was lowered from 7.2 to 6.6, whereas K_m for phosphate was unaffected by the pH change. At optimum concentrations of both substrates (2 mg/ml glycogen and 50 mM phosphate), phosphorylase b activity was entirely dependent on the presence of AMP; virtually no activity was detected in the absence of activator. Maximal enzyme activity was reached at 0.8 mM AMP at both pH 7.2 and 6.6, but V_{max} of the enzyme at pH 6.6 was only 90% of the value at pH 7.2. The activator concentration that produced half-maximal enzyme activation (K_a), for AMP did not change when assay pH was lowered to 6.6. When kinetic studies were carried out under a suboptimal concentration of phosphate (20 mM) the enzyme showed a much higher K_m for glycogen, a 2.6-fold increase at pH 7.2 compared with the value at optimal phosphate concentration (Table 2.3). However, when assayed at pH 6.6, the K_m for glycogen was the same at both

TABLE 2.2**Kinetic properties of phosphorylase b at optimal cosubstrate concentrations**

Values are means $\pm$ SEM with n, the number of separate determinations, in brackets. K_m values, were determined at the optimal concentration of cosubstrate. K_a values for AMP were determined at 2 mg/ml glycogen and 50 mM potassium phosphate.

* Significantly different from the corresponding value at pH 7.2 by the Student's t-test, $P < 0.005$.

	pH 7.2	pH 6.6
K_m Glycogen, $\mu\text{g/ml}$	128.81 $\pm$ 8.11 (3)	376.23 $\pm$ 4.25* (3)
K_m Phosphate, mM	31.83 $\pm$ 2.54 (3)	36.11 $\pm$ 2.48 (3)
K_a AMP, μM	142.42 $\pm$ 7.21 (3)	143.43 $\pm$ 11.52 (3)

TABLE 2.3

Kinetic properties of phosphorylase b at suboptimal cosubstrate concentrations

Values are means $\pm$ SEM with n, the number of separate determinations, in brackets. K_m for glycogen was determined at 20 mM phosphate and 1.6 mM AMP; K_m for phosphate was determined at 0.5 mg/ml glycogen and 1.6 mM AMP; K_a for AMP was determined at 0.5 mg/ml glycogen and 20 mM phosphate.

* Significantly different from the value at pH 7.2, $P < 0.01$.

	pH 7.2	pH 6.6
K_m Glycogen, $\mu\text{g/ml}$	333.12 ± 7.41 (3)	$369.43 \pm 4.25^*$ (3)
K_m Phosphate, mM	41.96 ± 1.75 (3)	35.46 ± 3.30 (3)
K_a AMP, μM	74.38 ± 10.9 (3)	--

TABLE 2.4**Inhibition of trout phosphorylase b by glucose and ATP**

Values are means $\pm$ SEM with n, the number of separate determinations, in brackets. I_{50} values, the concentration required for a 50 % reduction of enzyme velocity, were determined at 2 mg/ml glycogen, 50 mM phosphate and 1.6 mM AMP.

* Significantly different from the value at pH 7.2, $P < 0.005$.

	pH 7.2	pH 6.6
I_{50} glucose, mM	2.16 ± 0.10 (3)	$3.72 \pm 0.10^*$ (3)
I_{50} ATP, mM	5.79 ± 0.13 (3)	6.17 ± 0.50 (3)

concentrations. Affinity of the enzyme for AMP at suboptimal concentrations of substrates increased two-fold (K_a decreased by 50%) compared with the value at optimal substrate concentrations.

Trout phosphorylase $\underline{b}$ was sensitive to allosteric inhibition by both glucose and ATP (Table 2.4). I_{50} values, measured at optimal substrates and AMP concentrations, were 2.16 mM for glucose and 5.79 mM for ATP at pH 7.2. The I_{50} for glucose increased by 72% when assay pH was dropped to 6.6.

C). *Effect of pH and temperature on enzyme activity*

The effect of pH on phosphorylase $\underline{b}$ activity was examined at 21°C under optimal substrate and AMP concentrations. The enzyme showed a relatively sharp pH optimum at 7.5, with only 70-80% of maximal activity at pH 6.5 or 8.0 and less than 10% of activity remaining at pH 5.5 or 9.0 (Fig. 2.2). The effect of temperature on enzyme activity was assayed at intervals between 0 and 48°C at both pH 7.2 and 6.6. At pH 7.2 the enzyme showed a very sharp optimum at 25°C, with only 75% of the maximum activity remaining at 20 or 30°C. However, a wider optimum temperature range was found at pH 6.6 (Fig. 2.3). The optimum temperature at this pH was 30°C and more than 90% of the maximum activity was retained at 24 or 35°C. Activity declined sharply at high temperatures and the enzyme at either pH was completely inactivated at 45-48°C.

GLYCOGEN PHOSPHORYLASE KINASE

A). *Fluorometric phosphorylase kinase assay*

The standard spectrophotometric assay for GPK as described by Uyttenhove et al. (1991) was modified to produce a fluorometric assay. The fluorometric assay was about 50 times more sensitive than the spectrophotometric assay and used less crude enzyme per assay. Activities of GPK in trout organs measured by the fluorometric assay are shown in Table 2.5; all activities were also measured by the

Fig. 2.2 Effect of pH on trout muscle glycogen phosphorylase b activity

Assays were carried out at 25°C under optimum assay conditions but with pH of the reaction mixture adjusted to different values with potassium phosphate.

Fig. 2.3 Effect of temperature on the activity of phosphorylase b from trout white muscle

The enzyme was assayed for 10 min at pH 7.2 (closed circles) and 6.6 (closed triangles).

Table 2.5**Phosphorylase kinase in organs of rainbow trout**

Data are expressed as units/gram wet weight, means $\pm$ SEM, n = 5 fish.

Muscle is white skeletal muscle.

* Significantly different from the corresponding control value by the Student's t-test, $P < 0.025$.

	Total activity (U/gww)	
	Resting, Control	Exhaustive exercise
Liver	0.48 ± 0.04	$0.74 \pm 0.07^*$
Heart	0.18 ± 0.01	0.25 ± 0.08
Gill	0.16 ± 0.03	0.22 ± 0.02
Muscle	0.16 ± 0.01	$0.22 \pm 0.01^*$

spectrophotometric assay but no significant difference in the activities determined by this assay, compared with the fluorometric assay, were found in any instance (data not shown). For example, compared with values in Table 2.5, values for liver GPK activity measured by the spectrophotometric assay were 0.46 ± 0.05 and 0.74 ± 0.08 U/gww for control and exercised fish, respectively. Compared with the spectrophotometric assay, the fluorometric assay also had a substantially better signal to noise ratio and a lower background reading, and gave improved results when enzyme activity was very low. Furthermore, by using a four position cuvette compartment, blank cuvettes that corrected for endogenous phosphorylase a activity in the crude GPK extracts could be monitored simultaneously. The fluorometric assay was also tested using commercial purified rabbit muscle GPK and rabbit muscle glycogen phosphorylase b; phosphorylase a activity increased linearly with increasing GPK concentration (data not shown).

Other authors have expressed concern over the use of the coupled enzyme assay for GPK with unpurified enzyme extracts due to the possibility of ATP breakdown into AMP. Allosteric activation of phosphorylase b by AMP could erroneously be interpreted as a GPK-mediated conversion of phosphorylase b to a (Stalmans and Hers 1975). To determine whether such a problem could arise in this assay system, the effects of 80 μ M AMP on the activities of trout liver phosphorylase a and b were tested in the presence of varying amounts of caffeine (an inhibitor of phosphorylase b) (Fig. 2.4). Caffeine at 5 mM completely inhibited phosphorylase b activity in the presence of 80 μ M AMP but had no significant effect on phosphorylase a; thus, all routine assays were performed with the addition of 5 mM caffeine. Equivalent results to those in Fig. 2.4 were obtained using trout muscle phosphorylase a and b (data not shown).

B). *Glycogen phosphorylase kinase in trout organs*

FIG. 2.4 Effects of caffeine, in the presence of 80 μ M AMP, on the activities of phosphorylase from trout liver

Phosphorylase a (filled circles) and b (open circles). Data are means $\pm$ SEM, n = 5 fish.

GPB activity was detected in all four rainbow trout tissues tested: liver, heart, gill and white skeletal muscle (Table 2.5). Enzyme activity was highest in liver, about 3-fold greater than in other tissues in both control (resting) and exercised fish. Exhaustive swimming exercise stimulated significant increases in GPB activity in two organs; activity increased by 54 % in liver and by 38 % in white muscle.

It is well established that PKA phosphorylates GPB in mammalian tissues, resulting in a marked increase in the catalytic activity of the enzyme. To determine whether GPB from trout tissues also responded to PKA, I incubated tissue extracts from both control and exercised trout with the catalytic subunit of rabbit muscle PKA. Fig. 2.5 shows that treatment with PKA stimulated GPB activity in liver, heart and white muscle but, surprisingly, had no effect on the gill enzyme. The fold increase in GPB activity as the result of PKA treatment was 6-7 fold in white skeletal muscle and 2-2.6 fold in liver and heart.

The high fold stimulation of trout white muscle GPB by the catalytic subunit of PKA, led us to further investigate the mechanism of GPB activation in this tissue. Extracts of white skeletal muscle were incubated with Mg.ATP plus known second messengers (cAMP, cGMP, Ca^{2+} + phorbol 12-myristate, 13-acetate) to stimulate the activities of endogenous protein kinases. Incubation with Mg.ATP alone had no effect on enzyme activity indicating the absence of second messenger independent protein kinase activity. The enzyme was strongly stimulated, however, by incubation with Mg.ATP plus cAMP; activity doubled in extracts from control, rested fish and quadrupled in extracts from exercised fish (Table 2.6). Furthermore, an even greater increase in enzyme activity was achieved by incubation with Mg.ATP plus the catalytic subunit of PKA. However, incubation with Mg.ATP plus either cGMP or second messengers of protein kinase C (Ca^{2+} , phorbol 12-myristate, 13-acetate) had no effect on GPB activity in muscle extracts from either control or exercised trout (data not shown).

FIG. 2.5 Effect of added rabbit skeletal muscle PKA catalytic subunit and MgATP on the activity of GPK in organs from control versus exercised (exhaustive swimming) trout

Symbols are as follows: (open) control minus PKA, (diagonal lines) control plus PKA, (crosshatch) exercised minus PKA, (filled) exercised plus PKA. Values are U/g wet weight, means $\pm$ SEM, n = 5 fish.

Table 2.6**Second messenger dependence of glycogen phosphorylase kinase activation in trout white muscle**

Data are means $\pm$ SEM, n = 5 fish. PKA is the catalytic subunit of rabbit muscle protein kinase A.

* Significantly different from the corresponding control value by the Student's t-test, $P < 0.01$.

Condition	Total activity (U/g wet weight)	
	Control	Exhaustive Exercise
EDTA, ATP (control)	0.15 $\pm$ 0.014	0.21 $\pm$ 0.024
Mg.ATP	0.17 $\pm$ 0.050	0.24 $\pm$ 0.040
Mg.ATP + cAMP	0.35 $\pm$ 0.024	1.03 $\pm$ 0.08*
Mg.ATP + PKA	0.99 $\pm$ 0.07	1.58 $\pm$ 0.14*

Table 2.7

Total protein kinase A activity and the amount of active enzyme in rainbow trout liver, skeletal muscle and heart under different experimental conditions

Values are pmol/mg protein, $\pm$ SEM; n = 4 animals in each groups and all assays are done in triplicate. Experimental conditions are: MS-222, anaesthetized with MS-222; E1, vigorous swimming exercise for 1 min; EE, exercised to exhaustion.

Organs	Condition	Total pmol/mg	Active pmol/mg	Percent Active
Liver:	Control	257.6 $\pm$ 37.5	30.9 $\pm$ 3.5	12
	MS-222	232.6 $\pm$ 26.8	35.7 $\pm$ 4.9	15
	E1	252.7 $\pm$ 7.7	46.9 $\pm$ 4.4*	19
	EE	234.8 $\pm$ 15.3	48.7 $\pm$ 4.2*	21
White Muscle:	Control	57.5 $\pm$ 15.4	18.5 $\pm$ 3.1	32
	MS-222	58.3 $\pm$ 1.1	17.5 $\pm$ 3.4	30
	E1	59.2 $\pm$ 1.4	29.3 $\pm$ 3.7*	50
	EE	67.3 $\pm$ 9.9	38.1 $\pm$ 3.2**	57
Heart:	Control	119.8 $\pm$ 13.7	23.6 $\pm$ 1.9	20
	MS-222	118.4 $\pm$ 13.9	24.7 $\pm$ 3.5	21
	E1	134.1 $\pm$ 14.5	22.7 $\pm$ 3.1	17
	EE	143.6 $\pm$ 16.5	34.9 $\pm$ 3.9*	24

* - Significantly different from the corresponding control value, $p < 0.05$,

** $p < 0.01$.

Table 2.8

Total protein kinase A activity and the amount of active enzyme in rainbow trout brain, gill and kidney under different experimental conditions

Details are as in Table 2.7

Organs	Condition	Total	Active	Percent
		pmol/mg	pmol/mg	active
Brain:	Control	64.5 ± 14.2	19.6 ± 3.4	30
	MS-222	62.8 ± 5.4	23.3 ± 2.8	37
	E1	49.3 ± 2.9	26.4 ± 2.7	54
	EE	47.8 ± 4.0	27.9 ± 3.8	58
Gill:	Control	84.4 ± 8.1	31.0 ± 1.3	37
	MS-222	84.7 ± 8.5	26.3 ± 1.1	31
	E1	84.2 ± 7.9	36.2 ± 4.9	43
	EE	95.3 ± 12.4	34.2 ± 5.4	36
Kidney:	Control	69.1 ± 4.5	12.7 ± 1.2	18
	MS-222	97.0 ± 18.4	19.2 ± 4.5	20
	E1	76.5 ± 10.0	13.0 ± 0.7	17
	EE	76.8 ± 8.9	14.1 ± 0.7	18

cAMP DEPENDENT PROTEIN KINASE IN TROUT ORGANS

The above results indicated the primary importance of PKA in controlling GPK, and thereby glycogen phosphorylase, in trout tissues. Therefore, we measured the activities of endogenous PKA in six trout organs (Tables 2.7, 2.8). Comparisons were made between 4 physiological states: resting, anaesthetized, 1 minute of vigorous swimming, and swimming to exhaustion (approx. 10 min). Total PKA activity was highest in liver, 2-4 fold higher than in any other organ. Total PKA activities did not change under different metabolic states in any of the organs examined. However, the amount of active PKA varied as a function of physiological state in liver, white skeletal muscle and heart (Table 2.7), active PKA increasing as a function of exercise in all three organs. In liver the amount of active (minus cAMP) PKA rose from 12 % of the total in controls to 19 % after brief exercise (1 min) and 21 % after exhaustive exercise. In muscle the active enzyme increased from 32 % in controls to 50 % after brief exercise and 57 % after exhaustive exercise. The amount of active PKA in heart showed a significant increase only after exhaustive exercise.

cAMP CONCENTRATIONS IN TROUT TISSUES

Table 2.9 shows the concentrations of cyclic AMP in six organs of rainbow trout. As a result of exhaustive exercise, cAMP levels increased by 50 % in liver and by 70 % in white muscle and brain. Brief exercise had no significant effect on organ cAMP levels. Anaesthesia with MS-222 affected cAMP levels only in brain, levels falling to 42 % of the control values.

DISCUSSION

Phosphorylase b was purified from trout white muscle to near homogeneity as judged by SDS-polyacrylamide gel electrophoresis. The enzyme was

Table 2.9**Levels of cyclic AMP in six organs of rainbow trout under different metabolic conditions**

Values are means $\pm$ SEM; n = 4 fish in each group and all assays are done in triplicate. Other details are as in Table 2.7.

cAMP (pmol/g wet weight)				
	Control	MS-222	E1	EE
Liver	1209 $\pm$ 158	1753 $\pm$ 227	1454 $\pm$ 128	1839 $\pm$ 70*
Muscle	226 $\pm$ 32	233 $\pm$ 32	345 $\pm$ 33	383 $\pm$ 39*
Heart	2395 $\pm$ 457	2441 $\pm$ 302	2654 $\pm$ 360	2276 $\pm$ 223
Brain	2446 $\pm$ 450	1032 $\pm$ 297*	3238 $\pm$ 184	4175 $\pm$ 418**
Gill	4360 $\pm$ 665	2750 $\pm$ 364	5637 $\pm$ 625	3340 $\pm$ 475
Kidney	3279 $\pm$ 860	3456 $\pm$ 176	4166 $\pm$ 740	3608 $\pm$ 217

* - Significantly different from the corresponding value for control fish, $P < 0.05$,

** $P < 0.01$.

purified by successive chromatography on glycogen-ConA, DEAE-Sephacel and 3',5' cAMP-Sepharose 4B columns resulting in a final enzyme preparation with a specific activity of 134 U/mg protein. The purification factor was more than 200 fold. The molecular weight of the enzyme obtained by gel filtration suggests that trout muscle phosphorylase b exists as a dimer in vivo, which is in agreement with the data on phosphorylase from most animal sources (Dombradi 1981). However, when the elution buffer contained 1 mM AMP, the molecular weight was estimated to be 335,000 indicating that the enzyme had become a tetramer. This suggests that the presence of AMP promotes an aggregated tetrameric form of the enzyme, as has also been reported for the mammalian enzyme (Kent et al. 1958).

In the crude extract, phosphorylase showed activity (10 % of the total) without the addition of AMP (phosphorylase a), but the AMP-independent activity decreased during the purification procedure, and the purified phosphorylase showed very weak activity in the absence of AMP (<2 % of the total). My results concerning the low activity of glycogen phosphorylase a in resting trout white muscle are in good agreement with those reported previously (Morata et al. 1982; Storey 1991).

The kinetic properties of purified phosphorylase b from trout white muscle show some similarities to those of skeletal muscle phosphorylase from other vertebrate sources (Madsen 1986). K_m for glycogen (Table 2.2 and 2.3) was lower than described for the Crucian carp enzyme (Schmidt and Wegner 1990) at pH 7.2, but at pH 6.6, as well as at suboptimal concentrations of phosphate at both pH 7.2 and 6.6, the K_m values were in agreement with those described for carp and mullet (Bonamusa and Banaate 1990; Schmidt and Wegner 1990). The K_a for AMP was very similar to those reported for mullet, shark, lamprey, hagfish, dogfish (Yonezawa and Hori 1977) and rabbit phosphorylase b (Cohen et al. 1971). Overall, then, these results suggest that the catalytic behavior in trout muscle phosphorylase b would not differ very much from that reported to date for phosphorylase from other vertebrate sources.

Analysis of the effect of exercise on phosphorylase activity *in vivo* in trout white muscle showed that the percent a form remained low even under conditions of exhaustive burst exercise; white muscle phosphorylase a content rose from control values of $3.6 \pm 0.8\%$ to only $16.1 \pm 2.5\%$ of the total activity in exercise muscle (Storey 1991), although this type of exercise virtually depleted all muscle glycogen reserves (Parkhouse et al. 1987). With such a low percentage of the a form in working muscle, it might be possible that phosphorylase b activity could also be contributing to muscle glycogenolysis. The K_m of phosphorylase b for phosphate is within the physiological range of phosphate *in vivo* (Table 2.2) and the rise in P_i concentration from about $20 \mu\text{mol/g}$ wet weight in resting muscle to $50 \mu\text{mol/g}$ wet weight after exhaustive exercise (as the result of creatine phosphate hydrolysis) (Parkhouse et al. 1987) would potentiate enzyme function by increasing substrate availability. The enzyme also retained greater than 70% of its maximal activity at pH 6.6 (Fig. 2.2) and so could maintain relatively high activity as pH falls in working muscle (Milligan and Wood 1986b; Parkhouse et al. 1987). Glucose and ATP inhibited phosphorylase b (Table 2.4), but glucose was a less effective inhibitor (I_{50} increases) as pH decreased and ATP concentrations dropped sharply during burst work in trout muscle (Parkhouse et al. 1987; Storey 1991). Both compounds would, therefore, be less inhibitory under the cellular conditions in working muscle. However, the activity of phosphorylase b is completely dependent on ligand activation by AMP and this is perhaps the governing factor in determining whether the activity of phosphorylase b could contribute significantly to glycogenolysis in working muscle. In the muscle at rest AMP levels are very low ($0.02 \mu\text{mol/g}$ wet weight) (Parkhouse et al. 1987) and well below the K_a values of 0.14 and 0.07 mM at optimal and suboptimal substrate concentrations, respectively (Table 2.2 and 2.3). AMP levels rise sharply, however, during exercise to 0.07 mM during burst swimming and to as high as 0.15 mM when the fish are near exhaustion (Parkhouse et al. 1987). Obviously, then, these amounts are sufficient to

allow a substantial rate of phosphorylase b activity in working muscle, particularly under conditions where P_i concentration is also rising and ATP levels are falling.

The increase in AMP in working muscle reflects only a portion of 2-4.5 $\mu\text{mol/g}$ wet weight decrease in muscle ATP levels during burst exercise (Parkhouse et al 1987; Storey 1991). This is because AMP is further catabolized by the enzyme AMP deaminase to form IMP plus NH_4^+ , a strategy that allows cellular energy charge to remain high at the expense of total adenylate levels. More than 95% of the decrease in white muscle ATP content during burst swimming in trout is accounted for by the increase in IMP levels. The potential for positive regulatory control of phosphorylase b by IMP seemed high, therefore. However, tests found that this was not the case; trout muscle phosphorylase b was not activated by the addition of IMP in amounts up to 15 mM (data not shown). This is in agreement with a previous report by Driedzic and Hochachka (1976) which showed that IMP activated phosphorylase a, but had no effect on the b form of phosphorylase from carp muscle. However, in mammals IMP is thought to be important in the activation of phosphorylase b in working muscle (Griffiths 1981).

Phosphorylase b activity was dependent on pH and temperature (Fig. 2.2 and 2.3). The sharp optimum pH suggested the strong influence that changing intracellular pH in trout muscle could have on the rate of glycogenolysis. Rainbow trout organs possess both GPK and PKA activities and these appear to provide a functional cascade mechanism for the activation of glycogen phosphorylase. Activities of both enzymes were highest in liver and were 3- and 4-fold higher than the corresponding activities in white skeletal muscle. This is opposite to the pattern of glycogen phosphorylase activities in these organs, phosphorylase activity being 7-fold higher in skeletal muscle than in liver of resting trout (Storey 1991). These relationships may suggest that the enzyme phosphorylation cascade system for the activation of glycogen phosphorylase is qualitatively much more important for the regulation of glycogenolysis in liver than it

is in muscle. This may reflect a greater dependence of glycogenolysis on hormonal controls in liver whereas muscle glycogenolysis is also typically linked to Ca^{2+} -stimulation during contraction, calmodulin being one of the four subunits of GPK (Cohen 1980). Surprisingly, however, we were unable to find an activation of white muscle GPK *in vitro* when extracts were incubated with Mg.ATP in the presence of Ca^{2+} . Studies of dogfish skeletal muscle GPK, however, found just the opposite; the elasmobranch enzyme could not be phosphorylated by mammalian PKA or as a result of incubation with various cyclic nucleotides but appeared to be regulated only by changes in Ca^{2+} (Pocinwong et al. 1981).

In a previous study, the effects of exhaustive swimming exercise on glycogen phosphorylase activities in trout organs were examined (Storey 1991). Liver showed an overall 5-fold increase in the activity of phosphorylase α during exercise and gill about 2-fold. The increase in liver phosphorylase is readily explained by the activation of both liver GPK and PKA, shown in the present study, stimulated by elevated cAMP levels. This supports an activation of glycogenolysis in liver that increases blood glucose supply for both working muscles and other internal organs (e.g. gill, heart) whose work load is increased during high speed swimming. Activation of gill phosphorylase could not be explained, however, by the present data since no change in either GPK or PKA activity was found in gill from exercised fish, and no increase in gill cAMP levels. Furthermore, mammalian PKA did not activate gill GPK as it did the enzyme from other trout organs. This result seemed surprising in light of the fact that various exercise-induced adjustments of gill function are mediated via catecholamines with cAMP as the second messenger (Wood and Perry 1985).

Exercise clearly activated all components of the phosphorylase cascade in trout white muscle. Exhaustive exercise resulted in an increase in the percent phosphorylase α from 3.6 to 16 % (Storey 1991), GPK activity rose by 38 %, active PKA increased 2-fold, and muscle cAMP concentration increased by 1.7-fold. Furthermore, GPK

clearly phosphorylated trout phosphorylase and GPK was, in turn, phosphorylated and activated *in vitro* when incubated with mammalian PKA catalytic subunit. Thus, the cascade is intact in this teleost fish. However, as was discussed above, the amount of phosphorylase α in working muscle (only 16% of total activity) seems low. One reason for this may be the capacity of the phosphorylation cascade which may be limiting in determining the amount of phosphorylase β that can be converted to phosphorylase α during burst swimming exercise. Thus, not only are the activities of GPK and PKA lower both in absolute terms and relative to phosphorylase, as compared to the situation in liver, but neither enzyme was maximally activated during exercise. Although GPK activity increased by 38 % in exercised muscle, the 6-7 fold increase in activity that was stimulated by *in vitro* incubation with the catalytic subunit of mammalian PKA (Fig. 2.5) indicated that only a fraction of the total potential enzyme activity was expressed even after strenuous exercise. Endogenous PKA activity also rose to only about 50 % in the active (minus cAMP) form in exercised muscle. In combination, then, these two enzymes may be responsible for the low percent phosphorylase α (16 %) measured in working trout muscle during burst swimming (Storey 1991).

Exhaustive exercise resulted in significantly increased levels of cyclic AMP in liver, muscle and brain. Changes in the levels of this second messenger are often the result of catecholamine stimulation and indeed, epinephrine and norepinephrine titres are known to increase many-fold in trout plasma during exhaustive exercise (Wood and Perry 1985; Butler et al. 1986; Tang and Boutilier 1988). During steady-state swimming, however, plasma catecholamines did not change (Butler et al. 1986) and this correlates with the present results that found no significant change in organ cAMP levels in fish subjected to only 1 min of burst swimming.

Overall, then it seems that two modes of phosphorylase regulation may be important in working fish muscle and liver. The β to α conversion is probably of prime importance in activating glycogenolysis in trout muscle and liver, as it is in most other

systems. However, whereas phosphorylase b would be virtually inactive under the cellular conditions present in resting muscle, the changes that occur in working muscle (AMP and P_i rise, ATP decreases, pH drops) could permit a substantial contribution to overall glycogenolysis to be made by AMP-activation of phosphorylase b.

CHAPTER 3
CHARACTERIZATION OF
 α -GLUCOSIDASE FROM
RAINBOW TROUT LIVER

INTRODUCTION

Glycogenolysis can occur in animal tissues by both phosphorolytic (producing glucose-1-phosphate) and glucosidic (producing glucose) pathways. The former, involving glycogen phosphorylase, is extremely well known and well studied, particularly because of its role in cellular energy metabolism and its sensitivity to hormonal regulation. Glycogen can also be hydrolyzed by enzymes that cleave glucose units off the polymer; these include α -amylase, amylo-1,6-glucosidase, acid α -glucosidase, and neutral α -glucosidase. The acid α -glucosidase (sometimes called gamma-amylase), with a pH optimum of 4-5, is found in the lysosomes and is believed to function to hydrolyze any glycogen or oligosaccharides that are trapped in lysosomes as a result of cellular autophagy (Hers and van Hoof 1973; Jeffrey et al. 1970). Its absence leads to the storage of a huge amount of glycogen in the lysosomes that is inaccessible to phosphorolytic glycogen metabolism in the cytoplasm; medically, this is known as type II glycogenosis (Pompe's disease), an inheritable trait that is fatal in infancy (Brown et al. 1970). The neutral α -glucosidase, with a pH optimum of 6-7, seems to be associated with the microsomes where it appears to be involved in trimming sugar residues in newly synthesized glycoproteins and/or cleaving saccharides into monomers for movement across membranes of the endoplasmic reticulum (Castilla et al. 1980; Imai et al. 1989).

Murat (1976) suggested that acid α -glucosidase may have an important role in regulating glycogenolysis in fish for he was unable to detect activity of glycogen phosphorylase in the liver of the carp, *Cyprinus carpio*, and liver α -glucosidase activity increased in response to hormones that stimulated glycogenolysis. Later work by Picukans and Umminger (1979), however, detected phosphorylase in carp liver but also high α -glucosidase activities. These were fully half of the values for phosphorylase, suggesting that α -glucosidase must play a major role in the liver glycogen metabolism of this species. The study described in this chapter examines the roles of acid and

neutral α -glucosidases in the liver of the rainbow trout (*Oncorhynchus mykiss*) assessing the enzyme forms present and their kinetic and physical properties to determine the function of α -glucosidases in liver glycogen metabolism.

MATERIALS AND METHODS

CHEMICALS AND ANIMALS

All chemicals were obtained from Boehringer Mannheim Corp. (Montreal, PQ) or Sigma Chemical Co., St. Louis, MO. Rainbow trout (*O. mykiss*) were kindly provided by Dr. T.W. Moon, University of Ottawa and were held in aerated circulating water at 12°C with a photoperiod 12L/12D until used. Fish were fed ad libitum on commercial trout pellets. Fish were exercised and treated as described previously (see Chapter 2; Mehrani and Storey 1993b).

TISSUE PREPARATION AND ENZYME EXTRACTION

Samples of frozen tissue were quickly weighed and then homogenized (1:4 w/v) in ice-cold buffer containing 50 mM imidazole-HCl (pH 7.0), 100 mM NaF, 2.5 mM EDTA, 2.5 mM EGTA, 30 mM β -mercaptoethanol, and 0.1 mM PMSF using 2 x 10 sec bursts at full speed of a Tekmar tissue homogenizer. Homogenates were centrifuged at 20,000 g for 15 min at 4°C. Supernatants was removed and layered onto small columns (5 ml, 0.5 ml supernatant per column) of Sephadex G-25 equilibrated with homogenization buffer. The columns were centrifuged in an IEC benchtop centrifuge for 1 min and the enzyme-containing filtrate was collected and stored at 4°C for enzyme assay.

For subcellular localization of enzyme activity, fresh hepatocyte subcellular fractionation was performed following the procedure of de Duve et al. (1955) with slight modification. Fish were killed and livers were immediately removed and placed in cold buffer (0.25 M sucrose, 10 mM imidazole, 5 mM EDTA, pH 7.0). The liver

was then blotted dry, trimmed of excess fat, weighed and minced in 4 vol of fresh cold buffer. The preparation was then homogenized in a hand held smooth-walled glass tube fitted with a Teflon pestle. The resulting crude homogenate was sedimented at 600 g for 10 min. The pellet (cell debris and nucleus) was resuspended in cold buffer and sedimented as before. Supernatants were combined and recentrifuged at 8,000 g for 10 min. The resulting pellet (mitochondrial fraction) was suspended in cold buffer and kept on ice for enzyme assay. The supernatant was centrifuged again, this time at 16,000 g for 30 min. The resulting pellet (lysosomal fraction) was resuspended in cold buffer and the supernatant (cytosolic fraction) was kept at 4°C for enzyme assay.

ENZYME AND PROTEIN ASSAYS

Both the acid and neutral activities of α -glucosidase were measured by two different methods. The first method used a modification of the fluorometric method of Salafsky and Nadler (1973) that is based on the hydrolysis of 4-methyl-umbelliferyl- α -glucoside (MU α G). Before assay, samples of crude homogenate were brought to a final dilution of 1/50 with assay buffers but purified enzyme was used without dilution. Sample (0.05 ml) was then added to reaction mixtures containing 0.15 ml of either 50 mM potassium acetate buffer (pH 4.0) or 1 M phosphate buffer (pH 7.0) and 0.1 ml of 2 mM MU α G (obtained from Sigma). In some cases, assays were run in the presence or absence of 40 mM turanose which inhibits acid α -glucosidase activity. The reaction mixtures were incubated at 21°C for 20 min. Reactions were stopped by the addition of 2 ml of ice-cold 1 M glycine-NaOH buffer pH 10.5 and fluorescence was measured with an Aminco-Bowman spectrofluorometer, using an excitation wavelength of 366 nm and an emission wavelength of 450 nm. Standards of 50 nM MU α G in glycine-NaOH buffer were used. One unit of enzyme activity is defined as the amount of enzyme that hydrolyzes 1 nmol of MU α G per minute at 21°C. In the second method glycogen and maltose were used as substrates and the glucose produced was determined

by the hexokinase/glucose-6-P dehydrogenase coupled enzyme assay (Jeffrey et al. 1970). The reaction mixture contained 50 mM maltose or 5 mg/ml oyster glycogen in 50 mM potassium acetate (pH 4.0) or 50 mM phosphate buffer (pH 7.0) plus a suitable amount of enzyme in a final total volume of 0.4 ml. After incubating for 30 min at 21°C, the reaction was stopped by the addition of 1 ml of 200 mM Tris-HCl buffer (pH 8.0) and then glucose output was quantified.

Glycogen phosphorylase activity was assayed as described by Storey (1991). Protein was determined as described in Chapter 2.

ISOELECTROFOCUSING

Isoelectric focusing to analyze α -glucosidase isoenzymes was performed at 4°C according to Vesterberg (1971). A gradient of pH 3.5-10 LKB ampholines was stabilized by a 0-30 % w/v linear sucrose gradient. One ml of 10-fold diluted crude extract was placed in the middle of the 110 ml column and the column was allowed to develop overnight at 300 V (constant voltage). Fractions were then collected and assayed for enzyme activity.

DE-52 COLUMN CHROMATOGRAPHY

Crude extract, after passage through Sephadex G-25, was applied to a column (2.5 x 20 cm) of DE-52 Sephadex (defined) equilibrated in potassium phosphate buffer (30 mM P_i , 2 mM EDTA pH 6.8). The column was washed with 30 ml of P_i buffer and then isozymes were eluted with a linear gradient of KCl (0-1 M, 100 ml). Fractions of 0.8 ml were collected and assayed; peak fractions containing α -glucosidase activity were pooled and dialyzed overnight against dialysis buffer (10 mM imidazole pH 7.0, 2 % v/v glycerol, 15 mM β -mercaptoethanol) before use for kinetic analysis or molecular weight determination.

GEL FILTRATION

Molecular weights of isoenzymes were determined by gel filtration on Sephacryl S-300 (Andrews 1964). The column (2.5 x 75 cm) was equilibrated and run in 50 mM phosphate buffer containing 10 % v/v glycerol, pH 7.0. The column was developed at a flow rate of 5 ml/hr at 7°C. Calibration standards, with molecular weights in brackets, were cytochrome c (12,384), hemoglobin (64,500), lactate dehydrogenase (140,000), pyruvate kinase (257,000), glutamate dehydrogenase (350,000) and glycogen phosphorylase a (370,000).

RESULTS

ENZYME ACTIVITIES AND ISOZYMIC FORMS

Table 3.1 shows the activities of α -glucosidase in trout liver. Activity was detected at both acidic and neutral pH values in crude extracts (G-25 filtered) and was substantially higher at pH 7.0 than at pH 4.0. Exhaustive swimming exercise led to a 60 % increase in measurable activity at pH 4.0 but did not affect the pH 7.0 activity. For comparison, the activities of liver glycogen phosphorylase a and b in crude extracts are also presented, the activity of active phosphorylase a increasing by 4.4-fold in liver of fish exercised to exhaustion. Phosphorylase activity in trout liver was substantially higher than total α -glucosidase activities; acid + neutral α -glucosidase activities summed to only 4-5 % of the total (a + b) phosphorylase activity.

DE-52 ion exchange chromatography revealed the presence of 3 enzyme forms of α -glucosidase in trout liver (Fig. 3.1 and 3.2). Two acidic isozymes were identified with activity at pH 4.0 (I) and (II) (detected in assays without turanose) and a neutral enzyme showed activity at pH 7.0 (assayed in the presence of 40 mM turanose). The effects of turanose further characterized the enzymes. Activities of the acid isozymes I and II were strongly inhibited by 40 mM turanose when assayed at either pH 4.0 or pH 7.0, as has been reported previously for the acidic enzyme from other sources (Jeffrey

TABLE 3.1

Comparison of activities of glycogen phosphorylase and α -glucosidase in trout liver (crude preparation)

Enzyme	Control		Exercised	
	mU/mg	mU/gww	mU/mg	mU/gww
Phosphorylase:				
a	4.40 $\pm$ 0.20	324 $\pm$ 15	19.3 $\pm$ 2.1*	427 $\pm$ 155**
b	47.6 $\pm$ 2.50	3503 $\pm$ 184	52.6 $\pm$ 3.2	3888 $\pm$ 235
α -Glucosidase:				
pH 4	0.56 $\pm$ 0.09	41.4 $\pm$ 6.6	0.91 $\pm$ 0.11**	67.3 $\pm$ 8.1*
pH 7	1.87 $\pm$ 0.12	137 $\pm$ 8.8	1.91 $\pm$ 0.14	141 $\pm$ 10

Data are means $\pm$ SEM, n=5 with activities for both enzymes expressed in milliunits (nmol substrate utilized per min) per mg protein or per gram wet weight (gww), assayed at 21°C.

* - Significantly different from the corresponding control value by the Student's t-test, $P < 0.05$; ** $P < 0.001$.

Fig. 3.1 Elution profile of control trout liver acidic and neutral α -glucosidase activities from DE-52 ion exchange chromatography

Enzymes were eluted with a linear gradient of KCl (0-1 M) in phosphate buffer pH 6.8 and were then assayed using MU α G as the substrate at pH 4.0 (closed circles) or pH 7.0 (open circles). Activities are relative to enzyme activity in the peak fraction for the neutral enzyme.

Fig. 3.2 Elution profile of exercised trout liver acidic and neutral α -glucosidase activities from DE52 ion exchange chromatography

Enzymes were eluted with a linear gradient of KCl (0-1 M) in phosphate buffer pH 6.8 and were then assayed using MU α G as the substrate at pH 4.0 (closed circles) or pH 7.0 (open circles). Activities are relative to enzyme activity in the peak fraction for the neutral enzyme.

et al 1970; Lejeune et al. 1963). However, turanose had no effect on the neutral enzyme, but activity of this enzyme was nearly undetectable when assays were performed at pH 4.0. Comparison of the peak areas in Fig. 3.1 and 3.2 again shows higher amounts of the neutral, versus acidic, enzymes in liver extracts in agreement with Table 3.1. Enzyme activity in each of pooled peak (in U/mg protein) is also shown in Table 3.2. It is clear from these data that the increase in acidic α -glucosidase activity seen in liver of exercised trout was due to changes in isozyme I activity, which was enriched nearly 2-fold in the pooled peak fractions from exercised trout, compared with controls. Exercise did not affect the activities of the acid type II enzyme or the neutral α -glucosidase. Pooled peak fractions of the three partially purified isozymes from DE-52 chromatography were used for further kinetic studies.

ISOELECTROFOCUSING

Isoelectric focusing of G-25 filtered crude extracts also showed three separate peaks of α -glucosidase activity in trout liver (Table 3.3). The acidic isozymes I and II had pI values of about 4.9 and 5.0, respectively, whereas the neutral enzyme had a higher isoelectric point (pH 5.6-5.7). Exercise did not change the pI value of any of the enzymes.

EFFECT OF pH ON ENZYME ACTIVITY

The effects of pH on the activity of each enzyme are shown in Fig. 3.3. The two acidic isozymes showed pH optima at 21°C of 4.5 for isozyme I and 5.0 for isozyme II whereas the neutral enzyme showed maximal activity at pH 6.5. Isozymes I and II both showed sharp optima and retained less than 10 % of their maximal activity at pH 7. The pH profile of neutral α -glucosidase was broader with near-maximal activity over the pH 6 to 7 range and 20-30 % of maximal activity still remaining at pH values as low as 5 and as high as 9.

TABLE 3.2

Activities of α -glucosidase in trout liver separated on by DE-52 column chromatography

	Control	Exercised
	-----	-----
Enzyme	mU/mg protein	mU/mg protein
Acid isozyme I (pH 4)	81 $\pm$ 7.8	147 $\pm$ 10.2*
Acid isozyme II (pH 4)	109 $\pm$ 9.9	110 $\pm$ 11.5
Neutral enzyme (pH 7)	66 $\pm$ 4.1	64 $\pm$ 5.2

Activities of α -glucosidase enzymes separated by DE-52 chromatography were assayed in pooled peak fractions after dialysis as described in Materials and Methods. Data are means $\pm$ SEM, n=5 with activity of the enzymes expressed in milliunits (nmol substrate utilized per min) per mg protein, assayed at 21°C.

* Significantly different from the corresponding control values, $p < 0.001$.

Table 3.3
Isoelectric focusing of trout liver α -glucosidases

Enzyme form	Isoelectric Point	
	Control	Exercised
Acid type I	4.97 $\pm$ 0.07	5.03 $\pm$ 0.06
Acid type II	4.89 $\pm$ 0.05	4.91 $\pm$ 0.08
Neutral	5.62 $\pm$ 0.02	5.72 $\pm$ 0.07

Aliquots of enzyme (10 μ l) from each fraction were assayed at both pH 7 and pH 4 using MU α G as the substrate. Data are means $\pm$ SEM, n=3 isoelectrofocusing runs.

Fig. 3.3 Effect of pH on the activity of α -glucosidases from trout liver

Assays were carried out at 21 °C in 50 mM phosphate buffer with standard assay conditions, using MU α G as the substrate, and the partially purified isozymes from DE-52 chromatography. Enzymes are: acid type I (triangles), acid type II (closed circles), and neutral α -glucosidase (open circles).

KINETIC PROPERTIES OF α -GLUCOSIDASE ISOZYMES FROM TROUT LIVER

Table 3.4 shows affinities of the liver isozymes for three different substrates. The acid isozymes utilized all three carbohydrates (glycogen, maltose, MU α G) as substrates but the neutral enzyme did not hydrolyze glycogen. Affinities of types I and II acid α -glucosidase for both maltose and MU α G were greater (K_m values lower) than the corresponding affinities displayed by neutral α -glucosidase. Isozyme I from liver of exercised fish showed a significant increase in affinity (decrease in K_m) for both glycogen and maltose in comparison with the enzyme from control, resting fish but substrate affinities of the other enzymes were unaffected by the metabolic state of liver.

Jeffrey et al. (1970) found that acid α -glucosidase from the lysosomal fraction of rat liver was subject to activation by sodium chloride. The sensitivity of trout liver acid α -glucosidase to anions, cations, and overall ionic strength was tested. The chloride and acetate salts of Na⁺, K⁺, NH₄⁺, Ca²⁺ and Mg²⁺ in equimolar cation concentrations, were all nearly equally effective in the activation of the enzymes. Fig. 3.4 shows the effect of increasing concentrations of potassium chloride on the hydrolysis of maltose and glycogen by isozymes I and II; other cations showed almost the same profile as for KCl and all had inhibitory effects on enzyme activity in higher concentrations (> 100 mM). Activity of isozyme II was more strongly stimulated by salt than was isozyme I; in the presence of 100 mM KCl isozyme II activity was increased by 29-35 % whereas activity of isozyme I increased only 18-22 %. In both cases, KCl caused a greater stimulation of glycogen- compared with maltose-hydrolyzing activity.

MOLECULAR WEIGHT

The enzymes separated by DE-52 chromatography were applied to S-300 columns to determine molecular weights. The three enzymes showed distinctly different

TABLE 3.4

K_m values for different substrates of α -glucosidases from trout liver

Enzyme	Glycogen (mg/ml)	Maltose (mM)	MU α G (μ M)
Acid type I:			
Control	7.92 $\pm$ 0.29	2.33 $\pm$ 0.27	37.4 $\pm$ 6.8
Exercised	4.20 $\pm$ 0.39**	1.29 $\pm$ 0.15*	23.2 $\pm$ 8.1
Acid type II:			
Control	4.31 $\pm$ 0.37	2.03 $\pm$ 0.23	38.2 $\pm$ 9.7
Exercised	4.11 $\pm$ 0.24	1.86 $\pm$ 0.21	32.1 $\pm$ 5.4
Neutral:			
Control	ND	3.40 $\pm$ 0.27	108 $\pm$ 9.5
Exercised	ND	3.20 $\pm$ 0.53	116 $\pm$ 8.6

Values are means $\pm$ SEM, n = 5, assayed at 21°C.

* Significantly different from the corresponding control value P < 0.01;

** P < 0.001. ND = No activity detected for the neutral enzyme assayed with glycogen.

Fig. 3.4 Effect of KCl on acid α -glucosidase activity

Standard assay conditions were used except that potassium acetate buffer (pH 4.0) was 0.01 M. Results are expressed as the percentage stimulation of enzyme activity compared with activity in the absence of KCl. Symbols are: acid isozyme I (triangles) and isozyme II (circles) with closed symbols showing activity with maltose and open symbols activity with glycogen as substrate.

molecular masses of about 180, 130, and 365 kD, for acid isozymes I and II and neutral α -glucosidase, respectively (Table 3.5). There were no differences in the molecular weights of the isozymes from liver of control versus exercised trout.

SUBCELLULAR DISTRIBUTION

Subcellular fractionation via differential centrifugation was used to localize the α -glucosidase activities (Fig. 3.5). About 75 % of the acidic α -glucosidase activity was found in the lysosomal fraction (16,000 g pellet) fraction with additional small amounts in the mitochondrial and cytosolic fractions. The neutral enzyme activity, however, was present in nearly equal amounts in the lysosomal and microsomal/cytosolic (16,000 g supernatant) fractions with smaller amounts in both mitochondrial and nuclear fractions.

DISCUSSION

Three forms of α -glucosidase occur in trout liver. The two forms with acidic pH optima are localized primarily in the lysosomes whereas the neutral enzyme has a broader distribution that includes a large microsomal component. These subcellular distributions agree with previous reports on the location of the acid and neutral α -glucosidases in liver of other species (Jeffrey et al. 1970a; Imai et al. 1989; Lejeune et al. 1963; Hers 1963; Hers 1972; Castilla et al. 1978). In trout liver the neutral α -glucosidase activity was 2-3 fold greater than that of the total acid α -glucosidase.

The two acidic forms of α -glucosidase were readily separable by DE-52 ion exchange chromatography, isoelectrofocusing, and gel filtration. They differed slightly in their pH optima (pH 4.5 for type I, pH 5.0 for type II) but had generally similar affinities for the three substrates tested, glycogen, maltose and MU α G.

Table 3.5

Estimated molecular weight of trout liver α -glucosidases determined by gel filtration

Condition	Enzyme form		
	Acid type I	Acid type II	Neutral
Control	181,000 $\pm$ 2,000	130,000 $\pm$ 1,500	365,000 $\pm$ 3,000
Exercised	178,000 $\pm$ 2,500	129,000 $\pm$ 2,000	360,000 $\pm$ 4,000

Data are means $\pm$ SEM of at least three separate determinations.

Fig. 3.5 Distribution of α -glucosidase in subcellular fractions of control trout liver

Enzyme activity is expressed as percentages relative to activity of the neutral enzyme in the crude homogenate. Subcellular fractions are (W) whole crude homogenate, (N) nuclear and cell debris, (M) mitochondrial, (L) lysosomal, and (C) cytosolic supernatant for the enzyme assayed at pH 7.0 (filled bars) and pH 4.0 (open bars) with MU α G as the substrate. Data are means of samples from 2 fish.

Affinities for glycogen and maltose as substrates are in good agreement with those reported for the mammalian enzyme (Brown et al. 1970; Jeffrey et al. 1970b; Palmer 1971). The ability to catabolize both polysaccharides and disaccharides is consistent with a physiological role in the lysosomal degradation of carbohydrates as has been proposed in mammals (Hers et al. 1973). The two isozymes had different molecular weights (180 kD for type I and 130 kD for type II), compared with a value of 114 kD for the rat liver enzyme (Jeffrey et al. 1970a). Both enzymes were also activated by KCl and other salts, with slightly greater activation seen with glycogen as the substrate, but the percent activation by cations observed for fish acid α -glucosidase isoenzymes was lower than that reported for mammalian enzymes (Jeffrey et al. 1970a; Palmer 1971).

The two acid α -glucosidases differed distinctly in their responses to physiological stress. The activity and properties of type I α -glucosidase were clearly modified as a response to exhaustive muscular exercise by the fish whereas type II enzyme activity was unaffected by this stress (Table 3.1, 3.2 and 3.4; Fig. 3.1 and 3.2). Other authors have shown differences in the amounts of liver acid α -glucosidase between species or between individuals with or without the genetic defect that causes type II glycogenosis (Brown et al. 1970; Castilla et al. 1980; Murat 1976; Picukans and Umminger 1979). However, to my knowledge, this is the first demonstration of a change in the maximal activity and kinetic properties of α -glucosidase as a result of a change in the physiological state of individuals - rest versus exercise. In response to exhaustive swimming exercise, the maximal activity of trout liver acid α -glucosidase I increased by nearly 2-fold and the K_m values for two physiological substrates, glycogen and maltose, each decreased by about 50 %. Both of these changes should support increased lysosomal glycogenolysis in liver during exercise and this suggests that the glucosidic degradation of glycogen in lysosomes may contribute, to some extent, to total liver glucose production and output during exercise. In comparison to

the phosphorolytic breakdown of glycogen in liver, the net contribution of glucosidic degradation may be low since cytosolic glycogen phosphorylase α activity was 8-fold higher than total acid α -glucosidase activity in liver of resting trout and phosphorylase α activity rose 4.4-fold during exercise (Table 3.1). Nonetheless, the coordinated activation in liver of both lysosomal α -glucosidase type I and cytosolic glycogen phosphorylase in response to the fuel demands of exercising muscle suggests that both enzymes have a role to play in hepatic glucose output and that the activities of both may respond to the same extracellular signal(s) during exercise. The most likely signal, perhaps, is catecholamines; plasma levels of both epinephrine and norepinephrine are elevated during exhaustive swimming in trout (Butler et al. 1986; Tang and Boutilier 1988) and injections of these stimulate hyperglycemia in trout (Wright et al. 1989). Plasma glucose is elevated during burst swimming (Ristori and Laurent 1985; Milligan and Wood 1986) and liver glycogenolysis is activated (Storey 1991; Ristori and Laurent 1985). To date, hormone-mediated increases in glycogenolysis have been linked to the activation of glycogen phosphorylase in trout liver (Storey 1991), as typically occurs in vertebrate species (Hems and Whitton 1980) but the present data indicate that an activation of lysosomal α -glucosidase type I in liver may also contribute to glucose output during exercise.

Furthermore, if acid α -glucosidase type I is indeed a regulatory enzyme that is responsive to external signals, then it may be functional in the same role as glycogen phosphorylase in some species. This may explain the wide, species-specific variation in the proportions of α -glucosidase and phosphorylase that have been reported in different fish species. Murat (1976) reported that no glycogen phosphorylase activity could be detected in carp *C. carpio* liver and proposed that α -glucosidase fulfilled the glycogenolytic role. However, a re-evaluation of this by Picukans and Umminger (1979), using a different population of carp, found a liver phosphorylase α to acid α -glucosidase activity ratio of 1.5:1. The ratio in goldfish *Carassius auratus* liver is 5.6:1

(Picukans and Umminger 1979) whereas the present values for trout *O. mykiss* liver were 8:1 in control and 21:1 in exercised fish. Such variation in the phosphorylase: α -glucosidase ratio suggests specific roles for α -glucosidase in liver, perhaps adapted to suit the different dietary and metabolic demands of different fish species.

Neutral α -glucosidase from trout liver is a distinctly different enzyme. It clearly differs from the acid enzyme forms in its subcellular distribution (a major portion is in the microsomes), physical properties (pH optimum, molecular weight, isoelectric point), and substrate affinity and specificity. Neutral α -glucosidase hydrolyzed MU α G and maltose but did not produce glucose from glycogen. Furthermore, HPLC analysis of the reaction mixture before and after incubation of neutral α -glucosidase with glycogen showed no production of other sugars or oligosaccharides from the polymer (data not shown). These data agree with previous reports on neutral α -glucosidase from mammals (Castilla et al. 1980; Imai et al. 1989; Burns and Touster 1982; Hettkamp et al. 1984). The molecular weight of the trout enzyme was 360 kD; values for other species have been reported variously as 262-288 kD, 320-350 kD and 417 kD (Imai et al. 1989; Burns and Touster 1982; Hettkamp et al. 1984). Different roles have been proposed for the neutral enzyme including a partnership with α -amylase for the production of glucose from cytosolic glycogen, a role in the transfer of carbohydrate moieties in the endoplasmic reticulum, and a function in the structuring of sugar-containing macromolecules (Castilla et al. 1980; Imai et al. 1989; Lejeune et al. 1963).

PART 2

CHAPTER 4

ANOXIA SURVIVAL AND THE CONTROL OF GLYCOGENOLYSIS IN ORGANS OF THE FRESHWATER TURTLE *TRACHEMYS SCRIPTA*

INTRODUCTION

Various species of freshwater turtles have a remarkable ability to live for extended periods of time without oxygen (Belkin, 1963). For example, members of the *Chrysemys* and *Trachemys* (formerly *Pseudemys*) genera can endure 1-4 months submerged in anoxic water at 3°C, an ability that supports winter hibernation under water (Herbert and Jackson, 1985; Ultsch, 1985). Key metabolic adaptations support anoxia survival including the ability to suppress anoxic metabolic rate in a coordinated manner to a level only 10-20% of the resting normoxic rate, the maintenance of large reserves of fermentable fuels in organs, and a capacity to deal effectively with the acid load associated with ATP production by anaerobic glycolysis alone (Clark and Miller, 1973; Herbert and Jackson, 1985; Jackson, 1968; Sick et al. 1993; Storey and Storey, 1990).

The primary fuel supporting anaerobic metabolism in turtles is glycogen; the greatest reserve is in the liver (Clark and Miller, 1973; Daw et al. 1967). During anoxia, hepatic glycogenolysis leads to an increase in blood glucose levels that supplies fuel to other organs, particularly brain (Penney, 1974). The control of glycogenolysis in energy metabolism is the function of glycogen phosphorylase, which itself is regulated by a complex cascade mechanism involving a variety of protein kinases and phosphatases. Glycogen phosphorylase is converted from an inactive (dephosphorylated) b to an active (phosphorylated) a form by the action of phosphorylase b kinase which is, in turn, activated by phosphorylation by cyclic AMP-dependent protein kinase or by calcium (Cohen, 1978; Krebs and Fischer, 1956; Newgard et al. 1989; Ozawa, 1973; Soldering and Park, 1974). The regulation of glycogen phosphorylase activity also includes ligand-induced conformational changes (Krebs, 1981; Madsen, 1986). The molecular mechanisms involved in controlling glycogen metabolism have been extensively characterized in mammalian systems (Cohen, 1978; Carleson et al. 1979; Cohen, 1980; Hems and Whitton, 1980), and are

largely assumed to apply to lower vertebrates although specific information about the various enzymes involved is incomplete.

Glycogen may also be catabolized in animal cells by enzymes that cleave glucose (glucosidic pathway), rather than glucose-1-phosphate (phosphorolytic pathway), units off the polymer. Glucosidic degradation of glycogen can be accomplished *in vivo* by α -amylase, amylo-1,6-glucosidase, acid α -glucosidase, and neutral α -glucosidase (Jeffrey et al. 1970). Furthermore, I have recently reported the first example of an activation of acid α -glucosidase in response to a physiological stress *in vivo*; the maximal activity of trout liver acid α -glucosidase increased by 80 % and the K_m for glycogen decreased by 50 % in response to exhaustive swimming exercise, a condition that promotes glucose export from liver (Chapter 3; Mehrani and Storey, 1993b).

The present study explores glycogenolysis in the anoxia tolerant turtle, *Trachemys scripta elegans*. Organ-specific effects of *in vivo* anoxia exposure on enzymes of both the phosphorolytic (glycogen phosphorylase, phosphorylase kinase, cAMP-dependent protein kinase) and glucosidic (acid and neutral α -glucosidases) pathways of carbohydrate degradation as well as the regulation of the phosphorolytic pathway via *in vitro* incubations with second messengers and cAMP-dependent protein kinase were assessed.

MATERIALS AND METHODS

CHEMICALS AND ANIMALS

All chemicals, coupling enzymes and rabbit muscle PKA catalytic subunit were obtained from Sigma Chemical Co. (St. Louis, MO) or Boehringer Mannheim (Montreal, PQ) and were of the highest quality available. The pH values of all buffers were adjusted at 21°C.

Adult red-eared slider turtles, *T. s. elegans*, of 8-12 inches shell length were purchased from Wards Natural Science (Mississauga, ON). Animals were held for at least 3 weeks before use in large tanks with running dechlorinated water at 7°C and a heat lamp positioned over a central dry island. Turtles were fed a diet of lettuce, trout pellets, and egg shells supplied *ad libitum*. Aerobic, control turtles were sampled from this condition. Animals were killed by decapitation, and tissues were immediately removed, frozen in liquid nitrogen, and then transferred to -80°C for storage until use.

To impose anoxia, other turtles were transferred to 40 l containers (three animals per tank) filled with water that had been previously bubbled with nitrogen gas for 9 h. A wire mesh was positioned about 10 cm below the water surface to keep the turtles from surfacing, and the lid was closed (except for a small hole to vent gas). Bubbling with nitrogen gas was continued for the duration of the 5 h anoxia exposure, or in time course experiments for 1, 5 or 20 h and the container was bathed in 7°C water throughout to maintain a constant temperature. Water pO₂ was always less than 10 Torr when measured at the end of the experimental submergence time. After anoxia exposure, turtles were rapidly removed and organs sampled as described above.

ANALYTICAL METHODS AND ENZYME ASSAYS

Frozen tissues were homogenized in 4 vol (w/v) of extraction buffer (50 mM imidazole pH 7.2, 30 mM 2-mercaptoethanol, 2.5 mM EDTA, 2.5 mM EGTA, 50 mM NaF) with 0.1 mM PMSF added immediately prior to homogenizing, and then desalted as described in Chapter 2.

The assays for glycogen phosphorylase and phosphorylase $\bar{b}$ kinase as well as the methods for *in vitro* activation of both enzymes and for the determination of cAMP concentrations were as described in Chapter 2 (Mehrani and Storey, 1993a; 1993c). Alpha-glucosidase was assayed as described in Chapter 3 (Mehrani and Storey, 1993b). cAMP-Dependent protein kinase assays were as described in Chapter 2 except that

magnesium acetate and unlabelled ATP concentrations were 3.43 and 0.24 mM, respectively. The total Mg^{2+} and ATP concentrations gave 0.206 mM $\text{Mg} \cdot \text{ATP}$ and 0.8 mM free Mg^{2+} in the assay mixture (Brooks and Storey, 1992). Higher concentrations of free Mg^{2+} were inhibitory to turtle PKA (data not shown).

DATA ANALYSIS

Assays on each sample were performed in triplicate and data show the means $\pm$ SEM of determinations on $n = 3$ samples from different animals. Statistical analysis used the Student's t-test, or one-way analysis of variance (ANOVA) followed by the Dunnett's test (two-tailed).

RESULTS

A) Glycogen phosphorylase

Table 4.1 shows the effect of 5 h of anoxia exposure *in vivo* at 7°C on the total activity of glycogen phosphorylase (a + b) and the calculated percentage of the enzyme in the active a form in five organs of turtles. Anoxia exposure for 5 h significantly increased total phosphorylase activity in heart but had no effect on total enzyme activity in the four other organs. Anoxia increased phosphorylase a content in anoxic heart and liver (Fig. 4.1), the net result being significant increases in the percentage of enzyme in the a form in both organs (Table 4.1). Overall, the content of active phosphorylase a increased by 2-fold in liver of anoxic animals (from 0.207 ± 0.016 U/g wet weight in controls to 0.413 ± 0.036 U/gww) and by 2.5-fold in heart (from 0.68 ± 0.06 U/gww to 1.68 ± 0.13 U/gww) (see control values, Fig. 4.1).

To assess the mechanism of phosphorylase activation in turtle organs, enzyme extracts were incubated with second messengers of protein kinases or with the purified catalytic subunit of protein kinase A (PKA). Cyclic AMP was the only second

Table 4.1**Glycogen phosphorylase from turtle organs**

Effect of anoxia exposure *in vivo* on total phosphorylase activity and the percentage of enzyme in the active a form.

Organ	Condition	U/gww	% <u>a</u>
Liver:	Aerobic	1.48 $\pm$ 0.12	14.0 $\pm$ 1.2
	Anoxic	1.59 $\pm$ 0.14	26.0 $\pm$ 2.3*
Heart:	Aerobic	5.56 $\pm$ 0.54	12.0 $\pm$ 1.5
	Anoxic	7.34 $\pm$ 0.57*	23.0 $\pm$ 2.5*
Brain:	Aerobic	3.83 $\pm$ 0.18	18.5 $\pm$ 1.9
	Anoxic	3.44 $\pm$ 0.27	17.0 $\pm$ 2.3
Red muscle:	Aerobic	9.92 $\pm$ 1.23	17.0 $\pm$ 2.5
	Anoxic	9.28 $\pm$ 1.13	19.0 $\pm$ 1.4
White muscle:	Aerobic	11.3 $\pm$ 1.36	19.0 $\pm$ 4.6
	Anoxic	11.0 $\pm$ 1.15	26.0 $\pm$ 3.6

Values are means $\pm$ SEM; n = 3 animals per group. Activity is expressed as μ mol glucose-1-phosphate produced per min per gram wet tissue. Phosphorylase a and total phosphorylase (a + b) were measured as described in Chapter 2 Materials and Methods and the percent a was calculated.

* - Significantly different from the corresponding aerobic value as determined by the Student's t-test, $P < 0.05$.

Figure 4.1 Effect of incubation with cyclic AMP or protein kinase A catalytic subunit on the activity of glycogen phosphorylase α in organs from normoxic (N) and anoxic (A) turtles

See Materials and Methods for Chapter 2 for details of the incubations. Additions were: ATP + EDTA, control (solid bars), Mg.ATP + 2.5 μ M cAMP (open bars), and Mg.ATP + 2 mg/ml rabbit skeletal muscle PKA catalytic subunit (hatched bars). The activity of phosphorylase α in control incubations was the same before and after incubation in all cases, demonstrating that *in vitro* incubation itself did not change phosphorylase activities. Data are units/g wet weight, means $\pm$ SEM, n = 3 animals in each group and all experiments were done in triplicate. Significantly different from the corresponding control value as determined by the Student's t-test, * P < 0.01, ** P < 0.001.

messenger found to alter phosphorylase activity; cGMP and Ca^{2+} + 12-O-tetradecanoylphorbol-13-acetate were also tested but had no effect on enzyme activity (data not shown). Fig. 4.1 shows glycogen phosphorylase a activity after *in vitro* incubation of organ extracts from normoxic and anoxic turtles with ATP + EDTA (control), Mg.ATP + cAMP, or Mg.ATP + PKA catalytic subunit. *In vitro* incubation with Mg.ATP + cAMP stimulated phosphorylase a activity in liver extracts from both aerobic and anoxic turtles but did not alter enzyme activity in extracts from other organs. However, phosphorylase a in extracts from all organs responded to incubation with Mg.ATP + the catalytic subunit of PKA. Treatment with PKA increased phosphorylase a activity by 3.2, 2.2, 2.3, 1.8 and 1.9-fold in extracts from normoxic liver, heart, brain, red muscle and white muscle, respectively (Fig. 4.1). Similar effects were seen with extracts from anoxic animals except that the high enzyme activity in anoxic heart was not further increased by *in vitro* incubation. The net effect of *in vitro* incubation with PKA on phosphorylase a activity in liver (increases of 11-fold in normoxic and 7.4-fold in anoxic extracts) was a combination of two factors: an increase in total enzyme activity and an increase in the percentage of enzyme in the a form (Table 4.2). Thus, PKA stimulation of liver extracts increased total phosphorylase activity by 3.5-fold in aerobic and 4.3-fold in anoxic extracts and also increased the percent phosphorylase a from 14 % in aerobic and 26 % in anoxic liver extracts to 45 % in both cases after incubation with PKA. In other organs, however, the increase in phosphorylase a after incubation with PKA was the result of increases in the percentage of phosphorylase in the a form only with no significant change in total phosphorylase activity (Table 4.1 and Table 4.2).

B) Phosphorylase b kinase

Table 4.3 shows the activities of phosphorylase b kinase in organs from aerobic and anoxic turtles as well as the effect of *in vitro* incubation with the catalytic subunit

Table 4.2**Glycogen phosphorylase from turtle organs**

Effect of incubation of organ extracts with Mg.ATP + the catalytic subunit of protein kinase A *in vitro* on total phosphorylase activity and the percentage of enzyme in the active a form.

Organ	Condition	U/gww	Percent <u>a</u>
Liver:	Aerobic	5.25 ± 0.75*	45.0 ± 3.0*
	Anoxic	6.78 ± 0.84*	45.0 ± 2.7*
Heart:	Aerobic	5.85 ± 0.87	27.0 ± 1.8*
	Anoxic	6.97 ± 0.74	24.0 ± 2.3
Brain:	Aerobic	3.80 ± 0.32	42.0 ± 7.5*
	Anoxic	3.77 ± 0.37	40.0 ± 4.2*
Red muscle:	Aerobic	13.2 ± 1.71	36.0 ± 4.4*
	Anoxic	11.9 ± 1.90	38.0 ± 6.5*
White muscle:	Aerobic	10.6 ± 1.20	36.0 ± 5.0*
	Anoxic	14.6 ± 1.85	46.0 ± 3.6*

Details are as in Table 4.1.

* - Significantly different from the corresponding *in vivo* value for the enzyme without PKA incubation as reported in Table 4.1, P < 0.05.

Table 4.3

Glycogen phosphorylase b kinase from turtle organs

Effect of *in vitro* incubation with PKA catalytic subunit in stimulating enzyme activity.

		Basal activity (<i>in vivo</i>)	Activated by PKA (<i>in vitro</i>)
Organs		U/gww	U/gww
Liver:	Aerobic	0.15 ± 0.011	0.25 ± 0.015*
	Anoxic	0.09 ± 0.013 ⁺	0.26 ± 0.015*
Heart:	Aerobic	0.14 ± 0.045	0.45 ± 0.049*
	Anoxic	0.13 ± 0.037	0.42 ± 0.036*
Brain:	Aerobic	0.13 ± 0.065	0.19 ± 0.068
	Anoxic	0.15 ± 0.078	0.21 ± 0.075
Red muscle:	Aerobic	0.59 ± 0.06	1.93 ± 0.18*
	Anoxic	0.65 ± 0.12	1.80 ± 0.23*
White muscle:	Aerobic	0.51 ± 0.11	0.81 ± 0.14
	Anoxic	0.51 ± 0.09	1.24 ± 0.12*, ⁺

Values are means ± SEM; n = 3 animals in each groups and all assays are done in triplicate. Activity is expressed as μ mol phosphorylase b converted to a per minute per gram wet tissue.

* - Significantly different from the corresponding basal activity *in vivo* for the enzyme without incubation with PKA as determined by the Student's t-test, $P < 0.05$.

⁺ - Significantly different from the corresponding aerobic value, $P < 0.05$.

of PKA in stimulating the maximal activity of the enzyme. Enzyme activities were highest in skeletal muscle tissues, 3-6 fold higher than in other organs. Anoxia exposure *in vivo* resulted in a significant decrease in phosphorylase kinase activity in turtle liver but did not affect enzyme activity in other organs. The activity of phosphorylase kinase was significantly increased over basal values by *in vitro* incubation with the catalytic subunit of PKA in all extracts except those from brain. The maximum PKA-activated phosphorylase kinase activity was the same in both aerobic and anoxic extracts in four organs; the fold activation by PKA was about 3-fold in heart and red muscle. However, white muscle extracts from anoxic turtles showed significantly higher PKA-activated phosphorylase kinase activity than did aerobic extracts, although the basal activities in both aerobic and anoxia extracts were the same.

C) cAMP-dependent protein kinase activity and cAMP levels

Table 4.4 shows the activity of cAMP-dependent protein kinase and the concentrations of cyclic AMP in liver of aerobic, control turtles versus turtles given anoxic submergence for 1, 5 or 20 h at 7°C. Total PKA activity (assayed in the presence of 10 μ M added cAMP) did not change with anoxia exposure. However, anoxia exposure altered the amount of the free catalytic subunit of PKA in liver. After 1 h of anoxia exposure the percentage of enzyme present as the free catalytic subunit had risen significantly from 6.4 % in controls to 14.7 %. Longer anoxia exposures times had the opposite effect and PKAc content decreased to 2.1 % after 5 h and 3.7 % after 20 h. The cAMP content of liver also increased significantly (by 57 %) after 1 h of anoxia exposure but returned to control levels in 5 and 20 h anoxic animals.

Table 4.5 shows the effects of anoxia exposure on PKA activity in four other organs of *T. s. elegans*. As in liver, anoxia had no effect on total PKA activity. However, after 1 h of anoxia exposure the percentage of free catalytic subunit had

Table 4.4

Effect of anoxia exposure on protein kinase A (total and free catalytic subunit) and cAMP concentrations in turtle liver

Condition	Total PKA pmol/gww	PKAc pmol/gww	% PKAc	[cAMP] pmol/gww
Control	15838 ± 1415	995 ± 130	6.4 ± 1.0	872 ± 76
Anoxic:				
1 h	17440 ± 2430	2327 ± 498 [*]	14.7 ± 2.8 [*]	1371 ± 71 [*]
5 h	22933 ± 3500	395 ± 126 ⁺	2.1 ± 0.8 ⁺	892 ± 50 ⁺
20 h	20313 ± 4350	607 ± 122 ⁺	3.7 ± 1.1 ⁺	907 ± 66 ⁺

Values are means ± SEM; n = 3 animals in each group and all assays were done in triplicate.

^{*} - Significantly different from the corresponding control values as determined by ANOVA and the Dunnett's test (two-tailed), P < 0.01; ⁺ - Significantly different from the 1 h anoxic value, P < 0.01.

Table 4.5

Effect of anoxia exposure on protein kinase A in turtle organs

Organ and Condition	Total activity pmol/gww	PKAc pmol/gww	% PKAc
Heart:			
Control	30447 ± 2324	2508 ± 331	8.1 ± 0.4
Anoxic 1 h	30394 ± 476	1502 ± 289	4.9 ± 1.1
Anoxic 5 h	34024 ± 7787	2843 ± 555	8.5 ± 0.5
Anoxic 20 h	37219 ± 2406	3108 ± 673	9.8 ± 2.2
Brain:			
Control	26801 ± 2426	3977 ± 766	15.1 ± 3.8
Anoxic 1 h	33279 ± 3144	4137 ± 206	12.7 ± 1.8
Anoxic 5 h	27111 ± 4991	5411 ± 857	21.2 ± 5.0
Anoxic 20 h	19527 ± 2321	3184 ± 40	16.8 ± 2.1
White Muscle:			
Control	9272 ± 198	1183 ± 47	12.7 ± 0.2
Anoxic 1 h	7377 ± 957	794 ± 127	10.8 ± 0.5
Anoxic 5 h	10895 ± 940	1034 ± 360	9.3 ± 2.8
Anoxic 20 h	7488 ± 1053	1021 ± 198	13.6 ± 7.0
Red Muscle:			
Control	6059 ± 323	509 ± 32	8.4 ± 0.5
Anoxic 1 h	6916 ± 1191	379 ± 36	5.7 ± 0.7*
Anoxic 5 h	7609 ± 792	407 ± 99	5.3 ± 0.9*
Anoxic 20 h	7858 ± 1208	355 ± 104	4.4 ± 0.8*

Details are as in Table 4.4.

* - Significantly different from the corresponding control value, $P < 0.05$.

decreased sharply in red skeletal muscle and was suppressed throughout the anoxia exposure. The content of PKAc was not affected by anoxia exposure in brain, heart and white muscle.

D) α -Glucosidase

Table 4.6 shows the activities of α -glucosidases in turtle organs; activities were measured at both the pH 7 and pH 4 using 4-methyl-umbelliferyl- α -glucoside (MU α G) as the substrate. Two enzyme forms are generally found in animal tissues, an acid α -glucosidase that occurs in the lysosomes and a neutral enzyme that is associated with the microsomes (Mehrani and Storey, 1993b; Chapter 3). Activities of both enzyme forms were found in turtle organs and were highest in the liver, intermediate in heart and brain, and lowest in skeletal muscles. In all organs activity of the neutral enzyme was higher than that of the acidic enzyme, the ratio neutral:acid being 7.6:1 in brain, about 5:1 in heart, and 3:1 in skeletal muscles. In liver the ratio was 7:1 in aerobic and 4:1 in anoxic tissue. Anoxia exposure resulted in significant increases in the activities of both the neutral (by 12 %) and acidic (by 100 %) enzyme forms in liver. Neutral α -glucosidase activity also decreased significantly in anoxic, compared with aerobic, heart but anoxia exposure had no effect on either of the α -glucosidase activities in the other three organs.

DISCUSSION

For facultative anaerobes, a general pattern of metabolic response to declining O₂ tension is emerging (Hochachka 1988; Storey and Storey, 1990). As conditions become hypoxic, the rate of anaerobic glycolysis increases to supplement ATP production in inverse proportion to the decrease in the rate of oxidative phosphorylation. Below a critical oxygen tension, however, the attempt to maintain normal rates of ATP generation is abandoned and organisms initiate an energy

Table 4.6

Neutral and acidic α -glucosidase activities in tissues from aerobic and anoxic turtles

Organs	Condition	Total activity mU/gww	
		pH 7.0	pH 4.0
Liver:	Aerobic	153.3 $\pm$ 3.9	21.1 $\pm$ 1.5
	Anoxic	172.0 $\pm$ 4.1 ⁺	41.7 $\pm$ 2.6*
Heart:	Aerobic	58.1 $\pm$ 1.2	11.7 $\pm$ 0.9
	Anoxic	45.8 $\pm$ 1.4*	9.8 $\pm$ 0.8
Brain:	Aerobic	65.6 $\pm$ 3.6	8.6 $\pm$ 1.2
	Anoxic	59.5 $\pm$ 1.9	7.8 $\pm$ 0.9
Red muscle:	Aerobic	21.9 $\pm$ 3.6	7.0 $\pm$ 0.8
	Anoxic	23.7 $\pm$ 1.6	6.7 $\pm$ 1.0
White muscle:	Aerobic	15.2 $\pm$ 1.7	5.3 $\pm$ 0.8
	Anoxic	16.8 $\pm$ 1.9	5.4 $\pm$ 0.9

Values are means $\pm$ SEM, n = 3 animals in each group (triplicate determination). Activity is expressed as nmol MU α G hydrolyzed per minute per gram wet tissue at 21°C. Significantly different from the corresponding aerobic value as determined by the Student's t-test, * P < 0.001, ⁺ P < 0.025.

conservation strategy. By coordinating a reduction in the rates of all metabolic processes, overall metabolic rate is suppressed to a value that can be sustained over the long term via fermentative pathways of ATP production alone. Turtles show such a pattern; during the hypoxia transition period, organs show metabolic changes indicative of an activation of anaerobic glycolysis but with longer periods of anoxia exposure, glycolytic rate depression is seen, concomitant with the suppression of overall metabolic rate to only 10-20 % of the resting, aerobic rate at the same temperature (Kelly and Storey, 1988). The present study assessed the effect on enzymes of glycogenolysis of anoxic submergence of turtles at 7°C. Anoxia exposure resulted in a significant rise in the activities of enzymes of both the phosphorolytic (glycogen phosphorylase) and glucosidic (acid α -glucosidase) routes of glycogen degradation in liver. Furthermore, phosphorylase activity was also increased in anoxic heart. The activation of phosphorylase in liver was expected from the role of this organ in maintaining plasma glucose supplies. Liver contains the major reserves of glycogen in the organism and these are mobilized during anoxia to provide plasma glucose to fuel anaerobic metabolism in most other organs. Heart also maintains a substantial supply of glycogen that is used to sustain its contractile activity during anoxia. Results for liver agree with a previous study that assessed the effects of anoxic submergence at a higher temperature (5 h at 18°C) on activities and kinetic properties of several glycolytic enzymes in turtle organs (Brooks and Storey, 1989); the net increase in active phosphorylase a in liver was 3.3-fold in that study compared with 2-fold in the present study. However, present results for heart (a 2.5-fold increase in phosphorylase a) differed from the previous study (a 50 % decrease) (Brooks and Storey, 1989) but this might be accounted for by a more rapid and profound imposition of metabolic arrest at the higher temperature. Other organs of turtles did not show changes in phosphorylase after 5 h of anoxia exposure at 5°C, sustaining a low % a (Table 4.1) that suggests that either a) endogenous glycogen is not their major fuel during anoxia (this is certainly

true for brain which relies heavily on plasma glucose), or b) anoxia-induced mechanisms of metabolic rate depression had suppressed the demand for ATP generation via anaerobic glycogenolysis. These results were largely the same as occurred during anoxia at 18°C except that brain showed a small decrease (30 %) in phosphorylase a in the previous study (Brooks and Storey 1988; 1989).

To examine the regulation of turtle glycogen phosphorylase and to determine the possible sites in the enzyme cascade that could mediate anoxia-induced changes in phosphorylase activity, the responsiveness of phosphorylase to treatment *in vitro* with second messengers or the catalytic subunit of PKA was examined and, in addition, the endogenous activities of phosphorylase b kinase and protein kinase A were compared in extracts from organs of aerobic versus anoxic turtles. Turtle glycogen phosphorylase responded to treatment *in vitro* with Mg.ATP plus the catalytic subunit of mammalian protein kinase A, showing a significant increase in the percent phosphorylase a in all organs as well as an increase in total enzyme activity in liver (Table 4.2, Fig. 4.1). This indicates that the phosphorylation cascade mechanism involving protein kinase A, phosphorylase kinase, and glycogen phosphorylase is present and active in turtle organs. However, *in vitro* treatment of tissue extracts with cyclic AMP + Mg.ATP stimulated phosphorylase activity only in the liver suggesting that endogenous protein kinase A activity may be limiting in the *in vitro* situation.

Phosphorylase b kinase was present in all five turtle organs tested with highest activities found in muscle tissues. The enzyme was sensitive to activation by the catalytic subunit of protein kinase A, as also occurs in mammals, and a comparison of the basal versus PKA-activated activities indicated that only a fraction of the maximal enzyme activity was expressed *in vivo*. Basal activity ranged from about one-third of the maximal PKA-activated activity in heart and red muscle to 70 % of the maximum in brain. All organs also exhibited activities of cyclic AMP-dependent protein kinase with highest levels in heart and lowest in skeletal muscles. The enzyme was maintained

in a largely inactive form in all organs with the percentage of free catalytic subunit (PKAc) ranging from only 2-21 %. Except for liver, anoxia exposure *in vivo* had very limited effects on the activities of phosphorylase kinase and protein kinase A. Thus, basal activities of phosphorylase kinase in nonhepatic organs were not affected by anoxia exposure and the maximum (PKA-activated) activity of phosphorylase kinase differed between aerobic and anoxia extracts only in white skeletal muscle. For protein kinase A in nonhepatic organs, anoxia exposure led to a decrease in the percentage of PKAc in red muscle only.

Liver was the only organ that showed consistent changes in enzyme activities as the result of anoxia exposure *in vivo*. Basal phosphorylase kinase activity decreased by 40 % in the anoxic organ whereas the amount of protein kinase A catalytic subunit fell from 6 % to 2 % of the total PKA after the 5 h anoxia exposure. Thus, both enzymes showed coordinated decreases in activity during anoxia. Despite this, glycogen phosphorylase α was 2-fold higher in 5 h anoxic, compared with aerobic, liver suggesting a higher rate of glycogenolysis in anoxic turtle liver. Thus, the data for the kinases seem to be at odds with that for glycogen phosphorylase. However, to raise glycogen phosphorylase activity to a new, higher steady-state rate in the anoxic organ may require only transient activations of phosphorylase kinase and PKA, activations that may not still be apparent after 5 h of anoxia exposure. Indeed, when a time course study was conducted, a peak in cyclic AMP concentration in turtle liver occurred after 1 h anoxia exposure (Table 4.4), which suggests that transient changes in phosphorylase kinase and protein kinase A would occur early in anoxia to elevate the rate of glycogenolysis. The role of this cascade system in the activation of glycogen phosphorylase in anoxia is also supported by the strong effect of propranolol (a β -blocker) in suppressing both phosphorylase activation and glucose production in anoxic turtles (Keiver and Hochachka, 1991); hence, stimulation of anoxia-induced, catecholamine-stimulated glycogenolysis in turtle liver appears to be cAMP-mediated.

In the time course study the amount of active PKA rose sharply by 2.3-fold within one hour of anoxia exposure, accompanied by an elevation of liver cAMP levels (Table 4.4). Although the activation was transient and the percentage of active PKAc subsequently fell from 14.7 after 1 h anoxia to 2 % after 5 h anoxia, a sustained activation of glycogen phosphorylase and of glucose output into the circulation is maintained throughout an anoxic excursion (Penney 1974; Brooks and Storey, 1989)

In contrast with the results for liver, other organs showed either no change in PKAc content in anoxia (brain, heart and white muscle) or a sustained decrease in the % PKAc during anoxia exposure (red skeletal muscle). These results correlate well with the general lack of activation of glycogenolysis in turtle organs during anoxia exposure. Hence, even though levels of plasma catecholamines (epinephrine, norepinephrine) rise by 50-60 fold during anoxia (Keiver and Hochachka, 1991)), the primary target enzyme of these hormones, PKA, was not activated in any organ except liver. This may suggest the presence of a modulating factor or factors that interferes with the normal response of organs to catecholamines during anoxia.

Alpha-glucosidase enzymes have been identified in numerous organisms and participate in the catabolism of poly- and oligosaccharides in cells. Acid α -glucosidases, with pH optima about 4-5 (sometimes called gamma-amylases) are located in the lysosomes (along with other degradative enzymes that act in an acidic environment) where they function to hydrolyze the glycogen and oligosaccharides that are trapped in the organelle as the result of cellular autophagy (Jeffrey et al. 1970). The absence of the enzyme leads to the storage of huge amounts of glycogen in the lysosomes that is inaccessible to the phosphorolytic pathway of glycogen catabolism in the cytoplasm; in humans this is known as type II glycogenosis or Pompe's disease, an inheritable trait that is fatal in infancy (Brown et al. 1970). Neutral α -glucosidase, with a pH optimum of 6-7, seems to be associated with the microsomes where it appears to be involved in trimming sugar residues in newly synthesized glycoproteins and/or

cleaving saccharides into monomers for movement across membranes of the endoplasmic reticulum (Castilla et al. 1978; Imai et al. 1989). Unlike the acid enzyme, neutral α -glucosidase does not appear to hydrolyze glycogen (Castilla et al. 1978; Imai et al. 1989; Mehrani and Storey, 1993b). Both α -glucosidase types were found in turtle organs with the highest activities of both in liver and the lowest in skeletal muscles. The maximum activities of each in turtle liver were very similar to the activities reported for trout liver (Mehrani and Storey, 1993b). The neutral activity exceeded the acid enzyme activity in all organs, as occurs in other organisms, with a ratio of neutral:acid activities that varied from 3:1 to 7.6:1 among the different organs.

Liver glycogen represents the primary reserve of fermentable fuel available to support anaerobiosis in the diving or hibernating turtle. Liver glycogenolysis, leading to glucose export into the blood, is critical to sustaining survival, particularly for organs, such as the brain, which have very limited endogenous reserves of glycogen. Phosphorolytic degradation of glycogen, via glycogen phosphorylase, has always been thought of as the means by which glucosyl units of the polymer are made available for energy metabolism or glucose export. However, studies of fish challenged this presumption when Murat (1976) was unable to detect glycogen phosphorylase activity in carp liver but found, instead, high activities of α -glucosidase. Substantial activities of acid α -glucosidase also occur in liver of other fish, the ratio of phosphorylase α to acid α -glucosidase being 5.6:1 in goldfish, 8:1 in trout, and 1.5:1 in another population of carp (Mehrani and Storey, 1993b; Picukans and Umminger, 1979). The comparable ratio in *T. s. elegans* liver was 10:1 under both aerobic and anoxic conditions (Table 4.1 and 4.6). Thus, a significant capacity for glucosidic glycogenolysis exists in turtle liver and this is even more evident when comparisons are made with the phosphorylase α : α -glucosidase activity ratios of other organs which ranged from 60:1 in aerobic heart to 400:1 in white muscle. Thus, the present study, together with my recent study on trout (Chapter 3; Mehrani and Storey, 1993b),

suggests that acid α -glucosidase could play more than just a scavenging role in cellular carbohydrate metabolism. Indeed, an involvement in net glucose production by the liver is strongly suggested by the present data that show a 2-fold increase in acid α -glucosidase activity in turtle liver in response to anoxia. Thus, the glucosidic route of glycogen degradation in liver is activated under the same conditions of anoxia that activate the phosphorolytic pathway. Similarly, the maximal activity of acid α -glucosidase type I from trout liver increased by 80 % and its K_m for glycogen dropped by 50 % in response to vigorous swimming exercise, another physiological stress that activates liver glycogenolysis. Thus, it is apparent that the activity of liver acid α -glucosidase, and by implication the rate of lysosomal glycogenolysis, responds to changes in the physiological state (exercise, anoxia) of organisms when there is a demand hepatic glucose export. It appears, then, that in both fish and reptiles the glucosidic degradation of lysosomal glycogen could make a significant contribution to total hepatic glucose output. The precise percentage of total hepatic glucose output resulting from lysosomal glycogen degradation remains to be investigated as do the regulatory mechanisms that control this pathway. Murat (1976) reported that carp liver α -glucosidase activity increased in response to hormones known to stimulate glycogenolysis but we found no change in the properties of the trout liver enzyme after *in vitro* incubation of partially purified enzyme with Mg.ATP + the catalytic subunit of protein kinase A (Mehrani and Storey, 1993b).

CHAPTER 5

**PURIFICATION AND KINETIC
PROPERTIES OF cAMP
DEPENDENT PROTEIN
KINASE FROM
TURTLE
LIVER**

INTRODUCTION

Phosphorylation of proteins at serine and threonine residues is one of the most frequent forms of post-translational modification leading to changes in biological function. Since the discovery of cAMP-dependent protein kinase (PKA) in rabbit muscle by Krebs and colleagues (Walsh et al., 1968), the mechanisms of action of a number of extracellular signals have been found to involve regulation of the formation of cAMP and, thereby, control of its target enzyme, PKA (Greengard, 1978).

In the absence of cAMP, PKA exists in an inactive tetrameric form consisting of two regulatory and two catalytic subunits. The binding of cAMP to the regulatory subunits leads to the dissociation of the active catalytic subunits. Since a highly purified catalytic subunit is particularly useful for studying the effects of phosphorylation on enzymes that are phosphorylated and regulated by this protein kinase, or for analysis of the kinetic properties of the enzyme itself, purification procedures have been developed for PKA from a number of mammalian systems (Sugden et al. 1976; Zoller et al. 1979; Carleson et al. 1979; First and Taylor, 1989; Okuno and Fujisawa, 1990). Although the purification procedures developed to date are satisfactory for mammalian systems, they may not equally apply to the purification of the enzyme from lower vertebrate systems which may have low enzyme content or different enzymatic characteristics.

The present study reports the purification and characterization of the catalytic subunit of PKA from turtle liver and an analysis of the kinetic and physical properties of the enzyme from a lower vertebrate.

MATERIALS AND METHODS

CHEMICALS AND ANIMALS

KT-5823 and H-89 were obtained from Biomol (Plymouth Meeting, PA), protein kinase C inhibitor peptide (19-36; PKC-I) was purchased from Peninsula Labs Inc. (Belmont, CA), and PKA inhibitor peptide (5-24; PKA-I) was from Sigma

Chemical Co. (St.Louis, MO). Other chemicals and column materials were purchased from Sigma.

ASSAY OF LIVER PKA

PKA activity was assayed essentially as described in Chapter 2 (without the addition of cAMP) with the modifications of Mg^{2+} and ATP concentrations for turtle PKA as described in Chapter 4. When measuring activity with either histone IIA or protamine as substrates, reactions were stopped by adding 1 ml of ice-cold 5 % trichloroacetic acid/10 mM phosphoric acid followed by incubating 10 min on ice. Samples were then filtered through Whatman GF/C filter papers and washed 4 times with the TCA/ H_3PO_4 solution. Radioactivity was counted using Cerenkov methodology. Activities are reported as picomol phosphate transferred per minute (PIU) at $21 \pm 1^\circ C$.

PURIFICATION OF LIVER PKA CATALYTIC SUBUNIT

All steps were performed at $4^\circ C$ unless otherwise indicated. Frozen liver from aerobic, control turtles was homogenized 1:5 w/v in homogenization buffer (10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 2 mM β -mercaptoethanol, 0.1% v/v Triton X 100, 1 mM PMSF, 1 $\mu g/ml$ aprotinin and 0.1 mM IBMX) containing 1 mM cAMP (to dissociate all catalytic subunit), and then centrifuged at 1000 g for 5 min. The supernatant was removed and poly(ethyleneglycol) 8000 (PEG) was slowly added to a final concentration of 5 % w/v. The mixture was stirred in an ice bath for 20 min and then centrifuged at 20,000 g for 20 min; the supernatant was saved and the pellet discarded. The supernatant was then loaded onto a DEAE-cellulose (DE-52) column (20 x 2.5 cm) which had been equilibrated in PEM buffer (10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 2mM β -mercaptoethanol, 0.1% v/v Triton X-100). The column was washed with 10 vol PEM buffer and then the enzyme was eluted using

a linear gradient of 0-400 mM NaCl in PEM buffer. One ml fractions were collected and assayed without cAMP. Peak fractions were pooled, diluted 1:1 with distilled water, and then loaded onto a hydroxylapatite column (10 x 2.5 cm) previously equilibrated with buffer A (PEM buffer containing 5 % v/v glycerol). PKA was then eluted with a linear gradient of 0-500 mM potassium phosphate in buffer A. Fractions of 1.5 ml were collected and assayed for PKA activity. Peak fractions were pooled and dialyzed for 4 h against 2 changes of buffer A. The dialyzed sample was then loaded onto a 4 ml column of Protamine-agarose equilibrated with buffer A. The column was washed with 5 vol of buffer A and then PKA activity was eluted with a linear gradient of 0-200 mM NaCl. Peak fractions were pooled and dialyzed as above and then used for kinetic analysis. Purified PKA was stored in 40 % v/v glycerol at -20°C and was stable for at least for 3 months. Protein concentrations were determined as described in Chapter 2.

ISOELECTROFOCUSING

Isoelectric focusing of liver PKA was performed at 4°C according to Vesterberg (1971). A gradient of LKB ampholines, pH 3.5 to pH 10, was stabilized by a 0-30 % w/v linear sucrose gradient. Tissue samples from control animals were homogenized 1:10 w/v in PEM buffer plus 1 mM cAMP and were then centrifuged for 20 min at 20,000 g at 4°C. An aliquot of this supernatant (1 ml) or of the partially purified enzyme after DE-52 chromatography (2 ml) was placed in the middle of a 110 ml isoelectrofocusing column. The column was developed for 18 h at 300 V and then one ml fractions were collected and assayed for activity.

NATIVE AND SUBUNIT MOLECULAR WEIGHTS

The native molecular weight of turtle liver PKAc was determined by Sephacryl S-300 gel filtration as described previously (Chapter 3; Mehrani and Storey, 1993b).

SDS-polyacrylamide electrophoresis was performed by the method of Laemmli (1970) with 10 % acrylamide separating gels and 5 % stacking gels. Proteins on gels were stained by silver nitrate as described by Nielsen and Brown (1984).

DATA ANALYSIS

Statistical analysis used by one-way analysis of variance (ANOVA) followed by the Dunnett's test (two-tailed).

RESULTS

PURIFICATION OF LIVER PKA

Purification of the catalytic subunit of turtle liver PKA is summarized in Table 5.1. The catalytic subunit eluted as a single peak off of each of the three chromatography columns used; peak activity eluted at about 180 mM NaCl for the DE-52 cellulose column, 280 mM phosphate for the hydroxylapatite column, and 80 mM NaCl for the protamine-agarose column. The purification scheme resulted in a 191-fold purification with a yield of 14 %. The final specific activity of the purified PKA catalytic subunit, when assayed with kemptide as the substrate (without cAMP) was 65,783 pmol phosphate transferred per minute per mg protein.

SDS-polyacrylamide gel electrophoresis of the purified catalytic subunit showed a single protein band with a molecular weight of 42 kD (Fig. 5.1). This agreed well with the results of S-300 molecular exclusion chromatography which showed a molecular weight of 43.2 ± 1.6 kD for the partially purified enzyme from either the 5 % PEG supernatant or the pooled peak after DE-52 cellulose chromatography (Fig. 5.2).

Column isoelectrofocusing of turtle liver PKA is shown in Fig. 5.3. When the supernatant from the 5 % PEG treatment was applied to the column two peaks of PKA activity were found, a major peak with an isoelectric point of 6.41 ± 0.02 SEM and a

Table 5.1

Purification of the catalytic subunit of cAMP-dependent protein kinase from turtle liver

Step	Total protein (mg)	Total activity (PIU)	Specific activity (PIU/mg)	Fold purifi- cation	Yield (%)
Crude extract	82	28,290	345	-	100
5% PEG	40	27,384	685	2	96
DE-52	4	25,078	6270	18	89
OH-apatite	1.6	18,012	11258	33	64
Protamine-Agarose	0.06	3,947	65783	191	14

The values represent the means of three separate purifications of the enzyme.

Activity was measured as pmol phosphate transferred per minute (PIU) by the standard assay conditions.

Figure 5.1 SDS-PAGE of various fractions from turtle liver PKA catalytic subunit purification

The standards from top to bottom are: bovine serum albumin (66 kD), ovalbumin (45 kD) and carbonic anhydrase (29 kD). Left lane shows the DE-52 pooled peak, middle lane shows the hydroxylapatite pooled peak, and right lane shows the active fraction from protamine-agarose chromatography.

Figure 5.2 Native molecular weight determination for the catalytic subunit of turtle liver PKA via Sephacryl S-300 gel exclusion chromatography

The column was eluted with 50 mM phosphate buffer, pH 7.0, containing 50 mM NaCl, 1 mM cAMP and 10 % v/v glycerol. A plot of $(K_d)^{1/3}$ versus $(\text{molecular weight})^{1/2}$ was constructed for the following standards (closed circles): pyruvate kinase (230 kD), lactate dehydrogenase (140 kD), bovine serum albumin (67 kD) hemoglobin (64.5 kD), and cytochrome c (12.4 kD). Turtle liver PKA catalytic subunit (open circle).

Figure 5.3 Isoelectric focusing of turtle PKA

Open circles show the enzyme activity in the 5 % PEG supernatant and closed circles show the enzyme after DE-52 cellulose chromatography. Activities were measured under optimal assay conditions. Filled triangles show the pH gradient.

minor peak with an isoelectric point of 5.16 ± 0.04 ($n=3$ runs). However, after DE-52 chromatography only the major peak (pI 6.41) remained.

Experiments with specific protein kinase inhibitors confirmed the identification of the turtle liver enzyme activity as a PKA catalytic subunit (PKAc) (Table 5.2). The inhibitors specific for protein kinase A, PKA-I and H-89 (Cheng et al. 1986; Chijiwa et al. 1990), strongly decreased enzyme activity and showed I_{50} values in the nanomolar range. By contrast, the specific inhibitor of cyclic GMP-dependent protein kinase, KT-5823 (Kase et al. 1987), and the inhibitor of protein kinase C, PKC-I (House and Kemp, 1987), inhibited the turtle enzyme only at much higher concentrations.

KINETIC CHARACTERIZATION OF PURIFIED TURTLE PKAc

The kinetic properties of the catalytic subunit of turtle liver protein kinase A are presented in Table 5.3. The enzyme phosphorylated three substrates that are commonly used for the assay of mammalian PKA. Kemptide was the preferred substrate, the maximal activities of the enzyme with histone and protamine being only about one-fifth and one-tenth of the corresponding value with kemptide. Michaelis constants for kemptide, histone IIA, and protamine were comparable to values for mammalian PKAc (Kemp et al. 1977; Zetterqvist et al. 1990). The enzyme had a K_m of $83 \mu\text{M}$ for Mg.ATP when assayed with kemptide as the substrate. Turtle PKAc showed an absolute requirement for Mg^{++} ions, but in contrast with mammalian PKAc (Cherrington et al. 1976), higher free magnesium concentrations were inhibitory (Fig. 5.4).

An Arrhenius plot of PKAc activity versus temperature over the range $0-45^\circ\text{C}$ is shown in Fig. 5.5. The plot showed two distinct linear segments with a break in the plot at $15-16^\circ\text{C}$. The activation energies for the two segments differed by 4-fold being $27.9 \pm 1.85 \text{ kJ/mol}$ for $T > 15^\circ\text{C}$ and $115 \pm 2.5 \text{ kJ/mol}$ for $T < 15^\circ\text{C}$.

Table 5.2

Effects of salts and specific protein kinase inhibitors on turtle liver PKA activity

Inhibitor	Target	I ₅₀ value
H-89	PKA	28.1 ± 2.4 nM
PKA-I	PKA	42.1 ± 7.2 nM
KT-5823	PKG	49.5 ± 9.8 μM
PKC-I	PKC	258 ± 23 μM
KCl	--	543 ± 22 mM
NaCl	--	394 ± 32 mM
NH ₄ Cl	--	218 ± 13 mM
KF	--	27 ± 5.0 mM
NaF	--	32 ± 6.1 mM

Values are means ± SEM; for determinations on n = 3 separate preparations of purified enzyme. The I₅₀ value is the concentration of inhibitor that reduces enzyme velocity by one-half. PKG is cyclic GMP-dependent protein kinase; PKC is Ca²⁺ and phospholipid dependent protein kinase C.

Table 5.3**Kinetic constants of turtle liver PKA catalytic subunit**

K_m Mg.ATP	83.3 ± 6.50 μM
K_m Kemptide	11.7 ± 0.53 μM
K_m Histone IIA	1.22 ± 0.30 mg/ml
K_m Protamine	1.57 ± 0.45 mg/ml
V_{max} ratio (H-IIA/kemptide)	0.21 ± 0.04
V_{max} ratio (protamine/kemptide)	0.11 ± 0.03

Values are means ± SEM, for n=3 separate preparations.

Figure 5.4 Effect of increasing magnesium acetate concentrations on the activity of purified liver PKA catalytic subunit

Increasing Mg^{2+} concentrations were added to the assay mixture while keeping the $\text{Mg} \cdot \text{ATP}$ concentration constant. Calculation of total Mg^{2+} and ATP was according to Brooks and Storey (1992). Values are means $\pm$ SEM, $n = 3$ separate preparations. Total Mg^{2+} concentration varied from 3.47 mM (0.8 mM free Mg^{2+}) to 15.4 mM (6.4 mM free Mg^{2+}).

Figure 5.5 Arrhenius plot for turtle liver PKA

The reaction mixture was pre-incubated for 10 min to allow temperature equilibration before initiating the reaction with the addition of PKA; optimal assay conditions were used and activity was assayed in 5°C increments between 0–45°C. Data are means of three separate determinations with less than 10 % variation between runs.

The effect of pH on enzyme activity is shown in Fig. 5.6. Although the optimum pH was 7.0, PKAc from turtle liver showed activities greater than 80 % of maximal over a broad range between pH 5.5 and 7.5. Fifty percent of maximal activity was still retained at pH 4 but activity fell off sharply at basic pH values.

The effects of various salts on PKAc activity are shown in Fig. 5.7 and I_{50} values are summarized in Table 5.2. The enzyme was largely unaffected by KCl or NaCl concentrations in the standard assay up to 100-200 mM and I_{50} values were high, 543 ± 22 mM and 394 ± 32 mM, respectively. However, inhibition by KF and NaF was very strong and apparently specific for the fluoride ion, with I_{50} values more than 10-fold lower than the corresponding values for the chloride salts.

DISCUSSION

The control of PKA has been well characterized in mammals. The enzyme responds to changes in cyclic AMP concentrations which are themselves regulated by the relative activities of adenylate cyclase and phosphodiesterase (Benovic et al. 1988; Ross and Gilman, 1980). Binding of cAMP to the regulatory subunits of the R_2C_2 holoenzyme causes these to dissociate and releases the catalytic subunit monomers which catalyze the ATP-dependent phosphorylation of numerous protein targets (Krebs, 1972; Rubin and Rosen, 1975).

The purification procedure developed for the catalytic subunit of PKA from turtle liver was a reliable and rapid technique that could be completed in one day. Tissue homogenization in buffer containing 1 mM cAMP was advantageous for later purification steps, which separate the catalytic subunit and regulatory subunits. Purification of mammalian PKAc typically involves a DEAE-cellulose step which separates the regulatory subunit (which binds to the column) from the catalytic subunit (which does not bind) (Livesy and Martin 1988). However, this procedure could not be used with turtle liver PKAc because the catalytic subunit also bound strongly to DEAE-

Figure 5.6 Profiles of pH versus activity for turtle liver PKA catalytic subunit

Total Mg^{2+} and ATP concentrations were varied to maintain constant concentrations of free Mg^{2+} and $\text{Mg}.\text{ATP}$ of 0.8 mM and 0.24 mM, respectively. Values are means $\pm$ SEM, $n = 3$ separate determinations. Data were normalized to the activity at pH 6.8.

Figure 5.7 Inhibition of PKA catalytic subunit activity by different neutral salts

Activity was assayed as described in Materials and Methods with added KCl (open triangles), NaCl (closed circles), NH_4Cl (filled triangles), NaF (closed squares) and KF (open squares). Values are means $\pm$ SEM, $n = 3$ separate determinations.

cellulose. The different binding behavior of turtle liver PKAc to DEAE cellulose suggests that the turtle catalytic subunit has a different isoelectric point than the mammalian catalytic subunit. Indeed, the isoelectric point shown in Fig. 5.3 is significantly lower than that reported for the mammalian enzyme (Sugden et al. 1976).

Kinetic characterization of turtle liver PKA catalytic subunit showed that it was similar to mammalian PKAc in several ways. The catalytic subunit of turtle liver PKA was a monomer and the molecular weights determined by SDS-PAGE (42 kD) and S-300 gel exclusion chromatography (43 kD) were similar to those reported for mammalian PKA catalytic subunit. The enzyme exhibited a high affinity for the artificial substrate kemptide whereas affinity for histone IIA and protamine was low. The enzyme also had a broad pH optimum, an absolute requirement for Mg^{2+} ions, and a high specific activity, as are common for the enzyme from other sources (Cherrington et al. 1976; Reimann and Beham, 1983; Okuno and Fujisawa, 1990). The enzyme was also strongly affected by inhibitors (H-89, PKA-I) known to inhibit mammalian PKA; this, along with other similarities to the mammalian enzyme, suggests that many properties of PKA are highly conserved among vertebrates. Turtle liver PKA was also susceptible to inhibition by inorganic ions. Significant effects of cations and Cl^- only occurred at very high concentrations (200-500 mM), well beyond the physiological range of these compounds. Fluoride anion, however, was a surprisingly strong inhibitor with I_{50} values of about 30 mM. Whereas fluoride effects may be of relatively little consequence *in vivo*, fluoride is often used in high concentrations (50-100 mM) in *in vitro* incubations in order to inhibit protein phosphatases while studying the effects of endogenous protein kinases on enzymes. Hence, with turtle liver preparations at least, careful design of *in vitro* studies would be necessary in order to avoid inhibiting both PKA and phosphatase activities.

Of potential importance for the regulation of metabolic rate depression in turtles was the effect of temperature on PKA activity. Below 15°C enzyme activity dropped

very sharply with decreasing temperature, resulting in a 4-fold increase in activation energy for the lower versus higher range of assay temperatures. Such strong low temperature effects on the enzyme might contribute to minimizing various PKA-mediated cellular responses during anoxic submergence at low environmental temperatures such as occur naturally during winter hibernation under water. Indeed, the extent anoxia-induced metabolic rate depression at temperatures below 10°C (measured by whole body calorimetry) is much stronger than at higher temperatures; anoxic metabolic rate at 3°C was only about 10 % of the corresponding aerobic rate at the same temperature, compared with values of about 20 % at higher temperatures (Herbert and Jackson, 1985). Thus, strong negative effects of low temperature on PKA activity, and perhaps also on other enzymes involved in signal transduction, may be one factor that contributes to an enhanced suppression of animal metabolic rate at low temperatures.

CHAPTER 6

EFFECTS OF ANOXIA ON PROTEIN PHOSPHATASE IN TURTLE ORGANS: Purification and properties of protein phosphatase type-1 from turtle liver

INTRODUCTION

Reversible protein phosphorylation is a widespread and powerful mechanism for the regulation of many cellular enzymes and functional proteins (Ballou and Fischer, 1986; Cohen, 1989; Barford, 1991). Protein phosphorylation is catalyzed by numerous specific protein kinases and is reversed by protein phosphatases. Many protein phosphatases show broad and overlapping substrate specificities *in vitro*, and their classifications are often based upon their responses to specific activators and inhibitors (Cohen and Cohen, 1989). Protein phosphatase type-1 (PP-1) is a widely distributed enzyme capable of removing covalently-attached phosphate from a variety of regulatory enzymes *in vitro* (Hubbard and Cohen, 1989). PP-1 is the major activity that dephosphorylates enzymes of glycogen metabolism, playing a key role in coordinating the rates of glycogen breakdown versus synthesis *in vivo*. Dephosphorylation by PP-1 activates glycogen synthetase and inactivates glycogen phosphorylase and phosphorylase kinase (Cohen, 1985).

In recent studies we have shown that anoxia exposure induces significant changes in the activities of key enzymes in turtle organs; these mediate metabolic arrest and, in liver, also regulate glycogenolysis to provide glucose for export to fuel fermentative metabolism in other organs. Anoxia-induced, organ-specific modifications of glycogen phosphorylase, phosphorylase kinase, protein kinase A, protein kinase C, phosphofructokinase, and pyruvate kinase have been documented (Chapters 4 and 5; Brooks and Storey, 1989; 1991; 1993). In liver, anoxia induces a rapid, transient elevation of protein kinase A activity and cAMP levels within the first hour that leads to a 2-fold increase in phosphorylase a activity (Chapters 4 and 5). However, when anoxia exposure is prolonged, and correlated with metabolic arrest, both protein kinase A and phosphorylase kinase activities are reduced but phosphorylase a activity is maintained to sustain glucose output throughout anoxia.

Given the importance of liver glycogen as the major reserve sustaining the blood glucose supply that fuels anaerobic survival for all organs, the enzymatic regulation of glycogen metabolism during anoxia becomes critical. The present study analyzes the organ-specific changes in PP-1 activities during anoxic submergence in the freshwater turtle, *Trachemys scripta elegans*, to determine whether control over protein phosphatases is a factor in anoxia-induced metabolic arrest, in general, and/or in glycogen metabolism, in particular. Furthermore, liver PP-1 was purified to homogeneity and the physical and kinetic properties of the enzyme from a lower vertebrate are described.

MATERIALS AND METHODS

CHEMICALS AND ANIMALS

Okadaic acid was from CalBioChem. Rabbit muscle phosphorylase b, phosphorylase b kinase, and all other reagents, column materials and enzymes were from Sigma. Adult turtles, *Trachemys scripta elegans*, of 8-12 inches shell length were supplied and treated as described in Chapter 4.

PREPARATION OF ^{32}P -LABELLED SUBSTRATES

^{32}P -Labelled phosphorylase a (10^6 cpm/nmol) was prepared according to Cohen et al. (1988) with following modifications: 30 mg phosphorylase b were incubated for 60 min at 30°C with 2 mg/ml phosphorylase kinase in a 1.0 ml mixture containing 100 mM Tris-HCl/100 mM sodium glycerol 1-phosphate, pH 8.2, 0.1 mM CaCl_2 , 10 mM magnesium acetate and 0.2 mM [γ - ^{32}P] ATP (10^6 cpm/nmol). Then an equal volume of 90 % saturated ammonium sulfate was added, and after standing for 30 min on ice, the suspension was centrifuged for 10 min at 10,000 g. The supernatant was discarded and the precipitate was resuspended in 1 ml of freshly prepared TMS buffer (20 mM Tris-HCl pH 7.0, 50 mM β -mercaptoethanol, 100 mM

NaCl) at 30°C. To remove excess ATP, the solution was then passed through 0.7 x 5 cm disposable plastic columns equilibrated at 30°C with TMS buffer containing a 0.5 ml layer of Norite A activated charcoal sandwiched between 1 ml layers of Sephadex G-25 (fine) above and below. The protein eluate was transferred into 6 mm dialysis tubing and dialysed against TMG buffer (20 mM Tris-HCl pH 7.0, 50 mM β -mercaptoethanol, 10 % v/v glycerol) overnight at 5°C. Aliquots (containing about 3 mg or 30 nmol phosphorylase *a*) were combined with an equal volume of 100 % glycerol and stored in 1.5 ml plastic tubes at -20°C until use.

TISSUE EXTRACTION AND ENZYME ASSAY

For analysis of protein phosphatase activity, frozen tissues were rapidly weighed and then immediately homogenized 1:10 w/v for liver and heart and 1:5 w/v for other organs using a Tekmar homogenizer and ice-cold Buffer A (20 mM Tris-HCl pH 7.4, 2 mM EDTA, 2 mM EGTA, 15 mM β -mercaptoethanol) containing the following freshly prepared protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 0.1 mM tosylphenylalanylchloromethane, 1 μ g/ml aprotinin, and 5 mM benzamidine. Homogenates were centrifuged in an IEC microfuge at 13,000 x g for 2 min. Supernatants were removed and layered onto small columns (5 ml, 0.5 ml supernatant per column) of Sephadex G-25 equilibrated in Buffer A at room temperature in order to remove low molecular weight metabolites that might interfere with protein phosphatase assays. The columns were centrifuged in an IEC benchtop centrifuge for 1 min and the desalted enzyme filtrate was collected. Dilution buffer (50 mM Tris-HCl pH 7.0, 0.1 mM EGTA, 15 mM β -mercaptoethanol 2 mg/ml bovine serum albumin) was then added to prepare dilutions of 1:100 v/v for liver and heart extracts and 1:25 v/v for other tissues; high dilutions are used to minimize the effects of inhibitor-1 or -2 which may be present in crude extracts.

Protein phosphatase activity in diluted extracts was measured at 30°C essentially as described by Ingebritsen et al. (1983). Unless otherwise indicated, the standard reaction mixture contained in a 50 μ l volume: 20 mM Tris-HCl pH 7.0, 0.1 mM EGTA, 10 mM β -mercaptoethanol, 5 mM theophylline, 2.5 nM okadaic acid, and 10 μ l of enzyme preparation. Reactions were initiated by the addition of 20 μ l of 32 P-labelled phosphorylase α (6×10^8 counts.min $^{-1}$. μ mol $^{-1}$) and terminated after 10 min by addition of 100 μ l ice cold 25 % w/v trichloroacetic acid/10 mM phosphoric acid. After standing in an ice bath for 5 min, all samples were centrifuged and then 100 μ l of each supernatant was removed, mixed with 1.0 ml dioxane-based scintillant, and counted. Two control reactions were always included and 32 P released under these conditions was subtracted as a blank: 1) zero time control - reaction stopped immediately by addition of the TCA/phosphoric acid solution, and 2) minus enzyme control - 10 μ l of buffer replaced the enzyme extract. Both controls gave similar results. Release of 32 P was restricted to less than 30% of the total radiolabelled substrate available to ensure linear reaction rates. Initial studies showed that 32 P-labelled phosphorylase was more stable in 50 % v/v glycerol at -20°C rather than storing at 4°C. One unit of activity is defined as the amount of enzyme that catalyses the release of 1.0 nmol phosphate from 32 P-labelled phosphorylase per min at 30°C. Assays of phosphatase activity with paranitrophenyl phosphate as the substrate were performed as described by Silberman et al. (1983).

PURIFICATION OF PP-1 FROM TURTLE LIVER

All steps were performed at 4°C unless otherwise indicated. Frozen liver from control turtles was homogenized 1:5 w/v in Buffer A containing protease inhibitors (see above) and then centrifuged at 5,000 g for 10 min. The supernatant was removed and poly(ethyleneglycol) 8000 was added to a final 10 % (w/v). After stirring on ice for 20 min, the suspension was centrifuged at 15,000 g for 15 min and then the supernatant

was discarded. The pellet was resuspended in the same volume of Buffer A (containing protease inhibitors) using a loosely fitting glass/glass homogenizer.

DEAE-cellulose chromatography

The resuspended extract was loaded onto a DEAE-cellulose (DE-52) column (2.5 x 20 cm) which had been equilibrated with buffer B (50 mM Tris-HCl pH 7.4, 0.1 mM EGTA, 15 mM β -MeSH, 10 % v/v glycerol). The column was washed with 10 vol of buffer B and then the enzyme was eluted using a linear gradient of 0-500 mM NaCl in buffer B. Two ml fractions were collected and assayed and peak fractions were pooled.

Blue Dextran chromatography

The pooled peak fractions were diluted 1:1 v/v with buffer B and then applied to a Blue Dextran column (2x 20 cm) equilibrated in buffer B. The column was washed with buffer B and then eluted with a linear gradient of 0-500 mM NaCl in buffer B. Peak fractions were pooled and dialyzed against buffer B with three change of buffer.

Second DEAE column chromatography

The enzyme was then loaded onto a second DEAE column equilibrated in buffer B and washed with 10 column volumes of the same buffer. Phosphatase was again eluted with a linear gradient of 0-500 mM NaCl. The enzyme from this column eluted as two separate peaks and the second, major peak was collected for further purification.

Sephacryl S-200 chromatography

Pooled peak II (4-6 ml) was then loaded onto a Sephacryl S-200 column (2.0 x 80 cm) equilibrated with buffer C (buffer B but with 20 % v/v glycerol) containing 200

mM NaCl. Fractions of 2 ml were collected, assayed for phosphatase activity and the active fractions were pooled and dialyzed against buffer B with two change of buffer.

ADP-Agarose affinity chromatography

For the final step in the purification, the pooled peak fractions from gel filtration were applied to an ADP-agarose column (1 x 4 cm) equilibrated with buffer C. The column was then washed with buffer C and the enzyme was eluted with a gradient of 0-300 mM NaCl in buffer C. Fractions containing phosphatase activity were pooled, dialyzed against buffer D (20 mM Tris-HCl, pH 7.2, 0.1 mM EGTA, 15 mM β -MeSH, 40 % v/v glycerol), and then used for kinetic analysis.

SDS-PAGE, PROTEIN ASSAY AND MOLECULAR WEIGHT DETERMINATION

Techniques for SDS-polyacrylamide gel electrophoresis, protein quantification, and molecular weight determination were as described in Chapters 3 and 5.

DATA AND STATISTICS

Data on organ activities of phosphatase (means $\pm$ SEM, n=4 animals per group) were assessed via analysis of variance followed by the Dunnett's test, two-tailed. Kinetic differences between peak I and peak II phosphatase were analyzed using the Student's t-test.

RESULTS

PP-1 and protein phosphatase 2A are the only phosphatases that show significant activity towards glycogen phosphorylase α . In order to measure PP-1 alone, assays were carried out in the absence of divalent cations (and with 0.1 mM EGTA added) in order to inhibit the cation-dependent protein phosphatase and with 2.5 nM okadaic acid

added, a level of the inhibitor that fully inactivates protein phosphatase-2A (Cohen, 1991). Assays conducted in the presence versus absence of 2.5 nM okadaic acid showed that 92 ± 3 % of the total phosphorylase α dephosphorylating activity in liver remained in the presence of the inhibitor, indicating that most of the phosphatase activity was type-1. Phosphatase activity remaining in the presence of 2.5 nM okadaic acid was also over 90 % of total in white and red skeletal muscle extracts and averaged 87 ± 5 % in heart and brain extracts.

Tables 6.1 and 6.2 shows the activities of PP-1 in five organs from aerobic and anoxic turtles. Activities were quantified as both U/gram wet weight and U/mg soluble protein in order to provide comparisons with previous studies and give an indication of any possible changes in tissue hydration level should these occur over the course of anoxia. *In vivo* anoxic submergence at 7°C resulted in a significant decrease in liver protein phosphatase activity. Enzyme activity dropped to about 64 % of the aerobic, control value within 1 h of anoxic submergence and remained at this reduced level over the remainder of the 20 h anoxia exposure. The effect was the same when activity was expressed per gram wet weight or per mg soluble protein. Red skeletal muscle showed a similar pattern; activity had dropped to about 65% of the control value after 1 h of anoxic submergence and continued to decrease over time reaching 43 % of the control value after 20 h of anoxia exposure. Brain showed a transient decrease in activity to 56% of the control value after 1 h anoxia, but activity rose again with longer exposure times. Phosphatase activity in heart and white skeletal muscle did not change during anoxia exposure. Table 6.1 and 6.2 also show organ-specific variation in PP-1 activity in *T. s. elegans*. The highest phosphatase activity was found in liver, about 10-fold higher activity in white muscle. Activities in control organs ranked in the order liver > heart > red muscle > brain > white muscle.

PURIFICATION OF PP-1 FROM TURTLE LIVER

TABLE 6.1
Protein Phosphatase Type-1 Activities in Turtle Liver and Heart

Organ	Condition	U/gww	U/mg protein
Liver:	Aerobic	31.1 $\pm$ 0.7	0.73 $\pm$ 0.03
	Anoxic 1 h	19.8 $\pm$ 3.9**	0.45 $\pm$ 0.08**
	Anoxic 5 h	22.8 $\pm$ 0.8*	0.58 $\pm$ 0.04*
	Anoxic 20 h	19.4 $\pm$ 2.0**	0.53 $\pm$ 0.04*
Heart:	Aerobic	14.5 $\pm$ 1.8	0.51 $\pm$ 0.05
	Anoxic 1 h	12.6 $\pm$ 1.9	0.43 $\pm$ 0.08
	Anoxic 5 h	15.1 $\pm$ 2.2	0.56 $\pm$ 0.09
	Anoxic 20 h	12.8 $\pm$ 1.5	0.5 $\pm$ 0.07

Data are means $\pm$ SEM, n =4. Assays were performed using 1:100 v/v dilutions of the extracts. Specific activities are expressed as both U/gww and U/mg protein, where one unit of activity is the amount that catalyses the release of 1.0 nmol phosphate per minute. Assay of enzyme activity was carried out at 30°C using phosphorylase α (2.0 mg/ml) as the substrate and in the presence of 0.1 mM EGTA and 2.5 nM okadaic acid.

** - Significantly different from the corresponding control value, P < 0.01; *
- P < 0.05.

TABLE 6.2
Protein Phosphatase Type-1 Activities in Turtle Organs

Organs	Condition	U/gww	U/mg Protein
Brain:	Aerobic	6.5 ± 0.8	0.19 ± 0.02
	Anoxic 1 h	3.7 ± 0.4*	0.1 ± 0.01*
	Anoxic 5 h	5.4 ± 0.7	0.17 ± 0.03
	Anoxic 20 h	5.2 ± 0.8	0.17 ± 0.02
Red muscle:	Aerobic	8.7 ± 1.6	0.36 ± 0.06
	Anoxic 1 h	5.6 ± 0.7*	0.23 ± 0.02*
	Anoxic 5 h	4.7 ± 0.4*	0.21 ± 0.02*
	Anoxic 20 h	3.8 ± 0.3**	0.17 ± 0.01**
White Muscle:	Aerobic	2.3 ± 0.2	0.10 ± 0.01
	Anoxic 1 h	2.5 ± 0.2	0.11 ± 0.01
	Anoxic 5 h	2.1 ± 0.4	0.11 ± 0.01
	Anoxic 20 h	1.9 ± 0.1	0.10 ± 0.01

Assays were performed using 1:25 v/v dilutions of the extracts. Other conditions as in Table 6.1.

** - Significantly different from the corresponding control value, $P < 0.01$;

* - $P < 0.05$.

Turtle liver was chosen for the purification of PP-1 and a new purification protocol was developed, summarized in Table 6.3. The purification scheme resulted in a homogeneous enzyme preparation with a 3000-fold purification factor and a reasonable (10 %) final yield. Phosphatase activity eluted off both the first DEAE-cellulose column and the Blue Dextran column in single peaks at about 200 mM and 250 mM NaCl, respectively. Chromatography on the second DEAE-cellulose column resulted in two peaks of phosphatase activity, a minor one eluting at 100 mM NaCl and a major peak eluting at 220 mM NaCl (Fig. 6.1). The partially purified enzyme in both peaks was subjected to kinetic analysis (Table 6.4). The enzymes showed identical behavior with respect to inhibition by okadaic acid and nucleotides but differed in their relative activities towards two substrates. p-Nitrophenylphosphate phosphatase (PNPP) activity was relatively higher in peak I whereas phosphorylase α phosphatase (PP α) activity was higher in peak II, the ratio PP α :PNPP activity being 2.4 and 13.3 in peaks I and II, respectively. SDS-PAGE of the proteins from peak I and II indicated molecular weights of 36 and 37 kD, respectively, and in both cases doublet bands were seen (Fig. 6.2b). The pooled fractions from peak II were further purified by Sephacryl S-200 gel filtration and then ADP-agarose affinity chromatography. Enzyme was eluted from the final column at 125 mM NaCl. Final specific activity of the purified enzyme was 3156 U/mg protein and a single protein band was seen on SDS-PAGE (Fig. 6.2a). The purified enzyme was stored in 40 % v/v glycerol at -20°C and was stable for at least for one month. SDS-PAGE of the final purified enzyme indicated a subunit molecular weight of 37 kD. Sephacryl S-200 gel filtration (done prior to ADP-agarose chromatography) showed a native molecular weight of $38,000 \pm 2000$ daltons ($n = 3$) for peak II (Fig. 6.3) and the same 38 kD ($n=1$) for peak I. In initial studies chromatography on heparin-Sepharose or aminohexyl-agarose, which have been used for the purification of mammalian protein phosphatase-1 (Erdoli et al. 1985; Schuchard

TABLE 6.3**Purification of Turtle Liver Protein Phosphatase Type-1**

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purifi- cation (fold)	Yield (%)
Crude extract	2700	2835	1.05	-	100
10% PEG	1160	2192	1.89	1.8	77
1st DEAE	145	1880	13	12.4	66
Blue-Dextran	56	1360	24	23	48
2nd DEAE	11	879	80	76	31
Sephacryl S-200	3	454	151	144	16
ADP-agarose	0.09	284	3156	3006	10

Values are the means of three separate purifications of the enzyme. Activity was measured as nmol phosphate transferred per minute (U) by the standard assay conditions.

Fig. 6.1 Elution profile of turtle liver protein phosphatase type-1 from the second DEAE-cellulose ion exchange chromatography (step 4 of the purification)

Pooled dialysed enzyme from the Blue-Dextran step was loaded on the column and elution was with a linear gradient of NaCl (0-500 mM) in buffer B. Closed circles show enzyme activity and open circles show protein absorbance at 280 nm. The salt gradient is indicated by the dotted line.

TABLE 6.4**Activities and Kinetic Constants of Turtle Liver Protein Phosphatase Type-1**

	Peak I	Peak II	Final purified enzyme
K_i Okadaic acid (nM)	12.3 ± 1.2	11.5 ± 2.1	12.6 ± 1.4
K_i AMP (μ M)	26.6 ± 3.1	25.4 ± 5.1	23.2 ± 2.3
K_i ADP (μ M)	109 ± 7.5	106 ± 9.5	98.5 ± 5.6
K_i ATP (μ M)	577 ± 27	560 ± 31	525 ± 23
K_i IMP (μ M)	396 ± 36	356 ± 43	389 ± 29
PNPP activity*	3.3 ± 0.2	$1.6 \pm 0.3^{**}$	ND
PPa activity*	7.9 ± 1.1	$21.1 \pm 3.2^{**}$	345 ± 9.5

Data are means $\pm$ SEM, n = 3 separate determinations.

* - Units of activity for p-nitrophenylphosphate phosphatase (pNPP) and phosphorylase phosphatase (PPa) activities are in nmol phosphate released per min per ml enzyme.

ND = not determined. **. Significantly different from the corresponding peak I value, $p < 0.025$.

Fig. 6.2 SDS-PAGE of turtle liver PP-1

(A) SDS-PAGE with silver staining of samples taken from different steps in the purification procedure; lanes from left to right are standards (a), samples taken after the Blue Dextrane (b), and ADP-agarose [2 lanes, unconcentrated (c) and concentrated (d) enzyme] columns, respectively. Standards from top to bottom were: myosine (205 KD), β -galactosidase (116 KD), glycogen phosphorylase b (97 kD), bovine serum albumin (66 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD).

(B) SDS-PAGE with Coomassie blue staining of the partially purified phosphatase peak I (right lane) and peak II (left lane) from the second DEAE-cellulose column.

Fig. 6.3 Molecular weight determinations using S-200 size exclusion chromatography of peaks I and II protein phosphatase from the second DEAE column

The $(K_d)^{1/3}$ of each protein was plotted against $(\text{molecular weight})^{1/2}$. Standards (closed circles) were: pyruvate kinase (230 kD), alcohol dehydrogenase (150 kD), bovine serum albumin (66 kD), and cytochrome c (12.4 kD). Turtle liver protein phosphatase is shown by the open circle.

and Killilea, 1992), was tested but turtle liver phosphatase activity could be retrieved from these columns.

KINETIC PROPERTIES OF PURIFIED TURTLE LIVER PP-1

The effects of various divalent cations on PP-1 activity were analyzed using the partially purified peaks I and II as well as the purified enzyme. Neither the pure nor the partially purified enzymes were activated by divalent cations including MnCl_2 , MgCl_2 , CaCl_2 , and $\text{Co}(\text{CH}_3\text{COO})_2$, tested at concentrations ranging from 0-8 mM and, indeed, salts were inhibitory at levels over 2 mM (data not shown). The dephosphorylation of phosphorylase α by mammalian and starfish PP-1 is inhibited by both protamine and heparin (Doree et al. 1983; Pondaven and Cohen, 1987). Both peak I and II phosphatase were inhibited by basic proteins such as histone and protamine; heparin also had an inhibitory effect. I_{50} values for protamine were 43 ± 4 and 46 ± 5 $\mu\text{g/ml}$ for peaks I and II PP-1 whereas I_{50} values for histone were 84 ± 7 and 90 ± 8 $\mu\text{g/ml}$ for peaks I and II, respectively. I_{50} values for heparin were 83 ± 6 for peak I and 78 ± 2 $\mu\text{g/ml}$ for peak II PP-1. It was postulated that limited proteolysis during purification might have been responsible for the appearance of the two peaks I and II found after DEAE chromatography. In an attempt to test this both peak I and II enzymes were incubated with trypsin (20 $\mu\text{g/ml}$) and Mn^{++} (0.8 mM) for 5 to 20 min at 22°C but the treatment had no effect on phosphorylase phosphatase activity.

Effects of phosphatase inhibitors on the partially purified peak I and peak II phosphatases and the final purified enzyme are shown in Table 6.4. Okadaic acid inhibition of the enzyme was apparent in crude extracts (Fig. 6.4) and was retained throughout purification. All three adenine nucleotides inhibited the phosphatase, AMP being the most potent inhibitor with a K_i of 23 μM for the final purified enzyme. IMP was also inhibitory but far less so than AMP (K_i IMP was about 17-fold higher than K_i AMP). There were no significant differences in the kinetic constants for any

Fig. 6.4 Effect of okadaic acid on turtle liver protein phosphatase activity

Turtle liver extracts were assayed in 10-fold (open circles) and 100-fold (closed circles) dilutions with increasing concentrations of okadaic acid in the absence of divalent cations and in the presence of 0.1 mM EGTA. Activities are relative to enzyme activity in the absence of okadaic acid.

inhibitor between the peak I, peak II, and purified enzymes. Guanosine nucleotides (GMP, GDP GTP) as well as the cyclic nucleotides (cAMP, cGMP) had no effect on enzyme activity. Cohen et al. (1988) reported that caffeine blocked the inhibitory effect of AMP and also activated the mammalian enzyme. However, this effect was not seen with turtle PP-1. Indeed, as Fig. 6.5 shows, the addition of 7.5 mM caffeine or theophylline along with AMP actually increased the inhibition of the enzyme (higher concentrations of these compounds were also ineffective in overriding AMP inhibition). Furthermore, neither caffeine nor theophylline activated turtle liver PP-1 in the absence of AMP.

DISCUSSION

Studies reported in previous chapters have documented the importance of protein kinases and protein phosphorylation in the regulation of metabolism (particularly glycogen metabolism) during anoxia in turtles. A two-phase response by protein phosphorylation systems was also identified, which correlates with the two phases of metabolic response to submergence (a hypoxic transition period where glycolytic rate is strongly activated followed by metabolic rate depression) that have been documented by other types of studies (Kelly and Storey, 1988; Lutz et al. 1984). Thus, within the first hour of anoxic submergence cAMP levels rose significantly and the percentage of active protein kinase A rose from 6 to 15 % in liver (Chapter 4). By 5 h of anoxia, however, both protein kinase A and phosphorylase kinase activities in liver were suppressed although phosphorylase *a* activity remained elevated (Chapter 4). Given the changing patterns of protein kinase activities during anoxic submergence, I predicted that protein phosphatase activities should also be modified during anoxia. The present results show that coordinated regulation of PP-1 activity in response to anoxia exposure is, indeed, involved in regulating glycogenolytic rate. PP-1 activity dropped by 34 % in liver within the first hour of anoxic submergence (opposite to the activation

Fig. 6.5 Effect of AMP on protein phosphatase activity and interaction with caffeine and theophylline effects

Increasing concentrations of AMP were added to the assay mixture in the presence of 7.5 mM caffeine (open circles), 7.5 mM theophylline (closed circles), or AMP alone (triangles). Values are the means of $n = 3$ separate preparations. Activities were normalized to the activity in the absence of AMP.

of protein kinase A) and remained reduced throughout the remainder of anoxia exposure. The initial response seen after 1 h of anoxic submergence, protein kinase activation (Chapter 4) and PP-1 inactivation, would together initiate events that activate glycogenolysis and glucose export from liver. The mechanism involved in PP-1 inhibition may be cAMP-dependent protein kinase stimulated phosphorylation and activation of protein phosphatase inhibitor-1 (Huang and Glinesmann, 1976). Although protein kinase A and phosphorylase kinase activities subsequently fall during prolonged anoxia, phosphorylase α activity is sustained, probably because protein phosphatase is not oppositely activated during prolonged anoxia. Hence, the normally oppositely-directed responses of the kinases and phosphatases in regulating glycogenolysis appears to be interrupted by metabolic arrest mechanisms that suppress both during prolonged anaerobiosis. A similar net suppression of both phosphatase and kinase regulation of glycogen metabolism appears to occur in red muscle where both PP-1 (Table 6.2) and protein kinase A activities (Chapter 4) are decreased within 1 h and sustained throughout anoxia. A transient decrease in PP-1 activity in brain after 1 h anoxia exposure may also be important in the metabolic transition to anoxic function in that organ.

The purified phosphatase showed the characteristics of a protein phosphatase type-1 and lacked properties that are typical of a PP-2 (ie. activation by cations, heparin and protamine; very potent inhibition by okadaic acid) (Cohen, 1991; Doree et al. 1983). Enzyme properties of turtle liver PP-1 were similar to mammalian PP-1 in several ways. Like the mammalian enzyme (Cohen, 1991), turtle liver PP-1 showed high activity with glycogen phosphorylase α as its substrate and was inhibited by okadaic acid, adenosine nucleotides, and basic proteins such as histone and protamine. The I_{50} value for okadaic acid of 12 nM for turtle PP-1 was within the range of 10-15 nM reported for mammalian PP-1 (Cohen, 1991). The subunit molecular weight of turtle PP-1 was also similar to the mammalian enzyme (Ganapathi et al. 1981;

Hemmings et al. 1982). In mammalian tissues the PP-1 catalytic subunit may complexed to other proteins (a modulator subunit) that can mask its activity in crude extracts (Baliou and Fischer, 1986; Cohen, 1989). This association can be released by partial digestion with trypsin, transforming the catalytic subunit into a truncated form dependent on Mn^{2+} (Price et al. 1986). However, we did not observe any increase in phosphorylase phosphatase activity after treatment of liver extracts with trypsin and then incubation with Mn^{2+} . This suggests that PP-1 complexing may not occur in turtle organs or that such complexes are sufficiently labile as to be disrupted by the normal extraction procedures.

CHAPTER 7

PROTEIN KINASE C AND ANOXIA SURVIVAL IN TURTLES Purification And Properties Of Liver PKC Isozymes

INTRODUCTION

Phospholipid and calcium-dependent protein kinase (PKC) is involved in the regulation of physiological responses to different stimuli, ranging from cellular proliferation, differentiation, and exocytosis to the control of neural activity (Nishizuka, 1984; 1986; 1988; Woodegett et al. 1987; Kikkawa et al. 1989). The activation of PKC is believed to occur upon redistribution of the inactive cytosolic enzyme to the phospholipid environment of membranes (Kikkawa et al 1982; Anderson et al. 1986). This subcellular redistribution is a consequence of the increased phosphoinositide turnover elicited by the activation of phosphatidylinositol-specific phospholipase C as the result of extracellular signals that trigger specific membrane receptors (Berridge and Irvine, 1989). Protein kinase C is activated *in vitro* by phosphatidylserine (PS) in the presence of Ca^{2+} and diacylglycerol (DAG). The DAG is generated *in vivo* from the phosphatidylinositol (PI) and other glycerolipids by activation of phospholipase C or D (Augert et al. 1989; Berridge, 1987). DAG increases the affinity of protein kinase C for Ca^{2+} and PS (Kaibuchi et al. 1981). It is now known that PKC covers a family of serine and threonine specific protein kinases consisting of at least nine isozymes that have been identified by cDNA hybridization techniques from a minimum of seven genes (Nishizuka, 1988; Kikkawa et al. 1989; Pears and Parker, 1991).

In previous studies I have shown that cAMP levels increase in turtle liver during the early hours of anoxia exposure *in vivo* and PKA is activated; protein phosphatase, by contrast, is inhibited throughout the anoxia exposure (Chapter 4 and 6). This study now examines the possible role of PKC in mediating metabolic responses by turtle liver to environmental anoxia. Overall activity was monitored by following enzyme translocation and PKC isozymes distribution. Furthermore, PKC isozymes from turtle liver were purified and the kinetic properties of the enzymes from a lower vertebrate were analyzed.

MATERIAL AND METHODS

CHEMICALS AND ANIMALS

All phospholipids, diacylglycerol, and fatty acids were obtained from Serdary Research Laboratories (London, ON). Nitrocellulose sheets were from Schleicher and Schuell (Keene, NH). Antibody reagents were obtained from Gibco BRL. Histone H1, protamine, column materials and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Adult turtles, *T. s. elegans*, of 8-12 inches shell length were supplied and experimentally treated as described previously (Chapter 4).

PREPARATION OF LIVER HOMOGENATE

All steps were performed at 4°C. Samples of frozen liver were homogenized in 1:5 (w:v) in ice cold buffer A (0.25 M sucrose, 10 mM β -mercaptoethanol, 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 2 μ g/ml aprotinin, and 20 mM HEPES, pH 7.4) for 30 s in an Ultra-Turrax (Tekmar) tissue homogenizer. After centrifugation at 1800 x g for 8 min, the supernatant was removed and centrifuged at 100,000 g for 30 min to yield pellet (membrane) and supernatant (cytosol) fractions. The supernatant was immediately loaded onto a Whatman DE-52 ion-exchange column (1.0 ml of bed volume for 0.1 g tissue) equilibrated in buffer A and washed with 5 vol of buffer B (20 mM HEPES pH 7.4, 20 mM NaCl, 10 mM β -mercaptoethanol, 1 mM EGTA, 1 mM EDTA, 10% v/v glycerol), and then 5 vol of buffer B containing 50 mM NaCl, respectively. Neither the 20 nor 50 mM NaCl washes contained detectable PKC activity. Enzyme activity was then eluted by washing the column with 4 ml of 200 mM NaCl in buffer B; this concentration was optimal as determined by initial trials that monitored enzyme elution in a NaCl gradient. The pellet was rehomogenized in one-half the original volume of buffer A + 0.1 % v/v Triton X-100, incubated on ice for 20 min, and then centrifuged

at 100,000 g for 30 min. The resulting supernatant (containing the membrane associated fraction) was loaded onto a fresh DE-52 column and treated in an identical fashion as the cytosol fraction.

PKC isozymes from liver of control and anoxic turtles were identified by hydroxylapatite column chromatography. Samples of enzyme eluted from the DE-52 column by the 200 mM salt bump (2 ml of the 4 ml total) were diluted 1:4 v/v with buffer C (20 mM potassium phosphate, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β -mercaptoethanol, and 10% v/v glycerol) and then loaded onto hydroxylapatite columns (1 x 5 cm) pre-equilibrated with buffer C. The columns were washed with buffer C until eluent absorbance at 280 nm was zero. Then the PKC isozymes were eluted with linear gradient of 0-300 mM potassium phosphate in buffer C and 0.75 ml fractions were collected and assayed for enzyme activity.

PROTEIN KINASE C ASSAY

A) Standard assay

For the standard assay, the reaction medium contained 30 mM HEPES, pH 7.4, 5 mM MgCl_2 , 5 mM β -mercaptoethanol, 0.4 mM CaCl_2 , 20 μM ^{32}P -labelled ATP (4.5×10^5 cpm), 6.5 $\mu\text{g/ml}$ phosphatidylserine, 1 $\mu\text{g/ml}$ diacylglycerol (phospholipids were dissolved in assay buffer and sonicated for 1 min under nitrogen gas), and a phosphate-accepting substrate, either 40 $\mu\text{g/assay}$ of histone H1 (type III-S, Sigma) or 40 $\mu\text{g/assay}$ protamine or 75 μM FKKSFKL- NH_2 peptide substrate (the amino acid sequence believed to be the target of PKC in MARKS protein; Graff et al. 1989; Chakravathy et al. 1991), and suitable amounts of enzyme. The reaction was allowed to incubate at 30°C for 7 min; this time and temperature were optimal for the turtle liver enzyme as determined in preliminary studies. The reaction with histone or protamine was terminated by adding 2 ml of ice-cold 10% w/v trichloroacetic acid + 10 mM phosphoric acid and incubating 10 min on ice. Samples were then filtered through

Whatman GF/C filter papers and washed 4 times with the TCA/H₃PO₄ solution. The radioactivity retained on the GF/C glass fiber filters after washing was counted using Cerenkov radiation. Assays conducted with the peptide as the substrate (sometimes also for histone or protamine) were terminated by spotting samples (40 μ l) onto 2 x 2 cm pieces of Whatman P81 paper which were then washed 4 times with 75 mM phosphoric acid and counted as above. PKC activity was determined in the presence of calcium plus phosphatidylserine; nonspecific enzymatic activity was measured in the absence of phosphatidylserine and blank values were subtracted. One unit of PKC was defined as the amount of enzyme that catalyzes the incorporation of 1 pmol of phosphate onto a phosphate acceptor per minute at 30°C.

B) Mixed micelles assay.

The mixed micelles assay was conducted as described by Bell et al. (1985). To prepare micelles, the desired amounts of lipids were dissolved in chloroform/methanol (19:1, v/v), dried under a stream of nitrogen in a glass tube, and then solubilized in HEPES buffer containing 0.3 % v/v Triton X-100 by vortexing and incubating at 30°C for 10 min. Aliquots of these micelle preparations were added to the PKC assay previously described, in place of the sonicated lipids. The final concentration of Triton X-100 in the assay was 0.06 % (v/v).

C) Trypsin digestion experiments.

For the trypsin digestion experiments, samples of PKC peaks fractions from hydroxylapatite chromatography were incubated at 21°C in PKC assay buffer without Ca⁺⁺ and Mg⁺⁺ but containing 2 μ g/ml trypsin (Sigma). The digestion was terminated by adding soybean trypsin inhibitor (0.5 mg/ml) and putting the sample on ice. Then PKC activity was determined with the mixed micelles assay described above.

PURIFICATION OF PKC

Protein kinase C from liver of control turtles was purified as follows with all steps performed at 4°C unless otherwise stated. Previously frozen liver samples were homogenized with a Tekmar tissue homogenizer in 6 vol buffer A containing 0.1% v/v Triton-X 100. The homogenate was centrifuged for 5 min at 2000 x g at 4°C. The supernatant was removed to a new tube and then centrifuged at 100,000 x g at 4°C for 30 min. This supernatant was removed and applied to a DEAE-cellulose (DE-52, Whatman) column (3 x 20 cm) which was equilibrated with buffer B. The column was washed with 40 ml buffer B and then protein kinase C was eluted with a linear gradient of 0-400 mM NaCl in buffer B. The active fraction were pooled and brought to 1 M NaCl and were then applied to a phenyl-Sepharose column (3 x 17 cm), which was equilibrated with buffer B containing 1 M NaCl. After washing the column with 50 ml of buffer B + 1 M NaCl, PKC was eluted by decreasing the concentration of NaCl linearly from 1.0 to 0 M in 100 ml of buffer B, followed by washing with 50 ml of buffer B. The enzyme eluted in a single peak at 50 mM NaCl. The active fractions were collected and diluted with an equal volume of buffer C and then applied to a hand-packed hydroxylapatite column (1.5 x 10 cm) which was equilibrated with buffer C. The column was washed with 20 ml of buffer C and isozymes were separately eluted by application of a linear concentration gradient of potassium phosphate (20-300 mM) prepared in buffer C at a flow rate of 0.2 ml/min. Fractions of 1 ml were collected. Two distinct fractions of protein kinase C were obtained; peak I in fractions 28 through 35 eluting at a mean of 85 mM salt and peak II in fractions 42 through 47 eluting at a mean of 130 mM potassium phosphate. The fractions of each peak were pooled, dialyzed overnight against buffer B, and then used for kinetic analysis.

WESTERN BLOT ANALYSIS OF PKC ISOZYMES

The purified isoenzymes retrieved from the hydroxylapatite column were concentrated using Centricon-30 microconcentrators (Amicon, Danvers, MA). Proteins in these concentrated samples were then subjected to SDS-polyacrylamide gel electrophoresis with a 1.0 mm thick slab gel by the method of Laemmli (1970) with 8% acrylamide separating gels and 5% stacking gels. Proteins on the gel were electrophoretically transferred to a nitrocellulose membrane at 10 mA/cm² for 2 h by the method of Towbin et al. (1979) using a semi-dry electroblotter (Panther-TM) and were then processed by Western blot analysis as recommended by the supplier of the PKC antibodies, anti PKC α and anti PKC β (rabbit anti-peptide antibody; Gibco BRL). Immunoblots were developed by treatment with an alkaline phosphatase-conjugated goat anti-rabbit antibody and the color reaction was developed with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

DATA ANALYSIS

Statistical analysis used the Student's t-test (two-tailed) or one-way analysis of variance (ANOVA) followed by the Dunnett's test (two-tailed).

RESULTS

PKC ACTIVITIES AND TRANSLOCATION IN THE TURTLE LIVER

Table 7.1 shows the activity of PKC in liver of aerobic, control turtles versus turtles subjected to anoxic submergence for 1, 5 or 20 h at 7°C. The total PKC activity in turtle liver (cytosolic + membrane-associated, assayed with histone as substrate) did not change significantly during anoxia exposure. However, anoxia exposure altered the relative amount of PKC in liver membrane fractions. After 1 h of anoxia exposure the percentage of enzyme that was membrane-associated had risen from 21% in controls to 45% in anoxic liver. Longer anoxia exposures times caused membrane-associated enzyme activity to decrease again to the control level.

Table 7.1**Effect of anoxia on protein kinase C activity in turtle liver**

Condition	Cytosol pmol/mg protein	Membrane pmol/mg protein	% Membrane associated
Control	33.2 $\pm$ 2.79	9.25 $\pm$ 1.97	21
1 h Anoxic	27.4 $\pm$ 1.63	22.3 $\pm$ 1.45*	45
5 h Anoxic	32.2 $\pm$ 2.53	12.5 $\pm$ 1.27	28
20 h Anoxic	30.6 $\pm$ 3.44	7.61 $\pm$ 2.19	20

Values are means $\pm$ SEM, n=4 animals in each condition for assays with histone as the substrate. * - Significantly different from all other values for membrane-associated PKC as determined by one-way analysis of variance followed by the Dunnett's test (2-tailed), $P < 0.001$.

Hydroxylapatite chromatography of the PKC eluant from DE-52 columns revealed the presence of two PKC isozymes in turtle liver extracts (Fig. 7.1). These were identified by the relative position of their elution peaks, as PKC type II (β) and type III (α), using the nomenclature of Huang et al. (1987). Analysis by isozyme-specific antibodies (Makowske et al. 1988) also identified the two isoenzymes as the α and β forms (Fig. 7.2B). Comparison of the isozyme distribution pattern of cytosolic and membrane-associated PKC activities revealed that after 1 h of anoxia exposure 40% of the type II (β) and more than 80% of the type III (α) isozyme were translocated from the cytosol to the membrane (Fig 7.1B and D). The loss of enzyme from the cytosolic fractions was reflected in increased amounts detected in the membrane-associated fraction (Fig. 1A versus C). With longer anoxia exposures, the amount of membrane-associated activity decreased again, and the PKC distribution pattern was not different from that seen in control liver (data not shown).

PURIFICATION OF PKC ISOZYMES

The purification of the turtle liver PKC isozymes is summarized in Table 7.2. PKC activity was eluted as a single peak off both the DE-52 and phenyl Sepharose columns; the peaks eluted at about 150 mM NaCl and 50 mM NaCl, respectively. Hydroxylapatite column chromatography separated PKC activity into two peaks that eluted at about 85 and 130 mM phosphate. The purification scheme resulted in a 1381-fold purification with a yield of 17% for peak I (or type II PKC) and a 982-fold purification of peak II (type III PKC) with a yield of 16%. The final specific activity of the purified isozymes, when assayed with histone as the substrate, was 58,000 and 41,250 pmol phosphate transferred per minute per mg protein for type II (β) and type III (α), respectively. SDS-polyacrylamide gel electrophoresis of the purified peaks showed a single protein band for each type with a molecular weight of 80 ± 2 kD for

Fig. 7.1 Hydroxylapatite chromatography of liver PKC type II (β) and III (α) isozymes from normoxic and 1 h anoxic turtles

A) Normoxic cytosol, (B) 1 h anoxic cytosol, (C) normoxic membrane, and D) 1 h anoxic membrane fractions. Samples (2 ml) of eluant from DE-52 chromatography of cytosol or membrane fractions were diluted and loaded onto a hydroxylapatite column (5 x 1 cm) and developed as described in the Materials and Methods. Fractions (0.75 ml) were collected and assayed for PKC activity. Activities in the presence of phosphatidylserine, diacylglycerol and CaCl_2 (filled circles), or in the presence of 0.5 mM EGTA alone (open circles) are shown. Line shows the gradient of potassium phosphate.

Fig. 7.2 SDS-Polyacrylamide gel electrophoresis and immunoblot analysis of PKC isozymes from turtle liver

(A) SDS-PAGE (with Coomassie Blue staining) of various fractions from the purification: a) 100,000 g supernatant, b) DE-52 pooled peak, c) Phenyl Sepharose peak, d) hydroxylapatite peak I, and e) hydroxylapatite peak II. The standards from top to bottom were: phosphorylase α (97 kD), bovine serum albumin (66 kD), ovalbumin (45 kD) and carbonic anhydrase (29 kD).

(B) Centricon-concentrated enzyme fractions of peak I and peak II enzyme from hydroxylapatite column were submitted to SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. Lanes 1 and 3 contain turtle liver type II (β) PKC whereas lanes 2 and 4 have type III (α) PKC. Membranes were incubated with anti-PKC β (lanes 1 and 2) and anti-PKC α (lanes 3 and 4).

Table 7.2
Purification of protein kinase C isozymes from aerobic turtle liver

Steps	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (fold)	Yield (%)
Homogenate	500	21000	42	1.0	100
DE-52	54	24100	446	10.6	115
Phenyl Sepharose	11	10670	970	23.0	50
Hydroxylapatite:					
Peak I	0.06	3480	58000	1381	17
Peak II	0.08	3300	41250	982	16

These data are replicates of four experiments which used histone as the substrate in the standard assay.

Fig. 7.3 Activation of purified turtle liver protein kinase C by TPA: (A): type II (β), and (B): type III (α)

Assay conditions used 40 μ g histone H1S, varying concentrations of TPA and other conditions as follows: (filled circles) 50 μ M phosphatidylserine + 0.4 mM CaCl_2 ; (open circles) 50 μ M phosphatidylserine without CaCl_2 ; or (filled diamonds) 0.4 mM CaCl_2 without phosphatidylserine.

Data are presented relative to the maximum activity obtained in the presence of phosphatidylserine, CaCl_2 , and optimal 100 nM TPA. Values are means of three determinations on separate preparations of purified enzyme.

both peaks (Fig. 7.2A). Each purified protein band reacted with one of the anti-peptide antibodies specific for each isozyme, as shown in Fig. 7.2B.

Experiments with specific protein kinase C inhibitors confirmed the identification of the two enzyme activities in turtle liver as PKC isozymes (Table 7.3). The specific PKC inhibitors staurosporine, PKC-I (19-36), and calphostin C (House and Kemp, 1987; Koyabashi et al. 1989; Tamaoki et al. 1986) strongly decreased the activities of both the type II (β) and type III (α) enzyme with IC_{50} values in the submicromolar and micromolar range, respectively. H7 [1-(5-isoquinoliny) sulphonyl]-2-methyl-piperazin), another compound believed to be a specific inhibitor of PKC (Kawamoto and Hidaka, 1984), also inhibited the enzyme in the micromolar range.

KINETIC CHARACTERIZATION OF PURIFIED PKC ISOZYMES

A) Enzyme Activation

Both isozymes were activated by TPA (12-O-tetradecanoylphorbol-13-acetate) to different extents in the presence Ca^{2+} or phosphatidylserine (Fig. 7.3). In the presence of both Ca^{2+} and phosphatidylserine, TPA caused marked activation of both types. In the presence of phosphatidylserine alone, TPA activated type II (β) to 50 % of its maximum but TPA activation in the presence of Ca^{2+} alone reached only 30% of the maximal activity (Fig. 7.3A). By contrast, type III (α) PKC responded well to TPA activation in the presence of Ca^{2+} (but without phosphatidylserine), reaching about 60% of full activity, whereas in the presence of phosphatidylserine (but minus Ca^{2+}) enzyme activity reached only about 35% of maximum (Fig. 7.3B).

B) Phospholipid Specificity and Ca^{2+} Requirement.

Table 7.4 shows the effect of different phospholipids in activating both isozymes. Phosphatidylserine (PS) was the most effective activator of either isozyme, but substantial activation was also seen with phosphatidylinositol (PI) and

Table 7.3**Effect of different inhibitors on purified type II (β) and type III (α) protein kinase C from turtle liver**

Protein kinase C was assayed with 1,2-dioleoylglycerol (1 μ g/assay), CaCl_2 (0.4 mM), and phosphatidylserine (6.5 μ g/assay). Substrates used are histone H1 (40 μ g/assay) or peptide (75 μ M). Other assay conditions are as described in the Materials and Methods.

Inhibitor	Substrate	Type II (β)	Type III (α)
		$I_{50} \mu\text{M}$	$I_{50} \mu\text{M}$
Staurosporine	Histone H1	0.04 ± 0.002	0.08 ± 0.002
	Peptide	0.007 ± 0.001	0.009 ± 0.001
H7	Histone H1	4.74 ± 0.71	5.22 ± 0.31
	Peptide	1.23 ± 0.21	1.72 ± 0.12
PKC-I (19-36)	Histone H1	4.82 ± 0.70	6.32 ± 0.53
	Peptide	0.24 ± 0.03	0.28 ± 0.01
Calphostin C	Histone H1	9.60 ± 1.32	14.1 ± 3.50
	Peptide	4.82 ± 1.90	4.83 ± 0.93

Values are means $\pm$ SEM for $n = 4$ separate preparations of purified enzyme.

Fig. 7.4 Activation of purified turtle liver PKC by different phospholipids at various concentrations of CaCl_2 : (A): type II (β), and (B): type III (α)

PKC was assayed with 40 μg histone, 1 μg 1,2-dioleoylglycerol, varying CaCl_2 concentrations as indicated and phospholipids as follows: (filled circles) 50 μM phosphatidylserine; (open triangles) 50 μM phosphatidylinositol; (filled triangles) 50 μM lysophosphatidylinositol; or (open circles) no phospholipid added. Assays run with zero added CaCl_2 contained 0.5 mM EGTA.

Values are means of three determinations on separate preparations of purified enzyme.

Table 7.4**Effect of metabolite activators on the activities of type II (β) and type III (α) protein kinase C from turtle liver**

Protein kinase C was assayed with 1,2-dioleoylglycerol (1 μ g/assay), CaCl_2 (0.4 mM), peptide substrate (75 μ M) and phospholipids (50 μ M) as indicated. Data show activities relative to activity in the presence of PS.

	Protein kinase C activity	
	Type II (β)	Type III (α)
Phosphatidylserine	100	100
Phosphatidylinositol	69	98
Lysophosphatidylinositol	43	82
Lysophosphatidylcholine	20	15
Phosphatidylethanolamine	9	8
Phosphatidylcholine	1	14
Cardiolipin	15	21
Oleic acid	4	10
Inositol triphosphate	11	23
No additions	2	1

Values are means of three determinations on separate preparations of purified enzyme.

lysophosphatidylinositol (LPI). Phosphatidylethanolamine, phosphatidylcholine, lysophosphatidylcholine and cardiolipin showed lesser activation of the enzyme and oleic acid and inositol triphosphate also activated to low extents.

The effect of Ca^{2+} on PKC activity is shown in Fig. 7.4; the concentration of Ca^{2+} was varied between 1 nM to 1 mM and enzyme activity was analyzed in the presence of PS + DAG, PI + DAG, LPI + DAG, or DAG alone. The two PKC isoenzymes exhibited differences in phospholipid activation and both required Ca^{2+} ion for activation (Fig. 7.4). Type II showed low activation in the presence of DAG alone, even at high Ca^{2+} concentrations, but the type III isozyme with DAG alone showed increasing activity with higher Ca^{2+} concentrations. PI and LPI activated both isozymes and PI, in particular, gave maximal rates nearly equal to those with PS, but maximal rates with both compounds required much higher Ca^{2+} concentrations than those needed with PS. The two PKC isoenzymes showed different specificity for PI and LPI (Table 7.4 and Fig. 7.4). The type III (α) isozyme was highly activated by PI and LPI (Fig. 7.4B) but, type II (β) PKC was activated to a lower extent.

Fig. 7.5 and 7.6 show the effects of different phospholipids on PKC activity in the presence of two substrates, histone or peptide. At higher concentrations, PI was just as effective as PS in activating the type III (α) enzyme using either histone H1 (Fig. 7.5B) or peptide (Fig. 7.6B) as the substrate. PI also came close to the maximum activation with one of the type II substrates whereas LPI was a relatively poor activator under all circumstances, particularly with the type II enzyme. Phosphorylation of a third substrate, protamine, was phospholipid dependent but Ca^{2+} independent (Fig. 7.7). Both isozymes phosphorylated protamine, but the maximum reaction rates were lower than those for the other substrates. Relative V_{max} activities for protamine as the substrate were low; activity ratios for type II PKC were 100:64:22 for peptide vs. histone vs. protamine and 100:61:22 for type III PKC (Table 7.5). Table 7.5 also shows K_m values for the three substrates. K_m values for the peptide substrate were not

Fig. 7.5 Activation of purified turtle liver type II (β) and III (α) protein kinase C by phosphatidylserine, phosphatidylinositol and lysophosphatidylinositol

PKC was assayed in the presence of 1 μ g 1,2-dioleoylglycerol and 0.4 mM CaCl_2 with 40 μ g histone as the substrate. Panels A shows type II (β) PKC and panels B shows type III (α). Symbols show varying concentrations of: (open circles) phosphatidylserine; (open triangles) phosphatidylinositol; or (closed circles) lysophosphatidylinositol.

Values are means of three determinations on separate preparations of purified enzyme.

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PRECISIONSM RESOLUTION TARGETS

Fig. 7.6 Activation of purified turtle liver type II (β) and III (α) protein kinase C by phosphatidylserine, phosphatidylinositol and lysophosphatidylinositol

PKC was assayed in the presence of 1 μ g 1,2-dioleoylglycerol and 0.4 mM CaCl_2 with 75 μ M peptide as the substrate. Panels A shows type II (β) PKC and panels B shows type III (α). Symbols show varying concentrations of: (open circles) phosphatidylserine; (open triangles) phosphatidylinositol; or (closed circles) lysophosphatidylinositol.

Values are means of three determinations on separate preparations of purified enzyme.

Fig. 7.7 Activation of protein kinase C by phosphatidylserine and calcium using protamine as the substrate: (A): type II (β), and (B): type III (α)

PKC was assayed by the mixed micelle assay with 40 μ g protamine as the substrate, 1 μ g 1,2-dioleoylglycerol, varying concentrations of phosphatidylserine, and (filled circles) 0.4 mM CaCl_2 , or (open triangles) 0.5 mM EGTA.

Values are means of three determinations on separate preparations of purified enzyme.

Fig. 7.8 Effect of proteolysis by trypsin on purified protein kinase C: (A): type II (β), and (B): type III (α)

Purified type II (26 μg protein) or type III (28 μg protein) PKC was incubated in a final volume of 500 μl with 2 $\mu\text{g}/\text{ml}$ trypsin at 21°C. At the times indicated, 20 μl samples were withdrawn and PKC activity was assayed by the mixed-micelle method in the absence (open triangles) or presence (filled circles) of 6.5 μg phosphatidylserine + 1 μg 1,2-dioleoylglycerol. Activities are given relative to the activity of enzyme incubated without trypsin.

Values are means of three determinations on separate preparations of purified enzyme.

Table 7.5
Kinetic constants of turtle liver PKC isozymes

Substrate	type II (β)	Type III (α)
Histone H1:		
K_m , $\mu\text{g/ml}$	94.4 ± 4.2	$62.6 \pm 6.1^*$
$V_{\max}$, pmol/min/mg protein	47153 ± 1525	42028 ± 1736
Protamine:		
K_m , $\mu\text{g/ml}$	23.2 ± 1.9	$37.8 \pm 2.8^*$
$V_{\max}$, pmol/min/mg protein	16261 ± 712	15256 ± 623
Peptide:		
K_m , $\mu\text{mol/l}$	19.6 ± 2.5	15.6 ± 1.9
$V_{\max}$, pmol/min/mg protein	74165 ± 1028	68547 ± 1916

Values are means $\pm$ SEM, for n=3 separate preparations of purified enzyme.

* - Significantly different from the corresponding value for type II PKC as determined by the Student's t-test (two-tailed), $p < 0.025$.

significantly different between the two isozymes but type II (β) PKC showed a significantly greater affinity for protamine and a lower affinity for histone, as compared with type III (α).

C) Activation of PKC Isozymes by Tryptic Digestion.

After PKC is translocated to the membrane, it is cleaved at a hinge region between the regulatory and the catalytic domain by the Ca^{2+} dependent protease calpain (Nishizuka, 1988; Kikkawa et al. 1989). This leads to the removal of the regulatory subunit and releases the constitutively active phospholipid-independent catalytic subunit of PKC. To test for protease activation of turtle liver PKC, the purified isoenzymes were incubated with trypsin and the effect of proteolysis on enzyme activity was monitored over time (Fig. 7.8). Neither isozyme showed activation by tryptic proteolysis, and indeed, the activity of the type II (β) enzyme decreased sharply during the incubation (Fig. 7.8A). However, the activity of type III (α) PKC was resistant to the proteolysis (Fig. 7.8B).

DISCUSSION

Protein kinase C is found both in the cytosol and bound to the plasma membrane. Alterations in the subcellular distribution of protein kinase C appears to be an important regulatory mechanism to allow the preferential Ca^{2+} -mediated phosphorylation of specific endogenous protein substrates. The present work has analyzed the effects of anoxia on the subcellular distribution and activation of PKC in turtle liver. Analysis of PKC distribution over the time course of anoxia exposure, compared with normoxic control liver, showed that changes in PKC activity may be intimately associated with the metabolic reorganization that accompanies the anoxia response in turtle liver. Changes in PKC activity in anoxia were inferred from the changes in the percentage of enzyme that was associated with the membrane fraction

during anoxia (Table 7.1). In several mammalian cell lines (pineal cells, liver cells, skeletal muscle) increased enzyme activity is associated with a translocation of the enzyme from cytosol to the membrane fractions (Boneh and Tenenhouse 1988; Ho et al. 1988; Cleland et al. 1989). Changes in the subcellular compartmentalization of this Ca^{2+} -dependent kinase probably reflect the sites of action of different isozymes in regulating specific physiological functions. If so, the regulation of the association-dissociation of protein kinase C isozymes with membranes will be of critical importance in mediating biological processes influenced by this kinase. The data in Fig. 7.1 shows that the patterns of distribution of the two isozymes in turtle liver are different. After 1 h of anoxia exposure 80 % of PKC- α and 40% of PKC- β were translocated to the membrane fraction. These results suggest that either: (1) the different isozymes of turtle liver PKC have different metabolic functions in anoxia, or (2) depending on cell metabolic activity, changes in the levels of different fatty acids or phospholipids during anoxia may induce the redistribution of different PKC isozymes. For example, increased oleate concentration in isolated hepatocytes caused translocation of PKC- β from cytosol to the membrane (Diaz-Guerra et al. 1991).

Kinetic characterization of the turtle liver PKC isozymes showed that they were similar to their mammalian counterparts in several ways. The enzymes exhibited high affinity for phospholipids and were activated by micromolar concentrations of Ca^{2+} . The enzymes were also strongly affected by compounds known to specifically inhibit the mammalian enzyme: staurosporine, PKC-I, and calphostin C.

The variety of metabolites generated by inositol lipid metabolism (Downes et al. 1989) that might potentially be involved in cell signaling has raised the question of whether or not PKC isozymes may be activated *in vivo* by phospholipids other than just DAG and phosphatidylserine. Using the purified PKC isozymes, the present study showed that a full activation of PKC α could be achieved by phosphatidylinositol at low concentrations of Ca^{2+} (Fig. 7.4). Furthermore, PKC α was activated by

lysophosphatidylinositol to a greater extent than was PKC β (Fig. and Table 7.4). These findings suggest the possibility that the two isozymes can be differentially regulated by intermediates of inositol metabolism. This implies that PKC activity may depend not only on the production of DAG by phospholipase C, but also on the activity of the phosphatidylinositol kinases and phosphatases.

My previous results have documented an activation of PKA and an increase in cAMP levels during the first hour of anoxia in turtle liver. Combined with the present results for PKC, this information indicates that anoxia exposure modifies the activities of at least two protein kinase-mediated signalling systems in liver cells, each of which may regulate different and specific parts of the overall metabolic response to anoxia. Furthermore, the potential for crosstalk between these two protein kinase systems sets up the possibility of another level of metabolic control in coordinating cellular responses to anoxia that remains to be explored.

CHAPTER 8

GENERAL CONCLUSIONS

One of the most powerful mechanisms for producing coordinated changes in the activity states of proteins and enzymes in the cell is posttranslational modification via protein phosphorylation or dephosphorylation reactions. A single extracellular signal, transmitted through one or more membrane receptor types, changing the concentrations of intracellular second messenger molecules, and activating specific protein kinases, which in turn phosphorylate a variety of intracellular target enzymes/proteins, can alter the activities of diverse cellular processes in a coordinated fashion. In the studies described in this thesis I have used two different model animals to study the regulation of glycogenolytic enzymes via reversible protein phosphorylation during environmental stress conditions. Fish provided a useful model for analyzing reversible phosphorylation control of exercise metabolism because of their fast-start performance capabilities (Webb, 1978) in which the glycolytic pathway is rapidly activated. Freshwater turtles, which can endure prolonged submergence anoxia, provided an excellent model for studying the kinase/phosphatase control of metabolism in a situation where metabolic rate must be suppressed in a coordinated manner to only 10-20% of aerobic, control values (Herbert and Jackson, 1985).

Glycogen phosphorylase b from trout white muscle was purified to homogeneity with more than 204-fold purification. The enzyme was inhibited by glucose and ATP, with glucose inhibition decreasing at lower pH values. IMP did not affect the enzyme activity. The catalytic properties and Michaelis constants of trout phosphorylase b indicated that the enzyme would be virtually inactive at the physiological concentrations of substrates (glycogen and P_i) and activator (AMP) found in resting trout white muscle. The pH of the medium was also found to play an important role in the regulation of the enzyme both *in vitro* and physiologically. At low pH values, the enzyme appeared to be less active with a low affinity for glycogen at optimal substrate concentrations. However, in the presence of physiological concentrations of substrates, affinity for glycogen was not affected by pH, but affinity for AMP increased two-fold

at both pH 7.4 and 6.6. The data suggested that changes in cellular pH, ATP, P_i , and AMP levels during burst swimming exercise could allow phosphorylase b to augment phosphorylase a activity and make a substantial contribution to overall glycogenolysis in working trout muscle.

During exhaustive swimming exercise the complete cascade for activating glycogen phosphorylase was present in trout organs and all components were activated in white skeletal muscle and liver. GPK and PKA showed the highest activities in the liver, being three and four fold higher than corresponding activities in white muscle. Exercise stimulated a 60% increase in GPK activity in the liver and a 40% rise in white muscle. Furthermore, the amount of active PKA rose from 12% to 21% in the liver and from 32% to 57% in white muscle after exhaustive exercise, and at the same time the cellular levels of cAMP increased by 50% in liver and by 70% in white muscle of exercised fish. When extracts from both control and exercised animals were incubated with the catalytic subunits of mammalian PKA, GPK activity in liver, muscle and heart was strongly stimulated, activity rising 6 to 7-fold in white muscle and 2 to 2.6-fold in liver and heart, respectively. This occurred in extracts from both control and exercised fish and suggested that even in fish exercised to exhaustion, the maximal enzymatic potential for activation of glycogenolysis was not expressed. Other modes of GPK activation were not apparent, for the enzyme in the crude extracts was stimulated only by incubation with cAMP and did not respond to other second messengers such as cGMP and Ca^{2+} + TPA. Thus although GPK and PKA are activated in trout muscle during exercise the capacity of the phosphorylation cascade may be limiting in determining the amount of phosphorylase b that can be converted to phosphorylase a during swimming exercise. Not only are the activities of these enzymes lower both in absolute terms and relative to phosphorylase as compared with the situation in liver, but neither enzyme was maximally activated during exercise. Since glycogenolysis in working trout muscle is, in fact rapidly activated and glycogen reserves are virtually

fully depleted in fish swum to exhaustion (Parkhouse et al. 1987; Dobson et al. 1987), allosteric activation of glycogen phosphorylase **b** due to changes in the concentration of ATP, AMP, and inorganic phosphate, as well as pH, may also be key to glycogenolysis in working muscle.

The lysosomal enzyme α -glucosidase hydrolyzes both α -1,4 and α -1,6 linkages in glycogen, maltose and isomaltose. Deficiency of this enzyme in human and animal species results in glycogenosis type II or Pompe's disease (Hers, 1963; Jeffrey et al, 1970a; 1970b). Three forms of α -glucosidase were separable in trout liver by versatile methods of chromatography and isoelectric focusing procedures. Two forms showed acid pH optima, hydrolyzing glycogen, maltose and 4-methylumbelliferyl α -glucosidase (an artificial substrate) and these were associated with lysosomes. The third enzyme form was largely distributed in microsomes, with optimum activity at neutral pH and an inability to hydrolyze glycogen. The properties of liver acid α -glucosidase type I changed significantly in response to exercise; maximal activity increased by 80% and K_{in} values for both glycogen and maltose decreased by 50% in exercised, versus control, fish. Under the same conditions of exercise, liver glycogen phosphorylase **a** activity increased 4.4-fold. These changes in α -glucosidase type I are consistent with an activation of enzyme, in parallel with phosphorylase activation, under physiological stress conditions that promote glycogenolysis and glucose export from liver. These results are the first demonstration of the activation of the glucosidic route of glycogenolysis in response to a physiological stress and suggest that, in addition to the phosphorylase **b** to **a** conversion, the glucosidic route of glycogen breakdown could have a significant role to play in complementing the phosphorolytic pathway for glycogenolysis in the metabolic response by liver to the fuel demands of working muscle.

The effect of anoxia exposure on the behavior of phosphorolytic (glycogen phosphorylase, phosphorylase kinase, cAMP-dependent protein kinase, protein

phosphatase type 1) and glucosidic (α -glucosidase) pathways of glycogenolysis were studied in five organs of the anoxia-tolerant freshwater turtle. Activities of all four enzymes associated with the phosphorolytic pathway were found in liver, heart, brain, and red and white skeletal muscles and a functional cascade system for the activation of glycogen phosphorylase was demonstrated via *in vitro* incubation studies. However only liver and heart showed significantly elevated activities of phosphorylase α after 5 h anoxia exposure *in vivo* at 7 °C. PKA activity showed a rapid 2.3-fold increase in the liver within 1 h of anoxic submergence accompanied by a 60% increase in cAMP levels. At the same time PP-1 activity in liver had decreased to 63% of the control value and PP-1 remained suppressed over the subsequent 20 h of anoxia exposure. PP-1 and PKA activities were also suppressed in red skeletal muscle during anoxia and PP-1 activity dropped transiently in the brain. Furthermore, GPK and PKA activities in liver decreased below the control values and cAMP levels were again reduced to control levels after 5 h of anoxia. Activities of both the acidic and neutral forms of α -glucosidase were also detected in all five organs of turtles with highest levels in liver. Activity of the acid α -glucosidase increased by two-fold in liver during anoxia exposure. These results suggest that liver is the main organ that coordinates glucose export to provide other organs with fermentative substrates during long submergences and that both the cytoplasmic phosphorolytic and the lysosomal glucosidic pathways contribute to net glycogenolysis.

The catalytic subunit (PKAc) of turtle liver cAMP-dependent protein kinase was purified to homogeneity with a final specific activity of 65,783 pmol phosphate transferred/min/mg protein. The different binding behavior of turtle liver PKAc to DEAE cellulose suggested that the turtle catalytic subunit had a different isoelectric point than the mammalian catalytic subunit. Indeed, the isoelectric point (6.41 ± 0.02) was significantly lower than that of the mammalian enzyme. The purified enzyme showed a sharp break in the Arrhenius plot (0-45°C) with activation energy increasing

by 4-fold for temperatures below versus above 15°C. Temperature effects may be important in suppressing PKA function, and thereby PKA-mediated responses, *in vivo* to enhance anoxic survival time during winter hibernation under water.

Protein phosphatase type-1 was purified from turtle liver. A 3000-fold purification was achieved by DEAE, blue dextran, Sephacryl S-200 and ADP-agarose chromatography. The enzyme was a monomer of molecular mass 37 kD by SDS-PAGE and gel filtration. Turtle PP-1 was inhibited by okadaic acid and AMP as well as by ADP, ATP, and IMP. Regulation of liver PP-1 appears to be an integral part of anoxia-induced changes in liver glycogenolysis and metabolic rate depression.

Anoxia exposure caused a change in the distribution of PKC isozymes in turtle liver. During the first hour of anoxia 40% of PKC β and more than 80% of PKC α were translocated from the cytosol to the membrane fraction. When the enzyme from control liver was purified, two different isozymes were identified using chromatographic procedures and isozyme specific antibodies. PKC α was more strongly activated by phospholipids, phosphatidylinositol and lysophosphatidylinositol, as compared to PKC β . This characteristic may be used as a biochemical distinction between two isozymes.

Taken together the data show that anoxia induced changes in the activities of two protein kinases in turtle organs. Protein kinase A activation was both directly measured and implied from the anoxia-induced changes in cAMP levels whereas protein kinase C activation was indicated by the translocation of the enzyme from the cytosol to the membrane. These two kinases may have independent roles in regulating different aspects of metabolism under anoxic conditions. For example, at least some of the actions of PKA are specific to regulating fuel (glycogen) metabolism and this might be the primary role of PKA. By contrast, PKC might be involved in other areas of metabolic response to anoxia such as in processes that contribute to the overall suppression of metabolic rate (e.g. regulation of ion channel activity, modulation of the

rates of biosynthetic and degradative processes in the cell). However, substantial evidence has accumulated recently that indicates complex, probably cell-specific, modulation of the cyclic AMP signaling pathway by protein kinase C action (see Houslay, 1991 for review). Hence, both kinases, through "crosstalk" interactions may be interdependently involved in regulating multiple metabolic responses to anoxia. Future studies still have much to reveal about the coordinated regulation of cellular metabolism to achieve sustained, noninjurious, and reversible metabolic rate depression for prolonged anoxia survival.

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