REGULATION OF ENZYMES OF ENERGY METABOLISM – AMP DEAMINASE AND CREATINE KINASE – IN AN ANOXIA TOLERANT TURTLE

By

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ABSTRACT

The red-eared slider turtle (*Trachemys scripta elegans*) is one of the few vertebrate species that can survive long term oxygen deprivation. Maintenance of viable cellular energetics is key to anoxia survival and, in response to low oxygen, most anoxia tolerant animals show a drop in total adenylate levels while energy charge remains stable. To better understand how turtles regulate their energy metabolism when deprived of oxygen, the present studies focused on the control of two important enzymes in muscle energy metabolism: AMP deaminase (AMPD) and creatine kinase (CK). AMPD activity increased under anoxia in turtle skeletal muscle and the effects of ATP·Mg and ions indicated that allosteric controls are part of the mechanism of AMPD regulation. *In vitro* incubations to stimulate the actions of endogenous protein kinases and phosphatases showed that AMPD is a phosphoenzyme and suggested that reversible phosphorylation has a central role in AMPD regulation under aerobic versus anoxic conditions. CK from turtle heart is also a phosphoprotein and anoxia-induced metabolic rate depression was accompanied by a strong increase in the fraction of dephosphorylated CK that showed increased affinity for creatine. Incubation studies implicated selected protein kinases (PKA, PKG, and AMPK) and phosphatases (PP1) as responsible for heart CK regulation. However, anoxia-responsive changes in kinetic properties of skeletal muscle CK did not appear to be caused by a change in phosphorylation state. Regulation of muscle CK under anoxia may be linked with changes in the binding of CK with myofibrils and the effects of binding on enzyme properties.

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LIST OF ABBREVIATIONS

2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
ADP	adenosine diphosphate
AEC	adenylate energy charge
AMP	adenosine monophosphate
AMPD	adenosine monophosphate deaminase
AP	alkaline phosphatase
ATP	adenosine triphosphate
cAMP	cyclic 3'5-adenosine monophosphate
cDNA	complementary DNA
cGMP	cyclic 3'5'-guanosine monophosphate
CK	Creatine kinase
Cr	Creatine
Ea	activation energy
EC	energy charge
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid
eIF2	eukaryotic initiation factor 2
ETC	electron transport chain
GDH	glutamate dehydrogenase
GP	glycogen phosphorylase
GTP	guanosine triphosphate

HIF-1	hypoxia-inducible factor-1
HK	hexokinase
I_{50}	concentration of inhibitor that lowers enzyme velocity by 50%
IMP	inosine monophosphate
Ka	concentration of activator that produces half maximal activation
kDa	kilo Dalton
K_m	Michaelis-Menten constant (substrate affinity constant)
LDK	lactate dehydrogenase
MAPK	mitogen-activated protein kinase
MOPS	2- (N- morpholine) proanesulfonic acid
MRD	metabolic rate depression
mRNA	messenger RNA
MW	molecular weight
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
NT5	5'nucleotidase
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PCr	phosphocreatine
PDK	pyruvate dehydrogenase kinase
PEP	phosphoenolpyruvate
PFK	phosphofructokinase
PK	pyruvate kinase

PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PMA	phorbol 12-myristate-13-acetate
PMSF	phenylmethanesulfonyl fluoride
PVDF	polyvinylidine fluoride
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse-transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
V_{max}	maximal enzyme velocity

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CHAPTER 1

GENERAL INTRODUCTION

Every animal faces different challenges throughout its life, such as to avoid predation, cope with a changing environment, maintain proper nutrition, reproduce and care for its young (Runtz, 1996). Animals have developed many physical, physiological and behavioural adaptations to cope with these challenges (Campbell and Reece, 2002). Most animals are dependent on oxygen to provide a rich supply of ATP to their cells via oxidative catabolism of fuels (Garrett and Grisham, 2005). Despite the need for oxygen by most organisms, there are many facultative anaerobes, which survive equally well in the presence or absence of oxygen and often live in environments where oxygen can become limited for extended periods of time (Storey, 2004a). There is also another class called obligate anaerobes which never use oxygen, such as microbes and some gastrointestinal parasites (Storey and Storey, 2001).

Oxygen deprivation is rapidly lethal for humans and many other organisms but the realities of environment or lifestyle have lead to the evolution of facultative anaerobiosis in diverse groups of animals. Among vertebrates, the premier facultative anaerobes are freshwater turtles of the *Trachemys* and *Chrysemys* genera and two species in particular have become major models for studies of anoxia tolerance, the red-eared slider *T. scripta elegans* and the Western painted turtle, *C. picta bellii* (Jackson, 2000a). These can make anoxic dives of many hours and can survive several weeks (or as long as 3–4 months for *C. picta bellii*) when submerged in cold deoxygenated water (Figure1.1). This capacity supports winter hibernation in ice-locked ponds and lakes that can become severely hypoxic or anoxic. Known molecular mechanisms of anoxia tolerance include: (a) large organ reserves of fermentable fuels, chiefly glycogen, (b) strategies for buffering or excreting end products, (c) alternative routes of anaerobic carbohydrate catabolism

with higher ATP yields than are achieved from glycolysis ending in lactate, (d) good antioxidant defenses to minimize oxidative stress when oxygen is reintroduced, (e) strong metabolic rate depression (MRD), and (f) up-regulation of selected genes whose protein products aid anoxia survival (Figure1.2). Of these, the most important factor is MRD. Maintenance of cellular energetics is the most pressing concern for anaerobic survival because fermentative pathways yield just a fraction of the ATP per mole of substrate catabolized compared with oxygen-based catabolism; e.g. net ATP output is just 2 mol ATP/mol glucose converted to lactate versus 36 ATP/mol for conversion to CO_2 and H_2O. Hence, by strongly suppressing metabolic rate in anoxia, animals lower ATP demand and greatly extend the time that endogenous carbohydrate reserves can support survival. For example, freshwater turtles typically suppress metabolic rate to just 10–20% of the aerobic rate at the same body temperatures (Herbert and Jackson, 1985). MRD involves coordinated controls on the rates of both ATP-producing and ATP utilizing cell reactions so that two outcomes are achieved (Figure 1.3): (a) net ATP turnover is strongly reduced, and (b) the priorities for ATP expenditure are reorganized (Storey, 2004b).

Indeed, these outcomes have been well documented in studies with isolated turtle hepatocytes. Incubation under anoxia decreased ATP turnover by 94% and dramatically changed the proportion of ATP turnover devoted to five main ATP-consuming processes (Figure1.4): ion motive ATPases, protein synthesis, protein degradation, gluconeogenesis and urea synthesis. As a result the Na^+K^+ATPase pump became the dominant energy sink in anoxic hepatocytes, consuming 62% of total ATP turnover compared with 28% in normoxia. Protein synthesis and degradation were largely shut down in anoxia (by >90%) and urea synthesis was halted (Hochachka et al., 1996).

Metabolic Rate Depression

Low oxygen stress causes an animal to either raise anaerobic ATP production to meet its metabolic needs or to strongly suppress its ATP needs and enter a hypometabolic state for the duration of the hypoxic or anoxic excursion. Increasing the rate of glycolysis increases ATP output, but this generally fails quickly because most animals rapidly deplete their internal fuel reserves. This is why metabolic rate depression (MRD) is the most common way that facultative anaerobes endure long-term anoxia (Larade and Storey, 2002b, Storey and Storey, 2004). MRD allows for glycolysis to meet ATP demands, for fuel stores to last up to ten times longer, reduces acidification and reduces end product build up.

MRD is the key to anoxia survival because it allows metabolic rates to be lowered to a level that can be supported by fermentative pathways alone (Table1.1). For example, turtles submerged in cold water can reduce metabolic rates to ~10% of their aerobic rate (Jackson, 1968; Buck et al., 1993). Several extrinsic factors contribute to this, such as limited voluntary movement by skeletal muscles, saving energy consumption associated with digestion and absorption by not eating, and also by reducing heart, breathing and kidney filtration rates (Storey, 2003). Also, many ectotherms seek cooler habitats to decrease their body temperatures and reduce oxygen demands under hypoxic or anoxic conditions. However, it is intrinsic mechanisms which provide over half the energy savings in the hypometabolic state. Furthermore, priorities can exist and MRD is not applied uniformly to all processes or organs within a cell or animal (Figure 1.4). The molecular mechanisms of MRD are currently under much study; much remains to be

discovered but some critical mechanisms are already known and are discussed below.

Reversible Protein Phosphorylation

Reversible protein phosphorylation is an important mechanism of MRD because it affects enzyme function in many ways such as "on-off" control over activity, changing kinetic properties, or altering binding interactions with partner proteins (Storey and Storey, 2004a). Evidence of its wide use as a mechanism of MRD comes from its use in anoxia-tolerant vertebrate animals such as goldfish and turtles, in estivating terrestrial snails and toads and during hibernation in mammals. Reversible phosphorylation regulates many cell functions during hypometabolism including enzymes of glycolysis, pyruvate dehydrogenase, ion motive ATPases and ribosomal machinery (Storey, 2004a).

For example, in anoxia tolerant marine molluscs, inhibition of pyruvate kinase (PK) via phosphorylation controls the PEP branch point and directs carbon into anaerobic routes (succinate synthesis) of carbohydrate catabolism (Storey, 2004b). Aerobic and anoxic variants of PK have been isolated from marine molluscs and it has been shown that the anoxic form has a lower affinity for PEP, is less sensitive to fructose-1, 6-bisphosphate activation, and is more susceptible to alanine end product inhibition. PK activity is virtually shut off under anoxia, allowing carbon to enter into the pathway of succinate synthesis. It has been shown that aerobic PK is the dephosphorylated form and anoxic PK is the phosphorylated form; the two are interconverted via the actions of protein kinases and protein phosphatases (Storey, 2004b). Under anoxic conditions reversible phosphorylation also regulates glycogen phosphorylase (GP) as well as phosphofructokinase (PFK)-1 and PFK-2 and, together with PK control, provides

coordinate control of glycolytic ATP production under anoxia

Reversible phosphorylation also helps control glycolysis in anoxia-tolerant vertebrates. The liver is the only major glycogen reserve which can export glucose and regulate its supply to the whole body. Reversible phosphorylation inhibition of glycolytic enzymes (GP, PFK-1, and PK) under anoxia in goldfish liver serves to direct carbon flow into the export of glucose to support anaerobic metabolism in all other organs. Anoxic forms of both PFK-1 and PK show changes in their isoelectric points, indicative of protein phosphorylation (Storey, 2003). Anoxia dependent changes in the activities and properties of GP and glycolytic enzymes have also been found in some organs of turtles (Storey, 1996).

The mechanisms that mediate anoxia-induced covalent modification of enzymes have been examined in some cases. For example, anoxia effects on the kinetic parameters of PK and PFK-1 in marine molluscs were mimicked by treatment of tissues in vitro with cyclic 3'5'guanosine monophosphate (cGMP). Treatments with cGMP raised the Km for PEP and lowered the I_{50} for alanine of PK, replicating the effects of anoxia. Such data linked PK control in marine molluscs, and MRD in general, to control by protein kinase G (PKG) mediated signal transduction pathways (Cowan and Storey, 2003). Since all arrested states are accompanied by a reduction in cellular pH, this is also another factor that helps to regulate MRD (Storey, 2004a). Reduced pH is also often associated with hypercapnia or high CO_2 which also frequently accompanies anoxia. Indeed, hypercapnia alone has been shown to decreases oxygen consumption by 50% in land snails and marine worms (Storey, 2004a). Furthermore, one of the first events in the recovery of hibernating mammals from their hypometabolic state is hyperventilation to clear

accumulated CO_2 (Storey and Storey, 2001a).

Controlling Membrane Transport

ATP consumption by ion-motive ATPases is major energy expenditure in cells, so anoxia tolerant animals reduce ATP expenditure on these ATPases by suppressing ion movement via "channel arrest". For example, in anoxic turtle brain, ion channel conductance is suppressed to reduce ATP consumption without affecting ion gradients by also lowering K^+ leakage (Bickler et al., 2001). This is also accompanied by a decrease in Na^+ channel abundance, Na^+K^+-ATPase pump activity, and silencing of NMDA receptors. NMDA receptors are glutamate receptors, whose permeability to Ca^{2+} is a major source of Ca^{2+}entry into anoxic/ischemic brains (Bickler et al., 2001).

Studies have shown that adenosine is a signalling molecule which prompts responses that provide tissue protection. For example, in ischemic mammalian heart, oxygen demand is reduced by negative inotropic and chronotropic effects, promoting glycolysis, reducing free radical release, and inhibiting platelet aggregation so blood vessels are not blocked (Storey and Storey, 2004a). The positive effects of adenosine on the heart during preconditioning are mediated by two signal transduction pathways: protein kinase C (PKC) and p38 mitogen-activated protein kinase (MAPK). Another result of adenosine signalling is the opening of the mitochondrial ATP-dependent K^+ (K_{ATP}) channels as well as the stimulation of NO release which causes vasodilation, PKC activation and further K_{ATP} channel opening (Cowan and Storey, 2003, Buck, 2004).

The mitochondria generate ATP via oxygen-dependent catabolism of fuels but under anoxia, the activity of the electron transport chain (ETC) is reduced, and the Krebs

cycle is suppressed and oxygen-dependent ATP synthesis stops (Larade and Storey, 2002a). The mitochondria must remain in a viable state so aerobic metabolism can quickly resume when oxygen returns and thus the proton-motive force must be maintained. Under anoxia, the reduced ETC no longer pumps H^+ out of the matrix but F_1F_0-ATPase continues to pump it back into the matrix using ATP imported from the cytosol via an adenine nucleotide translocator (Storey and Storey, 2004a). It would be advantageous to limit this mitochondrial ATP consumption by directly inhibiting F_1F_0–ATPase activity. This is observed in the anoxic frog mitochondria, where F_1F_0–ATPase ATP consumption is just 4% in anoxia of rate in the aerobic condition. Another consideration is the protons that leak back into the mitochondrial matrix without passing through the F_1F_0–ATPase reduce energy conservation efficiency (Storey, 2003). For hypoxic systems, a 50% reduction in proton leak seems to be caused by a decrease in ETC activity and not a change in inner membrane proton conductance.

Controlling Protein Synthesis

Protein synthesis expends much ATP and is quickly suppressed under many stresses, becoming an integral part of the MRD that supports anoxia tolerance, hibernation and estivation (Storey and Storey, 2004a). For example, protein synthesis is reduced by >90% during anoxia in vertebrates such as turtle and fish. Suppression of protein synthesis could be regulated by reducing mRNA substrate availability or by inhibiting the ribosomal translational machinery. The latter is the primary method and is accomplished in two main ways: by phosphorylating selected translational machinery proteins or physically regulating ribosome assembly. Furthermore, although most mRNA

transcripts are maintained during anoxia, some are up-regulated and encode survival proteins that continue to be translated under anoxia (Storey and Storey, 2004b). In general, there are three objectives for controlling translation in anoxia: (1) the overall rate of protein synthesis must be reduced, (2) existing mRNA must be stabilized and stored to be available when normal conditions return, and (3) specific stress proteins must be synthesized (Storey and Storey, 2004a).

Translation initiation and elongation are both inhibited by reversible protein phosphorylation. The eukaryotic initiation factor 2 (eIF2), which introduces the initiator methionine tRNA to the 40S ribosomal subunit, is inhibited by phosphorylation (Rhoads, 1993, Kedersha et al., 1999, Mikulits et al., 2000). This mechanism, seen in all eukaryotes, is a response to many stresses such as viral infection, heat shock, nutrient deprivation and hypoxia/ischemia. Inhibition of protein synthesis during mammal ischemia or starvation also involves controls on other initiation factors (DeGracia et al., 2002). For example, eIF5 which promotes GTP hydrolysis within the 40S initiation complex is also regulated by reversible protein phosphorylation as is subunit E of eukaryotic initiation factor 4 (eIF4E) and its binding protein (4E-BP) (Storey and Storey, 2004b). Furthermore, reversible phosphorylation also controls peptide elongation; a variety of cellular protein kinases inhibit the eukaryotic elongation factor 2 (eEF2).

Active translation occurs on polysomes, which are aggregates of ribosomes moving along mRNA; whereas monosomes do not conduct translation. Hence, factors that cause polysome dissociation inhibit translation and produce ATP savings. Polysome dissociation is a response to stresses such as hypoxia and starvation, and is now known to be integral to MRD (Larade and Storey, 2002b). For example, extracts from anoxia

tolerant marine snails were separated on a sucrose gradient. Under aerobic conditions, most ribosomes were in the high density fractions that contained polysomes (Larade and Storey, 2002b). Under anoxic conditions, however, more and more ribosomes moved to the lower density monosome fractions until after 72 hours of anoxia exposure no polysomes remained. Furthermore, most mRNA transcripts also shifted to the monosome fractions; hence, mRNA transcripts of constitutive genes are conserved under anoxia but not translated (Larade and Storey, 2002b). The process reverses during aerobic recovery; polysomes are reassembled and mRNA translation restarts (Storey and Storey, 2004a).

All systems of MRD show increased gene expression of a few selected proteins under the stress conditions and one way to regulate the translation of only these proteins is by message selection. Under stress conditions, eIF4G undergoes proteolytic fragmentation and this change the mRNA types that can be translated (Storey and Storey, 2004a). Only messages containing an internal ribosome entry site (IRES) can be translated, allowing for very selective gene up-regulation during anaerobiosis, such as the hypoxia inducible factor, HIF-1 (Storey and Storey, 2004a). In turn, this transcription factor turns on a selected suite of genes that benefit anoxia survival.

Protein Degradation

If protein synthesis is suppressed under anoxic conditions, then rates of protein degradation must be similarly reduced or cell homeostasis will be compromised. Indeed, studies have shown that protein degradation rates are also reduced under anoxia resulting in reduced protein turnover and increased protein longevity in hypometabolic states (Storey and Storey, 2004a). For example, the half-life of cytochrome oxidase increased

by 77-fold in anoxic brine shrimp embryos (Anchordoguy et al., 1993, Anchordoguy and Hand, 1995). Suppressing protein degradation also minimizes the accumulation and processing of end products, as seen in anoxic hepatocytes by a 70% drop in urea production (Hochachka et al., 1996). Unfortunately to date, little is known about proteolytic suppression mechanisms during MRD. One possible mechanism is regulation of protein ubiquitination. Ubiquitin has a major role in cellular proteolysis, where polyubiquitinated proteins are degraded by the 26S proteasome and monoubiquitinated proteins are targeted for endocytosis and degraded in lysosomes (Pickart, 2001). Anoxic brine shrimp showed a 93% reduction in ubiquitin-protein levels and this was also seen in anoxic turtles (Anchordoguy and Hand, 1994). However, in hibernating mammals, ubiquitin-protein levels rose, suggesting protein inhibition in this case might be directed towards proteolysis machinery.

Enzymes of adenylate energy metabolism

MRD involves the coordinated suppression of the rates of all energy-consuming and energy-producing reactions in cells to rebalance metabolism at a very much lower rate of ATP turnover, often 10% or less of the normal resting value under unstressed conditions (Storey and Storey, 2004b). As discussed above, this involves regulation of many enzymes and functional proteins that are involved in pathways of ATP synthesis (e.g. glycolysis) and ATP consumption (e.g. transmembrane ion movements, protein synthesis, etc.). The transition to a hypometabolic state also involves much reduced rates of muscle work; e.g. animals (including anoxic turtles) show much lower heart rates (bradycardia) and little or no skeletal muscle movement. Hence, the molecular

mechanisms of metabolic arrest probably also extend to coordinating the suppression of activities of enzymes involved in muscle work which could include enzymes associated with ATP supply to working myofibrils and the regulation of adenylate metabolism. This thesis focuses on two enzymes that are involved in muscle energy metabolism.

Creatine kinase (CK) catalyzes a reversible reaction that synthesizes versus catabolizes the muscle phosphagen, creatine phosphate:

Creatine + ATP $\leftrightarrow$ creatine-P + ADP

Creatine phosphate is present in high concentrations in muscle tissues and acts as a storage form of energy that can be mobilized to buffer ATP levels when the rate of ATP consumption by working muscles is very high. In working muscle, PCr hydrolysis is the first source of energy that is mobilized, providing an instantaneous ATP supply to fuel contraction during the initial seconds of muscle work until a more sustained supply ATP from carbohydrate or lipid catabolism can be activated. Hence, during muscle work or under anoxia, the observed changes in [PCr] are much greater than those in [ATP] (Waarde et al., 1990).

CK is not only a fast ATP-regenerating enzyme but also a low-threshold sensor for ADP. Two products of cellular ATPases, ADP and H^+, are substrates of the CK reaction and are consumed during the CK catalyzed regeneration of ATP. Thus, the CK reaction also prevents local acidification near cellular ATPases as well as a build-up of ADP which is inhibitory to many ATP-utilizing reactions. Cells attempt to maintain a high and relatively constant adenylate energy charge (AEC) which is defined as:

[ATP + ½ ADP] / [ATP + ADP + AMP].

A high AEC promotes normal metabolic functions but when ATP production is

compromised, ADP and AMP build up and the AEC drops. Anabolic processes such as protein synthesis are quickly inhibited by a falling AEC and this helps to preserve remaining ATP for use by critical functions (such as ion motive ATPases) but if ATP levels drop too low, then injury or cell death occurs. One of the ways to temporarily boost ATP supply is to retrieve the energy trapped in ADP by using the adenylate kinase reaction (Figure 1.5) (Noda, 1973; Hamada and Kuby, 1978):

$$2\ ADP \rightarrow ATP + AMP.$$

However, this results in a build-up of AMP which still disrupts the AEC. To stabilize the AEC, AMP is often removed from the adenylate pool so that the total adenylate pool size drops but the AEC remains relatively constant. A decrease in the total adenylate pool commonly occurs both during intense muscle work and when animals enter a hypometabolic state. The key enzyme involved in removing AMP from the adenylate pool is AMP deaminase:

$$AMP \rightarrow IMP + NH_4^+$$

In working muscle, the production of NH_4^+ has roles as both an activator of the rate-limiting glycolytic enzyme, PFK-1, and as a consumer of protons to help counteract the acidification of the cytoplasm that occurs when glycolysis runs at high rates (Mommsen and Hochachka, 1988). AMP and IMP can also be dephosphorylated by 5'-nucleotidase located on the sarcolemma (Bowditch et al., 1985) to give adenosine and inosine, respectively, both of which have important signalling roles. Adenosine has a well-established role in mediating MRD in turtles (Lutz and Nilsson, 2004) whereas new research is suggesting that inosine may mediate a different aspect of metabolic response to varying oxygen tension – antioxidant defense (Buckley et al., 2005).

The lives of facultative anaerobes are very interesting and should be researched in order to unlock the secrets of survival in environments where oxygen is limited. By determining how animals adapt to oxygen limitation, we can both gain insight into the unifying principles of metabolic design in nature and derive novel strategies that could be used to improve stress tolerance in medical conditions involving low oxygen. For example, low oxygen causes much of the damage to tissues during heart attack or stroke and in other situations, such as the preservation of tissues and organs for transplant, low oxygen stress is a primary cause of the loss of viability as storage time increases.

To better understand how vertebrate animals with high anoxia tolerance can regulate their energy metabolism when deprived of oxygen, the studies presented in this thesis have focused on the regulation of two important enzymes in muscle energy metabolism: AMPD and CK. Two hypotheses were tested.

Hypothesis 1: AMPD is differentially regulated under aerobic versus anoxic conditions in turtle muscle in order to regulate AMP catabolism for multiple important functions in anoxia. These functions could include maintaining a viable AEC and regulating levels of effectors of energy metabolism (AMP, NH_4^+) and of signalling metabolites (adenosine, inosine).

Hypothesis 2: CK is a regulated enzyme under aerobic and anoxic conditions with multiple mechanisms contributing to its control. Regulation in both skeletal muscle and heart occur in order to meet different needs for buffering by the PCr system.

Figure 1.1. Duration of anoxic submergences at various temperatures from which painted turtles (*Chrysemys picta bellii*) have been observed to recover fully. (Data are adapted from Herbert and Jackson, 1985).

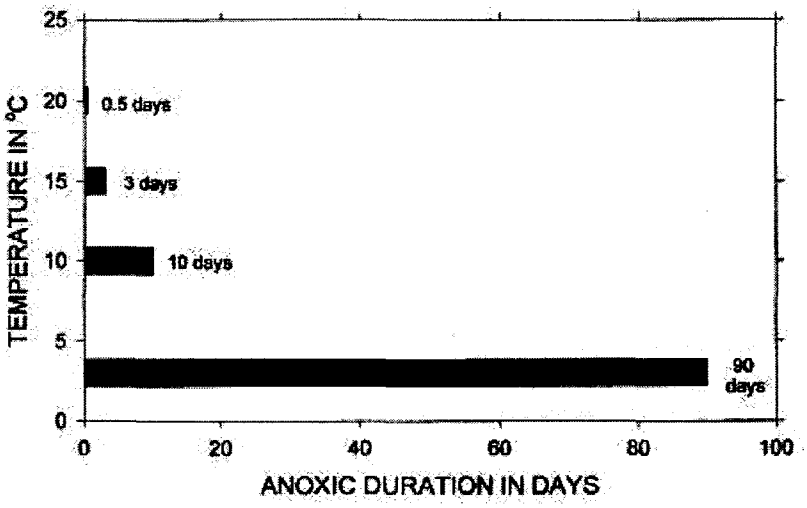
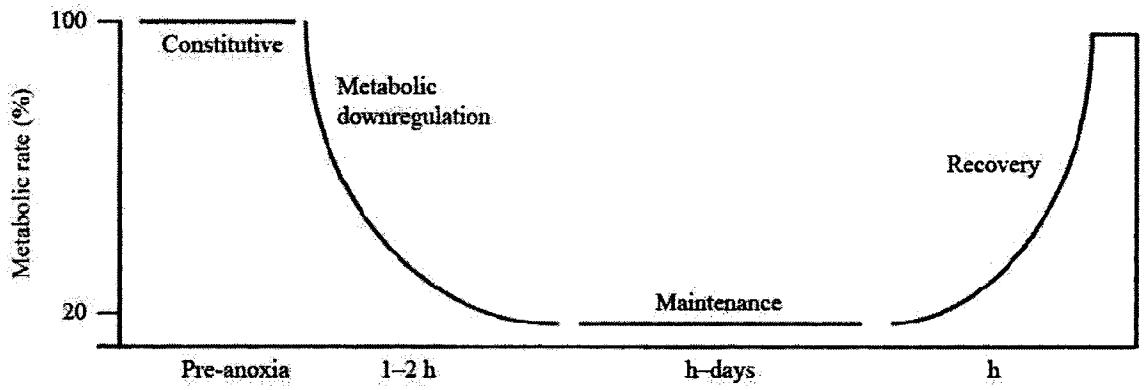


Figure 1.2. A diagrammatic overview of some of the factors involved in negotiating anoxia survival in the turtle brain.

Constitutive factors that predispose for anoxia tolerance include enhanced levels of glycogen stores, elevated heat shock proteins, increased densities of protective receptors and elevated antioxidant capacities. Three distinct phases are involved in surviving and recovering from anoxia. (1) A coordinated downregulation of ATP demand processes to ~20% of normoxic levels. This phase, which takes 1–2 h, includes a reduction in ion channel and electrical activities, a reduction in glutamate and dopamine release, a reduction in protein synthesis and a substantial increase in Hsp72 and Hsc73 levels. (2) Long-term survival (days) at basal levels of ATP expenditure. Neuronal network integrity is preserved through the continued operation of core activities. These include periodic electrical activity, an increased release of GABA and a reduced but continued release of glutamate and dopamine. There is a further increase in Hsc73, indicating a 'housekeeping' role for this protein during this period. (3) When oxygen becomes available there is a rapid upregulation of neuronal processes to restore full function, together with the activation of protection mechanisms against reperfusion-generated reactive oxygen species (ROS) (Data are adapted from Lutz and Milton, 2004)



Constitutive factors	Metabolic downregulation	Basal maintenance	Recovery
Glycogen	Hypoxia/energy failure sensing	ATP turnover rates minimal	Oxygen sensing
Opioid receptors	HIF-1α ↑, glycolysis ↑	Preserve network integrity	Ion channels ↑
GABA receptors	ATP ↓↑, adenosine ↑	Ion channel ↔	Electrical activity ↑
Hsp72, Hsc73	Ion channels ↓, electrical activity ↓	Electrical activity ↔	Protein synthesis ↑
NF-κB	Glutamate, dopamine release ↓	Glutamate release ↔	
PACAP 38	Protein synthesis ↓	Dopamine release ↔	ROS defenses ↑
Ascorbate	Hsp72 ↑, Hsc73 ↑	GABA release ↑	
SOD		GABA receptors ↑	
	Suppression of ATP turnover rates	Protein synthesis ↔	
		Hsp72 ↓, Hsc73 ↑	

Figure 1.3. Constant cellular ATP levels of anoxic turtles indicate that a coordinated decrease occurs in both ATP production and ATP utilization when a turtle makes the transition from a normoxic to an anoxic state. (Data are adapted from Jackson, 2000)

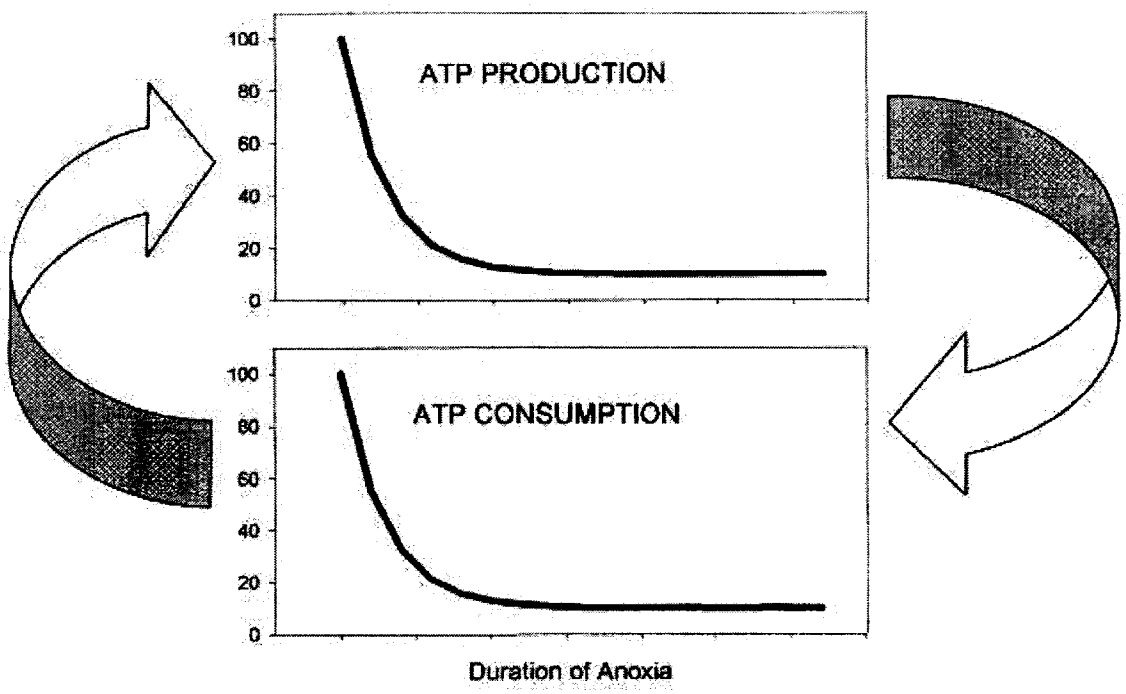


Table 1.1. The main ATP-demanding pathways during normoxia and anoxia in turtle hepatocytes. (adapted from Hochachka, et. al. 1996)

	ATP demand, μmol ATP $\times$ g^{-1} $\times$ h^{-1}		
Pathway	Normoxia	Anoxia	% suppression
Total	67.0	6.3	94
Na^+ pump	19.1	4.8	75
Protein synthesis	24.4	1.6	93
Protein breakdown	11.1	0.7	94
Urea synthesis	2.0	0.6	70
Gluconeogenesis	11.4	0.0	100

Figure 1.4. Cellular processes of isolated turtle hepatocytes that require ATP all decrease when the cells are made anoxic, and the overall cumulative decrease (~90%) is close to the decrease observed in the whole animal (Jackson, 1968; Herbert and Jackson, 1985) and in the total metabolic rate of hepatocytes (Buck et al., 1993) (Data shown are adapted from Hochachka et al., 1996).

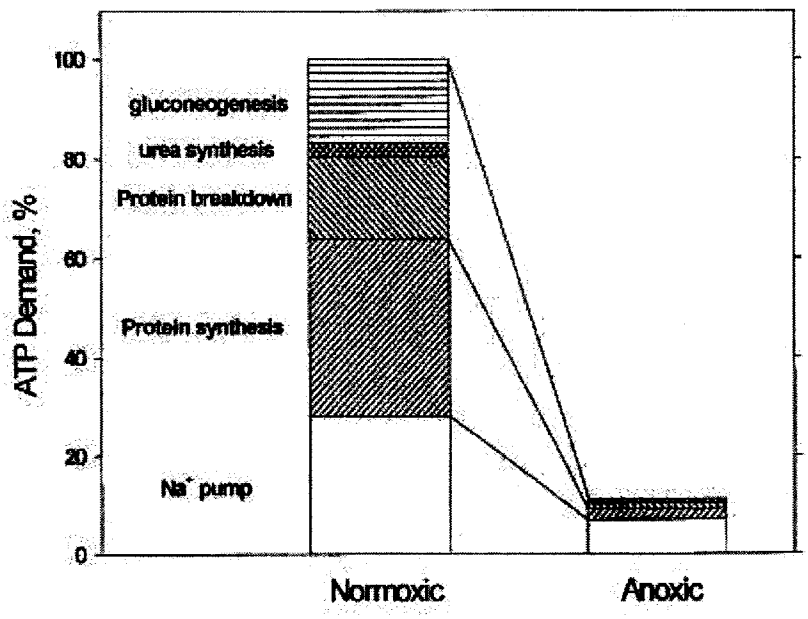
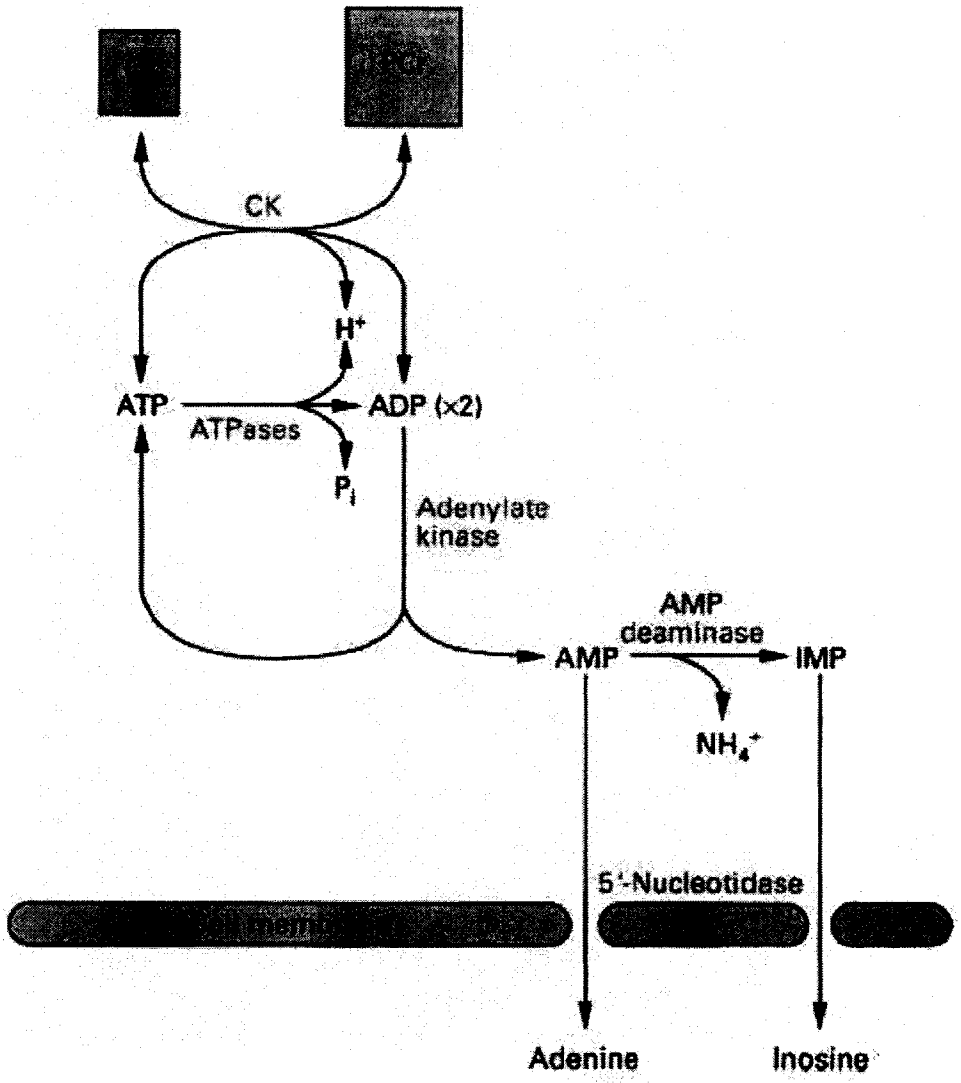


Figure 1.5. Creatine kinase involvement in adenine nucleotide metabolism

CK is a fast ATP-regenerating enzyme and a low-threshold sensor for ADP. Two products of cellular ATPases that is, ADP and H^+ are substrates of the CK reaction and are consumed during the CK-catalyzed regeneration of ATP. Thus, CK action prevents local acidification near cellular ATPases as well as a build-up of ADP. The latter would otherwise lead, after a series of enzymatic conversions, to a net loss of cellular adenine nucleotides, which would be deleterious for a cell. ADP (x 2): two ADP molecules are converted by adenylate kinase to give one molecule of ATP and AMP each (Adapted from Wallimann et al., 1992).



CHAPTER 2

REGULATION OF ADENOSINE MONOPHOSPHATE DEAMINASE FROM SKELETAL MUSCLE OF AN ANOXIA TOLERANT TURTLE

INTRODUCTION

5'- Adenosine monophosphate deaminase (AMPD, E.C. 3.5.4.6) is an enzyme that catalyses the irreversible hydrolytic cleavage of AMP to IMP and NH_4^+. The enzyme is widely distributed in animal tissues, but skeletal muscle has the highest activities (Lowenstein, 1972). In mammalian skeletal muscle under an energy stress (for example, during exercise), there is an inverse correlation between ATP depletion and IMP production. AMPD is also known to be a major source of ammonia production in vertebrate skeletal muscle, in particular in fish muscle (Walton, 1977). Therefore, a primary role for AMPD appears to be in the removal of AMP from the adenylate pool when the rate of ATP production is outstripped by the rate of ATP utilization. The most important consequence of this function is be the stabilization of cellular energy charge. By removing AMP from the energy charge equation, EC = [ATP + 0.5ADP] / [ATP + ADP + AMP], the overall energy charge can be stabilized at a high value, at the expense of a reduction in the total adenylate pool size. Additional consequences of this function in working muscle include: (1) to help push the adenylate kinase reaction (2 ADP $\leftrightarrow$ ATP + AMP) towards ATP generation, (2) to supply allosteric effectors of glycolytic enzymes (e.g. NH_4^+ activates phosphofructokinase during muscle work), (3) to remove some of the protons that accumulate during anaerobic glycolysis via the production of ammonium ions (Mommsen and Hochachka, 1988), and (4) to modulate the regulation of AMP-sensitive metabolic pathways (Lee and Wang, 1968).

There are several proposed mechanisms of AMPD regulation *in vivo*. AMPD is sensitive to various allosteric effectors (Smiley and Suelter, 1967; Raffin, 1983).

Inorganic phosphate is considered the primary inhibitor of AMPD *in vivo*, with K_i values of ~1-2 mM (Wheeler and Lowenstein, 1978). AMPD is also known to bind to myosin *in vivo* and this both physically positions AMPD close to the site of ATP hydrolysis by myosin ATPase and can alter enzyme properties. Shiraki et al. (1979) found that a 50-70% increase in maximal enzyme velocity was induced by myosin binding, whereas Ashby and Frieden (1978) and Barshop and Frieden (1984) reported only a slight activation. Myosin binding also decreases GTP inhibition (Ashby and Frieden, 1978), enhances activation by ADP (Barshop and Frieden, 1984), and lowers K_m AMP (Rundell et al., 1992b).

Of physiological relevance, the proportion of total AMPD that is bound to myosin increases significantly in intensely contracting muscle (Rundell et al., 1992a). AMPD is also controlled by the combined effects of allosteric modifiers and enzyme interactions with cellular structural elements (Lushchak and Storey, 1998). The interaction of AMPD with cellular structures, myofibrils and membranes (Shiraki, 1979) can also change in response to other physiological states besides exercise such as hypoxia (Lushchak and Storey, 1998), and changes in the distribution of AMPD between free and bound fractions can affect enzyme control (Lushchak and Storey, 1994; Rundell et al., 1993). AMPD may also be regulated by changes in temperature (English and Storey, 2000).

AMPD can also be regulated via reversible protein phosphorylation by the Ca^{2+} and phospholipid-dependent protein kinase C (PKC) (Hu et al., 1991; Tovmasian et al., 1990; Thakkar et al., 1993). The enzyme also is inhibited by phosphoinositides (Sims et

al, 1999). Terjung et al. (1998) indicated that a phosphorylation - dephosphorylation cycle was important in the regulation of AMPD in skeletal muscle of both mice and rats. Phosphorylation regulated the activity of AMPD by increasing enzyme affinity for AMP.

AMPD has been purified to apparent electrophoretic homogeneity from various vertebrate skeletal muscle sources (rat, rabbit, hen, frog, pikeperch) using analytical ultracentrifugation (Stankiewicz et al., 1979). The native molecular weight of the enzyme from these sources was close to 280 kDa and each consisted of four identical subunits of molecular weight ~70 kDa. However, some studies have revealed that AMPD is a tetramer of identical 80 kDa subunits controlled, at least in part, by AMP concentration, allosteric modulators such as inorganic phosphate (Terjung, 1991), and ATP turnover associated with muscle contraction (Edstrom, 1990).

To date, very little is known about reptile AMPD (Spychala, 1984; Spychala and Marszalek, 1986) and no studies have been done to assess AMPD regulation in an anoxia-tolerant reptile and its influence on muscle adenylate metabolism in the anoxic state. This chapter reports the purification and characterization of AMPD from red skeletal muscle of adult red-eared sliders (*Trachemys scripta elegans*). Kinetic parameters of the enzyme from both aerobic control and anoxic muscle were assessed and compared. Presented are pH optima, substrate affinity coefficients, K_a values for ATP activation, I_{50} values for inhibitors, and both native and subunit molecular weights. The effects of modifying enzyme phosphorylation state were also evaluated for AMPD activity from both aerobic and anoxic muscle.

MATERIALS AND METHODS

Animals and Biochemicals

Adult red eared slider turtles, *T. s. elegans*, were purchased from Carolina Biological. Animals were 8-12 inches shell length, 5-7 inches shell width; thirteen male turtles and fifteen female turtles were used. Turtles were held for 2 weeks before use in large tanks with running dechlorinated water at 11°C and were fed Wardley Reptile Ten Floating Food Stick (New Jersey, U.S.A.) ad libitum. Turtles were provided with a basking platform but no heat lamp. Animals were in good health with a few minor superficial scrapes and scratches on the skin. Just prior to experimentation, turtles were transferred a few at a time to the lab in large, covered plastic containers (2 turtles per box, no water added) and were placed in the 4°C fridge for 2 h before experimentation. Nail polish was used to number the shells of turtles to be used for anoxia experiments so that start/end points of anoxia exposure could be staggered for individuals.

Aerobic control turtles were maintained at 4°C for 2 hours prior to sacrifice and were then killed by rapid decapitation. The shells were opened using a hammer and chisel and then internal tissues were removed as quickly as possible and tissues were immediately frozen in liquid nitrogen and transferred to labeled plastic jars and placed at -69 °C for storage until use.

To impose anoxia, other turtles were held first at 4 °C in air for 2 hours and were then moved into 40 liter containers (2-3 animals per tank) filled with dechlorinated water that had been previously bubbled with 100 % extra dry nitrogen gas for at least 6 h; containers were held in incubators set to 4 °C ± 1 °C. After turtles were placed in a

tank a wire mesh was fitted about 10 cm below the water line so that turtles could not surface during the anoxic episode. Bubbling with gas was not continued throughout the experiment. Submergence anoxia was continued for 1, 5 and 20 h. Anoxic turtles were then rapidly killed and dissected as above.

The results presented in the thesis represent independent replicates on separate preparations of enzyme from different animals.

All biochemicals and coupling enzymes were obtained from Sigma Chemical Co., St. Louis, MO. or Boehringer Mannheim, Montreal, PQ.

Tissue homogenization and preparation

Samples of frozen red skeletal muscle were ground to a powder under liquid N_2 and then homogenized 1:5 (w:v) with a Diamed Pro 200 homogenizer in ice-cold homogenization buffer containing protein kinase, protein phosphatase, and protease inhibitors: 50 mM Tris-HCl buffer pH 7.2, 10mM β-mercaptoethanol, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerol phosphate, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The addition of chelating agents (EDTA, EGTA) that inhibit protein kinases and β-glycerol phosphate that inhibits protein phosphatases allows the native phosphorylation state of AMPD to be maintained during tissue extraction. After centrifugation at 10,000 g for 30 min at 4°C, supernatant was collected and stored on ice until use.

To study the kinetic properties of AMPD which was bound to myofibrils and to determine the proportion of free versus myofibril-bound AMPD, in some cases the

pellets were re-suspended in homogenization buffer and centrifuged to remove remaining free enzyme; this was repeated 3 times to make sure there was no unbound AMPD left. The pellets were then resuspended in 2 ml of homogenization buffer. The activity of AMPD bound in the pellet was measured using the coupled enzyme assay to assay the enzyme while it was still bound to myosin.

AMP-Deaminase (E.C. 3.5.4.6) Assay

AMPD activity can be measured in several different ways (Raffin and Thebault, 1991). Standard assays were conducted at 22°C and monitored the increase in absorbance at 285 nm corresponding to the formation of the product IMP. Optimal conditions for maximal activity using this assay were: 50 mM Tris-HCl buffer, pH 7.2 and 5 mM AMP.

Standard curves were prepared to determine the relationship between absorbance and concentration for both AMP and IMP. The slope obtained for the relationship between IMP absorbance and its concentration was 0.332 mM^{-1}, whereas that obtained for AMP was 0.194 mM^{-1}, both with identical y-intercepts of 0.07. To determine the molar absorptivity for IMP alone, in a reaction assay consisting of both product IMP, and substrate AMP, the molar absorptivity of AMP was subtracted from that of IMP, resulting in a value of 0.138 mM^{-1}. This corrected value was then applied to all conversions from absorbance to concentration. One unit of AMPD activity is defined as the amount that produced 1 µmol of IMP per minute at 22°C.

Due to high background absorbance by protein in resuspended pellets, activity

of bound AMPD was measured in a coupled enzyme assay where the production of NH_4^+ was quantified in the presence of α-ketoglutarate (α-KG), NADH, and glutamate dehydrogenase (GDH); the consumption of NADH was measured at 340 nm. Optimal conditions for this assay were: 50 mM Tris-HCl buffer, pH 7.2, 5 mM AMP, 2 mM α-KG, 0.15 mM NADH, and 1 U GDH. In this assay, one unit of AMPD activity is defined as the amount that produced 1 μmol of NH_4^+ per minute at 22°C.

Enzyme activity in both assays was monitored using a Multiskan Spectrum Microplate Reader (kinetic mode, reading interval = 21 s) with the Microplate Analysis (MPA) and Kinetics 3.51 computer programs used to analyze the data (Brooks, 1992).

In vitro incubations to stimulate endogenous kinases and phosphatases

For these experiments only, muscle extracts were prepared in 50 mM Tris-HCl buffer, pH 7.0 containing 10 mM β-mercaptoethanol, with homogenization and centrifugation as stated previously. To test the effects on AMPD of stimulating the activities of either endogenous protein kinases or protein phosphatases, aliquots of enzyme extract were incubated in 50 mM Tris-HCl buffer, pH 7.0, 10 mM β-mercaptoethanol containing the following:

(a) for the control (stop) situation, additions were 5 mM EDTA, 5 mM EGTA, 30 mM β-glycerol phosphate and 5 mM Na_3VO_4 (sodium orthovanadate which inhibits tyrosine phosphatases) to inhibit endogenous kinases and phosphatases and maintain the native phosphorylation state of the enzyme,

(b) to stimulate total endogenous protein kinases, additions were 30 mM

β-glycerol phosphate, 1 mM cyclic 3'5'adenosine monophosphate (cAMP), 1 mM cyclic 3'5' guanosine monophosphate (cGMP), 5 mM ATP, 5 mM $MgCl_2$, 1.3 mM $CaCl_2$, and 1 μg/ml phorbol 12-myristate-13-acetate (PMA),

(c) to stimulate total protein phosphatases, additions were 5 mM $MgCl_2$ and 5 mM $CaCl_2$.

(d) to stimulate individual protein kinases, the additions for each of the incubations were:

PKA: 1 mM cAMP, 5 mM ATP, 10 mM $MgCl_2$, 30 mM β-glycerol phosphate and 5 mM Na_3VO_4.

PKG: 1 mM cGMP, 5 mM ATP, 10 mM $MgCl_2$, 30 mM β-glycerol phosphate and 5 mM Na_3VO_4.

PKC: 1.3 mM $CaCl_2$, 7 ug/mL PMA (phorbol 12-myristate 13-acetate), 5 mM ATP, 5 mM $MgCl_2$, 30 mM β-glycerol phosphate and 5 mM Na_3VO_4.

AMPK: 1 mM AMP, 5 mM ATP, 10 mM $MgCl_2$, 30 mM β-glycerol phosphate and 5 mM Na_3VO_4.

(e) to stimulate individual phosphatases, the additions for each of the incubations were:

Tyr PPases only: 30 mM β-glycerol phosphate

Ser/Thr PPases only: 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 5 mM Na_3VO_4

PP1: 2.5 nM okadaic acid (inhibits PP2A), 5 mM Na_3VO_4, 2 mM EDTA and 2 mM EGTA. (note: no ions added, therefore no PP2B or PP2C activity)

PP2B: 5 mM $CaCl_2$, 2 mM EDTA (chelates Mg^{2+}, inhibits PP2C), 1 μM okadaic acid

(inhibits PP1/PP2A), and 5 mM Na_3VO_4

PP2C: 5 mM $MgCl_2$, 2 mM EGTA (chelates Ca^{2+}, inhibits PP2B), 1 μM okadaic acid (inhibits PP1/PP2A), 5 mM Na_3VO_4, and 1 nM cypermethrin (inhibits PP2B).

After incubation for 4 h at 4°C, AMPD activity was measured at 285 nm under optimal assay conditions as described above.

Enzyme Purification

AMPD was purified from red skeletal muscle of aerobic control turtles according to the method of Smiley et al. (1967) with modifications. Tissue homogenization and centrifugation was as described above except using a 1:3 (w:v) ratio for homogenization. Crude supernatant was collected and stored on ice. A 1 mL aliquot of phosphocellulose slurry (previously equilibrated in homogenization buffer) was then added to the supernatant and stirred for 10 min followed by centrifugation at 1000 g for 5 min. The supernatant was separated and the pellet was saved; AMPD activity remaining in the supernatant was measured. The above procedure was repeated until no AMPD activity was detected in the supernatant. The supernatant was discarded and the phosphocellulose pellets were combined and washed several times with homogenization buffer (followed by centrifugation as above) and then AMPD was released from the phosphocellulose by 3 x 1 mL washes with homogenization buffer containing 1 M KCl. The 3 washes were pooled and concentrated using a Centricon-30. Centricons were centrifuged at 5,000 x g at 4°C until sample volume was reduced to about 500 μL. Retentate in the Centricon was then washed with the addition of 2 mL of

homogenizing buffer followed by re-centrifugation, again until volume was reduced to about 500 μL. This was repeated three times to remove KCl from the retentate. The final retentate solution was then loaded onto a 2 cm x 1.8 cm (h x d) column of DEAE G50 Sephadex (equilibrated in homogenization buffer). The column was washed with 10 mL of the same buffer to remove unbound protein and then AMPD was eluted with a 0-1.2 M gradient of KCl in the same buffer; 200 μL fractions were collected and assayed for activity. Peak fractions were pooled and concentrated using a Centricon as described above.

Protein concentration

Total soluble protein content was measured by the Coomassie blue dye-binding method (Bradford, 1976), using the Bio-Rad prepared reagent and the micro assay procedure with bovine serum albumin as the standard; absorbance was measured at 595 nm on the microplate reader.

SDS-Polyacrylamide gel electrophoresis

Purity and subunit molecular weight of purified AMPD were determined using SDS-PAGE; an 8% separating gel was used with a 5% stacking gel. Protein samples were mixed 1:1 (v:v) with 2x SDS-PAGE sample buffer containing 100 mM Tris-HCl (pH 6.8), 4% SDS (w/v), 20% glycerol (v/v), 5% β-mercaptoethanol (v/v) and 0.2% bromophenol blue (w/v) and boiled for 5 min. Samples were then loaded onto a gel with the same amount of protein (previously measured using the Coomassie blue

dye-binding method) loaded into each well. Electrophoresis was carried out on a Bio-Rad mini-gel apparatus at 180 V for 40-50 min at room temperature with 1x running buffer containing 3.03 g Tris base, 14.4 g glycine and 1 g SDS per liter, pH ~8.3. Gels were run until the bromophenol blue tracking dye reached the bottom of the gel and were then fixed for 30 min in a solution of 10% acetic acid and 25% methanol (v/v) followed by incubation in 50% methanol for 15 min. The gel was washed with Milli-Q distilled; deionized water 5 times for 5 min each and then incubated in sodium thiosulfate solution (0.2 g/L solution) for 1 min. After washing the gel with Milli-Q water 2 times for 1 min each, the gel was incubated with chilled (4°C) silver nitrate solution for 25 min and then washed again with Milli-Q water 2 times for 1 min each. Bands were developed with the reducing agent solution for 10 min and then stopped by putting the gel in acetic acid (10 ml/L) for 10 min.

The subunit molecular weight of AMPD was determined from a plot of R_f versus log molecular weight of protein standards (kDa) run in a separate lane: myosin (205.7), β-galactosidase (133.1), bovine serum albumin (83.9), carbonic anhydrase (41.6), soybean trypsin inhibitor (31.4), lysozyme (17.3) and aprotinin (7.0).

Native Molecular weight estimation

The native molecular weight (M_w) of AMPD was estimated by gel filtration on a Sephacryl S-400 column (90 × 2.5 cm) (h × d) equilibrated in 50 mM Tris-HCl buffer pH 7.2, 10mM β-mercaptoethanol, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerol phosphate and 5% v:v glycerol. Fractions of ~700 µL (20 drops) were collected and

assayed for AMPD activity. The protein markers for gel filtration were blue dextran (M_w 2000 kDa), thyroglobulin (M_w 670 kDa), Jack Bean urease (M_w 545 kDa), *Bacillus pasteurii* urease (M_w 272 kDa), hemoglobin (M_w 64.5 kDa), and cytochrome c (M_w 12.5 kDa).

Data and Statistics

Enzyme kinetic parameters were analyzed by standard procedures using a nonlinear least squares regression computer program (Kinetics 3.51) developed for this purpose (Brooks, 1992). Data were expressed as mean ± S.E.M. with statistical testing performed using the Student's t-test.

RESULTS

Optimization of experimental conditions

Initial tests were used to optimize the composition of the homogenizing buffer to achieve maximal retrieval of AMPD activity from control turtle muscle. Four conditions were compared: (1) buffer alone (50 mM Tris-HCl, pH 7.2 containing 10 mM β-mercaptoethanol), (2) buffer plus 2 mM EDTA, 2 mM EGTA and 30 mM NaF, (3) buffer plus 30 mM NaF, and (4) buffer plus 2 mM EDTA, 2 mM EGTA, and 50 mM β-glycerophosphate. The mean relative activities of AMPD retrieved were: 1.0 ± 0.05, 1.6 ± 0.06, 0.4 ± 0.03, and 2.4 ± 0.09, respectively. The inclusion of NaF as a protein phosphatase inhibitor clearly had a strong negative effect on activity whereas the substitution of β-glycerol phosphate was much better. Hence, the fourth

homogenization condition was used for all standard isolations of muscle AMPD.

Figure 2.1 shows the effect of pH on the activity of AMPD from skeletal muscle of control turtles. The optimum pH was 7.2; activity declined sharply below pH 7.0 but decreased more gradually as pH increased above 7.2. Hence, all standard assays were subsequently performed at pH 7.2.

Kinetic parameters of AMPD

Velocity versus AMP substrate concentration curves are shown in Figure 2.2(A) for soluble AMPD in crude extracts (supernatant) of turtle muscle. The enzyme displayed Michaelis-Menten (hyperbolic) kinetics with respect to AMP concentration. The maximal activity of AMPD in crude muscle extracts from anoxic turtles was 1.2-fold higher than the value in control turtles, whereas the Km AMP was 27% lower than in control turtle muscle (Table 2.1); both of these values for anoxic turtles were significantly different from the corresponding values for aerobic turtles ($P<0.05$).

AMPD binding

AMPD is known to bind to myofibrils *in vivo* and changes in the distribution between free and myofibril-bound states have been shown to occur in response to physiological stimuli (such as exercise) in other systems. To determine whether the transition to the anoxic state altered the percentage of AMPD bound to myofibrils in turtle skeletal muscle, the proportions of free versus bound AMPD were quantified in anoxic versus aerobic *T. s. elegans*. Free AMPD activity was measured in the

supernatant after homogenization/centrifugation of muscle extracts by the normal procedure whereas bound AMPD activity was measured directly using the re-suspended the pellet. The percentage of bound AMPD in red muscle of 20 h anoxic turtles was 32.9% ± 1.5 (n=4), significantly higher ($P<0.05$) than the percent bound, 28.7% ± 1.2 (n=4), in the muscle of control animals (Table 2.1). The V_{max} of AMPD bound in the pellet of anoxic animals was also significantly higher than in aerobic controls (by 44%) and the K_m AMP of bound AMPD was 33% lower for the anoxic bound enzyme compared with aerobic bound AMPD. In both the aerobic and anoxic situations, the K_m AMP of the bound enzyme was also significantly lower than the corresponding K_m value for free AMPD; values for bound AMPD were 33-39% lower than aerobic controls (Table 2.1). Velocity versus [AMP] curves for bound AMP maintained the hyperbolic pattern seen for the soluble enzyme (Figure 2.2 (B)).

Purification and SDS-PAGE of turtle muscle AMPD

AMPD from aerobic muscle was purified in a two-step procedure using phosphocellulose and DEAE Sephadex G50 chromatography. Phosphocellulose will bind a variety of enzymes that have phospho-substrates such as nucleotides and binding of AMPD to phosphocellulose slurry has been widely used for AMPD purification (Smiley, 1967; English and Storey, 2000). After AMPD elution from phosphocellulose, ion exchange chromatography on DEAE was used as a second purification step. AMPD was eluted from DEAE with a KCl gradient and the elution profile is shown in Figure 2.3(A). The enzyme eluted in a single sharp peak at ~0.4 M KCl and peak fractions

were pooled for subsequent studies. The recovery of AMPD activity was 74.8% ± 4.6 for the DEAE column (mean ± S.E.M, n=4 purification trials). A typical purification of AMPD from red muscle of aerobic control *T. s. elegans* is summarized in Table 2.2. This procedure typically gave a net purification factor of roughly 20-fold and resulted in a purified preparation with a final specific activity of approximately 1.45 U/mg protein (assayed under optimal conditions at ~22 °C).

The kinetics study was performed on the partially purified enzyme. The same velocity versus [AMP] relationship (hyperbolic kinetics) was seen with both crude extracts and partially purified preparations after DEAE G50 Sephadex chromatography (Figure 2.3(B)). The K_m AMP of the purified enzyme from aerobic muscle was determined; the value was 0.44 ± 0.04 mM, significantly lower than the value for crude AMPD ($P<0.05$).

The purity and subunit molecular weight of turtle red skeletal muscle AMPD was assessed by SDS-PAGE with silver staining. Figure 2.4(A) shows a very high purification of the enzyme after the DEAE step (lane 2) as compared with the crude extract (lane 1). The purified enzyme showed one strong band of protein with one minor band remaining; hence, AMPD was highly purified. Lane 3 shows the separation of molecular weight standards and Figure 2.4 (B) shows the graph of log MW versus R_f (relative migration distance) on the gel. The estimated subunit molecular weight of AMPD was 86.2 kDa (n=1). This is consistent with the MW of the enzyme from other sources; e.g. a value of 80 kDa was reported for Sprague-Dawley rat skeletal muscle AMPD (Terjung, 1991, Rush, 1998), a value of 83-85 kDa for AMPD from human

platelets (Ashby, 1981).

Native molecular weight estimation

The native molecular weight of turtle muscle AMPD was determined by size exclusion chromatography on a Sephacryl S-400 gel filtration column. The peak of enzyme activity eluted at around 72.8 mL (Figure 2.5 (A)). Figure 2.5 (B) shows the graph of log MW versus elution volume for standard proteins and from this the native molecular weight of turtle AMPD was estimated to be 344.8 ± 78.6 kDa (n=3). When compared with the subunit molecular weight of 86.2 kDa this indicates that turtle AMPD is a tetramer.

Thermal stability and sensitivity of AMPD

Initial tests showed that AMPD was relatively unstable when stored at 4°C in homogenizing buffer with only 10-20% remaining after just 1-2 days. In order to find conditions that would allow longer term storage of the enzyme, the stability of AMPD in crude extracts from aerobic control muscle was tested over storage time at 4°C in the presence of various additives that could potentially improve enzyme stability. Figure 2.6 shows that certain additives improved the stability of AMPD. The addition of 200 mM KCl or 500 mM sucrose provided the greatest improvement in stability with about 65% and 46% of initial activity remaining after 2 days of storage. By contrast, 2 mM ATP was ineffective in stabilizing AMPD, giving no improvement in stability as compared with the enzyme stored without additives. As the result of these tests, it was

determined that that the enzyme should be stored in a high salt buffer (330 mM KCl).

Higher concentrations of protein allow for greater thermal stability of the enzyme. Figure 2.7 shows the thermal sensitivity of AMPD to incubation at 40°C, comparing the enzyme from the crude supernatant, bound AMPD from the pellet, and semi-purified AMPD after DEAE chromatography. Activity of the partially purified AMPD declined very rapidly with more than 60% activity lost within 5 min. Both the free and bound crude enzymes were more stable, with approximately 10 and 22% of activity lost in 5 min, respectively. The calculated t½ (min) values were supernatant 17.1 ± 0.68, pellet 12.2 ± 0.80, and DEAE 4.8 ± 0.18, respectively. Overall, the data indicate that turtle AMPD has low stability at temperatures that are not too far above the physiological range.

Effectors of AMP deaminase

Table 2.3 shows the effects of potential activators and inhibitors on turtle muscle AMPD. ATP was an allosteric activator of both the crude and partially purified enzyme and increased AMPD activity by 1.5-1.8 fold at the optimal ATP·Mg concentration of 2 mM. The ATP·Mg concentration that gave half-maximal activation (K_a) was calculated to be 0.62 ± 0.04 mM for the crude enzyme and significantly lower, 0.29 ± 0.03 mM, for partially purified AMPD after DEAE chromatography.

The effects of selected cations (K^+, Na^+, NH_4^+) and anions (Cl^-, SO_4^{2-}, inorganic phosphate) on AMPD activity were tested. Monovalent cations were weak inhibitors of AMPD with I_{50} values for NH_4^+ and Na^+ of ~400 mM and no effect of K^+ on AMPD (all

tested as chloride salts). By contrast, the divalent cation, Mg^{2+}, was quite a strong inhibitor with I_{50} values of 13-15 mM for both chloride and sulfate salts. Among anions Cl^- was a weak inhibitor and SO_4^{2-} appeared to inhibit weakly as well (since I_{50} values for $MgCl_2$ and $MgSO_4$ were very similar). Phosphate anion was a very strong inhibitor with I_{50} values of just 4-5 mM for either sodium or potassium salts.

In vitro incubations to assess reversible phosphorylation modification of AMPD

Aliquots of crude muscle extract from control and 20 h anoxic turtles were incubated under conditions that stimulated the activities of endogenous protein kinases or protein phosphatases and the subsequent effects on AMPD maximal activity were assessed. Figure 2.8 (A) shows the effects of stimulating the activities of protein kinases on AMPD activity. All conditions tested stimulated the activity of both aerobic control AMPD and anoxic AMPD except that stimulation of PKG activity did not alter the activity of AMPD in anoxic extracts. This indicates the AMPD could be subject to regulation by several of the major protein kinase systems in cells including PKA, PKG, PKC and AMPK. For incubations of aerobic control AMPD, the percentage increases in enzyme activity under the different conditions were: total kinase 51.1%, PKA 49.3%, PKG 42.4%, PKC 52.5% and AMPK 56.9%. The comparable percentage increases in AMPD activity in extracts from anoxic muscle were: total kinase 13.9%, PKA 36%, PKG 14.4%, PKC 22.7% and AMPK 44.5%. Hence, overall, the increase in activity was substantially higher for kinase effects on control AMPD than it was for kinase effects on anoxic AMPD. This might suggest that the transition from aerobic to anoxic

conditions results in a partial phosphorylation of AMPD but that the anoxic enzyme can still be phosphorylated more when it was specifically incubated with kinases. Figure 2.8 (B) shows the comparable effects of stimulating protein phosphatase activities. AMPD V_{max} activities in preparations from aerobic control turtle muscle were not affected by any of the treatments that stimulated endogenous protein phosphatase activities. However, stimulation of several phosphatase classes significantly reduced AMPD activity in muscle extracts from anoxic turtles. This included stimulation of total serine/threonine phosphatase activities, PP1, PP2B and PP2C (Figure 2.8 (B)). Overall, the results of these treatments to stimulate phosphatases suggest that the aerobic control enzyme is a low phosphate (dephosphorylated) form of AMP whereas the anoxic enzyme is a higher phosphate form that can be affected by phosphatase treatment.

Figure 2.1. Effect of pH on the activity of AMPD.

Assay conditions were 50 mM MES buffer for pH 6.0~6.8, 50 mM Tris-HCl buffer for remaining pHs, and 5 mM AMP. Data are means $\pm$ SEM, n = 3 determinations. Optimal activity was achieved at pH 7.2.

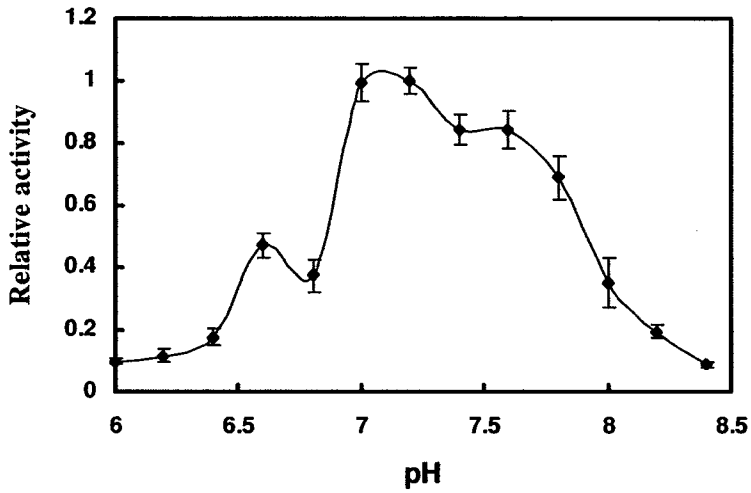


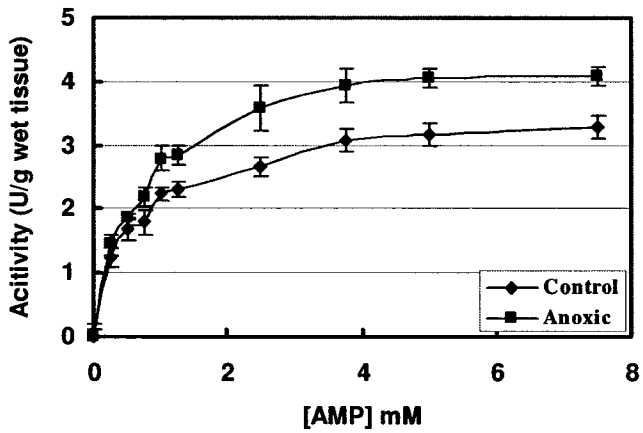
Figure2.2. Velocity versus AMP concentration for (A) soluble AMPD in the supernatant and (B) bound AMPD extracted from the pellet in muscle of aerobic control and anoxic turtles.

Data show mean ± SEM values for n = 4 independent trials.

For the supernatant, the K_m (0.57 ± 0.039 mM) of anoxic AMPD was significant lower than that of control AMPD (0.78 ± 0.043 mM) ($P<0.05$); the V_{max} (4.35 ± 0.17 U/g wet tissue) was significant higher than that of control AMPD (3.68 ± 0.19 U/g wet tissue) ($P<0.05$).

For the pellet, the K_m (0.35 ± 0.037 mM) of anoxic AMPD was significant lower than that of control AMPD (0.52 ± 0.049 mM) ($P<0.05$); the V_{max} (2.14 ± 0.13 U/g wet tissue) was significant higher than that of control AMPD (1.48 ± 0.11 U/g wet tissue) ($P<0.05$).

A



B

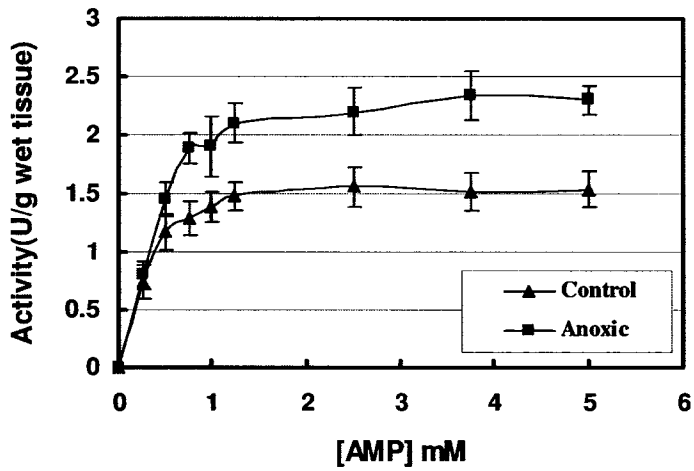


Table 2.1. Kinetic parameters of free (from supernatant) and bound (from pellet) forms of AMPD from muscle of aerobic control versus 20 h anoxia exposed turtles. Data are means ± SEM, n≥4. [a] - Significantly different from the corresponding aerobic control value, $P< 0.05$. [b] - Significantly different from the corresponding value for free AMPD in the supernatant, $P< 0.01$.

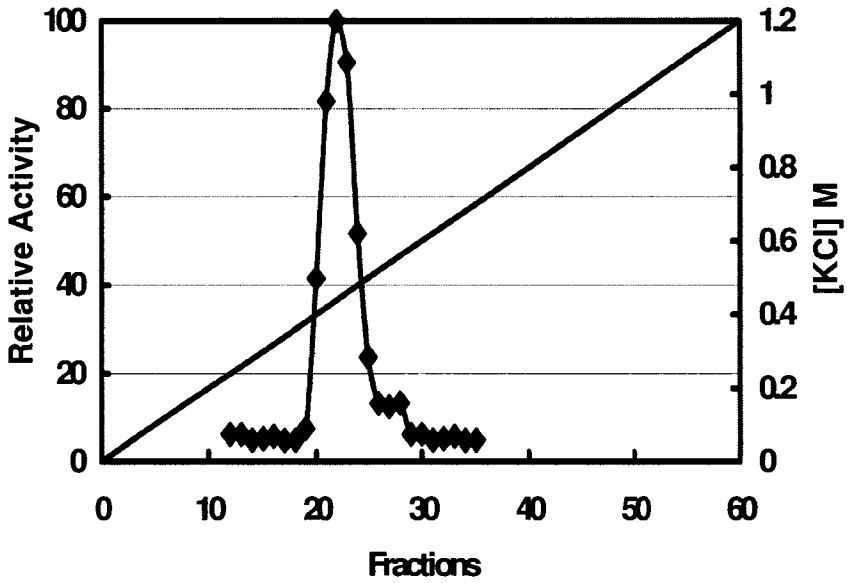
	Free		Bound		% bound
	K_m (mM)	V_{max} (U/g tissue)	K_m (mM)	V_{max} (U/g tissue)	
Aerobic	0.78 ±0.043	3.68±0.19	0.52±0.049[b]	1.48±0.11[b]	28.7%±1.2
20 h Anoxic	0.57±0.039[a]	4.35±0.17[a]	0.35±0.037[a,b]	2.14±0.13[a,b]	32.9%±1.5[a]

Figure2.3. Typical elution profile and kinetics analysis for control muscle AMPD from DEAE G50 Sephadex.

(A) Typical elution profile for control muscle AMPD from DEAE G50 Sephadex. Fractions were assayed under optimal conditions. The enzyme eluted at about 0.4 M KCl and recovery of activity in pooled peak fractions was 74.8%.

(B) Kinetic analysis of the DEAE partially purified enzyme showed a hyperbolic relationship between velocity and [AMP] with a calculated K_m AMP of 0.44 ± 0.04 mM, significantly lower than the value for crude AMPD ($P<0.05$).

A



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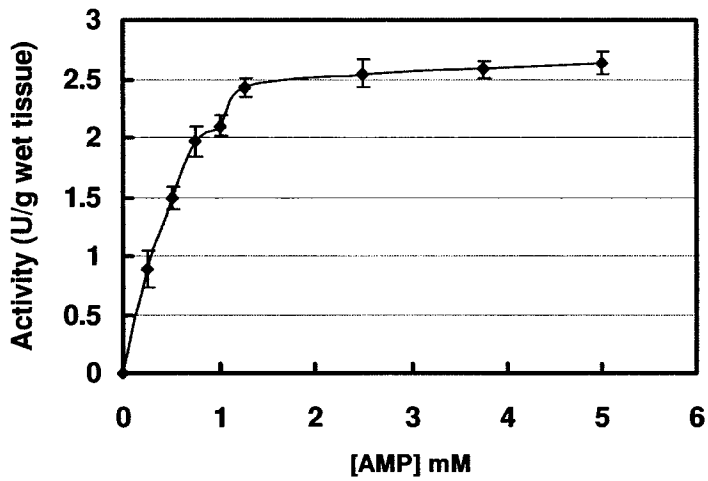


Table 2.2: Typical purification of AMPD from red muscle of aerobic control *T. s. elegans*. This procedure typically gave a net purification factor of ~20 fold and resulted in a purified preparation with a final specific activity of ~1.45 U/mg protein (assayed under optimal conditions at 22°C).

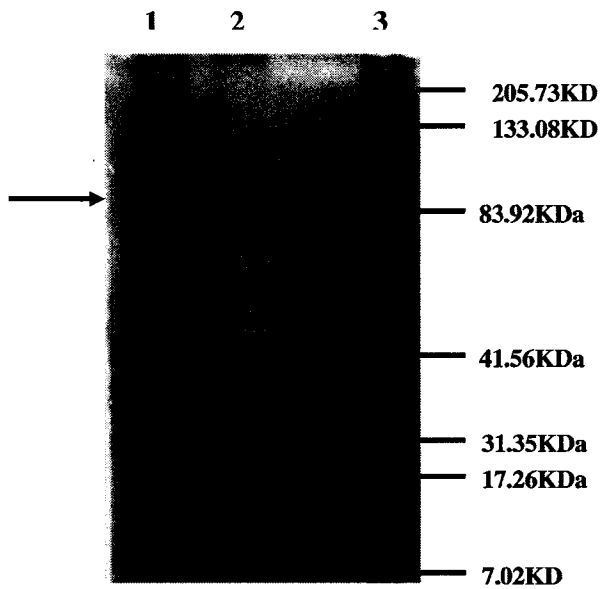
Purification Step	Total Protein (mg)	Total Activity (U)	Specific A. (U/mg)	Purification fold	Yield (%)
Supernatant	17.71	1.22	0.069	1	100
Phosphocellulose	2.21	0.65	0.294	4.26	53.6
Ion exchange chromatography	0.49	0.71	1.45	21.01	74.8

Figure 2.4. SDS-PAGE of purification AMPD from aerobic turtle muscle.

(A) SDS-polyacrylamide gel electrophoresis showing the purification of AMPD from aerobic turtle muscle. The gel was stained with silver. Lane 1: crude extract; Lane 2: after DEAE ion exchange chromatography; Lane 3: molecular weight standards.

(B) Standard curve relating log MW and Rf for the protein standards: myosin (205.7), β-galactosidase (133.1), bovine serum albumin (83.9), carbonic anhydrase (41.6), soybean trypsin inhibitor (31.4), lysozyme (17.3) and aprotinin (7.0). The Rf (0.82 cm) for AMPD is indicated by the arrow.

A



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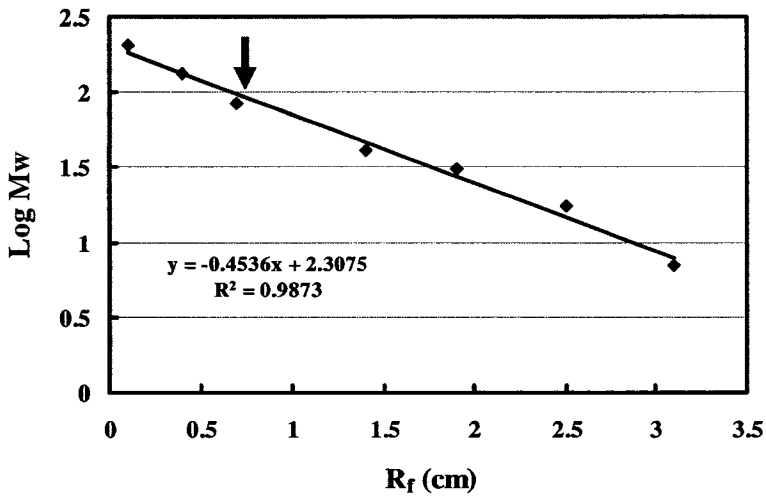
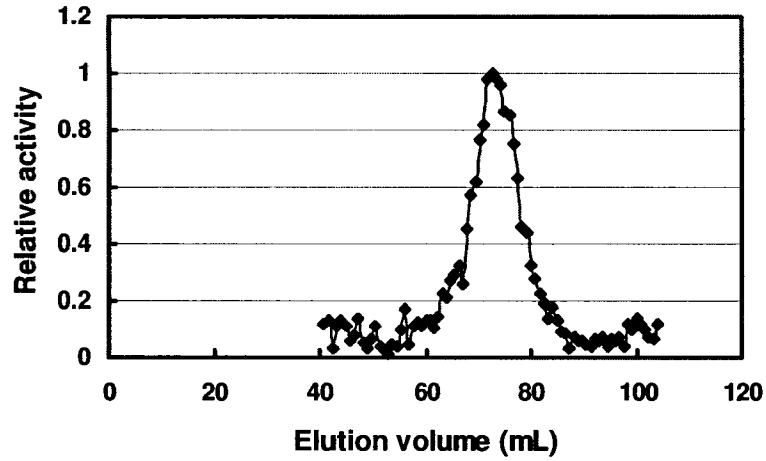


Figure 2.5. Sephacryl S-400 gel-filtration elution profile for soluble AMPD from control muscle.

(A) Sephacryl S-400 gel-filtration elution profile for soluble AMPD from control muscle. Fractions of 20 drops (approximately 700 μL were collected and assayed under standard conditions; the peak eluted at 72.8 mL.

(B) Standard curve for the Sephacryl S-400 column showing the elution positions of standard proteins and turtle AMPD (indicated with an arrow). The standards and their molecular weights are: blue dextran (M_w 2000 kDa), thyroglobulin (M_w 670 kDa), Jack Bean urease (M_w 545 kDa), *Bacillus pasteurii* urease (M_w 272 kDa), hemoglobin (M_w 64.5 kDa), Cytochrome (M_w 12.5 kDa). Data show means $\pm$ SEM, n = 3 individual determinations.

A



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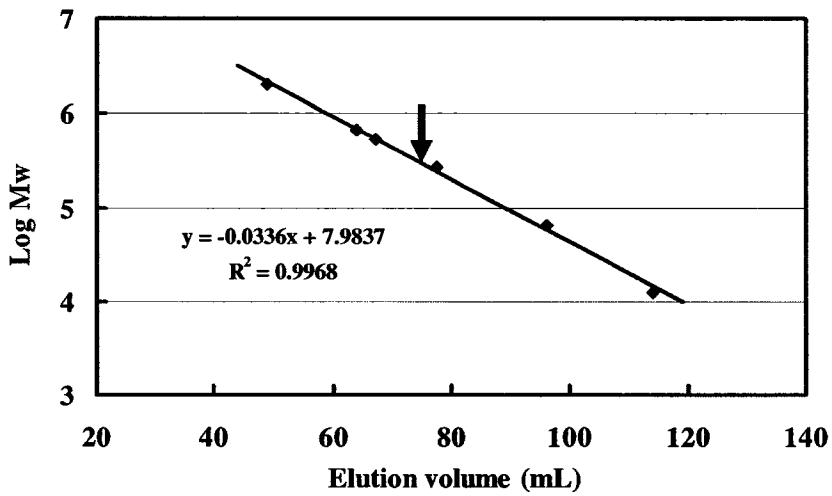
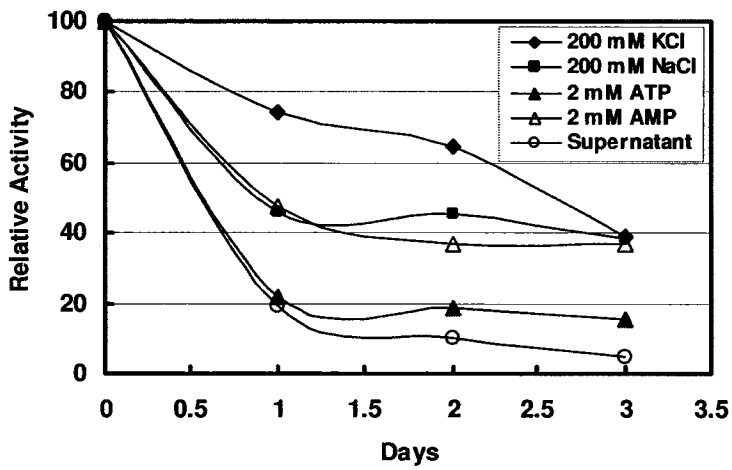


Figure 2.6. The effect of adding different compounds on the stability of crude aerobic muscle AMPD over storage time at 4°C in homogenizing buffer.

(A) Effects of storing in the presence of 200 mM KCl, 200 mM NaCl, 2 mM ATP, or 2 mM AMP versus the crude supernatant.

(B) Effects of storing in the presence of: 500 mM sucrose, 5% v:v glycerol, 10% v:v glycerol, and 10 mM $MgCl_2$. Data show the percentage of original activity remaining.

A



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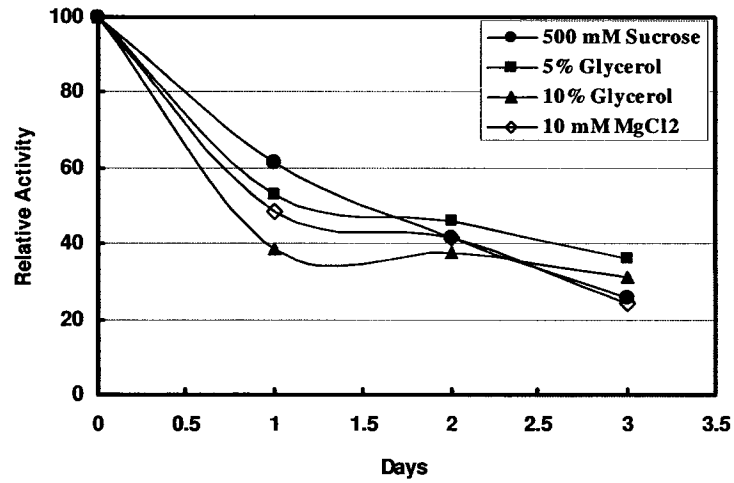


Figure 2.7. Thermal sensitivity of AMPD during incubation at 40°C comparing AMPD in the crude supernatant, bound AMPD released from the pellet, and partially-purified AMPD after DEAE chromatography.

At different times, aliquots of AMPD were removed and assayed under optimal conditions; relative activity remaining is shown over time. Data show the percentage of original activity remaining, means $\pm$ SEM, n = 3 individual determinations.

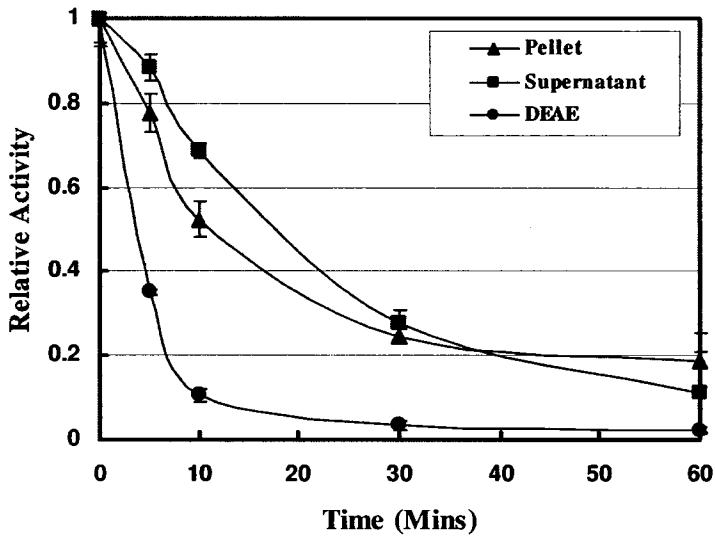


Table 2.3. Activators and inhibitors of turtle control muscle AMPD. K_a is the concentration of activator that gives half-maximal activation of AMPD; I_{50} is the concentration of inhibitor that decreases activity by 50%. Stock ATP was prepared and tested as a 1:1 molar mixture with $MgCl_2$. Pi is inorganic phosphate (potassium and sodium salt). K^+, NH_4^+ and Na^+ were tested as chloride salts, whereas anions were tested as sodium, potassium and magnesium salts. KCl was without effect up to the highest (500 mM) concentration tested.

	Crude extract		DEAE semi-purified	
Activator	K_a (mM)	Fold Activation	K_a (mM)	Fold Activation
ATP. Mg^{2+}	0.62 ± 0.04	1.5	0.29 ± 0.03	1.8

Inhibitor	I_{50} (mM)
KCl	No effect
NH_4Cl	392 ± 30
NaCl	447 ± 9
$MgCl_2$	15.2 ± 0.68
$MgSO_4$	12.7 ± 0.63
NaH_2PO_4	4.15 ± 0.24
KH_2PO_4	5.33 ± 0.35

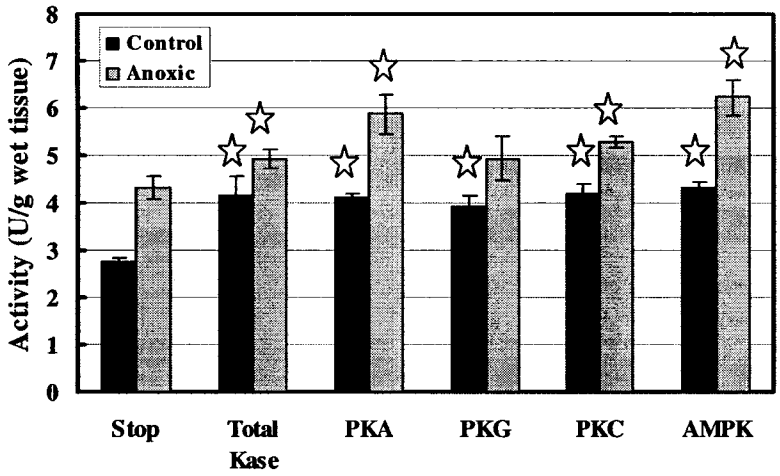
Figure 2.8. Effect of *in vitro* stimulation of the activities of endogenous protein kinases and phosphatases on AMPD activity in extracts from control (aerobic) versus anoxic muscle.

(A) Effect of *in vitro* stimulation of the activities of endogenous protein kinases on AMPD activity in extracts from control (aerobic) versus anoxic muscle. Incubation was for 4 h at 4°C under control conditions that stopped the activity of all phosphatases and kinases (STOP) or conditions that stimulated total protein kinase activity or stimulated individual kinases: PKA, PKG, PKC, or AMPK.

(B) Effect of *in vitro* stimulation of the activities of endogenous protein phosphatases on AMPD activity in extracts from control versus anoxic muscle. Incubation was for 4 h at 4°C under control conditions that stopped the activity of all phosphatases and kinases (STOP) or conditions that stimulated groups of phosphatases (total protein phosphatases, Tyr phosphatases, Ser/Thr phosphatases) or specific individual phosphatases (PP1, PP2B or PP2C).

* - Significantly different from the corresponding activity under the STOP condition, $P<0.05$.

A



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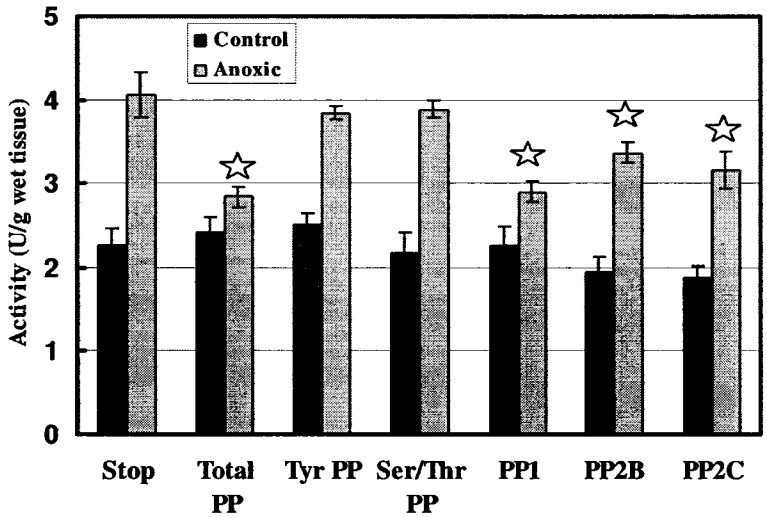


Discussion

AMP deaminase, an enzyme that consumes AMP to produce IMP and ammonia, has been implicated in the stabilization of cellular energy status under stressed conditions that cause high ATP breakdown by effectively minimizing the resulting increase in AMP concentration.

The optimal pH for AMPD activity was pH 7.2, which indicates that the enzyme works best under physiological pH conditions. The results presented in Figure 2.2 and Table 2.1 show that turtle muscle AMPD follows hyperbolic substrate saturation kinetics. Kinetic parameters, K_m and V_{max} changed significantly when the enzyme was isolated from anoxic muscle; the K_m was lower and the V_{max} was higher than that measured for the enzyme in control muscle. These stable changes in kinetic parameters suggest that the enzyme may be modified in response to low oxygen conditions and imply that AMPD has an altered and necessary function under stressed conditions. Stabilization of cellular energy charge is the key to the survival of all cells and requires particular attention under stress conditions, such as anoxia that compromise the ability to produce ATP. Stabilization of energy charge is one of the physiological functions proposed for AMPD (Chapman and Atkinson, 1973). In addition, Raffin and colleagues (Izem, Raffin and Thebault, 1993) showed that energy charge also affected AMPD activity. They found that the enzyme activity of the AMP deaminase increased linearly as the energy charge decreased from 0.9 to 0.6, and then continued to increase slowly until maximum activity was reached at energy charge value of about 0.2. This means that the activity of AMPD *in vivo* would increase quickly whenever energy charge was

reduced, such as during burst swimming (Kaletha and Zydowo, 1979). Whenever the rate of ATP hydrolysis exceeds the rate of its synthesis, AMP accumulates and AMPD is activated to convert AMP to IMP. By removing AMP from the total adenylate pool, energy charge is again elevated. Therefore, the system is self-regulated and energy charge is stabilized.

The percentage of bound AMPD in red muscle of 20 h anoxic turtles was 32.9% ± 1.5 (n=4), significantly higher ($P<0.05$) than the percent bound, 28.7% ± 1.2 (n=4), in the muscle of control animals (Table 2.1). In both the aerobic and anoxic situations, the K_m AMP of the bound enzyme was also significantly lower than the corresponding K_m value for free AMPD; values for bound AMPD were 33-39% lower than aerobic controls (Table 2.1). This could make AMPD a more effective catalyst in the bound state. However, velocity versus [AMP] curves for bound AMP maintained the hyperbolic pattern seen for the soluble enzyme (Figure 2.2(B)).

A change in the binding of AMPD to myosin implies that association of the enzyme with myosin is physiologically relevant (Shiraki et al., 1981). Lushchak and Storey (1998) showed AMPD distribution between free and bound states in fish muscle was modified by the extent of hypoxia. The present data indicate that a comparable effect of anoxia on AMPD binding occurs in turtle muscle. With lengthy hypoxia exposure, AMPD binding to myofibrils in the particulate fraction increased, presumably as a mechanism that helps to regulate adenylate metabolism in the muscle when energy charge declined with progressive hypoxia. It is well known that hypoxia leads to a decline in energy charge in fish tissues (Jorgensen and Mustafa, 1980).

Energy charge is an important intracellular regulator of many processes. Myosin ATPase is the major consumer of ATP in the skeletal muscle; ADP is its product and via the myokinase reaction AMP accumulates (2 ADP → ATP + AMP). Rising AMP and ADP concentrations reduce the energy charge. In this situation, removal of AMP from the adenylate pool via the AMPD reaction provides a mechanism for sustaining energy charge at a high value for as long as possible. Not only does increased binding of AMPD to myofibrils place the enzyme in closer physical contact with the source of ATP hydrolysis (ADP production) but the bound enzyme from turtle muscle has a higher affinity for AMP than does free AMPD and this affinity increases even more (K_m decreases) in the anoxic muscle. Hence, bound AMPD in anoxic muscle is poised to effective deaminate AMP as it is made.

AMPD from the skeletal muscle of aerobic control *T. s .elegans* was purified to electrophoretic homogeneity. A typical purification of AMPD is summarized in Table 2.2. This procedure typically gave a net purification factor of roughly 20 fold and resulted in a purified preparation with a final specific activity of approximately 1.45 U/mg protein. This purification procedure which was modified from Smiley et al. (1967) was rapid and reproducible and, as illustrated in Figure 2.3(A), AMPD was eluted in one peak from the DEAE G50 column.

The subunit molecular weight of *T. s .elegans* AMPD, as determined by SDS-PAGE (Figure 2.4(A)), was 86.2 kDa (n=1). This is consistent with the MW of the enzyme from other vertebrate sources; e.g. a value of 80 kDa was reported for Sprague-Dawley rat skeletal muscle AMPD (Terjung, 1991; Rush, 1998), and a value

of 83-85 kDa for the human platelet enzyme (Ashby, 1981). Native AMPD exists as a tetramer with subunits of 43-88 kDa (Hohl and Hohl, 1999) and two forms of the enzyme are found in some species (Spychala, 1984; Spychala and Marszalek, 1986). Hence, gel filtration on a Sephacryl S-400 column was used to determine both the native size and to determine whether *T. s .elegans* muscle AMPD existed as single or multiple isoforms. The results in Figure 2.4 and 2.5 show that turtle AMPD is a tetramer with only one form present.

The stability study showed that turtle AMPD was relatively unstable when stored at 4 °C. However, stability could be improved with the addition of certain additives (Figure 2.6). A high concentration of salt (such as KCl and NaCl) increased AMPD stability; this is consistent with findings by other authors (Raffin et al., 1987). Thermal stability at high temperature (40°C) was also assessed and was found to be quite poor. The t½ (min) values for loss of AMPD activity were 17.1 ± 0.68 min for the crude soluble enzyme, 12.2 ± 0.80 min for bound AMPD, and just 4.8 ± 0.18 min for the DEAE purified enzyme. The decrease in stability of the purified enzyme is undoubtedly due to a reduced protein concentration since previous studies have shown that high protein concentration increases the stability of AMPD in solution (Smiley et al., 1967; Lushchak and Storey, 1994). These data indicate that turtle AMPD has low stability at temperatures that are not too far above the physiological range.

Turtle AMPD was also tested for its response to an allosteric effector, ATP·Mg, whose concentration is known to vary between control and anoxic states. Interestingly, the free form of AMPD in control muscle was activated by ATP·Mg by 1.2-1.8 fold at

the optimal ATP·Mg concentration of 2 mM. Reasons given for ATP activation of the enzyme from other sources are obscure, but it has been proposed that as ATP levels decline physiologically, the activation would be lifted, perhaps as a mechanism to prevent under loss of adenylates from the total pool (Chapman et al., 1973). Spychala and his colleges (Spychala, 1984; Spychala and Marszalek, 1986) found ATP also activate the AMP deaminase in the lizard liver and other vertebrate liver tissue.

The inhibiting effects of cations and anions (Table 2.3) may be connected with a modification of enzyme structure or neutralization of either the substrate or active site of the enzyme although at present it is hard to differentiate between those two possibilities. Monovalent cations, K^+, Na^+, and NH_4^+ had very little effect on the enzyme. In particular, the very similar I_{50} values for NaCl and NH_4Cl indicates that there is virtually no product inhibition of AMPD by NH_4^+ since physiological levels of NH_4^+ are very low in animal cells (<5 mM). Mg^{2+} was a much stronger cation inhibitor. Among anions Cl^- was a weak inhibitor and SO_4^{2-} appeared to inhibit weakly as well since the I_{50} values for $MgCl_2$ and $MgSO_4$ were very similar. Phosphate anion was a very strong inhibitor with I_{50} values of just 4-5 mM for either sodium or potassium salts. It is quite likely that this effect is a competitive one resulting from P_i interference with AMP binding to the enzyme. For the evaluation of the mechanism by which phosphate and sulfate affects the enzyme, a detailed kinetic analysis needs to be undertaken.

Analysis of the effects of protein kinases and protein phosphatases on turtle AMPD was undertaken. The data suggest the transition from aerobic to anoxia conditions

results in a partial phosphorylation of AMPD. However, the anoxic enzyme could still be phosphorylated more when it was specifically incubated with kinases. The activity of aerobic control AMPD was increased about 50% after stimulation of total protein kinase activity, whereas the activity of the anoxic enzyme only increased about 14% under the same conditions. Stimulation of individual protein kinases also increased AMPD activity except that AMPD in anoxic extracts was not stimulated by PKG. This indicates the AMPD could be subject to regulation by several of the major protein kinase systems in cells including PKA, PKG, PKC and AMPK. Which one regulates turtle AMPD with respect to different physiological situations (e.g. anoxia, exercise) remains to be determined? The data on the role of protein kinases in the control of AMPD in other systems is also limited although there is good evidence for the involvement of PKC in mammalian muscle. For example, the results of Hohl and Hohl (1999) showed that the piglet cardiac AMPD was regulated by reversible phosphorylation mediated by PKC, but cAMP-dependent protein kinase (PKA) did not affect enzyme kinetics. Rat skeletal muscle AMPD was also phosphorylated by PKC (Tovmasian, 1990). Dephosphorylation of AMPD (Figure 2.2(B)) was also evident in incubations that stimulated endogenous i) total phosphatase activity; ii) PP1 activity; or iii) PP1, PP2B and PP2C activity. These results suggest that one of the serine/threonine phosphatases is probably responsible for AMPD dephosphorylation during the recovery from anoxic conditions.

The data presented in this chapter provide evidence of enhanced AMPD activity under anoxic conditions in turtle muscle. The results of the kinetic study support a role

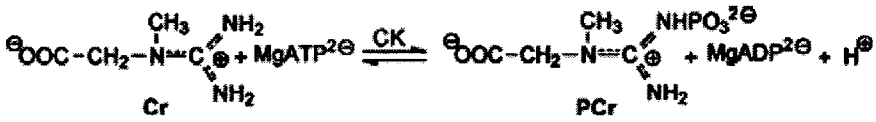
for the enzyme in the maintenance of energy charge during anoxia. The effects of ATP·Mg and salts indicate that allosteric controls are part of the mechanism of AMPD regulation in the turtle. The incubations to stimulate endogenous protein kinases and phosphatases strongly indicate that turtle muscle AMPD is a phosphoenzyme and that reversible phosphorylation has a central role to play in the differential regulation of AMPD under aerobic versus anoxic conditions.

CHAPTER 3

REGULATION OF CREATINE KINASE FROM SKELETAL MUSCLE AND HEART OF AN ANOXIA TOLERANT TURTLE

INTRODUCTION

Creatine kinase (CK; adenosine-5'-triphosphate: creatine phosphotransferase; creatine phosphokinase; phosphocreatine phosphokinase; creatine *N*-phosphotransferase; EC 2.7.3.2) catalyzes the reversible transfer of a phosphoryl group from MgATP to creatine (Cr), producing phosphocreatine (PCr) and MgADP.



Reaction catalyzed by creatine kinase.

Phosphocreatine was initially identified in skeletal muscle (Eggleton, 1928). At that time, it was thought to be the chemical source for the energy required for muscle contraction. According to McLeish and Kenyon (2005), the enzyme now known as creatine kinase was first identified by Lohman in 1934 and it was subsequently shown that by transfer of a phosphoryl group from PCr it formed ATP, which was discovered by Lehmann in 1936. ATP is now known as the mediator of all energy-requiring processes within cells.

There are four major CK isozymes, named for the tissues from which they were historically isolated. The isozymes have been characterized on the basis of differences in gene and amino acid sequence, tissue localization and immunogenicity. There are two cytosolic forms, the muscle (MM-CK) and brain (BB-CK) forms, which exist as dimers under physiological conditions. Under some circumstances, cytosolic CK can exist as a MB heterodimer (Eppenberger et al., 1967). There are also two mitochondrial forms of

the enzyme, the ubiquitous (Mi_u-CK) and the sarcomeric (Mi_s-CK) forms which, based on their isoelectric points, are sometimes referred to as acidic (Mi_a-CK) and basic (Mi_b-CK) mitochondrial CK, respectively (Wyse et al., 1992). The mitochondrial isoforms (MtCK) generally exist as octamers but can be readily dissociated into dimers (Wyse et al., 1992).

For many years only the soluble form of creatine kinase was known, and the main physiological role ascribed to CK was the maintenance of energy homeostasis at sites of high-energy turnover such as rapidly contracting skeletal muscle. The high levels of CK ensured that ADP and ATP levels remained almost constant, effectively buffering the cell against rapid depletion of ATP. The discovery of the mitochondrial isozymes showed that CK was also located in individual "compartments" and the concept of a creatine-phosphocreatine shuttle was developed. Here distinct isozymes are associated with sites of ATP production versus consumption, and they fulfil the role of a transport mechanism for high-energy phosphates (Wallimann et al., 1992).

The equilibrium creatine kinase reaction and the PCr pool are usually thought of as serving the purpose of maintaining an adequate ATP/ADP ratio in cells under increased energy demand (McGilvery and Murray, 1974). In addition to this usual concept of an energy buffer system, it has been proposed more recently that coupled creatine kinase systems are involved in effective metabolic regulation of energy fluxes and oxidative phosphorylation, besides their energy transfer function (Saks et al., 1996).

An important property of the creatine kinase system is that total activity, isoform distribution, and Cr concentration are highly variable (Ventura-Clapier et al., 1998). This indicated that in addition to a high variation of total activity and isoform expression, the

role of the CK system also critically depends on its intracellular organization and interaction with energy producing and utilizing pathways. Grayb and Skorkowski (2005) found two CK isoforms in herring (*Clupea harengus*) skeletal muscle: cytosolic CK and MtCK and the native M_r of these two were 86,000 and 345,000, respectively. This result indicated that one of the isoforms found in herring skeletal muscle is a cytosolic dimer and the other one is a mitochondria octamer. Total CK activity shows great species variation (Brirkedal and Gesser, 2003; Christensen et al., 1994). Christensen and his coworkers measured the total CK activity in heart muscle of 11 animals, showing highest activities in rat and pigeon, whereas cod had by far the lowest value. The CK activity of cod also fell below the range of the other ectothermic species, being about 14 times lower than that of turtle which had the highest CK activity of ectotherms examined. The other ectothermic species displayed CK activities two or five times below that of turtle (Christensen et al., 1994).

Heart muscle is of particular interest with regard to O_2 lack and temperature; heart muscle relative to other muscles may be particularly sensitive to O_2 limitation but it still has a key role to play in blood pumping even when oxygen is low. Hence, it is important to know how heart CK is regulated in anoxia tolerant organisms. Most studies identify metabolic adaptations that support anoxia survival by assessing changes in the maximal activities of CK in response to the stress. For example, some work has been done on anoxic fish heart (Hartmund and Gesser, 1992) and creatine kinase, energy-rich phosphates and energy metabolism in the heart of different vertebrates (Christensen et al., 1994). Little is known about the kinetic and physical properties of CK in heart of anoxia tolerant species. Storey (1975) studied CK in the heart of turtles (*Pseudemys*

scripta) and found two major electrophoretic forms. NADH activated the purified cytosolic enzyme by lowering the Km of PCr almost 3-fold.

Some studies have examined the role of protein phosphorylation in the control of CK (Quest et al., 1990; Hemmer, 1995; Stolz, 2002). These studies found that Thr-282, Thr-289 and Ser-285 are involved in the autophosphorylation of CK. Thr-282 and Ser-285 are located close to the reactive Cys-283. Thr-289 is located within a conserved glycine-rich region highly homologous to the glycine-rich loop of protein kinases which is known to be important for ATP binding. Thus, it seems likely that the described region constitutes an essential part of the active site of CK. Analysis of enzyme kinetic parameters also indicated that autophosphorylation of CK exerts a modulatory effect on substrate binding and the equilibrium constant, rather than on the catalytic mechanism itself. Theories of how CK interacts with ATP-producing reactions to capture the high-energy phosphate were developed because it was believed that the CK reaction was far from equilibrium. This conclusion was based on total ATP, ADP, Cr and PCr concentrations measured cells. However, new calculations based on the free level of those metabolites, revealed that the CK reaction was close to equilibrium (Brooks, 2004).

Although, CK has been widely studied, to our best knowledge, there has been no prior analysis of the molecular mechanism(s) by which CK may be altered and regulated in response to anoxia stress. The role of CK in turtle myocardium during underwater hibernation is of particular interest for two reasons, firstly, because a high myocardial CK activity seems to be associated with a high tolerance to hypoxia (Christensen et al., 1994), and secondly, because CK may be reorganized in response to changes in metabolic pathways (De Sousa et al., 1999).

The present study assessed multiple parameters of CK in skeletal muscle and heart of the freshwater turtle (*T. s. elegans*). This is the first study to compare multiple enzyme parameters between aerobic control and anoxic states and to try to identify the mechanism of enzyme modification in response to the stress. Activity of CK in skeletal muscle and heart of aerobic versus 20 h anoxic turtles was measured and kinetic properties of the aerobic and anoxic enzyme forms were characterized. The amount of CK protein, assessed via Western blotting, was also compared between aerobic and anoxic conditions to determine if changes in CK protein content were a factor in the response to anoxia. The stability of CK with respect to urea denaturation was also compared for aerobic versus anoxic forms of CK using the method of pulse proteolysis. Finally, the phosphorylation state of CK was analysed to determine whether CK was regulated by protein kinases and protein phosphatases and whether changes in the phosphorylation state could underlie enzyme regulation in anoxia.

MATERIALS AND METHODS

Animals and Biochemicals

Adult turtles (*T. s. elegans*) were treated as previously described in Chapter 2. All chemicals came from the same sources as described in Chapter 2.

Homogenization

Turtle heart or red skeletal muscle was homogenized 1:10 (w:v) in homogenization buffer (50 mM Tris buffer, pH 8.0, 50 mM β-glycerol phosphate, 10 mM β-mercaptoethanol, 2 mM EGTA, 2 mM EDTA and a few crystals of PMSF). Crude

homogenate was centrifuged at 10,000 ×g for 30 minutes. The supernatant was diluted 10 times with homogenization buffer for kinetic studies and held in the ice until use. For purification studies the ratio of tissue to buffer was 1:5 (w:v) for homogenization. To study the kinetic profile of bound CK in muscle, pellets were resuspended in 2 ml of homogenisation buffer and centrifuged as above. The supernatant was discarded and the pellet was washed a further 3 times to make sure that there was no unbound CK left. The pellet was then resuspended in buffer containing 500 mM KCl, stirred for few minutes to dissociate the enzyme from the pellet and after re-centrifugation, CK was assayed using supernatant for kinetic studies. The calculated Vmax for the bound enzyme fraction was then compared to the Vmax of soluble CK to determine the percentage of bound enzyme.

Enzyme assay

The activity of CK was measured using a Multiskan Spectrum Microplate Reader (at 340 nm) by measuring the production of ADP in a coupled enzyme assay in the presence of phosphoenolpyruvate, NADH, pyruvate kinase and lactate dehydrogenase.

CK $\quad Cr + ATP \rightarrow PCr + ADP + H^+$

PK $\quad ADP + PEP \rightarrow ATP + pyruvate$

LDH $\quad pyruvate + NADH \rightarrow lactate + NAD$

Optimal assay conditions for CK were 50 mM Tris buffer, pH 8.4, 5 mM ATP·Mg, 10 mM creatine, 5 mM $MgCl_2$, 20 mM KCl, 4 mM PEP, 0.225 mM NADH, and 1 U/assay of both LDH and PK. Standard assays used 10 µl of the diluted enzyme extract. The Km Cr was determined by measuring enzyme velocity at 5 mM ATP·Mg with different Cr concentrations (0, 0.5, 1, 2, 4, 6, 10, 14, 20, 25 and 30 mM). The Km

ATP was determined by measuring enzyme activity at 15 mM Cr with different ATP·Mg concentrations (0, 0.25, 0.5, 0.75, 1, 1.25, 2.5, 3.75, 5 and 7.5 mM).

DEAE ion exchange chromatography of CK

Ion exchange chromatography on DEAE Sephadex G50 was used to characterize CK from turtle red skeletal muscle and heart. The column (1.5 cm x 5 cm) was equilibrated in diluted homogenization buffer (10 mM Tris buffer, pH 9.0, 0.04 mM EDTA, 0.04 mM EGTA, 10 mM β-glycerol phosphate, 10 mM β-mecapthoethanol); 100 µl of homogenate was applied to the column and then the column was washed with 10 ml of the same buffer to remove unbound proteins. The column was eluted with a linear KCl gradient (0-400 mM) in diluted homogenization buffer. CK activity in each fraction was determined under optimal assay conditions.

Arrhenius analysis

Enzyme assays were performed under V_{max} conditions over a temperature range from 2°C to 35°C for both muscle and heart CK. Activation energy (Ea) was determined in KJ/mol for linear portions of the relationship. For temperature control, the Multiskan Spectrum Microplate reader was equilibrated to the appropriate temperature in a Precision 815 low temperature incubator. Assay plates were equilibrated in the incubator for ~10 min before reactions were initiated by the addition of enzyme.

Incubation to Stimulate Phosphatases and Kinases

Aliquots of enzyme extract were incubated for 4 hours at 4°C with specific inhibitors and stimulators as described previously (Chapter 2) to stimulate endogenous protein kinases and phosphatases. For these incubations, 50 mM β-glycerol phosphate was used instead of 30 mM NaF, because NaF inhibited CK activity. In addition to the conditions used previously, commercial alkaline phosphatase (AP) was also used to stimulate dephosphorylation by adding 10 mM $MgCl_2$, 5 mM EDTA and 1 U AP.

Western blotting

Frozen tissue samples were homogenized (1:5 w/v) in homogenization buffer containing 50 mM Tris buffer, pH 8.0, 50 mM β-glycerol phosphate, 10 mM β-mercaptoethanol and a few crystals of PMSF added just prior to homogenization. Samples were centrifuged at 10,000 x g for 30 min at 4°C. The supernatant was collected and soluble protein concentration was determined using the BioRad protein assay with bovine serum albumin as a standard. Samples were then diluted to the desired concentration in sample buffer containing 100 mM Tris-HCl (pH 6.8), 4% w/v SDS, 20% v/v glycerol, 5% v/v β-mercaptoethanol, and 0.2% w/v bromophenol blue. Samples were boiled for 5 min, cooled and then frozen at -20°C until use.

SDS-polyacrylamide gel electrophoresis was carried out using 12% acrylamide gels. Aliquots of 20 μg protein were loaded into each well. Proteins were separated on gels using a BioRad Mini-PROTEAN 3 system. Gels were run at 180 V until the desired separation of colored molecular weight markers (Kaleidoscope marker, BIORAD) was

achieved. Proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (Pall) by wet transfer in cold 25 mM Tris buffer, pH 8.5, 192 mM glycine and 20% v/v methanol. Transfer was carried out at 160mA for ~90 min.

PVDF membranes were incubated in blocking solution composed of TBST buffer (10 mM Tris buffer, pH 7.5, 150 mM NaCl, and 0.05% v/v Tween-20) and 5% (w:v) non-fat dry milk for 60 min at room temperature. The blocking solution was then discarded and the membrane was incubated in fresh TBST containing primary antibody against creatine kinase (CK-M C-14 goat polyclonal IgG Sc-15164; Santa Cruz Biotechnology), diluted according to manufacturer's instructions (1:500 v/v). Membranes were then incubated overnight at 4°C to allow primary antibody to bind. After incubation with primary antibody, membranes were washed with TBST in three short washes of five minutes each. Membranes were then incubated with 1:2000 secondary antibody (donkey anti-goat IgG-HRP Sc-2035) for two hours at room temperature in TBST. After incubation the membranes were washed again in TBST for 3 washes of 5 min. Final detection of the desired protein was carried out using an enhanced chemiluminescence assay with Super Signal West Pico Chemiluminescent substrate (PIERCE). The blots were scanned and quantified with the ChemiGenius Bio Imaging System (SynGene) using GeneTools software. Antibody specificity was always checked by using the immunizing peptide when commercially available.

To confirm that a consistent amount of protein was added to each well, membranes were subsequently stained with Coomassie blue. Membranes were rescanned and selected strong bands that showed constant intensities between samples (and were not near the MW of the protein of interest) were chosen as control bands. They were

quantified by densitometry and used to normalize the intensity of the immuno-reacted bands between lanes.

A minimum of 4 independent isolations from pooled tissue was performed per treatment. Control:anoxia ratios were calculated by first normalizing the control and anoxic band intensities with Coomassie stained blots, and then calculating the ratios of anoxic versus control values. The mean of these values was calculated ± SEM and the Student's t-test was employed to assess significant differences between control and anoxic states.

Pulse proteolysis:

Park and Marqusee (2005) reported a simple new method for assessing protein stability in response to denaturant treatment by using pulse proteolysis, which is designed to digest only unfolded proteins that relax on a time scale longer than the proteolytic pulse. Previous studies indicated that rat muscle CK is completely unfolded when treated with either 6 M urea or 3 M guanidine hydrochloride (Zhou et al., 2002).

To apply this technique to turtle CK, homogenates of red skeletal muscle and heart were prepared 1:2.5 w:v in homogenization buffer, centrifuged as described earlier, and then 20 µl aliquots of supernatant extract were incubated overnight at room temperature with 100 µl of each of the different concentrations of urea. Activity in each sample was then measured under optimum conditions as described previously. Pulse proteolysis was initiated by adding thermolysin (Sigma) to a final concentration of 0.40 mg/ml (the stock solution of thermolysin was prepared in a 2.5 M NaCl and 10 mM $CaCl_2$). After 10 min incubation, the reaction was quenched by adding 18 µl of 50 mM

EDTA (pH 8.0). CK protein content was then measured by Western blotting. Parallel experiments were performed to determine enzyme activity as a function of urea concentration. Parameters of protein unfolding and activity inhibition due to urea were calculated using the Kinetics program.

Data and Statistics

Enzyme kinetic parameters were analyzed by standard procedures as previously described in Chapter 2. Data are expressed as mean ± S.E.M. with statistical testing performed using the Student's t-test.

RESULTS

Optimization of experimental conditions

Optimization of homogenization buffer composition was undertaken for CK activity from red skeletal muscle and heart of aerobic control turtles. The presence of NaF (a protein phosphatase inhibitor) in the buffer clearly had a strong negative effect on CK activity whereas substitution of β-glycerol phosphate gave better results. Hence, the standard homogenization buffer used for CK isolation contained 50 mM Tris-base buffer, pH 8.0, 50 mM β-glycerol phosphate, 10 mM β- mercaptoethanol, 2 mM EGTA, 2 mM EDTA and a few crystals of PMSF.

Figure 3.1 (A) shows the effect of pH on the activity of skeletal muscle CK from control turtles. The highest activity was at pH 8.6. Activity declined sharply above pH 8.6 and below pH 6.6, but changed much more gradually between pH 6.6 and 8.6. There was no significant difference between activities measured at pH 8.2, 8.4 and 8.6; hence, all

standard assays were subsequently performed at pH 8.4. Heart CK was also assayed at pH 8.4.

Figure 3.1 (B) shows that crude CK stored at 4°C was very stable; no significant loss of CK activity was seen over 5 days (day 5 activity was still 98 ± 3% of the original).

Kinetic parameters of CK

Velocity versus ATP or creatine concentration curves are shown in Figure 3.2 (A) and (B), respectively, for soluble CK in crude extracts (supernatant) of turtle muscle. The enzyme displayed Michaelis-Menten (hyperbolic) kinetics with respect to both substrates. The maximal activity of CK in crude muscle extracts from anoxic turtles was 1.2-fold higher than the value in control turtles, but the Km (ATP) was not significantly different between the two conditions. However, Km (Cr) for the enzyme from anoxic turtles was 34% lower than the control value (Table 3.1(A)) ($P<0.05$).

Velocity versus substrate concentration curves are shown in Figure 3.3 (A) for ATP and Figure 3.3 (B) for Cr for insoluble CK in the pellet from turtle skeletal muscle. After several washings of the pellet (resuspension in buffer and re-centrifugation) to remove any remaining free enzyme, the pellet was resuspended in buffer containing 500 mM KCl to dissociated the bound enzyme from the pellet; after a final centrifugation, CK was then assayed using supernatant. The bound enzyme displayed Michaelis-Menten (hyperbolic) kinetics with respect to both substrates. The maximum activity of CK in the pellet from anoxic turtles was 65-66% of the value in control turtles for ATP and creatine, respectively. Table 3.1 (B) shows the properties of bound CK. As also occurred for soluble CK, the Km (Cr) of bound CK was 29% higher under anoxia than in the

aerobic control condition and the Km (ATP) was also significantly higher (by 22%) ($P<0.05$). The values for the percentage of CK bound in anoxic turtles were also significantly different from the corresponding values for aerobic turtles (Table 3.1(B)) ($P<0.01$). Although data derived from velocity versus [ATP] curves gave a higher estimate of overall % bound than did data derived from V vs. [Cr], the percentage decrease in the amount of bound enzyme during anoxia was the same, being 46 ± 3% and 44 ± 2% decreases, estimated from the ATP versus Cr data, respectively. CK substrate affinities also differed significantly between the bound and soluble states in both aerobic and anoxic turtles. Km (ATP) values of bound CK were 23% and 34% higher ($P<0.005$) for the aerobic and anoxic enzymes, respectively, than the corresponding values for soluble CK. The Km (Cr) of bound CK in anoxia was also significantly higher (by 18%; $P<0.005$) than the value for soluble CK in anoxia. However, the opposite effect was seen for aerobic CK; bound CK showed a 40% lower Km (Cr) than the soluble enzyme ($P<0.005$).

Velocity versus substrate concentration curves are shown in Figure3.4 (A) for ATP and Figure 3.4 (B) for Cr for soluble CK from crude extracts (supernatant) of turtle heart. The enzyme displayed Michaelis-Menten (hyperbolic) kinetics with respect to ATP and creatine concentration. The maximal activity of soluble CK in the heart from anoxic turtles was ~1.25 fold higher than the value for control CK, but the Km (ATP) was not different between the aerobic and anoxic states (Figure 3.5). Km for creatine was affected by anoxia exposure, falling to 72.6% of the value in control turtle heart ($P<0.01$).

Salt, pH and lactate effects on the CK from the heart of turtle

The results in Table 3.2 (A) show the effects of pH (pH 8.4, 7.2 and 6.5) on the Km values for ATP and creatine of turtle heart CK. The Km for ATP of control heart CK increased significantly with decreasing pH; compared with the value at pH 8.4 (0.50 ± 0.01 mM), the Km (ATP) rose by 1.35 fold with the decrease to pH 7.2 and by 3.1 fold at pH 6.5. However, Km (ATP) of CK from 20 h anoxic turtles showed a significant decrease from pH 8.4 (0.52 ± 0.029) to 7.2 (0.42 ± 0.021), whereas at pH 6.5, the Km (ATP) increased to 0.99 ± 0.025 ($P<0.05$), a ~2 fold increase compared with the value at higher pH's. The Km (Cr) responded oppositely with Km decreasing significantly as pH decreased; the value at pH 6.5 was 78.5% of the value at pH 8.4. However, CK from anoxic turtles showed very little effect of pH on Km (Cr) with only a small (9%) increase in Km at pH 6.5 compared with pH 8.4. Notably, Km (Cr) values of CK from aerobic and anoxic turtles, although significantly different at pH 8.4, were not significantly different at pH 6.5.

The effect of lactate on CK was tested at pH 7.2, the physiological pH. Table 3.2(A) shows that lactate decreased the affinity for ATP; Km (ATP) in the presence of 200 mM lactate at pH 7.2 rose by about 2.5-fold under both control and anoxic conditions as compared with the value at pH 7.2 without lactate. However, for the Km (Cr), the results showed that in the presence of 200 mM lactate, the Km decreased by 67.9% for the control and 42.6% for the anoxic turtle heart.

The effects of selected salts (KCl, NaCl, NH_4Cl, and K_2SO_4) on CK activity were also tested (Table 3.2B). Monovalent cations (K^+, Na^+, NH_4^+) with the same anion (Cl^-)

were inhibitors of CK. NaCl was the strongest inhibitor with I_{50} values of ~0.5 M, whereas NH_4^+ showed intermediate effects and KCl was the weakest inhibitor. Anion effects (Cl^-, SO_4^{2-}) on CK activity were tested using the same cation (K^+). The anions were also inhibitors of CK with I_{50} values for KCl being substantially higher (1.1-1.4 M) than the values for K_2SO_4 (0.2-0.3 M), so SO_4 is the stronger inhibitor. The I_{50} values for KCl, NH_4Cl and K_2SO_4 were significantly different between control and anoxic states.

Arrhenius plots

Figure 3.6 (A) and (B) shows Arrhenius plots for CK from red skeletal muscle and heart, respectively, with activities measured between 5 and 35°C for both aerobic control and anoxic conditions. The calculated activation energy, Ea, for CK from aerobic red skeletal muscle was 18.96 ± 1.13 KJ/mol, not significantly different from the value for CK in anoxic muscle, 19.12 ± 1.26 KJ/mol (Table 3.3). The Ea of CK from aerobic heart was 22.78 ± 1.76 KJ/mol and was significantly lower in anoxic heart, 18.27 ± 1.32 KJ/mol.

DEAE ion exchange chromatography of turtle muscle and heart CK

CK from aerobic and anoxic muscle and heart was subjected to ion exchange chromatography on DEAE Sephadex-G50. Figure3.7 (A) shows typical elution profiles for CK from skeletal muscle. CK in both aerobic and anoxic extracts eluted in single peaks that overlapped. The peaks eluted at about 0.16 M KCl and the recovery of activity was 83.2 % and 81.9 % for aerobic and anoxic CK. The overlap of the elution profiles indicates that CK from skeletal muscle of aerobic and anoxic turtles has the same

electrical charge and, therefore, suggests that the two may also have similar phosphorylation states. However, Figure 3.7(B) shows that CK in turtle heart extracts behaved differently with the enzyme from aerobic and anoxic heart showing distinctly different elution profiles. The elution peak for aerobic CK was at about ~0.18 M whereas the peak for anoxic CK was at ~0.12 M. This shows that CK has different charge states under aerobic versus anoxic conditions and these differences may be due to changes in the phosphorylation state of the enzyme. The recovery of heart CK activity from the columns was 79.2 % and 76.8 % for the aerobic and anoxic extracts, respectively.

In vitro incubations to assess reversible phosphorylation modification of CK

In vitro incubations of turtle muscle or heart extracts were used to stimulate the actions of endogenous protein kinases or protein phosphatases in order to determine whether CK maximum activity was altered in response to a change in protein phosphorylation state. Figure 3.8 (A) shows that stimulation of total protein kinases did not change the maximum activity of red skeletal muscle CK in extracts from aerobic control animals. However, incubation conditions that stimulated endogenous total protein phosphatases resulted in a significant increase in the V_{max} of CK in muscle extracts from both aerobic and anoxic turtles ($P<0.05$). Incubations that stimulated endogenous total protein kinases in heart extracts did not change the V_{max} of CK in aerobic extracts, but significantly decreased the V_{max} of CK in extracts from anoxic turtle heart (Figure 3.8 (B)) ($P< 0.05$). By contrast, incubations that stimulated endogenous total protein phosphatase activity in heart extracts significantly increased the V_{max} of CK from both aerobic ($P<0.01$) and anoxic turtles ($P<0.05$).

Heart CK was further subjected to incubations that tested the effects of individual types of protein kinases and phosphatases on the enzyme. Figure 3.9(A) shows the effects of incubating heart extracts from aerobic and anoxic turtles under conditions that stimulated the individual activities of PKA, PKG, PKC and AMPK. Stimulation PKA, PKG or AMPK significantly reduced the V_{max} of CK from anoxic turtles ($P<0.05$), but PKC did not change CK activity. However, equivalent incubations of heart extracts from aerobic turtles had no effect on CK V_{max}. Incubations that stimulated protein phosphatase activities were also conducted (Figure 3.9(B)). Conditions that stimulated total Ser/Thr phosphatases or individually stimulated PP1 or PP2C significantly increased CK V_{max} in extracts of both aerobic and anoxic heart ($P< 0.05$). Notably, the percent increase in activity was greater (about 25.6~40.8%) for aerobic CK than for anoxic CK (9.4~17.3%). Furthermore, addition of exogenous alkaline phosphatase (AP) strongly increased CK activity in both aerobic and anoxic extracts, by 88.8% for aerobic and 25% for anoxic CK. By contrast, stimulation of tyrosine phosphatases or PP2B had no effect on V_{max} of CK from either experimental condition.

Western blotting of CK in both muscle and heart

The amount of CK protein found turtle muscle and heart under aerobic versus anoxic states was examined via Western blotting. Antibodies recognizing the mammalian muscle isozyme of CK crossreacted well with the turtle protein producing a single strong band on SDS-PAGE gels at a molecular weight of ~43 kD which is consistent with the known subunit size of CK (Figure 3.10A). The data in Figure 3.10 (B, C) show that CK protein content increased significantly by about 30% in skeletal muscle during anoxia

whereas a significant decrease (~20%) in CK protein content occurred in anoxic heart ($P<0.05$).

Structural analysis of CK by urea denaturation and pulse proteolysis:

The structural integrity of enzymes can be changed by covalent modification (or by the presence of allosteric modifiers) to make a protein more or less susceptible to denaturation by urea and subsequent proteolytic attack on the denatured (unfolded) enzyme by thermolysin. Hence, the pulse proteolysis technique is an effective way to determine whether there are structural or conformational differences in a protein between two different physiological states. Extracts of muscle and heart from aerobic and anoxic states were incubated with different concentrations of urea to achieve different degrees of CK denaturation and then both the enzymatic activity remaining and the susceptibility to thermolysin proteolysis were assessed.

Figure 3.11 shows the results of urea denaturation and pulse proteolysis on CK from skeletal muscle of aerobic versus anoxic turtles. Figure 3.11 (A) shows representative Western blots of the amount of CK protein remaining (in the band at ~43 kD) after pulse proteolysis of the enzyme treated with different concentrations of urea. Figure 3.11(B) shows the mean protein amount remaining for n=3 trials. The patterns of protein degradation were nearly identical for CK from control and anoxic states and the calculated C_m values (the concentration of urea required to unfold 50% of the protein, rendering it susceptible to thermolysin proteolysis) were 5.11 ± 0.16 M urea for aerobic control CK and 4.86 ± 0.21 M urea for anoxic CK (these values were not significantly different) (Table 3.3). The effect of urea on CK activity is shown in Figure 3.11(C) and

again aerobic and anoxic CK behaved the same. The calculated I_{50} values (the concentration of urea required to inhibit 50% of activity) were 5.58 ± 0.26 and 5.51 ± 0.31 M for aerobic and anoxic CK, respectively.

Comparable experiments with heart CK showed somewhat different results. Figure 3.12 (A) shows the Western blot images after pulse proteolysis and Figure 3.12(B) shows the mean protein content remaining after thermolysin treatment as a function of urea concentration. Aerobic control CK showed a significantly higher resistance to unfolding and denaturation by urea. Calculated C_m values for urea were 4.68 ± 0.16 M for CK from anoxic heart and 12% higher at 5.31 ± 0.18 M for CK in the control heart (Table 3.3); the values were significantly different, $P<0.05$. The I_{50} values for urea effects on CK activity were also significantly different between aerobic and anoxic states; I_{50} values were 4.66 ± 0.13 M for the anoxic enzyme and 10% higher at 5.17 ± 0.15 M for CK in aerobic heart (Figure 3.12 C).

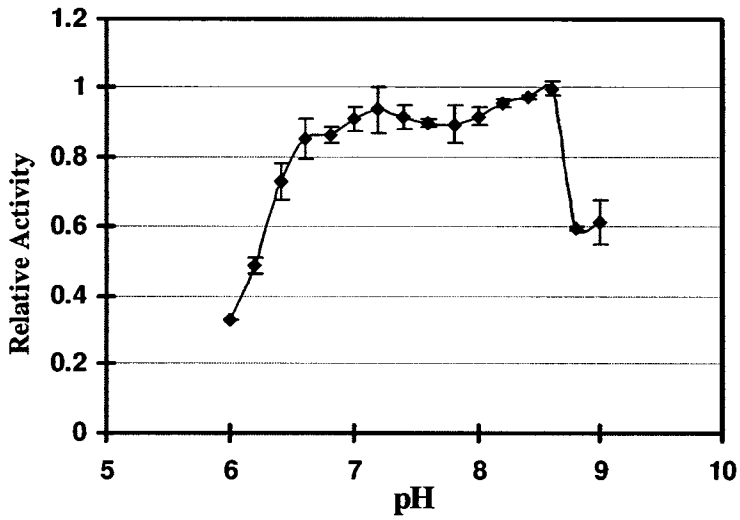
Figure 3.1. pH optimization of CK and stability study in the red skeletal muscle

(A) Effect of pH on the activity of CK. Assay conditions were 50 mM MES buffer for pH 6.0-6.8 and 50 mM Tris-HCl buffer for remaining pH values with 5 mM ATP and 15 mM Cr substrate concentrations. Optimal activity was achieved at pH 8.4.

(B) Stability of CK during storage at 4 °C; activity was tested at optimal pH with 5 mM ATP and 15 mM Cr.

Data are means ± SEM, n = 4 determinations on separate preparations of enzyme.

A



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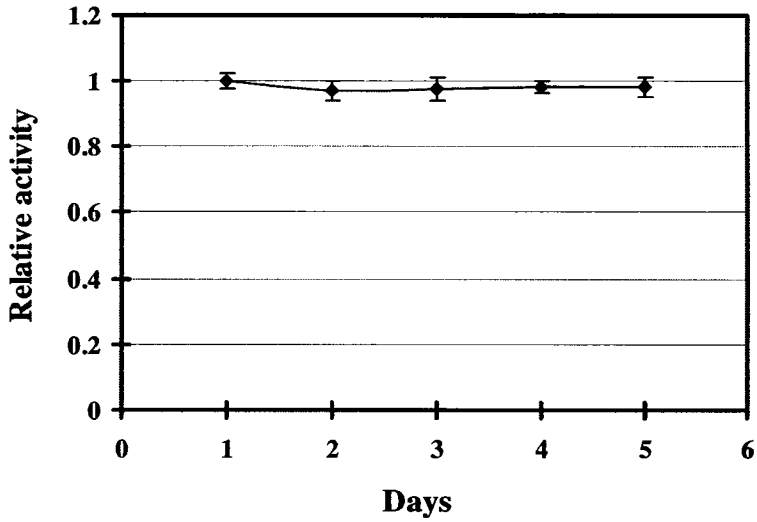


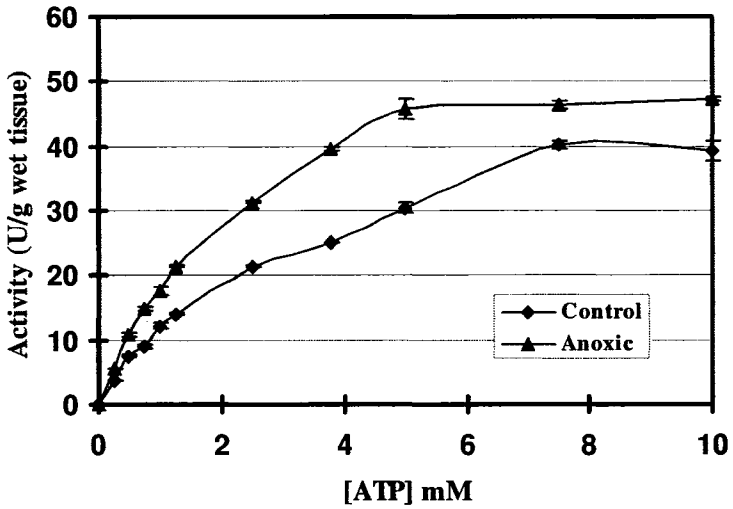
Figure 3.2. Velocity versus substrate concentration for supernatant CK activity from red skeletal muscle of aerobic control and 20 h anoxic *T. s. elegans*

(A) Activity as a function of ATP concentration at 15 mM Cr.

(B) Activity as a function of Cr concentration at 5 mM ATP.

Results are for assays on crude extracts; data are means ± SEM, n = 4.

A



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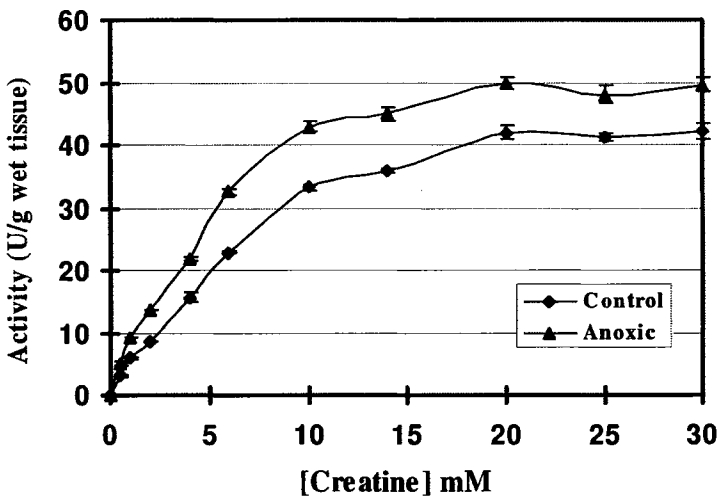


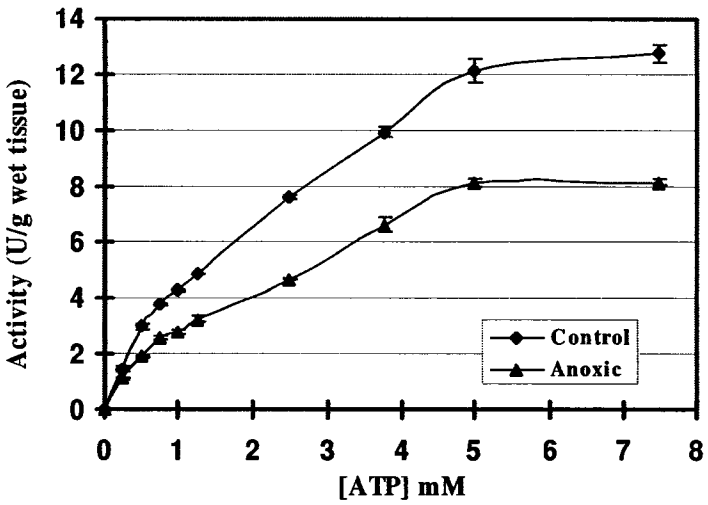
Figure 3.3. Velocity versus substrate concentration for pellet CK activity from red skeletal muscle of aerobic control and 20 h anoxic *T. s. elegans*

(A) Activity as a function of ATP concentration at 15 mM Cr.

(B) Activity as a function of Cr concentration at 5 mM ATP.

Results are for assays on crude extracts; data are means ± SEM, n = 4.

A



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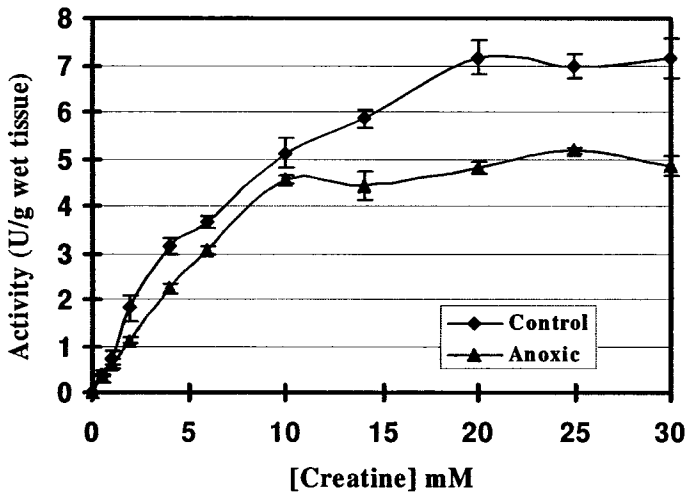


Table 3.1. Kinetic parameters of free and bound forms of CK from skeletal muscle of aerobic control versus 20 h anoxia exposed turtles.

(A) Supernatant

(B) Pellet and percentage of bound

Data are means ± SEM, n≥4. [a] - Significantly different from the corresponding aerobic control value, $P< 0.05$. [b] - Significantly different from the corresponding value for free CK in the supernatant, $P< 0.005$.

A

Muscle	Supernatant			
	K_m (mM)		V_{max} (U/g tissue)	
	ATP	Creatine	ATP	Creatine
Aerobic	3.04±0.14	8.78±0.15	39.2±2.75	40.3±2.83
20h Anoxic	3.39±0.17	5.79±0.12[a]	47.4±3.16[a]	48.2±3.29[a]

B

Muscle	Pellet				% Bound	
	K_m (mM)		V_{max} (U/g tissue)		ATP	Creatine
	ATP	Creatine	ATP	Creatine		
Aerobic	3.74±0.14[b]	5.29±0.12[b]	12.5±1.24	7.75±1.14	31.9±3.14	19.2±1.78
20h Anoxic	4.55±0.16[ab]	6.84±0.15[ab]	8.14±0.14[ab]	5.14±0.93[a]	17.2±2.95[a]	10.7±1.23[a]

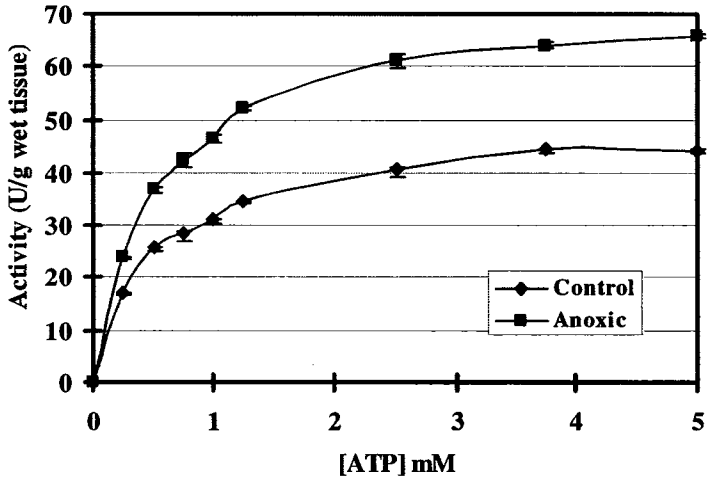
Figure 3.4. Velocity versus substrate concentration for supernatant CK activity from heart of aerobic control and 20 h anoxic *T. s. elegans*

(A) Activity as a function of ATP concentration at 15 mM Cr.

(B) Activity as a function of Cr concentration at 5 mM ATP.

Results are for assays on crude extracts; data are means ± SEM, n = 4.

A



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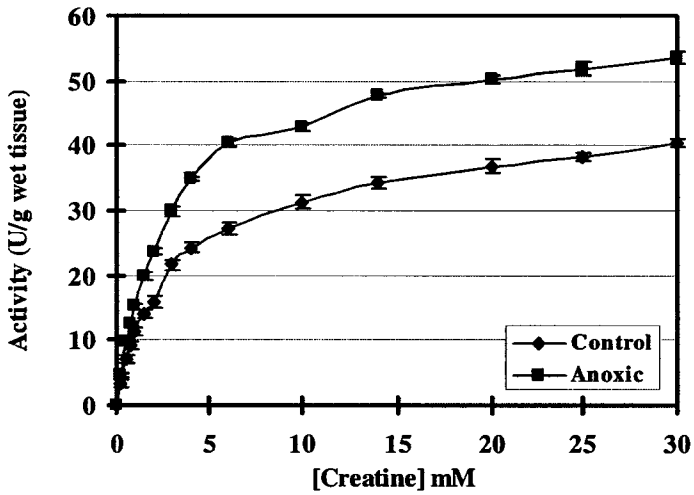


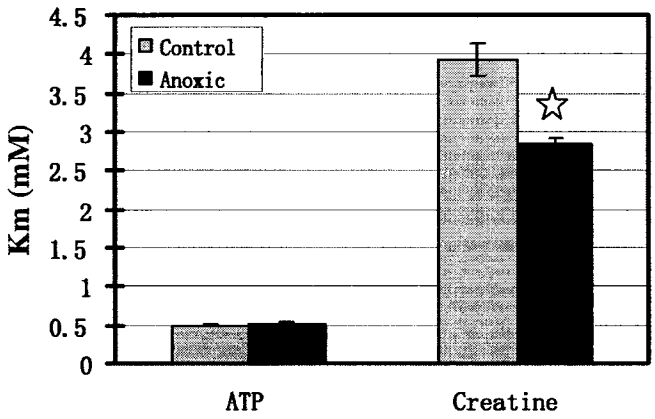
Figure 3.5. Summary of the Km and V_{max} for supernatant CK activity from heart of aerobic control and 20 h anoxic *T. s. elegans*

(A) Km (ATP) and Km (Cr)

(B) V_{max} of ATP and Cr.

Results are for assays on crude extracts; data are means ± SEM, n = 4. * - Significantly different from the corresponding aerobic control value, $P < 0.05$.

A



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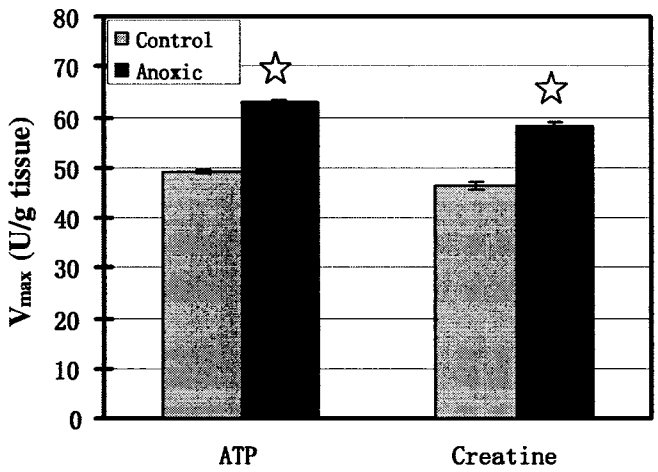


Table 3.2. (A) Effects of pH and lactate on the Km values of CK from turtle heart (B) I_{50} values for the effects of different salts on turtle heart CK.

Analysis of pH effects was done using 50 mM Tris buffer with the full assay mixture adjusted to each pH value (except for the coupling enzyme solution which was added as a 10 µl amount in pH 8.4 buffer). Lactate stock solution was made up in 50 mM Tris base and neutralized to pH 7.2 with 1 M NaOH solution. Data are means ± SEM, n≥4. [a] - Significantly different from the corresponding aerobic control value, $P< 0.05$. [b] - Significantly different from the corresponding value at pH 8.4. [c] – Significantly different from the value at pH 7.2 without lactate.

A

pH		8.2	7.2	6.5	Lactate (pH 7.2)
K_m (mM) for ATP	Control	0.503±0.013	0.68±0.015[a,b]	1.64±0.046[a,b]	1.76±0.033[a,b]
	20h Anoxic	0.518±0.021	0.42±0.029[a,b]	0.99±0.025[a,b]	1.21±0.049[a,b]
K_m (mM) for Cr	Control	3.922±0.084[a]	1.58±0.058[a,b]	3.08±0.077	1.15±0.031[a,b]
	20h Anoxic	2.848±0.065[a]	2.62±0.074[a,b]	3.12±0.081	1.56±0.026[a,b]

B

Salt	I_{50} (mM)	
	Control	Anoxic
KCl	1.38±0.05	1.14±0.04[a]
NaCl	0.52±0.01	0.48±0.02
NH_4Cl	0.66±0.01	1.03±0.02[a]
K_2SO_4	0.31±0.01	0.20±0.02[a]

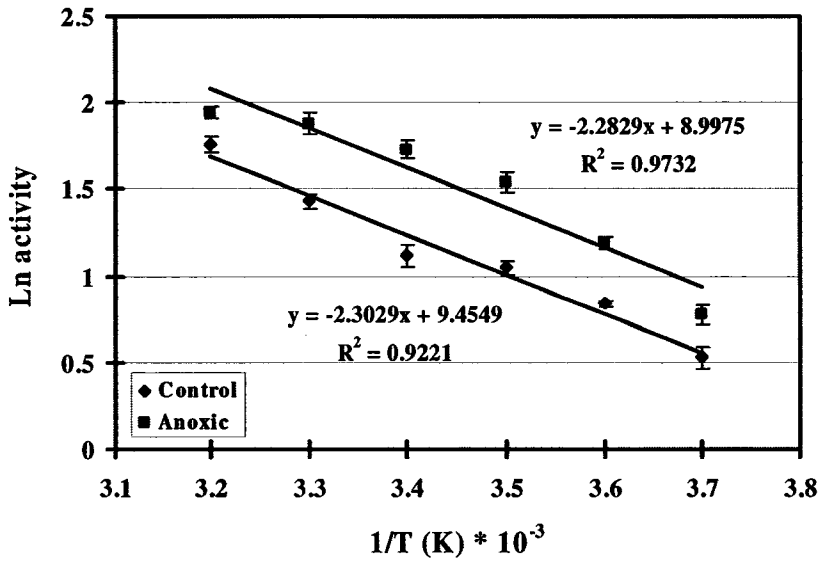
Figure 3.6. Representative Arrhenius plots for CK comparing the enzyme from crude extracts of red skeletal muscle and heart of aerobic control and 20 h anoxic *T .s. elegans*

(A) Arrhenius plots for skeletal muscle

(B) Arrhenius plots for heart

Activities were measured under optimal conditions between 3 and 35 °C. Results are for assays on crude extracts; data are means ± SEM, n = 3 independent determinations on separate preparations of enzyme.

A



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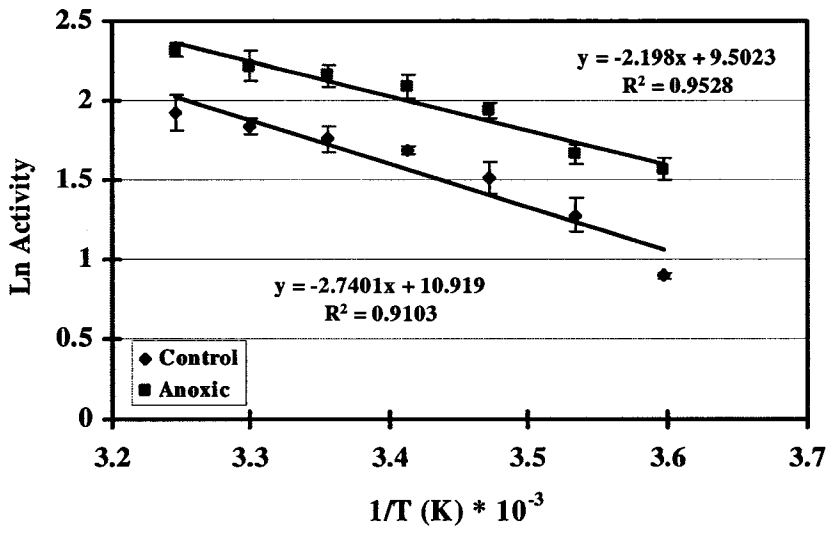


Table 3.3. Kinetic constants for CK from muscle and heart of aerobic control and 20 h anoxia *T. s. elegans*.

Data are means + S.E.M. (n=3). I_{50} is the concentration of urea that reduces enzyme activity by 50%. C_m is the concentration of urea required to unfold 50% of CK protein. Assays were performed under optimal substrate concentrations. Significance testing used the Student's t-test. [a]- Significantly different from the corresponding value for aerobic control turtles, $P<0.05$.

	Muscle		Heart	
	Aerobic control	**20 h anoxic**	**Aerobic control**	**20 h anoxic**
Ea(KJ/mol)	18.96 ± 1.13	19.12 ± 1.26	22.78 ± 1.76	18.27 ± 1.32[a]
I_{50} urea (M)	5.58 ± 0.26	5.51 ± 0.31	5.17 ± 0.15	4.66 ± 0.13[a]
C_m urea (M)	5.11 ± 0.16	4.86 ± 0.21	5.31 ± 0.18	4.68 ± 0.16[a]

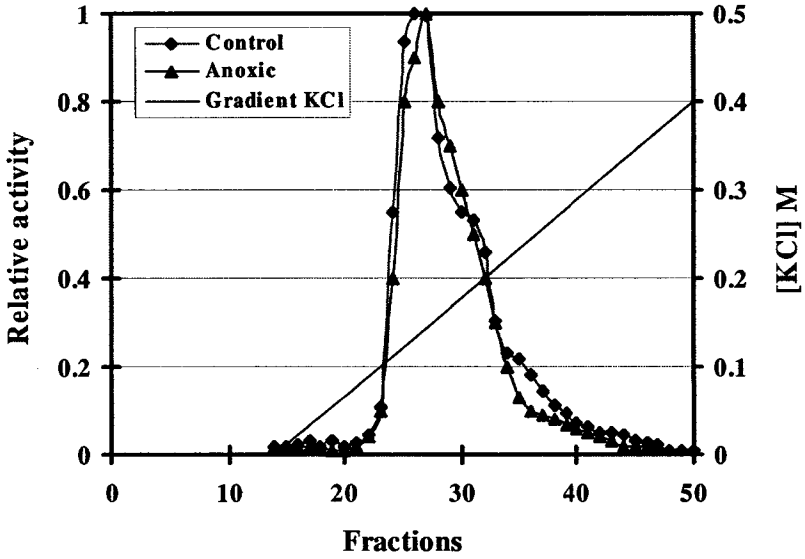
Figure 3.7. Typical elution profiles for CK from DEAE G50 Sephadex.

(A) Elution profiles for red skeletal muscle CK from aerobic and 20 h anoxic turtles. The recovery of CK activity was 83.2 % and 81.9 % for aerobic and anoxic samples.

(B) Elution profiles for heart CK from aerobic and anoxic turtles. The recovery of CK activity was 79.2 % and 76.8 % for the aerobic and anoxic samples.

The columns were eluted with a 0-0.4 M gradient of KCl, as described in the Materials and Methods. Fractions were assayed under optimal conditions and activities are shown relative to activity in the peak tube.

A



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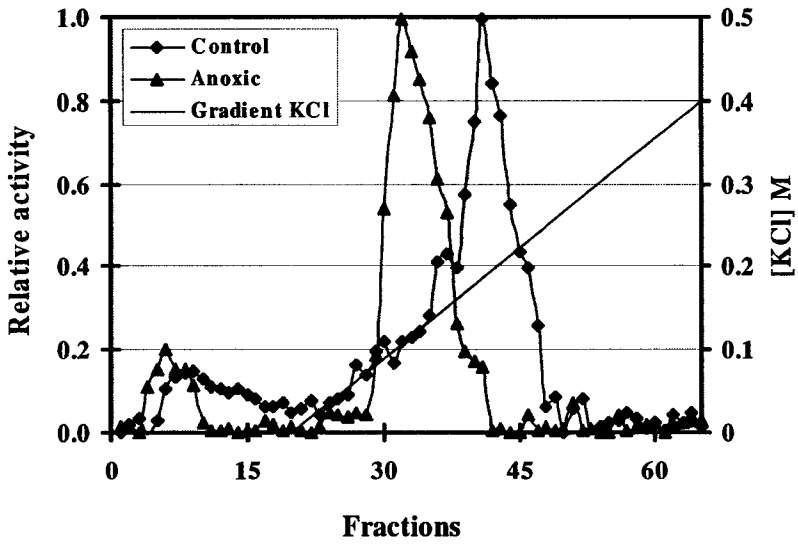

Figure 3.8. Effect of *in vitro* stimulation of total endogenous protein kinases or protein phosphatases on CK activities in extracts from control (aerobic) versus anoxic turtles.

(A) Red skeletal muscle CK

(B) Heart CK

Incubation was for 4 h at 4°C under one of three conditions: (a) STOP conditions that inhibited all phosphatases and kinases, b) conditions that stimulated total protein kinases, or (c) conditions that stimulated total phosphatases. * - Significantly different from the corresponding activity under the STOP conditions, $P<0.05$.

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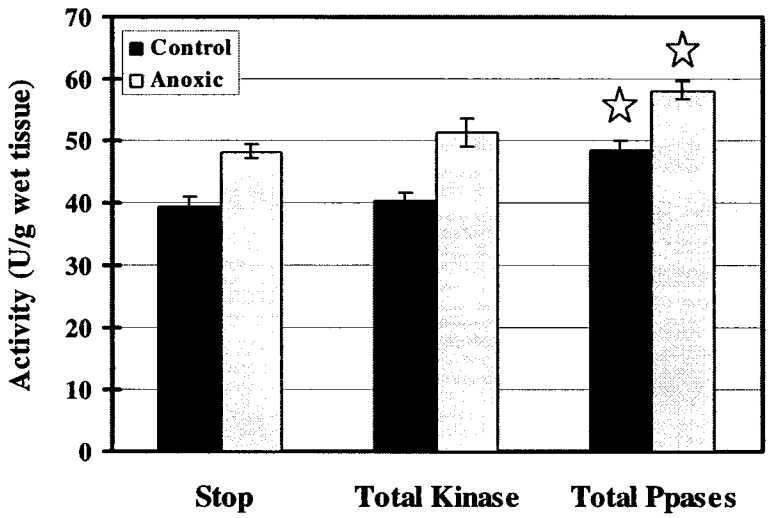

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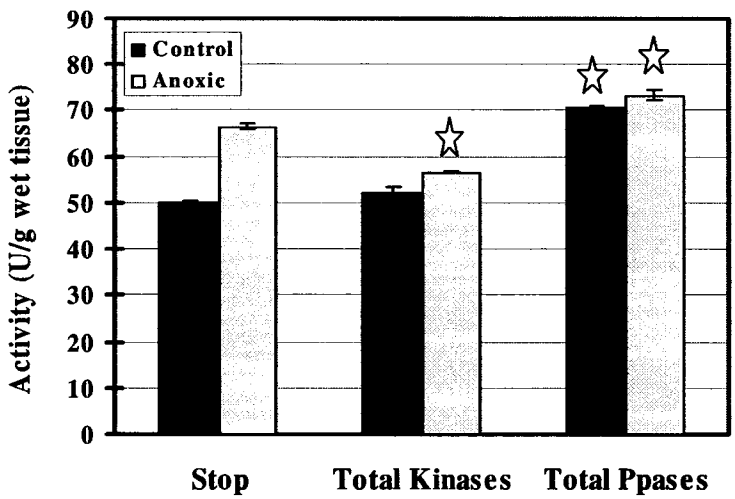

Figure 3.9. Effects of *in vitro* stimulation of the activities of endogenous total and specific individual protein kinases and phosphatases on CK activity in extracts from control (aerobic) versus anoxic heart.

(A) Effect of *in vitro* stimulation of the activities of endogenous protein kinases. Incubation was for 4 h at 4°C under (a) STOP conditions that inhibited the activities of all phosphatases and kinases, (b) conditions that stimulated total endogenous protein kinases, or (c) conditions that stimulated individual kinases: PKA, PKG, PKC, or AMPK.

(B) Effect of *in vitro* stimulation of the activities of protein phosphatases. Incubation was for 4 h at 4°C under (a) STOP conditions that inhibited the activities of all phosphatases and kinases, (b) conditions that stimulated total endogenous protein phosphatases, (c) conditions that stimulated Tyr phosphatases, (d) conditions that stimulated Ser/Thr phosphatases, (e) conditions that stimulated specific individual phosphatases: PP1, PP2B, PP2C, or (f) incubation with exogenous alkaline phosphatase (AP).

* - Significantly different from the corresponding activity under STOP conditions, $P<0.05$.

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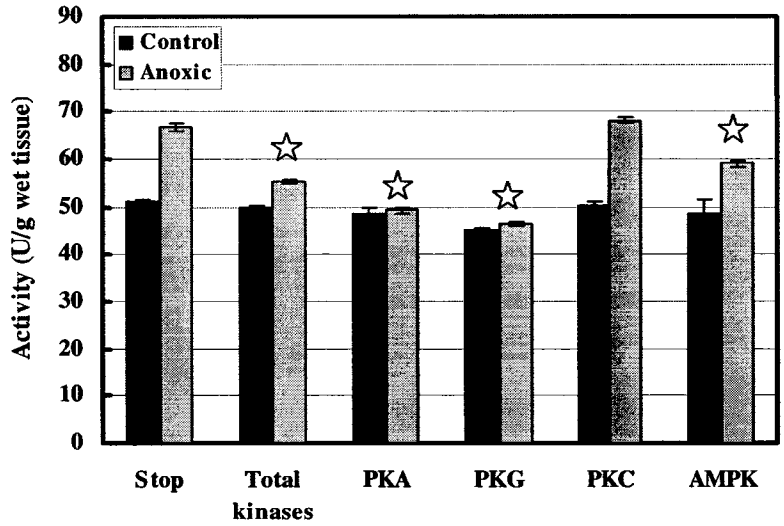

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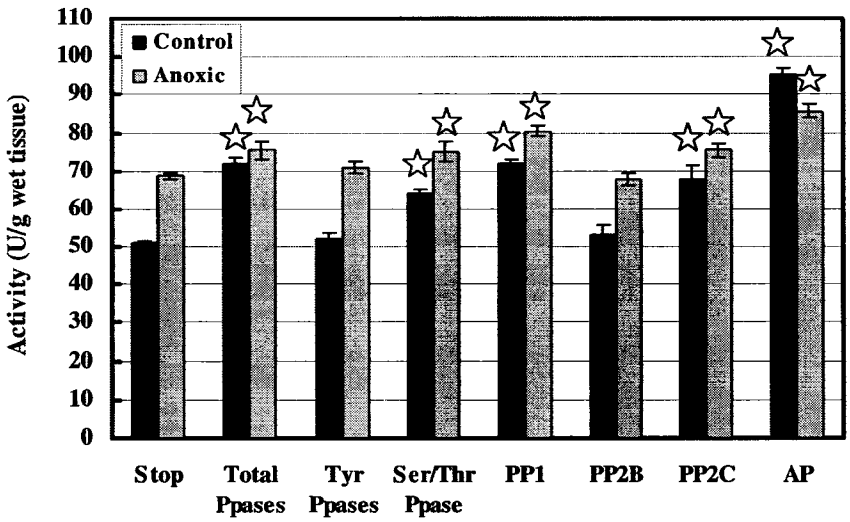

Figure 3.10. Profile of protein expression of CK in extracts from control (aerobic) versus 20 h anoxic muscle and heart.

(A) Western blots showing CK bands for control (aerobic) versus anoxic muscle and heart. Single bands crossreacted with the CK antibody at ~43 kDa MW, consistent with the expected subunit size of CK.

(B) Histogram showing relative levels of CK protein in control (aerobic) versus anoxic turtle red skeletal muscle.

(C) Histogram showing relative levels of CK protein in control (aerobic) versus anoxic turtle heart.

Data are means ± SEM, n = 3 independent determinations on separate muscle extracts.

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

A

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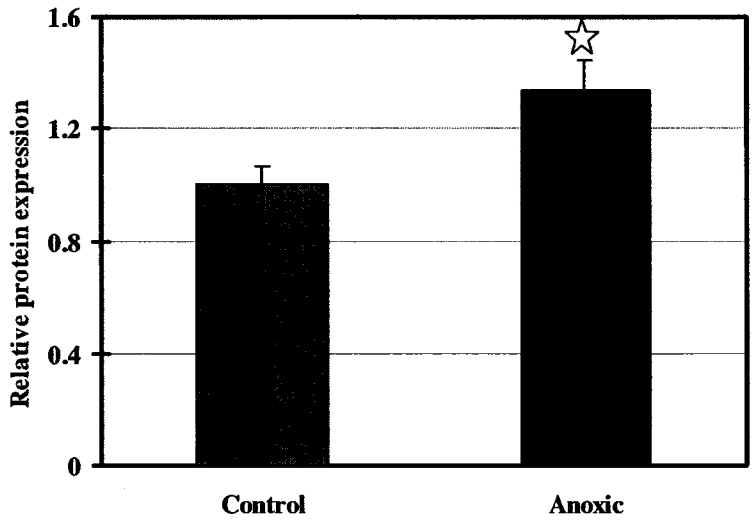

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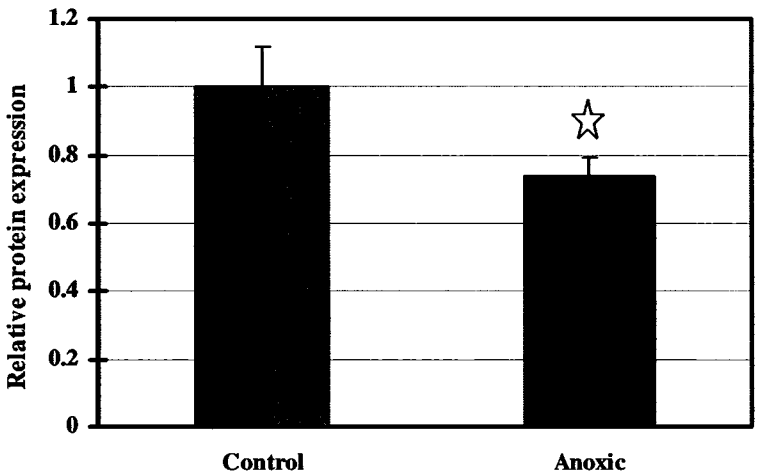

Figure 3.11. Assessment of urea denaturation of CK in extracts from control (aerobic) versus anoxic skeletal muscle as detected after pulse proteolysis

(A) Western blots showing the CK protein band at ~ 43 kDa MW after overnight incubation with different concentrations of urea followed by 10 min pulse proteolysis with thermolysin.

(B) Relative amount of CK protein remaining after pulse proteolysis as a function of urea concentration for control (aerobic) versus anoxic skeletal muscle extracts.

(C) Relative CK activity remaining as a function of urea concentration in control (aerobic) versus anoxic muscle extracts.

Data are means ± SEM (n = 3). Activity assays were conducted at 22 °C.

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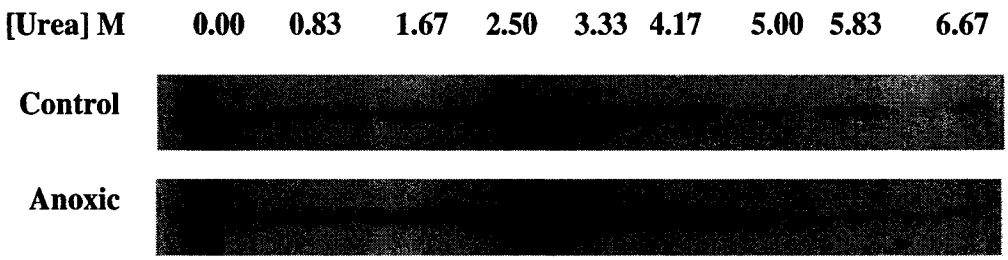

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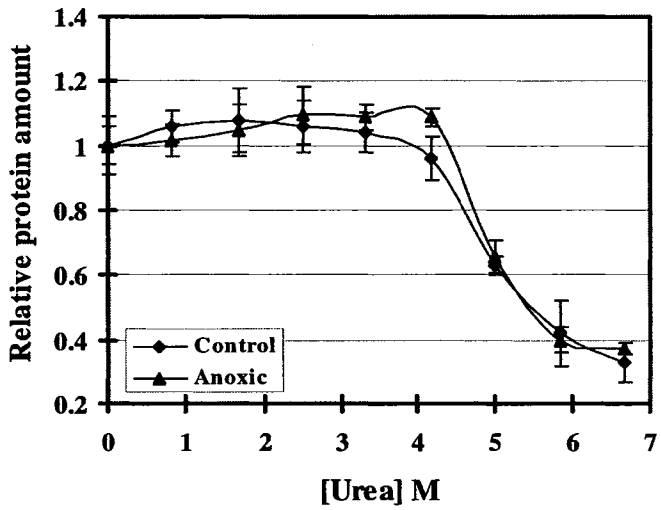

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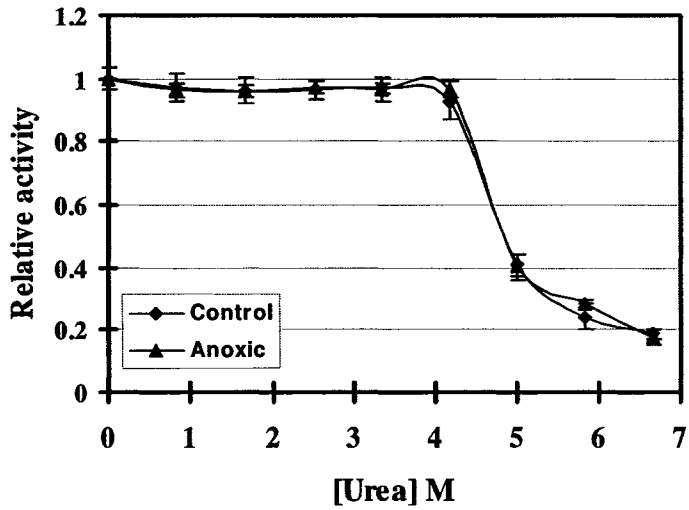

Figure 3.12. Assessment of urea denaturation of CK in extracts from control (aerobic) versus anoxic heart as detected after pulse proteolysis

(A) Western blots showing the CK protein band at ~43kDa MW after overnight incubation with different concentrations of urea followed by 10 min pulse proteolysis with thermolysin.

(B) Relative amount of CK protein remaining after pulse proteolysis as a function of urea concentration for control (aerobic) versus anoxic heart extracts

(C) Relative activity of CK remaining after overnight incubation with different concentration of urea.

Data are means ± SEM, n = 3. Activity assays were conducted at 22 °C.

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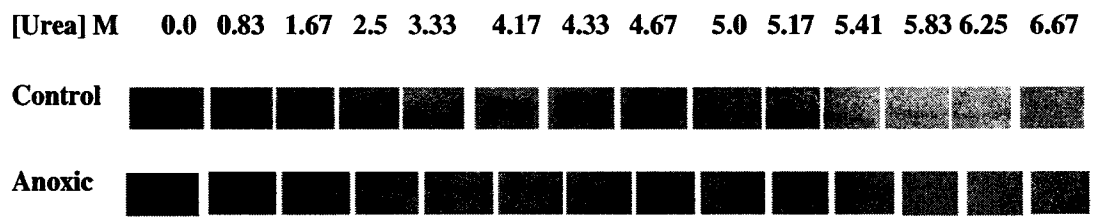

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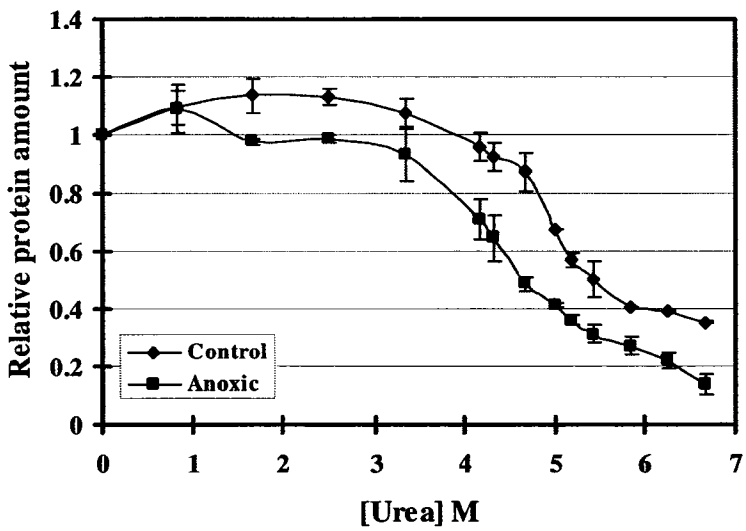

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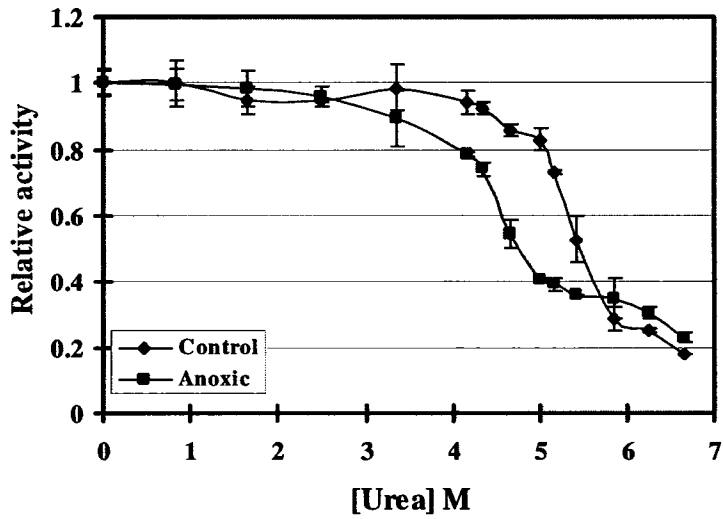

DISCUSSION

Creatine kinase is an enzyme that participates in ATP homeostasis. It acts to buffer ATP levels by using the pool of creatine phosphate to replenish ATP under cellular conditions where ATP consumption is greater than ATP production by glycolysis and/or oxidative phosphorylation. CK also functions to synthesize creatine phosphate when ATP availability is high and has an integral role in shuttling high energy phosphate in cells. Because of this buffering role, CK is typically present in very high activities in muscle tissues. For example, in the present study, the activity of CK was 80 U/g in heart and 50 U/g in red muscle when measured at pH 8.4 in the ATP + Cr → ADP + PCr direction. This agrees well with a previous study in our lab that found ~70 U/g in heart and ~80 U/g in red muscle for the same reaction measured at pH 8.2 (Duncan, 1988). Other studies have measured CK activities at pH 7.1-7.2 in the direction of ADP + PCr → ATP + Cr; activities are always much higher in this direction. Christiansen et al. (1994) found 1167 ± 126 ($\mu mol \cdot g^{-1} \cdot min^{-1}$) in heart of *Pseudemys (=Trachemys) scripta* whereas Willmore et al. (2001) found 225 ± 25.9 in heart and 742 ± 2.18 in red muscle at pH 7.0 in the same ATP-producing direction.

It is well known that pH has significant effects on enzymatic reactions. The optimal pH of CK from turtle red skeletal muscle was 8.6 (Figure 3.1(A)) but activity was at least 85% of maximal over a broad range from 7.0 to 8.4. Because the equilibrium of the CK reaction depends to a great extent on the pH, in the weakly acid range it favours the formation of creatine and in the weakly alkaline range it favours creatine phosphate formation. The cytosolic CK isozyme from a number of species has a pH optimum between 6.6-6.8 for the PCr + ADP direction (Storey, 1975; Kuby et al., 1954;

Dawson et al., 1965) whereas, pH 9.0 was the optimum for the Cr + ATP direction of rabbit muscle CK (Tanzer and Gilvarg, 1959). In the present study, kinetic analysis was carried out at pH 8.4 (in the weakly alkaline range) for the Cr + ATP direction.

Kinetic parameters, K_m (Cr) and V_{max} were significantly different for soluble CK isolated from anoxic red skeletal muscle (Figure 3.2 and Table 3.1) or heart (Figure 3.4 and 3.5) of turtles, as compared with aerobic controls. The K_m (Cr) was lower and the V_{max} was higher than that measured for the corresponding aerobic enzyme. This suggests a stable modification of the enzyme with the transition from the aerobic to anoxic state. Experimental evidence from studies of the effects of anoxia exposure on *T. s. elegans* and other freshwater turtles showed that a strong suppression of metabolic rate occurs under anoxia that includes a coordinated suppression of the rates of both ATP-utilizing and ATP-producing cellular reactions (Storey and Storey, 2004b). The mechanisms involved are still being resolved but it is clear that reversible phosphorylation of selected enzymes and functional proteins plays a major role (Storey, 2006). As a result of metabolic arrest, turtles can maintain stable cellular ATP levels over long periods of anoxia (Lutz et al., 1984; Kelly and Storey, 1998).

According to Christensen et al. (1994), CK activity showed no clear relationship to total creatine concentration. Among the ectothermic species, turtle and cod myocardia have similar total creatine concentration, although their CK activity differs by a factor of 14. According to Hochachka (1998), CK always operates near equilibrium and has access to, and reacts with, the entire pool of PCr and Cr, and the related ATP-ADP system. According to this concept, CK simply serves the purpose of maintaining an adequate ATP/ADP ratio in the cells. This means that the use of the near-equilibrium nature of the

CK reaction in estimating *in vivo* free ADP concentrations should be reconsidered. CK buffers the ATP pool in anoxic muscle, since the observed changes in [PCr] are much greater than those in [ATP] (Waarde, 1990). CK catalyzes the transphosphorylation of PCr to ADP to regenerate ATP, thus preventing a depletion of ATP levels. Thus, PCr is available as an immediate energy source, serving not only as an energy buffer but also as an energy transport vehicle (Guerrero-Ontiveros and Wallimann, 1998). Hence, CK is a key enzyme that buffers energy status in the cell, and this buffering ability is an important part of stabilizing energy reserves in anoxia.

The increase in CK activity under anoxia measured here correlated with similar findings by Birkedal and Gesser (2004) who found that CK activity in heart of cold anoxic painted turtles (*Chrysemys picta*) was significantly higher (by ~36%), compared with aerobic controls. Hence, the energy buffering capacity offered by CK seems to be enhanced under anoxic conditions. It might be speculated that the increased CK activity in the heart improves the energy buffering between sites of anaerobic energy production and energy consumption.

CK is well known to form binding associations with myofibrils (Hornemann et al., 2000). Binding functions to position CK where it is needed to replenish the ATP that is being consumed by the myosin ATPase but bound enzymes can also show altered enzymatic properties compared with soluble forms. To investigate this for turtle CK, an analysis of CK bound in the pellet of turtle skeletal muscle was undertaken. Overall, the amount of CK found in the pellet decreased by ~ 45% under anoxia as compared with aerobic muscle. This may be a consequence of metabolic rate depression; reduced rates of ATP turnover in anoxia would lead to a reduced need for CK to be associated with the

muscle fibers. Bound CK in the pellet also showed significantly different Km values for both ATP and Cr than the corresponding soluble enzyme in the supernatant. Km (ATP) values for both aerobic and anoxic CK were higher for the bound enzyme, as was Km (Cr) of anoxic CK. However, Km (Cr) of aerobic CK behaved oppositely. Hence, CK function may be altered when the enzyme binds to myofibrils. The effects seen here (generally higher Km values for the bound enzyme) would suggest that bound CK is less effective at catalyzing the Cr + ATP → PCr + ADP direction than is soluble CK. This makes sense because the bound enzyme should be mainly involved in catalyzing the reverse reaction to generate ATP from the ADP that is produced by myosin ATPase during muscle work.

Fine metabolic control of enzymes also involves variation in pH and allosteric effectors. The pH dependence of an enzyme is one factor that determines its overall activity in the cell. How a decrease in pulmonary oxygen partial pressure or arterial oxygen tension is translated to a decrease in metabolism has not been clearly established. One signal is likely to be intracellular acidification resulting from net ATP hydrolysis and anaerobic glycolysis, for example, in turtle brain and heart intracellular pH decreases by 0.5 unit or more during anoxia (Buck et al., 1998). Analysis of heart CK properties at different pH values showed that Km (ATP) increased (affinity decreased) with decreasing pH (Table 3.2 A) whereas Km (Cr) of aerobic CK decreased (affinity increased) with decreasing pH. By contrast, Km (Cr) of anoxic CK rose (affinity decreased) at pH 6.5 as compared with pH 8.4. This shows that pH in the alkaline range favours the Cr + ATP reaction of CK. Hence, as cellular pH drops under anoxia, conditions for PCr synthesis would be less favourable. This might be part of the overall metabolic rate depression in

anoxia and would also serve to promote the PCr hydrolysis reaction of CK as the primary direction of the enzyme under anoxia.

Despite profound metabolic depression, the product of anaerobic glycolysis, lactate, accumulates over time in anoxic turtles. Lactate can reach very high levels in turtles; for example, after 3-5 months of experimental submergence at 3°C, circulating lactate levels can reach 150-200 mM (Jackson, 2002). This amount of lactate greatly exceeds the normal buffering capacity of body fluids and requires supplemental buffering to prevent fatal acidosis. The shell and skeleton of turtles serve as the source of this additional buffering as well as a storage site for lactic acid, and these roles for the turtle's most distinctive structural feature (its shell) may be a main reason for its remarkable anoxic tolerance (Jackson, 2002). To determine whether high lactate had an effect on CK function, the Km values for CK were determined in the presence of 200 mM lactate at pH 7.2 (Table 3.2(A)). In the presence of lactate, Km (ATP) was significantly higher than without lactate whereas Km (Cr) was significantly lower. The net effect on these opposing changes on CK function is difficult to judge but they do indicate that rising lactate concentrations in anoxic tissues would influence CK function.

Many enzymes are sensitive to the levels of various salts within the natural range of concentrations of cations (K^+, Na^+, NH_4^+) and anions (Cl^-, SO_4^{2-}) in vivo. I_{50} values for K^+, Na^+ and NH_4^+ are shown in Table 3.2(B), where inhibition by monovalent cations is seen to be in the order of $Na^+ > NH_4^+ > K^+$. Between anions, the I_{50} values are in the order of I_{50} (Cl^-) > I_{50} (SO_4^{2-}), so the inhibition by SO_4^{2-} was stronger than that of Cl^-. I_{50} values for salt inhibition of CK are high but could have some influence on CK *in vivo* since

studies have shown that Na^+ and Cl^- concentrations generally fall during anoxic submergence of turtles, whereas the concentration of K^+ rises (Jackson, 2002).

CK from heart of aerobic versus anoxic turtles also showed significantly different activation energies (as determined from Arrhenius plots) and differed in the extent of urea-dependent protein unfolding as evidenced both by urea inhibition of CK activity and susceptibility to thermolysin treatment (Table 3.3; Figure 3.6 and 3.12). Both the C_m and the I_{50} value for urea were significantly higher for the aerobic control enzyme, indicating a more stable conformation compared with the anoxic form. These plus the Km and Vmax differences between the aerobic and anoxic forms of heart CK (Figure 3.5) suggest that there are stable physical differences between the two forms of the enzyme and other data presented here suggests that this probably derives from differences in the phosphorylation state of the protein under aerobic versus anoxic conditions.

One of the most common mechanisms that modify the conformation and activity states of enzymes is reversible protein phosphorylation – altering the amount of covalently bound phosphate on the enzyme due to the action of protein kinases or protein phosphatases. To determine whether CK was modified by reversible phosphorylation during aerobic-anoxic transitions, two types of analyses were done: (a) *in vitro* incubations under conditions that promoted protein kinase or protein phosphatase actions on the enzyme, and (b) DEAE ion exchange chromatography to look for changes in the elution profile of CK which result from a change in the net charge on the protein caused by an altered amount of covalently bound phosphate on the enzyme.

The elution patterns of heart CK from the DEAE column were very different in the aerobic versus anoxic conditions, the anoxic enzyme form eluting from the column at

a low salt concentration whereas the aerobic form eluted at a much higher [KCl]. Since a phosphorylated enzyme would show stronger binding to the DEAE resin, this suggests that aerobic control CK is the high phosphate form whereas the anoxic enzyme is the low phosphate (or dephosphorylated) form. Analysis of the effects of protein kinases and protein phosphatases on turtle heart CK was consistent with this interpretation. Incubation of aerobic CK under conditions that stimulated total protein kinases or individual protein kinases did not change enzyme activity, suggesting that aerobic CK was already phosphorylated. However, the activity of anoxic CK was significantly reduced to a level similar to that of aerobic CK by incubations that stimulated total protein kinases or the individual kinases PKA, PKG and AMPK. Oppositely, incubations that stimulated total protein phosphatase activities, serine/threonine phosphatases or the individual activities of PP1 and PP2C, as well as treatment with alkaline phosphatase all increased the activity of aerobic heart CK to values that were close to those of the anoxic enzyme. Activity of the anoxic enzyme was also increased by these same phosphatase treatments which suggest that the anoxic enzyme form is only partially dephosphorylated *in vivo* and can be more fully dephosphorylated by phosphatase treatment. Interestingly, the dephosphorylated (anoxic) form of heart CK seems to be the more active form with a higher V_{max} and a lower Km (Cr) than the aerobic phosphorylated form. This is consistent with previous studies of CK that have shown that phosphorylation inhibits CK activity (Quest et al., 1990; Hemmer, 1995; Stolz, 2002; Ingwall, 2002).

By contrast, aerobic and anoxic forms of muscle CK showed the same elution pattern off DEAE suggesting that they had the same phosphorylation state. Neither form of muscle CK was affected by incubations that stimulated protein kinases, but incubation

under dephosphorylating conditions increased CK activity in both the aerobic and anoxic situations. Hence, the incubation results suggest that the aerobic and anoxic forms of skeletal muscle CK are both partially phosphorylated.

In summary, the present study indicates that CK from *T. s. elegans* heart is a phosphoprotein and that entry into the hypometabolic state of anoxia results in a strong increase in the fraction of heart CK that is dephosphorylated and leads to increased affinity of the enzyme for creatine. The data implicates PKA, PKG or AMPK and PP1 as the protein kinase and phosphatase activities responsible for heart CK regulation. However, anoxia-responsive changes in the kinetic properties of skeletal muscle CK do not appear to be due to a change in the phosphorylation state of the enzyme. Anoxia-responsive regulation of muscle CK might instead be mainly due to changes in the binding association of the enzyme with myofibrils and the resulting effects of binding on the kinetic properties of the reaction.

CHAPTER 4

GENERAL DISCUSSION

The studies presented in this thesis used the red-eared slide turtle (*T. s. elegans*) as a model of vertebrate anoxia tolerance to address two aims: (1) to analyze the impact of anoxia on the kinetic behaviour of enzymes of adenylate metabolism, and (2) from the accumulated data, attempt to elucidate an enzymatic mechanism of adenylate regulation that supports anoxia tolerance. The enzymes studied, AMP deaminase and creatine kinase, are integral to the maintenance of cellular energy charge and buffering the energy supply for cells. According to Overgaad and Gesser (2004), hypoxia did not affect the myocardial concentration of ATP, ADP or total adenylates in turtles. However, hypoxia caused decreases in the concentration of PCr, the phosphorylation potential (PCr/Cr^2) and the sum of high-energy phosphates. The ability of the turtle heart to balance ATP production and consumption during hypoxia with small reductions in energetic state may be due to their superior ability to down-regulate metabolic requirements (Lutz and Nilsson, 1997; Jackson, 2000b, 2002).

Stabilization of cellular energy charge is the key to the survival of all cells and requires particular attention under stress conditions, such as anoxia, that compromise the ability to produce ATP. Stabilization of energy charge is one of the physiological functions proposed for AMPD (Chapman and Atkinson, 1973). Under anoxia in turtle skeletal muscle, AMPD showed increased activity and affinity for its substrate, hence, favoring the conversion of AMP to IMP. The action of AMPD in catabolizing AMP has multiple positive actions including (1) stabilizing the energy charge by removing AMP from the total adenylate pool, (2) pushing the adenylate kinase reaction toward ATP synthesis, (3) supplying allosteric effectors of glycolytic enzymes, and (4) removing some of the protons that accumulate from anaerobic glycolysis through the formation of

NH_4^+, all at the expense of the adenylate pool (Mommsen and Hochachka, 1988). Indeed, the consequence of AMPD action in stabilizing energy charge has been demonstrated in turtles; Kelly and Storey (1988) found a decrease of up to 32% decrease in total adenylates in turtle organs after 1 h of anoxic submergence but, despite this, AEC remained stable.

Creatine kinase is also involved in adenine nucleotide metabolism and a high level of CK can also regulate the adenylate kinase reaction and help to conserve the adenine nucleotide pools in the cell (Iyengar, 1984). Thus, if ADP is allowed to accumulate in a cell, e.g. by ischaemia or anoxia (McGilvery and Murray, 1974; Connett, 1988), it is transphosphorylated via adenylate kinase to yield ATP and AMP (Hamada and Kuby, 1978). AMP, an inhibitor of adenylate kinase and of metabolic pathways like gluconeogenesis (Uyeda and Racker, 1965), is converted into IMP and ammonia by AMPD (Kushmerick and Davies, 1969; Hamada and Kuby, 1978; Lowenstein, 1972) which in muscle is bound to the myofibrils (Ashby et at., 1979) at both ends of the A-band (Cooper and Trinick, 1984). IMP as well as AMP is dephosphorylated by 5'-nucleotidase located on the sarcolemma (Bowditch et at., 1985) to give inosine and adenosine, respectively, which both have actions as signalling molecules. Adenosine is well known to mediate metabolic arrest responses in anoxia/hypoxia tolerant species whereas recent evidence suggests that inosine may mediate antioxidant defense responses (Storey, 2006a). Since the sarcolemma is permeable to the latter two compounds, an accumulation of ADP for a prolonged period of time would ultimately lead to a loss of adenine nucleotides (Jennings et at., 1981). Indeed, there are at least three important metabolic consequences of keeping cellular ADP levels low by using the CK system: (i)

CK keeps the free intracellular [ADP] in a range where it may participate in the regulation of mitochondrial respiration, (ii) CK prevents the inactivation of ATPases by rising [ADP], and (iii) CK also prevents a net loss of cellular adenine nucleotides (Wallimann et al., 1992). Hence, along with AMPD, CK plays an important role in adenylate metabolism.

The molecular basis of metabolic rate depression is a controlled and coordinated suppression of the rates of all ATP-generating and ATP-utilizing metabolic functions so that a new lower net rate of ATP turnover is achieved (Storey and Storey, 1990, 2004b). In turtle brain, for example, the activity of Na^+K^+ATPase, the single greatest consumer of brain ATP, was reduced by 30-35% in different regions of the brain after 24 h anoxia (Hylland et al., 1997). Layered over the general suppression of metabolic functions are differential controls that reorganize the priorities for ATP use in the hypometabolic state. For example, when turtle hepatocytes were incubated under anoxia, overall ATP turnover decreased by 94% and the proportion of ATP use by different functions changed dramatically; protein synthesis and protein degradation were largely inactivated whereas ATP use by Na^+K^+ATPase rose to consume 62% of total ATP turnover compared with 28% in normoxia (Hochachka et al., 1996). Hence, both the rate of ATP turnover and the subcellular locations of ATP use change markedly under anoxia and this could require key adjustments by enzymes involved in adenylate metabolism. Indeed, as seen in the present thesis, anoxia exposure has significant effects on the activities, kinetic properties and binding interactions of AMPD and CK in both skeletal muscle and heart of turtles.

In general, we know that entry into a hypometabolic state does not involve major changes in the overall protein/enzyme make-up of cells, a logical occurrence since entry into an energy-limited state is not the time to undertake widespread energy-expensive protein synthesis or degradation (Storey, 2006b). For example, anoxic submergence had very little effect on the maximal activities of 21 metabolic enzymes in six organs of adult turtles (*T. s. elegans*), with changes in only 0-4 enzymes per tissue, in most cases suppressing activities (Willmore et al., 2001). Instead, constitutive activities of enzymes in anoxia-tolerant species are designed to meet the needs of anoxic excursions. This can be contrasted with the well-documented hypoxia-induced up-regulation of multiple glycolytic enzymes (coordinated by the hypoxia-inducible transcription factor, HIF-1) in hypoxia-sensitive species such as mammals (Wenger, 2002).

Instead of modifying the overall amounts of enzymes in response to hypoxia/anoxia, facultative anaerobes use post-translational mechanisms to alter the activity state and subcellular distribution of existing enzymes and functional proteins to facilitate the transitions to/from the hypometabolic state. For example, in turtle brain, the distribution of several glycolytic enzymes between soluble and particulate-bound states changed under anoxic conditions to increase enzyme association with the particulate fraction (Duncan and Storey, 1992). This could provide better physical positioning of the ATP-generating enzymes of glycolysis with the major ATP-utilizing processes, such as membrane ion pumps or, in muscle, changes in binding near the myosin ATPase. Post-translational modification by reversible protein phosphorylation is also the key to the overall suppression of the activity states of many enzymes not just during anoxia-induced hypometabolism but also in aerobic states of hypometabolism including hibernation,

estivation and torpor (Storey and Storey, 1990; 2004b). In turtles, reversible phosphorylation controls have been linked with the suppression of multiple cell functions during anoxia including glycolytic enzymes, voltage-gated ion channels (Na^+, Ca^{2+}, K^+), membrane receptors (e.g. *N*-methyl-D-aspartate-type glutamate receptor), and protein synthesis (e.g. ribosomal initiation and elongation factors) (Hochachka and Lutz, 2001; Bickler et al., 2001; Storey, 1996a, Storey and Storey, 2004b).

The studies in this thesis show evidence that these mechanisms also apply to the regulation of AMPD and CK in turtle heart and skeletal muscle. In turtle red muscle, AMPD and CK showed significant changes the percentages of bound enzyme under anoxic conditions. The percentage of bound AMPD increased significantly in skeletal muscle during anoxia. Furthermore, both anoxia and binding increased AMPD affinity for its AMP substrate so that the lowest Km AMP measured was for bound AMPD in anoxic muscle (0.35 mM), less than half of the value for free AMPD in aerobic muscle (0.78 mM). Both the changes in percent bound and the effects of binding on Km AMP would promote enhanced AMPD function under anoxia and suggest the importance of the enzyme in regulating adenylate metabolism by AMP removal when oxygen is limiting. Anoxia exposure also strongly affected the percentage of CK bound in turtle extracts with a 45 % reduction in the amount of bound activity in anoxic skeletal muscle. Both anoxia exposure and enzyme binding in the pellet reduced CK affinity for ATP (Km ATP increased significantly) whereas the results for Km creatine were mixed but with a net 22 % decrease in Km when comparing aerobic free CK with anoxic bound CK. Overall, then, the changes in CK kinetic parameters suggested that bound CK would be less effective at catalyzing the Cr + ATP → PCr + ADP direction than is soluble CK, an

effect that would be enhanced by the reduced amount of bound CK in anoxic muscle. The kinetic effects of anoxia on CK were interpreted as being designed to promote the reverse reaction (PCr + ADP → Cr + ATP) in anoxic muscle to generate ATP from phosphagen reserves. Furthermore, reduced CK binding in anoxia could be part of the overall metabolic rate depression, reflecting reduced ATP turnover and reduced muscle activity under anoxia. Hence, the data for the effects of anoxia exposure on the free versus bound distribution of both AMPD and CK seem to be consistent with the metabolic state of that would occur in anoxic, hypometabolic skeletal muscle and indicated that the changes in the binding of both of these enzymes has physiological relevance for muscle energy metabolism.

Regulation of both AMPD and CK with respect to anoxia survival is also linked with reversible phosphorylation of the enzymes. Reversible phosphorylation is known to mediate major changes in enzyme activity states between aerobic and anoxic conditions in many animals; including turtles (Storey and Storey, 2004b, Storey, 2004b). Through the regulated actions of protein kinases and protein phosphatases, a coordinated suite of changes can be achieved including (a) an overall suppression of metabolic rate (ie. strongly reduced ATP turnover), and (b) reordered priorities for cellular ATP use by targeted effects on key proteins and metabolic functions. Previous studies have shown that reversible phosphorylation controls mediate anoxia-dependent regulation of glycolytic enzymes, ion channels and ion motive ATPases, and protein synthesis machinery. The present data indicate that reversible phosphorylation also contributes to the regulation of enzymes of adenylate metabolism in response to anoxia exposure in turtles. The data for AMPD indicated that aerobic AMPD was the low phosphate form

(kinase treatments strongly increased activity; phosphatase treatments had no effect) whereas anoxic AMPD was a phosphorylated enzyme (kinase treatments had lesser effects on activity; phosphatase treatments reduced activity). Anoxia mediated phosphorylation of AMPD could potentially affect enzyme kinetic properties and/or enzyme binding to the subcellular fraction and help to coordinate changes in AMPD function in line with other aspects of energy metabolism in anoxia such as the control of ATP production by glycolysis. Interestingly, previous studies showed that PP1 activity in skeletal muscle was reduced by 50% within 5 hours when turtles were given anoxic submergence (Mehrani and Storey, 1995a) whereas total PKA activity was unaffected and the percentage of active PKA dropped only from ~8 % to 5% (Mehrani and Storey 1995b). This suggests that protein kinase activities may dominate over protein phosphatase activities in anoxic red skeletal muscle and this could contribute to the phosphorylation of AMPD in anoxia.

Although red skeletal muscle AMPD was phosphorylated under anoxia, muscle CK did not seem to undergo an anoxia-induced change in phosphorylation state although both the aerobic and anoxic enzyme forms proved to be partially phosphorylated enzymes that responded to phosphatase action. In turtle heart, however, clear evidence of an anoxia induced change in phosphorylation state was found. The aerobic form of CK proved to be the high phosphate form (eluted at high salt, not affected by protein kinase treatments but responsive to phosphatases) whereas the anoxic form was the low phosphate form (eluted at low salt, responsive to protein kinases). Susceptibility to anoxia-induced changes in phosphorylation state would allow CK activity, properties, and subcellular location to be coordinated with other enzymes of energy metabolism.

Unlike the situation in red skeletal muscle, however, previous studies found no change in PKA or PP1 activities in heart after 1, 5 or 24 h of anoxia which suggests that changes in the activities of other signal transduction enzymes are responsible for anoxia-induced changes in CK phosphorylation state.

In summary, the results presented here demonstrated that anoxia does affect the kinetic behavior of enzymes of adenylate metabolism by significant changing their activities and kinetic properties. The enzymatic mechanism of adenylate regulation includes binding interactions with the subcellular particulate fraction, reversible phosphorylation and allosteric regulation. The reversible phosphorylation mediates major changes in enzyme activity state between aerobic and anaerobic condition. Different enzymes have different phosphate forms; even the same enzyme shows different effects of anoxia on phosphorylation state in different tissues. The study also showed that not only posttranslational modification of enzymes but also the signal transduction is anoxia-induced. Through this study, more insight was gained into the mechanisms of regulation of two important enzymes of adenylate metabolism, therefore increasing our knowledge base of two very different metabolic states.

Perspectives:

The data in the present thesis has documented that AMPD and CK have key roles in the adenylate metabolism. Further studies could address the following topics:

1. Transcriptional regulation of AMPD and CK should be analyzed using PCR to measure changes in mRNA transcript levels in order to determine whether these proteins are also regulated by changes in gene expression under aerobic versus anoxic conditions.

Studies of protein turnover could also be done to determine if these enzymes are regulated by differential rates of protein synthesis versus degradation in aerobic versus anoxic states.

2. Phosphoproteomic analysis using 2-D gels could be used to gain a broad view of all the protein types that change phosphorylation state during anoxia in turtle organs. This will give us a better view of all the cell functions that need to be regulated for anoxia survival. Proteins are separate by isoelectofocusing in the first direction and by molecular weight in the second. Individual protein spots can then be excised, treated by proteolysis and fragments separated and identified by mass spectrometry based methods such as MALDI-TOF for peptide mass fingerprinting and tandem MS/MS for de novo peptide sequencing. Pretreatment of animals, tissues or cells with ^{32}P to label the ATP pool or addition of ^{32}P-ATP to cell extracts can be used to differentially label proteins in aerobic versus anoxic conditions followed by identification of strongly labelled proteins by the methods above. This will allow us to determine the proteins that change phosphorylation state and find out the pathways these proteins belong to; subsequently, the regulation of these key enzymes and their pathways can be studied.

3. AMPD makes IMP; this has roles that have already been discussed such as reducing [AMP] and other consequences like producing IMP and NH_4^+ that can affect metabolic enzymes (Mommsen and Hochachka, 1998). However, IMP can also be converted to inosine (similar to AMP conversion to adenosine) and recent studies are showing that inosine may have roles in regulating antioxidant defense (Storey, 2006a). Hence, a very interesting set of enzymes that are related to adenylate metabolism and would be good targets for future study in anoxia tolerant turtles would be the

5'nucleotidases (NT5C1 and NT5C2) – both the enzymes that make adenosine and the ones that make inosine. Adenosine may be formed intracellularly by cytosolic NT5C1 or by the membrane-bound ecto-NT5. Both routes help to raise adenosine levels in ischemia/hypoxia and low oxygen is also known to induce ecto-NT5 in mammals (Hunsucker et al., 2005). The NT5C2 isozyme has a different role; it is the Mg^{2+}-dependent cytosolic form that prefers (d) IMP or (d) GMP as substrates (Bretonnet et al., 2005). It can catabolise the IMP that is formed by AMPD and because of this role in intracellular metabolism, its regulated bifunctional nature, its elevated activity in some tumour cells, and its involvement in several alternative metabolic pathways. (Bretonnet et al., 2005), NT5C2 deserves particular interest. Because of the role of NT5C1 in producing adenosine which signals metabolic depression and NT5C2 in producing inosine which may have a role in triggering antioxidant defenses, both of these 5'nucleotidases deserve study in anoxia-tolerant turtles. Research should determine how are they regulated and how do they respond to anoxia stress, etc.

4. The demonstration that AMPD and CK can be regulated by both changes in binding associations and reversible phosphorylation during anoxia, suggests the idea that other enzymes of energy metabolism may also be controlled in this way and selected enzymes of glycolysis are subject to one or both of these mechanisms in anoxic turtles. Although glycolysis is a biochemical pathway that evolved under ancient anaerobic terrestrial conditions, recent studies have provided evidence that some glycolytic enzymes are complicated, multifaceted proteins with more functions than previously realized. One of these is hexokinase which catalyzes the first step of glycolysis. Hexokinase is controlled in part by binding to the mitochondrial outer membrane and

new studies have implicated hexokinase in the regulation of apoptosis (programmed cell death) (Kim and Dang, 2005) which is often triggered by changes in mitochondria. Low oxygen can cause apoptosis in anoxia intolerant animals but this response should be suppressed in anoxia tolerant species. So it will be very interesting and necessary to study the regulation of the hexokinase, its interactions with mitochondria, and its influence on apoptosis in the turtle model.

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