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PURIFICATION AND PROPERTIES OF TURTLE HEART CREATINE KINASE: ROLE FOR THE ENZYME IN GLYCOLYTIC CONTROL

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ABSTRACT

1. Adenosine triphosphate-creatine phosphotransferase (EC 2.7.3.2) from turtle (*Pseudemys scripta*) heart occurs in two major electrophoretic forms: one cytoplasmic (pI = 6·67) and one mitochondrial (pI = 7·37).

2. In the forward direction (phosphocreatine + ADP → ATP + creatine), NADH activates the purified cytosol enzyme by lowering the K_m for phosphocreatine almost thr e-fold.

3. From these studies it is concluded that *in vivo* the rising levels of NADH known to occur in the hypoxic heart of the diving turtle serve as the signal for rapid depletion of phosphocreatine and the subsequent autocatalytic activation of glycolysis.

4. The role if creatine kinase in the overall anaerobic metabolism of turtle heart is discussed.

Diving turtles as a group are several times more tolerant of anoxia than other reptiles, and are far more tolerant of anoxia than mammals. The green sea turtle, for example, can readily dive for at least two to several hours at a time (Hochachka, Owen, Allen & Whittow, 1974) and undergoes 'resting' periods under water for several hours. The red-eared turtle, *Pseudemys scripta*, survives dives as long as two weeks at 16–18° C despite a total lack of electron transport-mediated oxygen consumption (Robin, Vester, Murdough & Miller, 1964). Jackson (1968) has pointed out that after the first hour or so in a normal experimental 'dive' where the animal is fairly quiescent, all blood and tissue oxygen stores are fully depleted, yet the animal can continue its dive for many more hours on a glycogen-based fermentation whose measured capacity in calorific terms is about 15–20% of the animal's aerobic metabolism (Jackson, 1968). Under such extreme diving conditions, all the tissues of the body must be able to maintain their functions in anoxia. Since the vertebrate heart and central nervous system typically display an absolute oxygen dependence, special attention has been focused on the heart of the diving turtle as an ideal vertebrate organ in which to study evolutionary mechanisms of anoxia adaptation.

In the heart of the diving turtle a high glycolytic potential is seen both in glycogen and in enzyme levels. Thus, cardiac glycogen levels are 10 times higher in turtle than in terrestrial mammals and some five times higher than in diving mammals such as the seal (Beall & Privitera, 1973; Kerem, Hammond & Elsner, 1973). This feature in itself creates a higher glycolytic potential and it is reinforced by high quantities of glycolytic enzymes. Key enzymes such as hexokinase (HK), phosphorylase *a*, and phosphofructokinase (PFK) occur in substantially higher specific activities in turtle heart than in rat heart (Reeves, 1966; McNeill, Davis & La Rochelle, 1971). Their integrated function is so efficient that transient changes in ATP/ADP ratios are not detectable during aerobic-anaerobic

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transitions (Penney & Shemerdiak, 1973). Whereas a drop in ATP concentration is an important means for deinhibiting PFK and thus for ' turning on ' glycolysis in the rat heart during aerobic-anaerobic transition, it is not a useful signal in the heart of a diving turtle; not surprisingly, turtle heart PFK is remarkably insensitive to ATP (Storey & Hochachka, 1974). The usual regulatory role of ATP in this tissue is taken over by creatine phosphate, which is known to be rapidly depleted in anoxia (Clark & Miller, 1973), and which therefore supplies the tissue with a good measure of its glycolytic requirements. Under aerobic conditions, creatine phosphate levels are high and serve to hold glycolytic rates low by potent PFK inhibition. In the absence of any other metabolic controls, a drop in creatine phosphate would serve to deinhibit PFK and activate glycolysis. This deinhibition, potentiated by fructose diphosphate (FDP), appears central to glycolytic control in the anoxic turtle heart (Storey & Hochachka, 1974), and prompted an examination of creatine kinase, the enzyme activity controlling creatine phosphate concentrations.

Creatine kinase (EC 2.7.3.2) in mammals is thought to be controlled *in vivo* by two mechanisms (Kuby, Noda & Lardy, 1954): (1) the drop in muscle pH associated with the anoxic build-up of lactate serves to enhance phosphocreatine (P-creatine) breakdown and the production of ATP; (2) the rapid decline in ATP concentrations seen in most anoxic working muscles (Williamson, 1966) causes P-creatine depletion by mass-action. Neither of these control mechanisms could be operative in the turtle heart, firstly, because the pH drop during the first hours of anoxia is only about 0·1 pH units (Clark & Miller, 1973), and secondly, because the turtle heart displays no drop in ATP concentrations during aerobic-anaerobic transition when glycolysis is clearly activated (Penncy & Shemerdiak, 1973). Instead, turtle heart creatine kinase appears to be under NADH control. Very low NADH concentrations (0·04 mM) activate the reaction in the forward direction by lowering the K_m for creatine phosphate. Since turtle tissues are known to become highly reduced shortly after the onset of anoxia (Lai & Miller, 1973), our studies on creatine kinase point to a redox-based control of creatine phosphate levels which in turn control PFK and glycolytic activation.

MATERIALS AND METHODS

All substrates, cofactors, reagents and coupling enzymes were purchased from Sigma Chemical Co. (St. Louis, Missouri). Ampholines pH 3–10 and pH 5–8 were purchased from LKB Products (Stockholm, Brömvoa, Sweden). Starch for electrophoresis was purchased from Connaught Labs Ltd. (Toronto, Canada).

Turtles (*Pseudemys scripta*) used in this study were purchased from NASCO Co. Limited (Fort Atkinson, Wisconsin) and were kept at 10°C in cold water. Turtles were decapitated and hearts excised, blotted, weighed and then cut up into ice-cold homogenization buffer.

Hearts were homogenized in a Sorvall Omnimixer for 1 to 2 minutes with two volumes of 0·01 M Tris-HCl buffer pH 7·5, containing 20 mM β-mercaptoethanol, and 5 mM Mg^{2+}. The homogenate was then centrifuged at 12,900 *g* for 20 minutes and the pellet discarded. The supernatant was then brought to 55% saturation with solid ammonium sulfate and stirred for 1 hour at 4°C. The suspension was then centrifuged as above, and the supernatant was brought to 65% saturation with solid ammonium sulfate. After one hour with stirring the solution was centrifuged at 35,000 *g* for 20 minutes. The pellet was dissolved in a minimal volume of 1% glycine (pH 7·5) containing 20 mM β-mercaptoethanol. After dialysis against 1% glycine (pH 7·5) the enzyme was electrofocused according to the method of Häglund (1967). The enzyme was run at pH 5–8 (LKB-8133) at 700 volts for 40 hours. The temperature of the apparatus was maintained at 4°C ± 0·05°C. The purified enzyme had a specific activity of 5 μmoles of product formed per minute per mg. protein at 25°C, compared to a specific activity of 52–54 μmoles of product formed per minute per mg. protein at 30°C for crystalline (11-fold purified) creatine kinases from mammalian muscle (Wood, 1963). The preparation representing about 23-fold purification was free of any enzymes that would either interfere with the basic assay or remove or interconvert any of the added metabolites. Adenylate kinase activity was zero under the assay conditions.

Horizontal starch electrophoresis was performed on 13% gels in Tris-citrate buffer (pH 7·0) by the method of Biewer (1970). Gels were run at 250 volts and 10 amps for 18 hours at 4°C. Mitochondria were isolated by the method of Hogeboom (1955).

Enzyme activity was measured by the coupled

assay technique. For the 'forward' direction, ATP production was coupled to excess dialyzed Sigma HK and glucose 6-phosphate dehydrogenase (G6PDH) and the rate of creatine kinase activity was measured as the increase in A_{340} due to NADP reduction. For the 'reverse' direction ADP production was coupled to excess dialyzed Sigma pyruvate kinase and lactate dehydrogenase and the rate of oxidation of NADH was measured. Imidazole buffers were used in all assay reactions. Standard assay mixtures contained the following in a final volume of one ml.: 20 mM imidazole buffer, Mg^{2+}, P-creatine, ADP, NADP, HK, G6PDH at pH 6·7 for the 'forward' reaction. For the 'reverse' direction, in one ml.: 20 mM glycine-NaOH buffer (pH 9·0), Mg^{2+}, creatine, ATP, phosphoenolpyruvate (P-enolpyruvate), NADH, pyruvate kinase, and lactate dehydrogenase. All reactions were performed at 25°C and started by the addition of the creatine kinase preparation.

RESULTS

Electrophoretic Studies

Electrofocusing of a high-speed supernatant or a 55%–65% ammonium sulfate fraction of turtle heart reveals two major bands of creatine kinase activity with isoelectric points of 6·67 and 7·37 (Fig. 1). These two peaks correspond to the two heart bands found by other investigators (Dawson, Eppenberger & Kaplan, 1965), and are thought to correspond to the BB or brain isoenzyme and the MB hybrid, respectively. After electrofocusing the creatine kinase isoenzymes were separated, pooled separately and run on starch gels where it was seen that each was essentially free of the other. After collection, each form was dialyzed against imidazole buffer (pH 7·5) containing 20 mM β-mercaptoethanol to remove ampholines and sucrose and to stabilize the enzyme.

Fig. 2 shows the creatine kinase electrophoretic patterns obtained on starch gels.

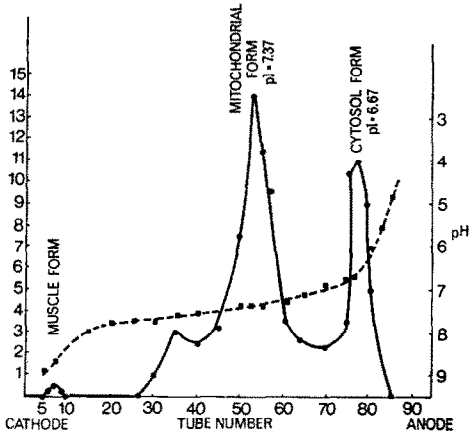

Fig. 1.—Electrofocusing run of a 55%–65% ammonium sulfate fraction of turtle heart assayed for creatine kinase. The electrophoresis was run as outlined in Materials and Methods. Assay reactant concentrations are: 20 mM imidazole buffer pH 6·7, 5 mM $Mg(Acetate)_2$, 1 mM ADP, 5 mM P-creatine, 0·2 mM NADP, 4 mM glucose and excess dialyzed Sigma HK and G6PDH. Fractions of one ml. were collected.

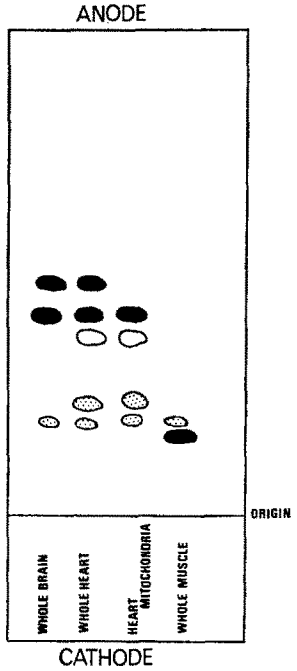

Fig. 2.—Electrophoretic patterns obtained with crude extracts and heart mitochondrial preparations (see Materials and Methods) of heart, skeletal muscle and brain from the adult turtle, *Pseudemys scripta*. The staining medium consisted of Tris buffer, 0·1 M, pH 7·5, 15 ml.; $MgCl_2$, 0·1 M, 1·0 ml.; glucose, 0·1 M, 1·0 ml.; excess HK and G6PDH; P-creatine, 0·1 M, 0·6 ml.; ADP, 0·05 M, 0·5 ml.; NADP, 0·035 M, 0·5ml.; tetra-nitro blue tetrazolium, 10 mg./ml., 1·0 ml.; and phenazine methosulfate, 1 mg./ml., 0·12 ml. In crude extracts of these tissues, adenylic kinase is also visualized by this method (indicated by hatched areas); it can be partially inhibited by the addition of AMP, 0·1 M, 1·0 ml., to the staining medium.

In common with other animals (Dawson *et al.*, 1965), turtle muscle shows one isoenzyme (designated MM), and the brain shows two other, faster migrating, forms (one BB and the other thought to be a hydrid, MB). The heart contains mainly the brain pattern with some of the muscle isoenzyme visible. FIG. 2 also shows that when isolated mitochondria from heart were run, only the slower major isoenzyme (MB) was present, indicating that this is the mitochondrial form and that the other isoenzyme is confined to the cytosol.

pH PROFILES

The cytosol isozyme has a pH optimum between 6·6 and 6·8 in the 'forward' direction which is in agreement with optimal pH 6·7 values from a number of different species (Kuby *et al.*, 1954; Dawson *et al.*, 1965). The mitochondrial form displays a very broad optimum from 5·7 to about 6·1 which is lower than the reported value for rat heart enzyme of 6·7 (Jacobus & Lehninger, 1973). The optimum for the mitochondrial enzyme in the opposite direction is about pH 9·0, compared to a previously reported value of pH 8·0 (Jacobus & Lehninger, 1973).

SUBSTRATE AFFINITIES

Cytosol enzyme

Michaelis constants at pH 6·7 are 0·33 mM for Mg^{2+} (the diacetate salt being used as it was found that anions like chloride and sulphate inhibited the enzyme), 1·25 mM for P-creatine, and 0·045 mM for ADP, compared to 3·6 mM for P-creatine and 0·12 mM for ADP from beef heart cytosol enzyme (Jacobus & Lehninger, 1973). The K_m of either substrate is unaffected by varying the concentration of the other. The K_m for P-creatine is lowered from 1·25 mM to 0·5 mM (a drop of nearly 3-fold) by the addition of 0·12 mM NADH (FIG. 3).

Mitochondrial enzyme

Michaelis constants are 0·71 mM for Mg^{2+}, 0·57 mM for P-creatine, and 0·022 mM for ADP at pH 6·7, compared to 0·72 mM for P-creatine and 0·035 mM for ADP for beef heart mitochondrial enzyme (Jacobus & Lehninger, 1973). FIG. 4 shows NADH to be an inhibitor of the mitochondrial enzyme

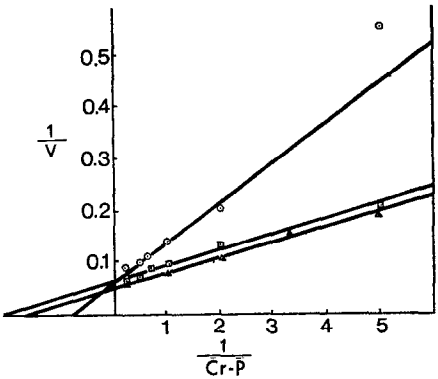

FIG. 3.—Double reciprocal plot of the effect of NADH on P-creatine saturation kinetics of the cytosol enzyme. Reaction concentrations are: 20 mM imidazole buffer, pH 6·7, 5 mM $Mg(Acetate)_2$, 0·8 mM ADP, 0·2 mM NADP, 4 mM glucose, excess dialyzed Sigma HK and G6PDH, varying P-creatine concentrations and NADH at the concentrations below: ○, no added NADH; △, plus 0·06 mM NADH; □, plus 0·12 mM NADH. Reaction velocity (V) is expressed in terms of A_{340} per minute.

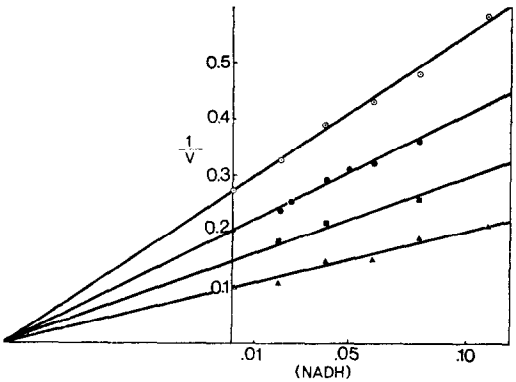

FIG. 4.—Dixon plot of NADH inhibition of P-creatine and ADP saturation kinetics of the mitochondrial enzyme. Reaction concentrations are: 20 mM imidazole buffer, pH 6·7, 5 mM $Mg(Acetate)_2$, 0·2 mM NADP, 4 mM glucose, excess dialyzed Sigma HK and G6PDH, varying concentrations of NADH and substrates at the concentrations below: ○, △, saturating ADP (0·2 mM) plus 1 mM and 5 mM P-creatine, respectively; ●, ■, saturating P-creatine (5 mM) plus 0·05 mM and 0·07 mM ADP, respectively.

with a K_i of 0·1 mM, non-competitive with either substrate.

'Reverse' Direction

At pH 9·0 the mitochondrial enzyme exhibits a K_m for creatine of 19 mM compared to 6 mM for the rat heart enzyme. The K_m for ATP for the turtle mitochondrial form is 0·6 mM compared to 0·1 mM for the rat heart enzyme. It was not possible to reverse the cytosol isoenzyme to any appreciable degree under these conditions. The beef heart cytosol isoenzyme (Jacobus & Lehninger, 1973) displays Michaelis constants for creatine of 33·3 mM and for ATP of 2·5 mM. The levels of creatine in mammalian tissue is between 20 and 30 mM (Kemp, 1973) and so the reported K_m for creatine is close to physiological concentrations. Since the turtle heart has levels of P-creatine approaching 4 mM (Clark & Miller, 1973), the cytosol enzyme can be considered irreversible under physiological conditions.

Other Effectors

A series of metabolites were checked for effects at K_m values of substrates for each enzyme and the following were seen to have no effect: 5 mM alanine, 5 mM aspartate, 2 mM FDP, 3 mM P-enolpyruvate, 3 mM 3-phosphoglyceric acid, 0.2 mM oxaloacetic acid, 4 mM glutamate, 3 mM pyruvate, 0·8 mM NAD, 3 mM malate, 0·2 mM guanidine triphosphate (GTP), 50 mM alpha-glycerophosphate, 5 mM succinate, 1 mM AMP, 50 mM lactate, 0·1 mM acetyl CoA. Mg_2Citrate is a non-competitive inhibitor with a K_i value of 17 mM, much higher that *in vivo* concentrations (Williamson, 1965). K^+ inhibits V_{max} activity by about 20% at 100 mM.

DISCUSSION

Regulatory enzymes typically display two characteristics in common: they are under tight (positive and/or negative) metabolite regulation and physiologically they behave as if they are irreversible (see Atkinson, 1966, for a review). In no previous study has creatine kinase been shown to display these characteristics. In the turtle heart, however, we had two convincing reasons for expecting the cytosolic isoenzyme to behave as a regulatory site. In the first place, one of the substrates for the reaction, creatine phosphate, has a profound inhibitory effect upon turtle heart PFK, a key control site in glycolysis (Storey & Hochachka, 1974). Hence, control of creatine phosphate concentrations (by control of creatine kinase activity) would appear to be fundamental to glycolytic control. And secondly, other workers have shown empirically that during the early part of hypoxic work the levels of creatine phosphate in turtle heart drop dramatically while the levels of ATP rise slightly, presumably due to creatine kinase facilitation (Penney & Shemerdiak, 1973). Hence our study of turtle heart creatine kinase basically became a search of its regulatory properties.

In other organisms, two mechanisms (changing H^+ and ATP concentrations) have been proposed for creatine kinase control in muscle. As we have pointed out above, neither a decreasing pH nor a decreasing ATP concentration can account for creatine kinase activation in the turtle heart, because neither 'signal' appears to be large enough to bring about significant effects on the enzyme. That is not the case for NADH, however. Lai & Miller (1973) have shown that when the oxygen supply to various tissues in the turtle becomes limiting, several oxidation-reduction (redox) systems in the cells become markedly reduced and in fact continue to become more and more reduced through the duration of the dive. In turtle heart, therefore, it is evident that NADH levels rise shortly after the outset of the dive, and these would serve to increase the affinity of creatine kinase for creatine phosphate, leading first to an activation of the enzyme, secondly to a drop in creatine phosphate concentrations, and subsequently to a deinhibition of PFK and a consequent activation of glycolysis at a time when it is in fact required.

Such a 'one on-signal' control on creatine kinase would appear to be adequate to closely integrate the activity of the cytosol

enzyme with the cell's glycolytic requirements, for the specificity of the NADH effect is high and the NADH concentrations required for full activation are well within expected physiological ranges (Chance, Williamson & Schoener, 1965). In contrast, 10-fold higher concentrations of NAD have no effect on enzyme catalytic rates and do not reverse the NADH activation. Similarly, NADP and NADPH do not affect the cytosol creatine kinase isoenzyme.

Physiologically, creatine kinase in the cytosol is thought to function in the direction of ATP production; hence, its apparent irreversibility is not difficult to understand. It is important to emphasize, however, that the K_{eq} for the reaction is the same irrespective of the isoenzymic catalyst present, and that the apparent irreversibility stems from kinetic properties of the enzyme. The extremely low affinity of the cytosol enzyme for creatine and ATP kinetically precludes any significant reverse function of the enzyme under physiological conditions. Moreover, catalytic function in the forward direction is also strongly favoured by the high affinities shown for creatine phosphate and ADP; in the absence of NADH, the turtle heart cytosolic isoenzyme displays a 3-fold greater affinity for both of these substrates than does the rat heart homologue, and this difference between the two enzymes is increased to nearly 9-fold in the presence of 0·1 mM NADH. Thus, an enzyme which on thermodynamic grounds is fully reversible in effect becomes a unidirectional catalyst on kinetic grounds. Other examples of such enzymes are known in the literature, a particularly well-worked out system being described by Barnes, McGuire & Atkinson (1972). In this property, the turtle cytosolic isoenzyme of creatine kinase differs fundamentally from its mammalian homologue (Kuby *et al.*, 1954). However, such behaviour is a hallmark of many regulatory enzymes and would appear to be necessary for a creatine kinase isoenzyme whose activity *in vivo* must be governed by mechanisms other than mass-action effects.

In contrast to the cytosolic isoenzyme, the mitochondrial form of creatine kinase is thought to buffer the respiration rate of heart mitochondria by immediate production of creatine phosphate from ATP being formed by oxidative phosphorylation (Jacobus & Lehninger, 1973). That is, this isoenzyme is thought to function in the backwards reaction in the cell. Interestingly, the kinetic constants for the turtle heart mitochondrial isoenzyme are between 3- and 6-fold higher than those for the rat heart analogue. The K_m found for creatine is 19 mM whereas the levels of this metabolite (measured as P-creatine) in the turtle heart is only about 4 mM compared to 10 mM for the rat heart mitochondria (Jacobus & Lehninger, 1973). This extremely high value for the turtle enzyme could reflect the fact that *in vivo* mitochondrial creatine kinase is thought to be membrane-bound and disruption of the system could affect the kinetic parameters (Hillmer & Gottschalk, 1974).

Finally, it should be noted that although the electrophoretic patterns in turtle muscle and heart found here seem similar to the patterns seen for other animals (Dawson *et al.*, 1965), turtle creatine kinase does not co-purify with any other creatine kinase so far studied. Standard methods for the purification of the muscle enzyme include one or more of the following: pH 9·0 plus ammonium ion, ethanol precipitation, ethanol precipitation plus heat (Kuby *et al.*, 1954), and acetone fractionation (Wood, 1963). None of the above methods individually or in combination allow purification of the enzyme.

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