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Effects of Ionic Strength and Sulfhydryl Reagents on the Binding of Creatine Phosphokinase to Heart Mitochondrial Inner Membranes

William C. Wenger,[1] Michael P. Murphy,[1] Gerald P. Brierley,[1] and Ruth A. Altschuld[1]

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Abstract

The concept that creatine phosphokinase is bound to the outer surface of the heart mitochondrial inner membrane originated from observations that the enzyme is retained by water-swollen heart mitochondria and by digitonin-treated heart mitochondria suspended in isotonic sucrose. The present study establishes that digitonin-treated mitochondria release creatine phosphokinase in isotonic KCl, and other investigators have reported an identical response for the water-swollen organelles. These observations suggest that mitochondrial creatine phosphokinase is not bound to the outer surface of the inner membrane at a site adjacent to the adenine nucleotide translocase under physiologic conditions.

Key Words: Heart mitochondria; creatine phosphokinase; adenylate kinase; privileged access; mitochondrial inner membranes; mitoplasts.

Introduction

Over the past two decades, there have been a number of conflicting reports dealing with the localization of the mitochondrial isozyme of creatine phosphokinase (EC 2.7.3.2). The earliest studies dealing with this issue placed CKm[2] in the intermembrane space on the basis of CKm extraction from isolated rat heart mitochondria in 0.1 M phosphate buffers (Pette, 1966; Klingenberg and Pfaff, 1966). The solubilization in 0.1 M potassium phosphate was subsequently shown to be inhibited by rotenone (Farrell *et al.*, 1972), and it was concluded that respiration-dependent matrix expansion sufficient

[1] Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio 43210.
[2] Abbreviations: CK, creatine phosphokinase; CKm, the mitochondrial isozyme of creatine phosphokinase.

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to rupture the mitochondrial outer membranes is a prerequisite for CKm extraction in this system. However, Sholte *et al.* (1973) demonstrated that CKm remains bound to heart mitochondria with outer membranes disrupted by digitonin, and Jacobus and Lehninger (1973) noted a similar CKm retention when isolated heart mitochondrial membranes were disrupted by osmotic swelling in distilled water. These data led to the hypothesis that CKm is "loosely bound" to the outer surface of the mitochondrial inner membrane.

This apparent binding of CKm to the inner membranes of heart mitochondria was subsequently incorporated into the privileged access model (Saks *et al.*, 1976) and eventually into the more generalized "phosphocreatine shuttle" (see Meyer *et al.*, 1984 for a discussion of this model). According to these models CKm is bound to the outer surface of the heart mitochondrial inner membrane at a site immediately adjacent to the adenine nucleotide translocase (Jacobus and Lehninger, 1973; Saks *et al.*, 1976). ATP produced by oxidative phosphorylation is transferred directly from the translocase to the active site of CKm, and ADP produced during the synthesis of phosphocreatine is immediately recycled to the matrix compartment through privileged access to the translocase. The adenine nucleotides are relegated to a catalytic role in the overall process, and phosphocreatine becomes the end product of heart mitochondrial oxidative phosphorylation and the primary means of energy transport in heart muscle.

The privileged access model has not gained universal acceptance, however (Altschuld and Brierley, 1977; Borrebaeck, 1980; Lipskaya *et al.*, 1980a), and the assumption that CKm is bound to the inner membrane under physiologic conditions has been questioned (Lipskaya *et al.*, 1982). These latter investigators demonstrated that CKm bound to water-swollen heart mitochondria can be salted off by a wide range of physiologic buffers, and attributed the binding in distilled water and other nonionic media to electrostatic interactions with inner membrane phospholipids. However, it has been suggested that matrix expansion during ion accumulation induces conformational changes in the inner membrane which facilitate solubilization of CKm (Font *et al.*, 1983), and a similar effect might be predicted during osmotic swelling. In view of the fact that CKm binding to the inner membrane was proposed originally on the basis of CKm retention by both water-swollen and digitonin-treated mitochondria, the present study was designed to examine the effects of ionic buffers on CKm binding to the digitonin-treated organelles.

Materials and Methods

Isolated beef heart mitochondria were prepared by a nagarse digestion in the presence of 2 mM EGTA, 5 mM Tris-Cl, 250 mM sucrose, pH 7.2, as

described by Jung *et al.* (1977). Mitoplasts were prepared by the method of Schnaitman and Greenawalt (1968). Briefly, beef heart mitochondria (25 mg · ml^{-1}) were incubated with 160 μg digitonin · mg^{-1} protein for 5 min at 0–4° C, reisolated by centrifugation, and suspended in 0.25 M sucrose at a concentration of 25 mg protein · ml^{-1}. Ultrastructural evaluation indicated that the mitoplasts were comparable to those described by other investigators (see Sholte *et al.*, 1973, for example). The outer membranes appeared fragmented, but the inner membranes remained intact and the matrix was condensed. Digitonin did not remove the outer membrane fragments, however, and there was no appreciable loss of monoamine oxidase activity. Oxygen consumption with glutamate-malate and ADP was equal to that of untreated beef heart mitochondria.

CK activity was determined at 25° C using commercially available kit reagents (Sigma Chemical Co., St. Louis, Missouri) on an ENI-Gemsaec centrifugal analyzer (Electro-Nucleonics, Inc.). Aspartate aminotransferase (EC 2.6.1.1) was assayed according to the method of Lott and Turner (1973) and adenylate kinase (EC 2.7.4.3) as described by Farrell *et al.* (1972). Control experiments established that the isolated beef heart mitochondria contained no detectable cytosolic isozymes of CK, and adenylate kinase activity measured by the above method was inhibited more than 90% by 10 μM diadenosine pentaphosphate. For studies of enzyme release, the mitochondria or mitoplasts were sedimented at 20,000 × *g* for 5 min and the supernate was decanted and reserved for analysis. The pellets were resuspended in normal saline and extracted with 1% Triton X-100. All samples were treated with 1 mM dithiothreitol and stored on ice prior to the enzyme assays.

Results

Light scattering measured at 540 nm did not decrease when the isolated beef heart mitochondria were incubated with digitonin, and transmission electron micrographs of randomly selected samples confirmed the absence of matrix expansion in the resulting mitoplasts. As shown in Table I, there was no loss of CKm when the mitoplasts were incubated for 10 min at 37° C in 0.25 M sucrose, but an average of 85% of the CKm was extracted when the mitoplasts were incubated with 0.125 M KCl or a Krebs–Ringer phosphate buffer. Intact heart mitochondria, on the other hand, released very little CKm when incubated under identical conditions. It has been suggested that an increase in cytosolic P_i during myocardial ischemia can cause a loss of CKm and interfere with energy transport reactions (Bittl *et al.*, 1985), but according to Table I, intact heart mitochondria did not release CKm in a 37° C physiologic salt solution containing 17 mM P_i. The 1.2 mM Mg^{2+}

Table I. Release of CKm from Isolated Heart Mitochondria and Mitoplasts[a]

Incubation buffer	CKm retained (I.U. mg^{-1} protein)	
	Mitochondria	Mitoplasts
Sucrose	2.0 ± 0.04	2.0 ± 0.06
KCl	1.7 ± 0.05	0.3 ± 0.05
Krebs–Ringer phosphate	1.8 ± 0.04	0.2 ± 0.05

[a]Intact and digitonin-treated mitochondria were incubated for 10 min, 37° C, pH 7.2, 0.5 mg protein · ml^{-1} in 250 mM sucrose, 125 mM KCl, or a Krebs–Ringer phosphate buffer containing 121 mM NaCl, 5 mM KCl, 16 mM Na_2HPO_4, 1 mM KH_2PO_4, and 1.2 mM $MgSO_4$. The data are mean ± S.E.M. (n = 3–4) for the CKm recovered in the mitochondrial or mitoplast pellets. Analysis of the reserved supernates indicated no net loss of CKm activity relative to the 2.0 I.U. mg^{-1} of unincubated mitochondria.

present in the Krebs–Ringer phosphate buffer appears adequate to inhibit phosphate-induced mitochondrial swelling and CKm release (Altschuld and Brierley, 1977; Font *et al.*, 1981). A similar effect of Mg probably accounts for the failure of anoxic, ATP-depleted isolated adult rat heart myocytes to release CKm over a 4-hr incubation at 37° C (Murphy *et al.*, 1982). It should be noted that 160 μg digitonin · mg^{-1} protein solubilized nearly all of the CKm contained in freshly isolated, viable, and metabolically competent isolated rat heart myocytes suspended in isotonic KCl or the Krebs–Ringer phosphate, but the CKm remained bound when the myocytes were suspended in 0.25 M sucrose, pH 7.2 (unpublished observations). These data indicate that CKm extraction in ionic buffers is not unique to beef heart mitoplasts.

In the case of water-swollen mitochondria, CKm extraction following salt addition was a function of ionic strength, except for the enhanced solubilization observed with adenine nucleotides (Lipskaya *et al.*, 1980b, 1982). These experiments were repeated using beef heart mitoplasts, and osmolarity was held constant throughout by appropriate additions of isotonic sucrose to prevent matrix expansion. Within the limits of experimental error, there were no differences in the extent of CKm solubilization when the data for water-swollen heart mitochondria and the nonswollen mitoplasts were compared (see Lipskaya *et al.*, 1980b for the experimental details). The effects of increasing KCl concentrations are summarized in Fig. 1. In these experiments, there was minimal release of matrix aspartate aminotransferase, but a salting off effect was observed for the 50–60% of total adenylate kinase retained by the reisolated mitoplasts in addition to the KCl-dependent extraction of CKm. It is also apparent that CKm solubilization occurs at the temperature commonly used to study mitochondrial respiratory activity (i.e., 25° C) as well as at a physiologic temperature of 37° C.

Although CKm does not appear to bind to the mitochondrial inner membrane in isotonic KCl buffers, many studies of the interaction between

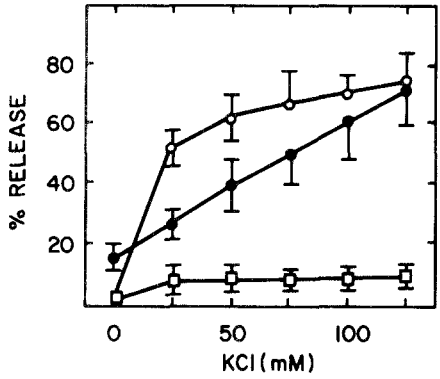

Fig. 1. Effects of increasing [KCl] on the release of CKm, adenylate kinase, and AST by beef heart mitoplasts. Mitoplasts were prepared as described in the text and incubated with varying concentrations of KCl for 10 min at 25° C. Osmolality was maintained with sucrose and pH held at 7.2 with 5 mM TES. Adenylate kinase (●), $n = 5$, AST (□), $n = 4$, and CKm (○), $n = 6$. Activity of the suspending medium is expressed as the percent of total activity measured in the mitoplasts prior to incubation. All values are mean ± S.E.M.

CKm activity and oxidative phosphorylation have been conducted in low ionic strength sucrose or mannitol buffers to overcome chloride inhibition of CKm activity (Hall *et al.*, 1979; Saks *et al.*, 1976). Under these conditions, one might predict that CKm would remain bound to the inner membrane. However, when 1.35 mM ATP, 5 mM P_i, 5 mM glutamate, 2 mM malate, 3 mM Mg acetate, 10 mM HEPES, and 20 mM creatine (pH 7.2) were added to 250 mM sucrose to initiate oxidative phosphorylation, the beef heart mitoplasts retained less than 10% of the initial CKm activity after reisolation to remove the CK substrates. The specific solubilizing effect of $MgATP^{2-}$ and the ionic strength produced by added P_i and the anionic respiratory substrates appeared to prevent CKm binding to the inner membrane (Lipskaya *et al.*, 1980b). It should be noted that this medium was comparable to that used to demonstrate an effect of respiratory activity on the kinetics of heart mitochondrial phosphocreatine synthesis (Saks *et al.*, 1976).

One of the more compelling lines of evidence for specific binding of CKm to the inner membrane has been the reversible solubilization achieved with high concentrations of organic mercurials (Font *et al.*, 1983). However, as shown in Table II, 500 μM *p*-hydroxymercuribenzoate solubilized both CKm and adenylate kinase bound to water-washed swollen heart mitochondria, and there was substantial rebinding of both enzymes after the addition of excess dithiothreitol. The effects of organic mercurials on the mitochondrial inner membrane are complex and involve multiple classes of sulfhydryl groups (Scott *et al.*, 1970). The concentrations necessary to solubilize CKm and adenylate kinase are several times higher than those required to alter ion

Table II. Effects of pHMB and DTT on Binding of CKm and Adenylate Kinase to Distilled Water-Washed Beef Heart Mitochondria[a]

	Bound CKm (I.U. mg^{-1})	Bound adenylate kinase (I.U. mg^{-1})
H_2O washed control	1.63 ± 0.08	0.23 ± 0.03
15 min pHMB	0.27 ± 0.06	0.08 ± 0.01
15 min pHMB + 15 min DTT	1.20 ± 0.01	0.19 ± 0.01

[a]The experiment was conducted exactly as described by Font *et al.* (1983) but the mitochondria were washed four times with distilled water to remove the 0.17 ± 0.01 I.U. mg^{-1} adenylate kinase released by osmotic shock. The washed mitochondria were incubated 15 min, 4° C, in distilled water containing 500 μM parahydroxymercuribenzoate (pHMB) and separated by centrifugation for analysis of liberated and bound CKm and adenyate kinase. An identical sample was treated with 3 mM dithiothreitol for an additional 15 min after incubation with pHMB. The pHMB (500 μM) caused inactivation of CK, but inclusion of 3 mM dithiothreitol in the assay buffer as recommended by Font *et al.* (1983) restored all but 30% of total activity. Adenylate kinase recovery exceeded 90% in all experiments. The data are mean values ± S.E.M. for three to four experiments.

transport reactions, and may involve nonspecific conformation changes. Regardless of the mechanism, the effects of sulfhydryl agents on the binding of proteins to the inner membrane are not specific for CKm.

Discussion

The retention of CKm activity by water-swollen heart mitochondria (Jacobus and Lehninger, 1973) and by digitonin-treated heart mitochondria suspended in isotonic sucrose (Scholte *et al.*, 1973) led originally to the concept that CKm is bound to the outer surface of the heart mitochondrial inner membrane. However, the results of the present study indicate that physiologic salt solutions extract the enzyme from digitonin-treated beef heart mitochondria, and an identical response has been reported for water-swollen mitochondria (Lipskaya *et al.*, 1980b). One could argue that matrix expansion alters inner membrane CKm binding sites, and a similar criticism has been leveled against nonswollen mitoplasts prepared with digitonin (Barbour *et al.*, 1984). However, these two models were responsible for the original assignment of CKm localization, and one would be forced to discount the early binding studies if there were substantial inner membrane alterations during matrix expansion or digitonin treatment.

The binding of CKm to heart mitochondrial inner membranes in nonionic buffers has been shown to involve cardiolipin but not the adenine nucleotide translocase (Müller *et al.*, 1985). CKm binds to liver mitochondria, which normally do not contain CKm activity (Hall and Deluca, 1980;

Müller *et al.*, 1985), to liposomes prepared from heart mitochondrial lipid extracts (Lipskaya *et al.*, 1982), and to cardiolipin-containing liposomes (Müller *et al.*, 1985). This binding is inhibited by adriamycin, which complexes membrane-bound cardiolipin, and by KCl, which disrupts electrostatic interactions (Müller *et al.*, 1985). CKm does not bind to phosphatidylcholine liposomes, and incorporation of the purified ADP/ATP translocator into these liposomes does not lead to CKm binding (Müller *et al.*, 1985). It is unlikely that the binding of CKm to heart mitochondrial inner membranes at low ionic strength involves a site adjacent to the translocase or that CKm binds to inner membrane cardiolipin at physiologic ionic strength.

Many of the phenomena attributed to the binding of CKm to a site adjacent to the adenine nucleotide translocase have been shown to result from subtle alterations in the extramitochondrial ATP/ADP ratios (Altschuld and Brierley, 1977), and a similar mechanism probably can account for the apparent effects of creatine kinase activity on the translocation of radiolabeled adenine nucleotides in isolated heart mitochondria (Barbour *et al.*, 1984). However, added pyruvate kinase and phosphoenolpyruvate have been shown to inhibit the increase in oxygen consumption produced by glucose plus hexokinase to a greater extent than that produced by 33 mM creatine in isolated rat heart mitochondria (Gellerich and Saks, 1982). A similar response was observed with the beef heart mitochondria used in the present study, despite nearly equivalent ATP and ADP concentrations in the bulk suspending medium (unpublished observations). One of the objectives of the present study was to determine if this phenomenon could be attributed to the exclusion of added pyruvate kinase from the intermembrane space by the protein-impermeable outer membrane rather than "privileged access." However, it was not possible to distinguish between the two alternative models by disrupting the outer membranes with digitonin, since the presence of respiratory substrates and adenine nucleotides solubilized the CKm bound to the mitoplasts. We can therefore only speculate that the heart mitochondrial outer membrane encloses a small unstirred compartment with ATP and ADP concentrations which differ slightly from those of the bulk extramitochondrial compartment during creatine-stimulated respiration in the presence of added pyruvate kinase.

It is possible that the intermembrane compartment of respiring mitochondria *in vivo* also contains ATP and ADP concentrations slightly different from the remainder of the cytosolic space, expecially if the diffusion of adenylates through the outer membrane pores is somewhat hindered relative to the rate of ATP turnover. However, in contrast to isolated heart mitochondria incubated with creatine and pyruvate kinase, the intermembrane ATP/ADP ratio would be increased relative to that of the extramitochondrial compartment because of the continual removal of ADP and export of ATP

by the translocase. A high intermembrane ATP/ADP ratio, combined with H^+ consumption by oxidative phosphorylation, would tend to drive the reversible CK reaction in the direction of phosphocreatine and ADP synthesis (Altschuld and Brierley, 1977). On the other hand, production of ADP and H^+ by cytosolic ATPases would favor the phosphorylation of ADP by phosphocreatine at sites spatially removed from the mitochondria. As a consequence, ADP need not diffuse to the mitochondria for rephosphorylation, and cytosolic phosphocreatine/creatine ratios and P_i concentrations will control the intermembrane phosphorylation potential and oxidative phosphorylation (Altschuld and Brierley, 1977). We tend to agree with Meyer *et al.* (1984) that the "phosphocreatine shuttle" can be viewed as an example of facilitated diffusion, but we consider the most physiologically relevant feature of this system to be the facilitated diffusion of the least abundant CK substrate, ADP (Altschuld and Brierley, 1977). Whether this constitutes a form of "privileged access" is a question of semantics.

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