Coles, C., Rawlings, J., Holm, R. H., Singer, T., & Orme-Johnson, W. H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3805–3808.
Erbes, D. L., Burris, R. H., & Orme-Johnson, W. H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4795–4799.
Ghazarian, J. G., Schnoes, H. K., & DeLuca, H. F. (1973) *Biochemistry 12*, 2555–2558.
Gillum, W. O., Mortenson, L. E., Chen, J.-S., & Holm, R. H. (1977) *J. Am. Chem. Soc. 99*, 584–595.
Gornall, A. G., Bardwill, C. J., & David, M. M. (1949) *J. Biol. Chem. 177*, 757–766.
Hill, C. L., Renaud, J., Holm, R. H., & Mortenson, L. E. (1977) *J. Am. Chem. Soc. 99*, 2549–2557.
Kurtz, D. M., Wong, C. B., & Holm, R. H. (1978) *J. Am. Chem. Soc. 100*, 6777–6779.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Omura, T., & Sato, R. (1967) *Methods Enzymol. 10*, 556–561.
Orme-Johnson, W. H., & Beinert, H. (1969) *J. Biol. Chem. 244*, 6143–6148.
Orme-Johnson, W. H., & Sands, R. H. (1973) in *Iron-Sulfur Proteins* (Lovenberg, W., Ed.) Vol. II, p 195, Academic Press, New York.
Orme-Johnson, W. H., & Holm, R. H. (1978) *Methods Enzymol. 53*, 268–274.
Pedersen, J. I., Ghazarian, J. G., Orme-Johnson, N. R., & DeLuca, H. F. (1976) *J. Biol. Chem. 251*, 3933–3941.
Stombaugh, N. A., Burris, R. H., & Orme-Johnson, W. H. (1973) *J. Biol. Chem. 248*, 7951–7956.
Sugiyama, T., & Yamano, T. (1975) *FEBS Lett. 52*, 145–148.
Yasunobu, K. T., & Tanaka, M. (1973) in *Iron-Sulfur Proteins* (Lovenberg, W., Ed.) Vol. II, p 27, Academic Press, New York.
Yoon, P. S., & DeLuca, H. F. (1980) *Biochemistry* (preceding paper in this issue).

Interaction of Creatine Kinase Isoenzymes with Beef Heart Mitochondrial Membrane: A Model for Association of Mitochondrial and Cytoplasmic Isoenzymes with Inner Membrane†

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ABSTRACT: A beef heart mitochondrial preparation characterized by a high degree of structural integrity, the absence of contaminating myofibrils, and a constant, relatively high level of creatine kinase was employed to study the location and quantitative distribution of the creatine kinase isoenzymes in various subfractions. Submitochondrial fractions studied were (I) the outer membrane–intermembrane space, (II) the total mitoplast, (III) the extrinsic surface (cytoplasmic side) of the inner membrane, and (IV) the inner membrane and matrix space. Determination of the isoenzymes in the fractions by electrophoresis in 0.025 M Tris–0.190 M glycine at pH 8.6 indicated that all fractions except I contained varying amounts of the cytoplasmic muscle-type isoenzyme (MM) and the cationic mitochondrial isoenzyme (MT), the highest proportion of MT creatine kinase being in fraction IV. Comparison of the detergent extracts of whole mitochondria with fraction IV indicated that the MT/MM ratio in the former is ~1:3 and in the latter is ~1:1. DEAE-cellulose chromatography of MM and MT mixtures suggests that the two forms of the enzyme can exist as a complex in solution. The free access of the membrane-impermeable phosphorylcreatine to the enzyme in the everted sonic particles as well as the submitochondrial partition of the enzyme supports the conclusion that the MT creatine kinase is very strongly associated with and probably traverses the inner membrane. A model for the association of beef heart creatine kinase isoenzymes with the inner membrane has been proposed by taking into account our results with those of Addink et al. [Addink, A. D. F., Boer, P., Akabayashi, T., & Green, D. E. (1972) *Eur. J. Biochem. 29*, 47–59] and the properties of the purified MT [Jacobs, H. K., & Graham, M. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 1574; Hall, N., Addis, P., & Deluca, M. (1979) *Biochemistry 18*, 1745–1751].

It is generally accepted that a significant portion of the cellular creatine kinase (CK,[1] EC 2.7.3.2) in the cardiac muscles of a variety of species of animals is in the mitochrondria (Jacobs et al., 1964; Jacobus & Lehninger, 1973; Sobel et al., 1972; Farrell et al., 1972; Scholte et al., 1973; Addink et al., 1972). However, widely divergent results have been obtained with respect to the quantitative distribution as well as to the location of the particulate enzyme in the myocardium. Thus, Jacobs et al. (1964) reported that the rat heart mitochondria contain 55% of the total organ CK. Subcellular fractionation of rat heart accompanied by careful monitoring of the fractions by means of marker enzymes led Scholte (1973a) to conclude that the mitochondrial compartment accounted for 19% whereas the myofibrils contained 33% of the total CK activity. The location of this enzyme in the

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[1] Abbreviations used: CK, creatine kinase; MM, muscle-type isoenzyme; BB, brain-type isoenzyme; MB, hybrid form of isoenzyme; MT, mitochondrial isoenzyme; Cr, creatine; PCr, phosphorylcreatine; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SMP, submitochondrial particles; 2-ME, 2-mercaptoethanol; A-P_5-A, diadenosine 5′-pentaphosphate; G6PDH, glucose-6-phosphate dehydrogenase; $NaDodSO_4$, sodium dodecyl sulfate; DNFB, 2,4-dinitro-1-fluorobenzene.

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mitochondria has been attributed to be the outer membrane (Farrell et al., 1972), the intermembrane space (Klingenberg, 1970), and the outer surface of the inner mitochrondrial membrane (Scholte et al., 1973). The limited solubilization of the beef heart mitochondrial CK by digitonin led Addink et al. (1972) to propose a bimodal distribution involving intermembrane space and the inner membrane matrix. The in vivo role of the mitochondrial CK is not known. Yang et al. (1977) have demonstrated that the mitochondrially bound enzyme can catalyze the direct transfer of phosphate from the ATP pool in the matrix to creatine (Cr), resulting in the formation of PCr. Extensive kinetic data have been compiled by Saks and co-workers [see Saks et al. (1977) for a review] to support their contention that the membrane-bound enzyme is involved in the transport of the energy generated during oxidative metabolism in the mitochondrion to the contractile proteins. On the basis of differences in electrophoretic mobility, a specific mitochondrial isoenzyme of CK (MT CK) has been postulated (Jacobs et al., 1964; Scholte, 1973a). Recently, two groups of investigators have described the properties of a basic form of creatine kinase purified from heart mitochondria (Jacobs & Graham, 1978; Hall et al., 1979), which seems to undergo facile aggregation on standing (Hall et al., 1979). The kinetic behavior of this MT is consistent with its role in the synthesis of PCr from ATP and Cr (Hall et al., 1979).

In this report we present evidence to support that the MT form of the enzyme is a firmly bound protein of the inner membrane of the beef heart mitochondria and that it occurs as a complex with the major cytoplasmic MM. A model for the lipid–protein interaction is proposed which accounts for the large number of disparate and contradictory results.

Methods and Materials

Mitochondria. Fresh beef hearts immediately after slaughter were diced into small (1 cm^3) pieces and transported in ice-cold isolation medium to the laboratory. In most of the experiments we employed 0.05 M sucrose, 0.270 M mannitol, and 0.001 M EDTA, pH 7.4 (A-SM medium). Some of the preliminary studies were done in 0.3 M sucrose, 0.001 M EDTA, 0.001 M buffer at pH 7.4 (medium A-S). A 10% (w/v) homogenate of the finely minced ventricle was prepared by disintegration of the tissue for 15 s in a blender followed by brief homogenization (5 strokes) in a loose glass–Teflon homogenizer at 4 °C. After the nuclear pellet was discarded by centrifugation at 600*g* for 10 min, the mitochondria were sedimented at 10000*g* for 10 min. Three successive washes each with 0.5 volume of the medium used in the previous step were done after the pellet was suspended with a Dounce homogenizer. Any light, fluffy layer was scraped off after the pellet was collected at 8000*g* for 10 min.

Digitonin Treatment. Fresh, medium A-SM washed mitochondria (8–10 mg of protein per mL) were treated with digitonin in medium A-SM by the procedure of Addink et al. (1972) using a final concentration of 0.2 mg/mg of protein. After 10 min at 0 °C, the detergent was diluted by the addition of 5 volumes of the medium A-SM and the suspension was centrifuged at 10000*g* for 20 min. The resulting mitoplasts were washed once with 10 volumes of the medium.

Submitochondrial Particles (SMP). Sonic particles from mitochondria were prepared by subjecting the mitochondrial suspension in 2 mL of medium A-S or A-SM to ultrasonic radiation in a Branson S75 sonifer with the sample immersed in ice water using the maximum energy output. The protein concentrations used were either 10 or 1–2 mg/mL (see Results). When sonication was continued for longer than 15 s, 5-s bursts of radiation were interspersed with 30-s cooling. Quantitative transfer to the centrifuge tubes was ensured by using 5-s sonication with 2 × 1 mL of the isolation medium. The sonic particles were separated by centrifugation at 144000*g* (max) for 1 h in a Beckman L-2 centrifuge and decanting the clear supernate.

Enzyme Extraction. Salt extraction of creatine kinase was done by using either sodium or potassium phosphate (80 mM, pH 7.4) or medium B (0.025 M Tris, 0.190 M glycine, and 0.001 M 2-mercaptoethanol, pH adjusted to 8.6). Whole mitochondria, mitoplasts, or SMP were suspended with a Vortex mixer at a concentration of 8–10 mg of protein per mL and kept at room temperature on a mechanical shaker for 30 min, and the residue was separated by centrifugation at 100000*g* for 30 min in a Beckman 40 rotor. Two additional extractions were done likewise.

Detergent extraction of the inner membrane was done by mixing the requisite quantities of either digitonin or Lubrol WX made up as indicated in the relevant sections of the Results. Both were more effective in the presence of ionic buffers than in medium A-SM. Lubrol solubilization was done for 15–30 min at 0 °C (Schnaitman & Greenawalt, 1968) to disrupt the inner membrane, and initial treatment for 30 min at 0 °C was followed by one to two cycles of freeze–thawing.

DEAE-cellulose Chromatography. Enzyme fractions containing both MT and MM obtained by Lubrol extraction of whole mitochondria were concentrated by precipitation with solid ammonium sulfate (30–70% saturation) and were exhaustively dialyzed against medium B. A DEAE-cellulose (Whatman DE32) column of 70 × 1.5 cm was equilibrated against the same buffer, and 12-mL fractions flowing at 16 mL/h were collected at 4 °C.

Cellulose Acetate Electrophoresis. Isoenzyme separation was done in a Beckman microzone electrophoresis system using medium B as the electrode buffer. Electrophoresis was done for 30 min at room temperature with a nominal voltage of 250 V. The enzyme was localized by the agar overlay technique (Rosalki, 1965). Positive identification of CK isoenzymes was made by using agar plates with and without PCr and also by a third run in the presence of 10^{-5} M diadenosine 5′-pentaphosphate ($A\text{-}P_5\text{-}A$) which specifically inhibits adenylate kinase (Lienhard & Secemski, 1973).

Creatine Kinase Assays. CK activity was measured in the direction of ATP synthesis by the procedure of Oliver (1955) in a Cary 14 double-beam spectrophotometer by following at 340 nm the rate of production of NADPH at 22–24 °C after initiation of the reaction with PCr. The Max-Pack Kit (Calbiochem, La Jolla, CA) or a mixture of similar composition was used. At the level of the coupling enzymes hexokinase–G6PDH (1700 milliunits of each), there was no significant interference by the mitochondrial ATPase. The absorbance readings at 340 nm were normalized to 30 °C by using the temperature coefficient of 1.6 (booklet with Max-Pack Kit). For conversion of the absorbance readings to units, a factor of $E_M{}^{1cm} = 6.2 \times 10^3$ for NADPH was used.

Protein was determined by the Lowry procedure (Lowry et al., 1951). The protein contents of the membrane fragments were in some cases estimated by direct spectrophotometry after solubilization with sodium lauryl sulfate (Clarke, 1976) by using a factor of $\Delta A_{1\%}{}^{1cm}(A_{280nm} - A_{310nm}) = 10.4$.

Electron Microscopy. Mitochondria were fixed immediately after isolation with 4% glutaraldehyde in 0.1 M sodium phosphate (pH 7.0). After 24 h, the pellets were washed in 0.1 M phosphate buffer and postfixed in 1% OsO_4 in 0.1 M phosphate buffer for 2 h. The pellets were successively washed

Table I: Creatine Kinase Activity of Heart Mitochondria[a]

	N[b]	act.[c] (milli-units/mg of protein)
rabbit	2	380
dog	7	490
lamb	4	730
rat	4	2180
beef	4	3090

[a] CK activity was measured as described under Methods and Materials in mitochondria isolated in medium A-S and washed twice with the same medium. Samples were diluted so that ΔA_{340}/min was in the range of 0.05–0.08. [b] N = the number of animals used. [c] 1 unit of activity = enzyme catalyzing the production of 1 μmol of NADPH per min at 30 °C.

with 0.1 M phosphate, distilled water, and 50 and 75% ethanol, followed by block staining with 4% uranyl acetate in 75% ethanol for 24 h. After dehydration in ethanol and infiltration with propylene oxide, the samples were imbedded in Epon. Silver to grey sections were mounted on formvar and carbon coated grids to be stained with 8% uranyl acetate and Reynolds lead citrate (Hayat, 1970). Transmission micrographs were obtained with an Hitachi HU-11C electron microscope.

All chemicals used were of reagent grade. The various biochemicals used in the CK activity assay were purchased from Sigma Chemical Co. (St. Louis, MO). The coupling enzyme, A-P_5-A, and the rabbit muscle CK were purchased from Bohringer-Mannheim, the Max-Pack Kits were from Calbiochem (La Jolla, CA), and enzyme grade ammonium sulfate was from Schwarz/Mann Research Laboratories, Inc. (Orangeburg, NY). The ingredients for NaDodSO$_4$–polyacrylamide gel electrophoresis were obtained from Bio-Rad Corp. (Richmond, CA). Osmium tetraoxide, glutaraldehyde, uranyl acetate, and lead citrate were obtained from Electron Microscopy Services (Ft. Washington, PA). Lubrol WX and digitonin were purchased from Sigma Chemicals (St. Louis, MO).

Results

In Table I is shown the CK activity of cardiac mitochondria isolated from five mammalian species as described under Methods and Materials and washed twice using medium A-S. Similar results (not shown here) were obtained with medium A-SM. These results show that under comparable conditions, the isolated mitochondria from rabbit, dog, and lamb hearts have a low CK activity (380, 490, and 730 milliunits/mg of protein, respectively) in comparison with rat and beef heart mitochondria which contained 2180 and 3090 milliunits/mg of protein of CK, respectively. The difference, in part, may be due to the ease with which the cytoplasmic component associated with the mitochondria (see Discussion) is released during preparation and washing. Numerous experiments indicated that three successive washes with the isolation medium were sufficient to remove the loosely associated cytoplasmic CK adhering to the intact mitochondria. Thus, with a typical beef heart mitochondrial pellet containing a total CK activity of ΔA_{340}/min of 190, three successive washes with medium A-SM removed enzyme activity equivalent to ΔA_{340}/min of 16.9, 4.8, and 0.19, respectively. A fourth wash contained negligible activity. Electron micrographs of beef cardiac mitochondria thus washed (Figure 1) show a high degree of structural integrity. Also noteworthy is the absence of myofibril which in the heart muscle is a major site of bound CK (Scholte, 1973; Zimmerman et al., 1979). Since the beef heart system provided structurally intact, myofibril-free mitochondria with a high and stable level of CK, it was chosen to study further the mode of interaction of CK with the mitochondrial membrane.

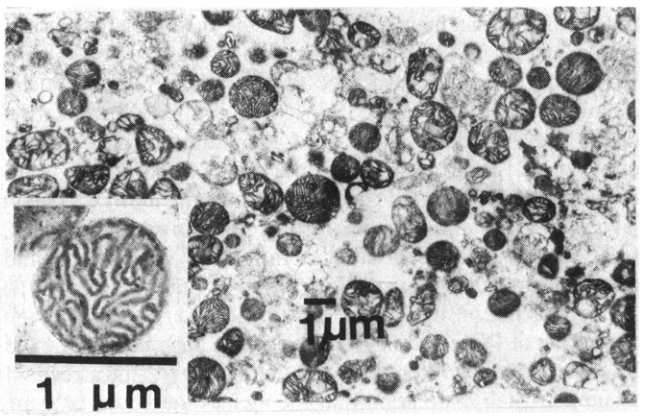

FIGURE 1: Electron micrograph of beef heart mitochondria isolated in medium A-SM: magnification ×5000. Inset: ×22 500 magnified micrograph illustrating the integrity of the inner and outer membranes.

Table II: Solubilization of Beef Heart Mitochondrial CK by Salts[a]

		no. of extractions		
		I	II	III
sodium phosphate (80 mM, pH 7.4)	CK[b]	15.60	3.60	0.10
	protein (mg)	1.62	0.56	0.46
Tris–glycine-2-ME, pH 8.6 (0.025 M: 0.19 M:0.01 M)	CK[b]	18.20	4.80	0.10
	protein (mg)	2.09	0.74	0.45

[a] Total enzyme activities of ΔA_{340}/min = 30–50 were extracted with 2 mL of buffers as described under Methods and Materials. [b] CK activity is represented as total A_{340}/min in the fraction.

The extent of solubilization of the bound CK with nonchaotropic electrolytes was investigated by extraction with potassium phosphate (80 mM, pH 7.4) and Tris–glycine buffer (0.025 M Tris, 0.190 M glycine, and 0.010 M 2-mercaptoethanol, pH 8.6). Phosphates have been used by other investigators as an effective solubilizer of bound CK (Klingenberg, 1970; Jacobus & Lehninger, 1973; Farrell et al., 1972). The Tris–glycine–mercaptoethanol medium was employed since we found it to be a highly suitable medium for electrophoretic analysis of CK isoenzymes. The results presented in Table II are averages of four determinations and show that two successive extractions under the conditions used resulted in the release of the entire extractable pool of CK in the case of sodium phosphate as well as that of Tris–glycine. The Tris-glycine buffer solubilized a total enzyme activity equivalent to ΔA_{340}/min of 23.1 in comparison to ΔA_{340}/min of 19.3 for the sodium phosphate. Results with potassium phosphate were essentially similar to those with sodium phosphate, the potassium counterion being only marginally less effective than sodium. Measurement of the activity in the residue at the end of three extractions on four separate preparations indicated that an average of 38% of the total activity was unextractable by the Tris–glycine–mercaptoethanol medium whereas 44% was unextractable by the phosphate medium. It is to be noted that since the membrane-bound enzyme is significantly activated on release by detergents like Lubrol WX (Figure 3) these values for the activity in the residue are minimal estimates. It has been shown that when heart mitochondria are treated with digitonin at a concentration of 0.2 mg/mg of protein in a nonhypotonic medium, the outer membrane is nearly dissolved, releasing a structurally intact mitoplast (Addink et al., 1972). Such preparations also show a high

Table III: CK Released by Digitonin Treatment of Beef Heart Mitochondria[a]

prepn	CK act. (ΔA_{340}/min) ppt[b]	supernate
I	25.9 (54)[c]	23.2 (46)
II	16.8 (42)	21.2 (58)
III	21.6 (49)	22.4 (51)

[a] Freshly prepared mitochondria were suspended in medium A-SM (~2 mg of protein per mL) and treated with digitonin (0.2 mg/mg) also in medium A-SM (2.0% stock solution) for 10 min at 0 °C. The mitoplast was centrifuged at 15000g for 10 min after dilution of digitonin by adding 5 volumes of medium A-SM. [b] The activities in the precipitates are uncorrected for membrane crypticity (see Figure 3). [c] Numbers in parentheses indicate percentage.

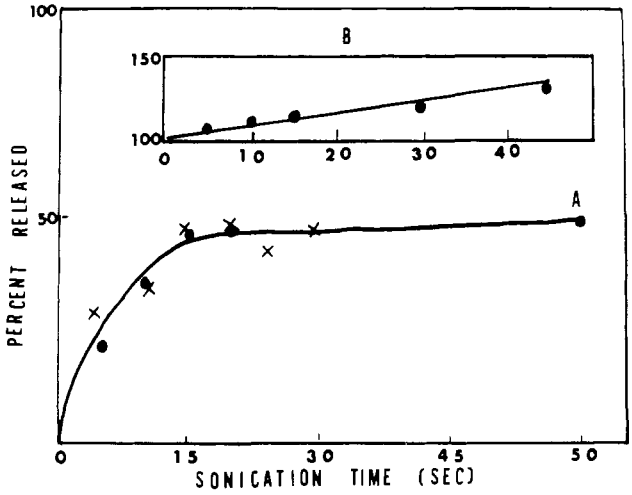

FIGURE 2: Release of creatine kinase by sonic radiation of beef heart mitochondria. Mitochondria washed 2 to 3 times with medium A-S or A-SM were suspended in 2 mL of medium (10 mg of protein per mL) and sonicated as described under Methods and Materials. A: enzyme activity in the 144000g × 60 min supernatant. B (inset): total CK activity obtained by adding the activity in part A and the activity measured in the precipitate suspended in medium A-SM. The activity in the precipitate has not been corrected for membrane constraint (see Results).

degree of respiratory control (Krebs et al., 1979). Results from three different preparations of digitonin particles are shown in Table III. The activity released by digitonin accounted for 46, 58, and 51% of the total. The inner membrane complex retained an average of 48% of the enzyme as measured without addition of Lubrol and hence this value is a lower estimate of the bound enzyme (see Discussion).

The rate of release of the mitochondrial CK by exposure to sonic radiation for various intervals of time from fresh mitochondria washed twice with medium A-SM at a concentration of 10 mg of protein per mL is shown in Figure 2A. The CK activity measured in the supernate rose sharply, reaching a plateau of ~45% of the total activity in 20 s. Further sonication resulted in a very slow increase in the soluble enzyme. The CK activity in the precipitate was measured as described under Methods and Materials. Such membrane fragments (results not shown) respond to added PCr in less than 20 s. They also exhibited a linearity of rate even at a relatively high rate of substrate utilization, indicating that there is no diffusional barrier between the enzyme and PCr in the everted sealed vesicles. Figure 2B represents the total activity (as the percent of unsonicated controls) recovered at various times of radiation in the sonic precipitate and supernatant fractions. The results indicate that there is ~30–40% activation of the membrane-bound enzyme by sonic

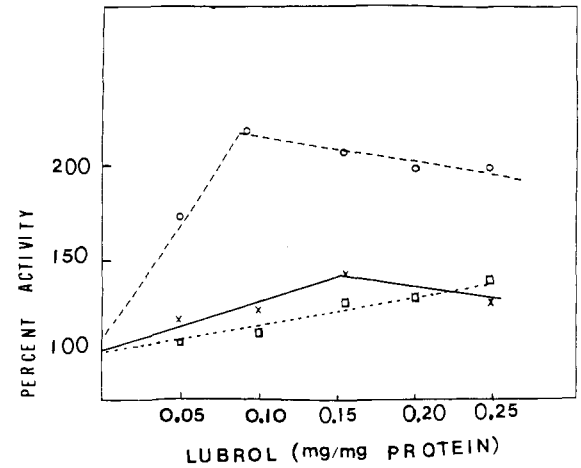

FIGURE 3: Different mitochondrial preparations were suspended in 2 mL of 0.003 M Tris–0.019 M glycine–0.010 M 2-mercaptoethanol, pH 8.6 (1.0 mg of protein per mL), at 0 °C. Lubrol WX was added from a stock solution of 1 mg/mL in the same medium, and the mixture was kept on ice for 15–30 min after mixing. (□) Sonic submitochondrial particles; (×) whole mitochondria; (○) digitonin particles (mitoplasts).

radiation. Subjecting the soluble cytosol CK to sonication under similar conditions established that the radiation had no direct effect on the enzyme. In experiments done using lower mitochondrial protein concentrations (1 to 2 mg of protein per mL) the partition between the soluble and membrane fractions was similar with 50% of the activity in either fraction (results not shown). The total activity recovered under these conditions ranged between 91 and 105%. In no case was there evidence of major internalization of the enzyme and concomitant latency.

Lubrol WX was used to release the membrane-constrained enzyme (Schnaitman & Greenawalt, 1968). Figure 3 summarizes the results obtained with the different mitochondrial particles when treated with increasing amounts of Lubrol in Tris–glycine–mercaptoethanol. When intact mitochondria were treated with Lubrol, the total creatine kinase activity increased by ~35%, the maximal effect being reached at 100–150 μg of the detergent. Higher levels of the detergent produced slight inhibition. With mitoplasts from which the outer membrane has been removed, the peak activation was reached at 80–100 μg of Lubrol and the degree of activation was ~100%. As with the whole mitochondria, there was some inhibition at higher levels of Lubrol. The submitochondrial particles which had been prepared by a 45-s sonication responded by an activation of ~25–35%.

Ion-exchange chromatography of the total extractable CK on DEAE-cellulose is shown in Figure 4. The enzyme extracted with Lubrol in medium B was precipitated by 65% saturation of ammonium sulfate and dialyzed exhaustively against medium B. Such extracts contain ~30% of MT CK as revealed by electrophoresis on cellulose acetate. In the experiments shown in Figure 4, a total enzyme activity equivalent to ΔA_{340}/min of 386 was applied to the column and ~60% of the activity was recovered under peaks I and II. No enzyme was detected under peak III, which accounted for the bulk of the protein, or under the other peaks. The CK activity in peak I represented 8% of the total eluted activity. In other preparations it accounted for 5–15%. Electrophoresis on cellulose polyacetate at pH 8.6 (Table IV) showed that peak I contained only the basic form of the isoenzyme whereas the peak II material contained both the basic (mitochondrial) and the predominant forms of the cardiac muscle isoenzyme, namely, the MM form (see Figure 5 and Table IV).

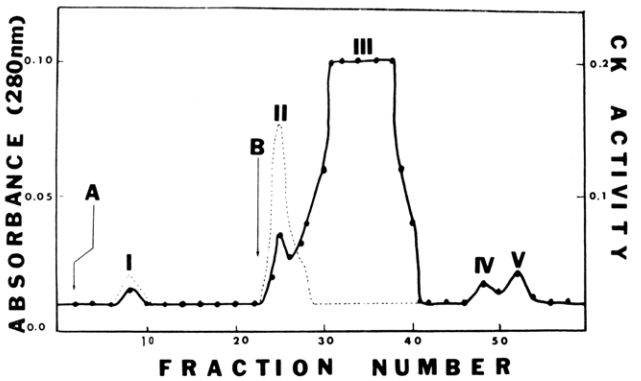

FIGURE 4: DEAE-cellulose chromatography of the total mitochondrial creatine kinase extracted and concentrated as described under Methods and Materials. A 70 × 1.5 cm column of Whatman DE32 was run at 4 °C at a flow rate of 16 mL/h. Step elution was done with (A) 0.025 M Tris–0.19 M glycine–0.001 M 2-mercaptoethanol and (B) 0.2 M KCl. Solid line = absorbance at 280 nm; dotted line = CK activity.

Table IV: Creatine Kinase Isoenzymes in Beef Heart Mitochondria[a]

fractions	isoenzymes (%) MT	MM	MB
(I) cytosol[b]	none	88–85	12–15
(II) nonionic wash of mitochondria	trace	95	ND[d]
(III) salt extraction of mitochondria	5–10	95–90	ND
(IV) submitochondrial particles (sonic)[c]	35–45	65–55	ND
(V) mitoplasts[c]	25–35	75–65	ND

[a] Electrophoresis on cellulose acetate paper was done at pH 8.6. The relative proportion of the different isoenzymes was determined by densitometry. [b] The 100000*g* × 60 min supernate of the whole heart homogenate was dialyzed against the electrophoresis buffer. [c] The enzyme was released by treatment with Lubrol (see Figure 3) or with digitonin. [d] ND = not detected.

$NaDodSO_4$–polyacrylamide gel electrophoretic analysis of the peak I material revealed the presence of two bands corresponding to molecular weights of 55 K and 28 K in addition to the main band of 35–40 K. Peak II contained a 40 K band and a band at 55 K. In this fraction six to seven minor bands were found which are probably spillovers from the major protein mixture eluted under peak III.

The isoenzymes of CK present in the different fractions were determined at pH 8.6. Positive identification of the active creatine kinase was ensured with each fraction by staining a duplicate strip on gels not containing PCr and a third strip on gels containing diadenosine 5′-pentaphosphate ($A-P_5-A$) to inhibit adenylate kinase but not creatine kinase (Lienhard & Secemski, 1973). The results of representative patterns are shown in Figure 5 along with rabbit skeletal muscle MM and the bovine uterus BB as references. The data for all fractions are summarized in Table IV. The cytosol (100000*g* for 60 min supernate of heart homogenate) contains two isoenzymes of CK, both migrating toward the anode, the major sport representing the MM and the more acidic MB was a minor component. No cationic form of CK was detectable in the cytosol (lane A, Figure 5). The pooled medium A-SM washes of mitochondria, the salt extracts (Table II) of mitochondria, and the peak II material of DEAE-cellulose chromatography contained a prominent spot corresponding to the MM form of CK and a minor spot migrating as a cationic protein at pH 8.6 (lane B, Figure 5). When mitoplasts or SMP were treated with high levels of digitonin (0.5 mg/mg of protein) for 1 h at 30 °C and overnight at 4 °C, an opalescent extract resulted. Electrophoresis of such extracts on cellulose acetate resulted in the pattern shown in lane C, Figure 5 (see also fraction IV, Table IV). Two distinct spots migrating toward the cathode (spots 1 and 2), one spot with the mobility of MM CK (spot 3), and a spot migrating more rapidly toward the positive role than the MB form (spot 4) were observed. Of the two basic forms, spot 2, which is also found in salt extracts of sucrose-washed intact mitochondria, respresents the normal MT form of CK. Spot 1, which appears only on the disintegration of the inner membrane with high digitonin or Lubrol, probably corresponds to the slowly aggregating but active form of MT CK observed by Hall et al. (1979) with a purified beef heart MT CK preparation. Spot 4 was present even in the absence of PCr and was absent when incubated in the presence of $A-P_5-A$ and hence is probably due to nucleoside diphosphate kinase activity released from the matrix. The intensity of staining in the two basic forms (spot 1 and 2) was equal to that due to the MM CK (spot 3). The peak I fraction from the DEAE-cellulose column contained only one basic form with the mobility of spot 2. The apparent isoelectric point for MT CK was ~9.4 and that for the MM form was 6.5 as determined by the pH of zero mobility on cellulose acetate.

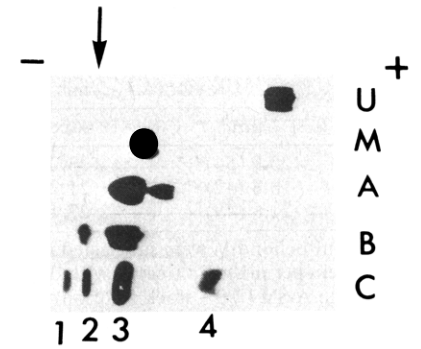

FIGURE 5: Electrophoretic identification of creatine kinase isoenzymes. Electrophoresis on cellulose acetate was done at room temperature for 30 min in 0.025 M Tris–0.190 M glycine–0.001 M 2-mercaptoethanol at pH 8.6. Active enzyme was visualized by overlaying of the strips on agar plates for 1 or 2 h at 37 °C. U = BB CK (bovine uterus); M = MM (rabbit skeletal muscle); A = beef cardiac cytosol, showing MM and MB; B = salt extract of mitochondria, showing MM (spot 3) and MT (spot 2); C = detergent extract of mitoplasts or SMP, showing two basic forms (spots 1 and 2; see Results), MM (spot 3), and unidentified nucleoside diphosphate kinase (spot 4).

Discussion

We have studied the nature and extent of the interaction of creatine kinase isoenzymes in heart mitochondria which have been extensively used in establishing the intraorganelle organization of the citric acid cycle enzymes (Addink et al., 1972), ATPase (Knowles & Penefsky, 1972) and the ATP–ADP porter (Boxer et al., 1977).

The results presented in Tables I and II demonstrate that mitochondria with a high level of CK are derived from beef heart after extensive washing with nonionic isotonic media. The enzyme extracted during these washes consists almost exclusively of the MM and probably represents the cytoplasmic contaminant attached to the outer membrane. Extraction of such mitochondrial preparations with salts leads to further release of creatine kinase. Electrophoresis of this fraction revealed the presence of both MT and MM. Optical estimation of the two forms by densitometry and by elution of the spots showed that the MT form represented ~15–25% of the total activity in this fraction. Precise quantitation of the enzyme activities by these procedures is difficult since the conditions for the agar overlay method (Rosalki, 1965) are

optimized for MM CK and might not necessarily hold for the MT form. Despite these reservations, the MT CK form in this fraction coexists with more than twice as much MM CK. Since the subunit molecular weights for the two isoenzymes are similar [32 K (Hall et al., 1979) and 44 K (Jacobs & Graham, 1978) reported for the MT CK; 41 K reported for the MM CK (Watts, 1973)], this represents a molar ratio of MM to MT of ≥2.

All three electrolytes solubilized only a part of the enzyme activity from the sucrose-washed mitochondria. The CK inaccessible to buffer salts accounts for 50–60% of the total by taking into account that the membrane-bound enzyme is not maximally active as indicated by the Lubrol effect (Figure 3). It is generally accepted that nonchaotropic salts like phosphate and KCl extract proteins loosely bound to the outer surface of the inner membrane (Klingenberg & Pfaff, 1966). When washed mitochondria were treated with digitonin (0.2 mg/mg of protein) under conditions known to yield intact mitoplasts from heart mitochondria (Addink et al., 1972), ~50% of the activity is retained in the inner membrane fraction. In SMP (sonic) prepared from washed intact mitochondria, the membrane vesicles were found to contain about one-half the total activity. The enzyme released during sonication consisted mostly of the cytoplasmic MM form. Thus, three different procedures demonstrated that 50% or more of the beef heart mitochondrial CK was very firmly anchored to the inner membrane.

The distribution pattern of cardiac mitochondrial CK reported here is in agreement with other reports (Jacobus & Lehninger, 1973; Klingenberg & Pfaff, 1966; Farrell et al., 1972) in that phosphate and other ionic buffers are quite effective as solubilizers of membrane-associated enzyme. The firm association of a significant proportion of CK with inner mitochondrial membrane in beef heart suggested by the results just discussed is supported by the results of Farrell et al. (1972) and Jacobus & Lehninger (1973), who found that phosphate released between 40 and 50% of CK activity, and by the study of Addink et al. (1972), who subjected beef heart mitochondria to digitonin (in the presence of bovine serum albumin as a stabilizer) and found that more than 70% of CK remained with the inner membrane particles even under conditions which resulted in the release of the citric acid cycle enzymes from the matrix. Klingenberg & Pfaff (1966) and Scholte et al. (1973) reported solubilization of 80 and 100% of CK, respectively, by treatment of rat heart mitochondria with phosphate. It is to be noted that Klingenberg and co-workers reported a very high proportion (55%) of the total cellular enzyme in the mitochondria (Jacobs et al., 1964), indicating that their preparation might have contained excessive amounts of cytoplasmic CK (Figure 6A) which led them to propose that the enzyme was located in the intermembrane space. This is not supported by any other submitochondrial fractionation studies. Scholte et al. (1973) employed treatment with trypsin to separate the inner and outer membranes of rat heart mitochondria which could have compromised the integrity of the membrane system. In fact, when more suitable procedures for the separation of the membranes were used, Sholte found that phosphate released less than 20% of the mitochondrial CK [see Scholte (1973b)] and further that its partition pattern was identical with that of cytochrome *c* oxidase which is an inner membrane enzyme.

The nature of CK forms that are in association with the inner membrane was investigated by releasing this pool by the use of detergents and separating the isoenzymes by electrophoresis. The results of such experiments (Figure 5, lane C; fraction IV, Table IV) demonstrated the presence of the basic form of the enzyme as well as the cytoplasmic form in approximately equimolar proportion. From these results we infer that the major portion of the MT CK imbedded in the inner membrane exists as a complex with the MM form. The MT–MM complex formation is also supported by the results of ion-exchange chromatography on DEAE-cellulose where only a portion of MT CK was eluted as a free enzyme (peak I of Figure 4). The major active peak (peak II of Figure 4) contained some MT CK along with MM CK.

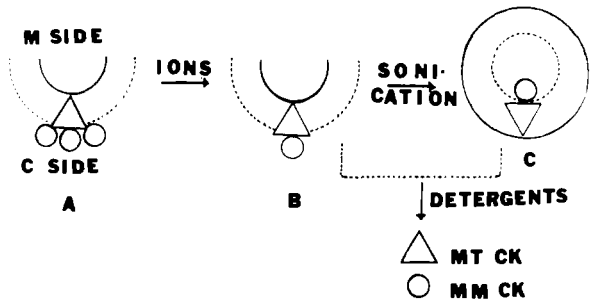

FIGURE 6: Schematic diagram of the association of the MT and MM CK with the mitochondrial inner membrane. A = inner membrane lipid bilayer with loosely associated MM CK along with strongly associated MT CK; B = MT–MM complex after salt wash; C = inside out vesicle produced by sonication. C side and M side represent the cytoplasmic and matrix sides, respectively.

Recovery of more than 100% of the activity in the total sonicates indicates that there is no encapsulation of the enzyme within the "inside out" vesicles and further that some enzyme is freed from the constraints imposed by the membrane (this conclusion is further supported by Lubrol activation). The instantaneous response of the sonic particles to PCr coupled with the fact that the inner mitochondrial membrane is impermeable to ionic compounds without specific transport systems (Chappell & Haarhoff, 1967; Klingenberg, 1970) indicates that the enzyme is available for interaction with the guanidinium substrates (Cr and PCr) in both the "inside out" and the "right side out" membranes.

Lubrol is known to activate enzymes that are embedded in the inner mitochondrial membrane as well as those in the matrix (Schnaitman & Greenawalt, 1968). The maximal effect of Lubrol was found with the mitoplast (Figure 3); some preparations showed a twofold activation after Lubrol treatment. The detergent produced a lesser degree of activation of whole mitochondria and of the submitochondrial particles. Scholte et al. (1973) found a significant degree of activation by Lubrol of rat heart CK in submitochondrial particles (sonic) but interpreted their results as being due to the production of 23% of inside out vesicles by sonication which trapped the enzyme in an unavailable form. Our results with SMP (total yield and accessibility of enzyme to PCr) do not support such an interpretation. The yield of only 23% of everted membrane particles in their preparations is at variance with results presented here as well as with other published work (Racker, 1962; Hackenbrock & Hammon, 1975; Klingenberg, 1977).

From the results presented here we propose a model for the mode of interaction of creatine kinase with the inner mitochondrial membrane in beef heart schematically represented in Figure 6. The basic MT CK is postulated to span the inner membrane, and on the cytoplasmic side it is postulated to associate with two or more molecules of the cytoplasmic MM (Figure 6A), of which one molecule is firmly bound, leading to the 1:1 complex (Figure 6B) when the organelles are washed especially with electrolytes (phosphate and KCl). The everted membrane would produce the structure shown in Figure 6C

in which MT CK is exposed on the cytoplasmic side. Nonionic detergents which disrupt the inner membrane relax the protein–lipid interaction and remove any constraint on the MT.

This proposed model takes into account in addition to our results the following facts. (1) The utilization of basic proteins of relatively low hydrophobicity for insertion into the mitochondrial inner membrane seems to be an important physiological phenomenon in the evolution of mammalian energy transducing systems. The ADP–ATP porter (p*I* of 10.3; Klingenberg et al., 1978), subunit 5 of ATPase (p*I* 10.4; Knowles & Penefsky, 1972), and subunits VI and VII of cytochrome *c* oxidase (p*I* values of 9.2 and 9.5, respectively; Freedman et al., 1979) are examples. (2) The wide variation in the estimates of the mitochondrial CK content is explained by the variable degree of association of the MT CK with the cytoplasmic component. (3) The preferential utilization of matrix ATP for the synthesis of PCr observed in Bessman's laboratory (Yang et al., 1977) can take place without the mediation of the ATP–ADP porter. The observations of Jacobus & Lehninger (1973) that MT CK activity is unaffected by the inhibition of the ATP–ADP porter by atractyloside are in agreement with this conclusion. (4) The contradictory results reported on the ability of DNFB, a proven inhibitor of the MM form of CK, to inhibit totally the mitochondrial CK activity are reconciled by the model. The system in Figure 6A in which MM CK completely blankets the MT component would be totally inhibited by DNFB (Yang & Dubick, 1977) whereas in the state represented by Figure 6B the MT CK can still be active (Sobel et al., 1972). (5) The high CK activity necessitated for the rapid transport of the metabolic energy by the scheme postulated by Saks et al. (1977) is possible by mobilization of the pool of the cytoplasmic CK. (6) Finally, the myocardium and the brain depend for their survival on constant, high uninterrupted mitochondrial respiration. Conservation of intramatrix ADP was suggested by Jacobus & Lehninger (1973) as the major function of the mitochondrial CK. Our model allows a mechanism by which a high level of ADP is maintained on the matrix side whereas the nucleotide translocase system necessitates the cycling of the adenine nucleotides in and out of the matrix.

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References

Addink, A. D. F., Boer, P., Wakabayashi, T., & Green, D. E. (1972) *Eur. J. Biochem. 29*, 47–59.

Boxer, D. H., Feckl, J., & Klingenberg, M. (1977) *FEBS Lett. 13*, 43–46.

Chappell, J. B., & Haarhoff, K. N. (1967) in *Biochemistry of Mitochondria* (Slater, E. C., Kaniuga, Z., & Wojtczak, L., Eds.) pp 75–91, Academic Press, New York.

Clarke, S. (1976) *J. Biol. Chem. 251*, 950–961.

Farrell, E. C., Baba, N., Brierley, G. P., & Grumer, H. D. (1972) *Lab. Invest. 27*, 209–213.

Freedman, J. A., Tracy, R. P., & Chain, S. H. P. (1979) *J. Biol. Chem. 254*, 4305–4308.

Hackenbrock, C. R., & Hammon, K. M. (1975) *J. Biol. Chem. 250*, 9185–9197.

Hall, N., Addis, P., & Deluca, M. (1979) *Biochemistry 18*, 1745–1751.

Hayat, M. A. (1970) *Princ. Tech. Electron Microsc. 1*, 262.

Jacobs, H. K., & Graham, M. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 1574.

Jacobs, H., Heldt, H. W., & Klingenberg, M. (1964) *Biochem. Biophys. Res. Commun. 16*, 516–521.

Jacobus, W. E., & Lehninger, A. L. (1973) *J. Biol. Chem. 248*, 4803–4810.

Klingenberg, M. (1970) *FEBS Lett. 6*, 145–154.

Klingenberg, M. (1977) *Eur. J. Biochem. 76*, 553–565.

Klingenberg, M., & Pfaff, E. (1966) *Regul. Metab. Processes Mitochondria, Proc. Symp., 1965 17*, 180–201.

Klingenberg, M., Riccio, P., & Aquila, H. (1978) *Biochim. Biophys. Acta 503*, 193–210.

Knowles, A., & Penefsky, H. S. (1972) *J. Biol. Chem. 247*, 6617–6623.

Krebs, J. J. R., Hauser, H., & Carafoli, E. (1979) *J. Biol. Chem. 254*, 5308–5316.

Lienhard, G. E., & Secemski, I. I. (1973) *J. Biol. Chem. 248*, 1121–1123.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Oliver, I. T. (1955) *Biochem. J. 61*, 116–122.

Racker, E. (1962) *Proc. Natl. Acad. Sci. U.S.A. 48*, 1659–1663.

Rosalki, S. B. (1965) *Nature (London) 207*, 414.

Saks, V. A., Seppet, E. K., & Lyulina, N. V. (1977) *Biokhimiya 42*, 445–452.

Schnaitman, C., & Greenawalt, J. W. (1968) *J. Cell Biol. 38*, 158–175.

Scholte, H. R. (1973a) *Biochim. Biophys. Acta 305*, 413–427.

Scholte, H. R. (1973b) *Biochim. Biophys. Acta 330*, 283–293.

Scholte, H. R., Weijers, P. J., & Wit-Peeters, E. M. (1973) *Biochim. Biophys. Acta 291*, 764–773.

Sobel, B. E., Shell, W. E., & Klein, M. S. (1972) *J. Mol. Cell. Cardiol. 4*, 367–380.

Watts, D. C. (1973) *Enzymes, 3rd Ed. 3*, 324–451.

Yang, W. C. T., & Dubick, M. (1977) *Life Sci. 21*, 1171–1178.

Yang, W. C. T., Geiger, P. J., & Bessman, S. P. (1977) *Biochem. Biophys. Res. Commun. 76*, 882–887.

Zimmerman, U. J. P., Iyengar, C. W. L., & Iyenger, M. R. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 339.