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Mitochondrial Creatine Kinase. Physical and Kinetic Properties of the Purified Enzyme from Beef Heart†

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ABSTRACT: An improved method for the purification of the mitochondrial isozyme of creatine kinase is reported. Studies of the properties of the purified enzyme from beef heart reveal pH optima of 8 and 6–6.5 for the forward and reverse reactions, respectively. The apparent K_m values for Mg-ATP and creatine are 0.056 and 4.5 mM and for Mg-ADP and phosphocreatine are 0.015 and 0.31 mM. The smallest active form of the enzyme is a dimer of 64 000 daltons. Two active forms are found in solution, one with a slightly cathodal mobility on cellulose polyacetate strip electrophoresis and a molecular weight of about 64 000 and another with a greater cathodal mobility on electrophoresis and with a molecular weight at least three times larger. The two forms can be separated by molecular seive chromatography but interconvert in solution, with the extent of the conversion dependent upon protein concentration and on the presence of a reducing agent. Intermolecular hybrids between subunits of the heart mitochondrial isozyme and subunits of other beef creatine kinase isozymes were not formed upon disruption and reannealing. The results of these studies are consistent with the hypothesis that mitochondrial creatine kinase is especially adapted to the formation of phosphocreatine in the mitochondrion and may have a very different structure from that of the MM, MB, and BB isozymes.

The enzyme creatine kinase (EC 2.7.3.2), which catalyzes the transfer of phosphate from ATP to creatine to produce ADP and phosphocreatine, has long been assumed to be involved in the regeneration of ATP in association with contractile or transport systems (Kuby & Noltman, 1962; Watts, 1973). Several different isozymes of creatine kinase have been isolated from mammalian tissues. The best characterized of these are commonly referred to as BB (CK-1, brain), MB (CK-2, hybrid, present in mammalian heart), and MM (CK-3, muscle). These enzymes have different kinetic parameters (Eppenberger et al., 1967; Dawson et al., 1967) and different amino acid compositions (Watts, 1973) and can be separated electrophoretically at pH 8.8 (Burger et al., 1964; Eppenberger et al., 1964). Treatment of a mixture of the MM and BB types

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Table I: Apparent Michaelis Constants Reported for Heart Mitochondrial Creatine Kinase[a]

	ref	Mg-ATP	creatine	Mg-ADP	PCr
intact pig heart mitochondria	Vial et al., 1972	0.35	10		
phosphate extract of rat heart mitochondria	Scholte, 1973		13		1.0
intact rat heart mitochondria	Jacobus & Lehninger, 1973	0.10	6	0.035	0.72
intact rat heart mitochondria	Saks et al., 1975	0.73	5.0	0.05	0.5
purified beef heart mitochondrial isozyme	this study	0.056	4.5	0.015	0.31

[a] K_m values are reported in millimoles per liter. In all cases values reflect the binding of one substrate in the presence of the other substrate in excess and therefore are the values for the last addition to the ternary complex.

with 6 M guanidine, followed by dialysis, results in the formation of the hybrid MB isozyme in vitro, demonstrating that these three forms are all dimers (Dawson et al., 1965). The molecular weight of rabbit MM is 82600 (Olson & Kuby, 1964), and experimentally determined values for other purified creatine kinases fall within the range 78 500–85 100 (Watts, 1973).

In 1964, Jacobs et al. reported the existence of another isozyme of creatine kinase associated with mitochondria isolated from the heart and brain of rats. This isozyme is separable from the other creatine kinases by electrophoresis and moves toward the cathode at pH 8.8, while the other isozymes move toward the anode (Sobel et al., 1972). Farrell et al. (1972) demonstrated that this isozyme could be extracted from beef heart mitochondria in sodium phosphate buffer. They isolated a form of the enzyme by centrifugation and estimated its molecular weight to be about 250000.

The affinities of the mitochondrial creatine kinase for each of its four substrates have been reported by several investigators (Table I), but in all cases these measurements have been made with intact mitochondria rather than the purified enzyme. Purification has proved to be difficult, chiefly due to the instability of the enzyme after extraction from the mitochondria (Farrell et al., 1972; Jacobs, 1974; Dmitrenko et al., 1975). We have recently reported a purification procedure (Hall et al., 1977) which results in a mixture of two interconvertible active forms of the creatine kinase from beef heart mitochondria. These appear to be an oxidized and reduced form of the enzyme, since the form of the enzyme which upon electrophoresis migrates more rapidly toward the cathode (m-2)[1] is slowly converted to the slower migrating form (m-1) upon incubation with high concentrations of β-mercaptoethanol or dithiothreitol.

This paper describes an improved purification procedure, including preparative separation of the two forms on the basis of molecular weight. The apparent K_m for each of the four substrates is reported as well as other kinetic properties. The molecular weights of the two forms were determined, and the amino acid composition is reported.

Materials and Methods

Purification of the Enzyme. The enzyme used in the experiments reported here was isolated by two methods, the first of which has been previously reported (Hall et al., 1977). This method produced enzyme with a specific activity of 111 units/mg, 37% m-1, and 63% m-2. Although it is possible to produce m-1 in its pure form by the method described in this paper, the specific activity is not significantly different, and the enzyme does not remain the pure m-1 form at high concentrations. For this reason, the kinetic studies were peformed by using the mixture of m-1 and m-2.

Assay Procedure. Routine assays for creatine kinase are carried out at 30 °C with the coupled reaction (Rosalki, 1967) by using Calbiochem CPK-Max Pak or CPK-Single Vial Reagent. Fractions from the agarose–ATP column which contain ATP must be diluted by at least a factor of 100, with the slope taken after the completion of the initial nonlinear reaction of the ATP in the sample.

In the kinetic studies, a potentiometric (pH-stat) procedure was employed by utilizing a Titrigraph SBR 2c, Titrator TTT 11, and pH meter 26 (Radiometer). The 8-mL reaction volume was magnetically stirred at room temperature. In addition to the substrates indicated in each experiment, the reaction mixture contained 1 mg/mL bovine serum albumin, 1 mM dithiothreitol, and, except where otherwise indicated, magnesium acetate in 1 mM excess of the concentration of ADP or ATP. Titration was with NaOH at 0.010 N in the case of the forward reaction (creatine and ATP as substrates) and with HCl at about the same concentration (0.0084 N) in the case of the reverse reaction (phosphocreatine and ADP as substrates). Full recorder scale (100%) is equivalent to 0.849 ± 0.001 mL so that at these concentrations, 1% full scale is equivalent to 0.085 μmol equiv of NaOH or 0.071 μmol equiv of HCl per 8-mL reaction mixture. Blanks were performed with and without substrates and were never more than 0.25%/min. Reaction rates were determined from the first 15% or less of titrant delivery, so that dilution error was less than 2% of the reaction volume. The reaction stoichiometry of a similar reaction mixture for the assay of rabbit MM has been reported to be 1.0 mol of ATP disappearing per mol of NaOH injected at pH 8.8 and 1.05 mol of ATP per mol of NaOH at pH 7.4 (Jacobs & Kuby, 1970). At pH 5.0 and 5.5 appropriate corrections were made for the fraction of H^+ produced or utilized.

Protein was assayed by the method of Bradford (1976) by using Coomassie Brilliant Blue G-250.

Electrophoresis. Separation and quantitation of creatine kinase isozymes on cellulose acetate strips were carried out as previously described (Hall & DeLuca, 1976).

Sephacryl Molecular Weight Studies. A 100 × 2.4 cm column of Sephacryl S-200 (Pharmacia), prepared in 20 mM sodium phosphate, pH 8.8, 10 mM β-mercaptoethanol, and 1 mM EDTA, was loaded with a 1-mL sample containing approximately 10 mg each of ovalbumin, ribonuclease, and chymotrypsinogen and 50 units of lactate dehydrogenase. The sample also contained about 30 units of either rabbit skeletal muscle creatine kinase (MM) or beef heart mitochondrial creatine kinase from the agarose–ATP affinity column (line 5 of Table II) or a mixture of the two. Fractions of about 1.4 mL were taken, OD_{280} was recorded, creatine kinase was assayed as described, and lactate dehydrogenase was assayed with a reagent consisting of 5.0×10^{-4} M sodium pyruvate and 0.1 mg/mL NADH in 0.1 M potassium phosphate, pH 7.5, by following the change in OD_{340} at 30 °C. Void volume was determined with 5–10-mg samples of blue dextran (av-

[1] Abbreviations used: m-1, the form of the mitochondrial isozyme of creatine kinase which on electrophoresis remains nearer to the origin; m-2, the form of the mitochondrial isozyme which moves farther toward the cathode; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Cr, creatine; PCr, phosphocreatine.

Table II: Purification of Mitochondrial Creatine Kinase from Beef Heart[a]

	total protein (mg)	electrophoresis, % CK act. m-2 (fast)	electrophoresis, % CK act. m-1 (slow)	total mitochondrial CK act. (IU)	mitochondrial CK sp act. (IU/mg)
(1) extract	946	62.4	37.6	2169	2.29
(2) ammonium sulfate fractionation, 35-40%	76.9	55.5	45.5	660	8.58
(3) dialysis and centrifugation: sedimented fraction	32.8	90.3	9.7	668	20.3
(4) DEAE-Sephacel chromatography	12.1	14.0	86.0	727	60.2
(5) agarose-ATP chromatography	4.81	36.8	63.2	461	96.3
(6) Sephacryl S-200 chromatography					
(a) first peak	3.00	84.5	15.5	290	96.7
(b) second peak	0.21	12.8	87.2	23.3	113.2

[a] In steps 1-4, some of the enzyme had the electrophoretic mobility characteristic of MM and some remained at the origin. These activities were subtracted from the total measured activity shown in these steps. The extract was from 63 g of mitochondria, wet weight.

erage molecular weight 2000000) after all the fractionations had been completed. This was necessary because the mitochondrial creatine kinase binds to the blue dextran.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis was performed by a modification of the procedure of Weber & Osborn (1969) which has been previously reported (Hall et al., 1977). The subunit molecular weight of the mitochondrial enzyme was estimated by comparing the relative mobilities of the purified enzyme and the proteins of the Pharmacia Low Molecular Weight Calibration Kit (rabbit phosphorylase *b*, bovine serum albumin, ovalbumin, bovine carbonic anhydrase, soy trypsin inhibitor, and α-lactalbumin).

Amino Acid Composition. Samples for amino acid analysis were hydrolyzed in vacuo in 6 N HCl for 24, 48, and 72 h and were analyzed according to the method of Spackman et al. (1958) by using a D 500 AA analyzer (Durrum Instruments).

Results and Discussion

Purification of the Enzyme. The results of a typical purification of mitochondrial creatine kinase are given in Table II. Beef heart mitochondria are isolated in 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA, and 1 mM β-mercaptoethanol. The mitochondria are washed three times in the same solution. They are suspended in 100 mM sodium phosphate, pH 8.0, containing 1 mM EDTA and 10 mM β-mercaptoethanol in 1 mL/g wet weight of mitochondria. The suspension is stirred gently at 40 °C overnight. Mitochondria and debris are removed by centrifugation, and the supernatant which contains the creatine kinase is further fractionated with ammonium sulfate. Solid ammonium sulfate is added to give first 30% saturation, followed by increasing cuts at 5% intervals to 55% saturation. Each of the precipitates is suspended in 25 mL of 50 mM Tris-HCl, pH 8.0, containing 10 mM β-mercaptoethanol and 1 mM EDTA (Tris–β-mercaptoethanol–EDTA). The most active fractions, usually the 30–35% and 35–40%, are dialyzed against the same buffer. After dialysis the fractions are centrifuged at 8000*g* for 15 min, and the precipitates are dissolved in about 200 mL of Tris buffer.

About 100 mg of protein (500–2000 units of activity) is loaded onto a 50 × 2.4 cm column of DEAE-Sephacel (Pharmacia) which has been equilibrated with the previously described Tris–β-mercaptoethanol–EDTA buffer, pH 8.0. This is followed by 100 mL of buffer and then a gradient, 600-mL total volume, 0–0.6 M NaCl in Tris–β-mercaptoethanol–EDTA buffer. A peak of activity elutes at about 0.4 M NaCl and is essentially all mitochondrial creatine kinase as determined by electrophoresis but also contains a small amount of MM creatine kinase (Table II, line 4).

The pooled fractions from the DEAE-Sephacel columns are dialyzed against Tris buffer. If the volume is too large, it may be concentrated first by pressure ultrafiltration (Amicon UM-10 filter). Up to 1000 units are then loaded onto a 5-mL column of agarose–hexane–adenosine 5′-triphosphate (P-L Biochemicals, type 3) and eluted with a 100-mL gradient, 0–10 mM ATP and 0–1 M NaCl in 20 mM sodium phosphate, pH 8.0, 10 mM β-mercaptoethanol, and 1 mM EDTA. Two peaks of activity are usually eluted, the first small peak at $^1/_3$ of the gradient, containing mostly MM isozyme, and the major peak, which elutes at about $^2/_3$ of the gradient, containing only m-1 and m-2 creatine kinase (Table II, line 5). In earlier preparations reported previously (Hall et al., 1977), smaller amounts of enzyme were processed on the agarose–ATP column and were found to elute entirely as the m-1 form, but this has been found to be the case only if the eluted enzyme is below 10 units/mL.

The peak fractions from the agarose–ATP are concentrated by ultrafiltration to give a few milliliters at 200–300 units/mL. As previously noted (Hall et al., 1977), most of the isozyme is converted to the m-2 form by concentration. This sample is then placed on a 100 × 2.4 cm column of Sephacryl S-200 (Pharmacia) prepared in 20 mM sodium phosphate, pH 8.0, 10 mM β-mercaptoethanol, and 1 mM EDTA. When the enzyme was used for SH-group analysis, the β-mercaptoethanol was omitted. This column is eluted with the same buffer, and two peaks of activity are found, the first at about 170 mL of elution, containing 90% or more of the activity, with about 85% of that activity as the faster migrating m-2 and 15% as m-1 (Table II, line 6a). The second, smaller peak, at about 215 mL of elution, has most of its activity as m-1 (Table II, line 6b).

The specific activities of these two fractions do not differ significantly from one another (96.7 and 113.2 units/mg in the example in Table II) and are similar to that produced by the earlier method (111 units/mg). The advantage of the new method, besides the capability of producing samples enriched for the two different forms, is an improved efficiency of recovery (14.4 vs. 4.6%), which can be further improved by reprocessing rather than discarding the lower specific activity samples after ammonium sulfate fractionation and sedimentation.

pH Optima. To determine the pH optima for each reaction, the reaction mixture, containing 20 mM creatine and 2.0 mM ATP in the case of the forward reaction and 4.0 mM phosphocreatine and 0.4 mM ADP in the case of the reverse reaction, was adjusted near to the required pH, enzyme was added, and the pH-stat end point setting was quickly adjusted to the measured pH to begin the recording. The results, shown in Figure 1, reveal optima at about pH 8.0 for the forward

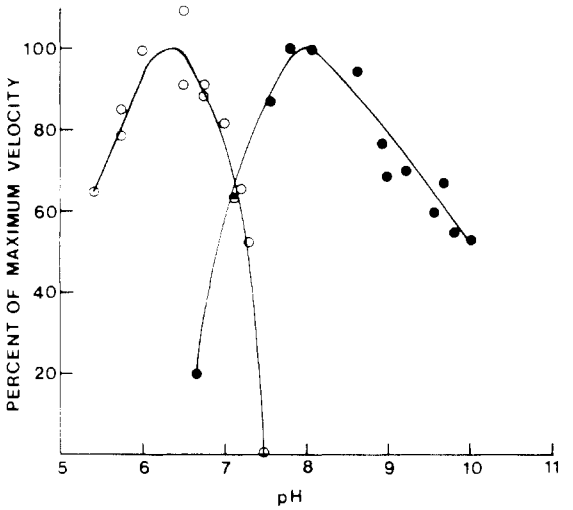

FIGURE 1: pH dependency of the forward (●) and reverse (○) reactions of mitochondrial creatine kinase in the pH-stat titrations. The values obtained at pH 5.0 and 5.5 have been corrected to account for the fact that less than 1 H^+ is released or consumed per ATP molecule (Alberty et al., 1951). Substrate concentrations are given in the text.

Table III: Relative Velocities of the Forward and Reverse Reactions[a]

	substrates (mM)					μmol of titrant
	ADP	PCr	ATP	Cr	pH	per min
beef MM			2.5	30.0	8.0	10
beef MM	0.25	10.0			6.0	41
beef mitochondrial			2.5	30.0	8.0	3.8
beef mitochondrial	0.25	1.0			6.0	4.2

[a] Assays were performed at room temperature (23 °C) in the pH-stat assay, by using 0.010 N NaOH and 0.0084 N HCl as titrants in the forward and reverse reactions, respectively.

reaction and at about pH 6.0–6.5 for the reverse reaction. The curves obtained are quite similar to those reported for intact mitochondria in coupled-enzyme assays (Jacobus & Lehninger, 1973), although the optima for the purified enzymes are lower by about 0.5–1.0 pH unit.

The relative rates of the forward and reverse reactions for the mitochondrial isozyme and for beef heart MM partially purified by DEAE chromatography (Witteveen et al., 1974) are shown in Table III. The conditions chosen are those found by us to be optimal for the mitochondrial isozyme and those reported by others to be optimal for MM isozymes (Witteveen et al., 1974; Eppenberger et al., 1967). The phosphocreatine concentrations are placed at 10 and 1 mM for the MM and mitochondrial isozymes, respectively, since substrate inhibition reduces the rate seen at higher concentrations. The observation that the maximal rate for the MM isozyme is four times greater in the direction of ATP formation, while the mitochondrial isozyme has approximately equal maximal rates in either direction, suggests that the latter is better adapted to the formation of phosphocreatine, while the MM isozyme is better adapted to the formation of ATP.

Kinetics. Measurements of the initial reaction velocities as a function of one substrate concentration, with the other held constant, result in double-reciprocal plots which are linear. (Figure 2A–D). At concentrations of phosphocreatine higher than 1 mM, some substrate inhibition is seen (Figure 2C), similar to that seen with cytoplasmic enzymes at concentrations higher than 10 mM (Dawson, 1970; Witteveen et al., 1974).

The fixed substrate levels are high enough that the measured

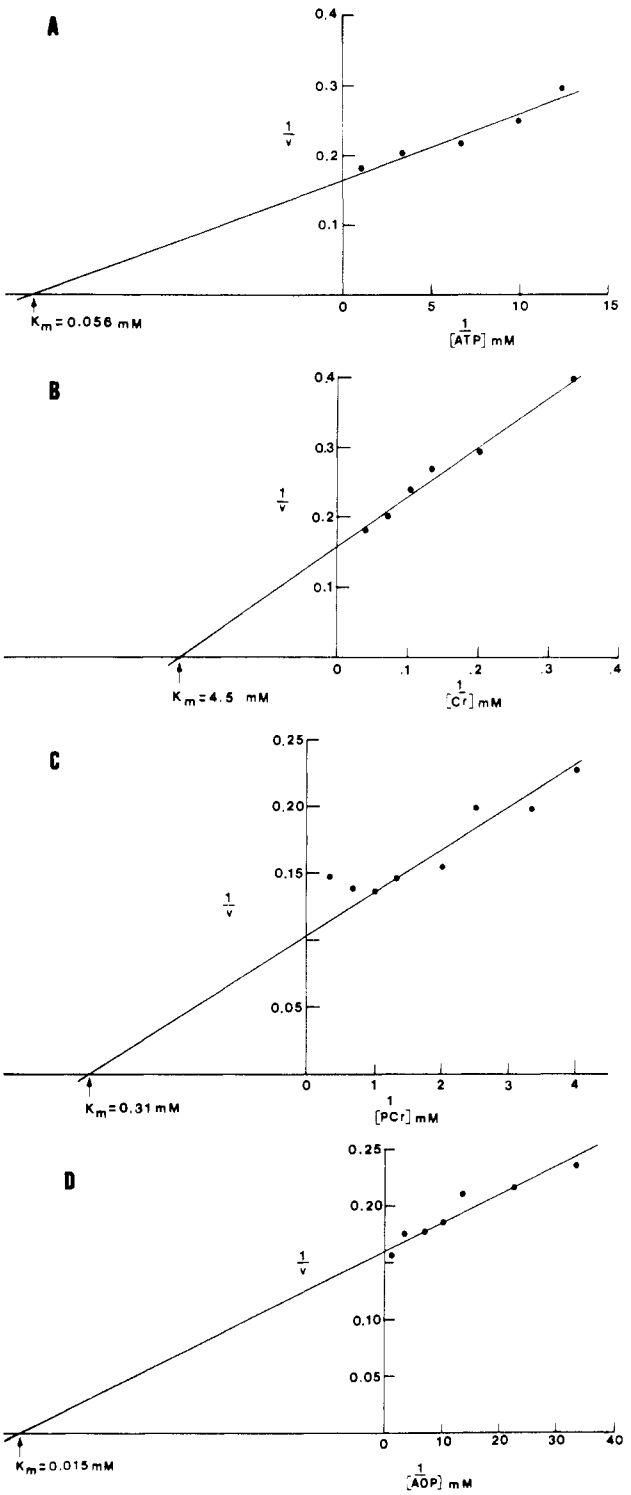

FIGURE 2: Lineweaver–Burk plots of the reactions of mitochondrial creatine kinase with (A) ATP (creatine = 30.0 mM), (B) creatine (ATP = 1.0 mM), (C) phosphocreatine (ADP = 1.0 mM), and (D) ADP (phosphocreatine = 3.0 mM). The pH-stat end point was 8.0 for the forward reaction (A and B) and 6.0 for the reverse (C and D). The reaction velocities are expressed as percent full scale on the titrigraph (see Materials and Methods). Each point is the mean of four determinations, and the K_m values are calculated for the least-squares best fit line.

apparent Michalis constants are those for the addition of the second substrate to form the ternary complex (Saks et al., 1975). The concentration of magnesium acetate was always maintained in 1 mM excess of the ATP or ADP concentrations, so that these should be present principally as Mg-ATP and Mg-ADP. The reactions in which ADP was the variable

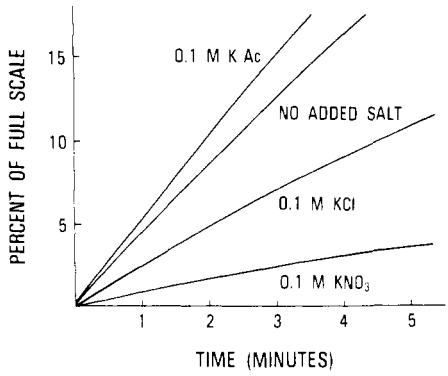

FIGURE 3: Titrigraph reaction curves for the forward reaction in the presence of KOAc, KCl, and KNO_3. Substrates were 2 mM ATP and 30 mM creatine.

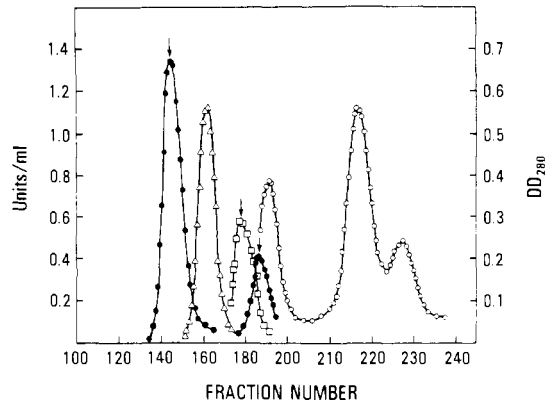

FIGURE 4: Separation of the mitochondrial creatine kinase m-1 and m-2 forms from proteins of known molecular weight on Sephacryl S-200. Fractions with creatine kinase activity were subjected to cellulose acetate strip electrophoresis to determine the distribution of the isozymes. The arrows indicate, from left to right, the peak fractions of the creatine kinase m-2, MM, and m-1 isozymes. The symbols indicate mitochondrial m-1 or m-2 creatine kinase (●), MM creatine kinase (□), lactate dehydrogenase (△), and OD_{280} (○). The peaks represented by OD_{280} are, from left to right, ovalbumin, chymotrypsinogen A, and ribonuclease A.

substrate (Figure 2D) were repeated with $Mg(OAc)_2$ at 5 mM with no significant change in the results. The K_m values (Table I) are all lower than those reported for the enzyme in situ in the mitochondria. Because of the wide separation of the pH optima for the two directions of the reaction and the fact that very little overlap in the pH-dependence curves was found, no attempt was made to measure the K_m values at the same pH, but rather, each was measured at the optimum pH for its respective reaction. Even under such optimal conditions for the reverse reaction, with the pH more than 1 unit below physiological pH, the measured K_m for ADP (1.5×10^{-5} M) is still significantly higher than the reported K_m for the adenine nucleotide translocator (1.7×10^{-6} M) (Vignais, 1976). At pH 8.0, the K_m for ATP (5.6×10^{-5} M) is well below that for the translocator under energy-rich conditions (1.8×10^{-4} M) (Souverijn et al., 1973). This supports the suggestion (Jacobs et al., 1964; Jacobus & Lehninger, 1973) that most if not all of the ATP exported from the matrix of the mitochondria by the translocator may immediately be taken up by the mitochondrial creatine kinase and that the creatine phosphate then formed may serve as the main high-energy phosphate product of heart mitochondria and serve to "shuttle" energy to sites of utilization for muscle contraction or for ion transport (Saks et al., 1977).

Analysis of the kinetics of the reaction in mitochondria under conditions of oxidative phosphorylation has led Saks et al. (1975) to suggest an intimate functional interaction of the mitochondrial creatine kinase with the ATP–ADP translocase of the inner membrane. In support of this are reports of Saks et al. (1976) and Yang et al. (1977) that the reaction of the membrane-bound enzyme with ATP supplied to the medium is less efficient than that with ATP synthesized by the oxidative phosphorylation of the mitochondria. Contrary results, suggesting that the ATP from the mitochondrial matrix has no privileged access to the creatine kinase, are reported by Altschuld & Brierley (1977).

The mitochondrial enzyme was found to be sensitive to the presence of chloride and nitrate anions in a manner similar to that of rabbit muscle creatine kinase (Milner-White & Watts, 1971). In contrast to the near-linear progress curve for the forward reaction in the presence of acetate (Figure 3), Cl^- and NO_3^- produce both an inhibition of the initial rate and a marked curvature of the progress curves of the pH-stat reaction. Such inhibition has been interpreted in the case of MM to be due to the binding of anions to the binding site for the transferable phosphoryl group, thus competitively inhibiting ATP binding and also stabilizing the nonreactive ternary complex of the enzyme with the creatine substrate and ADP produced by the forward reaction. The increased rate with potassium acetate over the control to which no salt is added may be due to increased ionic strength or may be some more specific effect of the acetate ion.

Molecular Weight Studies. Figure 4 shows the separation of the mitochondrial creatine kinase m-1 and m-2 forms from the other proteins, including rabbit MM creatine kinase, on a Sephacryl S-200 column. In the example shown, both the MM and mitochondrial isozymes were placed on the column, and the individual fractions were subjected to cellulose acetate strip electrophoresis to determine the relative quantities of the two isozymes. The m-2 creatine kinase isozyme elutes very close to the void volume, well ahead of lactate dehydrogenase. The m-1 is seen to elute between the rabbit MM and the ovalbumin, which have molecular weights of 83 000 and 45 000, respectively. Plots of the K_{av} values for three determinations on all of the proteins reveal an estimated molecular weight of 65 000 ± 2000 for the m-1 and 180 000–190 000 for the m-2. Since the latter form falls outside the molecular weights of the markers, the error may be greater than the range indicates, but it is clear that it is at least three times the size of the lower molecular weight form. Deviation of the larger m-2 form from a globular shape may be another source of error in the estimation of its molecular weight.

$NaDodSO_4$–polyacrylamide gels of the preparations from the agarose–ATP column, as well as those from the Sephacryl column, show only one band of protein. The subunit molecular weight was estimated from the plots of relative mobilities (R_f) of this band and of a standard protein mixture. Three such determinations gave a value of 35 000 daltons ± 4000. This strongly suggests that the lowest molecular weight form which shows enzymatic activity (m-1) is a dimer of about 64 000 made up of subunits of 32 000.

Hybridization. The cytoplasmic creatine kinase isozymes are known to be dimers of identical subunits (MM and BB) or hybrid dimers (MB) with one subunit of each kind (Dawson et al., 1965). Hybrids can be formed in vitro by disrupting and reannealing isozymes of differing electrophoretic mobilities isolated from different tissues or even from different species (Keutel et al., 1972). Attempts were made, therefore, to form hybrids in vitro between beef heart mitochondrial isozyme and beef MM, MB, and BB.

Mitochondrial isozymes, beef heart MM and MB partially purified by DEAE-Sephadex chromatography, and crude beef

Table IV: Amino Acid Composition of Bovine Creatine Kinases

mitochondrial[a]	MM (Thomson et al., 1968)	BB (Thomson et al., 1968)	mitochondrial[a]	MM (Thomson et al., 1968)	BB (Thomson et al., 1968)
Asp[b] 60	Asp 81	Asp 89	Met 10	Met 18	Met 19
Thr 26	Thr 30	Thr 38	Ile 32	Ile 30	Ile 30
Ser 32	Ser 35	Ser 43	Leu 48	Leu 68	Leu 79
Glu[b] 62	Glu 79	Glu 94	Tyr 16	Tyr 16	Tyr 20
Pro 34	Pro 39	Pro 52	Phe 18	Phe 32	Phe 32
Gly 52	Gly 65	Gly 69	Lys 34	Lys 65	Lys 48
Ala 28	Ala 38	Ala 55	His 14	His 29	His 25
Cys[c] 4	Cys	Cys	Arg 46	Arg 34	Arg 42
Val 40	Val 54	Val 50			

[a] Composition of the purified mitochondrial creatine kinase from beef heart is given in moles of amino acid residue per mole of protein, by assuming a molecular weight of 64 000. Tryptophan content was not analyzed. [b] Asp and Glu include Asn and Gln residues, respectively. [c] Cys was determined by Nbs_2 titration (see text).

brain extract were treated with 6.5 M guanidine and 0.1 M β-mercaptoethanol on ice for 1 h, by which time 90–95% of the activity had been lost. Samples were then dialyzed, singly and in mixture, against 0.025 M Tris-HCl, pH 7.5, and 1 mM β-mercaptoethanol, after which 30–35% of the original activity had been regained. The treated samples and the original untreated samples were then subjected to cellulose acetate strip electrophoresis. Although hybrids between MM and BB were formed and the MB subunits redistributed to form MM, MB, and BB isozymes, no bands were seen with electrophoretic mobilities intermediate between the mitochondrial forms and the MM or BB. Since hybrids can be formed between subunits of cytoplasmic creatine kinases from diverse species and even between mammalian creatine kinase and invertebrate arginine kinase (Watts et al., 1972), the absence of such an interaction with the mitochondrial isozyme may indicate a very different quaternary structure and perhaps a very long independent evolutionary history.

Determination of Free -SH Groups. Samples of the enzyme which had been prepared on a Sephacryl column with no β-mercaptoethanol or other reducing agent present were reacted with excess Nbs_2 in order to estimate the number of free sulfhydryl groups (Ellman, 1959). Three determinations on samples containing 85% m-2 and 15% m-1, which had been denatured in 5 M guanidine, and one determination on the native protein all gave values between 1.5 and 2.5 mol of -SH per mol of 32 000 molecular weight subunit. Because of the low protein concentration of the sample, the one determination which was obtained for the m-1 form cannot be considered as reliable, but it was higher than those for the m-2, as would be expected if the higher molecular weight m-2 is formed by intersubunit disulfide bonds. Jacobus & Lehninger (1973) reported that the rat heart mitochondrial creatine kinase is severely inhibited by *p*-(chloromercuri)benzenesulfonate and by mersalyl.

Stability. When samples enriched for the m-2 form were stored in 20 mM sodium phosphate buffer, pH 8.0, at 4 °C, they retained at least 90% of their activity after 2 weeks. The composition changed, however, with m-1 appearing as m-2 disappeared. This process was more rapid in the presence of 100 mM dithiothreitol, the amount of m-1 doubling in 1 week from 15 to 30% of the total in the presence of DTT and rising to only 20% in its absence. The initial rate of conversion was the same for samples with a 10-fold difference in protein concentration, but the total extent was slightly greater after 2 weeks for more dilute samples, consistent with the hypothesis that higher concentrations favor formation of the m-2 form from the m-1.

Amino Acid Composition. Amino acid composition of the purified enzyme (line 6a in Table II) normalized to an estimated molecular weight of 64 000 is given in Table IV. We have included the amino acid composition of bovine MM and BB creatine kinase for comparison. The value given for cysteine is the average result of the Nbs_2 titrations (2.0 mol/mol of subunit). Values for threonine, serine, valine, and isoleucine are corrected for destruction during hydrolysis and for incomplete hydrolysis (Reeck, 1970). Analysis for tryptophan was not done. The distribution of amino acids in the analysis compares well with that reported by Jacobs & Graham (1978), although their distribution was normalized to a subunit molecular weight of 44 000, which was determined by equilibrium sedimentation in 8 M urea. The reason for the discrepancy between our subunit molecular weight (32 000) and that of Jacobs and Graham (44 000) is not known.

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Formation of an Intermediate and Its Rate of Conversion to Pyruvate during the Tryptophanase-Catalyzed Degradation of *S*-*o*-Nitrophenyl-L-cysteine†

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ABSTRACT: The tryptophanase-catalyzed degradation of *S*-*o*-nitrophenyl-L-cysteine (SOPC) to *o*-nitrothiophenol, pyruvate, and ammonia was examined by noting changes in SOPC directly at 330 nm or by coupling the appearance of pyruvate to the oxidation of NADH catalyzed by lactic dehydrogenase. A discrepancy between these two rates suggested the appearance of a solution intermediate which was subsequently trapped by reduction with NaB^3H_4. The resulting [^{3}H]alanine was shown to be racemic by oxidative deamination with D-amino acid oxidase and L-glutamic dehydrogenase. When 200–400 μg of tryptophanase was used with 163–650 μg of lactic dehydrogenase in a coupled reaction mixture, the appearance of pyruvate followed first-order kinetics with the same first-order rate constant, $k = 27.1 \pm 1.08$ min^{-1} at 30 °C. Therefore, the rate-limiting step under these conditions is the nonenzymatic conversion of an intermediate to pyruvate. On the basis of these data and the experiments of Vederas et al. (1978) [Vederas, J. C., Schleicher, E., Tsai, M.-D., & Floss, H. G. (1978) *J. Biol. Chem. 253*, 5350–5354] which showed that the β carbon of pyruvate was stereospecifically protonated during the tryptophanase catalyzed degradation of tryptophan, it is concluded that α-iminopropionate or the carbinolamine of pyruvate and ammoia together with *o*-nitrothiophenol are the immediate products of the tryptophanase-catalyzed degradation of SOPC. The intermediate undergoes a measurable rate of conversion to pyruvate and ammonia.

Tryptophanase catalyzes a variety of reversible α,β-elimination reactions and β-replacement reactions as reviewed by Snell (1975). The reaction involves the enzymatic labilization of the α proton of the Schiff base complex of the amino acid and pyridoxal phosphate with elimination of the β substituent. The resulting aminoacrylate complex can be hydrolyzed or nucleophilic addition can result in β substitution. The reverse reaction appears to involve initial attack by ammonia on the coenzyme followed by addition of pyruvate to form the same aminoacrylate intermediate.

Since the degradation of tryptophan proceeds at a rate comparable to that of the reverse reaction (Watanabe & Snell, 1972), Snell (1975) suggested that the mechanism of degradation was likely the same as the synthesis reaction and, therefore, the hydrolysis of the aminoacrylate intermediate in the degradative pathway was enzyme catalyzed. This suggestion was recently supported by Vederas et al. (1978) when

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