Biochimica et Biophysica Acta, 1061 (1991) 215–225
 0005-2736/91/$03.50
ADONIS 0005273691000697

BBAMEM 75109

Location and regulation of octameric mitochondrial creatine kinase in the contact sites

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(Received 19 June 1990)
(Revised manuscript received 28 September 1990)

Key words: Creatine kinase; Mitochondrion; Oligomeric structure; (Rat brain)

Differential extraction of creatine kinase activity (CK, EC 2.7.3.2) from rat brain mitochondria by graded concentrations of digitonin all yielded supernates varying in CK activity. As analyzed by isozyme electrophoresis and gel permeation chromatography the extracts contained different species of creatine kinase: (i) one third of the total CK activity consisting of contaminating cytosolic brain-type CK (B-CK) was liberated by 100 μg digitonin/mg of mitochondrial protein, (ii) approx. 20% more CK activity consisting of B-CK, as above, plus dimeric and octameric mitochondrial CK (Mi-CK), was extracted by 300 μg/mg digitonin, whereas (iii) all CK activity, consisting of B-CK and mainly octameric Mi-CK, were liberated by 700 μg/mg digitonin. In contrast to Mi-CK, B-CK associated with contaminating synaptic vesicles was readily extracted even by low concentrations of digitonin, but on the other hand octameric Mi-CK was significantly more resistant to digitonin extraction than the dimeric enzyme species. It appeared that the Mi-CK resistant to treatment with 300 μg/mg digitonin consisted to a large percentage of octamers and was organized as a complex between the two envelope membranes, for its activity was latent and still remained regulated by the outer membrane pore, that is: (i) the Mi-CK activity in such mitoplasts could be inhibited reversibly by cessation of the adenine nucleotide transport through the outer membrane pore with a polyanion, (ii) the ADP produced by Mi-CK in mitoplasts was not available to external pyruvate kinase, (iii) approx. 50% of total CK activity was not susceptible to inhibition by iodo acetate and phosphocreatine. In agreement with these findings a preferential association of octameric Mi-CK was also found in isolated contact site fractions indicating a physiological role of Mi-CK in energy transfer and a structure-function relationship of Mi-CK octamers at these sites [1]. In addition some evidence for an interaction of Mi-CK with the adenylate translocator is presented.

Introduction

The mitochondrial creatine kinase (Mi-CK), in contrast to the cytosolic isozyme, exhibits an association-dissociation equilibrium between an octameric and dimeric structure [2–6]. This property is conserved in different isoforms of the mitochondrial enzyme which were isolated from chicken brain and cardiac muscle [4]. Since the octamer/dimer equilibrium is sensitive to substrates of CK [4] it was concluded that the shift between the two oligomeric structures may be physiologically important in regulation of mitochondrial energy exchange although in vitro it did not change significantly the specific activity of the free enzyme [4,5].

Analysis of the enzyme activity in intact, well coupled rat brain mitochondria revealed that a significant portion (40–50%) of the Mi-CK activity was latent. Because this latent activity was neither liberated nor made fully accessible to substrates by digitonin, a location of this enzyme activity inside the contact sites was postulated [1,7]. In agreement with this, Mi-CK by biochemical methods was found to be concentrated in isolated contact sites from brain [8] and kidney mitochondria [1]. It was suggested by Schnyder et al. [5] that the octameric species of the Mi-CK would be able to interact concomitantly with the inner and outer membrane. Based on these data we asked the question of whether the two fractions of creatine kinases (i.e., the freely accessible and the latent Mi-CK activity) differ in

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accessibility to substrates and location relative to the contacts and whether they may represent the dimeric and octameric structure, respectively.

Material and Methods

Chemicals. Chemicals were all purchased from Boehringer-Mannheim and Merck-Darmstadt, West Germany.

Enzyme assays. Adenylate kinase (EC 2.7.4.3), hexokinase (EC 2.7.1.1) and lactate dehydrogenase (EC 1.1.1.27) were measured in agreement with Bücher et al. [9]. Creatine kinase (EC 2.7.3.2) was determined with creatine and ATP in a coupled enzyme assay using pyruvate kinase/lactate dehydrogenase as indicator reaction to measure the ADP production [9]. To determine maximal activity in extracts or gradient fractions the assay system was hypotonic and contained 50 mM triethanolamine (pH 7.6), 5 mM EDTA, 8 mM $MgCl_2$, 16 mM KCl. To analyze the enzyme under isotonic conditions the mitochondria were suspended in 0.3 M sucrose, 10 Hepes (pH 7.4), 8 mM $MgCl_2$, 5 mM succinate. Both assay systems contained 0.2 mg NADH/ml, 1 mM phospho*enol*pyruvate, 2,5 mM ATP, 60 μM rotenone, 30 μg/ml oligomycin, 0.2 IU pyruvate kinase, 0.5 IU lactate dehydrogenase (free of $(NH_4)_2SO_4$), the reaction was started with 20 mM creatine and the oxidation of NADH was followed in a double beam spectrophotometer at 340 nm.

Preparation of mitochondria from rat brain. Mitochondria from rat brain were prepared by differential centrifugation according to Rehncrona et al. [10] in a medium containing 0.25 M mannitol, 0.075 M sucrose, 1 mM EGTA, 5 mM Hepes (pH 7.4) and 0.1% fatty acid free bovine serum albumin. The mitochondria were further purified by 35 min centrifugation at 38000 rpm in a 60 Ti rotor (Beckman) on a 20% percoll gradient. Percoll was removed from the mitochondrial fraction by washing two times with the above medium.

Respiration measurements. Respiration was determined by a Clark type oxygen electrode at 22°C according to Estabroock [11].

Treatment of mitochondria with digitonin. Aliquots of 0.2 ml of the mitochondrial suspension, containing 10 to 20 mg protein per ml, were incubated for 30 s at room temperature with concentrations of digitonin ranging from 100 to 700 μg/mg of protein. Aliquots of the suspension were directly used for determination of activity of creatine kinase and adenylate kinase. This resulted in a dilution of the digitonin in the enzyme assay by a factor of 100.

Treatment of isolated mitochondria with the polyanion. The mitochondrial fraction, suspended in isolation medium, contained a protein concentration of approx. 20 mg/ml. Aliquots of 0.2 ml of this mitochondrial suspension were incubated in the presence of 5 mM $MgCl_2$ for 5 min at room temperature with concentrations of the polyanion ranging from 5 to 40 μg/ml. The polyanion is a copolymer of metacrylate, maleate and styrene in a 1:2:3 proportion with a molecular weight of 10000 [12]. The suspension was subsequently centrifuged for 1 min in a table-top centrifuge. The supernate was removed and the sediment was resuspended in the original volume of isolation medium.

Isozyme electrophoresis. The isozymes were separated on cellulose acetate strips according to the method of Marcillat et al. [2] using a buffer of 50 mM sodium barbital and 2.7 mM EDTA (pH 8.8). The cellulose acetate strips were soaked in the buffer for 20 min and were then blotted free of excess buffer with filter paper. The samples were applied in the middle of the strips and were run for 45 min at 3 mA in a Boskamp chamber. After electrophoresis the isozymes were visualized by incubation of the strips in a medium containing 0.6 M glycyl-glycine (pH 6.0), 0.04 M Mg-acetate, 12 mM creatine phosphate, 3 mM ADP, 1 mM NADP, 10 mM AMP, 0.5 mM nitroblue tetrazolium, 0.2 mM phenazine methosulfate, 20 mM glucose, 5 IU hexokinase and 3 IU glucose-6-phosphate dehydrogenase.

Gel permeation chromatography. Gel filtration experiments were performed on a FPLC Superose 12 column (2.2 × 80 cm) Pharmacia in 50 mM sodium phosphate, 0.2 mM EGTA, 2.0 mM 2-mercaptoethanol and 2.0 mM sodium azide at pH 7.2. Thyreoglobulin (molecular mass 670 kDa), immunoglobulin (158 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa) were used as molecular weight standards. The void volume was determined by Blue dextran 2000. Activity of creatine kinase in each fraction was determined as described above.

Assay of protein concentration. Protein was determined by the method of Lowry et al. [13].

Incubation with [^{3}H]atractyloside. Of the mitochondrial subfractions 1 mg each was incubated for 10 min at room temperature with 11 nmol of [^{3}H]atractyloside. The membranes were washed with sucrose medium by centrifugation and the bound radioactivity was determined in a liquid scintillation counter.

Results

Fractional extraction of creatine kinase form mitochondria by digitonin

Digitonin treatment preferentially removes those parts of the outer mitochondrial membrane which are not in the contact sites and thus allows insight into the organization of peripheral kinases at these sites [8,14]. We therefore, analyzed the composition of the supernate and the pellet fraction of enriched brain mitochondria preparation after treatment with digitonin (Fig. 1A,B). Incubation of these isolated rat brain mitochondria with digitonin resulted in a step-wise extrac-

tion of the creatine kinase activity. One third of the total CK activity was liberated by 100 μg digitonin per mg mitochondrial protein. Some 10% more of the total activity became extractable at 300 μg/mg and almost all activity could be desorbed from the sediment by 700 or more μg of digitonin/mg when, according to electron microscopic observations, also the inner membrane was starting to disintegrate. In contrast to this sequential release of CK activity, 85% of the total activity of adenylate kinase (Fig. 1A) became already extracted in the first step which liberated also 90% of the lactate dehydrogenase activity (Fig. 1B). The latter activity represents free cytosolic enzymes, and indicated the presence of contaminating synaptosoms as determined recently by electron microscopy [8]. The integral outer mitochondrial membrane proteins, porin and r.i. NADH oxidase, were solulilized only in part by digitonin treatment and were recovered mostly in the pellets (Fig. 1B) which can be explained by the fact that brain mitochondria exhibit higher amounts of contacts. Thus pieces of the outer membrane remain attached to the mitoplasts via contact sites and co-sediment with the latter. The almost complete extraction of lactate dehydrogenase by 100 μg/mg of digitonin suggested the disruption of synaptosomal membranes at this low digitonin concentrations. This interpretation was also supported by electrophoretic CK isozyme analysis of the different fractions (Fig. 1C) which showed that almost all of the cytosolic brain type isozyme of creatine kinase (B-CK) became extracted together with the activity of lactate dehydrogenase upon incubation with 100 μg of digitonin per mg of protein (Fig. 1C, S100). Under these very same conditions, however, nearly all of the mitochondrial isozymes remained bound in the sediment (Fig. 1C, P100). Parts of this activity, representing mainly dimer but also some octamer, were extracted in the next step by applying 300 μg/mg of digitonin (Fig. 1C, S300), while most of the octamer still remained bound in the membrane fraction (Fig. 1C, P300). On the whole, a rough separation of three different fractions of creatine kinase from isolated brain mitochondria was possible by increasing digitonin concentrations: the first fraction containing cytosolic isozyme (B-type) from contaminating synaptosoms, the second fraction containing Mi-CK mostly in dimeric form and a third fraction containing Mi-CK mainly as octamer left in the sediment. Thus, Mi-CK octamers showed a significantly higher resistance to digitonin extraction compared to synaptosomal B-CK or mitochondrial Mi-CK dimers. Digitonin did not effect the self-association of Mi-CK. This can be seen by comparing the isozyme electrophoresis of P 300 and S 700 in Fig. 1, both exhibiting a 20%/80% relation between dimer and octamer. Furthermore, extraction of the isolated contact sites without digitonin, described below, led to comparable results.

Analysis of the oligomeric state of Mi-CK by gel permeation chromatography in brain mitochondria extract

Brain mitochondria were pretreated with 100 μg digitonin/mg to remove B-CK. The mitoplast fraction, with outer membrane still adhering via contacts, was subsequently extracted by 20 mM alkaline phosphate buffer in the presence of 1.5% deoxycholate and *p*-aminobenzamidine. The latter reagent was shown recently [2,3] to prevent the establishment of a new association equilibrium between octamer and dimer upon solubilization of the mitochondria. The two oligomeric states of creatine kinase in the mitoplast extracts were analyzed by gel filtration on FPLC Superose 12 columns (Fig. 2). Two activity peaks were eluted from the column corresponding to the molecular mass of 80 and 350 kDa, respectively [4]. The activity ratio between the high molecular, octameric and the low molecular, dimeric species of creatine kinase present in the mitoplast extract was 76 to 88% for the octamer. As already observed by electrophoretic analysis (Fig. 1C) Mi-CK octamers from brain mitochondria can be almost completely dissociated into dimers by 3 M urea. By FPLC chromatographic analysis on Superose 12 of an urea-treated extract from brain mitoplasts we observed a dissociation of Mi-CK into 85% of the dimeric species whereas 80% of total Mi-CK in the untreated extract was of the octameric form (Fig. 2).

Characterization of Mi-CK in the isolated contact fraction from brain mitochondria

In a second set of experiments we isolated contact sites from osmotically disrupted brain mitochondria as described recently [1,8]. The CK activity was extracted by hypotonic alkaline phosphate buffer from the contact fraction in the presence of *p*-aminobenzamidine and the isozymes were analyzed by native electrophoresis (Fig. 3). In agreement with the experiments employing digitonin extraction, we observed mainly the octameric species of Mi-CK in the contact site fractions (Fig. 3A and B, fractions 24 and 25) with an octamer to dimer ratio similar to that seen in intact mitochondria (compare Fig. 2 and Fig. 3).

Activation of state-3 respiration by creatine via mitochondrial creatine kinase in brain mitochondria

Having accepted that a significant portion of the Mi-CK octamers remained bound to the inner membrane, we proposed that a presumed organization in the contacts would result in control of the enzyme activity via the outer membrane pore. We therefore, compared the activity of Mi-CK determined (by optical Test) in intact mitochondria under isotonic conditions with the activation of the respiration by this enzyme. As was first described by Fonyo et Bessman [15] in heart mitochondria, state-3 respiration could be induced also in brain mitochondria in the presence of creatine to at

least 75% of the maximal activity (Fig. 4). There was no alteration of this effect by prior treatment with 100 or even 300 μg/mg digitonin. In all cases atractyloside efficiently inhibited the oxidative phosphorylation indicating that the mitochondrial oxidation was strictly controlled by the acceptor (ADP) import (Fig. 4, CAT). On the whole the experiments showed that, although one third of the total creatine kinase activity was extracted and parts of the outer membrane were removed by digitonin treatment, the enzyme activity which remained bound to the mitochondria was responsible for the direct ADP supply to the oxidative phosphorylation. Furthermore, the experiments suggested a higher efficiency of the Mi-CK in providing ADP to the adenylate translocator than to the extra-mitochondrial test system. This aspect became evident when the creatine

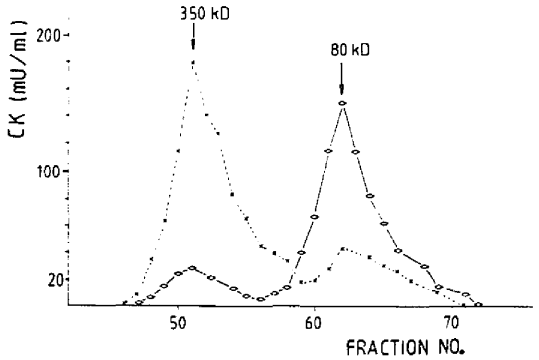


Fig. 2. Oligomeric state of mitochondrial creatine kinase in extracts from rat brain mitochondria as analyzed by gel chromatography. Brain mitochondria isolated in 0.25 M mannitol and 0.071 M sucrose were pretreated with 100 μg digitonin per mg. After centrifugation the mitochondria were resuspended in the same medium and 20 mM phosphate, 100 μM *p*-aminobenzamidine and 1.5% deoxycholate was added. The extract was chromatographed on a Superose 12 column. Elution profile of creatine kinase activity from an extract of untreated mitochondria (X), same extract after incubation with 3 M urea (◇). The activity of octamer and dimer was determined in the eluted fractions and is expressed as percent of the total activity which was eluted.

	% of total activity		
	untreated		urea
	Expt. 1	Expt. 2	
Octamer	76	85	12
Dimer	24	15	88

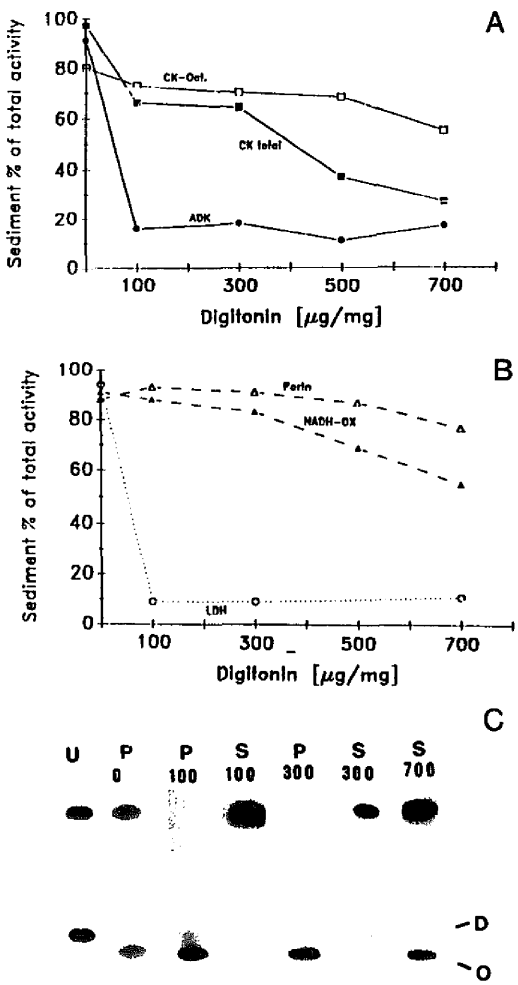
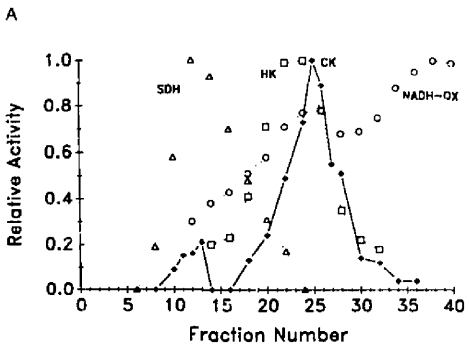


Fig. 1. Characterization of the oligomeric state of mitochondrial creatine kinase in different mitochondrial fractions. Isolated rat brain mitochondria were incubated with increasing concentrations of digitonin and were subsequently centrifuged. Panel A,B: the activity of lactate dehydrogenase (LDH, representing synaptosomes), rotenone insensitive NADH oxidase (NADH-OX, representing outer membrane) adenylate kinase (ADK) and creatine kinase (CK total) were determined in the sediments after solubilization by 1% Triton. The % activity of octamer (CK-Oct) was calculated from the isozyme electrophoresis and the total activity in the sediment. Panel B: the amount of porin was determined by SDS gel electrophoresis and subsequent immunodecoration of the trans-blots. Panel C: the supernates of mitochondria treated with 100 (S100), 300 (S300) and 700 (S700) μg/mg of digitonin and the Triton extracts of the corresponding sediments = P100, P300, as well as that of untreated mitochondria P0 were run in isozyme electrophoresis as described in methods. The fast anodically migrating band represents the cytosolic brain-type isozyme (B-CK). The slow cathodically migrating bands represent mitochondrial isozyme (Mi-CK): dimers (D) and octamers (O). To identify the dimer of the two cathodically migrating bands, a mitochondrial extract was treated with urea (U) as described in the Methods.



B

Fraction No	Octamer	Dimer
	% of total	
24	79	21
25	74	26

Fig. 3. Characterization of Mi-CK in isolated contacts. Rat brain mitochondria were disrupted by osmotic shock and sonification. The membrane subfractions were separated on a continuous 23 ml sucrose density gradient varying between 1.22 and 1.06 g/ml. The gradient, 6.5 ml overlay and 4 ml 70% sucrose cushion, was divided into 40 fractions, starting from the bottom. Panel A: in these fractions the distribution of hexokinase (HK) and creatine kinase (CK) was analyzed in relation to the activity profiles of marker enzymes of outer membrane (rotenone-insensitive NADH–cytochrome-*c* reductase = NADH-OX), and inner membrane (succinate dehydrogenase = SDH). The enzyme activities (U/ml) are expressed relative to the maximum activity in the peak fraction of the respective enzyme and are mean values of 2 different experiments. Panel B: samples of fractions 24 and 25 which contained the most CK activity were withdrawn from the density gradient shown in A and were run on a isozyme electrophoresis. The lanes were scanned on a soft laser densitometer (LKB Ultroscan). The intensity of the stained bands was determined by the integrator of the densitometer and is expressed as % of the total density of the octamer and dimer band.

kinase activity, determined by the optical test system, was compared with the creatine kinase-induced activity of oxidative phosphorylation (Table I). We observed in all cases, no matter whether the mitochondria were intact or had been pre-treated with 100–300 μg/mg of digitonin, that the creatine kinase activity, which was determined by the use of an extramitochondrial indicator enzyme assay, in the mitochondria or mitoplast fraction, was by far not sufficient to provide enough ADP for the corresponding activity of the oxidative phosphorylation (Table I, for example 19 nmol ADP produced versus 113 nmol ADP consumed/min per ml). Thus, to explain the discrepancy a direct coupling of the enzyme to the translocator had to be assumed which increases the transphosphorylation efficiency of Mi-CK and the export of high energy phosphate out of the mitochondria [16].

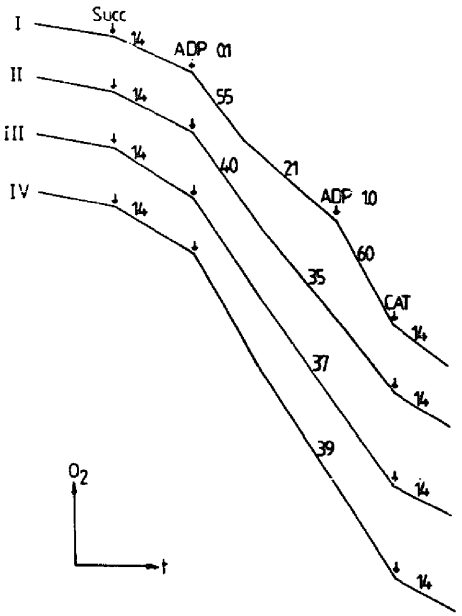


Fig. 4. Activation of state-3 respiration in brain mitochondria by creatine via internal creatine kinase. The oxygen consumption (nmol O_2/min per mg) of isolated mitochondria from brain was determined in isolation medium containing 5 mM succinate, 5 μM rotenone, 5 mM phosphate and $MgCl_2$. State 3 respiration was induced either by 0.1 mM ADP or 1.0 mM ADP (curve I), or by ADP regenerated by the reaction of mitochondrial creatine kinase in the presence of 25 mM creatine and 0.1 mM ADP, (curve II). Curves III and IV were obtained under the same conditions as curve II, but with mitochondria which were pretreated with 100 (III) or 300 μg/mg (IV) digitonin washed and resuspended in isolation medium. Carboxyatractyloside (CAT) 100 μM was added as indicated.

TABLE I

Functional coupling of mitochondrial creatine kinase to the adenylate translocator

Mitochondria from rat brain were incubated with increasing concentrations of digitonin as described in methods. The sedimented mitochondria were resuspended and the stimulation of the state-3 oxidation by the activity of mitochondrial creatine kinase was measured as in Fig. 4. The ADP consumed by the oxidative phosphorylation was calculated from the increas in oxidation rate after ADP addition and the P/O ratio. The activity of creatine kinase in the mitochondrial suspension was determined in isotonic isolation medium by optical test which employed pyruvate kinase/lactate dehydrogenase as indicator system to determine the ADP production of the enzyme. See Materials and Methods.

Digitonin	ADP production or consumption in nmol/min per ml by	
	mitochondrial creatine kinase	oxidative phosphorylation
0	60.4	113.0
100	40.0	133.0
300	19.0	113.0

TABLE II

Effect of pyruvate kinase on the activity of oxidative phosphorylation

The oxygen consumption (nmol O_2/min per mg) of intact mitochondria from brain (control) and mitochondria pretreated with 100 μg/mg digitonin was determined in isolation medium containing 5 mM succinate, 5 μM rotenone, 5 mM phosphate and $MgCl_2$. State-3 respiration was started by 0.1 mM ADP either in the presence or absence of 8 U pyruvate kinase (PK) and 0.5 mM phospo*enol*-pyruvate. Subsequent induction of state 3 respiration was performed by addition of 22 mM creatine. Mean values of 4 experiments.

Additions	% of max state-3 oxidation	
	control	digitonin
0.1 mM ADP	100	100
8 U PK	54± 5	70±10
8 U PK+22 mM creatine	100±15	100±10

Compartmentation of the ADP produced by mitochondrial creatine kinase

The above findings suggested that the ADP produced by Mi-CK was not available to the external indicator enzyme, pyruvate kinase, in the optical test system which agreed with the observations of Gellerich and Saks [17] in heart mitochondria. In a similar experiment, as the ones shown in Fig. 4, with digitonin-treated brain mitochondria we found that addition of pyruvate kinase could not inhibit the stimulation of oxidative phosphorylation by Mi-CK (Table II). In other words, although the outer membrane was disrupted by digitonin, the ADP did not exchange with external pyruvate kinase.

Control of creatine kinase activity by the outer membrane pore

As a further attempt to investigate the presumptive complex of Mi-CK with the two envelope membranes in the contacts we analyzed whether the enzyme activity remained controlled by the outer membrane pore also after disrupture of the outer membrane by digitonin. We employed an impermeable, amphiphilic, anionic polymer (of metacrylate, maleate and styrene in a 1:2:3 proportion) which, as shown earlier [18], was able to inhibit transport of adenine nucleotides across the outer membrane. After treatment with increasing concentrations of the polyanion the latency of Mi-CK activity was determined in intact mitochondria and mitoplasts by comparing these activities to those obtained after addition of Triton or sonication. The activity of creatine kinase in the mitoplast sediment was still reduced to 50 % such as in intact mitochondria [7,18] by 40 μg/mg polyanion, although prior treatment with 300 μg/mg of digitonin removed parts of the outer membrane (Fig. 5). Complete disruption of the mitoplast structure upon addition of Triton X-100 or sonification recovered the original activity suggesting that the octamer was not directly effected by the polyanion, but became inhibited because of its location behind the outer membrane pore (Fig. 5).

Accessibility of mitochondrial creatine kinase to external metabolites

As described previously [7] a significant part (approx. 50%) of Mi-CK activity was not measurable with the optical assay system under isotonic conditions in which brain mitochondria remain intact. It was assumed that this latent activity was part of the much higher effective activity of Mi-CK calculated from creatine dependent stimulation of the state-3 oxidation (Table I and Fig. 4). The assumption that the pore was regulating the Mi-CK activity let us to study the inhibitory effect of extramitochondrial phosphocreatine and iodoacetate on the enzyme reaction. We suggested that one would be able to study this effect of the inhibitors on the accessible and the latent activity by comparing the data of the optical assay system with the stimulation of the state-3 oxidation. The optical assay determines the extramitochondrial liberation of ADP, the registration of the state-3 oxidation rate determines the ADP production in the outer mitochondrial compartment. When measuring the freely accessible Mi-CK activity in intact and digitonin (100 μg/mg)-treated mitochondria by the optical test system we observed an inhibition by external phosphocreatine or iodo acetate which was comparable to that of the soluble enzyme (after addition of Triton X-100) as shown by the Dixon plot for iodo acetate (Fig. 6A,B). Yet, the Mi-CK activity which was accessible to the optical test was 2 to 3 times lower in control or digitonin-treated mitochondria compared to the soluble enzyme (see starting points of curves in

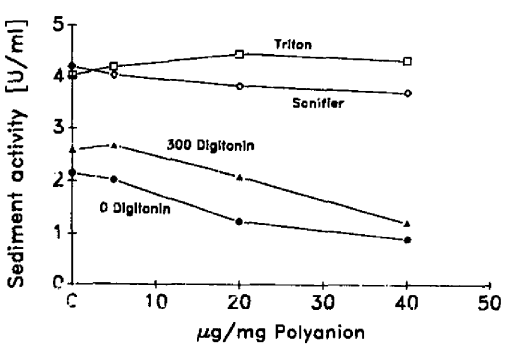


Fig. 5. Effect of outer membrane transport inhibition on the activity of mitochondrial creatine kinase. Brain mitochondria (0 Digitonin) were incubated with increasing concentrations of the polyanion (i.e., a copolymer of metacrylate, maleate and styrene in a 1:2:3 proportion) as indicated. The mitochondria were subsequently sedimented, resuspended in the same volume of sucrose medium and subjected to determination of enzyme activity. Aliquots of mitochondria were pretreated with 300 μg/mg digitonin (300 Digitonin). The total activity in all samples was determined in the presence of 1% Triton X-100 (Triton) or after sonification four times for 30 s (Sonifier).

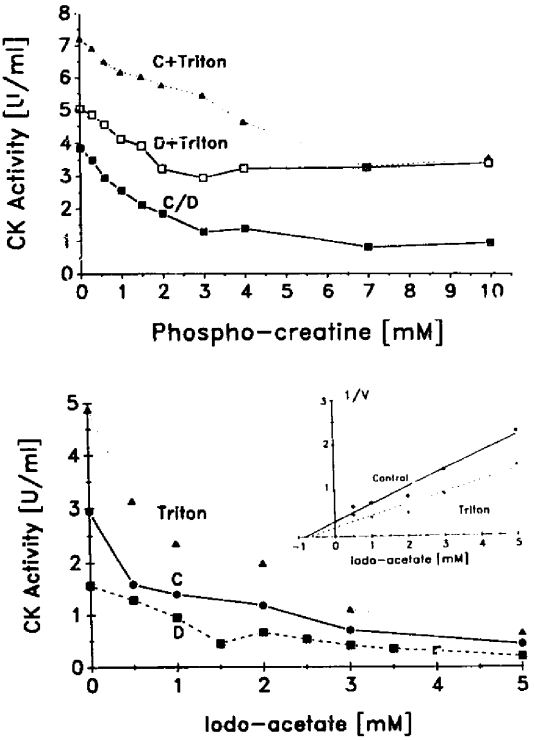


Fig. 6. Inhibition of mitochondrial creatine kinase by phosphocreatine or iodoacetate. The creatine kinase activity of brain mitochondria either intact (C) or after treatment with 100 μg/mg of digitonin (D) was determined in isotonic medium and after addition of 1% Triton X-100 both containing 5 mM succinate and 2 mM phosphate and $MgCl_2$ and 1 μM oligomycin. The enzyme reaction which was measured from creatine and ATP was inhibited either by increasing concentrations of creatine phosphate (Panel A) or iodoacetate (Panel B) as indicated. The data from Fig. 6B (Triton and C) were used for the Dixon plot shown as inset in the same fig.ure.

Fig. 6A, B). We suggested that only the accessible activity of Mi-CK became inhibited with the same K_i as the soluble enzyme. This was proved by studying the iodoacetate effect in two subsequent tests of the same mitochondrial sample: (i) the mitochondria were pre-incubated with increasing concentrations of iodoacetate, (ii) aliquots of these mitochondria were transferred to isotonic medium (without inhibitor) leaving the structure intact and the inhibition was measured, (iii) in a subsequent step the activity was recorded after solubilization of the membranes by addition of Triton X-100 to the same sample.

It appeared that about 50% of the total Mi-CK activity remained uneffected by iodoacetate (Fig. 8) and were uncovered after destroying the membranes. The fact that the inhibitors interact exclusively with the accessible activity explains why the stimulation of the state-3 oxidation via Mi-CK was not significantly reduced (Fig. 7A, B) by phosphocreatine and iodoacetate which was true also for mitoplasts (treated with 100 μg/mg digitonin). To summarize, the data suggest that the latent part of Mi-CK activity was not available to the inhibitors and that the octamer remaining in the contact sites did still not freely equilibrate with the external metabolites although parts of the outer membrane had been removed by digitonin.

Possible interaction of the mitochondrial creatine kinase with the adenylate translocator

Because of the functional coupling of mitochondrial creatine kinase to the ATP/ADP-translocator a specific binding between the two proteins has been postulated

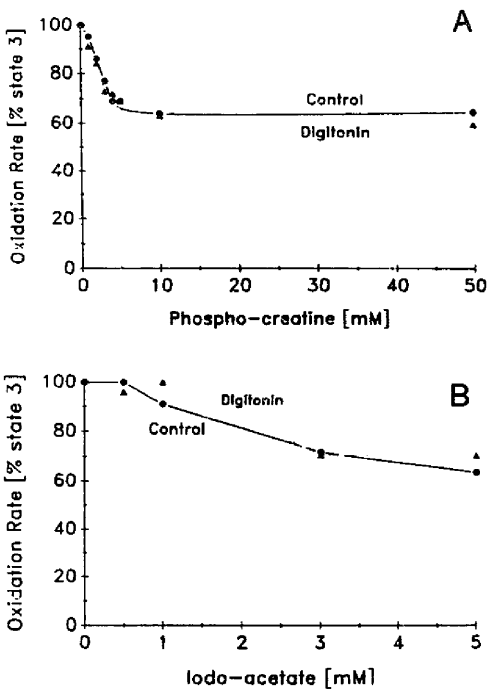


Fig. 7 Inhibition of creatine stimulated state-3 oxidation by phosphocreatine or iodoacetate. The oxygen consumption (nmol O_2/min per mg) of brain mitochondria was determined in isolation medium containing 5 mM succinate, 5 μM rotenone, 5 mM phosphate and $MgCl_2$. State 3 respiration was induced by the reaction of mitochondrial creatine kinase in the presence of 20 mM creatine and 0.1 mM ADP. The oxidation rate after addition of either phosphocreatine (Panel A) or iodoacetate (Panel B) is expressed as % of the maximal state 3 activity in the presence of 1 mM ADP. The latter state 3 activity was not effected by iodo acetate up to 5 mM. The same experiments were performed with mitochondria which were pretreated with 100 μg/mg digitonin, washed and resuspended in isolation medium. For a control these mitochondrial preparations were incubated with 100 μM carboxyatractyloside which fully blocked oxidative phosphorylation (see also Fig. 4).

TABLE III

Distribution of the adenylate translocator in subfractions of mitochondrial membranes

Contact sites, and inner and outer membrane fractions were isolated as described in [8]. The fractions were incubated in isolation medium in the presence of [^{3}H]atractyloside, centrifuged and analyzed for radioactivity.

	Inner membrane	Contact sites
Liver		
cpm/mg	14261.0	12616.0
cpm/nmol Cyt Ox	33114.0	90114.0
Kidney		
cpm/mg	3392.0	2664.0
cpm/nmol Cyt Ox	4978.0	3232.0

[16,19]. To explain the preferential binding of the creatine kinase in the contact sites we were interested to study whether the translocator might be located exclusively in these sites. We determined the concentration of the translocator by binding of [^{3}H]atractyloside to membrane subfractions of osmotically disrupted mitochondria (Table III). The data where based on the protein content of the respective fractions but also on cytochrome oxidase which in liver mitochondria was found to be concentrated in the crista membranes [20]. The outer membrane fraction contained only low contaminations of the adenylate translocator (not shown). However, the translocator (as a presumed binding site of Mi-CK) was not significantly enriched in the contact fraction of kidney mitochondria. By contrast, in liver mitochondria it appeared to be concentrated in the inner boundary membrane fraction when compared relative to the distribution of the cytochrome oxidase. The random distribution of the translocator in the inner membrane did not exclude a specific interaction between this protein and Mi-CK. Such an interaction is suggested here from the observation that the amount of octamer, determined by isozyme electrophoresis, decreased when brain mitochondria were incubated with atractyloside (Table IV). However, this effect of atractyloside was abolished, when the mitochondrial respiration was stimulated by creatine and ADP prior to the addition of atractyloside. It thus, appeared that atractyloside might compete with Mi-CK for the same binding site. In fact some 20% less [^{3}H]atractyloside became bound to the mitochondria under conditions favoring the formation of Mi-CK octamer (i.e., in this experiment the presence of substrate, creatine and ADP, see Table V).

TABLE IV

Influence of Mi-CK activity on binding of atractyloside

Isolated brain mitochondria were incubated in isolation medium, containing 5 mM phosphate and $MgCl_2$, in the presence 5 mM succinate and 20 mM creatine. The State 3 respiration was initiated by addition of 0.1 mM ADP. The ADP uptake ws inhibited by the addition of 0.1 mM atractyloside either before or after the reaction was started. The mitochondrial samples were extracted in the presence of 100 μM *p*-aminobenzamidine and Triton X-100. The extracts were run in isozyme electrophoresis and the different oligomers of creatine kinase were stained by enzymatic reaction with Nitro-blue tetrazolium as indicator. The activity was determined by densitometry. Means values of 2 experiments.

Atractyloside	% of total activity	
	octamer	dimer
no addition	86.2	13.8
addition prior to activation of state 3 oxidation	58.0	42.0
addition subsequent to activation of state 3 oxidation	83.6	16.4

TABLE V

CPM value

Coupled brain mitochondria were incubated in isolation medium with 11 nM [^{3}H]atractyloside/mg of protein before and after initiation of state 3 respiration by 0.1 mM ADP in the presence of 5 mM succinate, 4 mM $MgCl_2$ and phosphate, and 10 mM creatine. After 10 min of incubation 10 μl of the sample was applied onto Whatman filter paper saturated with 20% TCA. The filter paper was washed three times with 5%TCA and once with ethanol before it was immersed in scintillation medium and counted for radioactivity.

Expt. No.	cpm/mg				
	control	atract before start of state 3	% over control	atract after start of state 3	% over control
1	1930	1957	101.3	1362	70.5
2	2698	2893	107.2	2295	85.0
3	1514	1608	106.2	1292	85.3

Discussion

Two fractions of the mitochondrial creatine kinase

In the present investigation we report the existence of two fractions of mitochondrial creatine kinase which differ in affinity to the inner membrane, one fraction extractable with 300 μg digitonin/mg mitochondria consisting of Mi-CK dimers and some octamers and another fraction resistant to this treatment but extractable by 700 μg digitonin/mg consisting to a high percentage of octamers. Since 300 μg digitonin/mg, which removes parts of the outer membrane except at the contacts [21,22], desorbed only 10–20% of the total mitochondrial creatine kinase activity unlike adenylate kinase which was removed by 85% during this treatment (Fig. 1ABC) and since the isolated contact fraction of kidney and brain mitochondria contained significant amounts of Mi-CK activity [7,8] (Fig. 3), we concluded that Mi-CK octamers do not only show a higher affinity

to mitochondrial membranes compared to dimers [24], but are also present in significant amounts in isolated contact sites.

Characterization of creatine kinase in the contact fraction

The self-association of the dimer to an octamer has been described recently [2–5] but the regulatory function of this phenomenon remained unclear because it had no obvious effect on the specific enzyme activity itself in vitro [4]. However, in intact mitochondria the two molecular species of creatine kinase appeared to be organized with relation to the contact sites. We observed that it was mainly the octamer which remained bound to the membranes after digitonin treatment (Figs. 1,2) and in the isolated contact fraction about 80% of the total activity was of the octameric structure (Fig. 3). This agrees also with a recent study by Quemeneur et al [23] who suggested that in situ Mi-CK is octameric only, but nevertheless we always found a defined, albeit small portion of dimeric Mi-CK in intact mitochondria as well as in the contact sites (Fig. 2 and 3). The data may be explained by the finding of Schlegel et al. [24] that the octameric Mi-CK species was able to rebind to extracted mitoplasts over a broader pH range (pH 6.5–8.5) compared to the dimeric Mi-CK, and that rebinding of the dimer, which was possible at pH 7, to the inner membrane let to the formation of octamer on the inner membrane. In addition, here it appeared that the Mi-CK in the mitoplast fraction, after disrupture of the outer membrane, was associated with the outer membrane as well because it was still controlled by the outer membrane pore. We suggest that this binding occurs in the contact sites which still remain more or less intact after digitonin treatment.

Regulation of the creatine kinase by the outer membrane pore

The mitochondrial outer membrane pore, when reconstituted in artificial membranes, formed a voltage dependent anion selective channel (VADAC) [25]. This became evident at a voltage above 30 mV where the pore adopted a low conductance state [25,26]. It was recently observed that the pore in the latter state changed its ion selectivity from anions to cations [7]. It seemed plausible that a regulatory effect of the inner membrane potential on the outer membrane pore could only be expected in the contact sites, where the distance between inner and outer membrane, as observed by freeze etching [27], was below 2 nm. This view would on one hand presume a regulation of the pore, existing under physiological conditions, and on the other hand serve to explain the earlier observations of a specific structural organization of intermembranous kinases in the contact sites where creatine kinase, in contrast to adenylate kinase, appeared to be concentrated [8]. Taking into account that the pore protein is randomly distributed in the outer membrane [1], it appeared possible that two states of the pores might co-exist: those which would be influenced inside the contacts by the inner membrane potential and consequently would be of low conductance and cation selective, and other pores beyond the contacts which would be unregulated and therefore of high conductance and anion selective [7].

It has been shown in heart mitochondria, by competition experiments with external pyruvate kinase, that the ADP produced by mitochondrial creatine kinase does not equilibrate readily with the extramitochondrial ADP/ATP pool [17,28]. The same results were obtained here with digitonin-treated brain mitochondria (Table II). Even after outer membrane disrupture by digitonin, the ADP did not become available to pyruvate kinase. This suggested either an intimate contact between Mi-CK and the adenylate translocator as postulated by Saks [16,19] and/or a reduced permeability of the outer membrane pore for negatively charged metabolites. The latter would imply that, although digitonin treatment had removed parts of the outer membrane, the pore protein was nevertheless appropriate to regulate the Mi-CK. Indeed, we observed a reduction of Mi-CK activity also in digitonin treated mitochondria by the polyanion (Fig. 5), which changed the pore to the low conductance, cation selective state [7,18,29]. The results suggested that the octamer in the contacts formed a complex with the two mitochondrial envelope membranes.

Regulation in intact mitochondria of the creatine kinase activity by self-association of the enzyme

The location mainly of the Mi-CK octamer in the contacts resulted in the persistence of the regulatory

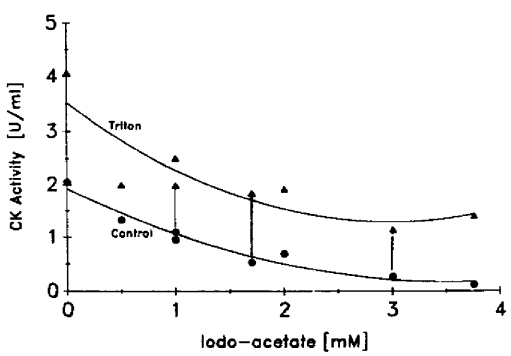


Fig. 8. Protection of latent Mi-CK activity against iodoacetate inhibition. Suspension of intact mitochondria were pre-incubated with increasing concentrations of iodoacetate. The activity of creatine kinase was determined in aliquots of the pretreated mitochondria in the absence of inhibitor either under isotonic conditions (Control) as in Fig. 6B or after subsequent addition of 1% Triton (Triton) to the same samples resulting in the liberation of uninhibited activity.

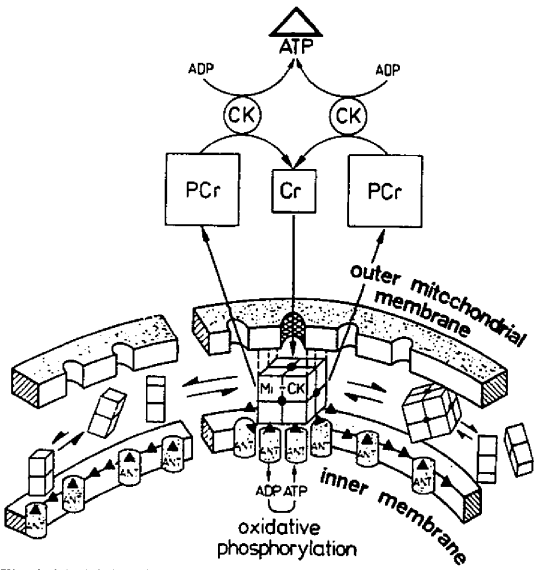


Fig. 9. Model describing the organization in the outer compartment of different oligomeric forms of mitochondrial creatine kinase. Upon formation of contact sites in respiratory state 3, the octamer of mitochondrial creatine kinase (Mi-CK) becomes bound in these sites to the adenylate translocator (ANT). Consequently, the equilibrium between octamer and dimer is shifted to the side of the octamer. The organization of the octamer in the contact sites and alignment to low conductance, cation selective pores results in exclusion of this creatine kinase activity from negatively charged cytosolic metabolites (ATP, phosphocreatine) while the dimer remains linked to unregulated pores which allow free access to the cytosol. The assembly of Mi-CK octamers with translocators (ANT), possibly facilitated via ANT-bound cardiolipin [40,41], as well as with the outer membrane pores, is thought to form a multi-enzyme microcompartment optimized for efficient energy channelling at the producing side of the phosphocreatine-circuit [24]. Phosphocreatine (PCr), the net product of the oxidative phosphorylation in mitochondria of cells with high energy demand (e.g. excitable tissues) is then made available at the receiving end of the PCr-circuit to cytosolic CK isozymes that are specifically associated at subcellular sites of high ATP requirement where CK is functionally coupled to ATPases or present at sites where ATP-levels have to be critically regulated [24,30].

effect of the pore even after disrupture of the outer membrane (Figs. 6,7,8). On the whole this would explain for the first time a physiologically relevant regulatory function for the octamer formation (Fig. 9), namely that by association of this Mi-CK species in the contacts the activity would become separated from the metabolites in the extramitochondrial compartment as was previously postulated [4,24,30]. Such a regulatory effect of the outer mitochondrial membrane on the enzyme kinetics was observed by Brooks and Suelter [31] and also in the present investigation. When the stimulation of the state-3 respiration by Mi-CK was studied about 70% of the ADP producing Mi-CK activity were not accessible to phosphocreatine and iodo acetate (Fig. 7A,B). Moreover, the determination of activity by an optical test system under isotonic conditions resulted in the observation of about 50% latent Mi-CK activity which was not susceptible to iodo acetate inhibition (Figs. 6,7,8). The observed latency of the enzyme is explained by the fact that under isotonic conditions, production of ADP (by Mi-CK activity) induces contact sites [32]. Mi-CK becomes fully accessible under hypotonic conditions in the presence of high ionic strength.

These results lent support to the assumption of an additional intracellular compartment for adenine nucleotides as has been previously postulated [28,33]. A separate compartment at the mitochondrial surface due to the existence of differentially regulated pores (Fig. 9) would support an asymmetric operation of the mitochondrial creatine kinase in that creatine would enter through the cation selective pores in the contacts, while creatine phosphate would leave the outer mitochondrial compartment via the unregulated pores which beyond the influence of the inner membrane potential are anion selective [7]. By this way the mitochondrial energy would be transferred to the cytosol in spite of the presence of a high cytosolic phosphorylation potential as postulated in the creatine phosphate shuttle [24,30,34–37].

The association of the octamer in the contact sites

The fact that the Mi-CK octamer is a highly symmetrical molecule with a cube-like shape [38] would favour an interaction of the two identical sides (top and bottom) of the molecule with the two mitochondrial membranes. This property of the octamer was recently demonstrated with artificial membranes where it was shown that Mi-CK was able to induce close contacts between a monolayer and unilamellar vesicles [39]. Considering that the contact sites are dynamic structures which change in frequency depending on the functional state of the mitochondria we prefer to postulate a mechanism (Fig. 9) which is initiated by the formation of contacts, namely that the association-dissociation equilibrium between the mitochondrial creatine kinase dimers and octamers would be shifted to the side of the octamer because only this structural species of the enzyme may be capable exclusively to interact with both membrane surfaces at once to form an "energy channelling" unit for efficient transfer of mitochondrial energy [24,30].

The mechanism of how this association of the octamer in the contacts is regulated is still unclear. In liver mitochondria the formation of contact sites was found to be regulated by ADP and also atractyloside induced these sites suggesting the adenylate translocator might be involved [32]. Based on the observation that Mi-CK is functionally coupled to the adenylate translocator [16,17,19] the latter protein could be the binding site. On the other hand, there are also arguments to assume cardiclipin as a possible receptor [40]. Since some

cardiolipin has been shown to be tightly bound to the translocator [41] it is reasonable to expect a higher concentration of both these structures in the contact sites. However, we observed a random distribution of the adenylate translocator in the inner membrane of kidney mitochondria which also contain Mi-CK. Nonetheless, our results would favour an interaction of the octamer with the translocator since atractyloside shifted the self-association equilibrium of Mi-CK to the dimer side (Table IV) and since less atractyloside was bound under conditions favouring the octamer formation (Table V).

Acknowledgements

We are grateful to Dr. Brandolin and Dr. Vignais Grenoble who provided the [^{3}H]atractyloside. Furthermore we would like to thank Dr. Jörg Schlegel, Markus Wyss and M. Rojo for discussion. Dr.Vijayia Kumar Nalam was recipient of a DAAD fellowship. This work was supported by a grants of the Deutsche Forschungsgemeinschaft (Br773/3-1) and Swiss National Fonds (SNF 3.376-086).

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