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Further characterization of contact sites from mitochondria of different tissues: topology of peripheral kinases

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A membrane fraction of intermediate density between inner and outer membrane was isolated by density gradient centrifugation from osmotically disrupted mitochondria of rat liver, brain, and kidney. The fraction was hexokinase rich and could therefore be further purified using specific antibodies against hexokinase and immunogold labelling techniques. In agreement with recent findings the gradient fraction which cosedimented with hexokinase contained the boundary membrane contact sites because it was composed of outer and inner membrane components and beside hexokinase, was enriched also by activity of creatine kinase and nucleoside diphosphate kinase. In contrast the activity of adenylate kinase appeared to be concentrated beyond the contact sites in the outer membrane fraction. By employing surface proteolysis analysis and specific blockers of the outer membrane pore we observed that the location of the kinases relative to the membrane components in the contact fraction resembled that of intact mitochondria. This specific organization of some peripheral kinases in the contact sites suggested an important role of the voltage dependence of the outer membrane pore, in that the pore may become limiting in anion exchange because of influence of the inner membrane potential on the closely attached outer membrane. Such control of anion exchange would lead to a dynamic compartmentation at the mitochondrial surface by the formation of contact sites, which may explain the preferential utilization of cytosolic creatine by the mitochondrial creatine kinase, as postulated in the phosphocreatine shuttle.

Introduction

Contact sites between the two mitochondrial boundary membranes were first described by Hackenbrock in thin sections of liver mitochondria [1]. They were further characterized in freeze-fractured mitochondria [2] and were found to increase during active oxidative phosphorylation [3]. The degree of contact formation upon transition into state 3 appeared to be regulated by metabolites such as fatty acids [4] and by hormones as observed in intact hepatocytes [5] and heart muscle [6]. In view of these findings we suggested that the contacts may play a principal role [7] in functional coupling of peripheral mitochondrial kinases, like hexokinase [8], to the inner mitochondrial compartment. We assumed that this organization would be of regulatory importance because depending on the bound or free state of the respective kinases they would either utilize glycolytic or mitochondrial ATP and thus affect the metabolic rate in the respective ATP producing system. In agreement with this suggestion the contacts were found to be absent in mitochondria of highly glycolytic adenocarcinoma cells [9]. The hexokinase bound to tumor mitochondria appeared to be unspecifically bound and was not coupled to the inner compartment [9]. In contrast to this, hexokinase in liver mitochondria bound preferentially to outer membrane pores located in the contact sites. The latter result was obtained by electron microscopic immunocytologic studies [10] and by isolation of the contact fraction from osmotically disrupted liver and brain mitochondria [11,12]. As observed for bound hexokinase, a functional coupling of mitochondrial creatine kinase to the oxidative phosphorylation was described by several authors [13,14]. Based on these results an interplay with the cytosolic isozyme in a phosphocreatine shuttle was postulated [15,16] which would be responsible for transfer of mitochondrial energy. To explain the asymmetric

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operation of the shuttle (i.e., the preferred access of the mitochondrial isozyme for creatine) it seemed reasonable to assume the existence of a third compartment for high energetic compounds at the mitochondrial periphery which would imply a restricted permeability of the outer membrane pore for example for phosphocreatine. In agreement to this idea, recent kinetic work of Brooks et al. [17] suggested the importance of the outer membrane for the regulation of the mitochondrial creatine kinase. Furthermore, subfractionation of brain mitochondria resulted in a concentration of creatine kinase in the contact fraction [12]. In addition, mitochondrial creatine kinase has been shown recently to form highly ordered octameric molecules consisting of four dimers arranged around a central negative stain filled cavity [18,19]. This square shaped structure with a central channel would suggest that mitochondrial creatine kinase may operate as an energy transferring molecule [19] at those sites where inner and outer mitochondrial membranes are in close proximity. Based on these facts the contact region appeared to be important in regulation also of other kinases than hexokinase, which led us to investigate the distribution of adenylate kinase, creatine kinase, and nucleoside-diphosphate kinase in relation to hexokinase in kidney, brain, and liver mitochondria. The localization was studied by isolation of the contact sites according to the method described for liver and brain mitochondria [11,12]. It was furthermore of interest to analyze the structure of the contact sites concerning the organization of the different kinases relative to the outer and inner membrane components by proteolytic analysis and inhibition of the metabolite exchange through the outer membrane pore by a polyanion [20].

Materials and Methods

Chemicals. All chemicals were purchased from Boehringer-Mannheim and Merck-Darmstadt, F.R.G.

Enzyme assays. The determination of monoamine oxidase (EC 1.4.3.4), succinate dehydrogenase (EC 1.3.99) was carried out as described earlier [21]. The activity of nucleoside-diphosphate kinase (EC 2.7.4.6) was analyzed according to Jacobus et al. [22]. The adenylate kinase (EC 2.7.4.3), the hexokinase (EC 2.7.1.1) and the creatine kinase (EC 2.7.3.2) was measured in agreement with Bücher et al. [23]. Rotenone-insensitive NADH–cytochrome-*c* reductase (EC 1.6.2.2) was determined according to Sottocasa et al. [24].

Preparation of mitochondria from rat kidney, liver and brain. Kidney and liver mitochondria from 10 rats (250 g body weight) were isolated by differential centrifugation in 0.25 M sucrose, buffered with 10 mM Hepes (pH 7.4) in case of liver and 3 mM Tris (pH 7.4) in case of kidney. The mitochondrial sediment was washed two times using 6000 × *g* and 3000 × *g* (Sorvall, rotor SS-34) for sedimentation. Mitochondria from rat brain were prepared according to Rehncrona et al. [25] in 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 34 min centrifugation at 38000 rpm in a rotor 60 Ti (Beckman) on a 20% percoll gradient. Percoll was removed from the mitochondrial fraction by washing two times with the above medium.

Preparation of contact sites. The purified mitochondria were exposed to a swelling and shrinking procedure by incubation in 30 ml of 10 mM phosphate buffer (pH 7.4), with subsequent addition (after 20 min incubation) of 12 ml 60% sucrose. After 20 min incubation portions of 25 ml were sonificated with a Branson Sonifier B-15 (three times 30 s at level 5.5), followed by centrifugation at 9000 rpm for 15 min in a Sorvall SS-34 rotor. The supernatant was layered above a 23 ml linear sucrose density gradient, varying from a density of 1.22 to 1.06 g/ml at 4 °C and was centrifuged for 20 h in a Sorvall Rotor TV-850 at 48000 rpm. Subsequently the gradient was divided into 40 fractions. The fractions were characterized by different marker enzymes. Pooled fractions 20–26 (the hexokinase peak), 28–36 (the NADH–cytochrome-*c* reductase peak) and 6–18 (the succinate dehydrogenase peak) from the original gradient were used for electrophoresis and immunogold labelling.

Indirect immunogold labelling of hexokinase I by antibody binding to the mitochondria. The contact site fraction of brain or kidney mitochondria was withdrawn from the density gradient and suspended in sucrose medium. Of this suspension, containing approx. 4 mg/ml mitochondrial protein, 300 µl were incubated for 10 min at room temperature either with 300 µl hexokinase antiserum or, as a control, with sucrose medium. After centrifugation (10 min at 400000 × *g* in a Beckman TL-100 ultracentrifuge) the samples were washed, resuspended in 300 µl sucrose medium containing protein-A-gold conjugate and were incubated as above. Subsequently both samples were recentrifuged on a discontinuous (40%/45%/50% (w/v)) sucrose density gradient for 2 h at 259000 × *g* with rotor TLS-55 in a Beckman TL-100 ultracentrifuge. The gradients were fractionated into eight fractions which were subjected to determination of marker enzymes.

Surface proteolysis analysis. To study the sidedness of the outer membrane and the orientation of the two membrane components in the contact fraction, the fraction was treated with different trypsin concentrations (0, 3.0, 15, 30, 60 µg trypsin/mg protein). The incubation for 15 min at 4 °C occurred in: 20 mM Hepes, 100 mM NaCl and 2 mM $MgCl_2$ (pH 7.4). The proteolysis was terminated by boiling the samples in 3% SDS. Subsequently the different samples underwent SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which was performed as described by Laemmli [26].

Incubation of subfractions with polyanion. 0.2 ml aliquots of the outer membrane and contact fractions suspended in sucrose isolation medium containing 5 mM $MgCl_2$ were incubated for 15 min at room temperature with 100 μg/mg of the polyanion, which is a polymer of methacrylate, maleate and styrene in a 1 : 2 : 3 proportion and was a gift of Dr. T. König, Budapest. The suspension was subsequently centrifuged for 15 min at 400000 × *g* with rotor TLA-100 in a Beckman TL-100 ultracentrifuge to remove the unbound polyanion. The supernatant was discarded and the sediment resuspended in sucrose medium.

Isozyme electrophoresis. The creatine kinase isozymes from rat brain were separated on cellulose acetate strips according to the method of Marcillat et al. [27] 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 from 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 magnesium acetate, 12 mM phosphocreatine, 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.

Specific antibodies. Antibodies were raised in rabbits as previously described [28] against hexokinase I purified from rat brain according to Chou and Wilson [29], cytosolic and mitochondrial creatine kinase from chicken brain purified as described recently [18] and porin prepared from rat liver mitochondria following the procedure of DePinto et al. [30].

Electrotransfer and immunodecoration. These were performed as described by Rott and Nelson [31].

Assay of protein concentration. Protein was determined by the method of Lowry et al. [32].

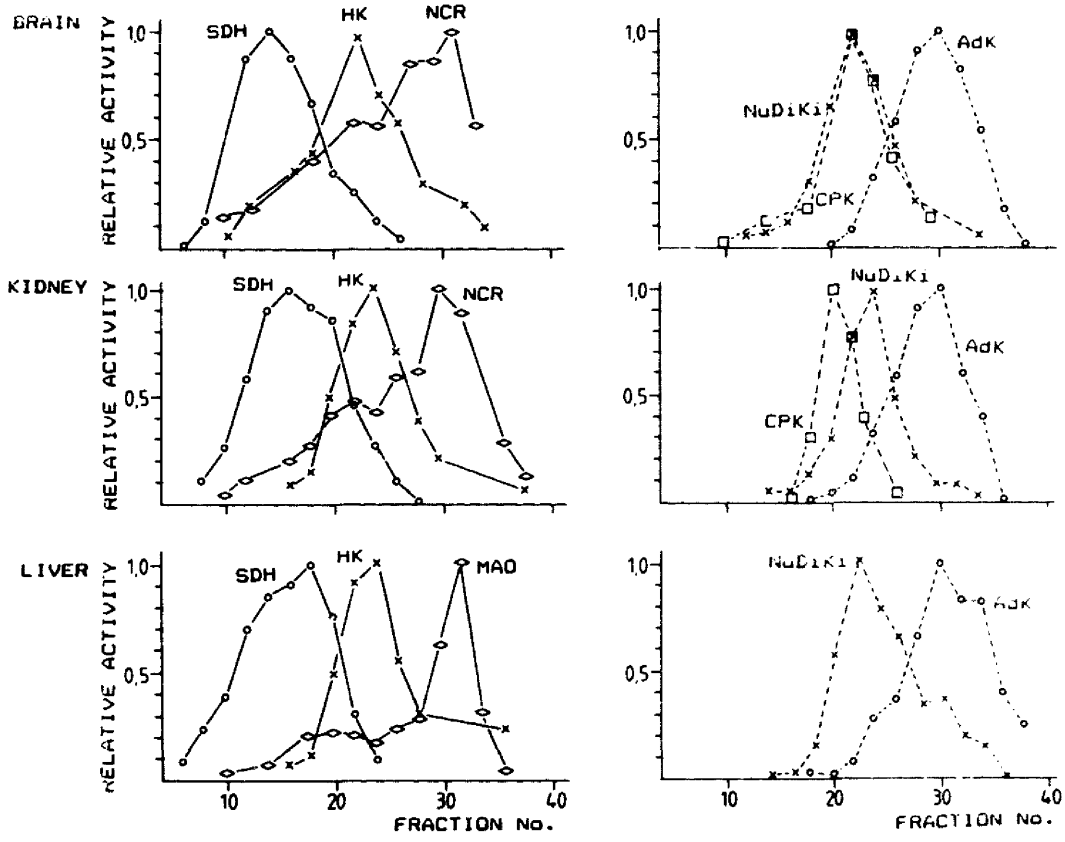

Fig. 1. Distribution of inner and outer membrane marker enzymes in a density gradient which separates subfractions of osmotically disrupted mitochondria. Rat brain, kidney and liver mitochondria were disrupted by osmotic shock and sonification. The subfractions were separated on a 23 ml continuous sucrose 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. In these fractions the distribution of hexokinase (HK) was analyzed in relation to the activity profiles of marker enzymes of outer membrane (rotenone-insensitive NADH-cytochrome-*c* reductase (NCR) or monoamine oxidase (MAO)), and inner membrane (succinate dehydrogenase (SDH)), left panel. The distribution of creatine kinase (CPK), adenylate kinase (ADK) and nucleoside-diphosphate kinase (NuDiKi), right panel, is shown after separation of subfractions of brain, kidney, and liver mitochondria in the same density gradient as above. 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 four different experiments.

Results

Distribution of inner and outer membrane enzymes in the density gradient

The membranes of osmotically-shocked kidney mitochondria can be separated into different fractions by centrifugation on a sucrose density gradient as was recently shown for liver and brain mitochondria [11,12]. The activity profiles of specific marker enzymes in the gradient (Fig. 1, left panel) revealed an outer membrane fraction (characterized by high activity of rotenone insensitive NADH–cytochrome-*c* reductase and monoamine oxidase) in the upper part of the gradient and an inner boundary and crista membrane-rich fraction (characterized by high activity of succinate dehydrogenase) at high density. In addition we observed a third fraction at intermediate density in which the highest activity of mitochondrial hexokinase was present. This region of the gradient contained low activity of inner and outer membrane marker enzymes. The gradient was divided into the three peak fractions (outer membrane 36–28, contact sites 26–20 and inner membrane 18–6) and the membranes were sedimented by centrifugation. The specific activity of inner and outer membrane markers was determined in these fractions relative to the mixture of mitochondrial membrane fragments which were loaded on the gradient (Fig. 2A). Inner and outer membrane markers appeared to be specifically enriched in the respective fractions while considerable amounts of both membranes were concentrated in the fraction of intermediate density.

Distribution of kinases in the density gradient

According to the distribution of inner and outer membrane marker enzymes the subfractionation of kidney and brain mitochondria like in liver revealed the separation of inner and outer membrane and enrichment of hexokinase in an intermediate fraction. We recently suggested that the presence of hexokinase in this membrane fraction might be representative for the contact fraction. It was, therefore, interesting to study the location of other peripheral mitochondrial kinases in subfractions from kidney and brain mitochondria and to compare it to that of liver mitochondria which don't contain creatine kinase. The kinase activity profiles in the density gradient derived from the different types of mitochondria (Fig. 1, right panel) agreed in that the activity of adenylate kinase remained on top of the gradients, whereas the activity of hexokinase, nucleoside-diphosphate kinase, and creatine kinase was concentrated in the presumptive contact fraction. In brain mitochondria hexokinase and creatine kinase were exclusively enriched in the contact fraction which became evident when the specific activity in the pooled inner-, outer-, and intermediate fractions was compared to the starting material (Fig. 2B). The contact fraction from kidney mitochondria also contained the highest specific activity of creatine kinase. However, the specific activity did not increase compared to the starting

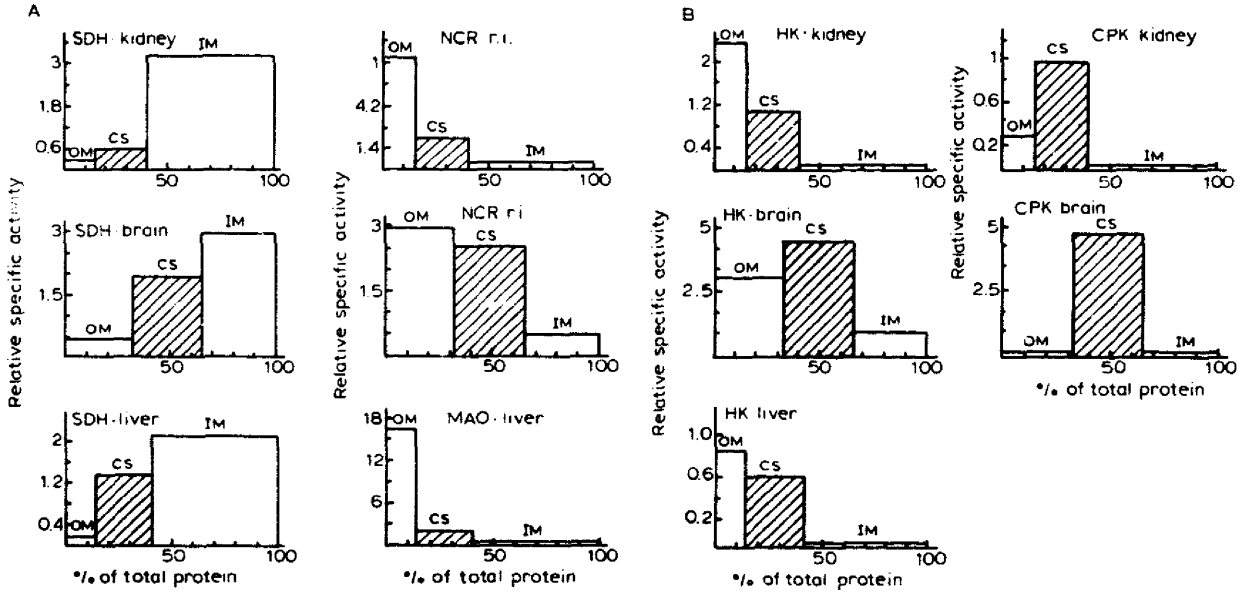

Fig. 2. (A and B). Specific activities of membrane marker enzymes and kinases in the subfractions obtained by density gradient centrifugation as in Fig. 1. The fractions containing high activity of SDH (Nos. 6–18), MAO or NCR (Nos. 28–36) and HK (Nos. 20–26) in the gradients shown in Fig. 1 were pooled, diluted with 10 mM Hepes (pH 7.4) and the membranes were sedimented by centrifugation for 60 min at 300000 × g. The resuspended pellets and an aliquot of the suspension which was loaded on the density gradient were subjected to enzyme and protein determination. The protein of the three fractions is expressed as % of total protein in the gradient. The specific activity of each enzyme is shown relative to the specific activity in the starting material which was loaded on the gradient. Abbreviations as in Fig. 1.

material, indicating that some activity was lost during the preparation. In contrast to creatine kinase the specific activity of hexokinase was higher in the outer membrane fraction from kidney and liver mitochondria as compared to the corresponding contact fraction. This was not astonishing because hexokinase can also bind to the outer membrane beyond the contacts but with a lower affinity. The specific activity of hexokinase in the outer membrane and intermediate fraction from liver mitochondria was lower than in the starting material. This may be explained by a stronger desorption of isozyme II which is the predominant hexokinase in the liver cell whereas kidney and brain contain mainly isozyme I.

Homogeneity of the contact site fraction as analyzed by specific antibodies against hexokinase

It could not be excluded that the presumptive contact fraction from the different mitochondria was accidentally composed of free inner and outer membrane vesicles of the same density and that free hexokinase and creatine kinase had migrated to the same position. In order to prove the formation of a complex between inner and outer membrane and the kinases, we used hexokinase as a tool. This enzyme according to the analysis of subfractions from liver and brain mitochondria and electron microscopy was exclusively located at the surface of mitochondria with preference for the contact sites [10–12]. By using the indirect immunogold assay for antibodies bound to hexokinase I, we increased the density of hexokinase in the contact fraction which was obtained from the density gradient. The antibody labelled fraction of brain (Fig. 3) and kidney (Fig. 4) mitochondria was subsequently re-centrifuged on a second discontinuous gradient containing 50%, 45% and 40% sucrose. The activity profiles of outer (rotenone-insensitive NADH-cytochrome-*c* reductase) and inner (succinate dehydrogenase) membrane marker enzymes, hexokinase, creatine kinase, and nucleoside-diphosphate kinase in these gradients coincided and were shifted to higher density compared to the gradients loaded with the unlabelled contact fraction (Figs. 3 and 4). This suggested that the two boundary membranes formed a homogeneous vesicle

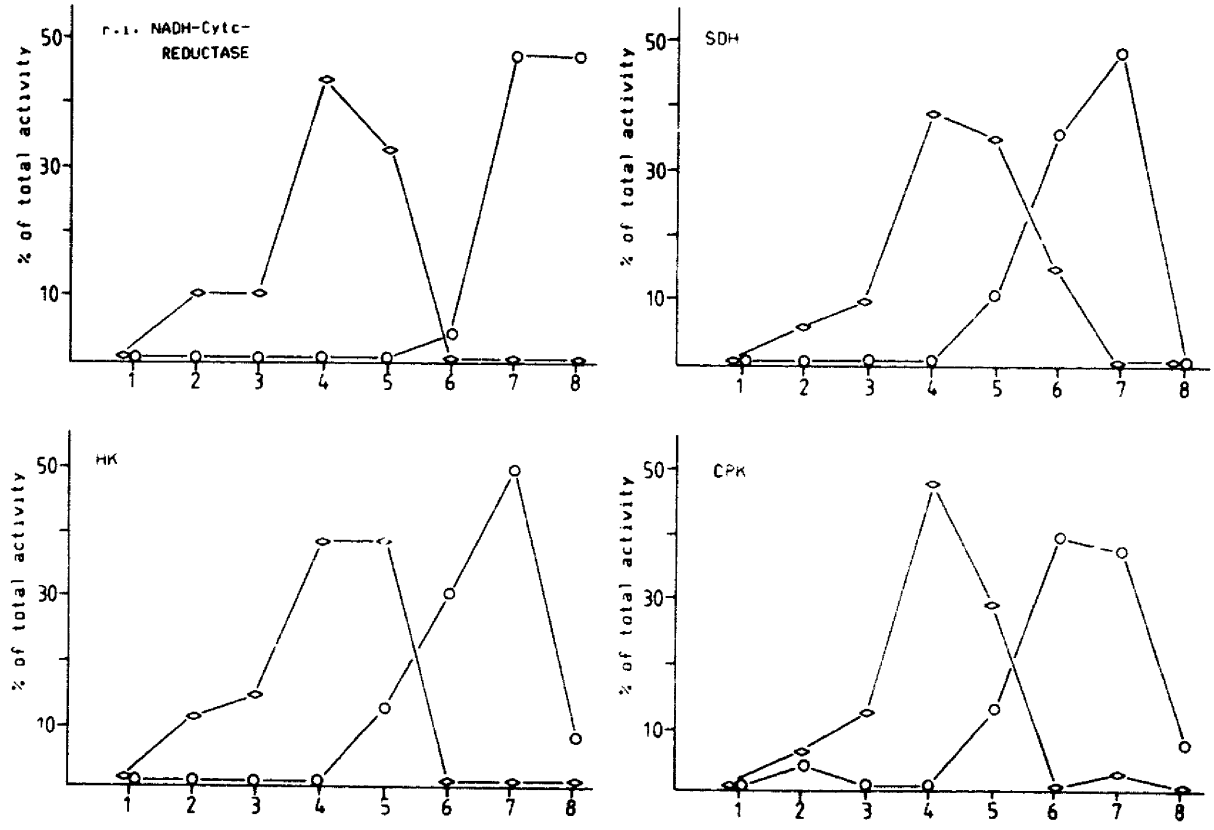

Fig. 3. Purification of contact sites from brain mitochondria by immunogold labelling of hexokinase. The isolated contact sites from brain mitochondria (fraction 20–26, Fig. 1) were incubated with antibodies against hexokinase I. This fraction as well as an undecorated one were subsequently treated with a protein-A-gold conjugate as described in Methods. Both fractions were centrifuged on a discontinuous density gradient containing 50% (fraction 2–3), 45% (fraction 4–5) and 40%), (fraction 6–7) sucrose (w/v). Fraction 1 represents the pellet fraction, fraction 8 corresponds to the volume loaded on top of the gradient. The activities of enzymes (names abbreviated as in Fig. 1) are expressed as % of total activity in the gradient. Open circles, enzyme activity profiles in the gradient loaded with unspecifically labelled contact fraction.

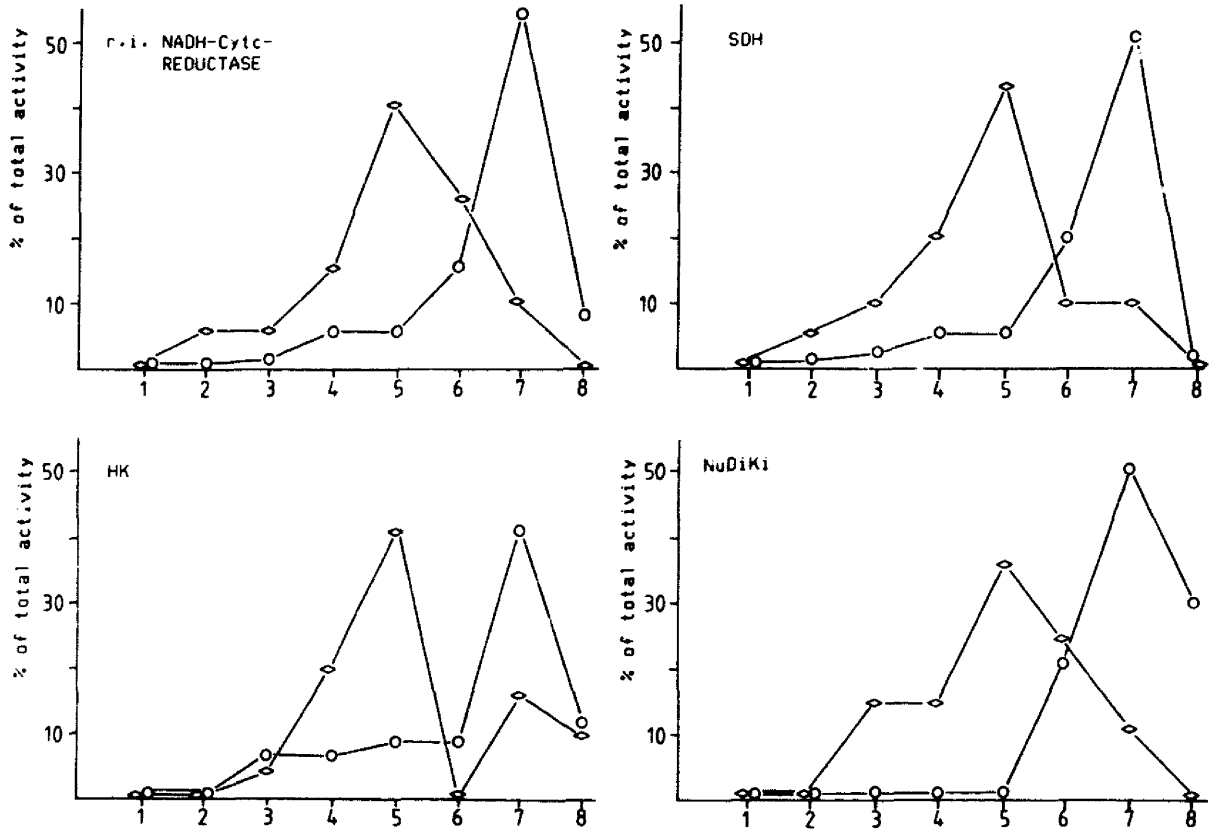

Fig. 4. Purification of contact sites from kidney mitochondria by immunogold labelling of hexokinase. The same experiment as described in Fig. 3 was performed with isolated contact sites from kidney mitochondria..

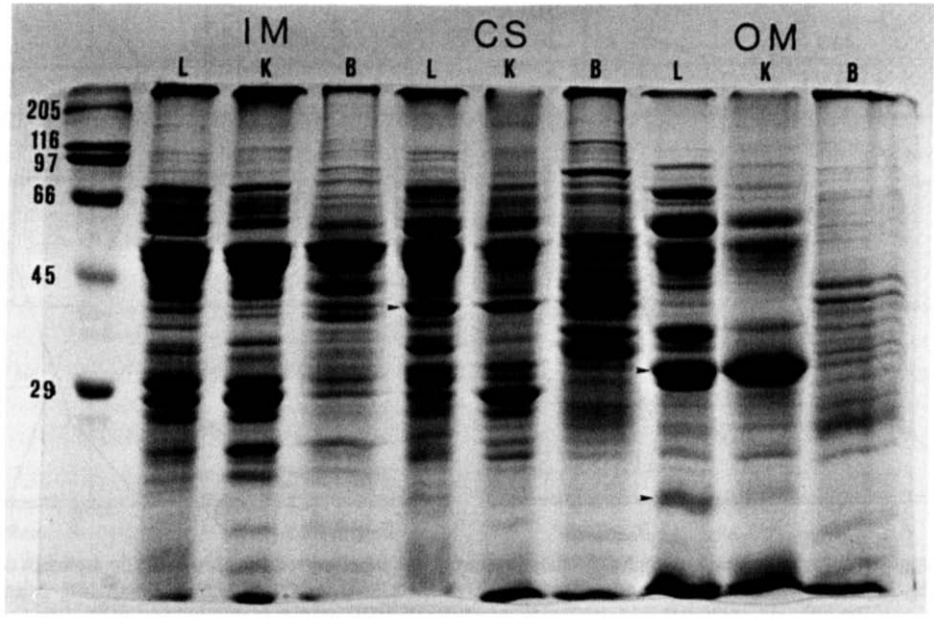

Fig. 5. SDS-polyacrylamide gel electrophoresis of mitochondrial membrane subfractions from liver, kidney, and brain. Samples of the inner membrane (IM), outer membrane (OM) and contact (CS) fraction were taken from a density gradient as in Fig. 1 and run on a SDS-polyacrylamide gel electrophoresis. The lanes from left to right represent mitochondrial membrane fractions from liver (L), kidney (K), and brain (B).

fraction and the three kinases were specific components of this fraction.

Electrophoretic characterization of the gradient fractions

SDS-polyacrylamide gel electrophoresis of the different gradient fractions revealed specific polypeptide patterns, which were very similar concerning the polypeptide bands present in the corresponding fractions of liver, and kidney (Fig. 5). The contact- and outer membrane fraction of brain mitochondria had a different composition. However, all subfractions of the three different mitochondria were comparable in that a 45 kDa polypeptide was enriched in the contact fractions while the 32 kDa polypeptide representing the pore protein, showed a larger band in the corresponding outer membranes. The 14 kDa polypeptide conferring glutathione transferase activity was present in a higher concentration in the outer membrane of liver mitochondria which agreed with a higher activity of this enzyme in liver compared to kidney and brain mitochondria.

Immunological identification of the pore protein in the different gradient fractions

The specific location of kinases in the contact fraction raised the question of the distribution of the pore protein in the outer membrane. As polypeptides in the range of 30 kDa and 40 kDa are numerous in mitochondrial membranes it was pertinent to identify the pore protein in the electrophoreses of the various gradient fractions by specific antibodies. After SDS electrophoresis of kidney mitochondrial subfractions the polypeptides were transblotted from the gels to cellulose nitrate sheets which were decorated with antibodies against porin. The immunological analysis of porin distribution in the different gradient fractions agreed with the intensity of protein staining in the electrophoresis (Fig. 5), in that porin was present in the contact fraction to a lower extent than in the outer membrane (Fig. 6).

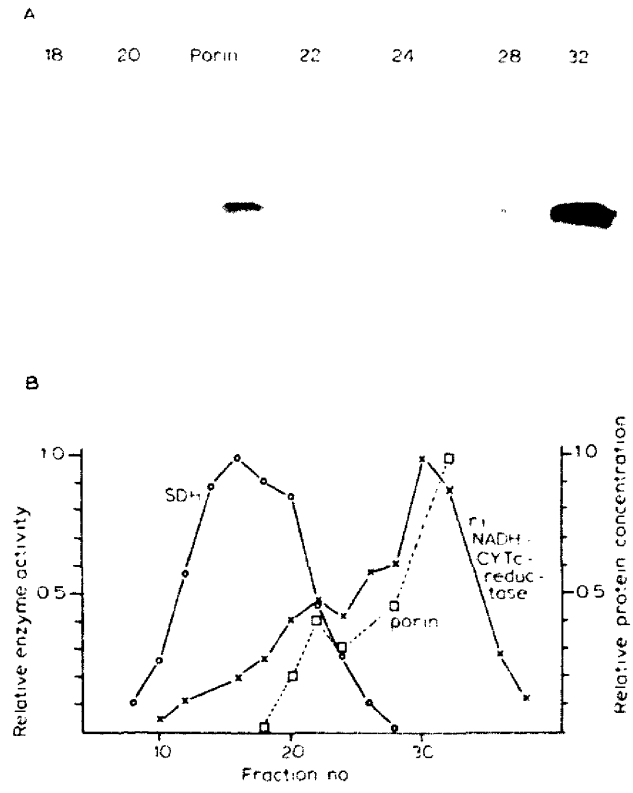

Fig. 6. Immunological identification of pore protein in different density gradient fractions form kidney mitochondria. Samples were withdrawn from the density gradient shown in Fig. 1 and were employed on a 12.0% Laemmli gel. The gel was transferred to a nitro cellulose sheet, which was decorated with antibodies against outer membrane pore protein. Panel A. the numbers of fractions are given on top of the sheet. Isolated porin (10 μg) was run on lane 'Porin'. 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 maximum density in the peak fraction. Panel B the data are shown relative to the enzyme activity profiles of outer and inner membrane marker enzymes as in Fig. 1.

Immunological identification of mitochondrial creatine kinase in the different gradient fractions

Because the antibodies we used were raised against the native mitochondrial creatine kinase from chicken brain, we had to exclude cross-reactivity with the cytosolic isozymes from rat tissues in the SDS-denatured form. We, therefore, prepared a mitochondrial and cytosolic extract from kidney. After isozyme electrophoresis on cellulose acetate strips, the isozymes were characterized by enzyme specific staining using Nitroblue tetrazolium as indicator (Fig. 7A). The same extracts were run on SDS gel electrophoresis and transblots of the gels were subsequently decorated with antibodies against mitochondrial and cytosolic creatine kinase (brain-type) (Figs. 7B, 7C). The upper band, representing the cytosolic isozyme, did not react with antibodies against the mitochondrial enzyme, but was highly reactive with antibodies against the isolated cytosolic enzyme. The lower band, representing the mitochondrial isozyme, reacted accordingly very specific with the respective antibodies. Having disproved cross-reactivity of the antibodies we used the latter to study the distribution of the mitochondrial creatine kinase in the different subfractions of kidney mitochondria (Fig. 8). The antigen distribution profile almost coincided with that of activity determined in the various fractions and both methods indicate the highest creatine kinase concentration in the contact fraction. Contamination of the fractions by cytosolic creatine kinase could be excluded by the absence of a specific immunoreactivity (not shown).

Surface proteolysis analysis

Proteolytic analysis was applied to study the sidedness of the outer membrane in the contact site fraction. As was known from earlier investigation of intact liver

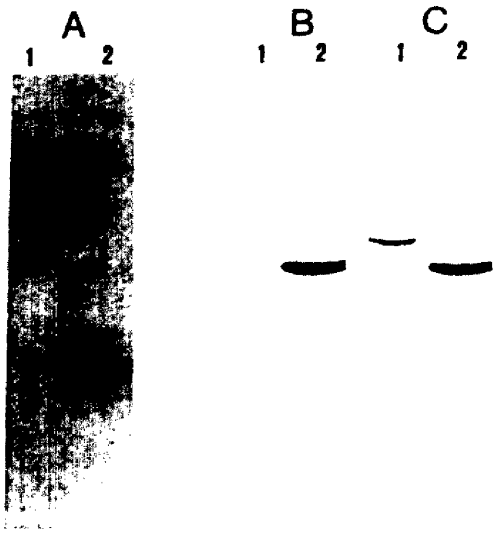

Fig. 7. Characterization of specificity of antibodies against mitochondrial and cytosolic isozymes of creatine kinase from kidney. A cytosolic (1) and mitochondrial (2) extract from kidney were run in isozyme electrophoresis (lanes A1, A2) and stained for enzymatic activity as described in Methods. The same samples were run on SDS gel electrophoresis. The polypeptides on the gels were transferred to nitro cellulose sheets which were subsequently incubated with specific antibodies against the mitochondrial isozyme (lanes B1, B2) and a combination of antibodies against both isozymes (lanes C1, C2). The polypeptide of rat mitochondrial creatine kinase migrates faster to the anode than that of the cytosolic isozyme. Thus, both isozymes can be identified also on SDS gels.

mitochondria, two polypeptides of 68 kDa and 14 kDa are susceptible to proteolysis [11]. The 14 kDa polypeptide representing the glutathione transferase [11] was present in a very low concentration in kidney mitochondria (Fig. 5). However, the 68 kDa polypeptide could be analyzed and was accessible to protease in the contact (Fig. 9B) and outer membrane fraction (Fig. 9A), indicating the outer membrane was exposed to the medium and had a regular right-side-out orientation in both fractions. The pore protein was neither accessible to protease in the contact fraction nor in the outer membrane of mitochondria.

Localization of kinases in the contact sites by inhibition of the pore permeability

Because of the regular, physiological orientation of the outer membrane in the contact sites it was assumed that the intermembrane kinases may be located in the vesicle fraction like in intact mitochondria, namely behind the outer membrane. If so, the inhibition of metabolite exchange through the outer membrane pore was expected to reduce the activity of the respective kinases. To perform the inhibition of the pore transport we used a synthetic polyanion (a copolymer of methacrylate, maleate and styrene in a 1 : 2 : 3 proportion) [20] which, as reported earlier, efficiently inhibited the ATP and ADP exchange across the outer membrane pore [33]. The activity of creatine kinases in the contact fraction in the presence of this inhibitor (Table I), was reduced by 60%, while no effect was observed on the activity of surface bound hexokinase with external ATP. The activity of adenylate kinase in the outer membrane fraction was also inhibited up to 50% by the polyanion suggesting that a significant portion of the enzyme protein was included in the outer membrane vesicles. The inhibitory effect of the polyanion was not due to a direct interaction with the kinases, because in all cases, the activity was completely regained after disrupture of the outer membrane by Triton. It is worth to note, that digitonin did not result in reactivation of creatine kinase, while it was effective concerning adenylate kinase. This finding agrees with the recent observation that in contrast to adenylate kinase, the activity of creatine kinase

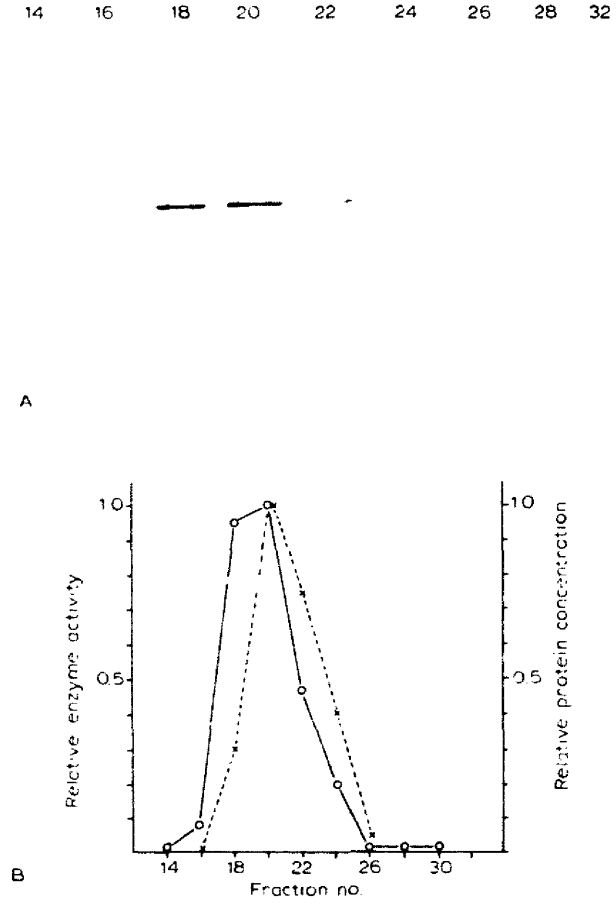

Fig. 8. Densitometer distribution profile of mitochondrial creatine kinase in the different density gradient fractions separated on SDS-polyacrylamide gel electrophoresis and decorated by specific antibodies after transblotting. The same experiment as described in Fig. 6 was performed to analyze the distribution of mitochondrial creatine kinase in the different gradient fractions from rat kidney mitochondria. Panel A shows the nitro cellulose sheet after immunodecoration. Panel B compares the activity- (×------×) with the enzyme protein distribution (o——o) profile of mitochondrial creatine kinase.

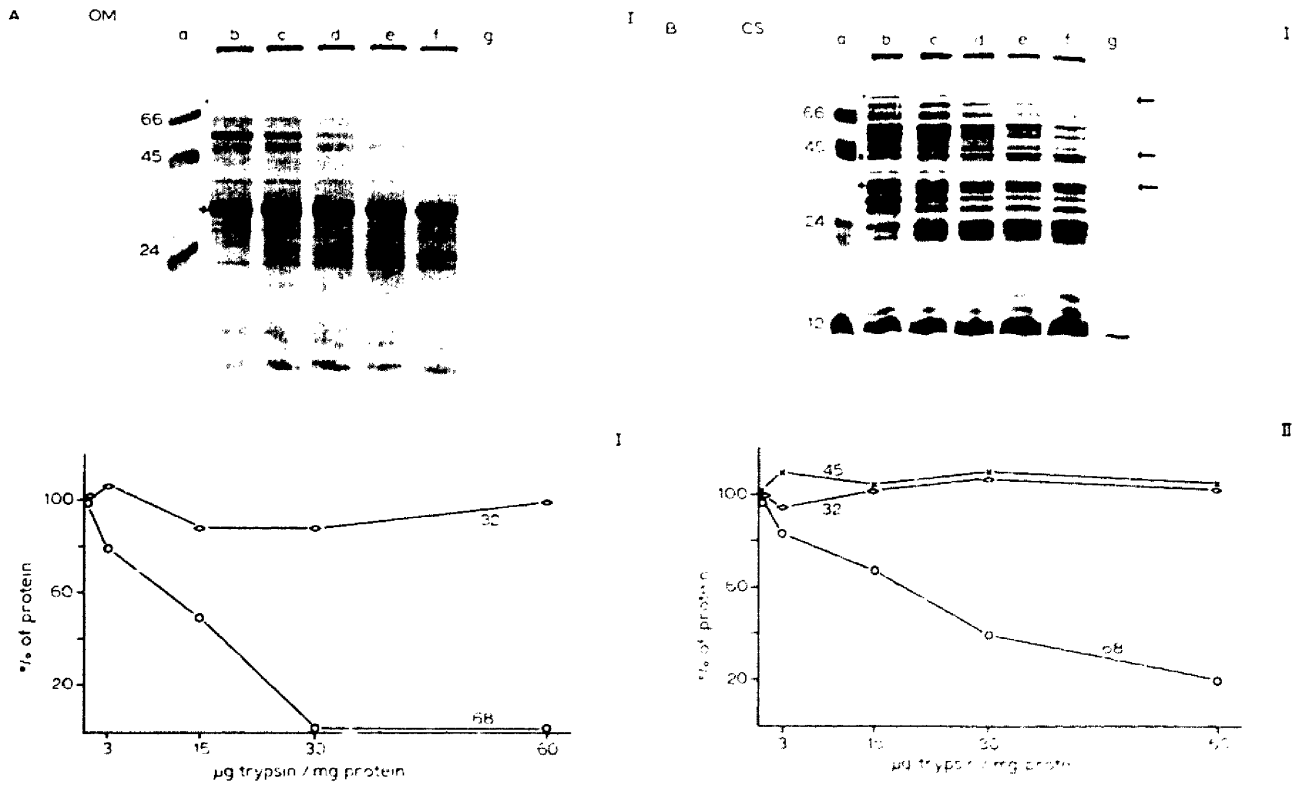

Fig. 9. (A and B). Proteolytic surface analysis of the contact- and outer membrane fraction. The outer membrane (OM, A) and contact fraction (CS, B) from a density gradient which separated kidney mitochondrial subfractions were subjected to tryptic digestion as described in methods. I: SDS-polyacrylamide gel electrophoresis of the fraction incubated with increasing trypsin concentrations as indicated in II (lane c–f). Lane a, molecular weight markers. (M_r in K); lane b, untreated fraction; lane g, trypsin. II: densitometric determination of the concentrations of 68 kDa, 45 kDa and 32 kDa (porin) polypeptides.

TABLE I

Effect of inhibition of outer membrane pore transport on the activity of different kinases in the outer membrane and contact fraction isolated from brain and kidney mitochondria

The outer membrane and contact site fraction obtained from kidney and brain mitochondria were, as described in Methods, incubated with a polyanion (PA) to block the pore transport. Brain mitochondria were subsequently treated with 300 µg/mg of digitonin and kidney mitochondria with 50 µg/mg to remove the outer membrane beyond the contacts. To solubilize all membranes 1% Triton X-100 was used. The activity of hexokinase (HK), creatine kinase (CPK), and adenylate kinase (AdK) was determined in the different fractions. n.d., not determined.

Sample	Brain CPK (U/ml)	Kidney HK (U/ml)	AdK
Contact sites			
Control	0.24	0.060	n.d.
+ PA	0.10	0.065	n.d.
+ PA + digitonin	0.10	n.d.	n.d.
+ PA + Triton	0.24		
Outer membrane			
Control	0.00	n.d.	1.01
+ PA	0.00	n.d.	0.68
+ PA + digitonin	0.00	n.d.	1.27
+ PA + Triton	n.d.	n.d.	1.21

could only partially be liberated by digitonin from brain mitochondria [12].

Localization of kinases in the contact sites by proteolytic analysis

The experiments above suggested a location of the mitochondrial creatine kinase between the two boundary membranes. However, unspecific reactions of the amphiphilic polyanion with the membrane and the enzymes could not be excluded. Supposing a location of the creatine kinase behind the outer membrane we, therefore, analyzed whether the enzyme was protected in the contact fraction against proteolysis. The contact fraction isolated from kidney mitochondria was incubated with different concentrations of trypsin. The membrane fraction was subsequently subjected to electrophoresis and immunodecoration with antibodies against mitochondrial creatine kinase (Fig. 10). The enzyme protein in the contact fraction was not digested by trypsin unless the membranes were solubilized by deoxycholate. This provided evidence that the above conclusion drawn from the polyanion effects was correct, namely that creatine kinase in the contact fraction was regularly located between the outer and inner membrane vesicles.

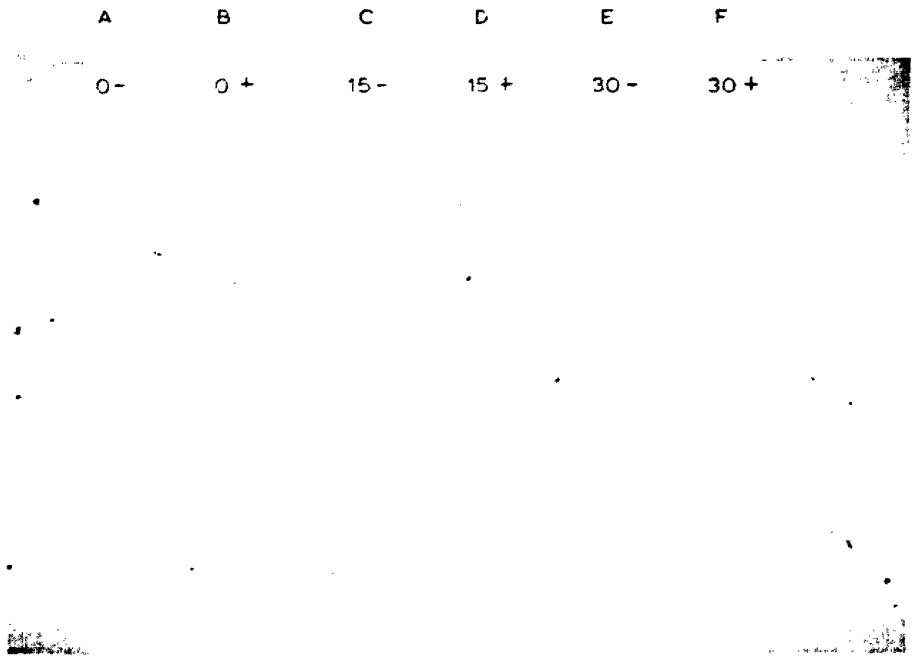

Fig. 10 Proteolytic analysis of the creatine kinase location in the contact fraction. The contact fraction from a density gradient which separated kidney mitochondrial subfractions was subjected to tryptic digestion as described in methods. SDS-polyacrylamide gel electrophoresis and immunodecoration was performed of the fraction after incubation without (lanes A, B), with 15 μg/mg (lanes C, D) or 30 μg/mg (lanes E, F) of trypsin. The presence or absence of 1.4% deoxycholate during the trypsin treatment is indicated by (+) or (−).

Discussion

Activity of kinases in the contact sites from mitochondria of different tissues

In agreement with recent findings [11,12] it was possible in the present investigation (Fig. 1) to separate a membrane fraction from disrupted mitochondria of liver, brain, and kidney which contained most of the hexokinase and mitochondrial creatine kinase activity. This fraction was located between the activity peaks of outer membrane (NADH–cytochrome-*c* reductase, rotenone insensitive, or monoamine oxidase) and inner membrane (succinate dehydrogenase) marker enzymes. This presumptive contact fraction from kidney and brain mitochondria was characterized to be a complex between hexokinase and the two boundary membranes by immunogold labelling of antibodies against the bound kinase (Fig. 3, 4). Based on these results we applied the method of contact site isolation to localize also other intermembranous kinases. In case of brain mitochondria we arrived at the result that, comparable to hexokinase, 80 to 90% of the creatine kinase activity which was loaded on the gradients was concentrated in the contact fraction, whereas adenylate kinase remained in the top fractions (partially soluble or included into outer membrane vesicles, Table I) and thus appeared to be located beyond the contacts (Figs. 1 and 2). This distribution of adenylate kinase was not due to inactivation of the enzyme in other fractions because more than 100% of the activity from kidney mitochondria was recovered in the top fractions. However, a significant amount of creatine kinase activity was lost from the kidney mitochondria during the isolation of the contact fraction as indicated by the unchanged specific activity in the contact sites compared to the starting material (Fig. 2). The activity profile of creatine kinase in the density gradient coincided with that of the enzyme protein (which was identified by specific antibodies against the mitochondrial isozyme, Fig. 8) suggesting that the concentration of the enzyme in the contact sites was not due to masking of the enzyme activity in fractions other than contacts. On the whole the results serve to conclude that the mitochondria contained always two fractions of hexokinase, creatine kinase and presumably nucleoside-diphosphate kinase: one part of the activity in the contact sites which remained bound to the membranes and another part of the creatine kinase and nucleoside-diphosphate kinase activity beyond the contact sites which was liberated during osmotic shock or in case of hexokinase was also bound to the free outer membrane. In contrast to the latter kinases the activity of adenylate kinase was exclusively located beyond the contact sites and was, therefore, either liberated or included into outer membrane vesicles upon swelling shrinking (Table I). This interpretation was in good agreement with the effect of digitonin on liver and brain mitochondria [11,12], where the activities of hexokinase and creatine kinase were only partially desorbed (30%

to 50%) by concentrations of digitonin which liberated most (80%) of the adenylate kinase.

Considering that the contact sites are dynamic structures which depend on the activity of the oxidative phosphorylation [3,4] it may be assumed that the amount of kinase activity which becomes organized in these sites depends as well on the metabolic state of the mitochondria. This has recently been observed for hexokinase [34].

Localization of peripheral kinases relative to the inner and outer membrane fragments

Alike contact sites from liver and brain [11,12] the contact fraction from kidney mitochondria appeared to be composed of closed vesicles in which the orientation of the inner and outer membrane resembled that in intact mitochondria (Figs. 9A and B). Accordingly, the location of kinases in the contact sites was like that known for intact mitochondria, namely hexokinase at the surface of the outer membrane [35] and creatine kinase [36], nucleoside-diphosphate kinase [22] and adenylate kinase [37] behind the outer membrane. These conclusions were drawn from the observed effects of the pore transport inhibition on the activity of the kinases (Table I) and the protection of creatine kinase against proteolysis (Fig. 10). In addition to this gross location, a specific organization of the surface bound and intermembranous kinases at the mitochondrial periphery was assumed because of the fact that adenylate kinase was enclosed into outer membrane vesicles while significant activity of the other three kinases was concentrated in the contact fraction. These conclusions concerning kinase organization based on biochemical work were recently supported by electron microscopy. In these experiments, which applied immunogold labelling with specific antibodies, a nonrandom distribution of hexokinase was also demonstrated in liver and brain mitochondria [10,12]. Furthermore, the distribution of creatine kinase in heart muscle mitochondria resembled that of hexokinase because it showed clusters of either Nitroblue tetrazolium [38] or specific antibodies [18] at the surface.

Metabolic functions of the specific organization of kinases at the mitochondrial periphery

Provided we accept the existence of a specific organization of kinases, a satisfying explanation for the preferential location in the contact sites may be given by the assumption of a different regulation of the outer membrane pore permeability inside these sites compared to pores beyond. In support to this idea, several authors [17,39] observed a regulatory effect of the outer membrane on creatine kinase activity. To explain the regulation of the outer membrane pore it seems pertinent to think of a property which is distinct from bacterial porins namely the voltage dependence. When reconstituted in planar bilayers the pore, at a voltage higher than 30 mV, is switched to a lower conducting state [40,41]. Taking into account the high ion conductivity of the pore, the existence of an electrochemical potential across the outer membrane can't be expected. However, in the contact sites it appears possible that the potential at the inner membrane influences the outer membrane, because, as analyzed by freeze-fracture, a distance between the two membranes of 1–2 nm can be assumed [3]. We can therefore think of a capacitative coupling between the two membranes like between two condensators. The transfer of positive charges through the inner membrane would than result in a field across the outer membrane with a polarity outside negative as proposed in Fig. 11.

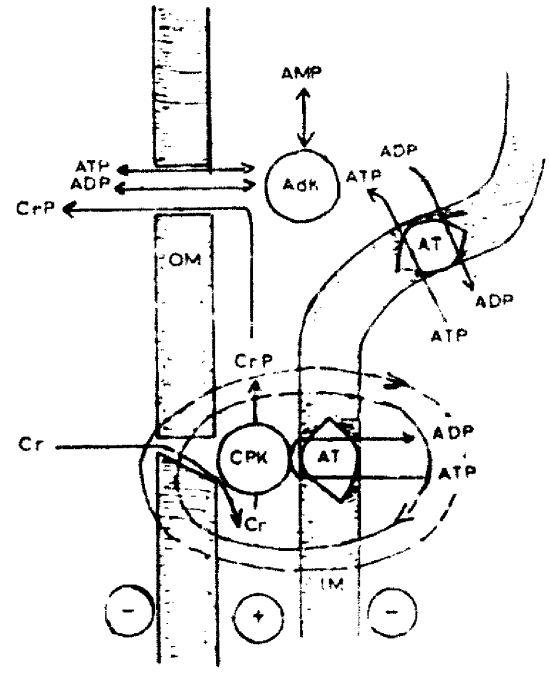

Fig. 11. Scheme showing the difference in transport properties of the pore inside and beyond the contact sites as regulated by the inner membrane potential. Abbreviations of enzyme names as in Fig. 1. AT, adenylate translocator; IM, inner membrane; OM, outer membrane; Cr, creatine; CrP, phosphocreatine. The pore inside the contact sites is influenced by the charge transfer across the inner membrane if one assumes a capacitative coupling between the two membranes like between two condensators. This changes the pore structure to the low conducting, cationically selective state, while the pores beyond the contacts remain high conducting and anionically selective.

We recently observed in intact mitochondria that the pore in the low conducting state becomes impermeable to ADP and ATP [33]. This means that the inner membrane potential might exert control on the adenine nucleotide and phosphocreatine transport in the contacts by switching the pore to the low conducting state and thereby prevent the equilibrium of anionic metabo-

lites with the cytosol. Supposing that low conducting pores are present in the contact sites we are then at the liberty to expect pores in the open state beyond the contacts which are characterized by high conductivity and low anion selectivity and would thus allow phosphocreatine to leave the outer mitochondrial compartment (Fig. 11). The regulation of the pore lends support to the concept of a separate compartment of adenine nucleotides in the intermembrane space [42] which would enhance the ATP translocation process because of the following reasons. The asymmetric ATP export peformed by the translocator is driven electrogenically by the mitochondrial membrane potential [43]. Thus, in competitive in vitro experiments a 13-times higher efflux of internal ATP could be maintained by the inner membrane potential [43]. If we consider the existence of an ATP/ADP quotient in the cytosol of 10 in liver [44] and much higher in muscle cells (because of binding of ADP to structure proteins [45]) it goes without saying that the electrogenic ATP translocation process would be at equilibrium and, therefore, would have low rates. However, it cannot be easily dismissed that under physiological conditions the flux rate of the mitochondrial ATP/ADP exchange is high, even in the presence of a high phosphorylation potential in the cytosol. A satisfying explanation to this problem could be given by a mechanism which would displace the energy driven ATP export from equilibrium. At this point the functional coupling of kinases to the translocator comes into effect. The existence of this interaction has been described for hexokinase in mitochondria from liver [7,46], muscle [47] and brain [48] and also for creatine kinase in muscle mitochondria by several authors [13–16,42]. Provided that we agree to the functional coupling between translocator and kinases it would be a promising mechanism to dislocate the electrogenic ATP export from equilibrium because the direct interaction with kinases would cause an immediate transfer of the phosphorylation energy from the exported ATP to metabolites which are not substrates for the translocator. The function of adenylate kinase is to stabilize the high cytosolic phosphorylation potential in case of ADP increase by converting it to ATP and AMP which is not substrate for the translocator. Therefore, the enzyme is aligned to unregulated pores outside the contacts in order to allow equilibration with the cytosolic ATP/ADP.

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References

1 Hackenbrock, C.R. (1968) Proc. Natl. Acad. Sci. USA 61, 598–605.
2 Van Venetie, R and Verkleij, A.J. (1982) Biochim. Biophys. Acta 692, 379–405.
3 Knoll, G. and Brdiczka, D. (1983) Biochim. Biophys. Acta 733, 102–110.
4 Klug, G., Krause, J., Östlund, A.K., Knoll, G. and Brdiczka, D. (1984) Biochim. Biophys. Acta 764.
5 Riesinger, I., Knoll, G., Brdiczka, D., Denis-Pouxviel, C., Murat, J.C. and Probst, I. (1985) Eur. J. Cell Biol. 36, 53.
6 Jacob, W. and Hertsens, R. (1986) 4th EBEC Short Reports, p. 208, Cambridge University Press, Cambridge.
7 Brdiczka, D., Knoll, G., Riesinger, I., Weiler, U., Klug, G., Benz, R. and Krause, J. (1986) in Myocardial and Skeletal Muscle Bioenergetics (Brautbar, N., ed.), pp. 55–69, Plenum Press, New York.
8 Bessman, S.P. (1966) Am. J. Med. 40, 740–749.
9 Denis-Pouxviel, C., Riesinger, I., Bühler, Ch., Brdiczka, D. and Murat, J.C. (1987) Biochim. Biophys. Acta 902, 335–348.
10 Weiler, U., Riesinger, I., Knoll, G. and Brdiczka, D. (1985) Biochem. Med. 33, 223–235.
11 Ohlendieck, K., Riesinger, I., Adams, V., Krause, J. and Brdiczka, D. (1986) Biochim. Biophys. Acta 860, 672–689.
12 Kottke, M., Adams, V., Riesinger, I., Bremm, G., Bosch, W., Brdiczka, D., Sandri, G. and Panfili, E. (1988) Biochim. Biophys. Acta 935, 87–102.
13 Saks, V.A., Rosenshtraukh, L.V., Smirnov, N. and Chazov, E.I. (1978) Can. J. Physiol. Pharmacol. 56, 691–706.
14 Jacobus, W.E. (1985) Annu. Rev. Physiol. 47, 707–725.
15 Bessman, S.P. and Carpenter, C.L. (1985) Annu. Rev. Biochem. 54, 831–862.
16 Wallimann, Th. and Eppenberger, H.M. (1985) in Cell and Muscle Motility, Vol. 6 (Shay, J.W., ed.), pp. 239–285, Plenum Press, New York.
17 Brooks, S.P.J. and Suelter, C.H. (1987) Arch. Biochem. Biophys. 257, 144–153.
18 Schlegel, J., Zurbriggen, B., Wegmann, B., Wyss, M., Eppenberger, H.M. and Wallimann, T. (1988) J. Biol. Chem. 263, 16963–16969.
19 Schnyder, T., Engel, A., Lustig, A. and Wallimann, T. (1988) J. Biol. Chem. 263, 16954–16962.
20 König, T., Kocsis, B., Mészaros, L., Nahm, K., Zoltán, S. and Horváth, I. (1977) Biochim. Biophys. Acta 462, 380–389.
21 Brdiczka, D., Dölken, G., Krebs, W. and Hofmann, D. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 731–743.
22 Jacobus, W.E. and Evans, J.J. (1977) J. Biol. Chem. 252, 4232–4241.
23 Bücher, Th., Luh, W. and Pette, D. (1964) Hoppe-Seyler Thierfelder Handbuch der physiologisch- und pathologischemischen Analyse, Bd VI/A, pp. 293–339, Springer, Berlin.
24 Sottocasa, G.L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) J. Cell Biol. 32, 415–438.
25 Rehncrona, S., Mela, L. and Siesjo, B.K. (1979) Stroke 10, 437–446.
26 Laemmli, U.K. (1970) Nature 227, 680–685.
27 Marcillat, O., Goldschmidt, D., Eichenberger, D. and Vial, C. (1987) Biochim. Biophys. Acta 890, 233–241.
28 Östlund, A.K., Göhring, U., Krause, J. and Brdiczka, D. (1983) Biochem. Med. 30, 231–245.
29 Chou, A.C. and Wilson, J.E. (1972) Arch. Biochem. Biophys. 151, 48–55.
30 De Pinto, V., Tommasino, M., Benz, R. and Palmieri, F. (1985) Biochim. Biophys. Acta 813, 230–242.
31 Rott, R. and Nelson, N. (1981) J. Biol. Chem. 256, 9224–9228.
32 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
33 Benz, R., Wojtczak, L., Bosch, W. and Brdiczka, D. (1988) FEBS Lett. 231, 75–80.

34 Adams, V., Brdiczka, D. and Bosch, W. (1988) 5th EBEC Short Reports, p. 281, Aberystwyth University College.
35 Felgner, P.L. and Wilson, J.E. (1977) Arch. Biochem. Biophys. 182, 282–294.
36 Jacobs, H., Heldt, H.W. and Klingenberg, M. (1964) Biochem. Biophys. Res. Commun. 16, 516–521.
37 Brdiczka, D., Pette, D., Brunner, G. and Miller, F. (1968) Eur. J. Biochem. 5, 294–304.
38 Bierman, W., Bernaert, I., De Blie, M., Bys, B. and Jacob, W. (1988) 5th EBEC Short Reports, p. 283, Aberystwyth University College.
39 Erickson-Viitanen, S., Viitanen, P., Geiger. P.J., Yang, W.C. and Bessman, S.P. (1982) J. Biol. Chem. 257, 14395–14404.
40 Colombini, M. (1979) Nature 279, 643–645.
41 Roos, N., Benz, R. and Brdiczka, D. (1982) Biochim. Biophys. Acta 686, 204–214.
42 Gellerich, F.N., Schlame, M., Bohnensack, R. and Kunz, W. (1987) Biochim. Biophys. Acta 890, 117–126.
43 Klingenberg, M. and Heldt, H.W. (1982) Metabolic Compartmentation (Sies, H., ed.), pp. 101–122, Academic Press, New York.
44 Soboll, S., Scholz, R. and Heldt, H.W. (1978) Eur. J. Biochem. 87, 377–390.
45 Hebisch, S., Sies, H. and Soboll, S. (1986) Pflügers Arch. 406, 20–24.
46 Gots, R.E. and Bessman, S.P. (1974) Arch. Biochem. Biophys. 163, 7–14.
47 Viitanen, P.V., Geiger, P.J., Erickson-Viitanen, S. and Bessman, S.P. (1984) J. Biol. Chem. 259, 9679–9684.
48 Inui, M. and Ishibashi, S (1979) J. Biochem. 85, 1151–1156.