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The role of contact sites between inner and outer mitochondrial membrane in energy transfer

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Three functions have been suggested to be localized in contact sites between the inner and the outer membrane of mitochondria from mammalian cells: (i) transfer of energy from matrix to cytosol through the action of peripheral kinases; (ii) import of mitochondrial precursor proteins; and (iii) transfer of lipids between outer and inner membrane. In the contact site-related energy transfer a number of kinases localized in the periphery of the mitochondrion play a crucial role. Two examples of such kinases are relevant here: (i) hexokinase isoenzyme I which is capable of binding to the outer aspect of the outer membrane; and (ii) the mitochondrial isoenzyme of creatine kinase which is localized in the intermembrane space. Recently, evidence was presented that both hexokinase and creatine kinase are preferentially localized in contact sites (Adams, V. et al. (1989) Biochim. Biophys. Acta 981, 213–225). The aim of the present experiments was two-fold. First, to establish methods which enable the bioenergetic aspects of energy transfer mediated by kinases in contact sites to be measured. In these experiments emphasis was on hexokinase, while ^{31}P-NMR was the major experimental technique. Second, we wanted to develop methods which can give insight into factors playing a role in the formation of contact sites involved in energy transfer. In the latter approach, mitochondrial creatine kinase was studied using monolayer techniques.

Introduction

Over 20 years ago, Hackenbrock [1] observed contacts between the inner and outer membrane in thin sections of rat liver mitochondria. He suggested that these sites of contact might serve a role in the coupling of the activity of certain kinases localized in the intermembrane space with the ATP-ADP carrier. At present, there is compelling evidence in support of Hackenbrock's original suggestion. Moreover, it has recently become evident that a number of additional mitochondrial functions are concentrated in intermembrane contact sites.

To date, three major processes have been proposed to be associated with contact sites. First, contact sites seem to create a functional micro-compartment in which a number of kinases localized in the mitochondrial periphery gain preferred access to ATP synthesized in the matrix through oxidative phosphorylation [2–7]. Secondly, contact sites are the site of import for most mitochondrial precursor proteins [8–10], especially of those precursor proteins which are imported in a membrane potential-dependent manner. Thirdly, it has been proposed that flow of phospholipids between the outer and the inner membrane, and vice versa, takes place at contact sites [11,12]. Import of precursor proteins and flow of phospholipids between the two mitochondrial boundary membranes are obligatory steps in the biogenesis of the mitochondrion.

As indicated above, close contacts between inner and outer membrane of mammalian mitochondria seem to create conditions which favor a rapid and efficient transfer of chemical energy from matrix ATP to a phosphoryl acceptor outside the inner membrane. The phosphotransferase reaction is catalyzed by kinases which can either be associated with the outer aspect of the outer membrane or be localized in the intermem-

Abbreviations: HK I, hexokinase isoenzyme I; Mi-CK, mitochondrial isoenzyme of creatine kinase; EM, electron microscopy; PCr, phosphocreatine; Cr, creatine.

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brane space. Examples of outer membrane-bound kinases are hexokinase isoenzyme I [5,18] and glycerol kinase [19]. Both are bound to the outer membrane pore protein, porin [19–21]. Examples of intermembrane space kinases which are relevant in this context are the mitochondrial isoenzyme of creatine kinase [5,7,15] and nucleoside-diphosphate kinase [5]. The above picture is based on two lines of evidence. First, functional experiments suggested a preferential use of matrix ATP over cytosolic ATP by bound hexokinase I [2,3,22] and mitochondrial creatine kinase [4,23–25]. Secondly, subfractionation of mitochondrial membranes using sucrose density centrifugation showed that bound hexokinase and mitochondrial creatine kinase were enriched in a membrane fraction with a density intermediate between that of the outer and the inner membrane [5,26]. Morphological analysis by EM revealed that the intermediate density fraction consisted of inner membrane vesicles inside outer membrane vesicles joined by a contact site [26]. The enrichment of hexokinase I in the contact fraction was further supported by the finding that remnants of the outer membrane remaining after digitonin treatment still contain the major part of the mitochondrion associated enzyme [6,26]. Also, immunogold labeling and cytochemical EM studies have provided evidence for a preferential localization of bound hexokinase I [27,28] and mitochondrial creatine kinase [7,15], respectively, in contact sites between inner and outer membrane.

We have recently started to develop experimental procedures which are aimed at getting a better understanding of the functional and structural properties of intermembrane contacts involved in energy transfer from matrix to cytosol. An adequate description of the functional properties of contact sites requires knowledge of both kinase activity and mitochondrial energy status. Results are presented on the development of a ^{31}P-NMR method, which allows the simultaneous measurement of these two parameters. The procedure was optimized using yeast hexokinase, which shows no binding to (rat liver) mitochondria.

Essentially nothing is known about the factors involved in the formation of contact sites. For mitochondrial creatine kinase, Wallimann and co-workers [30,34] recently proposed an important role for the octameric form of the enzyme itself in creating an intermembrane contact involved in energy transfer by simultaneously interacting with the inner leaflet of the outer membrane and the outer leaflet of the inner membrane. To test this hypothesis, monolayer experiments were performed. Recently, Demel et al. [16] have used the monolayer technique to demonstrate the formation of contacts between vesicles prepared from inner mitochondrial membrane lipids and monolayers of outer mitochondrial membrane lipids by the mitochondrial precursor protein apocytochrome *c*. The present experiments indicate that octameric Mi-CK is also capable of forming a tight monolayer-vesicle contact.

Materials and Methods

Materials

Mitochondria were isolated from rat liver as described elsewhere [13]. Hexokinase I was purified from rat brain according to the procedure developed by Wilson and co-workers [14]. The mitochondrial isoenzyme of creatine kinase was isolated from chicken heart as described [15]. Yeast hexokinase, creatine kinase (type III from bovine heart) and α-chymotrypsin were from Sigma. Glucose-6-phosphate dehydrogenase was from Boehringer. All other biochemicals used were of the highest grade available and obtained from regular sources.

Methods

Enzyme activity tests. Hexokinase activity was measured at 25 °C by a coupled spectrophotometric assay using glucose-6-phosphate dehydrogenase [14]. Creatine kinase activity was measured as described in Ref. 15. Enzyme activities are expressed in units, i.e., μmol of substrate consumed/min.

Binding of hexokinase I to rat liver mitochondria. Rat brain hexokinase I was rebound to rat liver mitochondria by the following procedure. First, the enzyme was desalted on a Sephadex G-25 column to remove components of the storage buffer and reconcentrated by centrifugation in a Centricon-10 microconcentrator (Amicon). Appropriate amounts of hexokinase I were added to mitochondrial suspensions in 0.25 M sucrose supplemented with 5 mM $MgCl_2$. Binding was for 20 min at 0 °C. The extent of binding was quantified by spinning down the mitochondria and measuring hexokinase activity in pellet and supernatant.

Preparation of nonbindable hexokinase I. Upon purification, rat brain hexokinase I showed more than 90% rebindability to rat liver mitochondria. Bindable hexokinase I was converted to its nonbindable form by mild treatment with α-chymotrypsin as described in Ref. 29.

^{31}P-NMR. ^{31}P-NMR experiments of mitochondrial bioenergetics were performed at 121.49 MHz on a Bruker MSL-300 spectrometer. The medium (final vol. 8 ml) was: 200 mM mannitol, 30 mM sucrose, 25 mM Hepes, 10 mM succinate, 10 mM phosphate, 10 mM $MgCl_2$, 20 mM glucose, 1 mM EDTA and 2 μM rotenone at pH 7.4. A flat-bottom, 20 mm diameter NMR tube was equipped with an insert allowing both stirring of the suspension by an air-driven turbine and supply of oxygen. Acquisition parameters were: (i) radio frequency pulses of 20 μs (60° pulse angle); (ii) recycle delay of 2 s; and (iii) spectral width of 5000 Hz.

Monolayer experiments

Interfacial measurements were performed at 25 °C in a thermostatically controlled box essentially as described earlier [16]. A Teflon dish of 5 ml was used and a subphase of 10 mM sodium phosphate (pH 7.0). Phospholipids were extracted from outer and inner membranes of rat liver mitochondria as described elsewhere [13]. Large unilamellar vesicles were prepared by an extrusion technique using filters with 400 nm pore size [17].

Results and Discussion

Consequences of hexokinase activity for mitochondrial bioenergetics

^{31}P-NMR offers attractive possibilities to determine concentrations of phosphorylated metabolites, mainly because of its non-invasive nature and the fact that all phosphorylated metabolites are measured simultaneously in a single spectrum. We have designed a set-up to measure mitochondrial bioenergetics in parallel with kinase activity. Yeast hexokinase, which is not able to bind to mitochondria (see below), was used to optimize the procedure.

Figs. 1 and 2 show a number of representative ^{31}P-NMR experiments. The concentration of ADP maintained by mitochondrial suspensions is often too low to be detectable by ^{31}P-NMR directly (e.g., Fig. 1D and 1E). Therefore, exogenously added creatine kinase (CK) was used to indirectly measure free ADP levels from its apparent equilibrium constant. Fig. 1A shows the result of CK (Sigma, type III from bovine heart) addition to the basic medium supplemented with 5 mM ATP and 8 mM Cr. The formation of phosphocreatine (PCr) and ADP was due to the establishment of CK equilibrium. Due to the (relatively) large amount of CK added, equilibrium was reached rapidly. An apparent equilibrium constant of CK (i.e., [ATP][Cr]/[PCr][ADP]) amounting to 207 was calculated from the spectrum in Fig. 1A. The series of spectra shown in Fig. 1B–E were obtained upon subsequent addition of rat liver mitochondria. Since the final density of the suspension was only 0.8 mg protein/ml, the very low internal volume of the mitochondria excludes a significant contribution from intramitochondrial metabolites to the spectra. Interestingly, a time-dependent increase in PCr and a concomitant decrease in ADP and inorganic phosphate were observed. Fig. 2A shows the time course of the concentrations of the various metabolites. After about

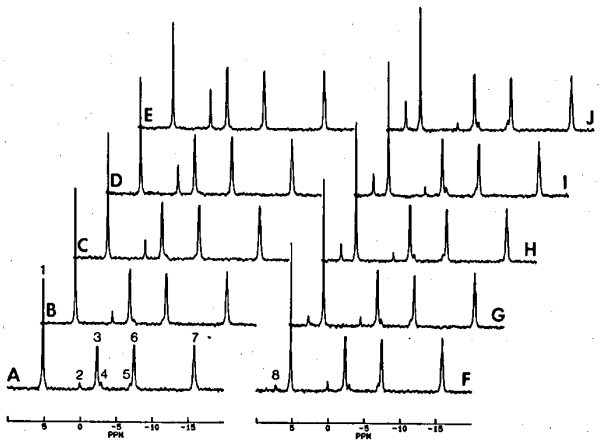

Fig. 1. ^{31}P-NMR of mitochondrial energetics. The NMR medium (see Materials and Methods) was supplemented with 8 mM creatine and 5 mM ATP. (A) After addition of 12 units bovine heart creatine kinase; (B–E) spectra obtained 1.1, 5.3, 9.6 and 20.3 min, respectively, after addition of 12 units creatine kinase and rat liver mitochondria to a final concentration of 0.8 mg protein/ml; (F–J) spectra obtained 1.1, 3.2, 5.3, 7.5 and 9.6 min, respectively, after addition of 12 units creatine kinase, 0.8 mg/ml rat liver mitochondria and 1.75 units yeast hexokinase. All spectra are the result of 64 scans. Peak assignments: 1, inorganic phosphate; 2, phosphocreatine; 3, 6 and 7, γ-, α- and β-phosphates in ATP, respectively; 4 and 5, β- and α-phosphates in ADP; 8, glucose 6-phosphate.

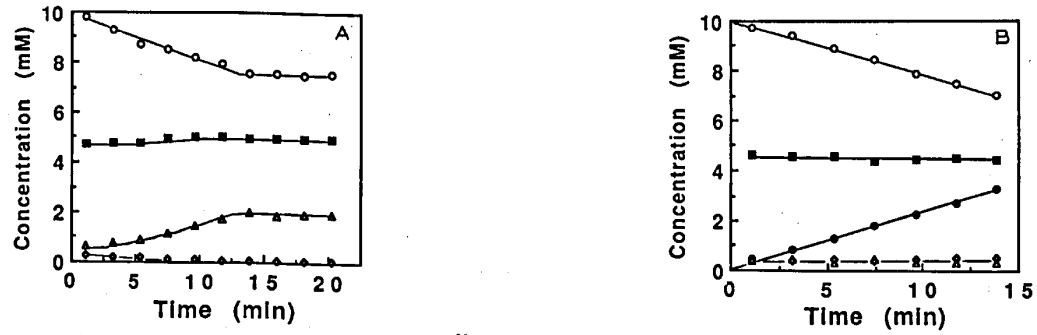

Fig. 2. Time-course of metabolite concentrations as measured by ^{31}P-NMR. (A) Evaluation of series shown partly in Figs. 1B–E; (B) evaluation of series shown partly in Figs. 1F–J. Peak intensities obtained by integration were converted to concentrations by comparison with the peak intensities of known amounts of the appropriate metabolite obtained under identical experimental conditions, except that hexokinase and creatine kinase were omitted. ADP concentration was either measured as above or (when very low) calculated from the creatine kinase equilibrium (see text). Symbols: ○, inorganic phosphate; ■, ATP; △, PCr; ◇, ADP; and ●, glucose 6-phosphate.

13 min, a new steady state was established. Synthesis of ATP through oxidative phosphorylation led to reduced levels of ADP. In parallel, restoration of the CK equilibrium resulted in additional formation of PCr. The major net effect of these activities is phosphorylation of Cr to PCr at the expense of inorganic phosphate.

A completely different picture arises when the above experiment is conducted in the presence of yeast hexokinase, as demonstrated in Fig. 1F–J and Fig. 2B. First, hexokinase activity resulted in the formation of glucose 6-phosphate. Its rate was linear with time and amounted to 0.22 $mM \cdot min^{-1}$. This is equivalent to a total activity of 1.76 units hexokinase, which is virtually identical to the activity of 1.75 units as determined by the spectrophotometric assay. Second, the levels of PCr and ADP remained essentially unchanged, indicative of a substantially lower extramitochondrial phosphate potential as compared to the absence of hexokinase. Glucose 6-phosphate formation exclusively made use of ATP from oxidative phosphorylation. This was evidenced by the equimolarity between the synthesis of glucose 6-phosphate and the decrease of inorganic phosphate, while the levels of ATP, ADP and PCr (and Cr) remained constant.

The above experiments demonstrate that quantitative information on both hexokinase activity and on the extramitochondrial energy status can be obtained simultaneously by ^{31}P-NMR. We are currently extending these studies to rat brain hexokinase I, using a combination of oxygraph, enzyme kinetic and ^{31}P-NMR techniques. As illustrated in Fig. 3, rat brain HK I showed a high degree of binding to rat liver mitochondria. Mild proteolysis with chymotrypsin eliminated binding completely. The loss in bindability of HK I is due to removal of a small N-terminal fragment [29], which is crucial for binding. The activity of the enzyme remained unaffected. Therefore, chymotrypsin-treated HK I can serve as a perfect control for highly bindable HK I. Yeast hexokinase also shows no binding to rat liver mitochondria (Fig. 3), but its kinetic parameters are too different from those of HK I to represent a legitimate control. Preliminary studies indicate that the experimental conditions (e.g., concentrations of nucleotides) are largely decisive for the expression of functional differences between bindable and nonbindable HK I.

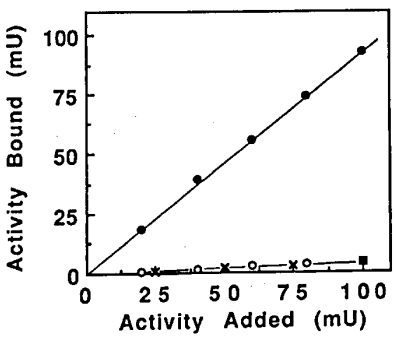

Fig. 3. Binding of hexokinase to rat liver mitochondria. For experimental details see Materials and Methods. Symbols: ●, rat brain hexokinase I; ○, α-chymotrypsin-treated rat brain hexokinase I; ×, yeast hexokinase.

Membrane contacts induced by mitochondrial creatine kinase

The mitochondrial isoenzyme of creatine kinase (Mi-CK) exists in two oligomeric forms, i.e., as an octamer and as a dimer [15,30]. The octameric species is the most abundant one. The enzyme seems to be enriched in intermembrane contact sites. Here we address the possibility that octameric Mi-CK is capable of interacting with two opposing membrane interfaces simultaneously, thus creating an intermembrane contact by itself.

Since Mi-CK is a cationic protein electrostatic interactions with negatively charged components have been implied to explain its binding to the inner mitochondrial membrane [31,32] and to cardiolipin-containing model membranes [33]. The latter study led to the postulation that cardiolipin is the receptor for Mi-CK at the inner mitochondrial membrane. It is interesting to note that cardiolipin is found in significant amounts in the outer membrane as well [13].

The monolayer technique was used to study the interaction of octameric chicken heart Mi-CK with various mitochondrial lipid systems. Both in monolayers of inner and outer membrane phospholipids, Mi-CK caused an increase in surface pressure indicative of penetration of the enzyme in between phospholipid molecules (data not shown). This implies that Mi-CK can in principle interact with both the inner and the outer membrane. Next we investigated whether Mi-CK is able to form contacts between a phospholipid monolayer and large unilamellar vesicles added to the subphase. Surface pressure served as a measure of the insertion of Mi-CK into the monolayer, while surface radioactivity measurements were used to detect the binding of the radiolabeled vesicles to the monolayer. A typical experiment is illustrated in Fig. 4. Injection of Mi-CK led to an increase in surface pressure. Replacing the subphase by protein-free buffer was without significant effect on the surface pressure. The same holds for the subsequent addition of vesicles. Interestingly, however, surface radioactivity started to increase immediately upon vesicle addition, reaching a maximum after approx. 50 min. Washing the subphase only slightly reduced the surface radioactivity, demonstrating a strong octameric Mi-CK mediated monolayer-vesicle contact. It is important to note that control experiments performed in the absence of Mi-CK showed no significant

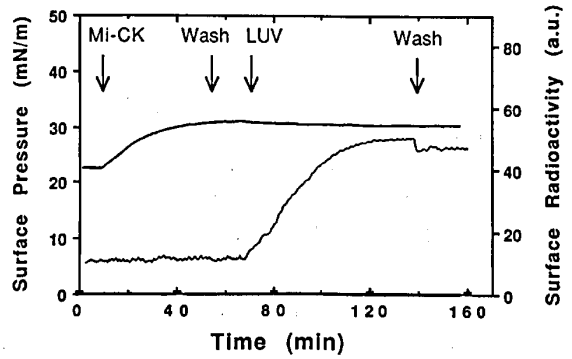

Fig. 4. Mi-CK-mediated contact formation between a monolayer of outer membrane phospholipids and large unilamellar vesicles of inner membrane phospholipids. Inner membrane phospholipid vesicles were labeled with 1-palmitoyl-2-[1-^{14}C-oleoyl]phosphatidylethanolamine. At the indicated points, 50 μg octameric Mi-CK/ml or 50 μM vesicles were added to the subphase. Where indicated the subphase was washed with 10 vols. of 10 mM NaP_i (pH 7.0) buffer. Upper trace, surface pressure; lower trace, surface radioactivity. For other details see Materials and Methods.

increase in surface radioactivity upon vesicle addition.

The monolayer experiments seem to indicate that Mi-CK indeed has the capacity to create a contact between two membrane interfaces. At present, we are investigating the specificity of the observed Mi-CK-induced contact, both in model and mitochondrial membranes. These experiments will give further insight into the physiological relevance of the present data for the formation of functional contact sites involving mitochondrial creatine kinase.

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