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Mitochondrial Creatine Kinase Mediates Contact Formation between Mitochondrial Membranes*

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Purified mitochondrial creatine kinase (Mi-CK) (EC 2.7.3.2) from chicken heart was shown to interact simultaneously with purified inner and outer mitochondrial membranes, thereby creating an intermembrane contact. Inner and outer mitochondrial membranes were purified from rat liver and thus were fully devoid of Mi-CK. Intermembrane contact formation was demonstrated by measuring the binding of inner membrane vesicles to outer membranes spread at the air-water interface. Mi-CK also mediated intermembrane adhesion when membranes formed with total lipid extracts of both membranes were used, pointing to the role of lipids as potential membrane anchors of Mi-CK in the mitochondrial intermembrane space. Other enzymes of the intermembrane space that (like Mi-CK) are also cationic, as well as cytosolic isoenzymes of creatine kinase, failed to induce contact formation. Thus, of the proteins tested, membrane contact formation was specific for Mi-CK. The two oligomeric forms of Mi-CK (octamer and dimer) differed in their ability to mediate intermembrane adhesion, the octamer being more potent. Highly basic peptides, *i.e.* poly-L-lysines, were shown to strongly interact with membranes formed with lipid extracts of mitochondrial membranes: they both induced intermembrane binding and fusion. Interestingly, the extent of contact formation mediated by poly-L-lysines was lower than that of octameric Mi-CK. The implications of these findings on the function and localization of Mi-CK and on the structure of the mitochondrial intermembrane compartment are discussed.

Creatine kinase (EC 2.7.3.2) isoenzymes catalyze the reversible transfer of the phosphoryl group from phosphocreatine to ADP, thus regenerating ATP. Creatine kinase genes are expressed in several tissues with high fluctuating energy turnover, *e.g.* skeletal and cardiac muscle, brain and photoreceptor cells, and spermatozoa (1). Several isoenzymes of creatine kinase have been characterized: brain-type and muscle-type as well as hybrid muscle/brain creatine kinase isoenzymes are mainly cytosolic and are always dimeric (see Refs. 1 and 23). The mitochondrial creatine kinase (Mi-CK)[1] isoenzymes are restricted to mitochondria and have been shown to exist in two oligomeric forms: as a dimeric and an octameric molecule (2). The specific subcellular location of creatine kinase isoenzymes has been interpreted in terms of the phosphocreatine/creatine circuit (1) as a means to ensure a high phosphorylation potential at sites of ATP consumption and to facilitate phosphocreatine synthesis at sites of ATP production.

Evidence has accumulated that the interaction of the various creatine kinase isoenzymes with cellular structures plays an important role in their function (reviewed in Ref. 1). For example, a physiologically significant portion of muscle-type creatine kinase is specifically bound at the myofibrillar M-line, where it is the principal component of the m-bridges and acts as a potent intramyofibrillar ATP-regenerating system (see Ref. 3).

Mi-CK is located in the mitochondrial intermembrane space, where it binds to the outer leaflet of the inner mitochondrial membrane (4) and is functionally coupled to oxidative phosphorylation (5). Mi-CK was shown to be enriched in the fraction of contact sites between the inner and outer membranes of disrupted mitochondria (6). In the contact sites, a simultaneous interaction of Mi-CK with the inner membrane adenine nucleotide translocator and the outer membrane porin has been proposed (7, 8). Since it was recently established that Mi-CK is indeed able to interact with outer mitochondrial membranes (9), we decided to investigate whether Mi-CK molecules can interact simultaneously with inner and outer mitochondrial membranes.

This work reports the specific ability of Mi-CK to interact simultaneously with inner and outer mitochondrial membranes, thereby creating an intermembrane contact by itself. The consequences of this property on the function, localization, and regulation of the enzyme *in vivo* and the possible structural role of Mi-CK in the formation of mitochondrial contact sites between the inner and outer mitochondrial membranes are discussed.

EXPERIMENTAL PROCEDURES

All reagents used were of analytical grade quality.

Enzymes and Peptides—All proteins were stored at −70 °C in 25 mM sodium phosphate, pH 7.0, 150 mM NaCl, 2 mM β-mercaptoethanol, 0.2 mM EDTA (protein buffer) and were rapidly thawed directly before the experiment. Creatine kinase isoenzymes were purified as described (2, 10, 11). Octameric Mi-CK was taken from a sample at

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[1] The abbreviations used are: Mi-CK, mitochondrial creatine kinase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LUVs, large unilamellar vesicles; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

high protein concentration (≥3 mg/ml) that consisted of ≥90% octameric Mi-CK. A dimeric Mi-CK sample containing 85–90% dimeric enzyme was generated directly before the experiment by incubation of octameric Mi-CK for ≥8 h at low protein concentration (0.4 mg/ml) with a mixture of 4 mM ADP, 5 mM $MgCl_2$, 20 mM creatine, 50 mM KNO_3, 150 mM NaCl, 25 mM sodium phosphate, pH 7.2 (dimerizing buffer), which causes octamers to dissociate into dimers (7). Dimeric Mi-CK was reoctamerized by dialysis against protein buffer and concentration by ultrafiltration with an Amicon Centricon microconcentrator. Purified mitochondrial adenylate kinase (12) was a gift of Prof. G. E. Schulz (Universität Freiburg, Federal Republic of Germany). Poly-L-lysines and cytochrome *c* (horse heart type VI) were purchased from Sigma. Apocytochrome *c* was prepared from cytochrome *c* by removal of the heme moiety (13) and subjection to a renaturation procedure (14).

Membranes and Lipids—Isolation and subfractionation of rat liver mitochondria were performed according to Ref. 15. Membrane fractions were finally suspended in 220 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4 (MSH buffer) at a concentration of 5–10 mM lipid phosphorus. Lipids were extracted from the different membrane fractions as described (15), dissolved in $CHCl_3/CH_3OH$ (4:1, v/v) to a concentration of 1–5 mM lipid phosphorus, and stored under N_2 at −20 °C. Lipid phosphorus was measured by the method of Fiske and SubbaRow (16) after destruction of phospholipids with 70% perchloric acid. Inner mitochondrial membrane vesicles were labeled with di[^{14}C]oleoylphosphatidylcholine (Amersham Corp.) using the phosphatidylcholine transfer protein (17) (final transfer of (labeled and unlabeled) phosphatidylcholine: 3–5 mol % mitochondrial lipid). Large unilamellar vesicles (LUVs) were labeled by adding di[^{14}C] oleoylphosphatidylcholine to the lipid extract of inner mitochondrial membranes (maximum of 1 mol %) and were produced in 10 mM sodium phosphate, pH 7.0, by the extrusion procedure (18) using 0.4-μm filters.

Monolayer Techniques—All monolayer experiments were carried out in Teflon dishes at 25 °C in a thermostatically controlled box. Dishes, etc. are described in detail in Ref. 9. The surface pressure was measured by the Wilhelmy method (see Ref. 19). The amount of radiolabel at the interface was determined by recording the surface radioactivity with a gas-flow detector (19) or by collection of the monolayer followed by scintillation counting. Monomolecular lipid layers were spread from a $CHCl_3/CH_3OH$ (4:1, v/v) lipid solution to give the desired initial surface pressure on a subphase of 10 mM sodium phosphate, pH 7.0. Outer mitochondrial membranes were spread at the air-water interface by the method of Verger and Pattus (20) as described (9) on a subphase of MSH buffer. In all experiments, the subphase was continuously stirred with a magnetic bar. The subphase was washed by injecting and ejecting 6 subphase volumes of buffer solution at opposite sides of the dish. Proteins and vesicles were added through a hole in the Teflon chamber connected to the subphase. Proteins and peptides were added to the subphase in the following concentrations: 0.13 μM octameric Mi-CK, 0.52 μM dimeric Mi-CK, 0.2 μM mitochondrial adenylate kinase, 0.2 μM cytochrome *c*, 0.2 μM apocytochrome *c*, 0.73 μM brain-type creatine kinase, 0.38 μM muscle-type creatine kinase, 0.2 μM poly-L-lysine. Concentrations were chosen to be higher than those at which maximal increase in surface pressure occurs (Refs. 9 and 21 and this work), except for mitochondrial adenylate kinase, for which the concentration inducing maximal surface pressure increase is not known. Inner membrane vesicles and LUVs were injected into the subphase to a final concentration of 50 μM lipid phosphorus. The volumes of actual injections into the subphase were always ≤2% of the total subphase volume, except for dimeric Mi-CK, for which the subphase volume was increased by ≤10%. In the latter case, original subphase volume was restored after equilibration of surface pressure. The interaction of dimeric and octameric Mi-CKs with monolayers has been shown not to be influenced by this manipulation (9).

Other Methods—Protein was determined by the bicinchoninic acid assay of Pierce Chemical Co. including 0.1% (w/v) SDS and using bovine serum albumin as a standard. SDS-PAGE was performed with Phastgel gradient 10–15% polyacrylamide (PhastSystem, Pharmacia LKB Biotechnology Inc.). Proteins were stained by the diamine silver staining method (40). The oligomeric state of Mi-CK was determined by gel filtration on a fast protein liquid chromatography Superose 12 HR 10/30 column (Pharmacia LKB Biotechnology Inc.) as described (7). Two-dimensional thin-layer chromatography was performed according to Ref. 15.

RESULTS

Mediation of Intermembrane Adhesion by Mi-CK—The aim of this work was to assess the ability of Mi-CK and other proteins to interact simultaneously with two mitochondrial membranes. In the experiments performed, one membrane is represented either by a monolayer of outer mitochondrial membrane lipids or by outer mitochondrial membranes spread at the air-water interface (Fig. 1*A*, *OM*), whereas the other membrane is represented by vesicles (LUVs of inner mitochondrial membrane lipids or inner mitochondrial membrane vesicles) added to the buffer compartment (Fig. 1*C*, *IM*). Experiments were performed as follows (Figs. 1 and 3). The particular protein was injected below a spread membrane or a phospholipid monolayer (Figs. 1*A* and 3, *octameric Mi-CK*, *adenylate kinase*, and *dimeric Mi-ck arrows*). An increase in surface pressure is indicative of protein-membrane interaction. After equilibration of surface pressure, free and loosely bound proteins were removed by washing the subphase with protein-free buffer (Figs. 1*B* and 3, *subphase wash arrow*). Radioactively labeled vesicles were then injected into the subphase (Figs. 1*C* and 3, *IM-ves.* or *LUVs arrow*); an increase in surface radioactivity is a measure of the interaction of monolayer-bound protein with the vesicles. Finally, the subphase was washed with vesicle-free buffer (Figs. 1*D* and 3, *wash arrow*) to allow quantification of specific vesicle binding.

Outer mitochondrial membranes were spread at the air-water interface and collected for analysis on SDS-PAGE. Fig. 2 shows that the SDS-PAGE patterns of purified outer mitochondrial membrane vesicles before (Fig. 2, *lane a*) and after (*lane b*) spreading at the interface were almost indistinguishable. Addition of octameric Mi-CK to the subphase below a spread outer membrane led to an increase in surface pressure (Fig. 3*A*), indicative of the interaction of Mi-CK with the spread outer membranes (9). The subphase was replaced with protein-free buffer, and radioactively labeled

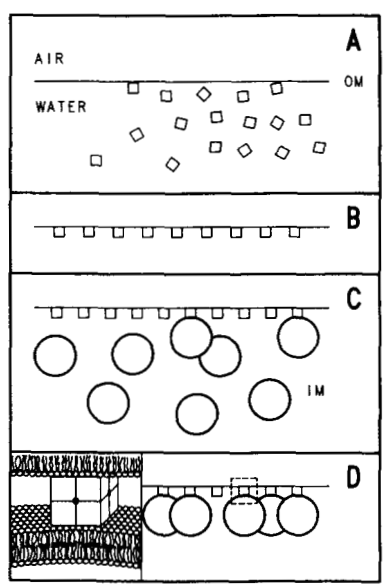

FIG. 1. **Scheme of experimental protocol.** A monolayer (*OM*) has been spread at the air-water interface, and proteins (*squares*) were injected into the subphase (*A*); the subphase has been washed with fresh buffer (*B*); vesicles (*IM*) have been injected into the subphase (*C*); and the subphase has been washed (*D*). *D*, *inset*, magnification of the region shown with *dashed lines* in *D* depicting a model of the simultaneous interaction of octameric Mi-CK with inner and outer mitochondrial membranes. For simplicity, only the lipid components of the membranes are shown.

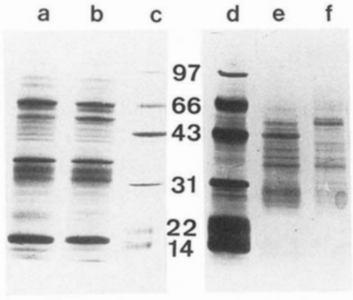

FIG. 2. **SDS-PAGE analysis of purified inner and outer membranes and of membranes at air-water interface.** SDS-PAGE was performed and proteins were stained as described under "Experimental Procedures." *Lanes a* and *b*, outer membrane vesicles (200 ng of protein) before and after spreading at the air-water interface, respectively; *lanes c* and *d*, low molecular mass standards (in kilodaltons) from Bio-Rad; *lane e*, polypeptide pattern (50 ng of protein) of the air-water interface after Mi-CK-mediated binding of inner membrane vesicles to a monolayer formed with lipid extracts of outer mitochondrial membranes; *lane f*, inner membrane vesicles (50 ng of protein). Note the strong similarity between *lanes a* and *b* and between *lanes e* and *f*. The band appearing in *lane e* around 43 kDa represents monolayer-bound Mi-CK and is not present in *lane f* since liver mitochondria are fully devoid of Mi-CK.

inner membrane vesicles were injected into the subphase. Surface radioactivity increased immediately, reaching a stable level after 60–80 min and largely remaining at the interface after subphase wash (Fig. 3*A*). To ensure that the increase in surface radioactivity reflects the binding of labeled vesicles to the spread membrane and not the transfer of label to the air-water interface by other means (*e.g.* due to enzyme activities present in the membrane fractions), the same experiment was performed using a monolayer of outer membrane phospholipids instead of spread outer membranes. As expected, surface radioactivity increased upon vesicle injection and was retained after the subsequence subphase wash (Fig. 4, *inset*, *octameric Mi-CK* (*A*) *column*). This interface was collected and analyzed by SDS-PAGE and two-dimensional thin-layer chromatography. The SDS-PAGE pattern (Fig. 2, *lane e*) revealed that both Mi-CK and inner membrane vesicles were present at the interface. Autoradiography of the thin-layer plate revealed a single spot at the phosphatidylcholine position (not shown). The results of the analysis confirmed that surface radioactivity corresponds to the presence of entire inner membrane vesicles at the air-water interface.

Octameric Mi-CK also mediated the formation of close contacts between membrane interfaces formed with total lipid extracts from mitochondrial membranes: radioactively labeled LUVs of inner membrane phospholipids were bound to a monolayer of outer membrane phospholipids in a Mi-CK-dependent manner (Figs. 3*C* and 4, *octameric Mi-CK column*). Experiments performed in the absence of protein showed no increase in surface radioactivity (Fig. 4, *no protein column*), demonstrating that the formation of a close intermembrane contact is indeed due to the ability of octameric Mi-CK to interact simultaneously with two membranes.

Specificity of Membrane Contact Formation Induced by Mi-CK—To elucidate the specificity of the Mi-CK-induced contact formation, the behavior of other enzymes was investigated in the same system (Figs. 3*B* and 4). The enzymes were cationic enzymes from the mitochondrial intermembrane space and anionic cytosolic isoenzymes of creatine kinase: (*i*) mitochondrial adenylate kinase, which is found free in the intermembrane space, and, in contrast to Mi-CK, is not accumulated at contact sites (6); (*ii*) cytochrome *c*, which is located at the outer leaflet of the inner mitochondrial membrane (22); (*iii*) apocytochrome *c*, the heme-free and almost

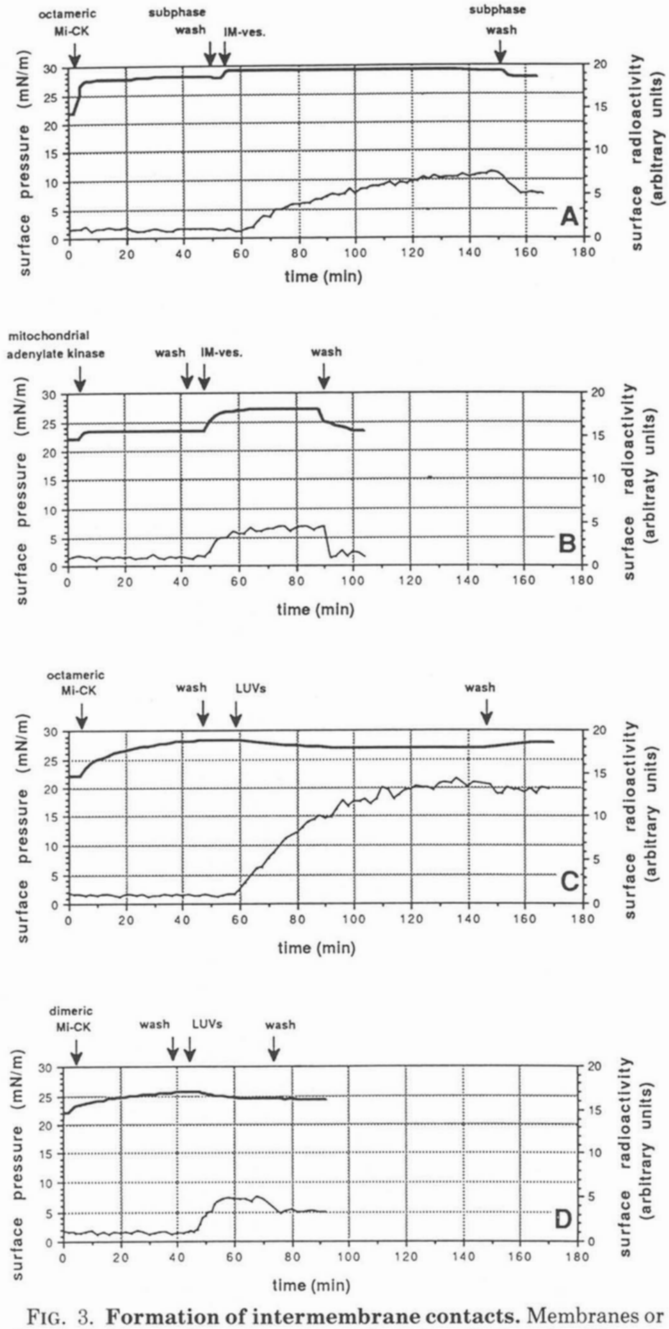

FIG. 3. **Formation of intermembrane contacts.** Membranes or phospholipid monolayers were spread as described under "Experimental Procedures" up to a surface pressure of 22 millinewtons/m. Additions to the subphase and subphase washes are indicated by *arrows*. Actual tracings are shown of surface pressure (*upper lines*) and surface radioactivity (*lower lines*) during the interaction of inner mitochondrial membrane vesicles (*IM-ves*) with spread outer mitochondrial membranes (*A* and *B*) and of inner membrane phospholipid LUVs with outer membrane phospholipid monolayers (*C* and *D*) as mediated by octameric Mi-CK (*A* and *C*), mitochondrial adenylate kinase (*B*), and dimeric Mi-CK (*D*). The surface radioactivity signal *minus* background remaining at the final stage of the experiment is a quantitative measure of protein-induced monolayer-vesicle contact.

completely unfolded precursor of cytochrome *c* (13); (*iv*) brain-type and (*v*) muscle-type cytosolic isoenzymes of creatine kinase (23). Most experiments were performed using membranes formed with lipid extracts from inner and outer mitochondrial membranes. As judged from the surface radioactivity retained at the air-water interface after washing the subphase with vesicle-free buffer, the capacity of these enzymes to bring membranes into close contact was negligible

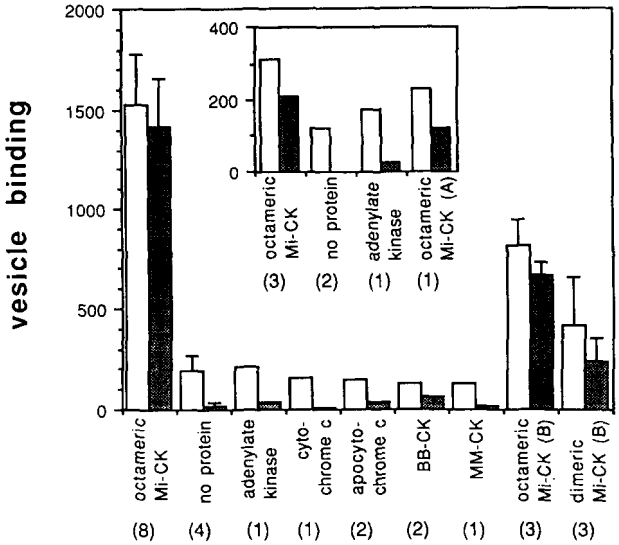

FIG. 4. **Extent of intermembrane contact formation displayed by different proteins.** Experiments were performed as described under "Experimental Procedures." The amount of lipid bound to the monolayer was calculated from the surface radioactivity signal at the final stage of the experiment as described for Fig. 3. Columns show the binding of inner membrane phospholipid LUVs to outer membrane phospholipid monolayers (*main figure*) and of inner mitochondrial membrane vesicles to spread outer membranes (*inset*) as mediated by different proteins before (*white columns*) and after (*shaded columns*) the second subphase wash. Vesicle binding is expressed as picomoles of total lipid phosphorus/square centimeter of monolayer. Values in *parentheses* indicate the number of experiments performed. *Bars* indicate the standard deviation. (*A*, *inset* at *right*), binding of inner mitochondrial membrane vesicles to a monolayer of outer membrane phospholipids; (*B*, *main figure* at *right*), the subphase contained, additionally, 0.4 mM MgADP, 2 mM creatine, 5 mM KNO_3, 15 mM NaCl before the first subphase wash. *BB-CK* and *MM-CK*, brain-type and muscle-type creatine kinases, respectively.

when compared to that of Mi-CK (Figs. 3*B* and 4).

Differential Ability of Octameric and Dimeric Mi-CKs to Induce Intermembrane Adhesion—Mi-CK exists in two oligomeric forms: octamer and dimer (2, 24). The octamer is the most abundant oligomer *in vivo* (7, 25). Since both oligomeric forms differ in their ability to interact with mitochondrial and model membranes (7, 9), we investigated whether the dimer and octamer differ in their ability to bring membranes into close contact. The dimer showed a much lower ability to induce membrane contact formation than the octamer (Figs. 3*D* and 4, *dimeric Mi-CK* (*B*) *column*). Because injection of dimeric Mi-CK implies the addition of appreciable amounts of dimerizing buffer to the subphase, experiments with octameric Mi-CK in a subphase of this particular composition were performed. Also, in this case, the amount of LUVs bound to the monolayer was higher than in experiments with dimeric Mi-CK, but lower than in experiments without the addition of dimerizing buffer (Fig. 4, *octameric Mi-CK* (*B*) *column*). This result is not due to a dissociation of octamers into dimers since analysis of the subphases by gel permeation chromatography revealed that the octamer/dimer ratio did not change during experiments (not shown). In the next series of experiments, octameric Mi-CK was subjected to different treatments prior to injection into the subphase: (*i*) dissociation into dimers and reoctamerization, (*ii*) incubation at low protein concentration (0.4 mg/ml) in protein buffer; and (*iii*) incubation at high protein concentration (≥5 mg/ml) in dimerizing buffer (treatments *ii* and *iii* do not result in dimerization of octameric Mi-CK because both the presence of dimerizing agents and a low protein concentration are required for dimerization) (2, 26). The extent of contact formation induced by octameric Mi-CK was not influenced by the different treatments (not shown) (data included in Fig. 4, *octameric Mi-CK column*), confirming that the extent of contact formation is a function of the oligomeric state of Mi-CK and not the consequence of other modifications of the enzyme occurring during the dimerization procedure.

Interaction of Poly-L-lysines with Membranes: Different Mechanism of Membrane Contact Formation—Electrostatic interactions of cationic Mi-CK with negatively charged lipids are thought to promote the binding of Mi-CK to mitochondrial membranes (9, 27). Therefore, we investigated whether highly basic peptides, *i.e.* poly-L-lysines, can also induce membrane contact formation. Experiments were performed with poly-L-lysine of different molecular mass: 289, 36.5, and 13 kDa. In preliminary experiments, we established that subphase concentrations ≥0.1 μM poly-L-lysine induce maximal surface pressure increase (not shown). Upon injection underneath a monolayer of outer membrane phospholipids, all poly-L-lysine species tested induced a small increase in surface pressure (Fig. 5). Subsequent addition of inner membrane phospholipid LUVs resulted in: 1) an increase in surface radioactivity with kinetics similar to that observed in experiments with octameric Mi-CK and 2) an immediate and rapid increase in surface pressure up to the maximal surface pressure of phospholipid monolayers (Fig. 5). The latter observation indicates that fusion of LUVs to the monolayer occurred (28). The amount of lipid transferred to the monolayer by vesicle fusion (~50 pmol of lipid phosphorus/cm^2) is far too low to account for the increase in surface radioactivity (Fig. 5, *inset*). This fact led to the conclusion that poly-L-lysine mediated both fusion and binding of LUVs to the monolayer. Interestingly, the extent of vesicle binding was a function of the size of the particular poly-L-lysine (Fig. 5, *inset*). This finding is corroborated by the observation that injection of a

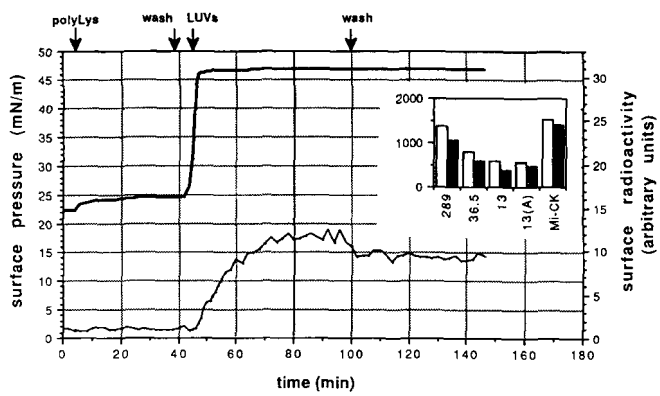

FIG. 5. **Intermembrane fusion and adhesion mediated by poly-L-lysine.** Phospholipid monolayers were spread as described under "Experimental Procedures" up to a surface pressure of 22 millinewtons/m. Additions to the subphase and subphase washes are indicated by *arrows*. Actual tracings are shown of surface pressure (*upper line*) and surface radioactivity (*lower line*) during the interaction between inner membrane phospholipid LUVs and outer membrane phospholipid monolayers mediated by 289-kDa poly-L-lysine (*polyLys*) (*main figure*). The *inset* shows the binding of LUVs to outer membrane phospholipid monolayers mediated by poly-L-lysine of different sizes and octameric Mi-CK before (*white columns*) and after (*shaded columns*) the second subphase wash. The amount of lipid bound to the monolayer was calculated from the surface radioactivity signal at the final stage of the experiment as described for Fig. 4 and is expressed as picomoles of total lipid phosphorus/square centimeter of monolayer. 289, 36.5, and 13, 289-, 36.5-, and 13-kDa poly-L-lysine; Mi-CK, octameric Mi-CK; (*A*), subphase concentration was 1 μM instead of 0.2 μM poly-L-lysine.

5-fold higher concentration of poly-L-lysine below the monolayer did not result in an increase in the amount of vesicles bound (Fig. 5, *inset, column 13(A)*). Poly-L-lysine showed a lower capacity to induce membrane contact formation than octameric Mi-CK (Fig. 5, *inset*).

DISCUSSION

In a previous study (9), it was shown that Mi-CK is able to interact equally well with both the inner and outer mitochondrial membranes. In this study, we demonstrated that Mi-CK molecules are able to interact simultaneously with two membrane interfaces, thereby creating an intermembrane contact.

Earlier work suggests that phospholipids are involved in the attachment of Mi-CK to mitochondrial membranes *in vivo* (9, 27). This study supports that notion since Mi-CK induced contact formation not only between freshly purified mitochondrial membranes, but also between membranes formed with total lipid extracts of mitochondrial membranes.

Several other enzymes (cytosolic isoenzymes of creatine kinase and basic enzymes of the mitochondrial intermembrane space) were examined in the experimental system described. Since none of them could induce membrane contact formation, the ability of Mi-CK to mediate intermembrane adhesion seems specific.

Additionally, there was a marked difference between octameric and dimeric Mi-CKs in their ability to mediate intermembrane adhesion. The capacity of dimeric Mi-CK to bind vesicles to a second membrane interface was substantially lower than that of octameric Mi-CK. It is possible that the capacity of dimeric Mi-CK to mediate membrane contact formation is even lower than was measured here since a small percentage ($\leq$15%) of octameric Mi-CK is always present in dimeric Mi-CK samples.

Earlier studies on poly-L-lysine-membrane interactions have shown that randomly coiled poly-L-lysine molecules undergo conformational changes upon interaction with acidic phospholipid vesicles (29) and that these compounds induce structural rearrangements of cardiolipin liposomes that result in the formation of tightly packed multilayered structures (30). The behavior of poly-L-lysine in our experimental system, in which natural lipid mixtures were used, is reminiscent of the above observations. Besides mediating vesicle binding to the monolayer, poly-L-lysine molecules also induced fusion of vesicles to the monolayer (Fig. 5). It is remarkable that octameric Mi-CK has an even higher capacity to mediate contact formation than the largest poly-L-lysine studied (Fig. 5, *inset*) in spite of the strong interaction of poly-L-lysine with lipids.

We propose that Mi-CK molecules behave similarly in the intermembrane space of mitochondria and in our *in vitro* system, *i.e.* that they interact simultaneously with the inner and outer membranes of mitochondria and possibly also with apposed crista membranes *in vivo*. It is very likely that the *in vivo* behavior of Mi-CK parallels the *in vitro* behavior because substantial parts of this study were done with membranes that closely resemble mitochondrial membranes (Fig 2 and Refs. 9, 15, and 31).

The results of this study have interesting implications for the structure of the mitochondrial intermembrane compartment. There is strong evidence that *in vivo*, all mitochondrial membranes are closely apposed and that the extensive intermembrane space normally seen in mitochondria is an artifact of the conventional specimen preparation for electron microscopy (*i.e.* aldehyde fixation and post-fixation in OsO_4) (32, 33). Additionally, several processes have been shown to occur at specific sites at which inner and outer mitochondrial membranes are in direct contact. These contact sites: (*i*) are involved in the exchange of energy metabolites between the mitochondrial and cytosolic compartments (34), (*ii*) are required for the import of mitochondrial precursor proteins (35, 36), and (*iii*) have been proposed to participate in the intramitochondrial translocation of phospholipids (37). The question of whether these processes occur at different or polyfunctional contact sites remains unanswered. Additionally, the structural nature of contact sites is virtually unknown. Mi-CK is enriched in the contact site fraction of disrupted mitochondria (6). Given the apposition of the inner and outer mitochondrial membranes *in vivo*, the membrane-binding properties of Mi-CK would almost inevitably lead to a simultaneous interaction with the apposed membranes. Thus, this study provides experimental evidence for a mechanism by which contact sites involved in energy transfer may be formed and/or stabilized.

The membrane-binding properties of Mi-CK also have implications for the *in vivo* function of the enzyme. The differences observed between dimeric and octameric Mi-CKs with respect to the formation of intermembrane contacts are in agreement with previous findings on the differential interaction of the oligomeric forms with membrane interfaces. Assuming that both oligomeric forms are physiologically relevant, octameric Mi-CK emerges as the oligomeric form that preferentially interacts with mitochondrial membranes. Octameric Mi-CK: (*i*) has a higher capacity to induce membrane contact formation than dimeric Mi-CK (this work); (*ii*) interacts with phospholipid monolayers with a higher affinity than dimeric Mi-CK, inducing a higher increase in surface pressure (9); and (*iii*) binds to mitoplasts over a wider pH range than dimeric Mi-CK (7).

The structure of octameric Mi-CK is consistent with its role in mediating contact formation. Structural analysis of octameric Mi-CK by electron microscopy shows a highly symmetric cube-like molecule with a side length of ~10 nm (24, 38). The octamer's top and bottom faces have similar binding characteristics as judged from the high adsorption potential to a variety of support films used in specimen preparation for electron microscopy, but they are distinct from the four side faces (38). We propose, on the basis of these experiments, that the top and bottom faces of octameric Mi-CK interact simultaneously with the two apposing membranes (Fig. 1*D*, *inset*). This would enable Mi-CK to be in direct contact with the inner membrane adenine nucleotide translocator and the outer membrane porin. These enzymes transport the creatine kinase substrates across the inner (ATP, ADP) and outer (creatine, phosphocreatine) membranes of the mitochondria as described in terms of the phosphocreatine/creatine circuit (1). A direct interaction of Mi-CK with these membrane proteins was already suggested by earlier studies, in which it was shown that Mi-CK is functionally coupled to oxidative phosphorylation (5, 39) and is regulated by the outer membrane porin (8). Finally, the different behavior of dimeric and octameric Mi-CKs toward membranes would enable regulation of the "coupled" creatine kinase reaction by shifts in the octamer/dimer ratio (7). This hypothesis could be proven if factors were discovered that influence not only the octamer/dimer ratio, but also the coupled creatine kinase reaction in mitochondria.

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