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In Vitro Complex Formation between the Octamer of Mitochondrial Creatine Kinase and Porin*

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An interaction of mitochondrial creatine kinase with purified outer mitochondrial porin (voltage-dependent anion channel) was shown by co-sedimentation assays as well as by gel permeation chromatography. Porin formed high M_r complexes with wild-type mitochondrial creatine kinase as well as with an N-terminal deletion mutant, lacking the first five N-terminal amino acids. The complexes were identified by creatine kinase activity in parallel with immunoblotting using specific antibodies against the two proteins. In addition, porin induced octamerization of the N-terminal creatine kinase mutant, which under the same conditions without porin, did not polymerize but remained more than 90% dimeric. Furthermore, binding of mitochondrial creatine kinase to porin affected the conductance of porin when reconstituted in "black membranes." At 10 mV the pore in the complex adopted a low conductance (1.5–2 nanosiemens) state, compared to the high conductance state (3–4 nanosiemens) of the free incorporated pores. The former state of the pore is known to be cationically selective. Thus, besides a specific structural interaction, a defined physiological function is assumed of the mitochondrial creatine kinase-porin complexes that are discussed here.

Creatine kinase (CK, EC 2.7.3.2)[1] catalyzes the reversible transfer of the high energy *N*-phosphoryl group from phosphocreatine to ADP. Three cytosolic and two mitochondrial isoforms of CK are known (for reviews, see Refs. 1 and 2). Part of the cytosolic isozyme activity associates with ATPases in subcellular structures such as the sarcoplasmic reticulum or the myofibrillar M-band, where CK is functionally coupled to Ca^{2+} pump (3, 4) and the actomyosin ATPase (5, 6), respectively. The CK isozymes are expressed in a tissue specific manner. CK mitochondrial isozymes (Mi-CK) are located in the mitochondrial intermembrane space (7) and are associated with the outer side of the inner membrane (8, 9). A functional interplay between mitochondrial and cytosolic isoforms of CK is thought to be important for the regulation of cellular energy homeostasis. The cytosolic enzymes re-phosphorylate the locally produced free ADP and increase creatine globally. The mitochondrial enzyme catalyses the back reaction of creatine to phosphocreatine at the expense of mitochondrial ATP (1, 2, 10–12).

The Mi-CK differs from its cytosolic counterparts in that it can form a highly ordered octameric oligomer from four dimers, whereas the cytosolic enzymes have a dimeric structure only. The Mi-CK octamers display a cubelike structure with identical top and bottom faces that are suited to interact with the two mitochondrial boundary membranes. Indeed, Rojo *et al.* (13) demonstrated *in vitro* that the octameric form of Mi-CK was preferentially able to connect two artificial membranes. In agreement with this Kottke *et al.* (14) observed that mainly the octameric form of the enzyme was present in isolated contact site fractions of brain mitochondria. Furthermore, it was shown by electron microscopy that the enzyme was indeed frequently located *in situ* between adhering outer membrane vesicles and the inner envelope membrane (15).

Qualitative evidence for compartmentation of Mi-CK in the inter-membrane space was obtained by radiochemical (16) and kinetic (9, 17) measurements. These experiments with isolated mitochondria suggest that the outer membrane restricts permeation of the enzyme substrates and therefore is important in regulating the Mi-CK activity. In addition, Saks and co-workers (18) presented evidence in myocytes that the outer mitochondrial membrane is an important diffusion barrier for ADP. In a previous study, we showed that Mi-CK in digitonin-treated brain mitochondria was not freely accessible to negatively charged inhibitors such as iodoacetate or phosphocreatine (14). This suggests that, even without observable outer membrane remnants in the electron microscope (15), Mi-CK seems to be somehow masked by direct interaction with a membrane structure such as the outer membrane pore. The pore protein (voltage-dependent anion channel) forms a voltage-dependent anion selective channel (19, 20) in the outer mitochondrial membrane. It was observed that the pore above 30 mV adopts a low conducting state that is cation-selective, restricting permeation of adenine nucleotides (21, 22).

Considering the results described above, we assumed that Mi-CK and porin might interact and that this interaction may be tested experimentally. We investigated the ability to interact with porin *in vitro* of two types of Mi-CK: wild-type, mainly octameric form, as well as an N-terminal deletion mutant of the same enzyme, mainly present as dimer.

MATERIALS AND METHODS

Chemicals

The chemical used were all bought from Boehringer Mannheim and Merck-Darmstadt, Germany.

Antibodies

The polyclonal antiserum against the N terminus of porin was a gift of Dr. F. Thinnes, MPI for Experimental Medicine Göttingen. Preparation and characterization of the antibodies are described in Thinnes *et*

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[1] The abbreviations used are: CK, creatine kinase; Mi, mitochondrial isozyme; PAGE, polyacrylamide gel electrophoresis; nS, nanosiemen(s).

al. (23). Polyclonal antiserum against purified Mi-CK from chicken heart was raised in rabbits after taking a preimmune serum for control experiments (24).

Enzyme Assays

CK was measured in agreement with Bücher *et al.* (25) with creatine and ATP in a coupled enzyme assay using pyruvate kinase/lactate dehydrogenase as an indicator reaction to measure ADP production.

Isolation of Porin

Porin was prepared from rat brain as described by DePinto *et al.* (26). The total membrane fraction from homogenized rat brain was extracted by Triton X-100.

Wild-type and N-terminal Deletion Mutant of Mi-CK

A deletion mutant of Mi-CK (Δ5-Mi-CK), lacking the first five amino acids, was created as described recently (27). Wild-type and Δ5-Mi-CK were expressed as described previously (27). Δ5-Mi-CK was diluted to a final protein concentration of 0.1 mg/ml in buffer A (50 mM sodium phosphate, pH 7.2, 0.5 mM EDTA, 2 mM mercaptoethanol) and stored at 4 °C for 40 h. After this incubation, the enzyme was dissociated completely into dimers showing a similar specific activity as the octamers.

Analysis of the Porin·Mi-CK Complex by Centrifugation

It was possible to separate the two oligomeric structures of Mi-CK by high speed centrifugation due to the significantly different molecular mass of the octamer (350 kDa) and the dimer (80 kDa). The sedimentation coefficient for the octamer is 12.8 *s* and that of the dimer 5 *s* (28). In our experiments we used a Beckman TL-100 centrifuge with a rotor TLA 100. The *k* factor of this rotor at 100,000 rpm is $k = 12$. The sedimentation time of the different molecules in the centrifuge was calculated by the relation $t = k/S$ (S = Svedberg units). This means that the sedimentation of the Mi-CK octamer needs 1 h, while the time for the dimer would be 2.4 h and that needed for porin (which has a sedimentation coefficient of 2.5 S (29)) would be 4.8 h. For determination of the interaction of porin with Δ5-Mi-CK by centrifugation the enzyme was completely dissociated into dimers by dilution. Dimeric Δ5-Mi-CK was then incubated for 1 h at room temperature with different amounts of porin. The incubated mixture contained: 25 μg of Δ5-Mi-CK, in buffer B (20 mM phosphate buffer, pH 7.2, 0.8 mM EDTA, 0.8 mM mercaptoethanol, 2% sucrose, 1.8% Triton X-100) and different concentrations of porin varying between 0 and 3 mg/mg of Δ5-Mi-CK. The samples were centrifuged for 1 h at 436,000 × *g* in a Beckman Rotor TLA 100.2. The sediments were resuspended in 50 μl of buffer A, and the CK activity was determined therein as well as in the supernatants.

Analysis of the Porin·Mi-CK Complex by Column Chromatography

Column Chromatography of Δ5-Mi-CK—100–200 μg of un-dissociated mainly octameric Δ5-Mi-CK were incubated for 1 h at room temperature with 300 μg of porin from rat brain in the presence of 3% Triton X-100, 1 mM EDTA, and 10 mM Tris, pH 7.0. As a control, Δ5-Mi-CK was incubated without porin under identical conditions. The samples were loaded on a 90-ml Superose 6B column (Pharmacia Biotech Inc.) and were eluted with buffer A. The eluate was collected in 100 fractions of 650 μl in which the activity of CK was monitored by optical test. The peaks of the elution profile were additionally concentrated by centrifugation filtration and were then subjected to SDS-PAGE. The polypeptides of the gels were blotted onto nitrocellulose sheets and subsequently decorated with specific antibodies against porin.

Column Chromatography of Wild-type Mi-CK—330 μg of wild-type enzyme were incubated with 330 μg of porin from rat brain for 10 min at room temperature in buffer B. The sample was centrifuged for 1 h at 436,000 × *g* in a Beckman Rotor TLA 100.2. The sediment was resuspended in 200 μl of buffer A and loaded on a 24-ml Superose 6B column. As a control, the same amount of wild-type enzyme was incubated without porin in buffer A and chromatographed on the same column using buffer A as eluant. The activity of CK was determined by optical test in 100-μl fractions. Activity of porin in the high molecular weight fractions was determined by conductance measurements as described below.

Reconstitution of Porin in Lipid Bilayers

The methods used for the black lipid bilayer experiments were described previously (21, 30). Phospholipid membranes were made of a 1% (w/v) solution of diphytanoyl phosphatidylcholine (Avanti Biochemicals, Birmingham, AL) in *n*-decane. The membranes were formed across a circular hole (surface area about 1 mm^2) in the thin wall of a Teflon cell that separates two aqueous compartments filled with 1 M KCl solution. The samples were added to both sides after the membranes had turned optically black in reflected light. The current through the membranes was measured by two silver electrodes switched in series with a voltage source and current amplifier. The amplified signal was monitored by a storage oscilloscope and recorded on a chart recorder.

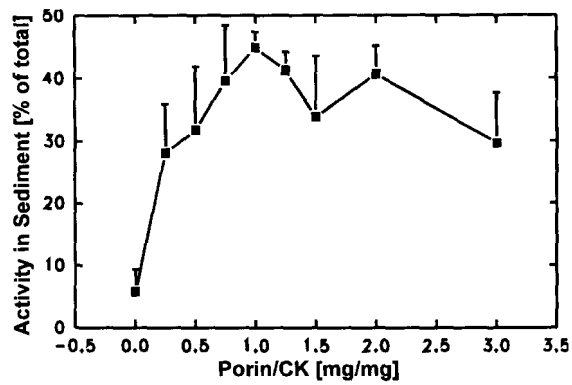

FIG. 1. **Sedimentation by ultracentrifugation of a porin·Mi-CK complex.** Δ5-Mi-CK was completely dissociated into dimers as described in methods. Of this enzyme 25-μg aliquots were incubated for 1 h at room temperature in buffer B (20 mM phosphate buffer, pH 7.2, 0.8 mM EDTA, 0.8 mM mercaptoethanol, 2% sucrose, 1.8% Triton X-100) with different concentrations of porin varying between 0 and 3 mg. The final concentration of Triton X-100 was adjusted in all samples to 1.8%. After centrifugation for 1 h at 436,000 × *g* in a Beckman Rotor TLA 100.2, the activity of Mi-CK was determined in the resuspended sediments and corresponding supernatants. The total yield in the different samples varied by 10%. The sedimented CK activity is plotted as a function of the porin/CK ratio. The data are means and S.D. of three experiments.

RESULTS

Analysis of Porin·Δ5-Mi-CK Complex Formation by Centrifugation—We used a mutant Δ5-Mi-CK in the experiments that was missing the first 5 amino acids at the N terminus. This deletion reduced the ability to associate into the octameric form (27). Upon dilution to 100 μg/ml in phosphate buffer and incubation for 40 h at 4 °C, Δ5-Mi-CK dissociated almost completely into dimers. Thus, only 5% of the total dimer (80 kDa) activity could be sedimented by the applied G force during centrifugation (Fig. 1). When Δ5-Mi-CK was incubated with porin, isolated from rat brain mitochondria, the activity of sedimented CK increased depending on the amount of porin added (Fig. 1). Compared to the control, the sedimented CK activity was 5 times higher at a ratio of 0.25 μg porin/mg of CK and 10 times higher at a porin/CK ratio of 1:1 (mg/mg). At higher ratios than 1:1 we observed a slight decrease of CK activity in the sediment. The recovery of the activity in the different samples was between 90 and 97%.

Neither dissociated Δ5-Mi-CK alone nor free porin alone could be sedimented in the experiment. Therefore, the sedimentation of Mi-CK in the presence of porin was indicating a complex formation between the two proteins.

Isolation of a Porin·Δ5-Mi-CK Complex by Gel Permeation Chromatography—Δ5-Mi-CK was incubated with porin at a ratio of 1:3 (mg/mg) for 1 h at room temperature. The sample was subsequently analyzed on a Superose 6B column. Three main activity peaks were eluted from the column (Fig. 2, *upper panel*). The large peak in the 350-kDa region presumably represents Mi-CK octamer, whereas the peaks in the 150- and 80-kDa region may correspond to the tetramer and dimer of the enzyme (Fig. 2, *upper panel*). In the control experiment performed without porin the 350-kDa peak was absent. This result indicates that porin induced and stabilized Mi-CK in the octameric structure.

The distribution of porin in the different column fractions

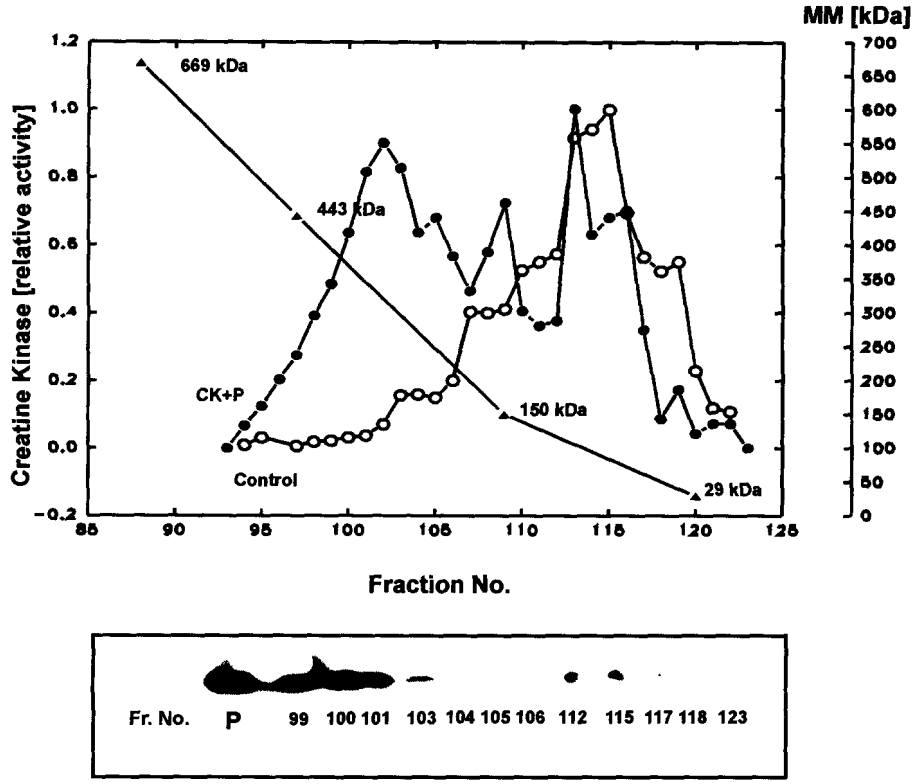

FIG. 2. **Isolation and characterization of a porin·Δ5-Mi-CK complex by gel permeation chromatography.** *Upper panel*, 100–200 μg of un-dissociated Δ5-Mi-CK were incubated for 1 h at room temperature with 300 μg of porin in the presence of 3% Triton X-100, 1 mM EDTA, and 10 mM Tris, pH 7.0 = *CK+P* (●). As control the enzyme was incubated without porin under identical conditions = *Control* (○). The samples were loaded on a 90-ml Superose 6B column and were eluted with 50 mM phosphate, pH 7.2, 0.5 mM EDTA, 2 mM mercaptoethanol. The eluate was collected in 130 fractions of 650 μl in which the CK activity was determined by optical test. The activity profiles are presented as enzyme activity relative to the maximal activity in the eluted peak fractions. Thyroglobulin, apoferritin, alcohol dehydrogenase, and carbonic anhydrase were used as molecular mass (*MM*) standards corresponding to 669, 443, 150, and 29 kDa, respectively. *Lower panel*, fractions containing CK activity, as indicated by *Fraction No.*, were concentrated by centrifugation filtration and were subjected to SDS-PAGE. The gels were blotted on nitro-cellulose sheets and decorated with specific antibodies against porin. P = isolated free porin.

was determined. Samples of all fractions were run on SDS-polyacrylamide gel electrophoresis and subsequently blotted on nitrocellulose sheets (Fig. 2, *lower panel*). After decoration with antibodies against porin, it was observed that porin was concentrated in those fractions containing octamers of Δ5-Mi-CK. Small amounts of porin were also found in fractions containing polypeptides of M_r 80,000. The latter fractions might represent porin dimer with bound Triton.

It is noteworthy that the porin distribution in the column fractions did not coincide with the Mi-CK octamer peak but was moving in front of it in fractions that correspond to a M_r of approximately 400,000.

Complex Formation of Porin with Wild-type Mi-CK—Porin and wild-type Mi-CK were incubated at a ratio of 1:1 (mg/mg) and the complex was sedimented by ultracentrifugation. The resuspended sediment was analyzed by gel permeation chromatography on a smaller, analytical Superose 6B column (Fig. 3). One main Mi-CK activity peak was observed at 350 kDa. The activity profile of the control experiment without porin showed only a small peak in the M_r 350,000 region while most of the activity eluted in fractions corresponding to 80 kDa, corresponding to the Mi-CK dimers.

The presence of porin in the peak at 350 kDa was confirmed by conductance measurements in artificial membranes. We observed a stepwise increase in conductance after the addition of the fractions (Fig. 4, *arrows*) that indicated incorporation of single pores. The conductance of 100 single steps each from two preparations of the complex was analyzed. The mean values are shown as histogram compared to free porin as control (Fig. 5). At 10 mV about 60% of the complex channels had a conductance of 1 to 2 nS. At this voltage the average conductance of free porin from brain was 2 to 3 nS (Fig. 5) (30). Thus the channels in the complex with Mi-CK appeared to adopt the lower conducting state of 2 nS.

DISCUSSION

Influence of Porin on the Octamer-Dimer Equilibrium of Mi-CK—We analyzed the interaction of porin with the wild-type Mi-CK present mainly as octamer and with a mutant of the same enzyme dissociated almost completely into dimers under the conditions used.

In gel permeation chromatography experiments, wild-type Mi-CK and the mutant Δ5-Mi-CK incubated with porin both resulted in a CK activity peak at 350 kDa. This peak was absent or much smaller in the controls without porin. The experiments indicate firstly that porin interacts with Mi-CK and secondly that this interaction shifts the dimer-octamer equilibrium of Mi-CK toward the octamer (Figs. 2 and 3). To investigate the latter phenomenon in more detail, the interaction of porin with the Δ5-Mi-CK mutant, lacking the first 5 N-terminal amino acids was analyzed. This mutant has a destabilized octamer; that is the dimer-octamer equilibrium shifts toward the dimer and Δ5-Mi-CK upon dilution dissociates quickly into dimers (27). Depending on the concentration, porin influenced the association-dissociation equilibrium of the mutated Mi-CK toward octamer formation (Fig. 1). These results suggest that porin interacts with a domain of Mi-CK, different from the N terminus. In experiments with artificial membranes, it has been described that negative charged phospholipids are necessary for the binding of the Mi-CK octamer (13, 31). Since the negative charges at the N-terminal end of porin are not accessible in Triton micelles (32), Mi-CK is unlikely to react with this domain of porin. Thus, the interaction of the octamer with porin may be of hydrophobic nature or involve other charged regions beyond the N terminus.

Influence of Wild-type Mi-CK on the Conductance of Porin—The mitochondrial outer membrane pore can be incorporated into artificial membranes. In these membranes, the average conductance of a single pore at 10 mV is between 3 and 4 nS (19). In the present experiments the conductance of the control pores was somewhat lower between 2.5 and 4 nS. The reconstituted pores are voltage dependent, meaning that at a voltage above 30 mV, the conductance decreases to about half of the value measured at 10 mV (20). In the latter, low conductance state the ion-selectivity changes from anion to cation selectivity

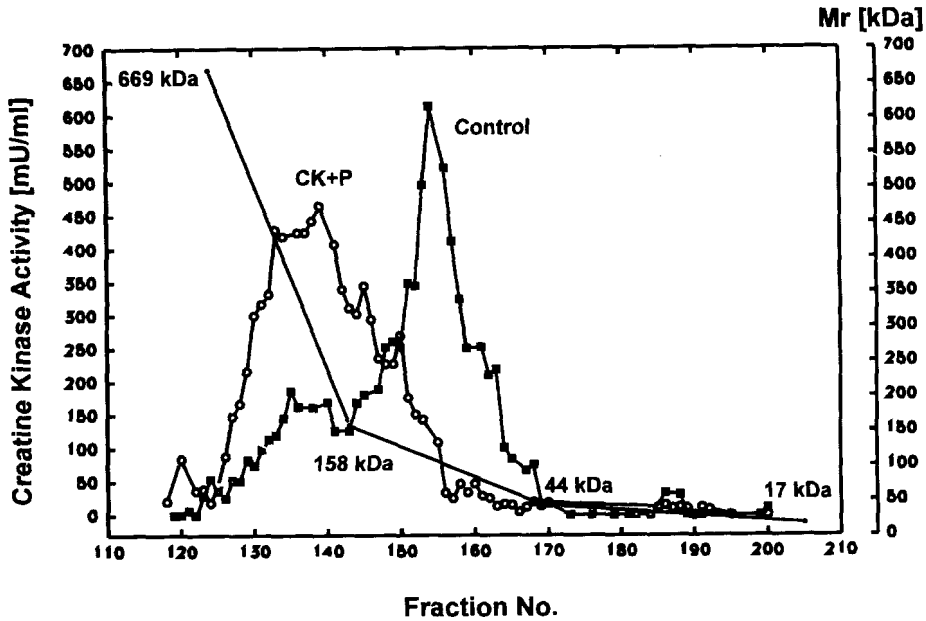

FIG. 3. **Isolation and characterization of a porin-wild-type Mi-CK complex by gel permeation chromatography.** Wild-type Mi-CK and porin from brain mitochondria were incubated at a ratio of 1:1 (mg/mg) and centrifuged as described under "Material and Methods." The sediments were resuspended and analyzed on a 24-ml Superose 6B column. Fractions of 0.1 ml were collected and the activity of Mi-CK was determined therein by optical test (○). As control the same amount of CK without porin was chromatographed under the identical conditions (■). Thyroglobulin, immunoglobulin, ovalbumin, and myoglobulin were used as molecular mass standards corresponding to 669, 158, 44, and 17 kDa, respectively.

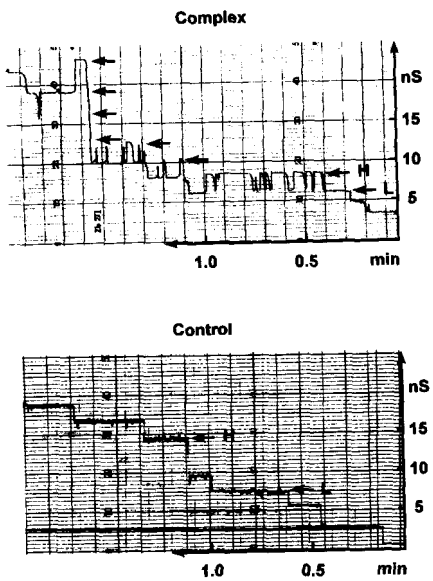

FIG. 4. **Chart recording of single-channel conductance after addition of porin·CK complex to a lipid bilayer membrane.** Fractions 130–140 from the chromatography of the porin·Mi-CK complex shown in Fig. 3 were combined. Of this sample, and of free porin as control, 5 μl were added to both sides of an artificial membrane formed of diphytanoyl phosphatidylcholine/*n*-decane in 1 M KCl solution. The current across the membrane was recorded at 10 mV. The conductance increased in discrete steps (*arrows*) representing incorporation of a single channel. The incorporated channels in the complex with Mi-CK appeared to switch frequently between the low (*L*) and high (*H*) conductance state.

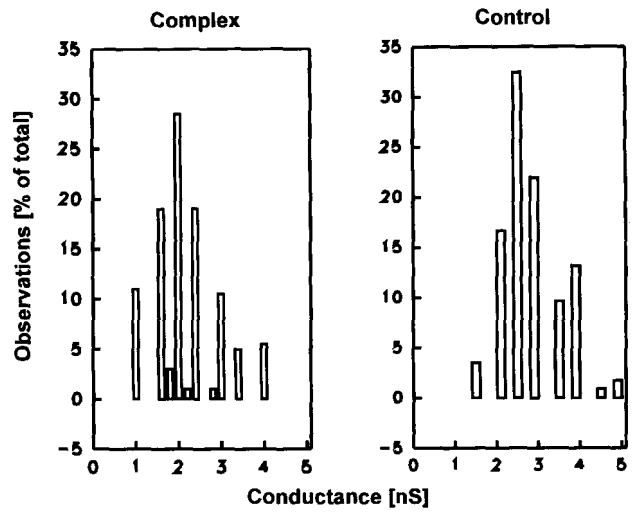

FIG. 5. **Histograms of conductance fluctuations of pores formed by the porin-wild-type Mi-CK complex isolated by gel permeation chromatography.** The artificial membranes were made of diphytanoyl phosphatidylcholine/*n*-decane in 1 M KCl solution. Of the combined high M_r fractions containing the porin·Mi-CK complex (130–140, shown in Fig. 3) 20 μl were added to both sides of the membranes, and a voltage of 10 mV was applied. The figure depicts the histograms of mean values from two separate experiments in which conductance of 100 single events was analyzed. As control 5 μl of free porin were added to the membrane.

(21). This cation selective state excludes permeation of adenine nucleotides, suggesting a physiological importance of the pore in regulation of the mitochondrial energy metabolism (22). Furthermore, kinetic analyses of Mi-CK in intact and digitonin-treated mitochondria showed that the enzyme was not freely accessible to substrates (16, 17) such as ATP, phosphocreatine, or negatively charged inhibitors (iodoacetate) (14). The resistance against digitonin treatment suggested the presence of complexes between porin and Mi-CK. Moreover, the determination that the enzyme was not freely accessible to negatively charged substrates and inhibitors indicated that porin in the complex might be in the cationically selective state. We analyzed the pores within the complex with wild-type Mi-CK obtained by chromatography. The pores that were incorporating into the bilayer membrane appeared to be of low conductance of 1–2 nS at 10 mV (Figs. 4 and 5) which might indicate the cation selective state. Porin in the cation selective state would explain the reduced permeability of the outer membrane for negatively charged Mi-CK metabolites in mitochondria.

Thus, a function for the complex between porin and the octamer of Mi-CK can be assumed. The complex might restrict the equilibrium of the enzyme with extramitochondrial adenine nucleotides and phosphocreatine but allow permeation of creatine (which is not charged under physiological conditions). This regulation would drive the reaction catalyzed by the mitochondrial enzyme toward phosphocreatine production. This is consistent with experimental observations with isolated mi-

tochondria and with our hypothesis of the phosphocreatine circuit (1, 2, 33).

Properties of the Porin·Mi-CK Complex—It was observed during chromatography that most of the porin migrated with the Mi-CK octamer peak suggesting a stable interaction between porin and CK. The distribution of porin in the fraction from the column was not coincident with the octamer peak at 340–350 kDa, but was concentrated in fractions representing a higher molecular mass of 400 kDa (Fig. 2). This molecular mass would be expected for a complex between the Mi-CK octamer (350 kDa) and a porin dimer (60 kDa).

In this study, we have not only shown that porin interacts with Mi-CK but we also demonstrated that this interaction exerts specific effects on both proteins. In the case of porin, the interaction influences the conductance of the channel. In the case of Mi-CK, the interaction with porin shifts the dimer-octamer equilibrium toward the octamer.

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