Mitochondrial creatine kinase and mitochondrial outer membrane porin show a direct interaction that is modulated by calcium.*

Uwe Schlattner**, Max Dolder, Theo Wallimann, and Malgorzata Tokarska-Schlattner

Institute of Cell Biology, Swiss Federal Institute of Technology (ETH), Hönggerberg HPM, CH-8093 Zürich, Switzerland

Running title: Mitochondrial creatine kinase interacts with porin

Number of pages: 23

Correspondence should be addressed to:

Dr. Uwe Schlattner,
Institute of Cell Biology, Swiss Federal Institute of Technology (ETH), Hönggerberg HPM, CH-8093 Zürich, Switzerland,
Tel.: +41-1-633 33 91; Fax: +41-1-633 10 69; E-mail: schlattn@cell.biol.ethz.ch.
SUMMARY

Mitochondrial creatine kinase (MtCK) co-localizes with mitochondrial porin (VDAC, voltage dependent anion channel) and adenine nucleotide translocator (ANT) in mitochondrial contact sites. A specific, direct protein-protein interaction between MtCK and mitochondrial porin was demonstrated using surface plasmon resonance spectroscopy. This interaction was independent of the immobilized binding partner (porin reconstituted in liposomes or MtCK) or the analyzed isoform (chicken sMtCK or human uMtCK, human recombinant porin or purified bovine porin). Increased ionic strength reduced the binding of MtCK to porin, suggesting predominantly ionic interactions. By contrast, micromolar concentrations of Ca\(^{2+}\) increased the amount of bound MtCK, indicating a physiological regulation of complex formation. No interaction of MtCK with reconstituted ANT was detectable in our experimental setup. The relevance of these findings for structure and function of mitochondrial contact sites is discussed.
INTRODUCTION

The mitochondrial isoenzymes of creatine kinase (MtCK)\(^1\), sarcomeric sMtCK and ubiquitous uMtCK are strictly localized within the cristae and the intermembrane space of mitochondria, where they convert mitochondrially produced ATP into PCr, another "high-energy" compound. MtCK, as a peripheral membrane protein, binds to the outer leaflet of the entire inner mitochondrial membrane and is specifically enriched in the so-called contact sites, where inner and outer membrane are in close proximity. Here, MtCK forms a functional microcompartment together with the transmembrane proteins adenine nucleotide translocator (ANT) in the inner membrane and mitochondrial porin (VDAC, voltage dependent anion channel) in the outer membrane (reviewed in Brdiczka \textit{et al.}, 1998; Schlattner \textit{et al.}, 1998; Wyss \textit{et al.}, 1992). The three proteins maintain a privileged exchange of MtCK substrates and products, called functional coupling or substrate channeling (Jacobus, 1985; Saks \textit{et al.}, 1985). Contact sites are therefore considered to play an important role in dynamic compartmentation of adenine nucleotides in the intermembrane space (Gellerich \textit{et al.}, 1993) and in high-energy phosphate channeling. It is also suggested that contact site complexes can constitute a functional and structural element of the permeability transition pore (PTP). Opening of PTP, possibly regulated by MtCK oligomers (O'Gorman \textit{et al.}, 1997), is considered a key event in the mitochondrial pathway leading to cellular apoptosis (Crompton, 1999).

Despite of the multiple functional interactions of contact site components, their exact topology and putative structural interactions have remained elusive. Chemical crosslinking of contact site proteins failed so far (Schnyder \textit{et al.}, 1994; Font \textit{et al.}, 1987). Co-purification of MtCK, ANT and porin in proteolipid contact site complexes only indicated a close proximity of these proteins (Beutner \textit{et al.}, 1998). Finally,
octamerization of a partially octamerization-incompetent MtCK mutant by addition of porin suggested that porin interacts with MtCK at some stage of octamer formation (Brdiczka et al., 1994; Kaldis et al., 1994). However, no unambiguous proof for a permanent direct protein-protein interaction between these two proteins has been reported yet.

The recent introduction of surface plasmon resonance (SPR) spectroscopy for the analysis of MtCK binding to membranes (Schlattner and Wallimann, 1999, 2000a, b) has opened the avenue for a detailed evaluation of the postulated MtCK/porin interaction. Here, by using BIAcore SPR technology, we compare binding of MtCK to pure phosphatidylcholine vesicles with its binding to vesicles containing reconstituted mitochondrial porin. We can show a specific MtCK/porin interaction that depends on porin concentration, involves ionic interactions and is modulated by calcium.
EXPERIMENTAL PROCEDURES

Proteins—Chicken sMtCK and human uMtCK were expressed in *E. coli* and purified to homogeneity according to Furter *et al.* (1992) and Schlattner *et al.* (2000). Purified proteins were stored at -80°C at 3-6 mg/ml in 50 mM NaP, at pH 7.0, 150 mM NaCl, 0.2 mM EDTA, and 2 mM β-mercaptoethanol. Before use, dilutions were made in 10 mM TES, pH 7.0, and 50 mM NaCl. Recombinant human mitochondrial porin was expressed in *E. coli*, purified as described earlier (Dolder *et al.*, 1999), and stored at a concentration of 6-10 mg/ml at 4 °C in 10 mM TES, pH 7.0, 50 mM NaCl, 1% octyl-polyoxyethylene, and 10% glycerol. Bovine porin was purified to homogeneity from beef heart mitochondria. In short, 40 mg mitochondria were solubilized on ice with 4 ml of 150 mM Na₂SO₄, 50 mM HEPES, pH 7.0, 1 mM EDTA, 3% CHAPS (buffer A), vortexed, and centrifuged in an air-driven ultracentrifuge (Beckman Instruments, Palo Alto, CA 94304 USA) at 30 PSI for 5 min. The clear supernatant was loaded on a column containing 2 g of dry hydroxyapatite and proteins were eluted with buffer A. The first 4 ml of the eluate were diluted with 20 mM MES, pH 6.0, 0.2 mM EDTA, and 1% CHAPS (buffer B) in a ratio of 1:3 (v/v) and loaded on a 1 ml cation exchange column (HiTrap SP, Pharmacia, Uppsala, Sweden). Under these conditions, porin does not bind to the column matrix and was collected in the flow-through, whereas contaminating proteins (mainly ANT) were eluted with 1 M NaCl in buffer B. Porin containing fractions were concentrated to 0.17-0.35 mg/ml using Centriprep-30 devices (Amicon Inc., Beverly, MA 01915, USA) checked for purity by SDS-PAGE, and used for reconstitution into phosphatidylcholine (PC) vesicles.

Other chemicals—Egg yolk PC was from Lipid Products (South Nutfield, Great Britain), hydroxyapatite from BioRad (Reinach, Switzerland), avidin and cardiolipin
from Sigma (Buchs, Switzerland), and all other chemicals from Fluka (Buchs, Switzerland).

Reconstitution of porin and preparation of liposomes—A lipid stock suspension at 5 mg/ml containing PC (99.9% w/w) and Biotin-X-DHPE (0.1% w/w) in 10 mM TES, pH 7.0, and 50 mM NaCl was prepared as described by Schlattner and Wallimann (2000b). Porin was reconstituted after Bathori et al. (1993) with minor modifications. Briefly, PC suspension and porin preparation were mixed at lipid:protein ratios of 10:1, 4:1 or 2:1 (w/w). Equivalent amounts of porin storage buffer were added to the control PC suspension. The mixtures were vortexed,sonicated and dialysed for 40 h against 10 mM TES, pH 7.0, and 50 mM NaCl at 4 °C to remove detergent. Liposomes were produced by a combination of freeze/thawing and extrusion techniques (Schlattner and Wallimann, 2000b), stored at 4 °C and used within two days. The quality and reproducibility of preparations was checked by transmission electron microscopy.

Surface plasmon resonance (SPR) analysis—Binding studies were done using surface plasmon resonance spectroscopy with a BIAcore 2000™ (Biacore, Uppsala, Sweden) according to Schlattner and Wallimann (2000a, b). Binding is expressed in arbitrary resonance units (RU). Interaction between MtCK and PC vesicles with or without reconstituted porin was determined in two different setups: (A) biotinylated lipid vesicles were immobilized for each measurement on an avidin coated CM5 sensorchip and MtCK was injected in the flow, or (B) MtCK was covalently immobilized on a CM5 sensorchip using routine amine coupling and lipid vesicles were injected. Programmed measurement cycles (immobilization of liposomes in setup (A), contact phase and dissociation phase of 170 - 240 s each, regeneration with 1% SDS) were performed at 25 °C and a flow rate of 0.3 ml h⁻¹ in running buffer (10 mM TES,
pH 7.0, 50 mM NaCl), if not stated otherwise. Additional experiments in setup (B) were performed with vesicles preincubated in TES buffer supplemented with 3.0 mM MgCl₂, 0.1 mM EGTA, and CaCl₂ to obtain 10 µM free calcium. Control and porin containing samples were injected in random order. Each cycle was repeated at least once to demonstrate that binding responses were reproducible. SPR data were corrected for background binding and refractive index changes using the signal from reference lanes either coated with avidin (setup A) or a blank lane (setup B).

*Extraction of lipids*—Lipids that co-purified with porin from beef mitochondria were isolated by precipitating protein and extracting lipids with chloroform according to De Pinto *et al.* (1989). The lipid fraction remaining after evaporation of chloroform was resuspended in porin storage buffer (1:3 (v/v) mixture of buffers A and B) to obtain the initial volume. This preparation was incorporated into PC vesicles in the same way as the bovine porin preparation and used in control experiments to assess binding of MtCK to phospholipids that were co-purified with bovine porin.
RESULTS AND DISCUSSION

Purified porin can be reconstituted into liposomes at high density—The high quality and the reproducibility of the liposome preparations were confirmed by electron microscopy (Fig. 1). Pure PC vesicles and vesicles with reconstituted porin were very similar in size with diameters of 100-400 nm when observed by negative stain electron microscopy. Porin containing vesicles were less smooth than pure PC vesicles, indicating the presence of reconstituted protein. Higher magnification revealed porin particles at very high density (Fig. 1d). Human recombinant porin at higher protein:l lipid ratios protein was sometimes present as crystalline patches (see Dolder et al., 1999).

MtCK directly interacts with mitochondrial porin but not with ANT—Interaction of MtCK with porin was analyzed by surface plasmon resonance with two different experimental setups: either using immobilized liposomes for analysis of MtCK binding (Fig. 2), or an inverse system with immobilized MtCK to analyze liposome binding (Fig. 3). We also applied different MtCK isoenzymes (human uMtCK or chicken sMtCK, both recombinant), as well as different porin preparations (recombinant human porin or purified bovine porin). Under all experimental conditions, the interaction between MtCK and porin-containing PC vesicles was significantly above the background binding to pure PC vesicles.

In parallel experiments with reconstituted ANT purified from beef heart mitochondria, we could not detect any signal above background, indicating the absence of a direct MtCK/ANT interaction under our experimental conditions (data not shown). These results confirm an earlier study that was unable to detect an interaction between MtCK and reconstituted ANT (Müller et al., 1985). In addition, both MtCK and ANT have a basic pI which makes a direct interaction rather unlikely (Lipskaya et al., 1980).
In vivo, an interaction between these two proteins may be mediated by cardiolipin patches surrounding ANT. However, the amount of co-purified cardiolipin in our preparations was probably too small to allow such an interaction.

MtCK binding depends on porin concentration, but not on the experimental setup—The binding of chicken sMtCK or human uMtCK (Fig. 2) to immobilized PC vesicles containing different amount of bovine porin showed typical saturation kinetics. End-association and end-dissociation responses depended on porin concentration as seen with the SPR data corrected for background binding to PC (Fig. 2b). The very slow dissociation of the MtCK/porin complex indicated a rather high affinity. However, because of this kinetic stability of the complex, reliable $k_{off}$ values cannot be calculated from the SPR kinetics. Since this would be necessary to verify the consistency of parameters derived from SPR data (Schuck and Minton, 1996), we were reluctant to calculate exact kinetic and equilibrium constants. Calculations based on the association kinetics alone yielded affinity values more than one order of magnitude lower than for the MtCK/cardioplin interaction, which are about 16-86 nM, depending on the isoenzyme (Schlattner and Wallimann, 2000a).

We verified the MtCK/porin interaction in an inverse system with immobilized MtCK and recombinant human porin (Fig. 3). Again, the presence of reconstituted porin increased the binding of vesicles to MtCK, albeit the binding kinetics was quite different than in the set-up with immobilized vesicles. The linear character of association kinetics and the pronounced dissociation as compared to the first setup (Fig. 3b) indicates a more complex binding mechanism, which is most probably due to a dissociation of the covalently immobilized MtCK octamers into dimers. Dimeric MtCK displays weak binding with quasi-linear association kinetics to cardiolipin-containing
PC vesicles as shown in an earlier study (Fig. 3 in Schlattner and Wallimann, 2000b). In addition, the SPR signal in this setup may be influenced by coalescent vesicles, changes in vesicle shape, or detachment of the very bulky liposomes from the surface by shearing forces of the flow. Porin containing vesicles, in contrast to pure PC vesicles, also interacted with immobilized cytochrome c and hexokinase (data not shown). This is in accordance with published data (Kottke et al., 1988; Mannella, 1998) and further supports the reliability of our experimental system.

Co-purified lipids in porin preparations are not responsible for MtCK binding—Porin preparations from beef heart mitochondria contained remnants of lipids, mainly PC, phosphatidylethanolamine, and cholesterol, but also smaller amounts of cardiolipin, as shown by thin layer chromatography (data not shown). The presence of cardiolipin is critical, since it is the high affinity receptor of MtCK in the inner mitochondrial membrane (Muller et al., 1985; Rojo et al., 1991) and it might contribute to the observed interaction of MtCK with porin-containing vesicles. Therefore, we compared MtCK binding to immobilized vesicles either the full porin preparation or the extracted protein-free lipid fraction (Fig. 4). Although the presence of the lipid fraction increased MtCK binding to some extent, binding was much weaker and did not show concentration-dependence as in case of porin-containing vesicles. This clearly confirms that interaction mainly occurs between MtCK and porin.

Binding partners and binding mode of MtCK in mitochondria—Cardiolipin and other phospholipids with negative net charge have been identified as binding partners of MtCK (Muller et al., 1985; Rojo et al., 1991) and a detailed quantitative characterization of these interactions is available (Schlattner and Wallimann, 1999, 2000a, b; Vacheron et al., 1997). Other binding partners have been ruled out so far on
the basis of crosslinking studies (Schnyder et al., 1994; Font et al., 1987). Now, in addition to phospholipids, porin can be classed as a new binding partner for MtCK in the outer mitochondrial membrane. Exact quantitation of kinetic constants was not possible, but the kinetic stability of the MtCK/porin complex indicates a rather high affinity (Fig. 2). By contrast, porin vesicles seem to offer much less binding sites per surface unit as compared to cardiolipin vesicles. With immobilized vesicles, the maximal equilibrium response of 0.15 μM human uMtCK is about 900 RU for 16% cardiolipin vesicles (500 RU immobilized; Schlattner and Wallimann, 2000a), while it is less than 100 RU for vesicles with comparably low porin content (i.e. lipid:protein ratio (w/w) of 10:1, 1300 RU immobilized; see Fig. 2b). Possibly, not every reconstituted porin molecule can contribute to binding of MtCK due to wrong orientation in the membrane or misfolding. Buffers with higher ionic strength (100 mM salt) reduced binding of MtCK to porin vesicles (data not shown). This suggests the participation of electrostatic forces, similar to the known MtCK/cardiolipin interaction, rather than the presence of hydrophobic interactions postulated earlier (Brdiczka et al., 1994). A candidate for the MtCK-binding domain of porin would be the flexible N-terminus, which is known to interact with cytochrome c (Mannella, 1998), a protein very similar to MtCK in its basic pI and its high affinity to cardiolipin.

_Calcium increases MtCK/porin interaction_—Out of several effectors examined, micromolar concentrations of calcium (10 μM) in the presence of magnesium (0.3 mM) increased binding of porin-containing vesicles to immobilized human uMtCK (Fig. 5). The same concentrations of Ca^{2+} and Mg^{2+} had little or no influence on the SPR signal when porin containing vesicles were injected onto immobilized cytochrome c or BSA (data not shown). This finding could be physiologically relevant, if contact site
formation is considered a dynamic process. Raising intracellular free calcium levels, e.g. upon activation of muscle contraction, would trigger the formation and/or stabilization of MtCK/porin complexes. In fact, a study in perfused heart suggested a link between calcium concentration and contact site formation (Bakker et al., 1994).

Significance of MtCK/porin interaction in mitochondrial contact sites—The structural basis for the substrate channeling between ANT, MtCK and porin in peripheral contact sites of mitochondria has long remained enigmatic. A major difficulty has been to explain the interaction of the highly symmetrical, cuboidal MtCK octamer with the outer and inner membranes, which differ substantially in their properties. While MtCK has two identical four-fold symmetry faces that interact with membranes (Fritz-Wolf et al., 1996), the two mitochondrial membranes differ entirely in their lipid and protein composition. The inner membrane contains high amounts of cardiolipin that will strongly interact with positively charged amino acid residues at the C-terminus of MtCK (Schlattner et al., unpublished data). Since cardiolipin also strongly binds to ANT (Beyer and Klingenberg, 1985; Drees and Beyer, 1988), the existance of cardiolipin patches in the inner membrane would be sufficient to bring ANT and MtCK in close vicinity as postulated for functional coupling (Saks et al., 1987, reviewed in Wallimann et al., 1992; Wyss et al., 1992) and to allow co-purification of ANT/MtCK/porin complexes (Beutner et al., 1998). For interaction of MtCK with porin in the outer membrane, which is almost deficient of cardiolipin, a similar indirect complex formation via other charged phospholipids has been proposed (Schnyder et al., 1994). However, our present data clearly show that in contrast to ANT, porin can directly interact with MtCK. This confirms previous indirect evidence for in vitro complex formation between MtCK and porin (Brdiczka et al., 1994).
Our finding on porin as a MtCK receptor in the outer membrane is not only important for contact site topology and metabolic channeling therein (reviewed in Schlattner et al., 1998), but seems also to be relevant for the potential implication of MtCK in apoptosis-related functions of contact sites. ANT and VDAC were proposed to trigger apoptosis by interacting with pro-apoptotic Bcl-2 family members (Marzo et al., 1998; Shimizu et al., 1999) and/or forming the mitochondrial permeability transition pore (PTP; reviewed in Crompton, 1999; Bernardi et al., 2001) as indicated by studies with reconstituted contact site complexes (Beutner et al., 1998). MtCK may interfere with these processes, as PTP opening was shown to be inhibited by octameric MtCK together with its substrates (O’Gorman et al., 1997).

Finally, our study extends the application of real-time SPR technology to the field of interactions between peripheral membrane proteins with reconstituted integral membrane proteins. This provides a very attractive tool for interaction proteomics, which will be especially important for the molecular analysis of metabolic channeling and cellular signal transduction.

Acknowledgment—The authors would like to thank Magda Livingstone for reading the manuscript.
REFERENCES


FOOTNOTES

* This work was supported by grants from the Sandoz Family Office (to M.T.-S. and U.S.), Novartis Stiftung, Schweizerische Herzstiftung and Wolfermann-Nägeli Stiftung (to T.W. and M.T.-S.).

** To whom correspondence should be addressed: Institute of Cell Biology, Swiss Federal Institute of Technology (ETH), Hönggerberg HPM, CH-8093 Zürich, Switzerland; Tel.: +41-1-633 33 91; Fax: +41-1-633 10 69; E-mail: schlattn@cell.biol.ethz.ch.

1 The abbreviations used are: ANT, adenine nucleotide translocator; CK, creatine kinase; MtCK, mitochondrial creatine kinase; PC, phosphatidylcholine; PCr, phosphocreatine; PTP, permeability transition pore; sMtCK, sarcomeric MtCK; SPR, surface plasmon resonance; uMtCK, ubiquitous MtCK; VDAC, voltage dependent anion channel.
FIGURE LEGENDS

FIG. 1. Electron micrographs of lipid vesicles. Pure PC vesicles (a,c) and PC vesicles with reconstituted recombinant human porin at a lipid:protein ratio of 10:1 (b,d); (a,b) general overview and (c,d) high-magnification view showing a single vesicle. Lipid concentrations of the preparations were 25 µg/ml. Bars correspond to 0.2 µm (a,b) and 0.1 µm (c,d). Samples were stained with 1% uranylacetate.

FIG. 2. Interaction of MtCK with immobilized PC vesicles containing reconstituted porin isolated from beef heart mitochondria. Two representative sets of SPR kinetics showing contact phase (black bar) and dissociation phase (white bar). (a) Data set corrected for background binding, and (b) data set corrected for non-specific binding to PC. Pure PC vesicles (⋯⋯⋯⋯) and vesicles containing porin at lipid:protein ratios (w/w) of 10:1 (−−−−), 4:1 (− − −) and 2:1 (----). Human uMtCK at 50 µg/ml was injected in the running buffer (10 mM TES, pH 7.0, 50 mM NaCl) onto immobilized vesicles (1300 RU).

FIG. 3. Interaction of PC vesicles containing reconstituted human recombinant porin with MtCK. Representative SPR traces of contact phase (black bar) and dissociation phase (white bar). (a) Data corrected for background binding, and (b) data corrected for non-specific binding of PC-vesicles. Pure PC vesicles (⋯⋯⋯⋯) and vesicles containing porin at lipid:protein ratios (w/w) of 10:1 (----). Vesicles at 500 µg/ml were injected in the running buffer (10 mM TES, pH 7.0, 50 mM NaCl) onto immobilized chicken sMtCK (23000 RU).
**FIG. 4.** *Contribution of lipids co-purified with porin from beef heart mitochondria on MtCK interaction.* The SPR response was measured for chicken sMtCK at 50 µg/ml injected in the running buffer (10 mM TES, pH 7.0, 50 mM NaCl) onto immobilized PC vesicles (1000 RU) and response values were taken at 120 s of dissociation phase. The vesicles contained either only PC or were supplemented with increasing amounts of a porin preparation from beef mitochondria (black bars) or corresponding volumes of a protein-free lipid extract from the same porin preparation (white bars). Response values were normalized in relation to the response with pure PC vesicles which was taken as 100%. Values are given as mean ± SEM (n=3).

**FIG. 5.** *Influence of calcium on porin/MtCK interaction.* SPR difference traces representing the additional SPR response in presence of 10 µM Ca²⁺ as compared to Ca²⁺-free buffer (see Materials and Methods), contact phase (black bar) and dissociation phase (white bar). Measurements were done using pure PC vesicles (— — —) or PC vesicles containing human recombinant porin at lipid:protein ratios (w/w) of 10:1 (-----). Vesicles at 25 µg/ml were injected in the running buffer (10 mM TES, pH 7.0, 50 mM NaCl) onto immobilized chicken sMtCK (25000 RU).
Figure 1
Figure 3
Figure 4
Figure 5
Mitochondrial creatine kinase and mitochondrial outer membrane porin show a direct interaction that is modulated by calcium
Uwe Schlattner, Max Dolder, Theo Wallimann and Małgorzata Tokarska-Schlattner

J. Biol. Chem. published online October 15, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106524200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts