Eur Biophys J (2010) 39:1649–1655
DOI 10.1007/s00249-010-0600-4

BIOPHYSICS LETTER

Mitochondrial creatine kinase interaction with cardiolipin-containing biomimetic membranes is a two-step process involving adsorption and insertion

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Received: 14 December 2009 / Revised: 9 March 2010 / Accepted: 16 March 2010 / Published online: 2 April 2010

Abstract Mitochondrial creatine kinase (mtCK) binding to the mitochondrial inner membrane largely determines its biological functions in cellular energy homeostasis, mitochondrial physiology, and dynamics. The membrane binding mechanism is, however, not completely understood. Recent data suggest that a hydrophobic component is involved in mtCK binding to cardiolipin at the outer face of the inner mitochondrial membrane, in addition to the well known electrostatically driven process. In this manuscript, using an electrochemical method derived from alternating current polarography for differential capacity measurements, we distinctly reveal that protein–cardiolipin interaction has a two-step mechanism. For short incubation time, protein adsorption to the phospholipid charged headgroup was the only process detected, whereas on a longer time scale evidence of protein insertion was observed.

Keywords Differential capacity · Mitochondrial creatine kinase · Cardiolipin · Langmuir monolayers · Protein-membrane binding

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Abbreviations

CK	Creatine kinase
mtCK	Mitochondrial creatine kinase
CL	Cardiolipin
ac	Alternating current
C	Differential capacity
E	Electrical potential
pzc	Potential of zero charge

Introduction

In mitochondrial intermembrane space, mitochondrial creatine kinase (mtCK) has been extensively studied because of its role in energy management within cells with high and fluctuating energy needs (Wallimann et al. 1992). MtCK uses mitochondrially synthesized ATP to phosphorylate creatine into phosphocreatine. This phosphagen then diffuses into the cytosol where it can be used by cytosolic CK to phosphorylate ADP back to ATP in the immediate vicinity of ATP binding sites (Wallimann et al. 1992, 1998a). It is also known that mtCK plays a role in mitochondrial morphology, as shown both by inhibition of mtCK expression (Lenz et al. 2007) and by analysis of mitochondrial ultrastructure of transgenic mice expressing mtCK in liver cells, a tissue normally devoid of this enzyme (Miller et al. 1997). MtCK exists as two interconvertible forms, a dimer and an octamer (Marcillat et al. 1987), but only the octamer is able to bind to the outer face of the inner mitochondrial membrane (Marcillat et al. 1987). Membrane binding occurs mainly via electrostatic interactions between cardiolipin (CL) (Muller et al. 1985; Schlame and Augustin 1985) and positively charged residues of the protein

C-terminal end (Lys 380, Lys 379 and Lys 369) (Schlattner et al. 2004). Interaction with cardiolipin model membranes induces CL-clustering (Epand et al. 2007; Maniti et al. 2009b) and involves a certain degree of protein insertion into CL monolayers (Maniti et al. 2009b). Insertion is also observed with a phosphatidylcholine: phosphatidylethanolamine: cardiolipin (PC:PE:CL) (2:1:1) mixture, mimicking mitochondrial inner membrane composition, but not with zwitterionic PC monolayers; this confirms that insertion is a result of interaction with CL (Maniti et al. 2009b). Because mtCK biological function is related to membrane binding (Khuchua et al. 1998), it is of interest to better characterize the mtCK–CL binding mechanism.

In this report, an electrochemical method, derived from alternating current (ac) polarography for differential capacity measurements, was used to further explore mtCK interaction with CL monolayers. This biophysical method enabled us to characterize CL monolayers spread at the argon–electrolyte interface and to detect the conditions enabling formation of a condensed CL monolayer in which no "empty" spaces are available. Monitoring the interaction of mtCK with such a CL monolayer enabled us to delineate two distinct steps in the interaction process: a first one in which the protein was adsorbed by the lipid polar head group and a second one, detected at longer incubation time, involving insertion into the monolayer.

Materials and methods

Materials

DTT, EDTA, bovine heart cardiolipin (CL), and cytochrome c from equine heart were obtained from Sigma (Saint Quentin Fallavier, France) and Tris and NaCl from Merck. Ultrapure water was obtained from a Millipore system. Mercury was purified and doubly distilled under vacuum.

Protein expression and purification

Recombinant octameric rabbit heart mtCK (340 kDa) was purified as described elsewhere (Marcillat et al. 1999). The purified enzyme was obtained in 20 mM Tris–HCl, 0.1 mM EDTA, and 0.2 mM DTT, pH 7.4, at a protein concentration of 0.2 g/L. Protein concentration was determined by the Lowry method using bovine serum albumin as standard. Purified mtCK was mainly octameric, as verified by use of both electrophoresis under non-denaturing conditions and gel filtration chromatography.

Monolayer formation

Dried CL samples were dissolved in hexane. Increasing amounts of phospholipid were spread on the surface of a 50 mM NaCl, 20 mM Tris–HCl buffer at pH 7.4. MtCK was injected beneath the monolayer at final concentrations ranging between 0.4 and 4 nM. Cytochrome c was injected beneath the monolayer at 2 nM final concentration.

Electrochemical measurements

Differential capacity (C) measurements were carried out in a Metrohm polarographic cell as previously described (Lecompte et al. 1994). The frequency of the alternating current modulation (10 mV) was 80 Hz. This equipment is a home-made device as described previously (Clavilier 1966) in which the selective amplifier has been replaced by a synchronous detector.

Oxygen was displaced from solution by argon bubbling before CL was deposited on the surface and the experiments were carried out in a streaming argon atmosphere. The working electrode was a hanging mercury drop, formed at the extremity of a thin capillary tip. Differential capacity measurements were carried out by keeping the drop neck in contact with the lipid film on the surface of the aqueous electrolyte. The differential capacity of the electrode in the presence of mtCK and in the absence of lipids was recorded by placing the mercury-drop electrode in the solution containing mtCK, and was measured as a function of time until it remained constant. The value obtained is attributed to a completely protein-covered electrode as previously described (Lecompte et al. 1994). An Ag/AgCl saturated KCl electrode and a platinum gauge were, respectively, the reference and the counter electrodes. Potentials (E) are reported versus the Ag/AgCl, sat. KCl reference electrode. The starting potential was chosen within the stable region of the monolayer, namely at -0.2 V. The C vs. E curves, in the absence or presence of mtCK or cytochrome c, were recorded when equilibrium was reached. C vs. E curves plotted are representative of at least three independent measurements. Measurements were performed at 22°C.

Results

Formation of a condensed CL monolayer

Increasing amounts of CL were spread at the argon–electrolyte interface and, for each concentration, a C vs. E curve was recorded for potentials ranging from -0.2 V to more negative values. As shown in Fig. 1, important modifications of the C vs. E curves as function of amount of CL were observed. At the potential of zero charge (pzc) of the electrode (-0.45 V Ag/AgCl sat. KCl), C decreased with increasing amount of CL (Fig. 1). A minimum value of 1.9 μF cm^{-2} was recorded with 0.2 μg CL cm^{-2}

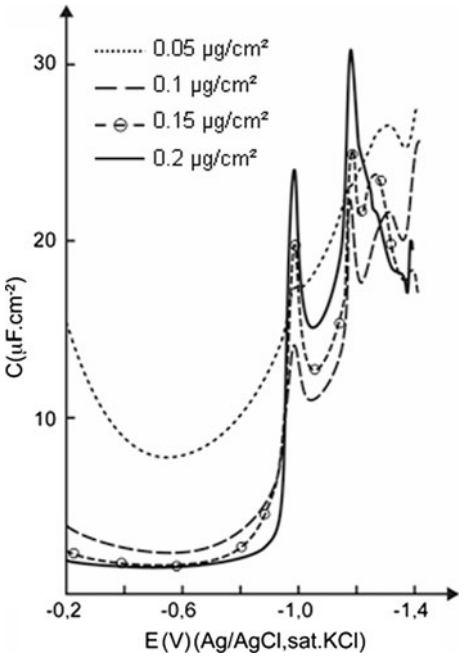

Fig. 1 Differential capacity vs. potential curves obtained with a hanging mercury-drop electrode in contact with CL monolayers obtained by spreading increasing amounts of CL at the Ar–electrolyte interface: 0.05 µg cm^{-2} (*dotted line*); 0.1 µg cm^{-2} (*dashed line*); 0.15 µg cm^{-2} (*dashed line with circles*); 0.2 µg cm^{-2} (*full line*). Curves are representative of at least three independent measurements

(Fig. 1, full line). Further increase in the amount of CL did not diminish this value, indicating that the entire available surface was already covered by lipids and that a condensed monolayer was formed (Lecompte et al. 1994, 1998). Moreover, *C* was constant over a large range of potential around the pzc, namely, between −0.2 and −0.8 V.

The second region of interest on the *C* vs. *E* curve corresponds to more negative potentials (−0.9 to −1.4 V) with two peaks at −1 and −1.2 V. At the lowest CL density used, 0.05 µg cm^{-2}, they were not well-defined, and appeared only as shoulders (Fig. 1, dotted line). However, when the amount of phospholipid spread at the interface was increased from 0.1 to 0.2 µg cm^{-2} (Fig. 1), peaks became sharper and higher. *C* vs. *E* curves recorded over longer incubation times (up to 2 h) were identical, attesting to monolayer stability.

Interaction of mtCK with a condensed CL monolayer

The condensed CL monolayer obtained by spreading 0.2 µg lipid cm^{-2} was then chosen to analyze mtCK binding. *C* vs. *E* curves were recorded periodically over a 2-h time period. Figure 2 shows the evolution of *C* at the pzc over this time period. No variation of *C* was observed for the first 30 min, but a strong increase was recorded 60 min after protein injection. At longer incubation times

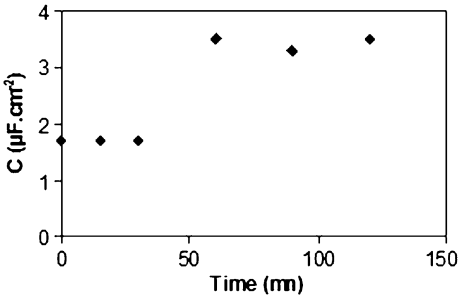

Fig. 2 Differential capacity of a condensed CL monolayer (0.2 µg lipid cm^{-2}) at the pzc after addition of 0.4 nM mtCK. *C* vs. *E* curves were recorded periodically over a 2-h time period. Values are representative of at least three independent measurements

C remained constant. Two crucial points over the incubation period, at 30 and 60 min after protein addition, were chosen to present the differential capacity of the monolayer as a function of potential variation in the absence (Fig. 3, full line) or presence of 0.4 nM mtCK (Fig. 3, dotted line and dashed line, respectively).

Thirty minutes after injection of 0.4 nM mtCK (Fig. 3, dotted line), no changes of the differential capacity measured around the pzc (1.9 µF cm^{-2}) were observed compared with that of the lipid alone (Fig. 3, arrow). However, several observations made on this curve attested to interaction of mtCK with the monolayer. First, in the presence of mtCK, *C* remained constant over a range of potential (−0.3 to −0.5 V) shorter than in its absence (−0.2 to −0.8 V). Second, the height of the peaks at −1 and −1.2 V distinctly decreased, from 27.5 to 17.5 µF cm^{-2} and from 29.4 to 24.3 µF cm^{-2}, respectively (Table 1). The *C* vs. *E* curve recorded 1 h after mtCK injection had a completely different profile (Fig. 3, dashed line). In this case, *C* distinctly increased around the pzc, from 1.9 to 3.1 µF cm^{-2}. Moreover, the height of the peaks observed at −1 and −1.2 V further decreased compared with the curve recorded 30 min after protein injection, to attain 15 and 20.8 µF cm^{-2}, respectively (Table 1).

At higher mtCK concentrations, the insertion phenomenon occurs more quickly and the adsorption process is "masked". Indeed, at concentrations tenfold higher than that used in this study only the insertion can be measured (Maniti et al. 2009b). However, the final *C* value was independent of the amount of protein added, as shown by measurements performed with mtCK concentrations ranging from 0.4 to 4 nM (Table 2).

The *C* vs. *E* profile of the CL monolayer in the presence of mtCK was then compared with that in the presence of cytochrome c, which is known to interact with CL-containing membranes. When Cytochrome c was introduced underneath the CL monolayer, *C* did not change at the pzc for at least 2 h; although adsorption can be attested to by changes in the *C* vs. *E* profile (Fig. 4).

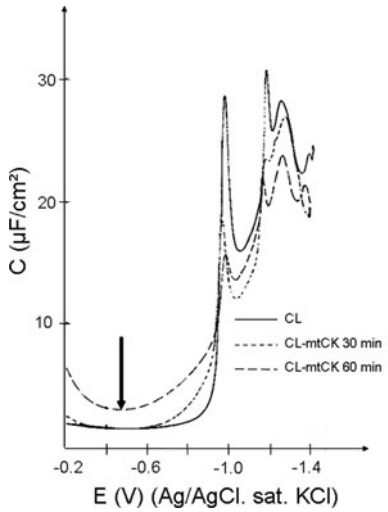

Fig. 3 Differential capacity vs. potential curves obtained with a hanging mercury-drop electrode in contact with a condensed CL monolayer before (*full line*) and 30 min (*dotted line*) or 60 min (*dashed line*) after addition of 0.4 nM mtCK. *Arrow*: potential of zero charge (pzc) of the mercury electrode. Curves are representative of at least three independent measurements

Table 1 Differential capacity values of a CL monolayer at the pzc or in the region of the rearrangement peaks in the absence or presence of mtCK, 30 or 60 min after protein injection

E (V)	C (µF/cm^2)		
	CL	CL-mtCK 0.4 nM 30 min	CL-mtCK 0.4 nM 60 min
−0.45	1.9	1.9	3.1
−1.0	27.5	17.5	15
−1.2	29.4	24.3	20.8

Values are representative of three independent measurements

Table 2 Maximum differential capacity values of a CL monolayer at the pzc after addition of increasing amounts of mtCK

[mtCK] (nM)	0.4	0.8	2	4
C_{max} (µF cm^{-2})	3.1	3.1	3.1	3.3

Discussion

This study obtained experimental evidence of a sequential process in mtCK–membrane binding. C is defined as the second derivative of the surface tension with respect to potential (Parsons 1954), and is, therefore, highly sensitive to minute variations of the surface pressure. That makes this property a valuable tool for analyzing the interaction of water-soluble molecules with phospholipid monolayers (Lecompte 2005; Lecompte et al. 1994, 2002, 2005). The differential capacity of the interface varies with the potential imposed between the working and the reference

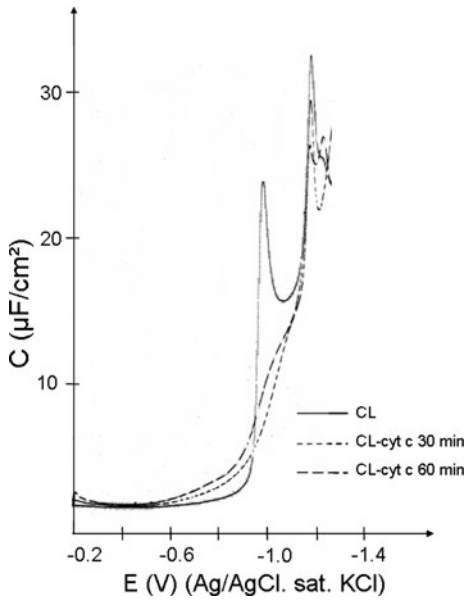

Fig. 4 Differential capacity vs. potential curves obtained with a hanging mercury-drop electrode in contact with a condensed CL monolayer before (*full line*) and 30 min (*dotted line*) or 60 min (*dashed line*) after addition of 2 nM cytochrome *c*. Curves are representative of at least three independent measurements

electrodes and depends on the type of molecules in contact with the mercury electrode (Lecompte et al. 1994); mainly, the highest value of C corresponds to the pure electrolyte (Lecompte et al. 1994).

Around the pzc (−0.45 V), C decreases when the amount of CL spread at the argon–electrolyte interface is increased (Fig. 1), indicating that electrolyte molecules at the electrode are gradually replaced by lipids. The lowest phospholipid density ensuring the formation of a compact cardiolipin monolayer is 0.2 µg cm^{-2}. The same surface concentration of lipids (0.2 µg CL cm^{-2}) was spread at the buffer surface in a Langmuir trough equipped with a pressure-sensing captor. The surface pressure was measured 20 min after spreading to ensure solvent evaporation and self-assembled monolayer formation and the values recorded were between 30 and 35 mN m^{-1} (not shown). Under these conditions, C is minimum and remains constant over a large range of potential around the pzc. A protein that perturbs the arrangement of phospholipid at the interface also modifies the differential capacity of the system. This electrochemical method is thus a powerful experimental tool that enables investigation of whether the protein is simply adsorbed by the lipid polar head or penetrates the monolayer. For a protein that is adsorbed by the condensed lipid monolayer, as illustrated in Fig. 5a, the capacity of the system (C) can be described by two condensers, the lipid membrane (C_L) and the protein layer (C_P), which are arrayed in series (Lecompte et al. 1994). The capacity of the system can thus be expressed as:

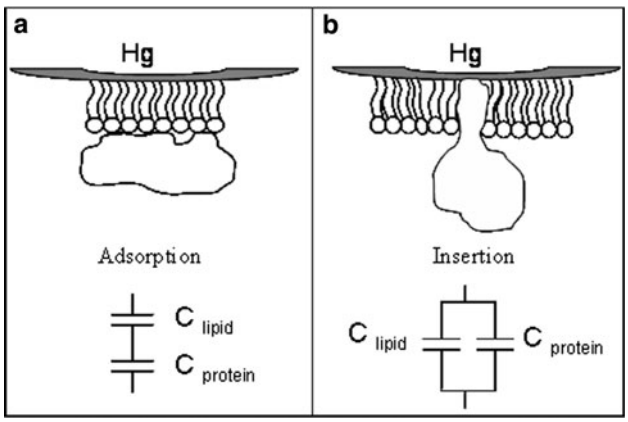

Fig. 5 Schematic illustration of a protein adsorbed by (**a**) or inserted into (**b**) a phospholipid monolayer in contact with a hanging mercury-drop electrode. The differential capacity of the system can be modeled by two condensers arrayed in series (**a**) or in parallel (**b**). Adapted from Lecompte et al. (1994)

$$1/C = 1/C_L + 1/C_P \quad (1)$$

where C_L and C_p are the differential capacities of condensed lipid and protein monolayers, respectively. In this case, the differential capacity is expected to decrease with the amount of protein adsorbed by the lipids. The extent of the decrease will depend on the proportion of phospholipid monolayer area covered by the protein, and on the values of C_L and C_P. As C_p is much higher than C_L, the decrease is not detectable. Indeed, the 12 μF cm^{-2} value measured for the differential capacity of the mercury drop fully covered by mtCK is much higher than that recorded for the CL monolayer (1.9 μF cm^{-2}).

For a protein that only penetrates into the monolayer, i.e. crosses the polar head–hydrocarbon chains interface (Fig. 5b), the circuit will exhibit properties that correspond to electrical condensers in parallel, with contributions from both the protein and the lipid monolayer. The value of C will depend on the amount of protein penetrating the membrane (Lecompte et al. 1994), following the equation:

$$C = C_L + \theta(C_P - C_L) \quad (2)$$

where C_L, and C_p are the differential capacities of condensed lipid and protein monolayers, respectively, and θ the fraction of lipid monolayer that has been penetrated by the protein, i.e., the fraction of phospholipids replaced by protein at the electrode.

According to Eq. 2, and because C_p is much higher than C_L, the expected effect is an increase in C. When both phenomena occur, the decrease in C induced by protein adsorption is masked by the greater increase in C because of protein insertion. In this case, the effect of protein insertion prevails.

In this study, a time-dependent effect of mtCK on the differential capacity of the interface was evidenced (Fig. 2). Of note, mtCK insertion was evidenced by differential capacity measurements over the same time range as morphological changes monitored by Brewster angle microscopy or using surface pressure measurements in a Langmuir trough with protein concentrations between 0.4 and 4 nM (Maniti et al. 2009a, b; Vernoux et al. 2006, 2007).

When measurement is done 30 min after protein injection, the value of C at the pzc remains constant indicating that the continuity of the dense phospholipid layer is not disrupted by protein insertion into the CL monolayer. Distinct changes in the C vs. E profile can, however, be seen (Fig. 3). Indeed, the range of potential over which C is constant is shorter than in the absence of protein and peaks that appear in the −0.9 to −1.4 V potential range are modified. The peak observed at −1.0 V on the C vs. E curve can be attributed to rearrangements of the CL monolayer in response to potential variations, as first proposed by Nelson and Leermakers for phosphatidylcholine (Nelson and Leermakers 1990). Whereas at the pzc the monolayer is oriented with the acyl chains toward the electrode and the polar headgroup toward the buffer subphase, at −1.0 V it is destabilized and competition may take place at the electrode between the polar heads and the acyl chains (Nelson and Leermakers 1990). The sharpness of the peak indicates that rearrangements occurred rapidly. The peak observed around −1.2 V is a desorption peak (Lecompte et al. 1998). Rearrangements are reversible, as identical C vs. E profiles are obtained when measurement is repeated. Modification in the intensity of these peaks reveals that mtCK binding to negatively charged polar heads affects monolayer behavior. All together, these observations indicate that mtCK–CL interaction is an adsorption process similar to that described for several molecules (Lecompte 2005; Lecompte et al. 1994, 1998, 2002).

However, for longer incubation with mtCK (60 min), the differential capacity at the pzc distinctly increases, evidencing perturbations of lipid layer continuity (Lecompte 2005; Lecompte et al. 1994, 1998, 2002) as a result of the replacement of phospholipids by protein, or part of the protein, inserting into the monolayer. Thus, the relative contributions of adsorbed and inserted protein to the differential capacity of the interface change during incubation. This change was not observed with cytochrome c, which also interacts with CL (Fig. 4). This suggests that the sequential two-step binding mechanism described here is specific for mtCK. It is difficult to measure mtCK binding kinetics in this experimental set up, but pseudo first-order binding rate constants over 4×10^{-3} s^{-1} have been derived from light-scattering experiments with CL vesicles (Schlattner et al. 2004). This would indicate that upon 30 min incubation most of the protein is adsorbed by the CL monolayer. Surface pressure measurements allowed us to

confirm this conclusion (not shown). The time-dependent change of the differential capacity of the interface, from adsorbed to inserted protein, thus reveals a two-step mtCK binding mechanism. The first step is the electrostatically driven adsorption of mtCK's positively charged Lys residues, exposed on the top and bottom faces of the octameric cube, by the negatively charged CL head (Schlattner et al. 2004). The second step involves protein insertion between lipids, which is generally associated with hydrophobic interactions. Our results are in agreement with the putative mtCK membrane binding model proposed by Schlattner et al. on the basis of a biphasic binding process involving two independent interaction sites (Schlame and Augustin 1985; Stachowiak et al. 1996) with fast and slow association, as seen with surface plasmon resonance spectroscopy (Schlattner and Wallimann 2000a, b). The fast binding had then been proposed to be purely electrostatic in nature and to occur via the C-terminus mtCK basic residues, whereas the slow binding would occur because of hydrophobic interaction leading to penetration of mtCK into the lipid membrane. Because of both the size of the mtCK octamer ($93 \times 93 \times 86$ Å) and the continuity of the lipid layer obtained under our experimental conditions, there would not be enough space between phospholipid head groups to allow insertion of the whole protein molecule. Therefore, the binding process is more likely to involve only partial insertion between the lipids of a hydrophobic segment of the protein in the vicinity of the residues involved in membrane recognition, as already proposed (Wallimann et al. 1998b). One candidate is the moderately hydrophobic proline-rich region situated between Lys 368 and Lys 380, which could form a loop and insert into the biomimetic membrane (Schlattner et al. 2006). Consequently, Lys 368 and 380 interacting with CL molecules would come closer, thus contributing to the previously reported CL-clustering (Maniti et al. 2009b), and to the decrease in membrane fluidity described on CL-containing liposomes (Granjon et al. 2001). Studies with proline mutant are currently under investigation to strengthen this hypothesis. This partial insertion of mtCK into the membrane would contribute to reinforcement of the mtCK–membrane interaction and explain its resistance against full detachment by high ionic strength (Schlattner et al. 2004).

Acknowledgments We are very grateful to Dr J. Clavilier for very fruitful suggestions and discussions. We acknowledge funding from University Lyon 1, CNRS, Région Rhône-Alpes and Agence Nationale de la Recherche.

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