Volume 281, number 1,2, 123-129 FEBS 09568 April 1991

ADONIS 001457939100278?

Interaction of mitochondrial creatine kinase with model membranes

A monolayer study

Manuel Rojo[1], Ruud Hovius[2,3], Rudy Demel[2], Theo Wallimann[1], Hans M. Eppenberger[1] and Klaas Nicolay[2,3]

[1]*Institute for Cell Biology, Eidgenössische Technische Hochschule, 8093-Zürich, Switzerland,* [2]*Institute of Molecular Biology and Medical Biotechnology* and [3]*Centre for Biomembranes and Lipid Enzymology, University of Utrecht, 3584 CH Utrecht, The Netherlands*

Received 1 February 1991

The interaction of mitochrondrial creatine kinase (Mi-CK; EC 2.7.3.2) with phospholipid monolayers and spread mitochondrial membranes at the air/water interface has been investigated. It appeared that Mi-CK penetrated into these monolayers as evidenced by an increase in surface pressure upon incorporation of Mi-CK. The increase in surface pressure was dependent on (1) the amount and (2) the oligomeric form of Mi-CK in the subphase, as well as on (3) the initial surface pressure and (4) the phospholipid composition of the monolayer. In this experimental system Mi-CK was able to interact equally well with both inner and outer mitochondrial membranes.

Creatine kinase; Model membrane; Protein-lipid interaction; Mitochondrion

1. INTRODUCTION

Creatine kinase (CK; EC 2.7.3.2) isoenzymes catalyze the reversible transfer of the phosphoryl group from phosphocreatine (PCr) to ADP, thus regenerating ATP. CK 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 CK have been characterized: brain-type (BB-), muscle-type (MM-), as well as the hybrid MB-CK isoenzymes are mainly cytosolic and are always dimeric (see [1] and references therein). The mitochondrial CK isoenzymes (Mi-CK) are restricted to mitochondria and have been shown to exist in two oligomeric forms, as a dimeric and as an octameric molecule [2].

Evidence has accumulated that the interaction of the various CK-isoenzymes with cellular structures plays an important role in their function. Cytosolic CK isoforms are associated with specific subcellular sites, e.g. in muscle, a physiologically significant portion of MM-CK is specifically bound at the myofibrillar M-line, while another portion of the same isoenzyme is loosely associated with the I-band. Some MM-CK is also specifically bound to the sarcolemma and the sarcoplasmic reticulum, while in Torpedo, a BB-like CK species was found to be associated with acetylcholine receptor-rich post-synaptic membranes (reviewed in [1]).

Mi-CK is located in the mitochondrial intermembrane space, where it is bound to the outer leaflet of the inner mitochondrial membrane [3]. In addition to the localization of Mi-CK along the cristae membranes [2], recently, subfractionation of disrupted mitochondria has shown that Mi-CK is accumulated in the fraction of contact sites between inner and outer membrane [4]. The presence of Mi-CK at contact sites was confirmed by 'in situ' histochemical staining, which showed CK activity localized at sites of close proximity between both mitochondrial membranes [5].

The specific subcellular location of CK isoenzymes can be interpreted in terms of the PCr/Cr circuit [1], as a means to ensure a high phosphorylation potential at sites of ATP consumption, and phosphocreatine synthesis at sites of ATP production. Functional coupling of the creatine kinase reaction and oxidative phosphorylation in mitochondria has been described [6]. Recently, we reported on the ability of Mi-CK to interact simultaneously with two phospholipid domains, thereby creating a contact between two membrane interfaces [7].

Correspondence address: T. Wallimann, Institute for Cell Biology, Eidgenössische Technische Hochschule, 8093-Zürich, Switzerland. Fax: (41) (1) 371 2894

Abbreviations: CK, creatine kinase; BB-CK, brain form of CK; MM-CK, muscle form of CK; Mi-CK, mitochondrial CK; Cr, creatine; PCr, creatine phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; BME, 2-mercaptoethanol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycol bis-(2-aminoethylether)-*N,N,N'N'*-tetraacetic acid; BSA, bovine serum albumin; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulfonic acid; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography

The aim of this work is to characterize more extensively the direct interaction of Mi-CK with membranes. The behaviour of different CK isoforms (MM-, BB- and Mi-CK) towards model membranes using the monolayer technique is described. The lipid specificity of the Mi-CK-membrane interaction is investigated using lipid extracts from various cellular membranes and pure lipid species.

2. MATERIALS AND METHODS

All reagents used were of analytical grade quality.

2.1. *Source of CK isoenzymes*

Chicken cardiac Mi-CK, BB-CK and skeletal muscle MM-CK were purified to homogeneity as described [2,8,9]. The isoenzymes (3.5–5 mg/ml) were aliquotted, rapidly frozen in liquid nitrogen and stored at −70°C in 25 or 50 mM sodium phosphate, pH 7–7.2, 150–250 mM NaCl, 2 mM BME, 0.2 mM EDTA. Octameric Mi-CK was taken from a sample at high protein concentration (see above), which consisted of ≥90% octameric Mi-CK. A dimeric Mi-CK sample, containing 85–90% dimeric enzyme, was generated by incubation of 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 Cr, 50 mM KNO_3 in 150 mM NaCl, 25 mM sodium phosphate, pH 7.2, known to induce a transition state-analog complex [2,10].

2.2. *Isolation of membrane fractions*

Isolation and subfractionation of rat liver mitochondria were performed according to [11]. Mitochondria from chicken heart were isolated as described [12] with an additional Percoll (Pharmacia) gradient centrifugation step. Microsomal membranes were isolated from rat liver as described [13].

2.3. *Lipids*

Lipids were extracted from the different membrane fractions by the method of Bligh and Dyer [14] as described [11], then dissolved in $CHCl_3/CH_3OH$ (4:1, v/v) to a concentration of 1–5 mM lipid phosphorus and stored at −20°C. Dioleoylphosphatidylserine and dioleoylphosphatidylcholine were synthesized according to established methods [15,16]. Cardiolipin from bovine heart was isolated and purified essentially as described [17]. Soybean phosphatidylinositol was purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Lipid phosphorus was measured after destruction of phospholipids with 70% perchloric acid by the method of Fiske and Subbarow [18].

2.4. *Radioactive methylation of Mi-CK*

Mi-CK was radiolabeled by reductive methylation with [^{14}C]formaldehyde (Du Pont-New England Nuclear, 55 Ci/mol) and sodium cyanoborohydride [19]. The net charge of the protein is not altered through this method. The reaction was carried out in 25 mM sodium phosphate, pH 7.0, 50 mM NaCl, 2 mM BME, 0.2 mM EDTA at a protein concentration of 1.9 mg/ml for 1.7 h at 25°C. The molar ratio of monomeric protein/formaldehyde was 1:140. After dialysis against the same buffer, the specific enzymatic activity, oligomeric form and dimerizing ability of methylated Mi-CK were indistinguishable from those of the unlabeled protein. The specific radioactivity of the labeled Mi-CK was 44 μCi/mg.

2.5. *Creatine kinase assay*

Creatine kinase activity was determined either in the direction of ATP synthesis in a pH stat [20] or in the direction of PCr synthesis according to Bücher et al. [21].

2.6. *Protein determination*

Protein was determined either by the BCA assay of Pierce Chemical Company (Rockford, USA) including 0.1% sodium dodecylsulfate (SDS) or by the method of Lowry [22] in the presence of 1% SDS, using BSA as a standard. Both methods gave identical results. The concentration of purified cardiac Mi-CK was determined measuring the absorption at 280 nm, using an absorbing index $A^{1\%}_{1cm}$ of 12, as determined by amino acid analysis of the pure enzyme.

2.7. *FPLC-Gel permeation chromatography*

Gel-filtration experiments were performed on a FPLC-Superose 12 HR 10/30 column (Pharmacia) as described [12].

2.8. *Monolayer techniques*

Interfacial measurements were performed at 25°C in a thermostatically controlled box under a nitrogen atmosphere. The surface pressure was measured by the Wilhelmy method [23]. The amount of radiolabel at the interface was determined by recording the surface radioactivity with a gas flow detector [23], 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. Two different Teflon dishes were used: one with a subphase volume of 5 ml and a surface area of 8.6 cm^2 for surface-pressure measurements, and one with a volume of 19 ml and a surface area of 29.6 cm^2 which allowed surface pressure and interface radioactivity to be monitored concomitantly.

Inner and outer mitochondrial membranes were spread at the nitrogen/water interface essentially as described by Verger and Pattus [24]. The teflon dish consisted of 3 chambers, each with a volume of 19 ml and a surface area of 35 cm^2. Vesicle suspensions (5–10 mM lipid phosphorus) were applied from a syringe at a constant speed between 3 and 40 μl/min to a rough glass plate partially submerged in the subphase. The application of vesicles was stopped when the surface pressure reached 6–8 mN/m. The film was then slowly compressed with a teflon bar to the desired surface pressure, and the interconnecting channel was closed. Subphase buffers were 10 mM sodium phosphate, pH 7.0 or 220 mM mannitol., 70 mM sucrose, 2 mM HEPES, pH 7.4. 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 at a rate of 10–20 ml/min. Proteins were added with a syringe through a hole in the teflon chamber connected to the subphase. The volumes of actual injections into the subphase were always ≤2% of the total subphase volume except for dimeric Mi-CK where the subphase volume was increased by maximally 10%. Due to the increased buoyancy of the Wilhelmy plate following these injections, corrections were necessary to obtain the true protein-induced increase in surface pressure.

2.9. *SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

SDS-PAGE was performed with Phastgel Gradient 10–15% polyacrylamide (PhastSystem, Pharmacia). Proteins were stained by the Diamine silver staining method (Development Technique File No. 210, Pharmacia).

3. RESULTS

3.1. *Interaction of octameric Mi-CK with phospholipid monolayers*

The interaction of Mi-CK with model membranes was studied by the monolayer technique. First the surface activity of the Mi-CK itself was measured by injecting increasing amounts of Mi-CK in 10 mM sodium phosphate buffer while recording the surface pressure. Mi-CK induced an increase in surface pressure that reached a maximum of 20.3 mN/m at a concentration of 0.1 μM octameric Mi-CK. All further experiments with lipid monolayers were performed at surface

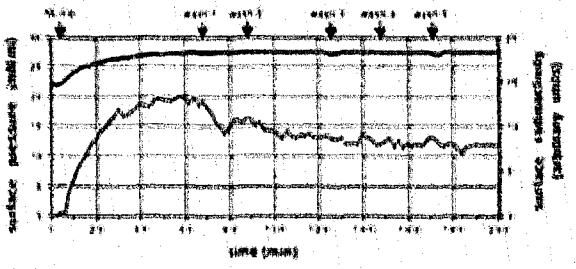

Fig. 1. Association of octameric Mi-CK with monolayers of mitochondrial inner membrane phospholipids. Changes in surface pressure (upper tracing: ═) and surface radioactivity (lower tracing: —) at the interface were monitored as a function of time. The monolayer was formed at 22 mN/m on a subphase of 10 mM sodium phosphate, pH 7.0. Protein concentration after injection (arrow: Mi-CK) was 0.12 μM of octameric Mi-CK. Subphase was replenished with 6 vols. of buffer at the points indicated. ^{14}C-labeled Mi-CK (44 μCi/mg) and non-radiolabeled Mi-CK were added in a mass ratio of 1 to 38. Wash buffers were 10 mM sodium phosphate pH 7.0 containing either 0 (1), 50 (2), 100 (3), 150 (4), or 500 (5) mM NaCl. The amount of Mi-CK at the interface before and after subphase wash with 10 mM sodium phosphate was 316 and 278 ng/cm^2 ($n = 2$).

pressures of ≥22 mN/m in order to avoid non-specific surface adsorption.

In the initial experiments with phospholipid monolayers, surface-pressure changes were measured and simultaneously the amount of ^{14}C-labeled Mi-CK at the interface was quantitated. ^{14}C-labeled Mi-CK was injected into the subphase below a stable monolayer of inner mitochondrial membrane phospholipids at an initial surface pressure of 22 mN/m (Fig. 1). The surface pressure increased slowly and equilibrated after 40–60 min at 27 mN/m. Surface radioactivity followed a parallel time-course, implying that Mi-CK was indeed causing the surface pressure increase. Labeled and unlabeled Mi-CK did not differ in their ability to interact with monolayers, since mixtures of native and ^{14}C-methylated Mi-CK in different proportions caused the same pressure increase and gave the expected proportional change in surface radioactivity (data not shown). After equilibration of surface pressure and radioactivity, the subphase was washed with sodium phosphate buffers (pH 7.0) containing increasing concentrations of NaCl. Even at 500 mM NaCl (Fig. 1) only a minor proportion (35%) of the bound Mi-CK was removed from the interface and no decrease in surface pressure was observed. Washing the subphase with 25 mM sodium phosphate pH 8.8 resulted in the removal of 25% of the bound Mi-CK (not shown).

The influence of the subphase composition on the initiation of the Mi-CK-monolayer interaction was also investigated. Octameric Mi-CK (0.12 μM) was injected underneath a monolayer of inner mitochondrial membrane phospholipids with an initial pressure of 22 mN/m into subphases of different composition. The surface-presence increase was found to be independent of ionic strength (0–150 mM NaCl), pH value (7.0–8.4) and the pressure or absence of 0.2 mM EGTA or 5 mM BME (not shown).

3.2. *Interaction of different CK-isoforms with phospholipid monolayers*

To investigate whether octameric Mi-CK exclusively induced a surface-pressure increase in a phospholipid monolayer, the interaction of other CK-isoforms with phospholipid monolayers was also studied.

All CK-isoenzymes tested induced a surface-pressure increase upon injection below monolayers of inner membrane phospholipids. The interaction of the different creatine kinases with the lipid interface was characterized by measuring the surface-pressure increase induced by the isoenzymes as a function of their subphase concentration (Fig. 2) and as a function of the initial surface pressure of the monolayer (Fig. 3). Injection of dimeric Mi-CK into a subphase implied the simultaneous addition of reagents inducing a transition state-analog complex to the subphase; therefore, in

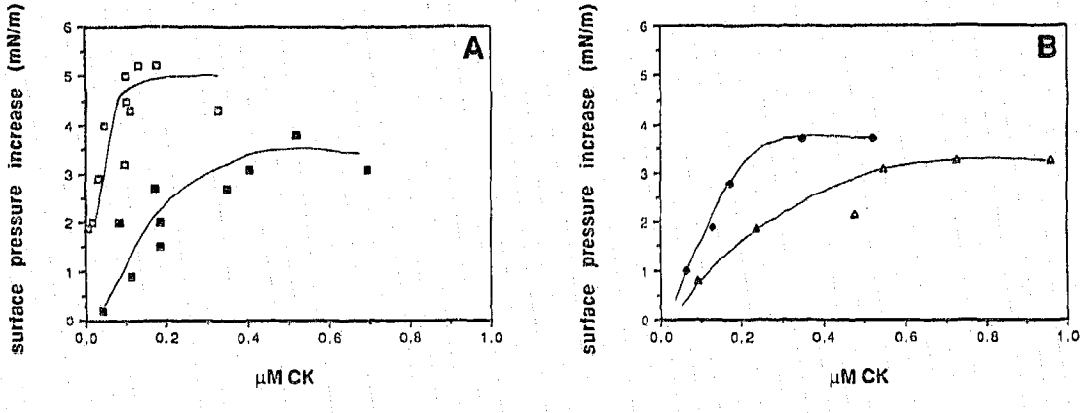

Fig. 2. Surface-pressure increase of inner mitochondrial membrane phospholipid monolayers as a function of the concentration of CK isoenzymes at an initial surface pressure of 22 mN/m. (A) octameric (□) and dimeric (■) Mi-CK; (B) MM; (◆) and BB-CK (△).

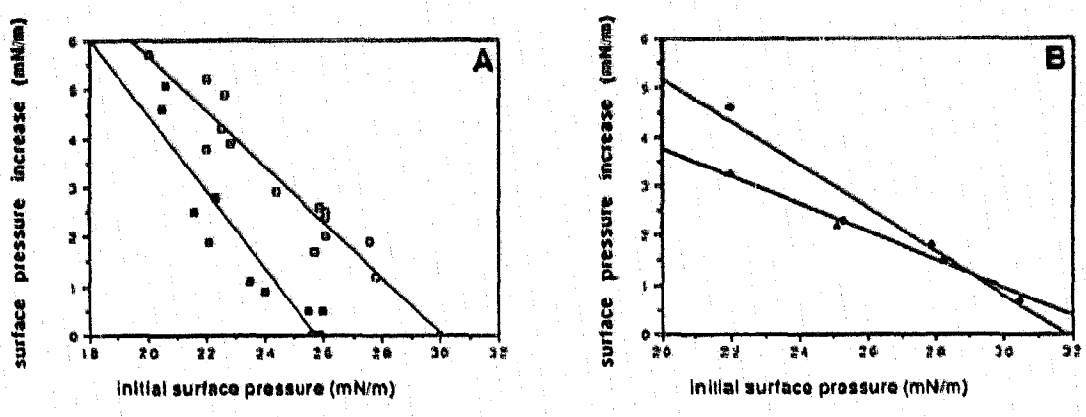

Fig. 3. Surface-pressure increase of inner mitochondrial membrane phospholipid monolayers at different intial surface pressures upon injection of (A) 0.12 μM octameric Mi-CK (□), 0.48 μM dimeric Mi-CK (■); (B) 0.38 μM MM- (♦) or 0.5 μM BB-CK (△) into the subphase. The lines drawn are the result of least-square fits to all the data points: $r = 0.96$ (octameric Mi-CK); 0.93 (dimeric Mi-CK); 0.97 (MM-CK); 0.99 (BB-CK).

order to compare the behaviour of dimeric and octameric Mi-CK towards phospholipid monolayers under identical subphase conditions, the same amount of reagents present in the dimer sample was added to the subphase prior to injection of octameric Mi-CK. Gel permeation analysis of the subphases of these experiments showed that no shifts of the dimer to octamer ratio occurred during the experiments (not shown). The magnitude of the pressure increase was saturable with all isoenzymes, but saturation was reached at different subphase concentrations of the particular isoproteins (Fig. 2). Octameric Mi-CK showed the highest affinity towards phospholipid monolayers, with saturation reached at 0.05–0.1 μM. The saturation concentration of octameric Mi-CK was lower than that of dimeric Mi-CK (0.2–0.4 μM). The two cytosolic isoenzymes also showed higher saturation concentrations than octameric Mi-CK and subtle differences in the saturation behaviour between both cytosolic proteins became evident: while MM-CK saturated at 0.2–0.3 μM, BB-CK did so at 0.3–0.5 μM.

The surface pressure increase induced by CK-isoenzymes was inversely proportional to the initial surface pressure of the monolayer (Fig. 3). For Mi-CK the amount of protein bound to the monolayer was measured as a function of the initial surface pressure using radiolabeled protein. It appeared that the two parameters displayed inverse proportionality (not shown). The so-called 'critical pressure' is the initial surface pressure of a monolayer above which a protein is no longer able to penetrate the monolayer and to cause a surface-pressure increase. With respect to this parameter, the different CK-isoforms also differed from each other (Fig. 3). The critical pressure of octameric Mi-CK was about 30 mN/m (Fig. 3A, Fig. 5), while that of dimeric Mi-CK was about 26 mN/m. The critical pressure of the cytosolic CK isoenzymes was higher (Fig. 3): about 33 mN/m for BB-CK and 32 mN/m for MM-CK.

3.3. *Phospholipid specificity of the Mi-CK-monolayer interaction*

It has been argued that anionic phospholipids, in particular cardiolipin, are essential for the interaction of Mi-CK with model and mitochondrial membranes [25]. Therefore, the lipid specificity of the surface-pressure increase induced by Mi-CK was investigated. Monolayers composed of dioleoylphosphatidylcholine plus varying amounts of anionic phospholipids which occur in mitochondria, that is, cardiolipin, phosphatidylserine and phosphatidylinositol, were used. The interaction of Mi-CK with monolayers of pure phosphatidylcholine at an initial pressure of 22 mN/m resulted in a lower pressure increase than with monolayers of total phospholipid extracts from different membrane fractions (Fig. 4, cf. Figs. 1, 2, 3 and 5). The surface-pressure increase was augmented with increasing content of anionic phospholipid in the

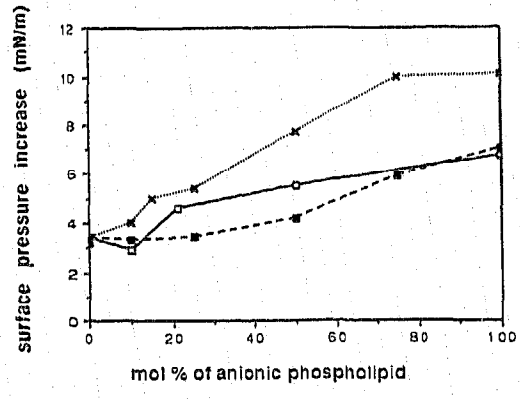

Fig. 4. Surface-pressure increase of phospholipid monolayers at an initial surface pressure of 22 mN/m as a function of the anionic phospholipid content of the monolayers. Monolayers were composed of dioleoylphosphatidylcholine plus the indicated mol% of phosphatidylinositol (■), dioleoylphosphatidylserine (□) or cardiolipin (×). Protein concentration was 0.12 μM octameric Mi-CK.

monolayer, the effect being most prominent for cardiolipin (Fig. 4).

The above findings imply that Mi-CK has a preference for monolayers containing anionic phospholipids. Moreover, it is suggested that the enzyme may not discriminate between membrane interfaces having a comparable anionic lipid content irrespective of the lipid species present. For this purpose, monolayers were prepared with phospholipids extracted from whole mitochondria, endoplasmic reticulum, as well as inner and outer mitochondrial membranes, which are known to have a similar anionic lipid content. The pressure increase induced by Mi-CK was measured as a function of the initial surface pressure with the various monolayers (Fig. 5). Mi-CK did not show any specificity for a particular phospholipid mixture, that is, the inverse proportionality between the Mi-CK induced surface pressure increase and the initial surface pressure of the monolayer was the same for all phospholipid extracts (Fig. 5).

3.4. *Interaction of Mi-CK with inner and outer mitochondrial membranes spread at the air/water interface*

Next, the effect of Mi-CK on monolayers spread from freshly isolated inner and outer mitochondrial membrane preparations was studied (for background see [26] and references therein).

First, the interface itself was investigated. Mitochondrial outer membranes were spread over a subphase of 220 mM mannitol, 70 mM sucrose, 2 mM HEPES at pH 7.4 and compressed to 22 mN/m. The interface was then collected and analysed for phospholipid and protein content. Spreading yield of protein was 53 ± 12% ($n = 4$), and the phospholipid to protein ratio of the interface (795 ± 65 nmol lipid phosphorus/mg protein, $n = 3$) was almost equal to that of the original vesicles applied (840 nmol/mg). The SDS-PAGE patterns of the interface and of the vesicles applied were undistinguishable (not shown). Second, the interaction of Mi-CK with these monolayers was studied. Inner and outer mitochondrial membranes were spread, compressed, and Mi-CK was injected in the subphase below. At an initial surface pressure of 22 mN/m, the injection of Mi-CK into the subphase resulted in a surface-pressure increase of about 5–6 mN/m (data not shown). Since the membranes spread at the air/water interface resemble mitochondrial membranes more closely than a pure phospholipid monolayer, this result confirms the ability of Mi-CK to interact similarly both with the inner and the outer mitochondrial membrane (cf. Fig. 5).

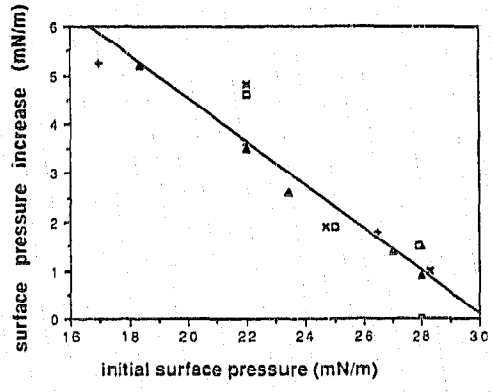

Fig. 5. Surface pressure increase of phospholipid monolayers at different initial pressures. Monolayers were made with phospholipid mixtures extracted from microsomal membranes (+), inner (▲) and outer mitochondrial membranes (□) from rat liver, and of whole mitochondria from chicken heart (×). Protein concentration was 0.12 μM of octameric Mi-CK. The line drawn is the result of a least-square fit to all the data points: $r = 0.94$.

4. DISCUSSION

In the work presented we showed that all CK isoenzymes (BB-, MM- and Mi-CK) induced a surface pressure increase when injected below a phospholipid monolayer (Figs. 2 and 3). This property is shared by many phospholipid-binding proteins (see e.g. [27] for review). As shown in Figs. 2 and 3, the cytosolic CK isoforms interacted with phospholipid monolayers with lower affinity than Mi-CK. The membrane-binding behaviour of the cytosolic CK isoenzymes is in agreement with the reported association of BB- and MM-CK with subcellular structures (reviewed in [1]) and with observations from many authors, in which cytosolic CK was shown to be partly co-enriched during purification of mitochondria (see e.g. [2]), but contradicts the reported inability of MM-CK to bind to heart mitochondria [28]. For Mi-CK, however, the data are straightforward and represent novel aspects of the membrane interaction of this mitochondrial enzyme.

A large proportion (± 65%) of the Mi-CK associated with the monolayer was not solubilized with high salt or alkaline pH buffer (Fig. 1), indicating that the interaction of the enzyme with the phospholipid molecules at the interface is strong and not only of ionic nature. By contrast, Mi-CK is effectively solubilized from mitochondrial inner membrane preparations under these conditions [2,29]. It is still a matter of debate whether ionic strength or salt composition is decisive for Mi-CK solubilization [29,30]. A spin-label electron spin resonance study on the binding of Mi-CK to cardiolipin-containing vesicles indicated that the enzyme binds to the surface region of the bilayer and does not penetrate into the hydrophobic region of the membrane [31], while our results suggest that domains of the Mi-CK molecule penetrate inbetween the phospholipid molecules of the monolayer.

Octameric and dimeric Mi-CK were shown to differ in their ability to interact with phospholipid monolayers. The octamer induced a higher surface-

pressure increase and showed a higher affinity as well as a higher critical pressure than the dimer (Figs. 2 and 3). Because of the latter facts, the pressure increase measured upon injection of a Mi-CK preparation containing mostly dimeric Mi-CK (≥ 85%) might be due to the small proportion of octameric Mi-CK (≤ 15%) still present in the sample.

The experiments with monolayers of different phospholipid composition confirmed the preferential interaction of Mi-CK with cardiolipin already established by Carafoli and co-workers [25]. However, the data shown here allow the conclusion that, beside cardiolipin, other anionic phospholipids can also function as membrane receptors of mitochondrial creatine kinase: Mi-CK interacted with various negatively-charged phospholipids (Fig. 4), a feature which was also observed by the above authors with phospholipid vesicles [32]. Mi-CK did not show any specificity in interacting with monolayers of total phospholipid extracts from different membranes (Fig. 5). Some of these membranes contain no cardiolipin at all, but other negatively-charged phospholipids in a similar proportion (see e.g. [33]). The ability of Mi-CK to interact equally well with monolayers spread from phospholipid extracts from inner and outer mitochondrial membrane suggests that the enzyme might in principle bind to both mitochondrial membranes. Indeed Mi-CK interacted with both inner and outer mitochondrial membranes spread at the air/water interface, inducing a similar surface-pressure increase.

The described membrane-binding properties of Mi-CK have several implications for the function and localization of the enzyme in vivo. Dimeric and octameric Mi-CK show somewhat different kinetic properties [34] as well as a differential pH-dependency of interaction with mitoplasts [12]. In this work dimeric and octameric Mi-CK differed in their behaviour towards monolayers especially at high surface pressures, with the octamer showing a higher critical pressure than the dimer (Fig. 3). Since the 'equivalent surface pressure' of biological membranes is thought to be in the range of 30-35 mN/m [35,36] it is tempting to say that, as proposed recently [12], mostly octameric Mi-CK interacts with mitochondrial membranes in vivo.

The ability of Mi-CK to interact equally well with both inner and outer mitochondrial membranes is especially relevant in view of the occurrence of Mi-CK at sites of close contact between inner and outer membrane of mitochondria, where a simultaneous interaction with both membranes has been proposed [12,37]. Over the last years evidence has accumulated that contact sites play a central role not only in the import of mitochondrial precursor proteins (see review [38]) and most probably in the intramitochondrial transport of phospholipids (see [33]), but also in energy metabolism [7,37]. Recently, we reported that Mi-CK mediated close contacts between two model membrane interfaces, a phospholipid monolayer and large unilamellar vesicles [7]. The present work sets the stage for further studies on Mi-CK-induced contacts between two membrane interfaces. The latter experimental system will resemble more closely the complex in vivo situation at contact sites and in the intermembrane space of mitochondria.

Acknowledgements: This work was supported by Grant No. 31-26384.89 from the Swiss National Science Foundation and a grant from the Swiss Society for Muscle Diseases. A short-term EMBO fellowship to M.R. is gratefully acknowledged. K.N. is a Christiaan and Constantijn Huygens fellow of the Dutch Organization for Scientific Research (NWO). We are indebted to W. Hemmer for generously providing large amounts of highly purified BB-CK. We also thank M. Wirt for performing the amino acid analysis of the Mi-CK, B. Brigot for expert technical assistance, Drs. R. and E. Furter-Graves and Dr. I. Riesinger for critical reading of the manuscript and Dr. B. de Kruijff for valuable discussions in the course of these studies.

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