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A Quantitative Approach to Membrane Binding of Human Ubiquitous Mitochondrial Creatine Kinase Using Surface Plasmon Resonance

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We have evaluated surface plasmon resonance with avidin–biotin immobilized liposomes to characterize membrane binding of ubiquitous mitochondrial creatine kinase (uMtCK). While the sarcomeric sMtCK isoform is well known to bind to negatively charged phospholipids, especially cardiolipin, this report provides the first experimental evidence on the membrane interaction of an uMtCK isoform. Qualitative measurements showed that liposomes containing 16% (w/w) cardiolipin bind octameric as well as dimeric human uMtCK and also cytochrome *c*, but not bovine serum albumin. Quantitative parameters could be derived only for the membrane interaction of octameric human uMtCK using an improved analytical approach. Association and dissociation kinetics of octameric uMtCK fit well to a model for heterogeneous interaction suggesting two independent binding sites. Rate constants of the two sites differed by one order of magnitude, while their affinity constants were both about 80–100 nM. The data obtained demonstrate that surface plasmon resonance with immobilized liposomes is a suitable approach to characterize the binding of peripheral proteins to a lipid bilayer and that this method yields consistent quantitative binding parameters.

KEY WORDS: Mitochondrial creatine kinase; protein–lipid interaction; membrane binding; cardiolipin; surface plasmon resonance.

INTRODUCTION

Mitochondrial isoforms of creatine kinase (MtCK) are localized between the inner and outer mitochondrial boundary membranes and in the cristae intermembrane space (reviewed in Wyss *et al.*, 1992). While sarcomeric MtCK (sMtCK) is restricted to muscle, ubiquitous MtCK (uMtCK) is found in many tissues, including brain, gut, kidney, and placenta (Payne *et al.*, 1991). Out of the two stable oligomeric forms of MtCK, dimers and octamers, the latter predominates *in vivo*. Octameric sMtCK is well known as a peripherally binding membrane protein with especially high affinity to the outer surface of the inner mitochondrial membrane (reviewed in Stachowiak *et al.*, 1998).

Membrane binding of MtCK is of considerable physiological relevance, since it creates a microenvironment containing octameric MtCK together with the integral membrane proteins adenylate translocator (ANT, inner mitochondrial membrane) and porin (outer mitochondrial membrane; Beutner *et al.*, 1996, 1998). These complexes allow the efficient export of energy in form of phosphocreatine (PCr) into the cytosol (reviewed in Wallimann *et al.*, 1992; Schlattner *et al.*, 1998) and, in addition, play a role in the formation and regulation of the mitochondrial permeability pore, which is involved in the execution pathway of apoptosis (Beutner *et al.*, 1996, 1998; O'Gorman *et al.*, 1997). Destabilization of octameric structure and membrane-binding capacity of MtCK lead to an impairment of these important functions (Khuchua *et al.*, 1998, O'Gorman *et al.*, 1997).

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In contrast to the uMtCK isoform, membrane binding of sMtCK isoforms has been studied extensively. It is generally accepted now that cardiolipin is the main binding partner of sMtCK *in vivo* and that binding is predominantly electrostatic (reviewed in Stachowiak *et al.*, 1998). However, it has been shown *in vitro* that the octameric enzyme can also bind to other negatively charged phospholipids (Rojo *et al.*, 1991a; Vacheron *et al.*, 1997), and is able to cross-link membranes (Rojo *et al.*, 1991b). Nevertheless, a number of problems still remain largely unsolved. The membrane-binding characteristics of the uMtCK isoform are unknown and inconsistent results were obtained for dimeric sMtCK (Marcillat *et al.*, 1987; Rojo *et al.*, 1991a). Various methods failed to detect an interaction between ANT and MtCK, although both proteins are present in isolated mitochondrial complexes (Beutner *et al.*, 1996, 1998). Further, some conflicting results were obtained for the binding mode of MtCK. In particular, it is not clear if MtCK is entirely surface-bound (Cheneval *et al.*, 1989) or penetrates partially into the lipid bilayer (Rojo *et al.*, 1991a; Vacheron *et al.*, 1997) and whether hydrophobic interactions play a significant role in the binding process (Brooks and Suelter, 1987; Saks *et al.*, 1986; Stachowiak *et al.*, 1996). Some candidates for membrane-binding epitopes of MtCK have been proposed (Fritz-Wolf *et al.*, 1996; Schlattner *et al.*, 1998), but they still await experimental verification.

A prerequisite to re-examine some of these pertinent questions, *e.g.*, by a site-directed mutagenesis approach, is an appropriate screening method to characterize membrane binding. Such a method should be efficient, very sensitive, applicable to membranes containing integral proteins, and capable of completely characterizing the interaction in terms of rate and equilibrium constants. Surface plasmon resonance (SPR) fulfills these conditions. It allows on-line biomolecular interaction analysis (BIA) of analyte binding to immobilized ligand without the necessity of labeling (Myszka, 1997; Salamon *et al.*, 1997). All constants characterizing a given interaction, *i.e.*, kinetic and equilibrium data, are provided by a single series of experiments. SPR analysis has been already successfully applied to protein–membrane interactions, *e.g.*, by using supported lipid bilayer (Heyse *et al.*, 1997; Salamon *et al.*, 1997) or lipid monolayer on hydrophobic surfaces (*e.g.*, BIAcore HPA sensorchip; Steffner and Markey 1997). However, both approaches have important disadvantages. While the first strategy is not yet easily implemented for BIAcore, the second does not allow one to measure interactions with transmembrane proteins. An alternative approach is the capturing of defined biotinylated liposomes (large unilamellar vesicles) on avidin-coated standard carboxymethyl sensorchips (Masson *et al.*, 1994; Stachowiak *et al.*, 1996). This method is very suitable for the characterization of MtCK–membrane interactions, since it allows the adjustment of properties of the sensorchip surface by changing vesicle (ligand) density, lipid composition or incorporation of reconstituted transmembrane proteins. Using such a SPR approach, the interaction of chicken sMtCK with cardiolipin-containing membranes has been studied to some extent (Stachowiak *et al.*, 1996). It has to be stressed that, while the SPR measurement itself can be entirely automated by commercially available instruments like the BIAcore, experimental design and mathematical treatment of the data are still crucial steps and must be carefully chosen (Myszka, 1997; Schuck and Minton, 1996).

The aim of this study was to validate our experimental SPR setup and to gather quantitative information on the binding of a ubiquitous MtCK isoform (human uMtCK) to liposomes mimicking the inner mitochondrial membrane. Our approach included improvement of data quality and kinetic analysis as well as control measurements for peripheral membrane binding. We could show that dimeric and octameric uMtCK, like cytochrome *c*, bind to CL-containing vesicles. Finally, SPR data for octameric human uMtCK analyzed by a heterogeneous interaction model yielded consistent rate and equilibrium constants.

MATERIAL AND METHODS

Materials

Egg yolk phosphatidylcholine was from Lipid Products (South Nutfield, Great Britain), avidin, cardiolipin, and cytochrome *c* from Sigma (Buchs, Switzerland), Biotin-X-DHPE from Molecular Probes (Leiden, Netherlands), and all other chemicals from Fluka (Buchs, Switzerland). Human ubiquitous MtCK (uMtCK) was expressed in *E. coli* and purified to homogeneity (Furter *et al.*, 1992; Schlattner *et al.*, unpublished).

Large Unilamellar Vesicles

A lipid stock solution containing 0.1% (w/w) Biotin-X-DHPE together with 16% (w/w) cardiolipin (CL)

and 83.9% (w/w) phosphatidylcholine (PC) or 99.9% (w/w) PC was produced as follows. Lipids dissolved in chloroform:methanol (2:1) were mixed in the appropriate ratios, dried in a rotary evaporator, and maintained under reduced pressure for at least 5 h. The lipid residue was resuspended in a running buffer (10 mM TES, 50 mM NaCl, pH 7.0) to a final concentration of 5 mg-ml^{-1} using glass beads to remove the lipid film from the round bottom flask. The suspension was finally vortexed for 15 min and could be stored at −20°C. Large unilamellar vesicles (liposomes) with a diameter of approximately 160 nm were produced by a combination of freeze/thawing and extrusion techniques (Stachowiak *et al.*, 1996). Briefly, 1-ml aliquot of stock suspension was subjected to 15 freeze thaw cycles using liquid nitrogen (5 min) and a water bath at 30°C (5 min). The suspension was then passed 21 times through a polycarbonate membrane (Avestin, Milsch Equipment, Germany) with a pore diameter of 200 nM mounted in a mini-extruder (Avestin). The liposome suspension was stocked at 4°C and used within 2 days.

Surface Plasmon Resonance

SPR was measured with BIAcore 2000 (Biacore, Uppsala, Sweden) and a carboxymethyl sensorchip CM5 (Biacore). The chip was coated with 20000 RU avidin using 1 mg-ml^{-1} avidin in water and routine amine coupling (Johnson *et al.*, 1991). A stable liposome surface of about 500 RU was generated according to Masson *et al.* (1994) by injection of biotinylated liposomes (1:250 dilution in running buffer). BIAcore response units (RU) are proportional to the amount of material bound at the sensorchip surface. Programmed measurement cycles at 25°C and a flow rate of 0.3 ml-h^{-1} (Fig. 1) consisted of vesicle immobilization (1:250 dilution of vesicles in running buffer), wash (running buffer), contact phase (different MtCK concentrations in running buffer), dissociation phase (running buffer), and chip regeneration (1% SDS).

MtCK and Control Protein Preparations

Measurement cycles were run with dilutions of MtCK stock solution (5 mg-ml^{-1}) in running buffer covering the range from 0.03 to 0.3 μM. Since such low concentrations favor spontaneous octamer dissociation, dilutions were prepared by the BIAcore autosampler just prior to injection. Octamer content in stock solutions and dilution series was routinely checked by gel filtration chromatography on a BioCad HPLC (Perseptive Biosystems, Perkin-Elmer, Rotkeuz, Switzerland) using a Superose 12 column (Pharmacia, Dubendorf, Switzerland). For qualitative control experiments, cytochrome *c* and BSA were used in higher molarities (1.2 μ*M*), since their much smaller M_r results in a weaker SPF signal upon binding as compared to octameric uMtCK. Protein was determined according to Bradford (1976) using the Bio-Rad assay (Glattbrugg, Switzerland) and BSA as standard.

General Data Analysis

Data were analyzed using BIAcore Evaluation Software (Biacore) for fitting and Sigma Plot (Jandel Scientific) for regression analysis. Kinetics were fitted for a time span of 100 s, which was determined as follows. A minimum delay of 10 s after addition or removal of uMtCK was necessary to avoid mixing effects and bulk refractive index changes. Additional problems with mass transport limitation might occur at low concentrations of bound analyte, i.e., at the beginning of association and at the end of dissociation. Thus, dissociation phase was fitted in the time interval from 10 to 110 s, after the end of MtCK injection. To determine the starting point for fitting during contact phase, we used the quality of our fit to the SPR data (see below). Since deviations from the applied model occurred between 0 and 20 s after uMtCK injection, contact phase was fitted in the time interval from 20 to 120 s. Only fits with residuals smaller than 1 RU were retained for further analysis. Resonance units at equilibrium (R_{eq}) were extrapolated from these fittings. For each MtCK concentration, at least three different data sets of different experiments were pooled and mean ± standard deviation (SD) were calculated using an M_r of 345 for octameric human uMtCK.

Calculation of Binding Constants

SPR data were fitted with double exponential integrated rate equations. They correspond to a heterologous interaction model comprising two independent binding sites, which are identified by the indexes 1 and 2. The dissociation kinetics is described by the rate equation

$$y = (R_0 - R_1) \cdot e^{-k_{d1} \cdot t} + R_1 \cdot e^{-k_{d2} \cdot t} \quad (1)$$

where k_{d1} and k_{d2} are the dissociation rate constants (off rates), R_0 is the response at the beginning of dissociation, and R_1 the response associated with k_{d2}. The contact phase, where association and dissociation of MtCK takes place, is described by the rate equation

$$y = R_{eq1} \cdot (1 - e^{-(k_{a1}c + k_{d1}) \cdot t}) + R_{eq2} \cdot (1 - e^{-(k_{a2} \cdot c + k_{d2}) \cdot t}) \quad (2)$$

where and k_{a1} and k_{a2} are the association rate constants (on rates), k_{d1} and k_{d2} the dissociation rate constants (off rates, already calculated from the dissociation phase), and R_{eq1} and R_{eq2} the equilibrium responses (R_{eq} is reached when bound and free analyte are in equilibrium or steady state). The dissociation equilibrium constants or affinity constants were calculated as

$$K_d = k_d/k_a \quad (3)$$

To verify the obtained rate and equilibrium constants, both were independently calculated with linear plots derived from contact phase kinetics. A modified equation (2), where k_a and k_d were replaced by the concentration dependent "apparent" rate constant

$$k_{obs} = k_a \cdot c + k_d \quad (4)$$

was fitted to contact phase kinetics. Slope and *y*-axis intercept of a linear fit to the plot k_{obs} versus c yielded estimates for the rate constants k_a and k_d. The slope of the plot R_{eq}/c versus c, analogous to a Scatchard plot, provided estimates for the equilibrium constant (or affinity constant) K_d.

RESULTS

We used immobilization of biotinylated liposomes on an avidin-coated surface to characterize membrane interaction of human ubiquitous MtCK (uMtCK) and other proteins with the surface plasmon resonance technique (BIAcore). The liposomes contained 84% phosphatidylcholine (PC) and 16% cardiolipin (CL), thus mimicking the mitochondrial inner membrane.

A typical measurement cycle with human uMtCK is shown in Fig. 1. Visual inspection of the kinetic data already reveals that the enzyme indeed interacts with the CL-containing vesicle surface. The quantity of immobilized liposomes and thus the binding capacity of the surface could be exactly adjusted to 500 RU. The SPR tracing also illustrates how washing with

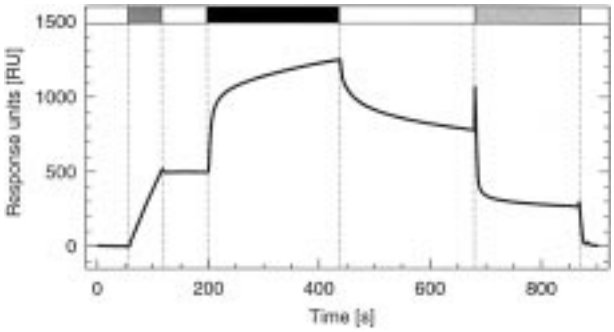

Fig. 1. A typical BIAcore measurement cycle of human uMtCK. The cycle consisted of vesicle immobilization (▨), buffer wash (□), contact (association) phase with 0.15 μM human uMtCK (■), dissociation phase (□), chip regeneration with 1% SDS (▨) and buffer wash (□). For details see Materials and Methods.

1% SDS removes the vesicles and recovers the avidin surface for repeated immobilization cycles. Avidin also minimizes nonspecific binding of basic analyte proteins like MtCK to the sensorchip surface. Since avidin has a basic isoelectric point, it neutralizes remaining negative charges of the carboxymethyl chip and leads to electrostatic repulsion of any basic analyte protein at the given pH of 7. However, unspecific binding increased with the number of measurement cycles and was then corrected by blank runs without immobilized vesicles.

Bovine serum albumin (BSA) and cytochrome *c* were used to check the specificity of our experimental setup for peripherally binding membrane proteins. BSA (1.2 μM) did not show any specific interaction with liposomes after correction for background binding (Fig. 2d). By contrast, injection of 1.2 μM cytochrome *c*, a basic peripheral membrane protein, resulted in pronounced association and dissociation kinetics (Fig. 2a). Using 16% CL-vesicles, we could estimate the dissociation equilibrium constant K_d of cytochrome *c* to be in the lower μM range (1–10 μM).

We further verified if the presence of CL is necessary for the strong interaction of human uMtCK octamers with liposomes. The results shown in Fig. 2 demonstrate that binding to pure PC vesicles was very weak (Fig. 2c) as compared to vesicles containing 16% CL (Fig. 2b). The binding properties of dimeric and octameric human uMtCK were compared at low protein concentrations (12.5 $\mu g \cdot ml^{-1}$). Under this condition, dimeric uMtCK can be obtained by simple dilution and incubation of octamers at 4°C for 24 h in running buffer. Dissociation of octameric MtCK with the transition state analog complex (TSAC, Milner-White and Watts, 1971) was avoided, since the latter is less efficient in case of human uMtCK (Schlattner

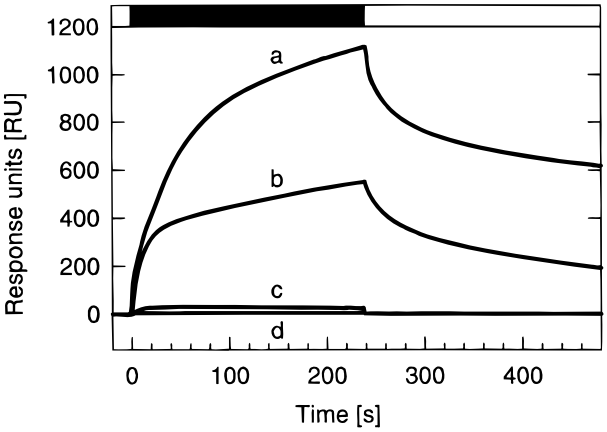

Fig. 2. Binding and dissociation kinetics of cytochrome *c*, BSA, and uMtCK. Contact phase (■) and dissociation phase (□) of 1.2 μM cytochrome *c* (a), 1.2 μM BSA (d) or 0.15 μM octameric uMtCK (b,c) with liposomes containing 16% CL and 84% PC (a,b,d) or 100% PC (c). Data, recorded at 25°C and a flow rate of 0.3 ml-h^{-1}, were corrected for unspecific binding. For details see Materials and Methods.

et al., unpublished) and since TSAC components may, in addition, interfere with the binding process. A fresh uMtCK dilution contained >95% octamers (control uMtCK); the octamer content was decreased to about 5% after 24 h of incubation (dissociated uMtCK). The SPR tracings, corrected for unspecific interactions, revealed binding of dissociated, dimeric uMtCK (Fig. 3b) during contact phase, albeit weaker than for the octameric control uMtCK (Fig. 3a). The response of dissociated uMtCK was clearly higher than could be expected from the remaining 5% octamers. We can, therefore, conclude that dimeric uMtCK also has bound to the 16% CL-vesicles.

To obtain exact binding parameters, association and dissociation kinetics were recorded in a concentration range of uMtCK from 0.03 to 0.30 μM (Fig. 4a, b). The obtained SPR kinetics did not follow a simple 1:1 interaction model, as already observed in other studies with immobilized vesicles (Stachowiak *et al.*, 1996; Lange and Koch, 1997). Therefore, we analyzed the data using a heterogeneous interaction model, which implies two independent binding sites. We have taken some precautions to avoid the possibility that heterogeneity is artificially introduced in our experimental system, *e.g.*, by mass transport limitations or rebinding effects during dissociation (Myszka, 1997; Schuck and Minton, 1996). We routinely used a low binding capacity (immobilization of only 500 RU vesicles) and analyzed only those parts of the kinetics that are least likely to be influenced by mass transport limitations (see solid lines in Figure 4a, b and Materials and Methods for details). Additional control experiments were performed at higher flow rate (0.9 ml-h^{-1}, not shown). Although these measures enhanced data quality, as judged by improved fitting, they did not allow description of the SPR kinetics by a model simpler than heterogeneous interaction. The integrated rate equations of such a model, consisting of double expo-

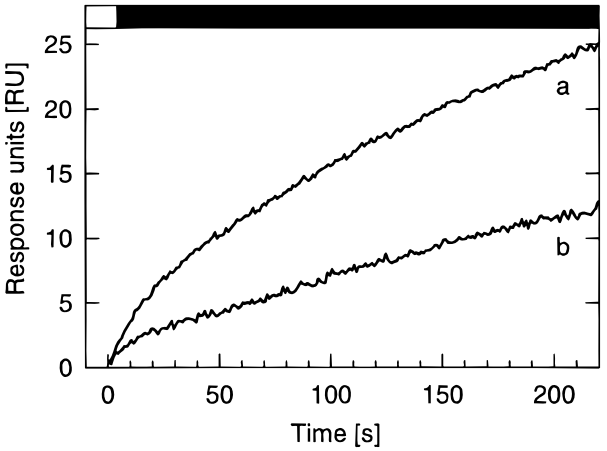

Fig. 3. Comparative binding kinetics of dimeric and octameric human uMtCK. Contact phase (■) of dilutions containing 12.5 μg ml^{-1} human uMtCK in (a) the octameric form (>95% octamers) or (b) the dimeric form (95% dimers), recorded at 25°C and a flow rate of 0.6 ml-h^{-1}. Data were corrected for unspecific binding. For details see Materials and Methods.

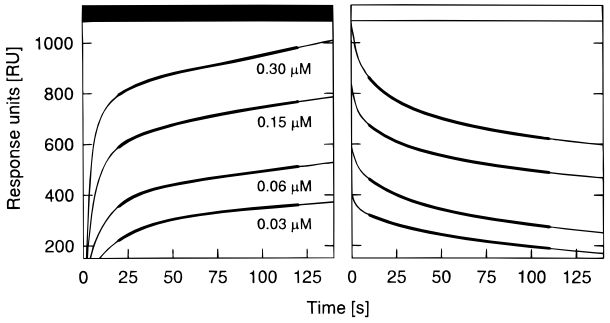

Fig. 4. Typical binding and dissociation kinetics of octameric human uMtCK. Contact phase (■) and dissociation phase (□) of octameric human uMtCK, recorded at 25°C and a flow rate of 0.3 ml-h^{-1}. MtCK was applied in running buffer (10 mM TES, 50 mM NaCl) at four different concentrations (for details see Materials and Methods). The response units (RU) are proportional to the amount of MtCK bound at the vesicle surface (faint lines). Parts of the kinetics unlikely to be influenced by refractive index changes, mass transport, or rebinding effects were used for mathematical analysis of rate and equilibrium constants (bold lines). For details see Materials and Methods.

nentials (see Materials and Methods) properly fitted most kinetic data with residuals below 1 RU.

Association and dissociation rate constants (k_a and k_d) as well as the dissociation equilibrium constants (K_d) were calculated by two independent methods and are summarized in Table I. First, rate constants were directly derived from the fit of the double exponential rate equations to SPR data of contact and dissociation phase [see Eqs. (1) and (2) in Materials and Methods]. Independently, both rate constants were also estimated from contact phase kinetics. The rate equation applied in the latter method uses the global apparent rate constants k_{obs1} and k_{obs2} with $k_{obs} = k_a \cdot c + k_d$ [see Eq. (4) in Materials and Methods]. As seen in a plot of k_{obs} versus c (Fig. 5a,b), k_{obs} indeed showed a linear dependency on analyte concentration. The rate constants of both interaction sites could thus be calculated from slope and y-axis intercept of a linear fit to this plot. Dissociation equilibrium constants (or affinity constants) were directly calculated from the above rate constants [see Eq. (3) in Materials and Methods]. Independently from rate constants, K_d was derived from the concentration dependency of the equilibrium response R_{eq}. Although equilibrium was not entirely reached in our experiments, it could be estimated from the extrapolation of the contact phase kinetics (Fig. 5a, b). K_d was then directly calculated from the slope of the Scatchard plot R_{eq}/c versus R_{eq} (Fig. 5c,d).

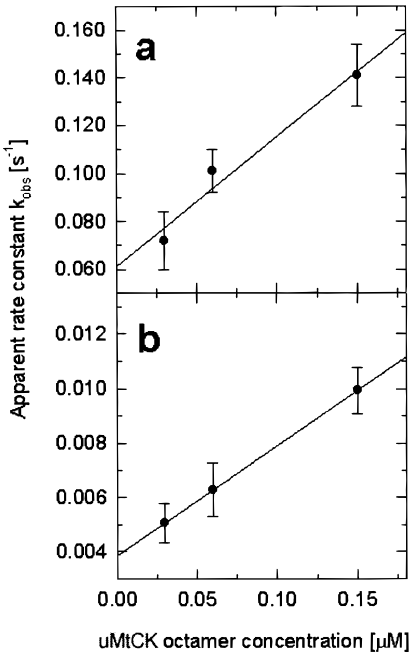

Fig. 5. Concentration dependence of the apparent rate k_{obs} during contact phase. Plots for (a) the first and (b) the second binding site, derived by fitting the contact phase kinetics of octameric human uMtCK (Fig. 2) to a heterogeneous interaction model. Each data point represents the mean value ± SD of at least three experiments.

DISCUSSION

This study presents first experimental evidence and provides quantitative data on the membrane-binding properties of the human ubiquitous mitochondrial CK isoform (uMtCK). It further provides an evaluation of surface plasmon resonance with biotin–avidin immobilized vesicles as a method to generally characterize the binding of peripheral membrane proteins to lipid bilayers.

Our experimental setup was validated with cytochrome *c* and BSA as positive and negative binding controls, respectively (Fig. 2). The interaction of the basic protein cytochrome *c* with lipids is considered a paradigm for the electrostatic binding of peripheral proteins to biological membranes (*e.g.*, Brown and Wuthrich, 1977; Heimburg and Marsh, 1995) and has been studied intensively (*e.g.*, DeKruijff and Cullis, 1980; Rytomaa, Mustonen, and Kinnunen, 1992; Salamon and Tollin, 1996). Similar to MtCK, cytochrome

Table I. Binding Parameters for the Interaction of Octameric Human uMtCK with 16% CL Vesicles

Rate constants				Dissociation equilibrium constants	
k_{a1}[M^{-1}-s^{-1}]	k_{a2}[M^{-1}-s^{-1}]	k_{d1}[s^{-1}]	k_{d2}[s^{-1}]	K_{d1}[nM]	K_{d2}[nM]
[a] 6.1 ± 2.2 10^5	3.8 ± 2.2 10^4	4.8 ± 0.1 10^{-2}	2.8 ± 0.9 10^{-3}	78 ± 37	74 ± 34
[b] 5.4 ± 1.1 10^5	4.1 ± 0.1 10^4	6.1 ± 1.0 10^{-2}	3.8 ± 0.1 10^{-3}	103 ± 22	84 ± 49

[a] Rate constants derived from the direct fit of SPR data to dissociation and association rate equations and K_d values calculated as $K_d = k_d/k_a$ (for details see Materials and Methods).
[b] Rate constants derived from linear plots of k_{obs} versus c (see Fig. 5) and K_d values derived from Scatchard plots (see Fig. 6c,d).

c binding to membranes shows highest affinity for CL (Rytomaa, Mustonen, and Kinnunen, 1992), but also includes some hydrophobic component (Salamon and Tollin 1996). Our SPR method detected pronounced binding to CL-containing membranes with cytochrome *c* and uMtCK and, as expected, no binding with the soluble protein BSA. Thus, the system is perfectly suited for qualitative analysis of peripheral membrane proteins binding to defined lipid bilayers. Furthermore, the K_d estimated for the cytochrome *c*–CL interaction is similar to data recently obtained with a different SPR method (Salmon and Tollin, 1996), indicating that our experimental setup is also capable of yielding meaningful quantitative binding parameters.

Human uMtCK octamers, while strongly binding to CL-containing vesicles, showed only a weak interaction with pure PC vesicles. This result confirms that binding of uMtCK depends on the presence of acidic phospholipids, as do several sMtCK isoforms (Muller *et al.*, 1985; Vacheron *et al.*, 1997; Schlattner *et al.*, 1998). In contrast to octamers, sMtCK dimers were claimed to be unable to interact with mitochondrial membranes (Marcillat *et al.*, 1987). Other studies detected a weak binding of sMtCK dimers, although the ability to cross-link membranes was drastically reduced compared to the octamer (Schlegel *et al.*, 1990, Rojo *et al.*, 1991a,b). In agreement with these latter results, we find a weak but specific binding of uMtCK dimers to CL-containing liposomes. Earlier reports probably failed to detect dimer binding because of insufficient sensitivity of the method and the use of basic pH, which is known to diminish membrane affinity of MtCK (Marcillat *et al.*, 1987). In fact, the weaker membrane interaction of sMtCK dimers as compared to octamers was often ascribed to the more acidic pI of dimers (Wyss *et al.*, 1992). However, in case of the human MtCK isoforms, the difference in pI amounts only to 0.3 pH units (Schlattner *et al.*, unpublished), which may be insufficient to explain the difference in membrane interaction observed here.

A different membrane binding behavior of the two oligomeric forms of MtCK would be in line with other diverging and physiologically relevant properties. sMtCK dimers, in contrast to octamers, are unable to maintain full creatine-stimulated respiration in isolated mitochondria (Khuchua *et al.*, 1998) and to delay the opening of the permeability transition pore *in vitro* (O'Gorman *et al.*, 1997). Recently, we have found an increase of sMtCK dimers in two different animal models of ischemic heart and proposed the octamer:dimer ratio of MtCK as a regulatory parameter *in vivo* (Soboll *et al.*, 1999). However, the dimer-specific properties may not only depend on a lack of membrane interaction, since differences in membrane binding of the two oligomeric forms are quantitative rather than qualitative.

The binding kinetics of human uMtCK dimers could neither be fitted with single nor double exponential rate equations, thus hampering the calculation of quantitative binding parameters and indicating a more complex binding mode. Such a binding might consist of multiple steps, *e.g.*, low-affinity attachment of dimers followed by membrane-mediated octamerization, which finally leads to high-affinity binding. An effect of membranes on MtCK octamerization has also been observed upon rebinding of sMtCK dimers to mitoplast membranes (Schlegel *et al.*, 1990).

Quantitative analysis of the interaction between human uMtCK octamer and 16% CL-containing liposomes suggested the presence of two independent binding sites and yielded rate and equilibrium (affinity) constants for both of them (summarized in Table I). It is important that constants calculated by a direct fit to the SPR data were the same as the values derived from the linear plots k_{obs} versus c and R_{eq}/c versus R_{eq}. This is an important prerequisite for internal consistency of SPR data analysis (Schuck and Minton 1996). Our results for human uMtCK do not, however, entirely coincide with the values obtained with SPR for binding of chicken sMtCK to cardiolipin membranes (Stachowiak *et al.*, 1996). This may be, in part, due to differences between the two isoforms. In addition, the present study used a refined analytical approach, taking into account the concentration dependence of MtCK binding and the influence of dissociation during the contact phase.

The dissociation equilibrium constants (K_{d1} and K_{d2} often also called affinity constants) are a measure of the binding strength or affinity of the interaction. All determined K_d values were in the range of 80 to 100 nM and, thus, we can consider that, within experimental error, both binding sites have the same affinity. The calculated values are in the same order of magnitude as the more recent literature data for sMtCK isoforms (Table II), especially the study of Vacheron *et al.* (1997) using very similar CL-containing liposomes.

Association and dissociation rate constants (k_a and k_d) are measures of the velocity of the interaction. In contrast to K_d, the two binding sites differed in their rate constants by one order of magnitude. Fast association (k_{a1}) was linked to a fast dissociation (k_{d1})

Table II. Dissociation Equilibrium Constants (K_d) for the Binding of Different MtCK Isoforms to Mitochondrial Membranes and Liposomes

MtCK isoform	Ligand	pH	Temp. (°C)	K_d (nM)	Reference
sMtCK	Heart mitochondria	7.2	10	4.5	Hall and DeLuca, 1980
sMtCK, beef	Heart mitochondria	7–7.4	0–5	36	Lipskaya *et al.*, 1980
sMtCK, chicken	Heart mitoplasts	7.4	25–30	100	Brooks and Suelter, 1987
sMtCK, beef	Heart mitochondria	7.4	5	15	Fedosov *et al.*, 1993
sMtCK, pig	PC/PG/CL liposomes	7.4	25	130	Vacheron *et al.*, 1998
uMtCK, human	PC/CL liposomes	7.0	25	80	Current study

and, conversely, slow association (k_{a2}) was linked to slow dissociation (k_{d2}) (Table I). We may therefore distinguish "fast" and "slow" binding sites. They contribute about 60 and 40%, respectively, to the total amount of binding sites, as estimated from the equilibrium response of both kinetics (Fig. 6a,b). At present, it is difficult to decide if the phenomenological occurrence of two binding sites is due to our experimental design or if it reflects the actual binding mode of human uMtCK. If the latter is true, fast and slow binding may be the key to elucidate the physical nature of the two binding sites. For example, they may represent two different binding sites at the MtCK surface or, vice versa, two different kinds of CL-patches on the liposomes. However, it is possible that both may be the case. While fast binding might be purely electrostatic, slow binding could involve specific CL patches, forming nonbilayer structures (hexagonal H_{II} phases; de Kruijff and Cullis, 1980) and MtCK undergoing structural changes to expose a hydrophobic stretch in the C-terminus (Schlattner *et al.*, 1998). This process would lead to partial penetration of MtCK into the lipid bilayer as already observed upon sMtCK binding to cardiolipin (Rojo *et al.*, 1991a; Vacheron *et al.*, 1997). Partial integration of MtCK into the membrane would also explain its resistance against full detachment by high ionic strength and the presence of such a bulky enzyme in the narrow mitochondrial intermembrane space (Schlattner *et al.*, 1998).

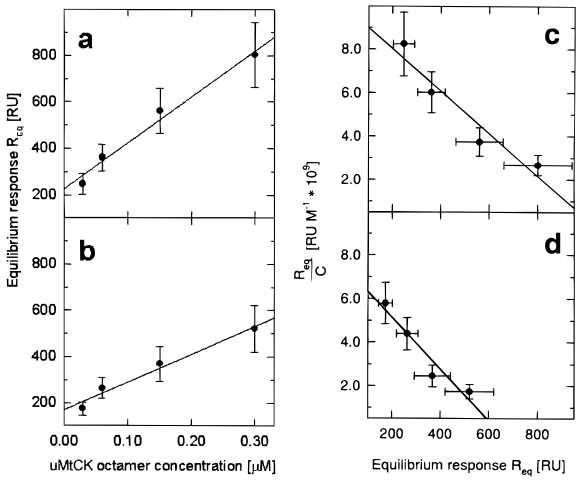

Fig. 6. Affinity of octameric human uMtCK for 16% cardiolipin vesicles: (a,b) concentration dependence of the equilibrium response R_{eq} and (c,d) corresponding Scatchard plots for (a,c) the first and (b,d) the second binding site. R_{eq} values for the two binding sites were derived from the contact phase fit. Each data point represents mean values ± SD of at least three experiments.

We conclude that SPR with immobilized liposomes, which we have presented here, is an appropriate method to discriminate the binding behavior of peripheral membrane proteins and is able to yield quantitative data, including rate and equilibrium constants. It will be suitable to describe the membrane binding of different CK isoforms or mutant proteins and to further examine the hydrophobic binding component. The experimental setup will also allow analysis of interactions with more complex membranes, including different lipid mixtures and reconstituted transmembrane proteins like the adenylate translocator. Schlame and Augustin (1985) described binding sites of MtCK on mitoplasts differing in their affinity by two orders of magnitude. The single K_d of the MtCK–CL interaction will be an experimental advantage when trying to resolve additional binding sites in more complex model membranes.

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