THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 23, Issue of June 4, pp. 24334–24342, 2004

*Printed in U.S.A.*

# C-terminal Lysines Determine Phospholipid Interaction of Sarcomeric Mitochondrial Creatine Kinase*

Received for publication, December 24, 2003, and in revised form, March 15, 2004
Published, JBC Papers in Press, March 25, 2004, DOI 10.1074/jbc.M314158200

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**High affinity interaction between octameric mitochondrial creatine kinase (MtCK) and the phospholipid cardiolipin in the inner mitochondrial membrane plays an important role in metabolite channeling between MtCK and inner membrane adenylate translocator, which itself is tightly bound to cardiolipin. Three C-terminal basic residues revealed as putative cardiolipin anchors in the x-ray structures of MtCK and corresponding to lysines in human sarcomeric MtCK (sMtCK) were exchanged by *in vitro* mutagenesis (K369A/E, K379Q/A/E, K380Q/A/E) to yield double and triple mutants. sMtCK proteins were bacterially expressed, purified to homogeneity, and verified for structural integrity by enzymatic activity, gel filtration chromatography, and CD spectroscopy. Interaction with cardiolipin and other acidic phospholipids was quantitatively analyzed by light scattering, surface plasmon resonance, and fluorescence spectroscopy. All mutant sMtCKs showed a strong decrease in vesicle cross-linking, membrane affinity, binding capacity, membrane ordering capability, and binding-induced changes in protein structure as compared with wild type. These effects did not depend on the nature of the replacing amino acid but on the number of exchanged lysines. They were moderate for Lys-379/Lys-380 double mutants but pronounced for triple mutants, with a 30-fold lower membrane affinity and an entire lack of alterations in protein structure compared with wild-type sMtCK. However, even triple mutants partially maintained an increased order of cardiolipin-containing membranes. Thus, the three C-terminal lysines determine high affinity sMtCK/cardiolipin interaction and its effects on MtCK structure, whereas low level binding and some effect on membrane fluidity depend on other structural components. These results are discussed in regard to MtCK microcompartments and evolution.**

Isoenzymes of creatine kinase (CK)[1] play a key role for energy homeostasis of cells with elevated or alternating energy requirements. Together with high intracellular concentrations of easily diffusible creatine and phosphocreatine, these CK isoenzymes maintain a unique cellular energy buffer and energy transport system, the CK/phosphocreatine-circuit (1–3). In most vertebrate tissues, cytosolic dimeric CK is coexpressed with a predominantly octameric mitochondrial isoenzyme (MtCK), sarcomeric MtCK (sMtCK) or ubiquitous uMtCK (2). This divergence is an early phylogenetic event since octameric MtCK is already present in the protostome marine worm *Chaetopterus variopedatus* (4, 5) and has preserved its sequence through evolution (6). These facts already point to a specific functional role of the mitochondrial CK octamer. In fact, MtCK is part of proteolipid complexes localized in the cristae and the peripheral intermembrane space of mitochondria (7, 8). These proteolipid complexes form microcompartments that maintain a privileged exchange of substrates and products, the so-called functional coupling (see Fig. 8) (9). ATP from oxidative phosphorylation in the matrix space is exported by adenylate translocator (ANT) in the inner membrane and immediately converted by MtCK to ADP and phosphocreatine. ADP is subsequently reimported into the matrix space via ANT, an obligatory ATP/ADP antiporter, and phosphocreatine is released into the cytosol via mitochondrial porin (or voltage-dependent anion channel, VDAC) in the outer mitochondrial membrane. This intimate exchange of substrates and products, the so-called functional coupling or metabolite channeling (10, 11), fulfills important functions that may vary among different tissues, species, and developmental states (12, 13): (i) phosphocreatine becomes the high energy intermediate that is exported from mitochondria into the cytosol (3); (ii) locally generated ADP stimulates oxidative phosphorylation (11); and (iii) ADP channeled through the MtCK/ANT interaction inhibits the $Ca^{2+}$-induced opening of the mitochondrial permeability transition pore (14, 15), a well known trigger of apoptosis (16, 17). Thus, overexpression of uMtCK in many malignant cancers with especially poor prognosis (18, 19) may be related to high energy turnover and failure to eliminate cancer cells via apoptosis. MtCK functions in energy buffering and transport, as well as permeability transition pore regulation may also explain the supportive and protective effects of the CK substrate creatine in many muscular, neurodegenerative, and age-related disorders (20–22). Thus, microcompartmentation of

* This work was supported by grants from the Swiss National Science Foundation (Grant 31-62024.00 to T. W. and U. S., and a Marie Heim-Vögtlin subsidy to M. T.-S.), the Swiss Cancer League and the Zentralschweizer Krebsstiftung (to U. S. and T. W.), as well as the Region Rhone Alpes (to N. V., O. M., and C. V.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: CK, creatine kinase; MtCK, mitochondrial CK; sMtCK, sarcomeric MtCK; uMtCK, ubiquitous MtCK; MM-CK, muscle-type CK; ANT, adenylate translocator; CL, cardiolipin; DMPC, dimyristoyl-phosphatidylcholine; DMPG, dimyristoyl-phosphatidylglycerol; GP, generalized polarization; GPex, excitation-generalized polarization; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-ethanesulfonic acid; WT, wild-type; SPR, surface plasmon resonance; REES, red edge excitation shift; VDAC, voltage-dependent anion channel.

MtCK and the involved molecular interaction mechanisms are increasingly recognized to be important in human health and disease.

Although MtCK has been characterized in quite some detail *in vivo* and *in vitro*, the regulation, dynamics, and topology of MtCK-containing microcompartments are far from being understood. Only recently, we provided evidence for a physical interaction between octameric MtCK and VDAC that is enhanced by physiological concentrations of $Ca^{2+}$ (23). The latter is characteristic for a lowered cellular energy state and thus of potential regulatory importance. VDAC may be the interaction partner sensing $Ca^{2+}$ via a recently identified specific binding site (24). By contrast, no direct binding of MtCK to ANT has been detected so far. Thus, the known high affinity ($K_d$ <100 nM) of both proteins, MtCK as well as ANT, to the acidic phospholipid cardiolipin (CL) of the inner mitochondrial membrane is of prime importance (25–29). It may allow for close co-localization of MtCK and ANT in proteolipid complexes, possibly through CL membrane patches. In support of this, mutations that reduce MtCK membrane binding through destabilization of its octameric structure also reduce creatine-stimulated respiration in cardiomyocyte mitochondria (30). In fact, it is well known that CL-protein interactions are important not only for activity and structural integrity of mitochondrial inner membrane proteins but also for subunit assembly and supercomplex formation (31–34). CL (diphosphatidyl glycerol) is unique in having a large dianionic headgroup that consists of three glycerol molecules connected by two phosphodiester linkages with the terminal glycerols bound to four acyl chains via their primary and secondary hydroxyl groups (31).

Despite the importance of the MtCK-CL interaction for metabolite channeling, the structural components involved in the binding process have remained elusive so far. The x-ray structures of MtCK (35, 36) revealed the formerly purported N-terminal CL-binding domain of MtCK (37) to be buried inside the cuboidal octamer, making this domain unlikely to take part in the binding process. On the other hand, based on the molecular structures of both MtCK isoenzymes (35, 36), the flexible C-terminal stretches were proposed as putative interaction domains (3, 38), but no evidence has been provided so far. Here, we set out to test the latter hypothesis by generating point mutants of C-terminal basic residues of human sMtCK and subjecting them to detailed quantitative, biophysical analysis. Light scattering, surface plasmon resonance, and fluorescence spectroscopy revealed the prime importance of three C-terminal lysines for kinetic and equilibrium constants of the sMtCK/phospholipid membrane interaction, as well as for the structural changes that are induced in both interaction partners upon binding (39).

## EXPERIMENTAL PROCEDURES

*Plasmids and Chemicals*—Plasmid pUS01 carrying the gene for human sMtCK between the NdeI and BamHI sites has been described before (40). Cytosolic rabbit MM-CK was from Roche Applied Science, and laurdan and biotin-X-dihexadecylphosphatidylethanolamine were from Molecular Probes (Leiden, Netherlands). Phospholipids were obtained from Lipid Products for light scattering and Biacore experiments (South Nutfield, UK) or from Sigma for fluorescence experiments. If not mentioned otherwise, all other chemicals were from Sigma/Fluka.

*Single-site Mutagenesis*—Mutation of C-terminal lysines into small, moderately hydrophobic alanine, polar glutamine, or negatively charged glutamate was performed with a convenient modular cloning system, using standard methods (41). A plasmid coding for C-terminally truncated human sMtCK (sMtCKΔ369–380, pFLO1) was obtained from pUS01 using inverse polymerase chain reaction as described (36). pFLO1 contains a new stop codon and unique restriction sites, namely EcoRV by silent mutation of the Ile-368 codon (ATT → ATC) and EcoRI inserted after the new stop codon. Synthetic oligonucleotide linkers coding for mutated C-terminal variants were then cloned in between EcoRI/EcoRV by ligation with a plasmid fragment (EcoRI/NdeI digest of pFLO1) and insert fragment (EcoRV/NdeI digest of pFLO1). The linkers maintained EcoRV and introduced a new unique KpnI site by silent mutation of the Val-370 codon (GTG → GTA), thus allowing us to screen for insert-containing vectors. Mutation of Lys-379/Lys-380 into alanines was initially performed by inverse polymerase chain reaction with mutagenic primers that were 5′-phosphorylated for religation of the linear product. To improve primer hybridization, different codons for alanine were used. All inserts were sequenced according to the chain termination method with an automated system (ABI PRISM 310, PerkinElmer Life Sciences) to assure correct *in vitro* mutagenesis and the absence of random mutations. All utilized oligonucleotide linkers and primers are summarized in Table I.

TABLE I
*Overview of mutagenic primers*

Mismatches with the template in the mutagenic primers and nucleotide exchanges as compared with the original sequence in the linkers are marked as bold and underlined. The silent mutations yielding the unique EcoRV and KpnI sites are indicated.

| Mutant | Primers |
|---|---|
| sMtCK-kaa | 5′-p-TAAACTTTCCCTTTCCCAATTTA-3′<br>3′-GGAGTCAAACCG**CG**T**CG**C-p-5′ |
| | **Oligonucleotide linkers** |
| | EcoRV KpnI Stop |
| sMtCK-kqq | 5′-AT**C**AAGGT**A**CCACCCCCTCTGCCTCAGTTTGGC**C**A**GC**AGTAA**G**-3′<br>3′-TA**G**TTCCA**T**GGTGGGGGAGACGGAGTCAAACCG**GA**C**G**TCATT**CTTAA**-5′ |
| sMtCK-kee | 5′-AT**C**AAGGT**A**CCACCCCCTCTGCCTCAGTTTGGC**G**AA**G**A**A**TAA**G**-3′<br>3′-Ta**G**TTCCA**T**GGTGGGGGAGACGGAGTCAAACCG**C**TT**C**T**T**ATT**CTTAA**-5′ |
| sMtCK-aaa | 5′-AT**CGC**GGT**A**CCACCCCCTCTGCCTCAGTTTGGC**GCGGC**GTAA**G**-3′<br>3′-TA**GCG**CCA**T**GGTGGGGGAGACGGAGTCAAACCG**CGCCG**CATT**CTTAA**-5′ |
| sMtCK-eee | 5′-AT**CG**AGGT**A**CCACCCCCTCTGCCTCAGTTTGGC**G**AA**G**A**A**TAA**G**-3′<br>3′-TA**GC**TCCA**T**GGTGGGGGAGACGGAGTCAAACCG**C**TT**C**T**T**ATT**CTTAA**-5′ |

*Protein Expression and Purification*—Wild-type (WT) and mutant sMtCK proteins were expressed in *Escherichia coli* BL21(DE3)pLysS and purified as described in detail elsewhere (36, 40). After purification on Blue Sepharose (Amersham Biosciences) and Resource S ion exchanger, the kinase was over 98% pure as revealed by SDS-PAGE. Fractions were routinely analyzed by standard 12% SDS-PAGE with a minigel apparatus and low $M_r$ markers (Bio-Rad), stained with Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany). Protein concentration was determined with the Bio-Rad reagent according to Bradford (42), using bovine serum albumin as a standard.

*Enzyme Kinetics, Gel Filtration, and CD Spectroscopy*—CK enzymatic activity was determined with a photometer using a coupled enzyme assay as described in detail in Schlattner *et al.* (40). Briefly, ATP production from phosphocreatine and ADP (reverse reaction) is coupled via hexokinase and glucose-6-phosphate dehydrogenase to NADPH generation. Quantitation of octameric and dimeric sMtCK by gel filtration chromatography was carried out as described in Schlattner and Wallimann (29). Far-UV (190–250 nm) and near-UV (250–320 nm) CD spectra of sMtCK were recorded on a JASCO J-715 dichrograph (Jasco, Great Dunmow, UK) at 25 °C and under constant nitrogen flow, using a quartz cell with a 1-cm optical path. sMtCK in 25 mM sodium phosphate (pH 6.75) was diluted in this buffer to about 0.2 mg/ml (far-UV) or 0.6 mg/ml (near-UV) and sterile-filtered.

*Generation of Liposomes*—Aliquots of the required lipids in chloroform solution were combined in the desired molar ratio, *i.e.* pure phosphatidylcholine (PC), PC/CL (10: 1), PC/PE/CL (2:1:1), or DMPC/DMPG (3:2). For fluorescence experiments, the amphiphilic membrane probe laurdan was added in a molar ratio phospholipids/laurdan of 400:1, whereas for Biacore experiments, 0.1% (w/w) biotin-X-dihexadecylphosphatidylethanolamine was incorporated. During liposome preparation, laurdan and laurdan-labeled liposomes were kept in darkness to avoid photobleaching, and all lipids were maintained above the gel to liquid-crystal transition temperature. Large unilamellar vesicles were prepared by hydration and a combination of freeze-thawing and extrusion

as described previously (28, 43). Briefly, dry lipids were hydrated (5–20 mg/ml) in 10 mM TES, pH 6.5, 50 mM potassium acetate (light scattering and Biacore experiments) or 20 mM Tris, pH 7.4, 0.1 mM EDTA (fluorescence experiments) with subsequent heating to 70 °C when containing laurdan and finally dispersed by vortexing to produce multilamellar vesicles. The lipid suspension was subjected to 6–10 freeze-thaw cycles and then extruded 19 times through polycarbonate membranes (Nucleopore) with 0.4- and then 0.2-$\mu$m diameter pores using a mini-extruder (Avanti Polar Lipids). The resulting large unilamellar vesicles have a diameter of ~150 nm as analyzed by light scattering and electron microscopy and were used within 3 days.

*Light Scattering and Surface Plasmon Resonance Spectroscopy*—Light scattering was measured at an angle of 90° using an SPEX fluorolog-2 instrument with a 450-watt xenon arc lamp as a light source as described in Stachowiak *et al.* (44). Intensity was measured using a single photon counter equipped with a Peltier-cooling unit. The wavelength for excitation and detection was 420 nm. All measurements were carried out at 25 °C in a stirred 1-cm quartz cuvette put into a thermostated sample holder. 50 $\mu$g of biotin-free vesicles and 10 mM $\beta$-mercaptoethanol were added to standard buffer (10 mM TES, pH 6.5, 50 mM potassium acetate, pH 6.5) and preincubated for at least 15 min to achieve a constant scattering signal. Vesicle cross-linking was induced by the addition of 30 nM sMtCK and followed for 1000 s. Data were normalized to the scattering signal just after the addition of sMtCK. Binding of sMtCK to a model lipid membrane was measured by surface plasmon resonance (SPR) with a Biacore 2000™ instrument (Biacore, Uppsala, Sweden) according to Schlattner and Wallimann (29, 43), using standard buffer with 2 mM $\beta$-mercaptoethanol. Briefly, a carboxymethyl sensorchip CM5 (Biacore) was covered with 20000 response units of avidin to immobilize 800–1000 response units of biotinylated liposomes. sMtCK-membrane association and dissociation kinetics were recorded at 25 °C and a flow rate of 0.3 ml $h^{-1}$ with sMtCK concentrations ranging from 30 to 300 nM and a final injection of 0.5% SDS to recover the chip-avidin surface. Kinetic data were corrected for background binding to the chip surface by subtracting the signal for pure PC liposomes. Parts of the kinetics unlikely to be influenced by refractive index changes, mass transport, or rebinding effects (43) were fitted with a double exponential, integrated rate equation. This model corresponds to a heterologous interaction comprising two independent binding sites (for a detailed discussion, see Ref. 43). Only fits with residuals smaller than 1% of the response unit signal were retained to determine rate constants and equilibrium response $R_{eq}$. The affinity $K_D$ (dissociation equilibrium constant) was derived from rate constants as $K_D = k_{off}/k_{on}$ and independently from the slope of a plot $R_{eq}$/c *versus* c, analogous to a Scatchard plot. Data are given as mean ± standard deviation (S.D.) of at least three data sets of different experiments.

*Fluorescence Spectroscopy*—Fluorescence measurements of laurdan and endogenous tryptophan residues in sMtCK were done as described in Granjon *et al.* (39). Laurdan measurements were performed with a Hitachi F4500 fluorometer. The excitation and emission band-pass values were 2.5 and 5 nm, respectively. All fluorescence spectra were corrected for spectra of the buffer solution containing liposomes without laurdan. The samples were stirred, and the temperature was controlled with a water thermostated bath. Laurdan emission spectra were recorded at 37 °C for DMPC/DMPG liposomes or 32 °C for PC/PE/CL liposomes by using a 355-nm excitation wavelength and either 60 $\mu$g of laurdan-liposomes or 60 $\mu$g of laurdan-liposomes in the presence of 60 $\mu$g of protein at a 500:1 phospholipids/protein molar ratio for DMPC/DMPG and 360:1 for PC/PE/CL. The final volume was adjusted to 800 $\mu$l with 20 mM Tris buffer, pH 7.4, 0.1 mM EDTA, 5 mM dithiothreitol. The excitation-generalized polarization (GPex) spectra were constructed by calculating the GP value for each emission wavelength as $GPex = (I_{440} - I_{490})/(I_{440} + I_{490})$ according to Parasassi *et al.* (45), where $I_{440}$ and $I_{490}$ are the emission intensities at each excitation wavelength. The choice of the emission wavelengths for the calculation of GP values was based on the characteristic emission wavelengths of pure gel and liquid-crystalline phases.

Endogenous tryptophan fluorescence measurements by red edge excitation shift (REES) were performed with a Spex Fluorolog fluorometer. Crossed polarizers were used to minimize the signal due to the diffusion (46). Excitation and emission band-pass values were 2 and 3 nm, respectively. Emission spectra were recorded 20 min after the addition of CK to the liposomes, using a 0.4 × 1 cm cuvette at 25 °C. The assays were carried out using 60 $\mu$g of protein in 800 $\mu$l of 20 mM Tris buffer, pH 7.4, 0.1 mM EDTA, 5 mM dithiothreitol, in the presence or absence of 60 $\mu$g of DMPC/DMPG or PC/PE/CL liposomes. The maximum emission wavelength was measured by using excitation wavelengths ranging from 275 to 307 nm.

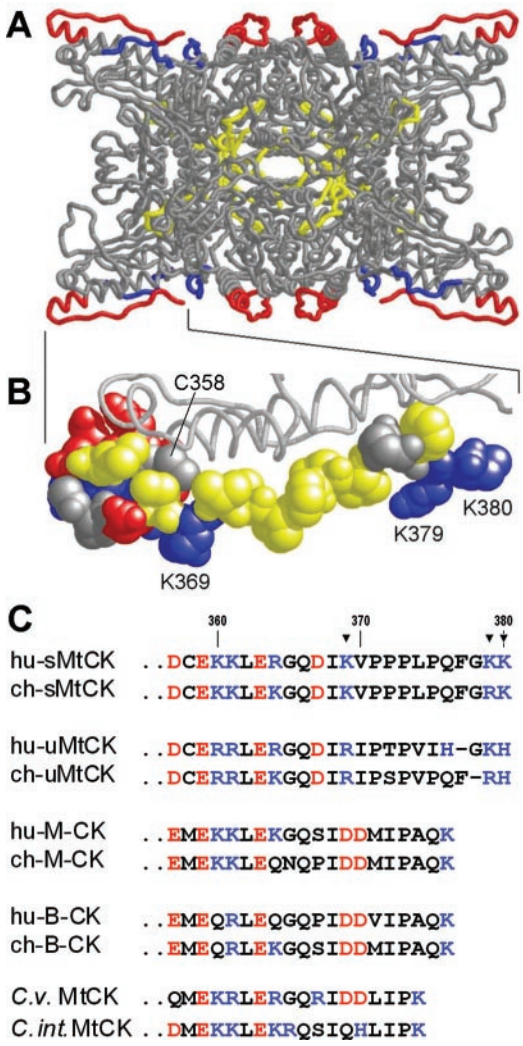


FIG. 1. **Putative membrane-binding domains of MtCK.** *A*, backbone representation of chicken sMtCK (Protein Data Bank code 1crk). Putative membrane-binding motifs are colored in *red* (C-terminal stretch, Asp-357–Lys-380, comprising six basic residues) and *blue* (short internal stretch, Ala-107–Gln-115, comprising one lysine). The N terminus (*yellow*, Thr-1–Met-25) is buried inside the octamer. *B*, enlarged C-terminal stretch with amino acids in space-fill representation (*red*, acidic residues; *blue*, basic residues; *yellow*, hydrophobic residues). *C*, sequence alignment of the C-terminal stretch of vertebrate CK isoenzymes from human (*hu*) and chicken (*ch*), as well as the ancient MtCK from the polychaete *C. variopedatus* (*C.v.*), a marine annelid worm, and the ascidian *C. intestinalis* (*C. int.*), a low marine chordate. Numbering is according to human sMtCK. The *arrows* in *C* indicate the three lysine residues of human sMtCK mutated in this study. (This figure was prepared with RASMOL v.2.6.)

## RESULTS

*Mutant Design*—As already suggested earlier, the C-terminal stretches of the cuboidal MtCK octamers are prime candidates for membrane interaction (3, 35). As we could show by solving the x-ray structures of sMtCK and uMtCK isoenzymes (35, 36), there are four well exposed but poorly ordered C-terminal stretches at both of the opposite, 4-fold symmetric faces of the octamer (Fig. 1*A*, "top" and "bottom" faces). Mainly, these faces were found attached to phospholipid membranes when analyzed by electron microscopy (47). The very C-terminal stretch of vertebrate MtCK contains three basic residues, the side chains of which protrude from the putative membrane binding face (Fig. 1*B*) and which are largely conserved among vertebrate MtCK but not in cytosolic CK isoenzymes (Fig. 1*C*). In human sMtCK, these basic amino acid residues correspond to Lys-369, Lys-379, and Lys-380. To analyze their role in

TABLE II
*Specific enzyme activities of sMtCK mutants*

Specific enzyme activities are averaged from at least three independent measurements, using a coupled enzyme assay (40).

| Mutant | Specific activity[a] | |
|---|---|---|
| | $V_{max}$ | Relative $V_{max}$ |
| | *units/mg*[b] | *% WT* |
| sMtCK-WT | 59.3 | 100 |
| sMtCK-kqq | 53.7 | 91 |
| sMtCK-kaa | 57.8 | 97 |
| sMtCK-kee | 60.8 | 103 |
| sMtCK-aaa | 52.3 | 88 |
| sMtCK-eee | 56.0 | 94 |

[a] Reverse reaction; see "Experimental Procedures."
[b] One unit of enzyme activity is equal to 1 μmol of ATP or phosphocreatine transphosphorylated per minute at 25 °C. Maximal standard deviation is ± 26%.

membrane binding, we have replaced both terminal positive charges (Lys-379/Lys-380) alone or in combination with the third lysine (Lys-369/Lys-379/Lys-380), with polar glutamine (sMtCK-kqq, lowercase letters for residues at positions 369, 379, and 380), moderately hydrophobic alanine (sMtCK-kaa, sMtCK-aaa), or negatively charged glutamate (sMtCK-kee, sMtCK-eee). The different replacement mutants should allow us to titrate C-terminal charges from positive to neutral (hydrophobic) and negative and to identify non-specific effects possibly occurring with individual replacement mutants. However, glutamine and glutamate with a size close to lysine are not expected to introduce steric hindrance or to otherwise affect C-terminal structure.

*Generation and Integrity of Mutant sMtCK*—Single-site mutations of C-terminal lysines were generated by using designed synthetic inserts (except for sMtCK-kqq). Human sMtCK WT and mutant proteins were expressed in *E. coli* and purified by a two-step chromatographic protocol (40) to over 98% purity as judged by SDS-PAGE (data not shown; see "Experimental Procedures"). All purified mutant proteins remained soluble and enzymatically active after storage at 4 °C, suggesting that stability and proper folding were not significantly affected by the mutations described. Structural and functional integrity were analyzed in more detail by enzyme activity assays, CD spectroscopy, and analytical gel filtration chromatography. All mutant proteins showed specific enzyme activities that were, within error, identical to wild type (Table II). Far-UV CD spectra of mutants were superimposable to those of wild-type enzyme, confirming the overall structural integrity (Fig. 2*A*). In the near-UV range (≥240 nm, Fig. 2*B*), the three Cotton bands (minima at ~280, 290, and 300 nm) characteristic for sMtCK (48) were conserved in all mutant proteins. The octamer/dimer ratio of mutant proteins determined by gel filtration chromatography was even slightly higher than with wild type, providing clear evidence for preservation of the oligomeric state (Fig. 3).

*Kinetic and Equilibrium Analysis of sMtCK-induced Liposome Cross-linking*—Negatively charged phospholipids, especially CL that is specific for the inner mitochondrial membrane (31), are the established lipid binding partners of mitochondrial CK (26, 28). Since MtCK octamers can simultaneously bind two membranes through their identical top and bottom faces (27), incubation with liposomes leads to cross-linking that can be measured as a decrease in light scattering (44). We used such an assay for a first screening of sMtCK mutants (Fig. 4). With pure PC liposomes, neither wild-type nor mutant sMtCK was able to decrease the scattering signal, *i.e.* to cross-link liposomes (Fig. 4*A*). In contrast, PC/CL liposomes (molar ratio 10:1) mimicking the inner mitochondrial membrane (Fig. 4*B*) or pure CL liposomes (Fig. 4*C*) were readily cross-linked by sMtCK-WT. The sMtCK mutant proteins showed a graded reduction in cross-linking capability, most pronounced with the sMtCK-eee and -aaa triple mutants. The apparent cross-linking kinetics could be fitted to a single exponential rate equation to obtain equilibrium response and pseudo first-order rate constants (Fig. 4, *D* and *E*). These are rather phenomenological parameters that describe a complex phenomenon, comprising MtCK binding to liposomes, cross-linking of individual liposomes, as well as cross-linking of liposome aggregates. The steady state response of all double mutants was identical within error and about half of wild type, whereas with the triple mutants, it was down to about 10% of wild type with PC/CL liposomes (Fig. 4*D*). Calculated rate constants revealed a clear difference between the two liposome preparations (Fig. 4*E*). With PC/CL liposomes, they were very similar for wild-type and mutant proteins, except for a somewhat lower rate with sMtCK-kaa and a faster rate for sMtCK-eee, which is, however, prone to large errors due to low signal change. When using pure CL liposomes, the cross-linking rate of mutants was also greatly reduced as compared with wild type (Fig. 4*E*).

*Kinetic and Equilibrium Analysis of sMtCK/Phospholipid Interaction*—To obtain quantitative binding parameters for membrane interaction of sMtCK mutants, we have applied SPR (29). In this approach, liposomes (PC/CL, molar ratio 10:1, and pure PC) were immobilized on a sensor chip to study on-line the association and dissociation kinetics of sMtCK. SPR kinetics recorded in a concentration range of 30–300 nM and corrected for background binding to pure PC liposomes already revealed clearly reduced binding capacity of mutant sMtCK as compared with wild type (Fig. 5, *A–C*). Similar to earlier studies (29, 43), SPR kinetics could only be fitted by two-exponential rate equations (Fig. 5, *A* and *B*). This was not due to mass transport limitations as verified by varying flow rate. Apparently, MtCK interaction with CL-containing liposomes involves two binding sites (29, 43). The calculated $k_{on}$ rate constants for these two sites (given as $10^3$ $M^{-1}s^{-1}$) were 830 (±210) and 140 (±40) for wild type but 90 (±50) and 17 (±7) for sMtCK-kqq and 80 (±50) and 15 (±7) for sMtCK-kee. For the other sMtCK mutant proteins, reliable kinetic data could not be obtained because of low binding signals, higher unspecific background binding, and a parallel decrease in dissociation rates. Instead, overall affinity constants ($K_D$) were derived from Scatchard plots based on the concentration dependence of the SPR equilibrium response (Fig. 5*D*). These values agreed quite well with affinities estimated from the measured rate constants ($K_D = k_{off}/k_{on}$) for sMtCK-WT (17–53 nM) and the two double mutants sMtCK-kqq (124–289 nM) and sMtCK-kee (147–362 nM), confirming the consistency of our analysis. As with liposome cross-linking, a similar $K_D$ was determined for mutants with the same number of exchanged lysines, and a graded reduction in affinity was observed from sMtCK-WT to double mutants (about 6-fold) and triple mutants (about 30-fold). Thus, the number of positive charges, but not the nature of the replacing amino acid, had significant influence on the CL affinity of sMtCK.

*Analysis of sMtCK/Phospholipid Interaction with Fluorescence Spectroscopy*—The interaction of sMtCK with lipid membranes is known to induce changes in the physical state of membranes as well as in the structure of sMtCK (39), resulting in a more rigid membrane bilayer and subtle changes in the sMtCK conformation (39). To determine how these changes are affected by elimination of C-terminal basic residues, we compared sMtCK-WT with a double mutant (sMtCK-kqq) and a triple mutant (sMtCK-eee). The lipid composition of the model membranes was chosen according to earlier fluorescence studies on MtCK/phospholipid interaction (28, 39): (i) PC/PE/CL

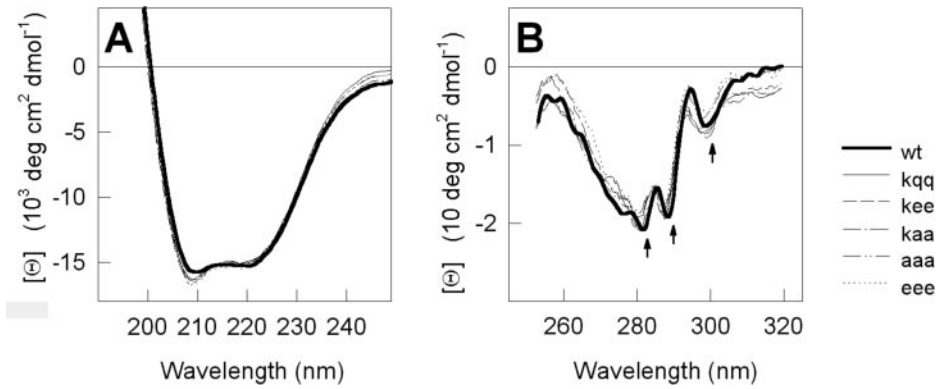


FIG. 2. **CD spectral patterns of wild-type and mutant sMtCK.** Far-UV CD spectra (*A*) and near-UV CD spectra (*B*) of 0.2 mg/ml protein in 25 mM sodium phosphate (pH 6.75) corrected with buffer spectra. The *arrows* in *B* indicate CK-characteristic Cotton bands at 281, 288, and 300 nm.

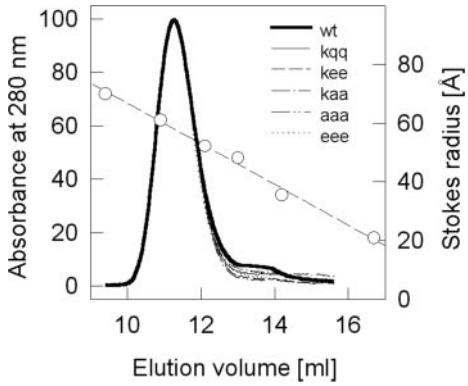


FIG. 3. **Gel filtration chromatography of wild-type and mutant sMtCK.** About 20 $\mu$g of protein (0.1 mg/ml) of each mutant was separated on a Superose 12 column (Amersham Biosciences) at a flow rate of 0.75 ml min$^{-1}$ and 22 °C. Elution profiles were normalized to the octamer peak of MtCK-WT. The column was calibrated for Stokes radii with the following marker proteins (Amersham Biosciences): chymotrypsinogen A (20.9 Å, 25 kDa), albumin (35.5 Å, 67 kDa), aldolase (48.1 Å, 158 kDa), catalase (52.2 Å, 232 kDa), ferritin (61 Å, 440 kDa) and thyroglobulin (70.0 Å, 669 kDa).

liposomes (molar ratio 2:1:1) containing purified lipids with saturated and unsaturated fatty acids such as PC/CL and resembling even more closely the inner mitochondrial membrane and (ii) DMPC/DMPG liposomes (molar ratio 3:2) representing an entirely different, non-natural lipid mixture with defined, saturated acyl chains that lead to a much higher phase transition temperature of the membrane. These liposomes lack CL but can bind MtCK via acidic DMPG (28), the metabolic precursor of CL.

Laurdan was used as an amphiphilic, fluorescent membrane probe that is sensitive to the polarity of the environment. Here, the GPex of laurdan was calculated as a measure for membrane dynamics and the phospholipid liquid-crystalline state (39). When temperature dependence and the GP excitation spectrum were determined by varying temperature or excitation wavelength, respectively, differences between sMtCK-WT-containing and protein-free liposomes were observed (data not shown), consistent with published data (39). CK-incubated liposomes displayed a slightly higher GPex spectrum as compared with protein-free liposomes (data not shown), and ΔGPex difference spectra could be calculated to quantify these changes (Fig. 6, *A* and *B*). These spectra showed a characteristic increase of GPex above a 400-nm excitation wavelength, indicative of decreased membrane fluidity (39), which was dependent on the C-terminal sequence of CK and the liposome composition. With PC/PE/CL liposomes mimicking the inner mitochondrial membrane, sMtCK-WT induced a strong increase of emission wavelength at 420 nm, whereas only about 80 and 45% of this increase were detectable with sMtCK-kqq and sMtCK-eee mutants, respectively, and almost no increase was seen with cytosolic rabbit MM-CK taken as a negative control (Fig. 6*A*). Thus, mutating C-terminal lysines not only strongly reduced membrane interaction of sMtCK but also decreased its influence on ΔGPex spectra and membrane fluidity. However, even the sMtCK-eee triple mutant persistently showed a large change in the ΔGPex spectrum as compared with MM-CK control, clearly indicating the presence of additional membrane-ordering component(s) in the sMtCK structure. An entirely different result was obtained when using the non-natural DMPC/DMPG liposomes (Fig. 6*B*). Already, the sMtCK double mutant decreased the ΔGPex spectrum to MM-CK control level. Thus, with these phospholipids, only an entirely preserved C-terminal stretch containing all three lysines could exert an additional influence on membrane order.

Mitochondrial CK contains several conserved tryptophan residues (48) that can be used as fluorescent probes to analyze conformational changes. Here, the emission wavelength peak of endogenous tryptophan fluorescence was analyzed when shifting excitation wavelength to the red edge of the absorbance band above 300 nm, the so-called REES. This gives a measure for subtle changes in the microenvironment of tryptophan residues that may occur upon sMtCK/membrane interaction. As compared with sMtCK in solution (Fig. 7*A*), the addition of liposomes led to a strong increase in emission wavelength above 300 nm when using sMtCK-WT or, to a lesser extent, sMtCK-kqq (Fig. 7, *B* and *C*), but not with the sMtCK-eee mutant. Here, results with DMPC/DMPG and PC/PE/CL liposomes were similar. This indicates that the environment of some tryptophan residues of sMtCK-WT and to a lower degree also of sMtCK-kqq became motionally restricted upon interaction with acidic phospholipids, whereas the structure of sMtCK-eee remained unchanged. In conclusion, whereas SPR and laurdan experiments suggest some degree of phospholipid interaction of sMtCK triple mutants, possibly linked to decreased membrane fluidity, the REES experiments indicate that this type of interaction has no consequence on the microenvironment of tryptophans.

## DISCUSSION

In the present study, we have used mutagenesis of single sites in combination with biophysical methods to identify the principal membrane-binding domain of octameric human sMtCK and to characterize its role in the binding process. The domain is located at the very C-terminal stretch of the monomers and comprises three main conserved basic residues, corresponding to Lys-369, Lys-379, and Lys-380 in human sMtCK

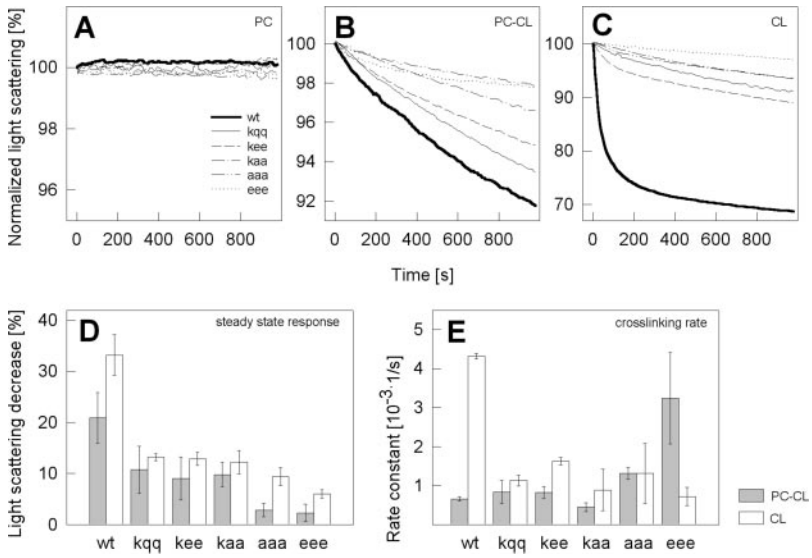


FIG. 4. **Kinetics of vesicle cross-linking induced by sMtCK.** Representative light scattering traces due to vesicle cross-linking by wild-type and mutant sMtCK with liposomes consisting of pure PC (*A*), PC/CL (molar ratio 10:1) (*B*), and pure CL (*C*) are shown. Fitting the kinetics of several experiments to a single exponential rate equation gives quantitative data for *D*, the steady state scattering signal, and *E*, the pseudo first-order cross-linking rate constant (data ± S.D., $n = 3$).

(3, 38). Mutation of these lysines drastically reduces the capacity of sMtCK to attach to and to cross-link mitochondrial model membranes containing CL by at least an order of magnitude. Exchange of lysines also affects the structural changes normally observed in both binding partners upon interaction. Lysine-mediated interactions are essential to induce conformational changes in the sMtCK structure, whereas they are only partially responsible for the decreased fluidity of mitochondrial model membranes.

Mutation of the three C-terminal lysines did not interfere with folding, structure, or stability of the sMtCK mutant proteins. Stability at 4 °C, enzymatic activity, near- and far-UV CD spectra, and octamer formation were identical to wild-type protein. In contrast, the binding behavior of mutant proteins with membranes containing acidic phospholipids that mimic the inner mitochondrial membrane was drastically changed. Two independent quantitative methods, namely SPR for directly monitoring membrane binding as well as light scattering for analyzing membrane cross-linking, consistently revealed a significantly altered interaction between sMtCK mutants and PC/CL liposomes. (i) Mutation of two and then three correlated with a gradual reduction in membrane binding. Replacing the C-terminal positive cluster (Lys-379/Lys-380) clearly reduced membrane binding parameters (affinity 7-fold, steady state cross-linking 2-fold), but only additional elimination of the third lysine (Lys-369/Lys-379/Lys-380) led to a strong decrease (affinity 30-fold, steady state cross-linking 10-fold). (ii) Whether lysine is replaced by glutamine, alanine, or glutamate was almost irrelevant for the observed effect on equilibrium response or affinity and had only minor consequences for the rate constants. (iii) However, even replacement of all three lysines by opposite charges, *i.e.* glutamates, did not entirely eliminate membrane interaction since up to about 10% of "binding capability" as compared with wild type persisted, depending on the parameter considered.

These results clearly show that it is the exchange of lysines against alanines and not the repulsive effect introduced by acidic glutamate that is responsible for the largely reduced CL affinity. Thus, high affinity interaction is entirely based on specific salt bridges between the three examined basic MtCK residues and the acidic, dianionic CL headgroup. This is in accordance with the few binding sites for CL headgroups known from the x-ray structure of bacterial photoreaction centers (49) and cytochrome $bc_1$ complex (32). They consistently show both negatively charged phosphatides of CL interacting directly or via water molecules with at least one arginine, lysine, or histidine, thus contributing to structural stability or subunit interaction. As in the case of MtCK, most of these basic residues are conserved in the respective protein family. In the known MtCK structures (35, 36), the distances between the C-terminal lysine side chains are compatible with the Lys-379/Lys-380 pair being bound to the two charged phosphodiester groups of a single CL molecule and Lys-369 being bound to another CL molecule. Our data support the notion that evolved from x-ray crystallography, phylogenetic conservation of CL-binding sites, as well as other mutagenesis studies (32, 50), namely that the unique large and charged headgroup of CL requires a specific and tightly interacting binding site. As far as residual membrane binding is concerned, it may involve the striking hydrophobic cluster in the C-terminal flexible stretch (Fig. 1*B*, *yellow*), basic residues located further downstream (Fig. 1*B*, *blue*), or even basic residues in the surface-exposed linker region between small and large MtCK domains (Fig. 1*A*, *blue*), as speculated earlier (3). Involvement of the more distant linker region could explain why even the repulsive effect introduced by replacing lysines with glutamates did not affect the residual membrane affinity of sMtCK. Both of these structures, the C-terminal stretch and domain linker, miss a defined secondary structure and are more flexible than the rest of the protein (35). This may help in docking of the lysine side chains of MtCK onto the charged phospholipid headgroups of the membrane lipids.

Interestingly, interaction of octameric MtCK with the phospholipid membrane has consequences for the structure of both binding partners. First, it decreases the fluidity of the lipid bilayer, and second, it leads to subtle conformational changes in the sMtCK structure itself as well (39). Consistent with the effect of the double and triple lysine mutants on membrane binding, the conformational changes of sMtCK were gradually decreased. With the triple mutant, endogenous tryptophan fluorescence REES was entirely eliminated. This points to an essential role of the C-terminal stretch in such structural modifications induced during the binding process. On the other hand, when analyzing the liquid-crystalline state of a mitochondrial model membrane (PC/PE/CL) with a laurdan fluorescent probe, the resulting GPex spectra were only partially changed with sMtCK mutant proteins as compared with wild type. Even the exchange of all three C-terminal lysines only partially abolished the ordering effect on PC/PE/CL membranes. Therefore, additional component(s) in the sMtCK structure must be involved, which most likely are those responsible for residual membrane binding of triple mutants as dis-

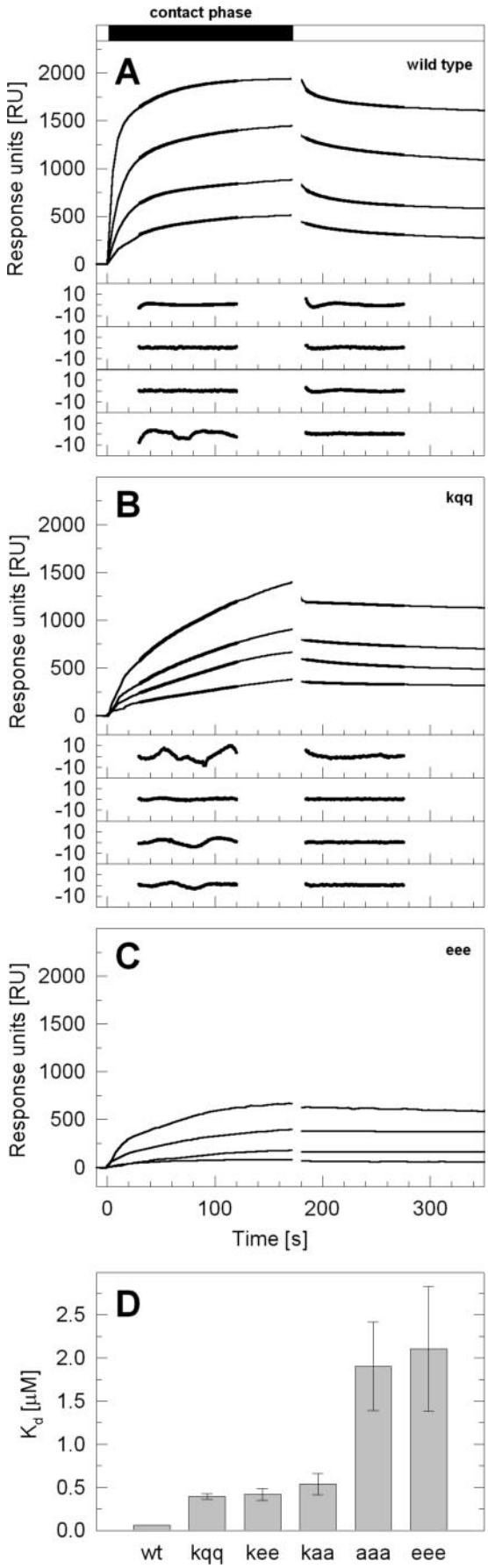


FIG. 5. **Kinetics of sMtCK membrane binding and dissociation.** Representative SPR traces of the contact and dissociation phase of sMtCK-WT (*A*), sMtCK-kqq (*B*), and sMtCK-eee (*C*) with PC/CL liposomes (molar ratio 10:1). Traces for 30, 60, 150, and 300 nM octameric human sMtCK (*thin lines*) are given together with the fitting to double exponential rate equations (*bold lines*) and the corresponding residuals (*below*). *D*, affinity constants $K_D$ calculated from Scatchard plots derived from the relationship between octamer concentration and SPR signal at equilibrium (43). SPR data were recorded at 25 °C and a flow rate of 0.3 ml $h^{-1}$ with 10 mM TES, pH 6.5, 50 mM potassium acetate, and 2 µM β-mercaptoethanol as running buffer. Response units are proportional to the amount of MtCK bound at the vesicle surface.

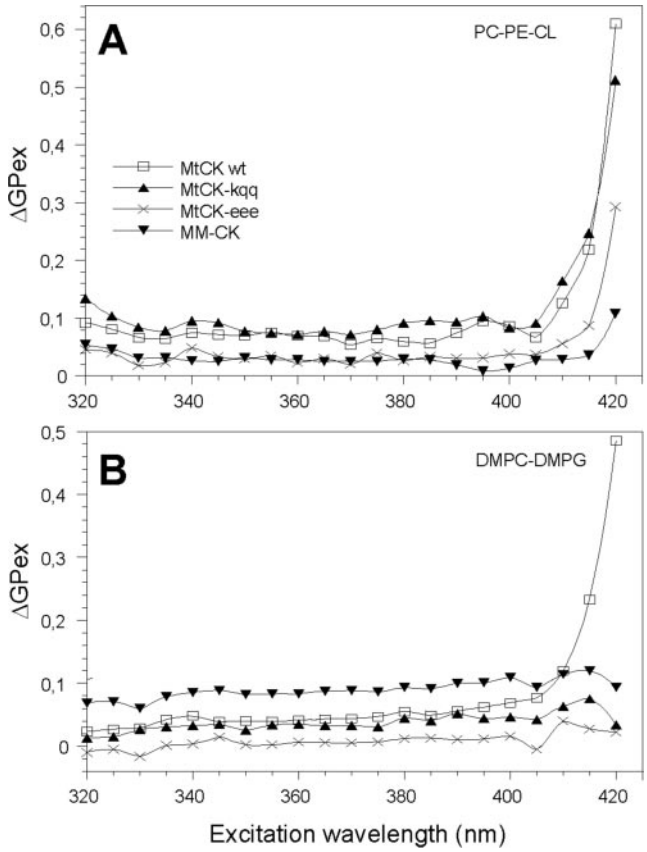


FIG. 6. **Difference spectra of laurdan excitation-generalized polarization.** The difference in the excitation GP (ΔGPex) between 60 µg of liposomes incubated with and without 60 µg of CK, using either PC/PE/CL (*A*) or DMPC/DMPG (*B*) liposomes, is shown. The CK species used were: sMtCK-WT (*open squares*), sMtCK-kqq (*filled triangles*) or sMtCK-eee (*crosses*), cytosolic rabbit MM-CK (*inversed triangles*). Samples were suspended 20 mM Tris (pH 7.4), 0.1 mM EDTA, 5 mM dithiothreitol and measured at 32 (*A*) or 37 °C (*B*). The molar ratio of laurdan/phospholipids was always 1:400.

cussed above. In contrast, sMtCK mutants do not affect the order of DMPC/DMPG membranes as determined with GPex spectra. This may be due to the exclusive presence of saturated acyl chains that already lead to more densely packed lipid bilayers and a higher phase transition temperature as with PC/PE/CL membranes. Here, an additional ordering effect of sMtCK may only occur in the presence of all C-terminal lysines. In addition, PG may have a different binding mode as CL, the preferred binding partner of MtCK. CL has unique properties, in particular the dianionic headgroup, a very high content of unsaturated C18 acyl chains, and the capability of phase polymorphism, *i.e.* to form inverted hexagonal structures (31).

Due to the conservation of C-terminal basic residues among vertebrate MtCKs, the results obtained here for human sMtCK should also apply for uMtCK isoenzymes that show a similar high affinity to CL (29, 43). However, sMtCK and uMtCK also show subtle differences in the very C-terminal sequence (Fig. 1*C*). In particular, Lys-380 in sMtCK is replaced by a histidine

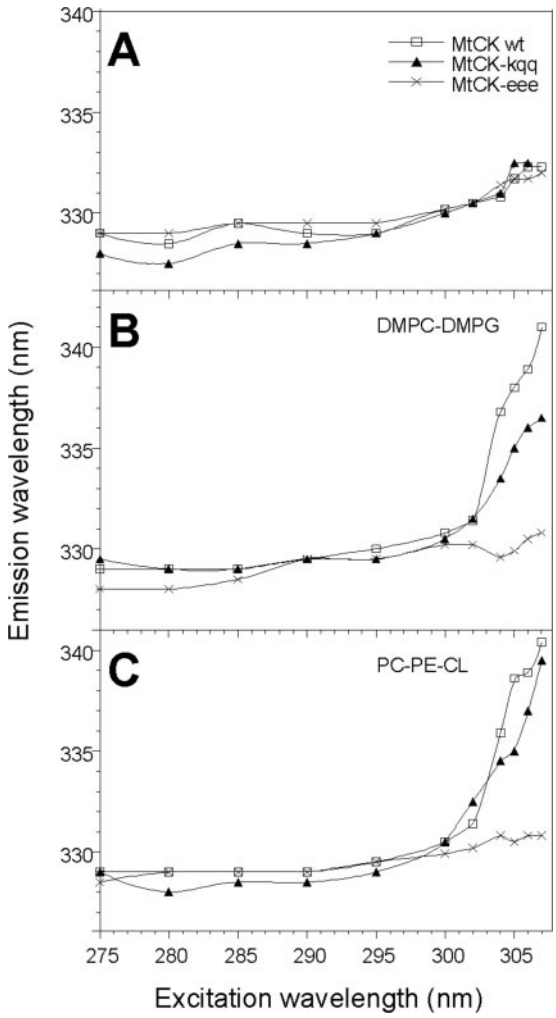


FIG. 7. **Red edge excitation shift.** The maximum wavelengths of the emission spectra at different excitation wavelengths, using 60 $\mu$g of sMtCK-WT (*open squares*), sMtCK-kqq (*filled triangles*), or sMtCK-eee (*crosses*) at 25 °C, are shown. *A*, without liposomes; *B*, in the presence of 60 $\mu$g of DMPC/DMPG liposomes; or *C*, in the presence of 60 $\mu$g of PC/PE/CL liposomes. Samples were suspended in 20 mM Tris, pH 7.4, 0.1 mM EDTA, 5 mM dithiothreitol.

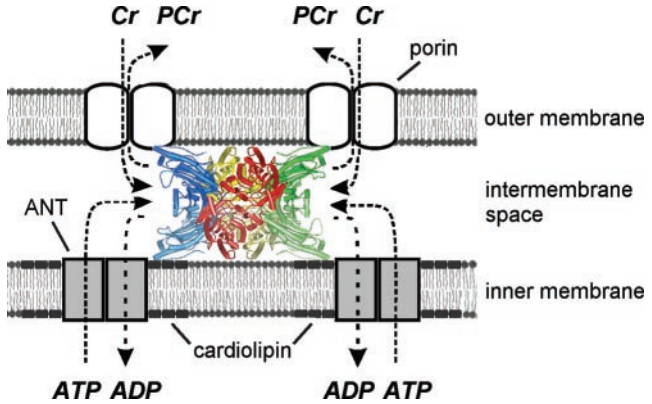


FIG. 8. **MtCK in mitochondrial contact sites.** A model of the topology of MtCK, ANT, and mitochondrial porin (VDAC) in the so-called peripheral contact sites, where MtCK cross-links outer and inner mitochondrial membranes, is shown. Note the direct interaction of MtCK with porin and CL, as well as the close vicinity to ANT. Substrate and product pathways are indicated. *Cr*, creatine; *PCr*, phosphocreatine.

in uMtCK and thus will contribute much less to a basic local surface charge at neutral pH. This may partially explain the slower association rate and lower affinity of uMtCK toward phospholipid membranes as compared with sMtCK (29).

From a phylogenetic perspective, it is interesting to compare vertebrate MtCK with the MtCK of *Ciona intestinalis*, a marine chordate,[2] and the very ancient octameric MtCK of *C. variopedatus*, an invertebrate annelid worm (4, 5, 51). *Chaetopterus* MtCK, the witness of a very early divergence of octameric mitochondrial and dimeric cytosolic CK occurring more than 670 million years ago, has preserved a high sequence identity of about 71% with vertebrate MtCK (6). Despite this similarity, some divergent properties have also been observed with *Chaetopterus* MtCK, as for example a higher stability of the octameric structure (4, 51). It has also been speculated that this ancient MtCK displays very tight binding to CL due to its high pI of 9.7 and several basic residues in the C-terminal domain (5). However, a sequence alignment with vertebrate CKs (Fig. 1*C*) clearly shows that *Chaetopterus* MtCK is missing the three basic residues that were shown in this study to determine phospholipid interaction. Instead, the C-terminal sequence shows a striking homology with cytosolic muscle-type CK and brain-type CK (Fig. 1*C*), which are known to bind only weakly to CL-containing membranes (28, 52). In particular, the C-terminal stretch of *Chaetopterus* MtCK is much shorter, has only one terminal basic charge, but two additional acidic aspartates, which will give rise to a rather neutral local electrostatic potential. Very likely, such octamers would not interact with CL in the inner mitochondrial membrane and should be unable to form functional complexes in the way vertebrate MtCK is doing. This would impair the functional coupling of invertebrate MtCK with ANT and limit the exchange of adenine nucleotides across the inner mitochondrial membrane (3) (Fig. 8). Such a lack of functional coupling is well known for ancient mitochondrial arginine kinase (53), a close homologue of CK occurring in some protostomian arthropods and mollusks, such as the tobacco horn worm, *Limulus polyphemus*, or some crustaceans (4). Interestingly, the lower chordate *Ciona* shows a C-terminal MtCK sequence that is intermediate between *Chaetopterus* and vertebrates. The tandem of aspartates is lost, but the basic residue at position 369 is still missing. Taken together, it may be speculated that CL binding of MtCK and metabolite channeling with ANT have been acquired by MtCK only later at the dawn of vertebrate evolution. At the same time, most of the hydrophobic membrane interactions occasionally observed with ancient MtCKs (51, 54) may have been lost.

In conclusion, the sMtCK mutants generated in this study provide evidence that the three lysines at the very C-terminal stretch largely determine the interaction of the octameric enzyme with CL, the main receptor for sMtCK in the mitochondrial inner membrane, as well as the conformational modifications induced in sMtCK upon binding. The membrane interaction domain identified here clearly confirms earlier speculations (3, 35) and explains how MtCK is able to attach to and to cross-link membranes containing acidic phospholipids (27, 28, 55) without the participation of the N terminus. The latter has been proposed earlier as interaction site (37) but is in fact buried inside the cuboidal MtCK octamer (Fig. 1*A*, *yellow*). Our results are in line with a proposed topology of MtCK and ANT (Fig. 8) in which MtCK C termini and ANT are brought into close vicinity by binding to a common CL patch in the inner mitochondrial membrane (3). In fact, CL can act as a glue for supercomplex formation between proteins in the inner mitochondrial membrane (33, 34). Since four C termini are exposed at one binding face of the cuboidal MtCK octamer, such a

[2] Available online at genome.jgi-psf.org/ciona4/ciona4.home.html.

topology could accommodate interaction with four ANT dimers, giving rise to a molar dimer ratio of about 1:1 in such a proteolipid complex as already speculated earlier (56). The CL interactions of MtCK, together with its octameric structure, are important prerequisites for the formation of the functional microcompartments with ANT (3, 30). These microcompartments control metabolite channeling between CK and ANT (Fig. 8), thus participating in the CK/phosphocreatine energy circuit (2) and possibly regulating mitochondrial permeability transition, one of the pathways leading to apoptosis (14, 15, 57). Under pathological conditions, these microcompartments can be disrupted, thus potentially leading to energy deficits and increased apoptosis (58–60). Alternatively, MtCK overexpression in creatine-depleted mice (61) or patients with mitochondrial myopathies (62) leads to the formation of crystalline sheets between inner and outer mitochondrial membranes, as well as between cristae membranes. This corroborates the potential of MtCK to bind simultaneously to two membranes (27). Very recently, cysteine 358, which is located near the C-terminal basic residues examined in this study (Fig. 1*B*), has been identified as a prime target of peroxynitrite-induced oxidation of MtCK (63). Earlier, sulfhydryl reagents such as parahydroxymercuribenzoate were shown to inhibit membrane binding of MtCK (28) at low concentrations, even before reacting with the active site cysteine Cys-278. These data suggest that oxidative modifications occurring in many pathological conditions will affect Cys-358, thereby reducing the membrane affinity of MtCK through local changes in mobility or the structure of the MtCK C-terminal stretch. Further experiments will be necessary to confirm this hypothesis.

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