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Cardiolipin Clusters and Membrane Domain Formation Induced by Mitochondrial Proteins

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We show in this study that mitochondrial creatine kinase promotes segregation and clustering of cardiolipin in mixed membranes, a phenomenon that has been proposed to occur at contact sites in the mitochondria. This property of mitochondrial creatine kinase is dependent on the native octameric structure of the protein and does not occur after heat-denaturation or with the native dimeric form of the protein. Cardiolipin segregation was demonstrated by differential scanning calorimetry using membranes containing cardiolipin and either dipalmitoylphosphatidylethanolamine or 1-palmitoyl-2-oleoylphosphatidylethanolamine. Addition of the ubiquitous form of mitochondrial creatine kinase leads to the formation of a phosphatidylethanolamine-rich domain as a result of the protein binding preferentially to the cardiolipin. Such phase separation does not occur if cardiolipin is replaced with dioleoyl phosphatidylglycerol. Lipid phase separation is observed with other cardiolipin-binding proteins, including cytochrome *c* and, to a very small extent, with truncated Bid (t-Bid), as well as with the cationic polypeptide poly-L-lysine, but among these proteins the octameric form of mitochondrial creatine kinase is by far the most effective in causing segregation and clustering of cardiolipin. The proteins included in this study are found at mitochondrial contact sites where they are known to associate with cardiolipin. Domains in mitochondria enriched in cardiolipin play an important role in apoptosis and in energy flux processes.

Keywords: membrane domains; cardiolipin; mitochondria; creatine kinase; contact sites

Introduction

There are locations in the mitochondria at which the inner and outer mitochondrial membranes come into close apposition. These sites have been referred to as contact sites and are enriched in both a mitochondrial isoform of creatine kinase (MtCK),[1] as well as cardiolipin (CL).[2] The importance of the assembly of lipids and proteins at this site has been linked to their biological function for energy channeling and mitochondrial permeability transition pore (MTP) opening, an early event in mitochondria-mediated apoptosis,[3,4] where domains in mitochondria that are enriched in CL play an important role.[5,6]

There are two forms of MtCK in vertebrates; the sarcomeric form (sMtCK) found in striated muscle, and the ubiquitous form (uMtCK) present in most other tissues, including brain and kidney.[7] It has long been known that cardiolipin (CL) is important for the binding of MtCK to the mitochondrial membrane.[8–11] MtCK undergoes a concentration-dependent reversible oligomerization between octameric and dimeric forms,[7] with octameric uMtCK dissociating 23–24 times slower, compared with sMtCK.[12] The crystal structures of both octameric

Abbreviations used: DPPE, dipalmitoylphosphatidylethanolamine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; DOPG, dioleoyl phosphatidylglycerol; CL, cardiolipin; CK, creatine kinase; MtCK, mitochondrial creatine kinase; uMtCK, ubiquitous mitochondrial creatine kinase; sMtCK, sarcomeric mitochondrial creatine kinase; DSC, differential scanning calorimetry; t-Bid, truncated Bid.

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MtCK's are known.[13–14] The octameric form binds rapidly to anionic phospholipids, while the dimeric forms binds much more slowly, possibly requiring prior octamerization at the membrane surface.[15] The octameric MtCK mediates intermembrane contact that is stabilized by electrostatic interactions between anionic lipids[16] and several C-terminal lysine residues of the protein.[17]

We present evidence that MtCK forms clusters of CL away from other lipids. We use differential scanning calorimetry (DSC) to assess the changes in miscibility of the lipid components of the membrane. The application of DSC for studying the formation of membrane domains induced by peptides or proteins has been discussed recently.[18] DSC has the advantage of not requiring

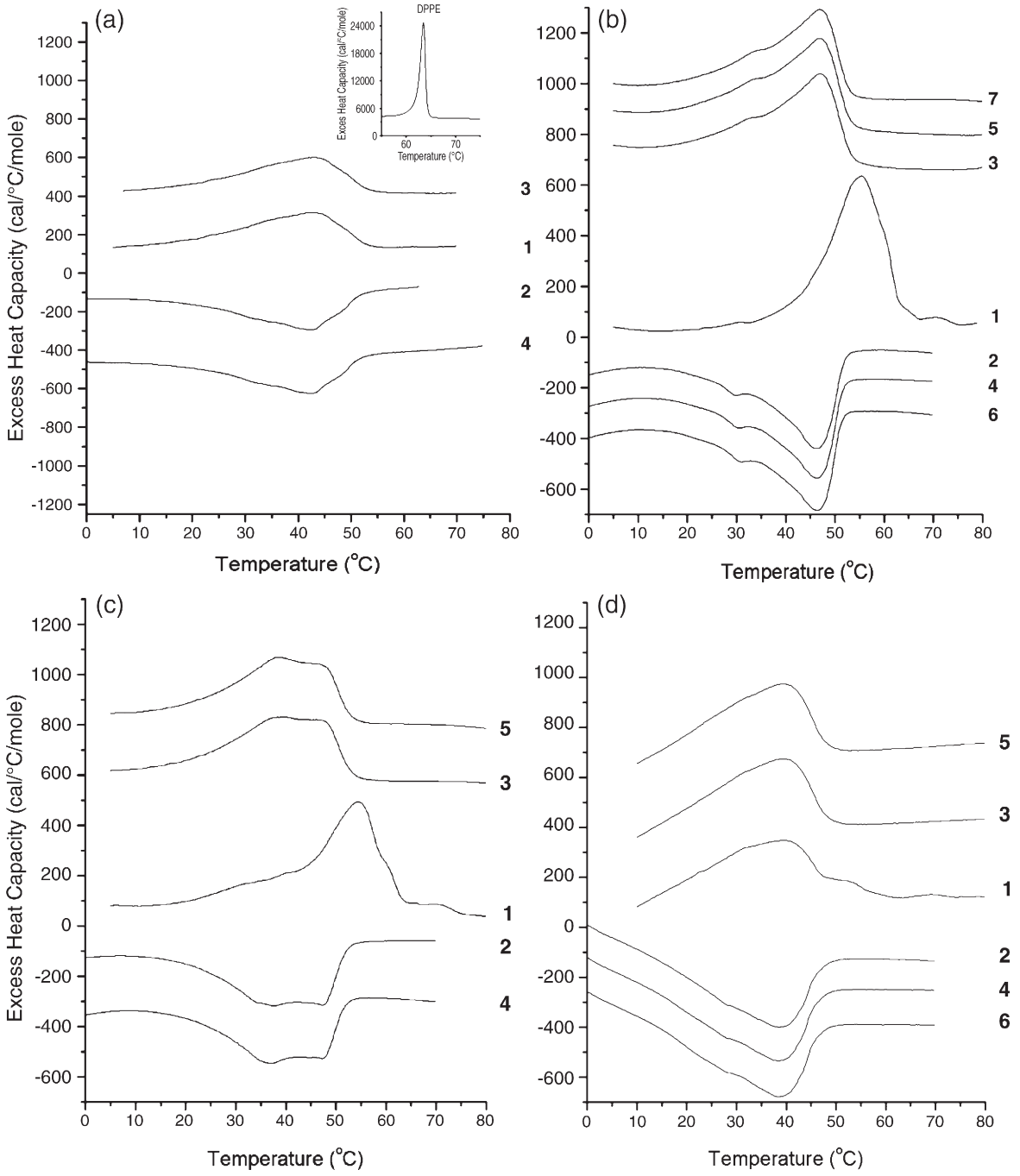

Figure 1. Differential scanning calorimetry (DSC). Scan rate 0.75 deg.C min^{-1}. Lipid concentration 2.6 mM in 20 mM Pipes (pH 7.40), 1 mM EDTA, 150 mM NaCl with 0.002% (w/v) NaN_3. Lipid mixture is DPPE:CL (0.65:0.35, mol:mol). Sequential heating and cooling scans. Numbers are the order in which the scans were carried out, with odd-numbered scans being heating scans, each of which was followed by one of the even-numbered cooling scans. Scans are displaced along the *y*-axis for clarity of presentation. (a) DPPE:CL (0.65:0.35, mol:mol) alone with an inset showing the DSC of pure DPPE; (b) and (c) Lipid with added octameric uMtCK at a lipid to octameric protein molar ratio of (b) 560 or (c) 910). (d) Lipid with added dimeric uMtCK at a lipid to dimeric protein molar ratio of 1000.

bulky labels that can modify the behavior of the system, and the method is widely applicable. This application of DSC is based on the fact that a pure lipid component will give rise to a sharper phase transition with a higher enthalpy than will the same lipid component present in a mixture. This is analogous to a pure crystal exhibiting a sharp melting behavior over a narrow range of temperature compared with an impure substance melting over a broad range of temperature. Lipid domain formation, i.e. the enrichment of a particular lipid component in one region of the membrane (a domain), will result in this lipid component exhibiting a sharper phase transition (melting behavior), together with an increase in the transition enthalpy. In addition, we have measured how this phenomenon is related to the conformation of the protein as monitored by circular dichroism (CD), a spectroscopic method sensitive to the secondary structure of the protein. We demonstrate that the ability of the ubiquitous mitochondrial creatine kinase (uMtCK) to recruit CL is dependent on its native structure as well as its octameric form. The capacity of uMtCK to recruit CL is greatly enhanced compared to that of other basic proteins, in particular cytochrome *c* and tBid, which are also important players in apoptosis and are known to have affinity for CL.

Results

DSC

A mixture of DPPE:CL (0.65:0.35, mol:mol) is composed of miscible lipids,[18] giving a single broad phase transition over the range of temperature of approximately 2–45 °C, intermediate between that of each of the individual lipid components. CL has a phase transition below 0 °C and is not observable by DSC, while DPPE has a transition at 63.3 °C as a pure hydrated lipid.[19] The transition of pure DPPE is very sharp, occurring over a narrow temperature range (Figure 1(a), inset). The transition of DPPE:CL (0.65:0.35, mol:mol) is reproducible on heating and cooling, exhibiting a small shift to lower temperatures on cooling (Figure 1(a)). Hysteresis between heating and cooling is common in DSC and occurs because of kinetic effects of the transition as well as instrumental factors. The enthalpies and temperatures of the transitions of DPPE:CL (0.65:0.35, mol:mol) in the absence and in the presence of proteins are given in Table 1 for the first heating scan. A higher temperature and enthalpy of a component is indicative of partial phase separation of the higher-melting DPPE component. In addition to lipid transitions, there could have been DSC peaks arising from protein denaturation. However, a protein thermal denaturation transition could not be detected by DSC below 70 °C. This thermal denaturation is not observed by DSC because the breadth of the transition is too great and/or its enthalpy is too small to give rise to an observable DSC peak at the concentrations of protein used. Nevertheless, we show by CD that some protein denaturation does occur in this temperature range. We ascribe the loss of protein effect on the lipid phase transitions after the first heating scan as being caused by irreversible thermal denaturation of the protein. Thus, all of the transitions observed by DSC below 70 °C correspond to phase transitions of the lipid. Addition of octameric uMtCK to hydrate a lipid film of DPPE:CL resulted in a marked change in the DSC profile on the first heating scan (Figure 1(b) and (c); Table 1). The protein was added from a concentrated solution and would thus be mostly octameric, having a slow dissociation rate.[7,12] The final molar ratio of lipid to octameric uMtCK was 560 (Figure 1(b)) or 910 (Figure 1(c)). As expected, the extent of separation of the DPPE component is greater at lower ratios of lipid to protein (Figure 1(b) and (c); Table 1), as indicated by the higher temperature and enthalpy of the high-melting component of the transition on the first scan. The dimeric uMtCK has much less affect on the phase transition behavior of the lipid (Figure 1(d); Table 1). There is a small component at 52.3 °C, but its enthalpy is only 0.3 kcal mol^{-1}. This shift in the transition of a portion of the lipid may be caused by the dimer itself or by a small amount of remaining octamer.

For experiments done with POPE:CL at 0.75:0.25 molar ratio (Figure 2(a), panel A) or at 0.8:0.2, mol:mol (not shown), octameric uMtCK causes a sharpening of the broad transition of the lipid mixture, with a partial separation of the POPE and CL components. Thus, the maximal excess heat capacity (height of the DSC peak) increases about twofold in the presence of the protein from about 200 cal deg.C^{-1} mol^{-1} to 400 cal deg.C^{-1} mol^{-1},

Table 1. Transition characteristics of DPPE:CL (0.65:0.35, mol:mol) in the presence and absence of proteins (first heating scan)

Protein added	Lipid to protein molar ratio	Major component		Minor components	
		T (°C)	Δ*H* (kcal mol^{-1})	*T* (°C)	Δ*H* (kcal mol^{-1})
None	–	42	3.5	25	1.6
uMtCK octamer	560	55	7.6	40	1.7
uMtCK octamer	910	53	5.0	38	2.0
uMtCK dimer	1000	39.4	3.2	27	3.0
Cytoch. *c*	200	54	3.2	35	3.5
Cytoch. *c*	430	51	1.4	35	4.3
Cytoch. *c*	870	38	3.7	22	1.1
t-Bid	200	42	2.0	32	2.5
t-Bid	410	39	4.6	19	1.3
Poly-L-lysine	450	59	2.1	50	3.5
Poly-L-lysine	900	53	2.5	38	4.4

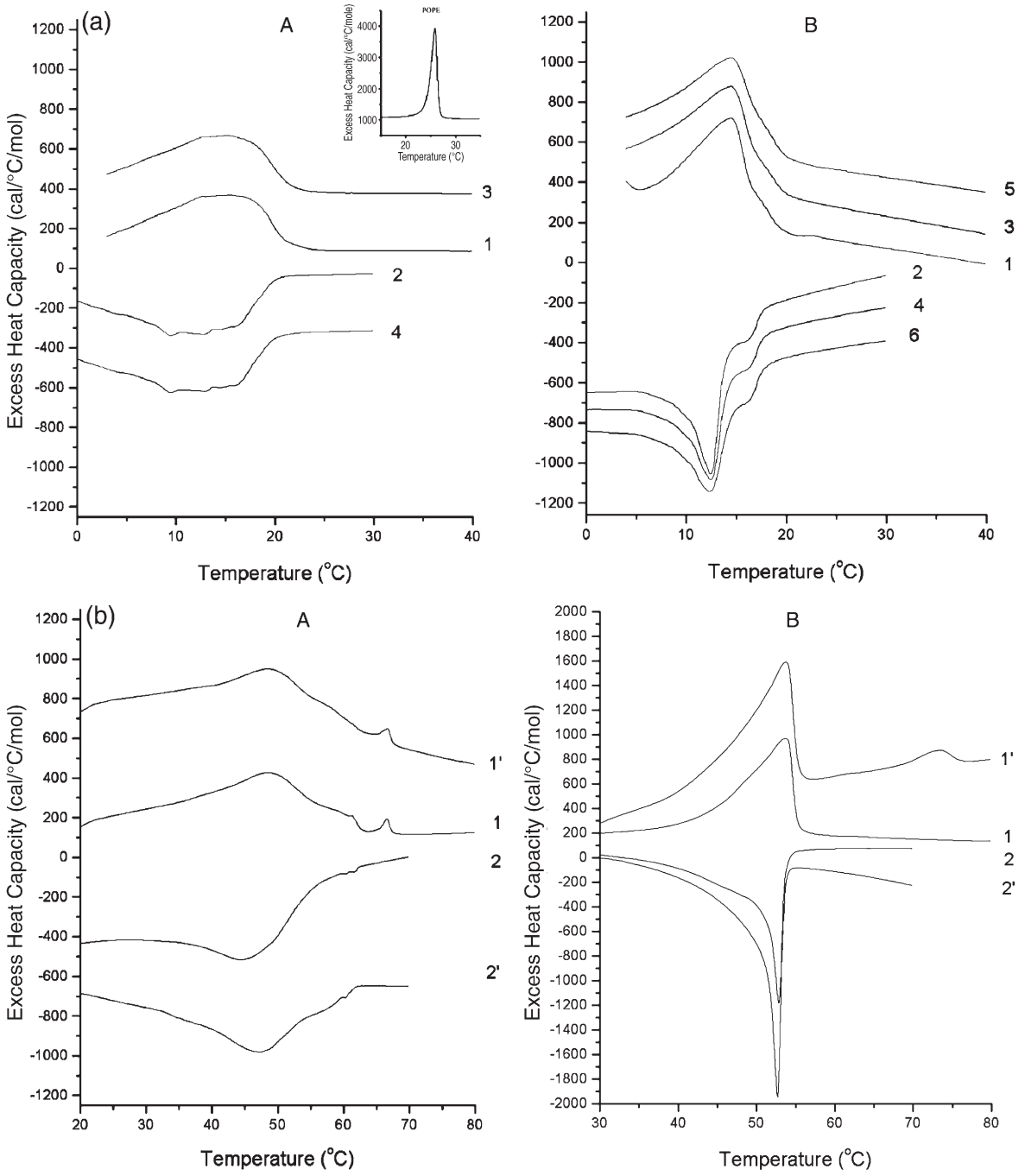

Figure 2. (a) DSC heating and cooling curves for POPE:CL (0.75:0.25, mol:mol); panel A, POPE:CL (0.75:0.25, mol:mol) alone with an inset showing the DSC of pure POPE; panel B, POPE:CL (0.75:0.25, mol:mol) with added octameric uMtCK at a lipid to octameric protein molar ratio of 360. Other details are as for Figure 1. (b) Panel A, DSC heating and cooling curves for DPPE:POPG (0.65:0.35, mol:mol) or with added octameric uMtCK at a lipid to octameric protein molar ratio of 970; panel B, DSC heating and cooling curves for DPPE:DOPG (0.65:0.35, mol:mol) or with added octameric uMtCK at a lipid to octameric protein molar ratio of 550. Other details as for Figure 1, except that results are given for two independent experiments. The second experiment is distinguished by a prime on the scan number.

indicating less mixing of the two lipid components and more segregation into domains. Pure POPE has a phase transition temperature of 25 °C (Figure 2(a), panel A, inset), considerably lower than that of DPPE, shown in Figure 1. Hence, the DSC scans in Figure 2 are over a lower temperature range. In addition, the peak of the POPE component is less well separated from that of the lipid mixture in the presence of certain proteins than was the case for mixtures with DPPE (Figure 1). The molar ratio of lipid to octameric uMtCK was 360 (Figure 2(a), panel B; Table 2). Less effect of protein was observed with DPPE:POPG (0.65:0.35, mol:mol) at a lipid to protein ratio of 970 or with DPPE:DOPG (0.65:0.35,

Table 2. Transition characteristics in the presence and absence of uMtCK (first and second heating scans)

Lipid mixture	Lipid to protein molar ratio	Major component		Minor components	
		T (°C)	ΔH (kcal mol^{-1})	T (°C)	ΔH (kcal mol^{-1})
POPE:CL (0.75:0.25, mol:mol) First scan	–	17	1.7	12	2.0
+uMtCK[a]	360	14.3	3.1	17.9	0.2
POPE:CL (0.75:0.25, mol:mol), Second scan	–	16	1.0	12.2	2.5
+uMtCK[a]	360	12.2	2.7	18	0.2
DPPE:DOPG (0.65:0.35, mol:mol) First scan	–	53.6	3.6	47.6	2.4
+uMtCK[a]	550	53.6	4.6	47.6	3.7
DPPE:DOPG (0.65:0.35, mol:mol) Second scan	–	52.5	3.1	47.6	3.5
+uMtCK[a]	550	52.5	4.7	47.3	3.0

[a] uMtCK added to lipid mixture described in the row above.

mol:mol) at a lipid to protein ratio of 550 (Table 2). However, with the DPPE:POPG mixture (Figure 2(b), panel A) there does appear to be a small component with the thermal transition temperature of about 65 °C, corresponding to a small amount of residual DPPE. The small peak at 73 °C (0.5 kcal mol^{-1}) corresponds to the denaturation of some of the free unbound uMtCK. The phase transition temperatures of pure POPG or DOPG are close to or below 0 °C.[20] No pure PG component is observed with the samples shown in Figure 2(b).

We compared the lateral phase separation of CL and DPPE caused by uMtCK with that of other basic mitochondrial proteins. Cytochrome *c* is known to bind specifically to anionic membranes,[21,22] and particularly to CL.[23–25] The interaction of this protein with CL may play an important role in apoptosis.[26–28] Cytochrome *c* is also thought to have a role in catalyzing the oxidation of CL, contributing to apoptosis.[29] The oxidized form of CL has less affinity for cytochrome *c*, allowing it to be liberated into the cytosol during apoptosis.[30] Although cytochrome *c* appears to induce some lateral phase separation of CL and DPPE (Figure 3), this effect is not as marked as that found with MtCK, even at higher molar concentrations of protein than that used for uMtCK (Table 1). At a lipid to protein ratio of 870, the observed lipid transitions are quite similar to that of the lipid in the absence of protein in contrast to the case of uMtCK.

Another protein that is believed to bind to CL is truncated Bid (t-Bid), which is a proapoptotic Bcl-2 protein that targets to mitochondria by binding to CL[31–34] at mitochondrial contact sites.[35,36] However, this protein has essentially no effect in promoting the formation of domains with anionic lipids (Figure 4; Table 1) at the molar concentrations used in this study.

A major driving force for the formation of membrane domains enriched in anionic lipids is through charge neutralization.[37] One would therefore expect poly-L-lysine to be effective in promoting charge segregation in this system, as it has been observed in other systems.[38] We find that poly-L-lysine can induce the segregation of DPPE in mixtures with CL (Figure 5; Table 1). Poly-L-lysine does not induce the formation of an inverted hexagonal phase with CL, but it can do so with some 65:35, mol:mol mixtures of PE and CL.[39] However, in the present study, the PE has only saturated palmitoyl chains and is therefore not likely to convert to an inverted phase. Furthermore, after the first heating scan, the heating and cooling scans are reversible, which is not seen for systems forming a hexagonal phase at high temperature.

CD

The DSC showed that, in general, there was a change in the behavior of the system after the first heating scan. A likely cause for this is the irreversible denaturation of the protein during the first heating scan. To determine the thermal denaturation properties of the proteins that were used, we measured the CD in the presence and in the absence of sonicated liposomes (SUVs) composed of DPPE:CL (0.65:0.35, mol:mol), of CL alone and of POPE:CL (0.75:0.25, mol:mol).

Solutions of uMtCK form aggregates on heating in the absence of lipid as observed visually and by the increase in dynode voltage in the CD instrument (not shown). In the presence of SUVs of CL there was a marked increase in turbidity at room temperature when uMtCK was added (Figure 6(a)). This turbidity increased gradually with temperature over the range 25–85 °C, at which point the protein was coagulated and no CD signal could be measured. This change was irreversible on recooling (Figure 6(a), panel B, red curve). With POPE:CL (0.75:0.25, mol:mol) SUVs, the protein retained its native helical content at room temperature (Figure 6(b)), as it did also in DPPE:DOPG SUVs (not shown).

The unfolding of cytochrome *c* was monitored by CD (Figure 7(a)). In the absence of lipid, the protein undergoes an unfolding process at about 85 °C (Figure 7(b)). This is in agreement with data reported in the literature using DSC,[40,41] or CD.[42] In the presence of SUVs of CL, the denaturation temperature is shifted markedly to lower values (Figure 7(b)). Similar results were observed using SUVs composed of DPPE:CL (0.65:0.35, mol:mol) as with CL alone. This is in accord with the results obtained by Spooner and Watts[43,44] using NMR, and Heimburg and Marsh, using FTIR,[45] showing that lipid binding loosens the protein structure. The shift to lower temperatures may be a consequence of the low surface pH in bilayers with anionic lipids.[46]

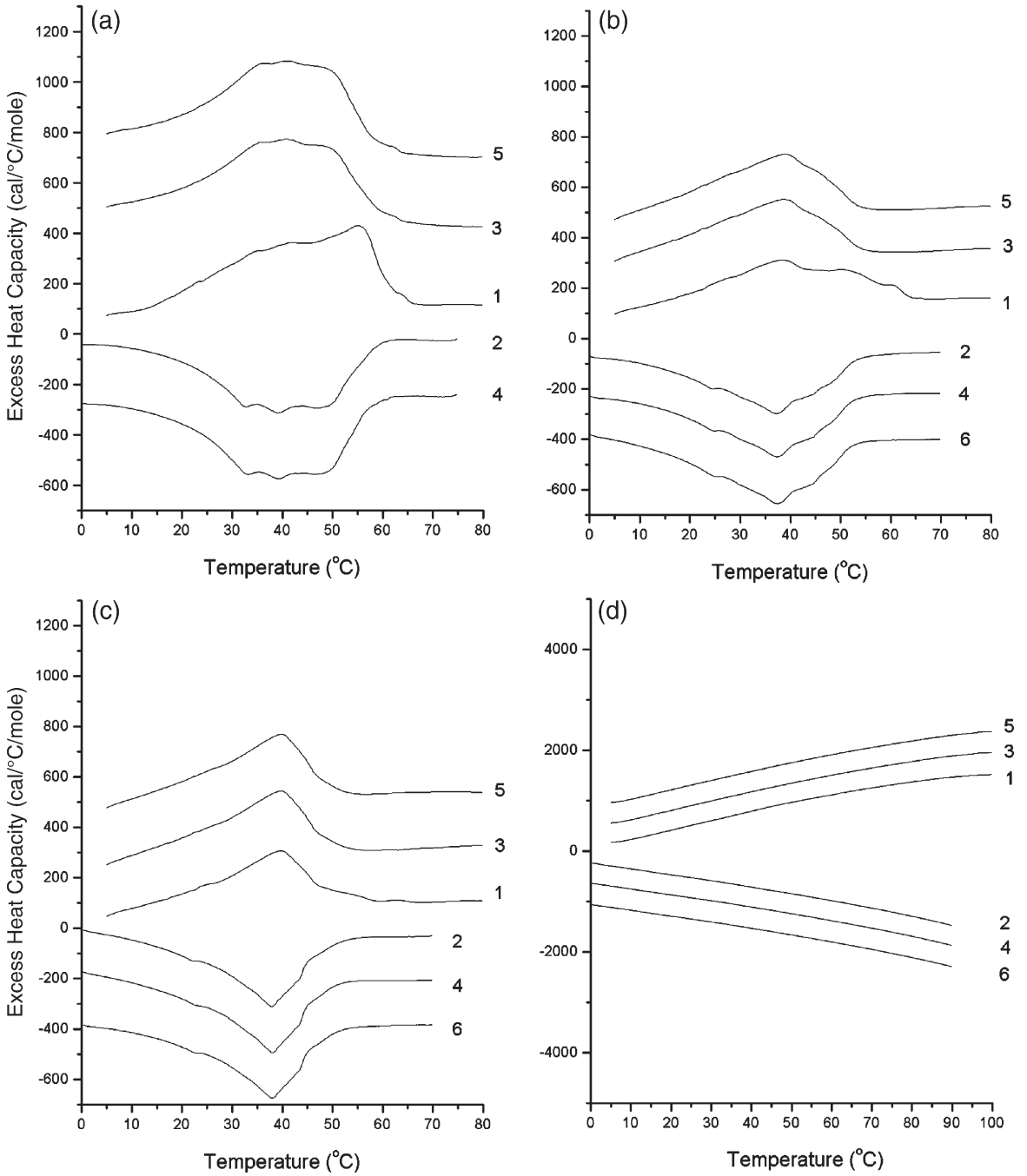

Figure 3. DSC heating and cooling curves for DPPE:CL (0.65:0.35, mol:mol) with added cytochrome *c* at a lipid to protein molar ratio of (a) 200, (b) 430, and (c) 870. (d) CL with added cytochrome *c* and CL at a lipid to protein molar ratio of 200 as in (a). Other details are as for Figure 1.

Although poly-L-lysine at neutral pH does not have much secondary structure in buffer, it has been shown to acquire enhanced secondary structure in the presence of anionic lipids.[47,48] This is the case also with SUVs of DPPE:CL (0.65:0.35, mol:mol) (Figure 8(a)). This structure is lost on heating (Figure 8(b), black curve) and is not regained completely on cooling (Figure 8(b), red curve).

For all of the proteins and poly-L-lysine, the CD spectra show that through the phase transition of the lipid mixture during the first heating scan, a significant fraction of the protein remains in its native state and is able to interact with CL and produce domains.

Discussion

The octameric form of uMtCK facilitates the appearance of a transition in DSC scans at a higher temperature and with higher enthalpy than that for the pure lipid mixture of DPPE:CL (0.65:0.35, mol:mol) alone (Table 1). At a lipid to protein molar ratio of 560, uMtCK induces the appearance of a

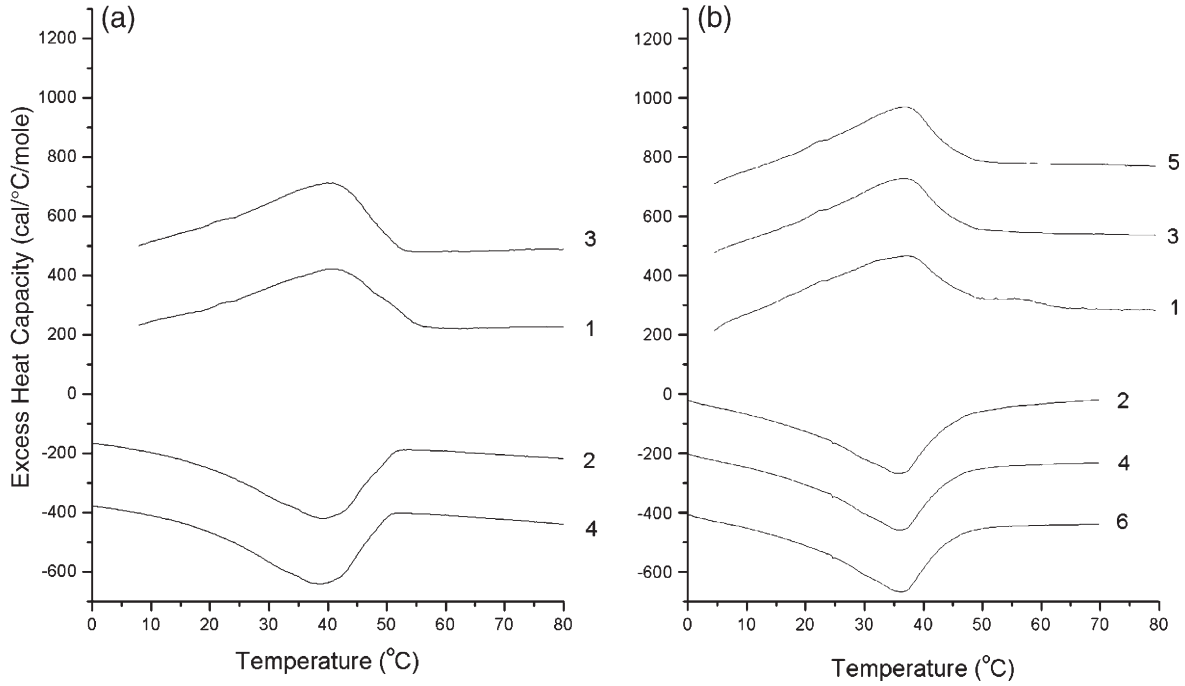

Figure 4. DSC heating and cooling curves for DPPE:CL (0.65:0.35, mol:mol) with added t-Bid at a lipid to protein molar ratio of (a) 200 and (b) 400. Other details are as for Figure 1.

peak at 55 °C with an enthalpy of 7.6 kcal mol^{-1}, as compared to 42 °C and 3.5 kcal mol^{-1} for the DPPE:CL mixture. The enthalpy of the transition for pure DPPE is 10.2 kcal mol^{-1} at 63.3 °C (Figure 1(a), inset), in agreement with literature values.[49] We therefore interpret the formation of the peak at 55° C when uMtCK is present (Figure 1, panel B, scan 1) to be a consequence of the lateral phase separation of most of the DPPE present from the mixture with CL. It is known that uMtCK binds only to liposomes with anionic lipid.[16] Thus, the protein interacts preferentially with the CL component, leaving DPPE as a domain almost completely separated from CL. It is interesting that the uMtCK has a large effect on the transition properties of the lipid mixture only in the first heating scan. We suggest

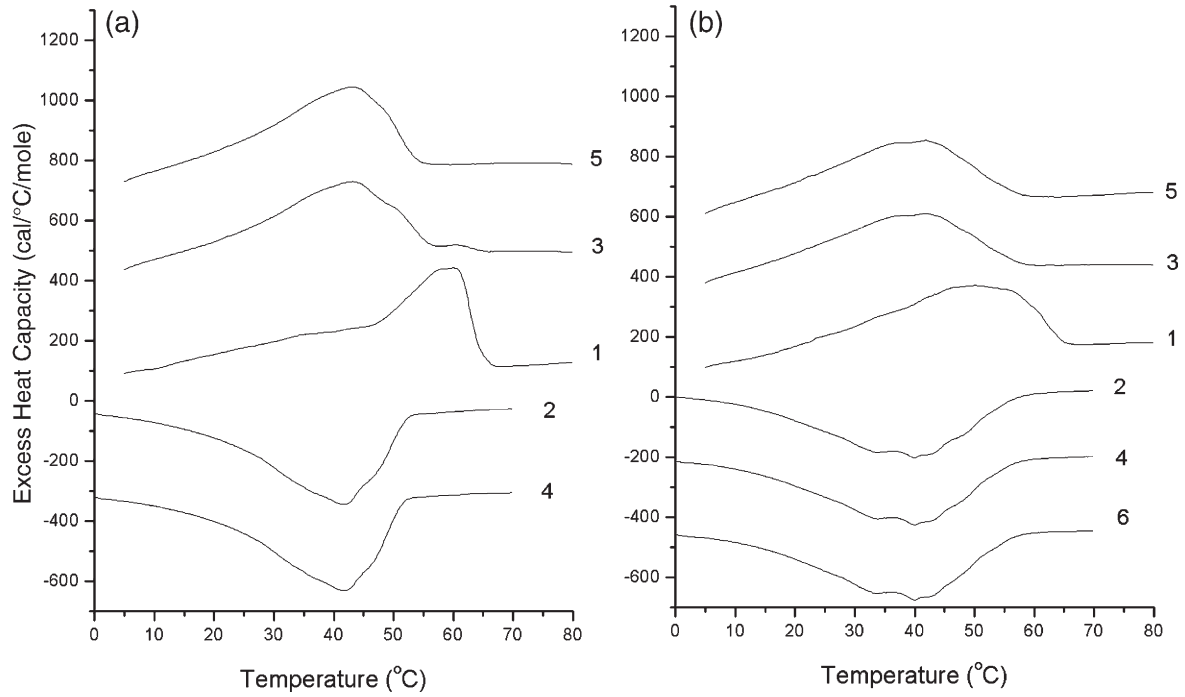

Figure 5. DSC heating and cooling curves for DPPE:CL (0.65:0.35, mol:mol) with added poly-L-lysine at a lipid to polymer molar ratio of (a) 450 and (b) 900. Other details are as for Figure 1.

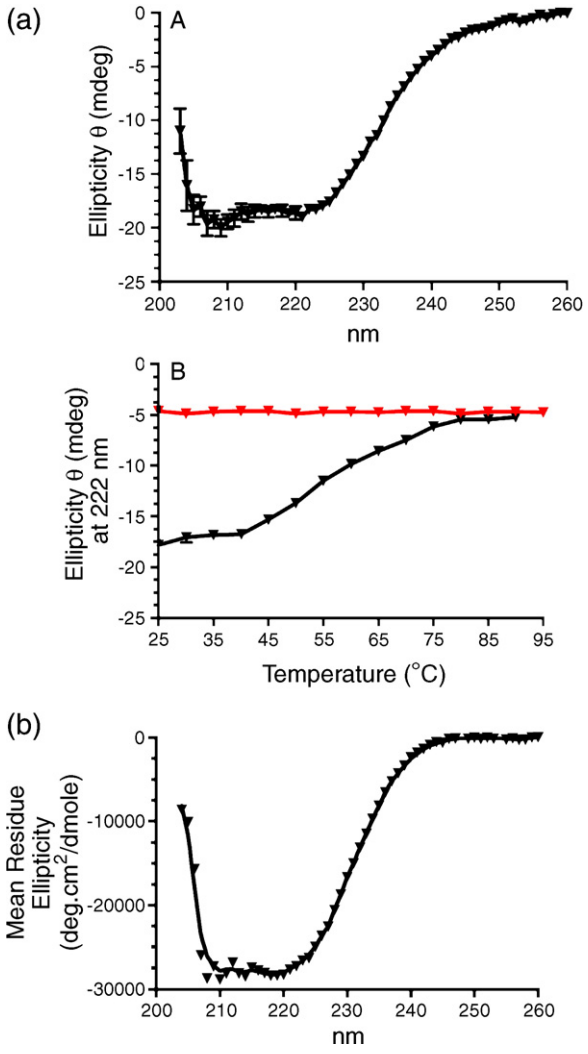

Figure 6. (a) Panel A, CD spectra of uMtCK at 25 °C in the presence of SUV of CL at a lipid to protein molar ratio of 190. Addition of protein to clear CL SUV produced intense turbidity, precluding the quantification in terms of molar ellipticity; panel B, temperature-dependence of the ellipticity at 222 nm of uMtCK in the presence of SUV of CL at a molar ratio of 190. Black curve, heating scan, red curve, cooling scan. (b). CD spectra of uMtCK at 25 °C in the presence of clear SUV of POPE:CL (0.75:0.25, mol:mol) at a lipid to protein molar ratio of 280.

this is caused by the total denaturation of the protein at higher temperatures. We have observed that the magnitude of the ellipticity of uMtCK in the presence of SUVs of CL is reduced markedly on heating, caused, in part, by an irreversible protein coagulation (Figure 6(b)). Thus, in the first heating scan with the DPPE:CL mixture, a major fraction of the protein is still in its native state in the temperature region of the lipid transition. However, at the end of the first heating scan the protein is almost completely denatured irreversibly. As a consequence, the uMtCK has much less effect on the lipid transitions in the next cooling scan and subsequent scans. This is fully in line with experiments on thermal inactivation of uMtCK enzymatic activity (not shown). We have observed that when uMtCK is in a dimeric form, there is almost no segregation of a high-temperature peak (Figure 1(d)). We therefore conclude that the ability of uMtCK to induce CL-rich domains is dependent on the native octameric structure of the protein forming a cluster of Lys residues near the carboxyl terminus that have been shown to interact with anionic lipid.[17] In addition, the octameric form of human MtCK has an isoelectric point (pI) of 7.7, compared with 7.5 for dimer,[50] thus providing an additional factor favoring the partitioning of the octamer to the interface of an anionic membrane.

We also tested the mixture of CL with a lower melting lipid, POPE. This lipid mixture gave a broad transition in the absence of protein (Figure 2(a), panel A), over a somewhat lower temperature range, as expected for this more unsaturated system. In this case also there is a marked alteration of the phase transition properties of the POPE mixture with CL, resulting in the sharpening of the transition and partial separation of the lipid components (Figure 2(a), panel B). The separation of the PE-enriched component is less complete than in the case of the CL/DPPE mixture, which is also driven by differences in the acyl chain composition of the two lipid components. Before the first heating scan, the lipid and protein mixture was kept at low temperatures to avoid denaturation of the protein. Indeed, the CD spectra of the protein in the presence of SUVs of POPE:CL (0.75:0.25, mol:mol) corroborates that the protein remains fully helical at 25 °C (Figure 6(b)).

The DPPE:DOPG mixtures exhibit little change in their phase transition behavior upon addition of uMtCK (Figure 2(b)), although uMtCK is known to bind to PG.[51] This points to specificity for CL in the phase separation phenomenon we are observing. This specificity has been observed before in the ability of uMtCK to promote lipid transfer between juxtaposed bilayers.[52]

We compared other basic mitochondrial proteins in their ability to induce phase separation of the specific lipid mixtures used above. The theoretical pI of cytochrome *c* is 9.6, contributing to its interactions with membranes containing anionic lipids.[22] Cytochrome *c* had been shown to promote the segregation of anionic lipids in both phospholipid bilayers,[53] as well as monolayers.[54] We find some separation of DPPE and CL with higher concentrations of cytochrome *c* (Table 1; Figure 3). The ability of cytochrome *c* to rearrange lipid is modified by heating because of the irreversible denaturation of the protein (Figure 7). Even though cytochrome *c* partly denatures in the temperature range of the lipid phase transition in the presence of anionic lipid, it is still able to perturb the lipid phase transition in the first heating scan (Figure 3). There is evidence that cytochrome *c* interacts with CL-containing membranes largely by electrostatic interactions when the protein is in its native state, but the unfolded form binds to membranes by hydrophobic

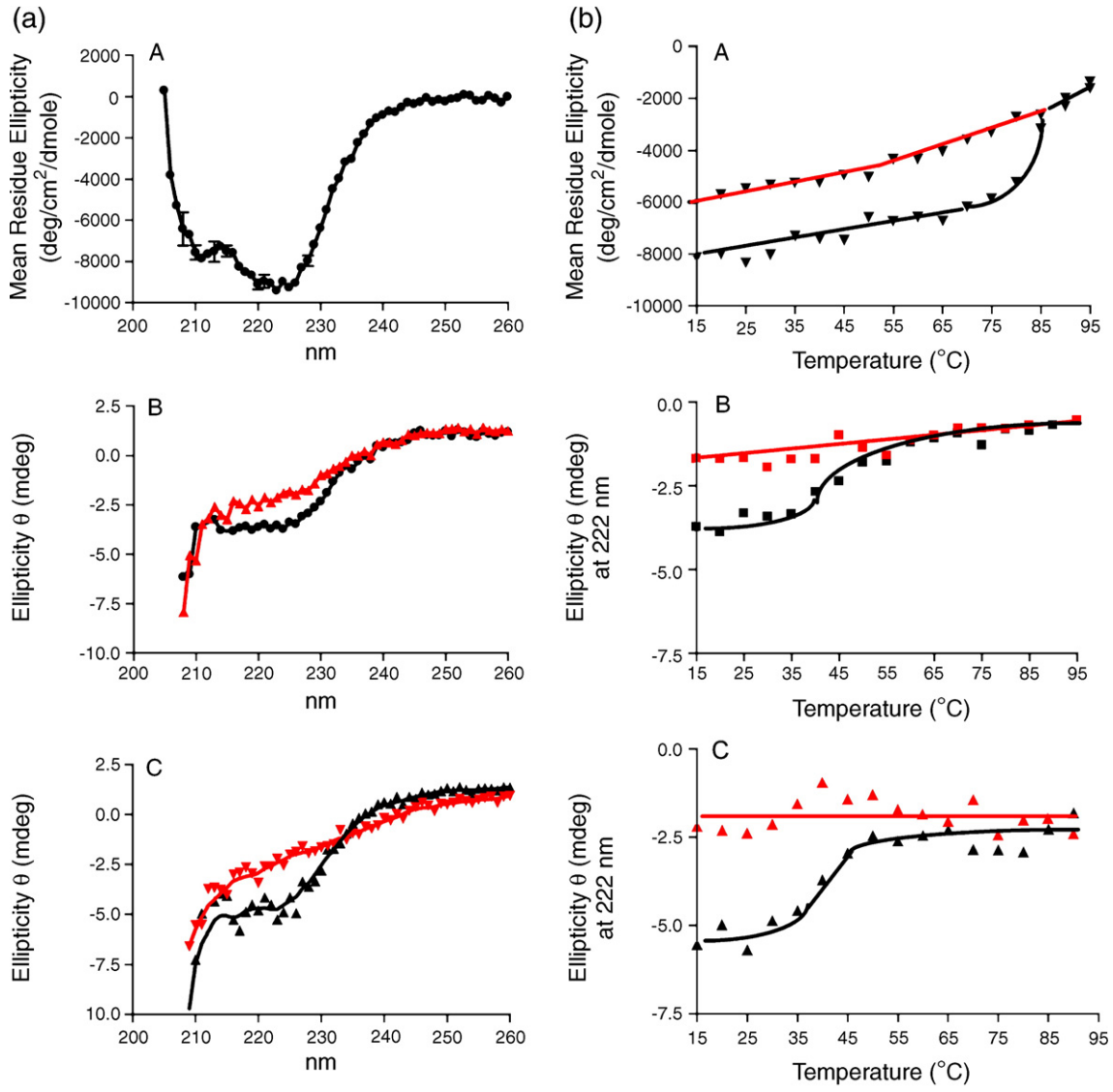

Figure 7. (a) CD spectra of cytochrome *c* measured at 25 °C: (A) 6 μM cytochrome *c* in buffer at pH 7.4; (B) with SUVs of CL at a lipid to protein molar ratio of 130 (black curve) and after heating to 95 °C (red curve); (C) with SUVs of DPPE:CL (65:35, mol:mol) at a lipid to protein molar ratio of 410 (black curve) and after heating to 95 °C (red curve). (b) Thermal denaturation of cytochrome *c* as measured by CD at 222 nm. Heating curves in black and cooling curves in red for (A) protein in Pipes buffer (pH 7.4); (B) in the presence of SUV of CL at a lipid to cytochrome *c* molar ratio of 130; and (C) in the presence of SUV of DPPC:CL (0.65:0.35, mol:mol) at a lipid to cytochrome *c* molar ratio of 410.

interactions.[27,55] Also, membrane domains formed by CL have been reported with cytochrome *c*.[56–58]

tBid also has a high pI, 8.4, and therefore would partition into the electrical double layer of a negatively charged membrane at neutral pH. In addition, tBid appears to have some specificity for interacting with CL. This protein targets to mitochondrial contact sites that are enriched in CL, but it is not very effective in inducing the formation of these domains, as indicated by our observation that tBid has very little ability in causing rearrangement of the DPPE:CL mixtures (Figure 4), under the conditions of this study.

Finally, we have studied the behavior of poly-L-lysine with DPPE:CL mixtures. This polymer is highly charged and binds preferentially to anionic lipids. It is known to induce formation of domains enriched in anionic lipid.[59] We find that this polypeptide can promote the rearrangement of DPPE and CL, thus confirming our interpretation of charge clustering. Heating to high temperatures causes some irreversible modification of the polypeptide secondary structure (Figure 8), so that the lipid transitions are different in the first DSC heating scan (Figure 5).

Mitochondria have contact sites that are enriched in both CL and in MtCK. uMtCK is particularly potent in inducing lipid phase separation between CL and zwitterionic lipids. Furthermore, this property is dependent on the oligomeric structure of the uMtCK. One would expect the same phenomenon to occur at contact sites at physiological temperature, since the lipid composition of these sites, although more complex, is still mostly the anionic lipid CL together with zwitterionic lipids. At contact sites,

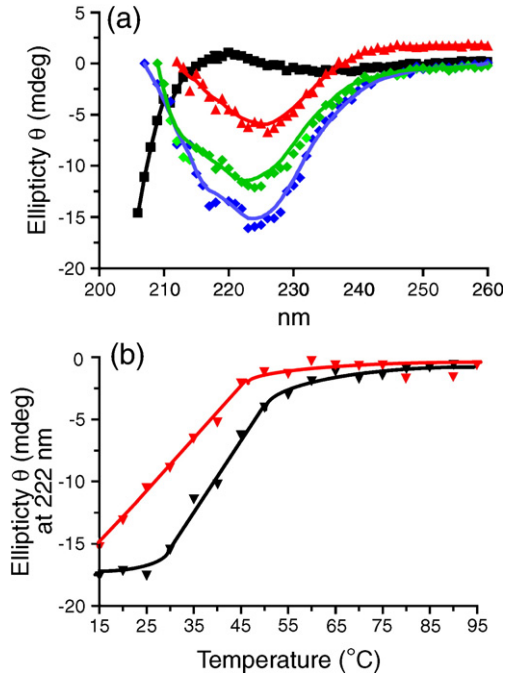

Figure 8. (a) CD spectra of poly-L-lysine at 25 °C in Pipes buffer at pH 7.4 (black), in the presence of SUV of CL at a molar ratio of 140 (red) and in the presence of SUV of DPPE:CL (0.65:0.35, mol:mol) at a molar ratio of 450 before (blue) and after (green) heating. (a) Temperature-dependence of ellipticity of poly-L-lysine at 222 nm in the presence of SUVs of CL.

uMtCK could bind to CL on the inner membrane, stabilizing a CL-rich domain that could bridge the inner and outer membranes by binding to the voltage-dependent anion channel on the outer membrane.[60] Thus, the lipid phase segregation facilitated by uMtCK could help in understanding the mechanism of contact site formation. The potency of uMtCK in clustering CL is a nucleation point for the formation of a CL-rich domain to which other proteins, such as cytochrome *c* and t-Bid, can bind. The binding to the voltage-dependent anion channel makes MtCK part of the protein complex forming the permeability transition pore. The sustained opening of this pore may be more important for necrosis,[61] and has important implications for energy flux processes taking place in mitochondria as well as in apoptosis.[4,62–64] This complex at contact sites is also highly enriched in CL, and its function is modulated by the presence of CL.[65,66] The work presented here indicates that MtCK may play an important role in recruiting CL to this membrane domain.

Materials and Methods

Materials

The phospholipids dipalmitoylphosphatidylethanolamine (DPPE), 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE), dioleoyl phosphatidylglycerol (DOPG) and tetraoleoyl cardiolipin (CL) were purchased from Avanti Polar Lipids (Alabaster, AL). Recombinant mature uMtCK was expressed and purified as described.[14] For some experiments, the final preparation of uMtCK was concentrated fourfold using a 10 kDa cut-off Microcon centrifugal filter YM-10 (Millipore) and was stored frozen at a concentration of 7 mg ml^{-1}; the protein is an octamer at this concentration.[7] Diluting the protein to a concentration below 0.4 mg ml^{-1} and keeping the diluted solution in the refrigerator for 24 h before addition to the lipid mixture causes dissociation of the octamer to the dimeric form. Horse heart cytochrome *c* and poly-L-lysine (28,200 Da) were purchased from Sigma-Aldrich (St. Louis, MO). tBid was prepared by caspase 8 cleavage of Bid as described.[67]

Preparation of samples for DSC

Lipid films were made by dissolving appropriate amounts of lipid in chloroform/methanol (2:1, v/v), followed by evaporation of the solvent under nitrogen to deposit the lipid as a film on the wall of a test-tube. Final traces of solvent were removed in a vacuum chamber attached to a liquid nitrogen trap for 5 h. Dried films were used immediately or kept under argon at −20 °C. Films of DPPE:CL, DPPE:POPG or DPPE:DOPG (0.65:0.35, mol:mol) were hydrated with buffer alone or protein solution in 20 mM Pipes (pH 7.40), 1 mM EDTA, 150 mM NaCl, 0.002% (w/v) NaN_3, and suspended by vigorous vortex mixing and warming for a few minutes below 50 °C to facilitate the hydration of the lipid mixture. For mixtures of POPE:CL, buffer or protein solution were kept at 4 °C and hydration was done by vortex mixing vigorously, while maintaining the temperature below 17 °C. This lipid mixture was more sensitive to the thermal history of the sample and the exact ratio of POPE to CL. In order to achieve an accurate comparison of the phase behavior with and without protein, care was taken to use the same hydration protocol for both samples, and to determine the lipid concentration of the stock solutions in organic solvent by phosphate analysis.[68]

Differential scanning calorimetry (DSC)

Measurements were made using a Nano II differential scanning calorimeter (Calorimetry Sciences Corporation, Lindon, UT). The scan rate was 0.75 deg.C min^{-1} with a delay of 5 min between sequential scans in a series to allow for thermal equilibration. The features of the design of this instrument have been described.[69] DSC curves were analyzed by the fitting program DA-2 provided by Microcal Inc. (Northampton, MA) and plotted with Origin, version 5.0. The concentration of MLV in the samples studied was maintained at 2.5 mg ml^{-1}. The cell volume is 340 μl.

Circular dichroism

CD spectra were recorded on an AVIV model 215 spectropolarimeter equipped with a Thermo Neslab M25 circulating bath. A quartz cell with a 0.1 cm path-length was placed in a thermally controlled cell holder. The temperature changes were computer-controlled and points were taken at 5 deg.C intervals. The machine was equipped with a Peltier junction thermal device and a Thermo Neslab M25 circulating bath. For samples

containing lipid, the lipid was first made into a dry film as described above, and then hydrated by vortex mixing with buffer. The lipid suspension was sonicated to clarity under argon in a bath-type sonicator to make small unilamellar vesicles (SUVs) and protein was then added to the SUVs. Addition of uMtCK, cytochrome *c* or poly-L-lysine caused the SUVs to become turbid.

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References

1. Speer, O., Back, N., Buerklen, T., Brdiczka, D., Koretsky, A., Wallimann, T. & Eriksson, O. (2005). Octameric mitochondrial creatine kinase induces and stabilizes contact sites between the inner and outer membrane. *Biochem. J.* **385**, 445–450.
2. Ardail, D., Privat, J. P., Egret-Charlier, M., Levrat, C., Lerme, F. & Louisot, P. (1990). Mitochondrial contact sites. Lipid composition and dynamics. *J. Biol. Chem.* **265**, 18797–18802.
3. Dolder, M., Wendt, S. & Wallimann, T. (2001). Mitochondrial creatine kinase in contact sites: interaction with porin and adenine nucleotide translocase, role in permeability transition and sensitivity to oxidative damage. *Biol. Signals Recept.* **10**, 93–111.
4. Schlattner, U., Tokarska-Schlattner, M. & Wallimann, T. (2006). Mitochondrial creatine kinase in human health and disease. *Biochim. Biophys. Acta*, **1762**, 164–180.
5. Kuwana, T., Mackey, M. R., Perkins, G., Ellisman, M. H., Latterich, M., Schneiter, R. *et al.* (2002). Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell*, **111**, 331–342.
6. Zamzami, N. & Kroemer, G. (2003). Apoptosis: mitochondrial membrane permeabilization—the (W)hole story? *Curr. Biol.* **13**, R71–R73.
7. Schlegel, J., Zurbriggen, B., Wegmann, G., Wyss, M., Eppenberger, H. M. & Wallimann, T. (1988). Native mitochondrial creatine kinase forms octameric structures. I. Isolation of two interconvertible mitochondrial creatine kinase forms, dimeric and octameric mitochondrial creatine kinase: characterization, localization, and structure-function relationships. *J. Biol. Chem.* **263**, 16942–16953.
8. Muller, M., Moser, R., Cheneval, D. & Carafoli, E. (1985). Cardiolipin is the membrane receptor for mitochondrial creatine phosphokinase. *J. Biol. Chem.* **260**, 3839–3843.
9. Muller, M., Cheneval, D. & Carafoli, E. (1986). The mitochondrial creatine phosphokinase is associated with inner membrane cardiolipin. *Advan. Expt. Med. Biol.* **194**, 151–156.
10. Schlame, M. & Augustin, W. (1985). Association of creatine kinase with rat heart mitochondria: high and low affinity binding sites and the involvement of phospholipids. *Biomed. Biochim. Acta*, **44**, 1083–1088.
11. Stachowiak, O., Dolder, M. & Wallimann, T. (1996). Membrane-binding and lipid vesicle cross-linking kinetics of the mitochondrial creatine kinase octamer. *Biochemistry*, **35**, 15522–15528.
12. Schlattner, U. & Wallimann, T. (2000). Octamers of mitochondrial creatine kinase isoenzymes differ in stability and membrane binding. *J. Biol. Chem.* **275**, 17314–17320.
13. Fritz-Wolf, K., Schnyder, T., Wallimann, T. & Kabsch, W. (1996). Structure of mitochondrial creatine kinase. *Natur*, **381**, 341–345.
14. Eder, M., Fritz-Wolf, K., Kabsch, W., Wallimann, T. & Schlattner, U. (2000). Crystal structure of human ubiquitous mitochondrial creatine kinase. *Proteins: Struct. Funct. Genet.* **39**, 216–225.
15. Rojo, M., Hovius, R., Demel, R., Wallimann, T., Eppenberger, H. M. & Nicolay, K. (1991). Interaction of mitochondrial creatine kinase with model membranes. A monolayer study. *FEBS. Letters*, **281**, 123–129.
16. Vacheron, M. J., Clottes, E., Chautard, C. & Vial, C. (1997). Mitochondrial creatine kinase interaction with phospholipid vesicles. *Arch. Biochem. Biophys.* **344**, 316–324.
17. Schlattner, U., Gehring, F., Vernoux, N., Tokarska-Schlattner, M., Neumann, D., Marcillat, O. *et al.* (2004). C-terminal lysines determine phospholipid interaction of sarcomeric mitochondrial creatine kinase. *J. Biol. Chem.* **279**, 24334–24342.
18. Epand, R. M. (2006). Detecting the presence of membrane domains using DSC. *Biophys. Chem.* In the press (doi:10.1016/j.bpc.2006.05.008).
19. Garidel, P. & Blume, A. (2000). Miscibility of phosphatidylethanolamine-phosphatidylglycerol mixtures as a function of pH and acyl chain length. *Eur. Biophys. J.* **28**, 629–638.
20. Wiedmann, T., Salmon, A. & Wong, V. (1993). Phase behavior of mixtures of DPPC and POPG. *Biochim. Biophys. Acta*, **1167**, 114–120.
21. Wainwright, P., Power, D. M., Thomas, E. W. & Davies, J. V. (1978). The interaction of cytochrome C with phospholipids. A pulse-radiolysis study. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.* **33**, 151–162.
22. Gorbenko, G. P., Molotkovsky, J. G. & Kinnunen, P. K. J. (2006). Cytochrome c interaction with cardiolipin/phosphatidylcholine model membranes: effect of cardiolipin protonation. *Biophys. J.* **90**, 4093–4103.
23. de Kruijff, B. & Cullis, P. R. (1980). Cytochrome c specifically induces non-bilayer structures in cardiolipin-containing model membranes. *Biochim. Biophys. Acta*, **602**, 477–490.
24. Demel, R. A., Jordi, W., Lambrechts, H., van, D. H., Hovius, R. & de Kruijff, B. (1989). Differential interactions of apo- and holocytochrome c with acidic membrane lipids in model systems and the implications for their import into mitochondria. *J. Biol. Chem.* **264**, 3988–3997.
25. Kimelberg, H. K. & Lee, C. P. (1969). Binding and electron transfer to cytochrome c in artificial phospholipid membranes. *Biochem. Biophys. Res. Commun.* **34**, 784–790.
26. Iverson, S. L. & Orrenius, S. (2004). The cardiolipin-cytochrome c interaction and the mitochondrial regulation of apoptosis. *Arch. Biochem. Biophys.* **423**, 37–46.
27. Belikova, N. A., Vladimirov, Y. A., Osipov, A. N.,

Kapralov, A. A., Tyurin, V. A., Potapovich, M. V. *et al.* (2006). Peroxidase activity and structural transitions of cytochrome c bound to cardiolipin-containing membranes. *Biochemistry*, **45**, 4998–5009.
28. Garrido, C., Galluzzi, L., Brunet, M., Puig, P. E., Didelot, C. & Kroemer, G. (2006). Mechanisms of cytochrome c release from mitochondria. *Cell Death. Differ.* **13**, 1423–1433.
29. Kagan, V. E., Tyurin, V. A., Jiang, J., Tyurina, Y. Y., Ritov, V. B., Amoscato, A. A. *et al.* (2005). Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nature Chem. Biol.* **1**, 223–232.
30. Orrenius, S. & Zhivotovsky, B. (2005). Cardiolipin oxidation sets cytochrome c free. *Nature Chem. Biol.* **1**, 188–189.
31. Lutter, M., Fang, M., Luo, X., Nishijima, M., Xie, X. S. & Wang, X. D. (2000). Cardiolipin provides specificity for targeting of tBid to mitochondria. *Nature Cell Biol.* **2**, 754–756.
32. Liu, J., Weiss, A., Durrant, D., Chi, N. W. & Lee, R. M. (2004). The cardiolipin-binding domain of Bid affects mitochondrial respiration and enhances cytochrome c release. *Apoptosis*, **9**, 533–541.
33. Van, M. N., Kajava, A. V., Bonfils, C., Martinou, J. C. & Harricane, M. C. (2005). Interactions of Bax and tBid with lipid monolayers. *J. Membr. Biol.* **207**, 1–9.
34. Gonzalvez, F., Pariselli, F., Dupaigne, P., Budihardjo, I., Lutter, M., Antonsson, B. *et al.* (2005). tBid interaction with cardiolipin primarily orchestrates mitochondrial dysfunctions and subsequently activates Bax and Bak. *Cell Death Differentiat.* **12**, 614–626.
35. Lutter, M., Perkins, G. A. & Wang, X. (2001). The pro-apoptotic Bcl-2 family member tBid localizes to mitochondrial contact sites. *BMC. Cell Biol.* **2**, 22.
36. Kim, T. H., Zhao, Y., Ding, W. X., Shin, J. N., He, X., Seo, Y. W. *et al.* (2004). Bid-cardiolipin interaction at mitochondrial contact site contributes to mitochondrial cristae reorganization and cytochrome *c* release. *Mol. Biol. Cell.* **15**, 3061–3072.
37. McLaughlin, S. & Murray, D. (2005). Plasma membrane phosphoinositide organization by protein electrostatics. *Nature*, **438**, 605–611.
38. Ben-Tal, N., Honig, B., Peitzsch, R. M., Denisov, G. & McLaughlin, S. (1996). Binding of small basic peptides to membranes containing acidic lipids: theoretical models and experimental results. *Biophys. J.* **71**, 561–575.
39. de Kruijff, B. & Cullis, P. R. (1980). The influence of poly(L-lysine) on phospholipid polymorphism. Evidence that electrostatic polypeptide-phospholipid interactions can modulate bilayer/non-bilayer transitions. *Biochim. Biophys. Acta*, **601**, 235–240.
40. Bagel'ova, J., Antalik, M. & Bona, M. (1994). Studies on cytochrome c-heparin interactions by differential scanning calorimetry. *Biochem. J.* **297**, 99–101.
41. Zhang, F. & Rowe, E. S. (1994). Calorimetric studies of the interactions of cytochrome c with dioleoylphosphatidylglycerol extruded vesicles: ionic strength effects. *Biochim. Biophys. Acta*, **1193**, 219–225.
42. Wang, L., Chen, R. X. & Kallenbach, N. R. (1998). Proteolysis as a probe of thermal unfolding of cytochrome *c*. *Proteins: Struct. Funct. Genet.* **30**, 435–441.
43. Spooner, P. J. & Watts, A. (1991). Reversible unfolding of cytochrome c upon interaction with cardiolipin bilayers. 2. Evidence from phosphorus-31 NMR measurements. *Biochemistry*, **30**, 3880–3885.
44. Spooner, P. J. & Watts, A. (1991). Reversible unfolding of cytochrome c upon interaction with cardiolipin bilayers. 1. Evidence from deuterium NMR measurements. *Biochemistry*, **30**, 3871–3879.
45. Heimburg, T. & Marsh, D. (1993). Investigation of secondary and tertiary structural changes of cytochrome c in complexes with anionic lipids using amide hydrogen exchange measurements: an FTIR study. *Biophys. J.* **65**, 2408–2417.
46. Pinheiro, T. J., Elove, G. A., Watts, A. & Roder, H. (1997). Structural and kinetic description of cytochrome *c* unfolding induced by the interaction with lipid vesicles. *Biochemistry*, **36**, 13122–13132.
47. Chittchang, M., Alur, H. H., Mitra, A. K. & Johnston, T. P. (2002). Poly(L-lysine) as a model drug macromolecule with which to investigate secondary structure and membrane transport. Part I: physicochemical and stability studies. *J. Pharm. Pharmacol.* **54**, 315–323.
48. Fukushima, K., Muraoka, Y., Inoue, T. & Shimozawa, R. (1989). Conformational study of poly(L-lysine) interacting with acidic phospholipid vesicles. *Biophys. Chem.* **34**, 83–90.
49. Caffrey, M. (1993). *Lipidat: A Database of Thermodynamic Data and Associated Information on Lipid Polymorphism and Polymorphic Transitions.* CRC Press, Boca Raton, FL.
50. Schlattner, U., Eder, M., Dolder, M., Khuchua, Z. A., Strauss, A. W. & Wallimann, T. (2000). Divergent enzyme kinetics and structural properties of the two human mitochondrial creatine kinase isoenzymes. *Biol. Chem.* **381**, 1063–1070.
51. Granjon, T., Vacheron, M. J., Vial, C. & Buchet, R. (2001). Mitochondrial creatine kinase binding to phospholipids decreases fluidity of membranes and promotes new lipid-induced beta structures as monitored by red edge excitation shift, laurdan fluorescence, and FTIR. *Biochemistry*, **40**, 6016–6026.
52. Epand, R. F., Schlattner, U., Wallimann, T., Lacombe, M.-L. & Epand, R. M. (2006). Novel lipid transfer property of two mitochondrial proteins that bridge the inner and outer membranes. *Biophys. J.* In the press (doi:10.1529/biophysj.106.092353).
53. Haverstick, D. M. & Glaser, M. (1989). Influence of proteins on the reorganization of phospholipid bilayers into large domains. *Biophys. J.* **55**, 677–682.
54. Kasbauer, M. & Bayerl, T. M. (1999). Formation of domains of cationic or anionic lipids in binary lipid mixtures increases the electrostatic coupling strength of water-soluble proteins to supported bilayers. *Biochemistry*, **38**, 15258–15263.
55. Piccotti, L., Buratta, M., Giannini, S., Gresele, P., Roberti, R. & Corazzi, L. (2004). Binding and release of cytochrome c in brain mitochondria is influenced by membrane potential and hydrophobic interactions with cardiolipin. *J. Membr. Biol.* **198**, 43–53.
56. Sennato, S., Bordi, F., Cametti, C., Coluzza, C., Desideri, A. & Rufini, S. (2005). Evidence of domain formation in cardiolipin-glycerophospholipid mixed monolayers. A thermodynamic and AFM study. *J. Phys. Chem. B*, **109**, 15950–15957.
57. Domenech, O., Morros, A., Cabanas, A., Teresa, M. M. & Hernandez-Borrell, J. (2006). Supported planar bilayers from hexagonal phases. *Biochim. Biophys. Acta.* In the press (doi:10.1016/j.bbamem.2006.06.012).
58. Domenech, O., Sanz, F., Montero, M. T. & Hernandez-Borrell, J. (2006). Thermodynamic and structural study of the main phospholipid components comprising the mitochondrial inner membrane. *Biochim. Biophys. Acta*, **1758**, 213–221.

59. Hartmann, W. & Galla, H. J. (1978). Binding of polylysine to charged bilayer membranes: molecular organization of a lipid.peptide complex. *Biochim. Biophys. Acta*, **509**, 474–490.
60. Vyssokikh, M., Zorova, L., Zorov, D., Heimlich, G., Jurgensmeier, J., Schreiner, D. & Brdiczka, D. (2004). The intra-mitochondrial cytochrome c distribution varies correlated to the formation of a complex between VDAC and the adenine nucleotide translocase: this affects Bax-dependent cytochrome c release. *Biochim. Biophys. Acta*, **1644**, 27–36.
61. Nakagawa, T., Shimizu, S., Watanabe, T., Yamaguchi, O., Otsu, K., Yamagata, H. *et al.* (2005). Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature*, **434**, 652–658.
62. Brdiczka, D. G., Zorov, D. B. & Sheu, S. S. (2006). Mitochondrial contact sites: their role in energy metabolism and apoptosis. *Biochim. Biophys. Acta*, **1762**, 148–163.
63. Saks, V., Dzeja, P., Schlattner, U., Vendelin, M., Terzic, A. & Wallimann, T. (2006). Cardiac system bioenergetics: metabolic basis of the Frank-Starling law. *J. Physiol.* **571**, 253–273.
64. Dolder, M., Walzel, B., Speer, O., Schlattner, U. & Wallimann, T. (2003). Inhibition of the mitochondrial permeability transition by creatine kinase substrates. Requirement for microcompartmentation. *J. Biol. Chem.* **278**, 17760–17766.
65. Rostovtseva, T. K., Kazemi, N. & Bezrukov, S. M. (2006). The role of Cardiolipin in VDAC channel regulation. *Biophys. J. Meeting Abstracts*, **89**, 333a.
66. Hoffmann, B., Stockl, A., Schlame, M., Beyer, K. & Klingenberg, M. (1994). The reconstituted ADP/ATP carrier activity has an absolute requirement for cardiolipin as shown in cysteine mutants. *J. Biol. Chem.* **269**, 1940–1944.
67. Li, H., Zhu, H., Xu, C. J. & Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*, **94**, 491–501.
68. Ames, B. N. (1966). Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol.* **8**, 115–118.
69. Privalov, G., Kavina, V., Freire, E. & Privalov, P. L. (1995). Precise scanning calorimeter for studying thermal properties of biological macromolecules in dilute solution. *Anal. Biochem.* **232**, 79–85.

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