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A spin-label electron spin resonance study of the binding of mitochondrial creatine kinase to cardiolipin

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The binding of the mitochondrial creatine kinase to aqueous dispersions of beef heart cardiolipin has been studied via the perturbation of the mobility of spin-labelled cardiolipin, using electron spin resonance (ESR) spectroscopy. In the presence of creatine kinase (1:1 protein/lipid ratio, by mass), the ESR spectra of cardiolipin labelled in a single acyl chain [*n*-(4,4-dimethyl-oxazolidinyl-*N*-oxy)stearoylcardiolipin] indicate a restriction of motion both at the C-5 and C-14 positions ($n = 5, 14$) of the lipid chains. The restriction in mobility was reversed by addition of phosphate or adriamycin, which are thought to inhibit the binding of creatine kinase to the mitochondrial membrane or to displace it from its binding site on the membrane. The effect of the protein on the chain mobility is consistent with surface binding of the protein; no positive evidence was obtained for penetration of the protein into the hydrophobic region of the membrane.

The mitochondrial isoform of creatine kinase was discovered in 1964 by Jacobs et al. [1] and located on the outer side of the inner mitochondrial membrane about 10 years later [2]. Subsequent work has shown that the enzyme can be released by salts or mercurials, [3–5] suggesting electrostatic binding to the membrane. The recent finding that adriamycin inhibits the binding of the released creatine kinase to mitochondria and to cardiolipin-(diphosphatidylglycerol)-containing liposomes [6] and the related finding that the enzyme is released by a cardiolipin-specific phospholipase but not by phospholipase digestion of other phospholipids [7], have led to the conclusion that cardiolipin of the inner membrane is the receptor for the enzyme [6]. The concentration of cardiolipin in the mitochondria is approximately 10 mol%, and is presumably higher in the inner membrane. Adriamycin is a general cardiolipin reagent [8], which inhibits a number of cardiolipin-dependent enzymes of the inner mitochondrial membrane [9–11].

Convincing evidence [4, 12–14] has shown that ATP newly synthesized in the mitochondrial matrix has privileged access to creatine kinase prior to mixing with the total ATP pool [15, 16]. Similarly, ADP formed in the kinase reaction is transported into the mitochondrial matrix in such close functional contact to the adenine nucleotide translocase as to overcome the inhibition by atractyloside [14]. As a result, close coupling between the translocase and the kinase, possibly resulting from direct physical interaction, has been postulated [17, 18].

The work presented here has been carried out to substantiate the conclusion that cardiolipin is the inner-membrane receptor for creatine kinase. The interaction of the enzyme with aqueous dispersions of beef heart cardiolipin has been studied using spin-label ESR spectroscopy. Spin labels have been used which bear the label group either on the C-5 atom (1-(3-*sn*-phosphatidyl)-3-{1-acyl-2-[5-(4,4-dimethyl-oxazolidine-*N*-oxyl)stearoyl)]glycerol 3-phospho}-*sn*-glycerol, 5-CLSL) or on the C-14 atom (14-CLSL) of the *sn*-2′ cardiolipin chain. In this way it was possible to probe the mobility of the lipid chain both close to the polar-apolar interface of the lipid bilayer and close to the terminal methyl ends of the lipid chains. The usefulness and sensitivity of the method has previously been demonstrated in a study of the binding of apo-(cytochrome *c*) to negatively-charged lipid dispersions [19]. The ESR spectra are sensitive to both the amplitude and rate of motion of the labelled lipid chains, and binding of the protein to the lipid was therefore detected by its perturbation of the lipid chain motion. From the size and nature of the perturbation it was possible to make predictions about the mode of association of the protein with the lipid membrane.

MATERIALS AND METHODS

Isolation and purification of mitochondrial creatine kinase

Mitochondrial creatine kinase was isolated from rat heart mitochondria prepared as described in [6]. The suspension of proteins released by the treatment of mitochondria with phosphate was brought to 25% ammonium sulfate saturation and then, after centrifugation at $100000 \times g$ for 40 min, to 50% saturation. After centrifugation the precipitate was dissolved in 10 mM sodium phosphate, 50 mM NaCl, 1 mM NaN_3 and 1 mM 2-mercaptoethanol, pH 6.8 (buffer A) and dialyzed against this buffer overnight. The protein was then loaded onto a Carboxymethyl-Sephadex C-50 (Pharmacia, Uppsala, Sweden) column and eluted with a salt gradient of 50–400 mM NaCl. After testing creatine kinase activity the pooled active fractions were concentrated by ammonium

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Abbreviation. *n*-CLSL, 1-(3-*sn*-phosphatidyl)-3-{1-acyl-2-[*n*-(4,4-dimethyl-oxazolidine-*N*-oxyl)stearoyl]glycero-3-phospho}-*sn*-glycerol.

Enzyme. Creatine kinase (EC 2.7.3.2).

sulfate precipitation at 50% saturation and loaded onto a DEAE-Sephacel (Pharmacia) column using buffer A. The activity was collected in the first fractions which failed to bind to the column. The purity of the eluted protein was examined by SDS/PAGE. It migrated as a single band in the gel.

Measurement of creatine kinase activity

The creatine kinase activity was assayed spectrophotometrically with an Aminco DW−2a spectrophotometer by a coupled enzyme system using 550 nm and 366 nm as the wavelength couple. The assay solution contained 250 mM sucrose, 10 mM Hepes/KOH, pH 7.4, 1 mM $MgCl_2$, 0.2 mM NADH, 0.5 mM phospho*enol*pyruvate, 1 unit lactate dehydrogenase/ml 1 unit pyruvate kinase/ml and 0.5 mM ATP. The reaction was started by the addition of creatine (4.8 mM final concentration) to the kinase-containing sample.

Gel electrophoresis

A slightly modified Laemmli gel electrophoresis system was used for SDS/PAGE, [20]. The acrylamide concentration was 12%.

Measurements of protein concentrations

Protein concentrations were determined by a biuret procedure for mitochondrial proteins [21] and according to [22] for all other samples. In all cases bovine serum albumin was used as the standard.

Preparation of spin-labelled cardiolipin

Cardiolipin, spin-labelled at C-5 of the *sn*-2′ chain, was synthesized by coupling phosphatidylglycerol to the corresponding spin-labelled phosphatidic acid (see [23]), using the method described in [24]. Cardiolipin spin-labelled at C-14 of the *sn*-2′ chain was synthesized from monolysocardiolipin according to the method described in [25].

Preparation of liposomes

The preparation of cardiolipin liposomes was essentially according to [19]. The phospholipid and the spin-label were co-dissolved in organic solvent, dried under a stream of nitrogen and subsequently under vacuum, and then dispersed in buffer.

ESR measurements

ESR spectra were recorded on a Varian E-12 Century Line spectrometer equipped with nitrogen gas flow temperature regulation. Samples were prepared as described in [19] after incubation of creatine kinase with the liposomes for 15 min at the indicated temperatures. The mixture were centrifuged to separate free creatine kinase from lipososome-associated creatine kinase and were contained in sealed 1-mm OD glass capillaries accommodated within a standard quartz ESR tube which contained silicon oil for thermal stability. The temperature was measured with a thermocouple situated in the silicon oil just above the top of the ESR cavity.The concentration of spin-labelled cardiolipin was 1 mol%.

The apparent order parameters were calculated according to

$$S(\text{app.}) = (A_{\parallel} - A)/[A_{zz} - (1/2)(A_{xx} + A_{yy})] \cdot (a_o'/a_o) \quad (1)$$

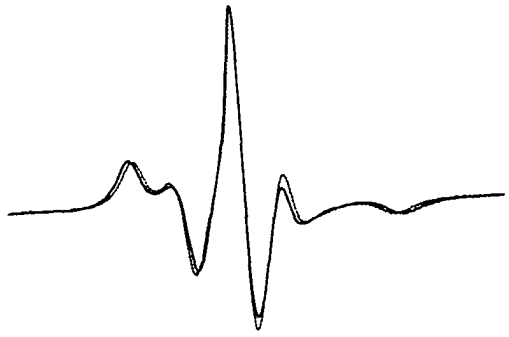

Fig. 1. *ESR spectra of the cardiolipin isomer spin labelled at position 5, 5-CLSL, in beef heart cardiolipin suspensions at 15°C.* Lipid alone (– – – –); lipid in the presence of 1:1 (by mass) creatine kinase (——). Total scan width, 10 mT (100 G).

where $2A_{\parallel}$ is identified with outer maximum hyperfine splitting, $2A_{max}$, and A is obtained from the inner, minimum hyperfine splitting, $2A_{min}$, using the following corrections [26, 27]

$$A = A_{min}(\text{G}) + 0.85\text{G} \qquad S(\text{app.}) < 0.45 \quad (2)$$

$$A = A_{min}(\text{G}) + 1.32\text{G} + (1.86\text{G})\log[1\text{-}S(\text{app.})] \qquad S(\text{app.}) > 0.45 .$$

The apparent isotropic hyperfine splitting constant is given by:

$$a_o = (1/3)(A_{\parallel} + 2A) \quad (3)$$

and that corresponding to the single-crystal environment in which the principal values of the hyperfine tensor, A_{xx}, A_{yy} and A_{zz}, were measured [28] is given by:

$$a_o' = (1/3)(A_{xx} + A_{yy} + A_{zz}) .$$

Detailed lineshape simulations have shown that the spectra, such as those in Fig. 1 contain important contributions from slow molecular motions [29]. Thus the order parameter calculated using Eqn (1), which assumes fast molecular motion, can only be considered as an effective value, but is nonetheless useful for making comparisons between lipid environments in the presence and absence of protein. Under these conditions A_{max} is also a useful empirical parameter which contains contributions from both the amplitude and the rate of motion and is also influenced by the polarity of the spin-label environment.

Materials

Beef heart cardiolipin and adriamycin were from Sigma Chemical Co, St Louis, MO, USA. All other reagents were of the highest purity grade commercially available.

RESULTS AND DISCUSSION

Binding of mitochondrial creatine kinase to aqueous dispersions of cardiolipin

Representative ESR spectra of the 5-CLSL cardiolipin spectra in beef cardiolipin dispersions, in the presence and absence of creatine kinase, are given in Fig. 1. The addition of the protein to the cardiolipin dispersions gives rise to a clear increase in the hyperfine anisotropy, as seen by the increase in splitting of the outermost peaks and the corresponding decrease in splitting of the innermost pair of peaks. The spectral

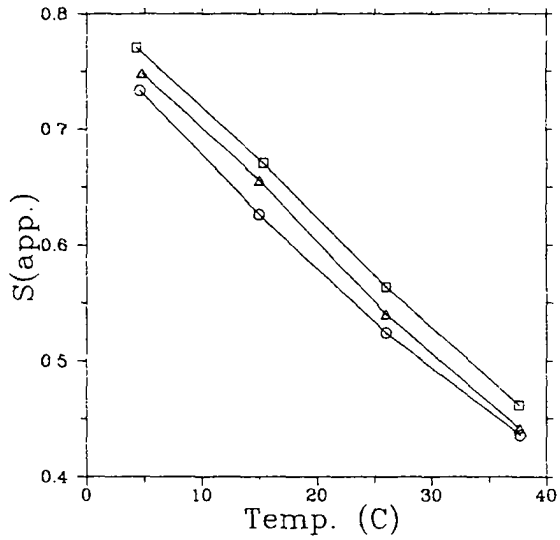

Fig. 2. *Temperature dependence of the apparent order parameter, S(app.), of the 5-CLSL cardiolipin spin label in beef heart cardiolipin dispersions.* (○) lipid alone; (□) lipid in the presence of 1:1 (by mass) creatine kinase; (△ lipid in the presence of 1:1 (by mass) creatine kinase with 80 mM phosphate.

Table 1. *Outer hyperfine splitting, A_{max}, apparent order parameter, S(app.), and apparent isotropic hyperfine splitting constant, a_o, at 26° C for the 5-CLSL cardiolipin spin label in aqueous dispersions of beef heart cardiolipin in the presence and absence of creatine kinase and 80 mM phosphate, or adriamycin, (100 nmol/mg lipid)*
The precision of the measurements is approximately ±0.5%. CL, cardiolipin; CrK, creatine kinase; ratios are given by mass

System	A_{max}	S(app.)	a_o
	mT (G)		mT (G)
CrK/CL (2:1)	2.59 (25.9)	0.582	1.51 (15.1)
CrK/CL (1:1)	2.58 (25.8)	0.564	1.52 (15.2)
CL alone	2.51 (25.1)	0.524	1.53 (15.3)
CrK/CL (1:1) + 80 mM P_i	2.53 (25.3)	0.541	1.52 (15.2)
CL + 80 mM P_i	2.54 (25.0)	0.539	1.52 (15.2)
CrK/CL (1:1) + adriamycin	2.52 (25.2)	0.547	1.51 (15.1)
CL + adriamycin	2.54 (25.4)	0.540	1.52 (15.2)

anisotropy can be quantified in terms of the apparent order parameter, S(app.), defined in Eqn (1).

The temperature dependence of the apparent order parameters is given in Fig. 2. In each case the apparent order parameter decreases with increasing temperature, corresponding to an increasing mobility of the lipid chains. The apparent order parameter is greater in the presence of creatine kinase than in its absence, over the entire temperature range studied. The increases in apparent order parameter correspond approximately to the changes observed on decreasing the temperature by 5°C, and provide a clear indication of binding of the protein to cardiolipin. This is in line with the proposal, also supported by very recent work on the isolation and characterization of the gene and cDNA encoding the human placental mitochondrial creatine kinase [30], that cardiolipin is the binding site for creatine kinase in the inner mitochondrial membrane [6].The finding that the ATP/ADP translocator contains tightly bound cardiolipin [18] suggests the interesting possibility that the latter is the species that interacts with creatine kinase (see also [31]). Preliminary binding of the kinase to bulk cardiolipin of the inner membrane could in this case favor its association with its ultimate binding site, i.e. the tightly bound cardiolipin of the ADP/ATP translocase.

Effects of phosphate and adriamycin on the binding of mitochondrial creatine kinase to cardiolipin

A control for the binding of the kinase to cardiolipin is given in Fig. 2 by the effect of 80 mM phosphate, which is known to displace the protein from the mitochondrial membrane. The addition of phosphate decreases the change in the apparent order parameter induced by the protein, corresponding to a reduction in binding of creatine kinase to the cardiolipin membranes.

Further controls are offered in Table 1, which shows the effects of increasing protein concentration and of adding adriamycin, which is known to prevent the binding of the released kinase to the mitochondrial membrane. The addition of a twofold higher concentration of creatine kinase produces an additional increase in the apparent order parameter and in the outer hyperfine splitting, A_{max}, over that seen at the 1:1 (by mass) protein/lipid ratio. Apparently, the binding is not yet saturated at the 1:1 (by mass) ratio. The effect of the increased protein concentration is also seen at 36°C, but less clearly at lower temperatures, presumably because the spectra are not very sensitive to motional perturbations, or because the binding decreases, at these lower temperatures.

As was the case with phosphate, the effect of adding adriamycin is also to decrease the spectral perturbation induced by the kinase. The binding of adriamycin itself produces a small decrease in the mobility of the lipid chains of cardiolipin. In fact, the apparent order parameters and outer splittings in the presence of adriamycin are very similar, both in the presence and absence of protein, throughout the temperature range studied. This suggests that the binding of creatine kinase is almost completely abolished in the presence of adriamycin.

The apparent isotropic splitting factors, a_o, in Table 1 are all indicative of a moderately polar environment [32], consistent with the position of the label in the hydrocarbon chain. Binding of both protein and adriamycin to the cardiolipin apparently reduces a_o somewhat, possibly indicating a reduction of the polarity at the surface of the cardiolipin liposomes. However, because of the possible complications of slow motional effects on the spectral interpretation, this point must be considered as tentative.

The temperature dependence of the ESR spectra of the 14-CLSL spin label in cardiolipin dispersions, in the presence and absence of creatine kinase, is indicated in Fig. 3. The spectra at the lower temperatures consist apparently of two components, both in the presence and absence of protein. One component corresponds approximately to the three sharp lines seen in the spectra at the higher temperatures, and the other component corresponds to the additional peaks seen in the outer wings of the spectra at the lower temperatures. The most likely explanation for this apparent two-component behaviour is incipient lateral phase separation into regions of fluid and less fluid lipid chains at the lower temperatures, on approaching closer to the gel-to-fluid phase transition of the cardiolipin bilayers. The alternative explanation, that the spectra consist of a single lipid component which experiences additional slower motion components, although less likely, cannot be totally excluded.

The effect of the addition of protein on the spectra of the 14-CLSL label is similar to that of a decrease in temperature,

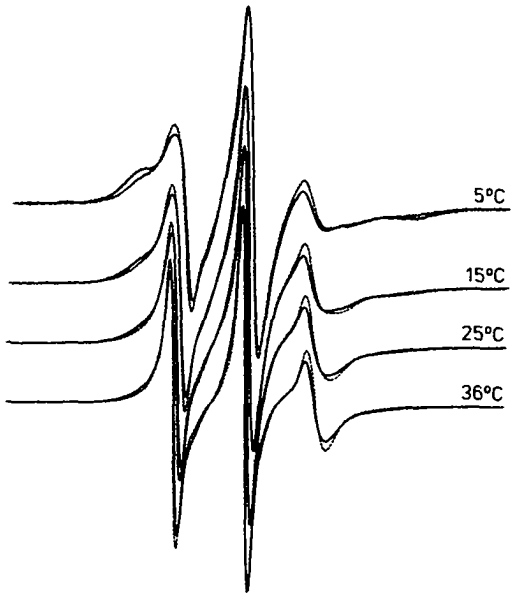

Fig. 3. *ESR spectra of the cardiolipin isomer spin labelled at position 14, 14-CLSL, in beef heart cardiolipin suspensions, as a function of temperature.* Lipid alone (– – –); lipid in the presence of 1:1 (by mass) creatine kinase (———). Total scan width, 10 mT (100 G).

i.e. in the sense that the proportion of the component with reduced mobility apparently increases. This is analogous to the behaviour of the 5-CLSL label, as previously noted. On increasing the temperature, the proportion of the lower mobility component decreases and the part of the spectra arising from the more fluid component becomes sharper, corresponding also to an increase in mobility. At 36°C the spectra essentially consist entirely of a single fluid component both in the presence and absence of kinase, although differences in the spectra are still discernable, corresponding to a decreased mobility in the presence of the protein.

It has been previously found that addition of apo-(cytochrome *c*) to phosphatidylglycerol or phosphatidylserine liposomes gave rise to two-component spectra of phospholipids labelled at C-14, which were rather similar to those of Fig. 3 [19]. However, in this case, the spectra of the lipid in the absence of protein gave no indication of being close to a region of lateral phase separation, or of containing components with much slower motion. For this reason, in analogy to the spectral effects induced by integral proteins, it was suggested that the two-component nature of the ESR spectra could be attributed to partial penetration of the protein into the hydrophobic region of the membrane. In the present case there is no evidence for such spectral effects in a region well away from the range of slow motion effects in the pure lipid system. Thus there is no positive evidence for penetration of the creatine kinase into the cardiolipin bilayer.

The extent of perturbation of the spectra of the 5-CLSL label also suggests that the protein-lipid interaction is not strong enough to implicate membrane penetration. For instance, a 2:1 (by mass) ratio of creatine kinase to cardiolipin induces an 11% increase in the apparent order parameter at 26°C. In comparison, at saturation binding, apo-(cytochrome *c*) induces corresponding increases of approximately 30% [19]. Indeed, cytochrome *c*, which does not penetrate lipid bilayers, also induces considerably larger perturbations of spin-label mobility on binding to negatively charged lipid membranes than those observed for creatine kinase (Görrissen, H. and Marsh, D., unpublished). This is probably due, at least in part, to the more strongly basic nature of cytochrome *c* which has a pI of approximately 10 as opposed to the pI of approximately 8.5 for the creatine kinase (Cheneval, D. and Carafoli, E., unpublished).

In summary, the ESR spectra presented here offer clear indications for the binding of creatine kinase to cardiolipin liposomes. The effects of binding on the mobility of the cardiolipin lipid chain are relatively minor and are consistent with a surface association of the protein with the lipid headgroups, which indirectly affects the motion of the chains.

The results of the adriamycin experiments are in line with a recently proposed model for the interaction of adriamycin with cardiolipin-containing membranes [11]. The model proposes that the stacking of the anthracycline moieties prevents adriamycin from penetrating into the lipid bilayer but allows its electrostatic binding in a 2:1 ratio to cardiolipin, resulting in the clustering of the latter. The ESR experiments presented here confirm that adriamycin does not significantly penetrate into the lipid bilayer at least at the concentration used (100 nmol/mg lipid, at molar ratios of 7:1), since only relatively small changes in the hyperfine splittings were seen. Thus, binding of adriamycin to membrane cardiolipin appears to be of the same electrostatic nature as that of creatine kinase. This conveniently rationalizes the competition of the two molecules for cardiolipin binding.

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