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Association of spin-labelled cardiolipin with dimyristoylphosphatidylcholine-substituted bovine heart cytochrome *c* oxidase. A generalized specificity increase rather than highly specific binding sites

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The endogeneous lipid of bovine heart cytochrome *c* oxidase has been replaced by dimyristoylphosphatidylcholine using cholate-mediated exchange. The lipid-substituted preparation contained less than 1 mole cardiolipin per mole enzyme and possessed full oxidative activity. The association of spin-labelled cardiolipin with such lipid-substituted cytochrome oxidase preparations has been assayed using ESR spectroscopy. An average relative association constant 5.4-times that for phosphatidylcholine is obtained for cardiolipin. Measurements on preparations with increasing contents of unlabelled cardiolipin, introduced during lipid exchange, reveal that this selectivity corresponds to a generalized increase in specificity for all lipid association sites on the protein.

Cardiolipin (diphosphatidylglycerol) is a unique four-chain phospholipid found at high abundance in the inner mitochondrial membrane. This acidic phospholipid has been found to co-purify with cytochrome *c* oxidase [1,2] and some evidence has also suggested that it was necessary for the activity of the bovine heart enzyme [3,4]. Previously we have demonstrated that it is possible to replace the endogenous lipids of yeast cytochrome oxidase (including cardiolipin) by dimyristoylphosphatidylcholine and still retain oxidative activity [5]. The question still remained open as to whether this would be possible for the bovine heart enzyme.

On the other hand spin-labelled cardiolipin has been shown to associate preferentially with both bovine heart and yeast cytochrome oxidase [6,7]. However, this preferential association could be due either to a generalized increase in cardiolipin selectivity for all 50 lipid association sites found on cytochrome oxidase [8,9], or to the existence of a smaller number of highly specific sites for cardiolipin [1,10].

In the present communication we show that the endogenous lipids of bovine heart cytochrome oxidase can be substituted by dimyristoylphosphatidylcholine in a manner similar to that used for the yeast enzyme, yielding a preparation which contains less than 1 mole of cardiolipin per mole of enzyme. Such preparations have then been used to investigate the site specificity of cytochrome oxidase using spin label ESR spectroscopy.

Cytochrome *c* oxidase was prepared from fresh bovine hearts [2] with modifications given in Ref. 6. Endogenous lipids were three times exchanged in cholate against a 1000-fold excess of dimyristoylphosphatidylcholine (DMPC; Fluka,

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Buchs) followed by dialysis according to Ref. 9, except that sucrose and KCl were omitted from the buffer. When required the appropriate amount of exogenous bovine heart cardiolipin (Sigma, Munich) was added to the DMPC after the third exchange. Enzyme activity was determined at 25°C in 40 mM phosphate buffer (pH 6.7) with 30 μM reduced cytochrome *c*, using a cholate dilution assay [9]. The molecular weight of the enzyme was taken as 200 000.

The amount of cardiolipin remaining in the exchanged preparation was estimated, after alkaline extraction, by thin-layer chromatography (TLC) and by gas-liquid chromatography (GLC) of the fatty acid methyl esters [5]. Bovine heart cardiolipin was found to contain 87% linoleate chains. The extraction efficiency was checked by digesting the protein residue with 6 M HCl at 120°C for 60 h, followed by phosphate and GLC analysis. A maximum of 0.3–0.4 mole cardiolipin per mole enzyme was unextracted.

Spin-labelled cardiolipin (1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-(2-(12-carboxydodecyl)-2-butyl-4,4-dimethyl-3-oxazolidinyloxy)-*sn*-glycero(3)-phospho]-*sn*-glycerol) was synthesized according to the method of Ref. 11. Spin labelling, ESR spectroscopy and spectral analysis were performed as described in Refs. 7 and 9.

Thin-layer chromatography of the lipid extracted from the three times DMPC-exchanged bovine heart cytochrome *c* oxidase (35 moles lipid phosphorus per mole enzyme) revealed only traces of cardiolipin and at least two other phospholipids than DMPC. Gas-liquid chromatography showed linoleate to be present at a level of 1.3% of the total fatty acids, corresponding to a maximum content of 0.3 mole cardiolipin per mole enzyme. Allowing for the unextracted lipid gives a total of $\leqslant 0.7$ mole residual cardiolipin per mole enzyme. In a separate experiment with a sample of lower lipid/protein ratio (14 mole lipid phosphorus per mole enzyme) no detectable linoleate was found by GLC. This degree of cardiolipin replacement is comparable to that obtained with the yeast enzyme [5], and compares very favourably with that previously obtained with the bovine heart enzyme [2,3]. The enzymatic activity, assayed in the presence of excess DMPC (1000 mol/mol enzyme), was 0.09 $min^{-1} \cdot mg^{-1}$ for the starting preparation and 0.10 $min^{-1} \cdot mg^{-1}$ for the three times lipid-exchanged enzyme. Thus, as for the yeast enzyme [5], DMPC-substituted bovine heart cytochrome *c* oxidase containing less than one mole of cardiolipin per mole of enzyme retains its oxidative activity.

The ESR spectra of cytochrome *c* oxidase-DMPC complexes reconstituted as above with varying amounts of exogenous cardiolipin added in the final reconstitution step are given in Fig. 1. The spectra consist of two components, one corresponding to normal fluid bilayer lipids and the other to more motionally restricted lipids interacting directly with the protein [6–9]. The ratio of fluid to motionally restricted spin-labelled lipids, n_f^*/n_b^*, is quantitated by difference spectroscopy. For the sample without added cardiolipin, the equilibrium equation for lipid-protein association is [9,12]:

$$n_f^*/n_b^* = (n_t/(N_1 K_r^{av})) - (1/K_r^{av}) \quad (1)$$

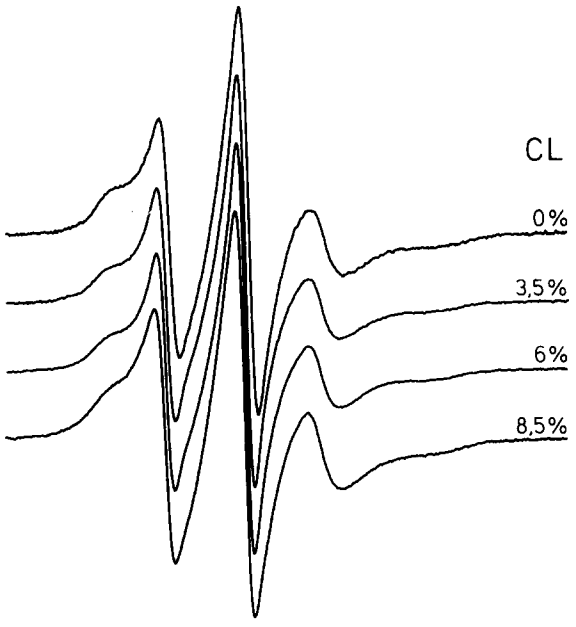

Fig. 1. ESR spectra of cardiolipin spin label in cytochrome *c* oxidase-DMPC complexes with increasing contents of unlabelled cardiolipin. The total lipid/protein ratio was maintained constant at 140 mole lipid phosphorus per mole protein, and mol% of cardiolipin is indicated. $T = 32$°C, total scan width = 100 gauss.

where n_t is the total lipid/protein mole ratio in the sample, N_1 is the total number of lipid association sites on the protein and K_r^{av} is the average association constant of the spin-labelled cardiolipin relative to DMPC *. Assuming $N_1 = 50$, as determined previously for cytochrome oxidase [7–9], yields a value of $K_r^{av} = 5.4$, the same as that obtained previously for yeast cytochrome oxidase [7].

By adding exogenous cardiolipin it is possible to test (by competition) whether this selectivity for cardiolipin corresponds to a generalized increase in specificity of all 50 lipid sites or to just a few sites with a much higher specificity. From Fig. 1 it is seen that the fraction of motionally restricted lipid does not vary greatly with increasing cardiolipin content, suggesting that there are no highly specific sites for cardiolipin on cytochrome oxidase. The results of spectral subtractions given in Fig. 2 are interpreted in terms of two classes of binding sites: n_1 specific sites of relative association constant, K_1, and n_2 non-specific sites with $K_2 = 1$, where $N_1 = n_1 + n_2$ and $K_r^{av} = (n_1K_1 + n_2K_2)/N_1$. The equilibrium association equation is [12]:

$$\frac{n_1K_1(n_f^*/n_b^*)}{(1+K_1(n_f^*/n_b^*))(1+(n_f^*/n_b^*))^{-1}n_t^* + n_t - N_1} + \frac{n_2K_2(n_f^*/n_b^*)}{(1+K_2(n_f^*/n_b^*))(1+(n_f^*/n_b^*))^{-1}n_t^* + n_t - N_1} = 1 \quad (2)$$

where n_t^* is the number of moles of added cardiolipin per mole of protein (determined by GLC analysis). Analytic solutions are plotted in terms of the fraction of protein-associated specific lipid: $f = (1 + (n_f^*/n_b^*))^{-1}$ for values of n_1 and K_1 consistent with $N_1 = 50$ and $K_r^{av} = 5.4$. Clearly the results are consistent with $n_1 \sim N_1$ and $K_1 \sim K_r^{av}$, demonstrating that the selectivity for cardiolipin arises from a generalized increase in specificity for nearly all sites on the protein. The application of Eqn. 2 assumes that there is no differential selectivity between spin-labelled and unlabelled cardiolipin, as shown to be the case for phosphatidylcholine [9,12]. Similar experiments to those of Fig. 2, but with increasing quantities of spin-labelled cardiolipin, rather than unlabelled cardiolipin, have yielded essentially identical conclusions, although the quantitation is more difficult because of spin-spin broadening at the higher spin label concentrations.

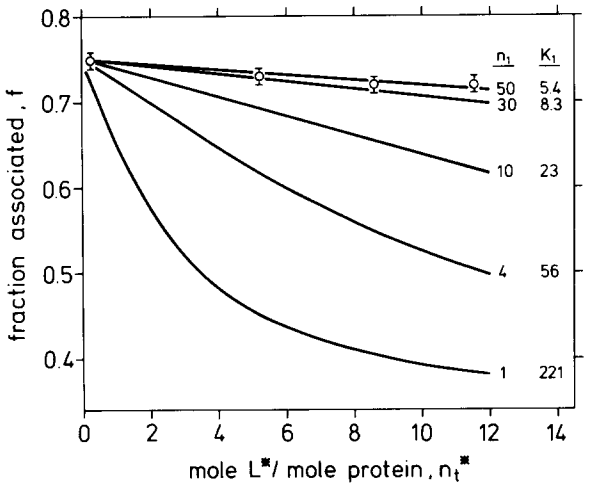

Fig. 2. Fraction, f, of motionally restricted cardiolipin spin label (O) in cytochrome c oxidase/DMPC as a function of cardiolipin content, n_t^*. The solid lines give the calculated values obtained from Eqn. 2 with $n_t = 140$, $N_1 = 50$, $K_r^{av} = 5.4$ and $K_2 = 1$ for various values of n_1 (and K_1).

In conclusion it is demonstrated that cardiolipin is not essential for the oxidative activity of bovine heart cytochrome c oxidase, and that there are no highly specific binding sites for spin-labelled cardiolipin on the enzyme. The generalized increase in specificity of the protein for cardiolipin over phosphatidylcholine most probably explains why it is preferentially concentrated in delipidated preparations and may also account for at least part of its enhanced effectiveness in restoring activity [1,2]. Finally, we cannot exclude that

* The model used in Eqns. 1 and 2 assumes a fixed number of lipid sites per protein independent of lipid composition and total lipid/protein ratio. It is thus independent of the degree of oligomerization or of aggregation of the protein, provided that the latter does not change with lipid/protein ratio. If N_1 is not constant with lipid/protein ratio, the formulation can still be used to compare degrees of association at a fixed lipid/protein ratio. However, experiments have shown that the stoichiometry of motionally restricted lipid sites does remain approximately constant, independent of lipid/protein ratio in the range applicable to the data of Fig. 2 [7–9].

cardiolipin is essential for the full, coupled activity of the enzyme.

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