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Apocytochrome *c* Binding to Negatively Charged Lipid Dispersions Studied by Spin-Label Electron Spin Resonance†

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ABSTRACT: The interaction of apocytochrome *c* with aqueous dispersions of phosphatidylserine from bovine spinal cord and with other negatively charged phospholipids has been studied as a function of pH and salt concentration by using spin-label electron spin resonance (ESR) spectroscopy and chemical binding assays. The ESR spectra of phospholipids spin-labeled at different positions on the *sn*-2 chain indicate a generalized decrease in mobility of the lipids, while the characteristic flexibility gradient toward the terminal methyl end of the chain is maintained, on binding of apocytochrome *c* to phosphatidylserine dispersions. This perturbation of the bulk lipid mobility or ordering is considerably greater than that observed on binding of cytochrome *c*. In addition, a second, more motionally restricted, lipid component is observed with lipids labeled close to the terminal methyl ends of the chains. This second component is not observed on binding of cytochrome *c* and can be taken as direct evidence for penetration of apocytochrome *c* into the lipid bilayer. It is less strongly motionally restricted than similar spectral components observed with integral membrane proteins and displays a steep flexibility gradient. The proportion of this second component increases with increasing protein-to-lipid ratio, but the stoichiometry per protein bound decreases from 4.5 lipids per 12 000-dalton protein at low protein contents to 2 lipids per protein at saturating amounts of protein. Apocytochrome *c* binding to phosphatidylserine dispersions decreases with increasing salt concentration from a saturation value corresponding to approximately 5 lipids per protein in the absence of salt to practically zero at 0.4 M NaCl. The perturbation of the spin-label mobility exhibits a parallel dependence on salt concentration. Binding of apocytochrome *c* to dimyristoylphosphatidylglycerol dispersions remains constant between pH 8 and pH 5.5, but then the number of lipids per protein bound increases by a factor of 2 on further decrease to pH 4. The overall spin-label mobility is little affected over the range pH 10-5.5 and exhibits a slight increase at lower pH. The number of motionally restricted spin-labeled lipids remains constant down to pH 5.5 and then increases abruptly. A comparison of the relative effects of apocytochrome *c* and cytochrome *c* binding in the presence and absence of salt yields very similar results with dimyristoylphosphatidylglycerol and beef heart cardiolipin to those obtained with phosphatidylserine. In particular, the maximum binding at a particular salt concentration is greater for the precursor apocytochrome *c* than for the mature protein cytochrome *c*.

The majority of mitochondrial proteins are synthesized on free ribosomes in the form of precursors which are subsequently imported into the organelle by a posttranslational transport step (Hay et al., 1984). Depending on the final destination of the protein, either insertion or translocation across one or two membranes has to occur. During this process the precursor (apoprotein) is converted into the mature holoprotein.

Apocytochrome *c*, the heme-free precursor of cytochrome *c*, has been extensively used to study the molecular details of posttranslational protein transport. Both the apoprotein and

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holoprotein have identical polypeptide chains, but they differ in secondary and tertiary structure. Cytochrome *c* is a highly structured, nearly spherical protein, whereas the apo form has virtually no structure (Fisher et al., 1973). After synthesis on free polysomes, apocytochrome *c* binds to the mitochondrial outer membrane, probably involving a specific receptor (Hennig et al., 1983). During transport across this membrane the heme group is attached (Hennig & Neupert, 1981), whereafter the cytochrome *c* reaches its final location at the outside of the inner mitochondrial membrane (Capaldi, 1982).

The molecular details of the actual insertion and translocation steps are obscure. Conceptually, it is difficult to imagine how these processes can proceed by solely involving protein type of structures without loss of vital membrane barrier function. The essential role for lipids in determining membrane permeability together with the potential of (local) regulation of lipid structure by lipid–protein interactions has led to suggestions that lipids are involved in protein insertion and translocation (de Kruijff et al., 1985; Nesmeyanova, 1982). Due to its basic character (9 net positive charges at neutral pH) apocytochrome *c* binds strongly and specifically to model membranes containing negatively charged lipids (Rietveld et al., 1983). The initial interaction is primarily electrostatic, but differential scanning calorimetry experiments (Rietveld et al., 1983, 1985) and changes in the fluorescence properties of the single tryptophan residue at position 59 (Rietveld et al., 1985) suggest a subsequent penetration of the protein into the hydrophobic core of the bilayer. Interestingly, when added to large unilamellar vesicles composed of negatively charged lipids, the apocytochrome *c* is able spontaneously to get across the lipid bilayer, as judged from its degradation by enclosed trypsin (Rietveld & de Kruijff, 1984; Dumont & Richards, 1984).

To get further insight into the nature of the apocytochrome *c*–lipid interaction, we have studied the effect of the protein on the motional properties of spin-labeled phospholipids in model membrane systems by using electron spin resonance (ESR).[1] With this technique it has been possible to get detailed information on the dynamics of lipids surrounding integral membrane proteins (Marsh, 1983, 1985). It will be shown that the interaction between apocytochrome *c* and negatively charged lipids results in motional restriction of the lipids, to a certain extent resembling integral membrane protein–lipid interactions, in accordance with a deep penetration of the protein into the lipid bilayer.

Materials and Methods

Materials. Apocytochrome *c* was prepared from horse heart cytochrome *c* (type VI, Sigma, St. Louis, MO) as described by Fisher et al. (1973) and then subjected to a renaturation procedure as previously described (Hennig & Neupert, 1983). The protein ran as a single band on a 15% polyacrylamide gel, and the amount of cytochrome *c* in the final preparation was less than 1%. The apocytochrome *c* concentration was determined by using a protein assay according to Lowry et al. (1951) with either apocytochrome *c* or BSA as a standard, and the protein was stored in 1-mL aliquots at a concentration of about 1.5 mg/mL at −20 °C in 10 mM Tris, pH 7.0, containing 0.01% mercaptoethanol either with or without 100 mM NaCl. The protein was always used immediately after thawing. For the pH titration the apocytochrome *c* was dialyzed after the renaturation procedure against 10 mM buffer solutions of Tris, PIPES, citric acid, or sodium borate, containing 0.01% mercaptoethanol. The pH was adjusted both before and after adding the protein to the lipid. Dimyristoylphosphatidylglycerol was prepared from dimyristoylphosphatidylcholine (Fluka, Buchs, Switzerland) by using transphosphatidylation catalyzed by phospholipase D (Comfurius & Zwaal, 1977). Bovine spinal cord phosphatidylserine was from Lipid Products, South Nutfield, U.K., and beef heart cardiolipin (diphosphatidylglycerol) was from Sigma, St. Louis, MO. Phosphatidylglycerols, *n*-PGSL,[1] spin-labeled at different positions on the *sn*-2 chain, and phosphatidylserine, 14-PSSL, spin-labeled on the C-14 atom of the *sn*-2 chain, were synthesized essentially as described in Marsh and Watts (1982).

Sample Preparation. A dry film of approximately 1 mg of lipid (containing 1% of spin-labeled lipid) was hydrated with buffer or with apocytochrome *c* solution in buffer, with salt added where required. To saturate all lipid binding sites, 4–6 mg of apocytochrome *c* was added to 1 mg of lipid. The mixtures were incubated for 30 min at 30 °C, or at 35 °C in the case of DMPG. The lipid–protein complex was centrifuged at room temperature (20 min, 3000*g*) and transferred to a sealed-off 100-μL capillary. The complex was further concentrated in the capillary by centrifugation at room temperature (20 min, 3000*g*) before ESR measurements, which were carried out at 30 °C. The exact lipid-protein ratio was afterwards determined by solving the complex in 1 drop of 1 N NaOH, followed by phosphate analysis according to Eibl and Lands (1969) and protein determination.

ESR Spectroscopy. ESR spectra were recorded on a Varian E-12 Century Line spectrometer with nitrogen gas flow temperature regulation. Samples were contained in 1 mm OD glass capillaries accommodated within a standard 4-mm quartz ESR tube, which contained silicon oil for thermal stability. Temperature was measured with a fine thermocouple situated in the silicon oil just above the top of the ESR cavity. ESR spectra were recorded at a nominal microwave power of 10 mW and a microwave frequency of 9.11 GHz, with a field sweep of 100 G centered at 3.24 kG and a 100-kHz field modulation amplitude of 0.8–1.25 G, depending on the spectral line widths. Spectra were digitized by using a Digital Equipment Corp. LPS system and dedicated PDP 11/10 computer with VT-11 display. Spectral subtraction and quantitation were performed with interactive graphics using software written by Dr. W. Möller of the Max-Planck-Institut. Further details of subtraction strategies and spectroscopic methods are given in Marsh (1982).

Results

The ESR spectra of various positional isomers of the phosphatidylglycerol spin-label in bovine phosphatidylserine dispersions in the presence and absence of saturating concentrations of apocytochrome *c* are given in Figure 1. The spectra are recorded at a temperature above the gel–fluid phase transition temperature of the lipid bilayer, as evidenced by the characteristic axially symmetric, averaged spectra [cf. Marsh (1982)]. Binding of apocytochrome *c* abolishes any chain-melting phase transition for the lipid–protein complex (Rietveld et al., 1983, 1985). For all positions of the spin-label group on the *sn*-2 phosphatidylglycerol chain it is seen that the interaction with apocytochrome *c* gives rise to spectra that both are broader and have larger splittings than the spectra for the lipid alone. This corresponds to a restriction of the motion

[1] Abbreviations: *n*-PGSL, 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoglycerol; 14-PSSL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoserine; PS, bovine spinal cord phosphatidylserine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; CL, beef heart diphosphatidylglycerol; ESR, electron spin resonance; BSA, bovine serum albumin; PIPES, 1,4-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

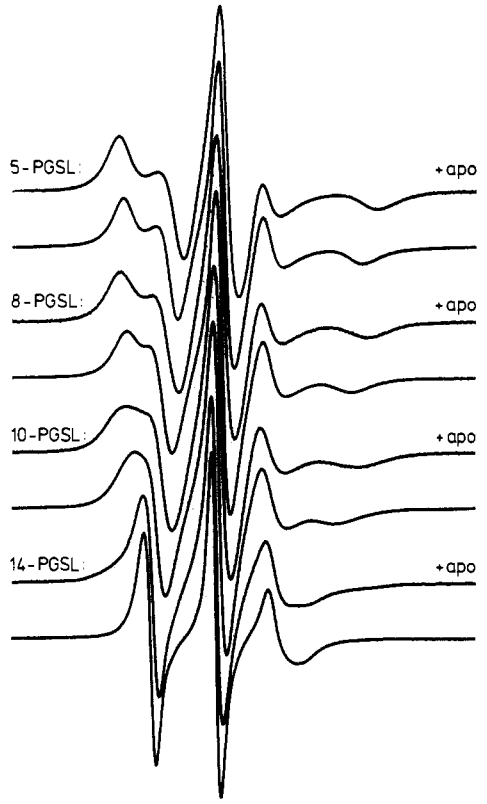

FIGURE 1: ESR spectra of phosphatidylglycerol spin-label positional isomers *n*-PGSL in bovine phosphatidylserine dispersions/0.1 M NaCl, 10 mM Tris, and 0.1 mM EDTA, pH 7.0, in the presence and absence of apocytochrome *c*. The upper spectrum of each pair (+apo) is with a saturating amount of apocytochrome *c* bound (4:1 w/w added apocytochrome *c*), and the lower spectrum of each pair is from the lipid alone. Total scan width = 100 G; T = 30 °C.

of the spin-labeled chains in the presence of the protein. The increase in spectral splittings is much greater than that observed on binding of cytochrome *c* (H. Görrissen and D. Marsh, unpublished results; see Figure 7 below). For those labels that are situated close to the terminal methyl end of the lipid chain and give rise to a narrow spectrum in the lipids alone, a second broader spectral component is observed in the outer wings of the narrow component. This resembles to some extent the induction of a motionally restricted spin-label spectral component by large integral proteins [see, e.g., Marsh and Watts (1982)], although there are important differences that will be described below. No such second motionally restricted lipid component is seen, resulting from the interaction of cytochrome *c* with negatively charged lipid dispersions (Görrissen and Marsh, unpublished results).

The two-component nature of the spectra in the presence of apocytochrome *c* is seen very clearly for the 12-PGSL spin-label in Figure 2a. The fluid component from the lipid–protein complex at 30 °C very closely resembles that obtained from the lipid alone at 22 °C (Figure 2c). Spectral subtraction of the spectrum of Figure 2c from that of Figure 2a yields the motionally restricted spectral component of the lipid–protein complex (Figure 2b).[2] The difference spectrum,

[2] The component for subtraction is chosen to be the spectrum of the pure lipid recorded at a temperature which gives best correspondence with the fluid component in the composite spectrum (Figure 2a). The criterion of good fit is then further assessed by the quality of the difference spectrum (Figure 2b) which must correspond to a single-component spectrum of reduced mobility. Subtraction from a single-component lipid spectrum, e.g., by using a similar spectrum at a different temperature, does not yield a realistic single-component end point as in Figure 2b.

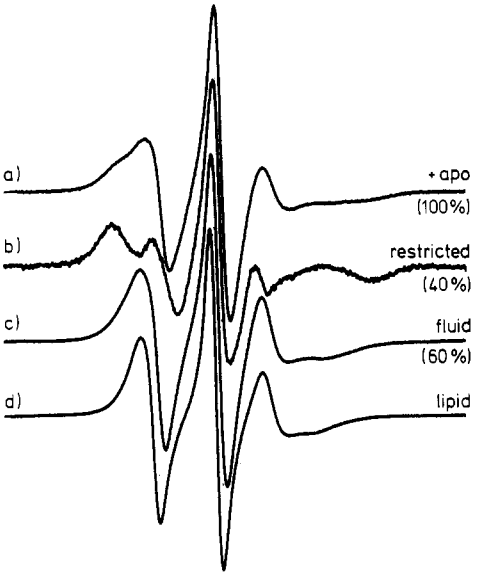

FIGURE 2: Spectral subtraction of the composite ESR spectrum of the 12-PGSL spin-label in bovine PS dispersions/10 mM Tris and 0.1 mM EDTA, pH 7.0, in the presence of a saturating amount of apocytochrome *c*: (a) 12-PGSL in bovine PS + apocytochrome *c*, 30 °C; (b) difference spectrum obtained by subtracting 60% from the double-integrated spectral intensity of composite spectrum a, using spectrum c for subtraction; (c) fluid spectral component used for the subtraction from composite spectrum a (12-PGSL in bovine PS/0.1 M NaCl, 10 mM Tris, and 0.1 mM EDTA, pH 7.0 at 22 °C); (d) 12-PGSL in bovine PS alone, 30 °C. Total scan width = 100 G.

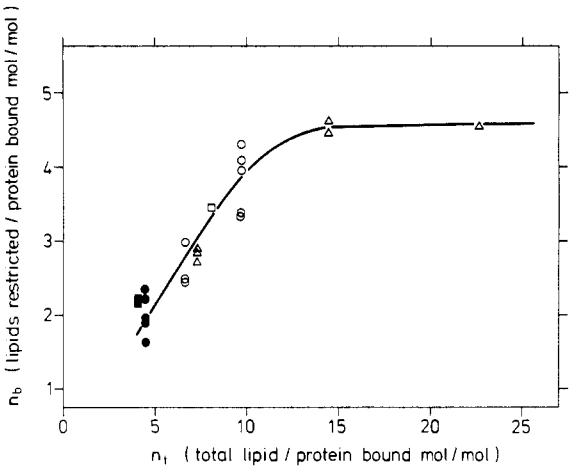

FIGURE 3: Number of motionally restricted spin-labeled lipids per protein, n_b, as a function of total number of PS lipid molecules per apocytochrome *c* molecule bound, n_t, in 10 mM Tris and 0.1 mM EDTA, pH 7.0 (filled symbols) and with 0.1 M NaCl (open symbols): (○ and ●) 12-PGSL label; (□ and ■) 14-PGSL; (△ and ▲) 14-PSSL.

Figure 2b, is a typical single-component spectrum, comparable to that observed, for example, with the 5-PGSL label in DMPG at 22 °C (spectrum not shown), and therefore corresponds to a considerably higher degree of motional restriction than that for the fluid component. The outer hyperfine splitting (56 G) is, however, appreciably smaller than that found for rigidly immobilized spin-labels (ca. 65 G) or for the motionally restricted component found with typical integral membrane proteins [ca. 60 G; see, eg, Marsh and Watts (1982)]. The degree of motional restriction of the fluid component is seen by comparison of Figure 2c (which matches the fluid component of the lipid–protein complex at 30 °C) with the spectrum of the lipids alone at 30 °C, which is given in Figure 2d. A clear increase in spectral anisotropy and line widths is observed.

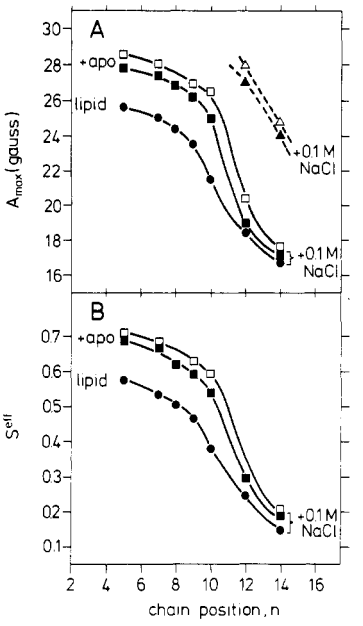

FIGURE 4: (A) Maximum hyperfine splitting, A_{max}, and (B) effective order parameter, S^{eff}, at 30 °C as a function of nitroxide position, n, in the chain for the n-PGSL phosphatidylglycerol spin-label isomers in bovine PS dispersions (10 mM Tris and 0.1 mM EDTA, pH 7.0): (□) in the presence of 5:1 w/w added apocytochrome c; (■) in the presence of 4:1 w/w added apocytochrome c and 0.1 M NaCl; (●) in the absence of apocytochrome c; (△) maximum hyperfine splitting of the motionally restricted spin-label component induced by apocytochrome c (open/filled symbols in the absence/presence of 0.1 M NaCl).

The spectral subtraction also yields the relative proportions of the fluid and motionally restricted components in the spectra of the lipid–protein complexes. In the example in Figure 2 the motionally restricted component constitutes 40% of the total integrated spectral intensity. The effective number of motionally restricted spin-labeled lipids, n_b, is given as a function of the total number of lipids per protein bound, n_t, by $n_b = fn_t$, where f is the fraction of motionally restricted component, and is shown in Figure 3. In this figure the data from spectral subtractions with different spin-labels are combined and also are given for samples with and without salt, the degree of protein binding being greater for the latter. The different data points for a given lipid/protein ratio correspond to different trial fluid components in the spectral subtraction and give an estimate of the reliability of the subtraction end points. The data for the different spin-labeled lipids lie on the same curve, suggesting that they reflect the true behavior of the unlabeled host lipids. At high lipid/protein ratios the number of motionally restricted lipids reaches an approximately constant value of 4.5 lipids/protein. As the lipid/protein ratio decreases (more protein bound), this number gradually decreases, reaching a limiting value of approximately 2 motionally restricted lipids/protein at the maximum binding of apocytochrome c (approximately 3:1 w/w protein/lipid; see Figure 5 below).

The spectral characteristics of the various lipid spin-label components are given in Figure 4. The parameters plotted are the outer maximum hyperfine splitting, $2A_{max}$, and the effective spin-label order parameter, S^{eff}, as a function of the position of the spin-label group on the phospholipid chain. The effective order parameter is defined by

$$S^{eff} = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})} \frac{a_0'}{a_0} \quad (1)$$

where $A_{\parallel}$ is identified with A_{max} and $A_{\perp}$ is obtained from the inner, minimum hyperfine splitting, $2A_{min}$ (cf. Figure 1) according to

$$A_{\perp}\,(G) = A_{min}\,(G) + 1.4\left[1 - \frac{A_{\parallel} - A_{min}}{A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})}\right] \quad (2)$$

The effective isotropic hyperfine splitting constant is given by $a_0 = \frac{1}{3}(A_{\parallel} + 2A_{\perp})$, 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 (Jost et al., 1971) is given by $a_0' = \frac{1}{3}(A_{xx} + A_{yy} + A_{zz})$. Recently, detailed line shape simulations have shown that the spectra such as those in Figure 1 contain important contributions from slow molecular motions (Lange et al., 1985). Thus the order parameter calculated by using eq 1, which assumes fast molecular motion, can only be considered as an effective value but is nevertheless useful for making intercomparisons between different spin-label positional isomers and between lipid environments in the presence and absence of protein. Under these conditions A_{max} is a useful empirical parameter which contains contributions from both the amplitude and rate of motion and is also influenced by the polarity of the spin-label environment. Similar dependences on label position and on the effect of protein for both A_{max} and S^{eff} are seen in Figure 4. The values of A_{max} and S^{eff} decrease as the spin-label position is stepped down the chain from locations close to the polar–apolar interface to positions near to the terminal methyl ends of the chains. This "flexibility gradient" is a characteristic signature of chain-labeled phospholipids in fluid lipid environments (R. D. Pates, A. Watts, and D. Marsh, unpublished results; Hardman, 1982; J. M. Seddon, H. Eibl, J.-H. Sachse, A. Watts, and D. Marsh, unpublished results). A considerable increase in both S^{eff} and A_{max} is seen in the presence of saturating amounts of apocytochrome c at all positions down the phospholipid chain. The effect is greater in the absence of salt than in the presence of salt, since in the former case the amount of protein bound is greater (see below). The effect is also greater at positions closer to the polar–apolar interface than at positions close to the terminal methyl ends of the chains, although it must be remembered that in the former case there is probably an unresolved motionally restricted spectrum underlying the fluid component spectrum.[3] For the 12- and 14-PGSL spin-label positional isomers the motionally restricted component is clearly resolved, and as seen from Figure 4A, this has a much greater splitting than the fluid component and also shows a strong dependence on the spin-label position in the chain. The latter is in contrast to the properties of the motionally restricted component observed with integral membrane proteins (Marsh & Barrantes, 1978; R. D. Pates and D. Marsh, unpublished results) which, being near to the limit of motional sensitivity, displays only a small dependence on spin-label position.

The dependence of the apocytochrome c binding, and concomitantly of the spectral parameters S^{eff} and A_{max} for the 5-PGSL spin-label, on added salt concentration is given in Figure 5. In all cases apocytochrome c was present in saturating quantities. The binding of apocytochrome c decreases steeply with increasing salt concentration, corresponding to a progressive screening of the phosphatidylserine surface charge by the counterions. Very little binding is observed for

[3] Extrapolation of the results for the motionally restricted component in Figure 4A suggests that this is most probably the case. Attempts at spectral subtraction to reveal a second component in the spectra of the 5- and 8-positional isomers were unsuccessful, possibly because the line splittings are very similar.

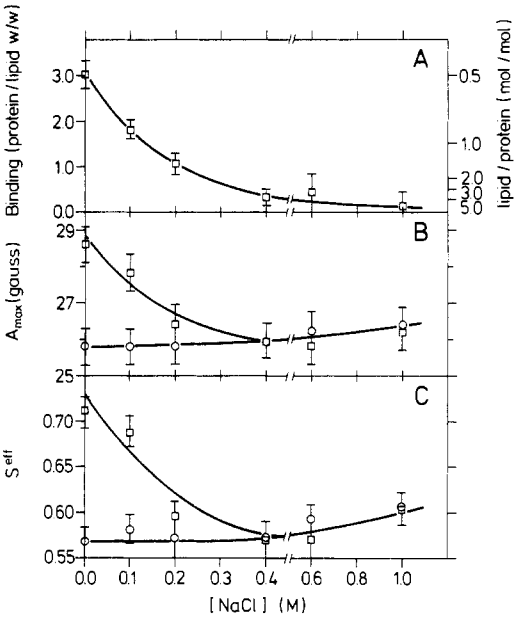

FIGURE 5: Salt dependence of (A) apocytochrome *c* binding, (B) outer hyperfine splitting, A_{max}, and (C) effective order parameter, S^{eff}, at 30 °C of the 5-PGSL spin-label in bovine PS dispersions (10 mM Tris and 0.1 mM EDTA, pH 7.0) in the presence (□) and in the absence (○) of a saturating amount (4–5:1 w/w protein/lipid) of added apocytochrome *c*.

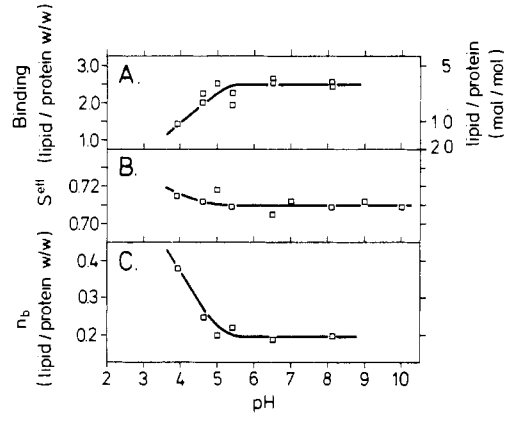

FIGURE 6: pH dependence of (A) apocytochrome *c* binding, (B) effective order parameter, S^{eff}, at 30 °C of the 5-PGSL spin label, (C) effective number of motionally restricted lipids, $n_b = fn_t$, deduced from the 12-PGSL spin-label, in DMPG dispersions (10 mM buffer, 0.01% β-mercaptoethanol) in the presence of 4:1 w/w added apocytochrome *c*.

salt concentrations greater than 0.4 M. The decrease in binding is paralleled by a corresponding decrease in perturbation of the lipid by the protein, as monitored by the spin-label parameters S^{eff} and A_{max}. At a NaCl concentration of 0.4 M both S^{eff} and A_{max} have decreased to the values observed for the lipid alone in the absence of protein. A slight increase is observed at higher salt concentrations, both in the presence and in the absence of protein, corresponding most probably to the onset of salt binding to the phospholipid headgroups [cf. Cevc et al. (1981)].

The pH dependence of binding of apocytochrome *c* to DMPG dispersions is given in Figure 6. Also included is the pH dependence of the effective order parameter of the 5-PGSL spin-label and the number of motionally restricted spin-labeled lipids determined from spectral subtractions with the 12-PGSL spin-label. The protein binding remains constant from pH ≳8 down to pH ≲6 and then drops rapidly from pH 5 to pH 4. DMPG has no titratable groups in this range; therefore, the

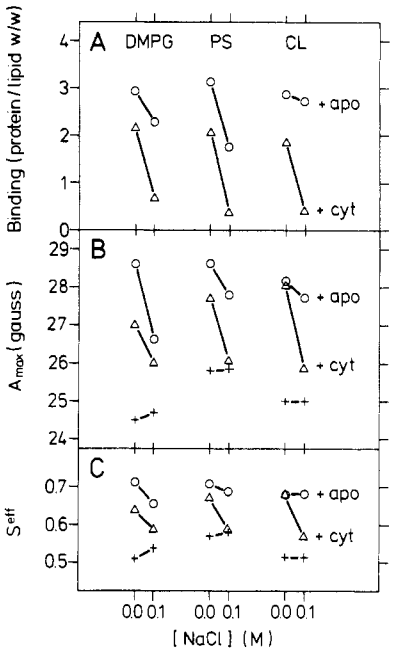

FIGURE 7: Interaction of apocytochrome *c* (○) and cytochrome *c* (△) with lipid dispersions of dimyristoylphosphatidylglycerol (DMPG), bovine brain phosphatidylserine (PS), and beef heart cardiolipin (CL), in the absence and in the presence of 0.1 M NaCl: (A) protein binding, (B) outer hyperfine splitting, A_{max}, and (C) effective order parameter, S^{eff}, at 30 °C of the 5-PGSL spin-label (10 mM Tris, 0.1 mM EDTA, and 0.01% β-mercaptoethanol, pH 8.0 for DMPG and pH 7.0 for PS and CL).

decrease in binding can be attributed to the titration of protein residues, most probably carboxylic amino acid side chains. The effective order parameter of the 5-PGSL spin-label remains approximately constant from pH 10 down to pH 5.5 with a possible small increase at pHs lower than this. The number of motionally restricted spin-labeled lipids remains constant down to approximately pH 5 and then increases abruptly on further lowering the pH.

The relative amounts of binding of cytochrome *c* and apocytochrome *c* to dispersions of three different negatively charged lipids, and the spectral perturbations of the 5-PGSL lipid spin-label induced on binding, are compared in Figure 7. Apocytochrome *c* binds to a greater extent than cytochrome *c* to all three lipids, both in the absence and in the presence of 0.1 M salt. In all cases the addition of salt leads to a reduction in protein binding in agreement with the results of Figure 5. To a great extent the values of the spectral parameters S^{eff} and A_{max} parallel the degree of binding. Both apocytochrome *c* binding and cytochrome *c* binding cause an increase in S^{eff} and A_{max}, the increase being somewhat greater for apocytochrome *c* than for cytochrome *c*. The one exception to the latter is the effect of cytochrome *c* binding to cardiolipin in the absence of salt, but in this case it is known that cytochrome *c* binding gives rise to partial formation of an inverted hexagonal (H_{II}) phase (de Kruijff & Cullis, 1980). It should also be remembered that the ESR spectral parameters are temperature-dependent, and temperatures have been chosen for the three different lipids so as to yield comparable values in the presence of protein.

Discussion

In this work the mode of binding of apocytochrome *c* to negatively charged lipid bilayers has been studied via the effects on the ESR spectrum of anionic phospholipid spin-labels incorporated into the bilayer, and via the salt and pH dependence of the binding. Comparison with the corresponding

data obtained with cytochrome *c* (Görrissen and Marsh, unpublished results) also provides valuable insights into the mechanism of binding.

Electrostatic interaction with the lipid surface charge constitutes the major and obligatory component of the binding, since the protein can be totally displaced by high concentrations of salt (Figure 5). It was previously found that the protein does not bind to phosphatidylcholine bilayers which bear no net surface charge (Rietveld et al., 1983). Supportive evidence for an electrostatic interaction also comes from the pH dependence: as the protein positive charge is increased on titration below pH 5, the number of lipids associated with each protein abruptly increases (Figure 6A). However, in contrast to the binding of the holoprotein, the binding of apocytochrome *c* most probably also has an additional hydrophobic component, since the protein partially penetrates into the apolar region of the lipid bilayer (see later discussion).

At neutral pH and in the absence of salt, saturation binding of apocytochrome *c* is obtained at a level of approximately 1 protein per 7 phospholipids. Although this represents a significantly greater extent of binding than found for cytochrome *c*, it is nonetheless of the same order of magnitude (Figure 7). The latter suggests that the random coil apocytochrome *c* assumes a more compact structure, characteristic of the native cytochrome, on binding to the negatively charged lipid. Evidence for such an increase in structure of the protein is provided by recent circular dichroism experiments (Rietveld et al., 1985).

On the other hand, very important differences in the mode of binding of the two proteins are indicated by the spin-label measurements. In general terms, the overall perturbation of the lipid mobility on protein binding is greater for apocytochrome *c* than for cytochrome *c*. This is true for a variety of negatively charged lipids (Figure 7) and reflects the fact that the charges on the more flexible apoprotein can more readily adapt to the charge distribution on the lipid surface than those of the holoprotein. A yet more significant difference is the appearance of a second, more motionally restricted spectral component for lipids labeled close to the terminal methyl ends of the chains. Such an effect is not observed with cytochrome *c* itself and provides strongly suggestive evidence for penetration of the apoprotein into the lipid bilayer.

The spectral subtraction presented in Figure 2 is similar in principle to those obtained in spin-label studies with membrane-spanning integral proteins [see, e.g., Marsh (1985)]. Taken alone, Figure 2 may not constitute unequivocal proof for the existence of two distinct spectral components. It is perhaps possible that a somewhat similar spectrum could be generated with a single-component model involving slow-motional components. However, the agreement between the results with the 12-PGSL spin-label (Figure 2) and those with the 14-PGSL positional isomer which has a quite different spectral line shape, taken together with the systematic variation with salt concentration and lipid/protein ratio (Figures 3 and 4), leaves little doubt that the spectra consist of two distinct lipid components. By analogy with the similar results obtained with integral proteins, the studies with apocytochrome *c* therefore provide a direct structural indication for a limited penetration of the apoprotein to at least the C-14 level of the hydrocarbon chains in the lipid bilayer.

The motionally restricted lipid component induced by apocytochrome *c* differs quantitatively from that seen with large integral membrane proteins (Marsh, 1985), however, in two ways. First, the degree of motional restriction evident from the spectra is less, and second, the number of lipids per protein associated with this component is considerably less. Both of these features are consistent with an only partial penetration of the apocytochrome into the hydrophobic region of the bilayer. At low lipid/protein ratios approximately 5 lipids per protein are motionally restricted. This closely corresponds with the number of lipids that could be arranged around a cylinder of 10-Å diameter that penetrates only half of the bilayer. (Such a structure corresponds approximately to the dimensions of a single α-helix.) Other models for the protein penetration are of course also possible. At high protein/lipid ratios the number of associated lipids per protein decreases, presumably corresponding to aggregation of the penetrant section of the protein within the bilayer. Aggregation of the protein upon integration with negatively charged lipids has previously been found to occur and can result in chemical cross-linking via cysteine residues (Rietveld et al., 1986).

As already mentioned, other lines of experimental evidence also suggest that apocytochrome *c* penetrates the lipid bilayer. Both monolayer studies and tryptophan fluorescence measurements have led to similar conclusions (Rietveld et al., 1985). The fluorescence studies suggest that the tryptophan of apocytochrome *c* is located in the glycerol backbone region of the lipid bilayer, whereas the spin-label measurements suggest penetration of (part of) the protein to the center of the bilayer. Yet further studies, involving accessibility to encapsulated trypsin, have demonstrated that apocytochrome *c* can (transiently) penetrate directly across the bilayer (Rietveld & de Kruijff, 1984). Clearly such studies could have very important implications for the mechanism of import of apocytochrome *c* into the mitochondrion, although it will be noted that the ionic conditions and the membrane lipid composition are far more complicated in the physiological situation than in the simplified model system studied here.

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NADPH Binding Induced Proton Ionization as a Cause of Nonlinear Heat Capacity Changes in Glutamate Dehydrogenase†

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ABSTRACT: Functional group interactions involved in the formation of the glutamate dehydrogenase–NADPH binary complex have been studied by three independent but complementary approaches: (1) the pH dependence of the overall dissociation constant measured by an improved differential spectroscopic technique; (2) the pH dependence of the enthalpy of complex formation measured by flow calorimetry; and (3) the pH dependence of the number of protons released to, or taken up from, the solvent in the complex formation reaction, measured by titration. We conclude that the coenzyme binds to the enzyme through three distinguishable interactions: a pH-independent process involving the binding of the reduced nicotinamide ring; a relatively weak "proton-stabilizing" process, occurring at low pH involving the shift at a pK of 6.3 in the free enzyme to 7.0 in the enzyme–NADPH complex; and a stronger "proton-destabilizing" process, occurring at a higher pH involving a shift of a pK of 8.5 in the enzyme down to 6.9 in the enzyme–NADPH complex. The proton ionization of the free enzyme involved in this third interaction exhibits some unusual thermodynamic parameters, having $\Delta G^\circ = +11.5 \pm 0.1$ kcal mol^{-1}, $\Delta H^\circ = +19 \pm 1$ kcal mol^{-1}, and $\Delta S^\circ = +23$ eu. We show here that this proton ionization step is directly related to and indeed constitutes the "implicit" shift in enzyme macrostates which we have shown to be responsible for the existence of large highly nonlinear ΔC_p° effects in the formation of this complex [Fisher, H. F., Colen, A. H., & Medary, R. T. (1981) *Nature (London) 292*, 271–272].

Most of the detailed chemical mechanisms currently written to explain the enzymatic catalysis of specific reactions feature the transfer of protons between the solvent and functional groups located on the various components of enzyme–substrate–(coenzyme complexes). Experimentally, this proton "traffic" expresses itself in the form of observed ligand-induced pK shifts of enzyme functional groups. Recent papers on the mechanisms of reactions catalyzed by L-glutamate dehydrogenase (Srinivasan & Fisher, 1984; Rife & Cleland, 1980) fit this description. In a previous report on calorimetric measurements of the enthalpies of formation of a ternary complex of L-glutamate dehydrogenase, we observed a complex pattern of just such proton transfer (Fisher et al., 1980). In order to determine the precise nature of these events in the active ternary complexes, and to explore their possible involvement in the catalytic process, it is first necessary to obtain a thorough understanding of protonic events in the enzyme–NADPH binary complex, whose formation precedes those of the higher order complexes. To this end, we have undertaken such a study using three complementary but independent experimental approaches: differential spectroscopy, calorimetry, and displaced-proton titration.

In an earlier study (Fisher et al., 1981), we had looked at the thermodynamics of the formation of this complex from a rather different point of view. The focus of that study was the unusual nonlinear temperature dependence of the enthalpy of the process. We concluded that the basis of the nonlinear ΔC_p° was the inclusion of a hidden step in the reaction course

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