J. Biochem. Mol. Biol. Vol. 29, No. 2 pp. 169~174 March 31, 1996

EPR Spectra of Spin-Labeled Cytochrome c Bound to Acidic Membranes: Implications for the Binding Site and Reversibility

Tongpil Min, Nan-hyang Park, Hee Young Park, Sun-Joo Hong and Sanghwa Han*

Department of Biochemistry, Kangwon National University, Chunchon 200-701, Korea
(Received January 5, 1995)

Abstract: Yeast cytochrome *c* (cyt *c*) was modified at cysteine-102 with a thiol-specific spin label and its interaction with liposomes containing acidic phospholipids was studied by electron paramagnetic resonance (EPR) spectroscopy. Association of cyt *c* with liposomes resulted in a significant reduction in the mobility of the spin label and a fraction of cyt *c* even seemed to be immobilized. Based on a large spectral change upon binding and the proximity of the spin-label to lysine-86 and -87, we propose these two residues to be the potential binding site at neutral pH. The interaction is electrostatic in nature because the spectral changes were reversed by addition of anions. Dissociation of the bound cyt *c* by anions, however, became less effective as the lipid/protein ratio increased. This suggests a repulsive lateral interaction among the bound cyt *c*. Unlabeled cyt *c* molecules added to preformed cyt *c*-liposome complex displaced the bound (spin labeled) cyt *c* and the process was competitive and reversible.
Key words: binding site, cytochrome c, EPR (electron paramagnetic resonance), membrane, spin label.

Located in the intermembrane space of mitochondria, cyt *c* is involved in energy transduction by shuttling electrons between cyt *c* reductase and cyt *c* oxidase. It contains a *c*-type heme covalently attached to two cysteinyl residues of the polypeptide chain. A methionine sulfur and a histidine nitrogen constitute the axial ligands for the heme iron.

Cyt *c* contains a number of lysine residues which, at physiological pH, confer a large positive charge on the protein. The inner membrane of a mitochondrion is rich in cardiolipin, a negatively charged phospholipid. Therefore cyt *c* is expected to interact with the membrane and is sometimes classified as a peripheral membrane protein despite its high solubility in water [see Pinheiro (1994) for a review]. The binding of cyt *c* to acidic membranes has been shown by a variety of spectroscopic techniques to induce conformational changes in the protein such as weakening of the heme-methionine bond (Hildebrand *et al.*, 1990; Spooner and Watts, 1991; 1992) and partial unfolding of the polypeptide chain (Heimburg and Marsh, 1993; de Jongh *et al.*, 1995). Recently in a series of work (Rytömaa *et al.*, 1992; Rytömaa and Kinnunen, 1994; 1995) based on the measurements of resonance energy transfer from a pyrene-labeled phospholipid to the heme of a bound cyt *c* molecule, Kinnunen and coworkers proposed a two-site model for the binding of cyt *c* to the membrane: an A-site interacts electrostatically with the membrane at neutral pH and a C-site participates in the binding at low pH forming hydrogen bonds with the protonated phosphate group of a phospholipid. Although the two sites are unequivocally distinguished by different behaviors upon addition of anions, information at the molecular level can be obtained by spectroscopic characterization of the different modes of binding.

Spin-label EPR techniques have been applied to the study of cyt *c*-membrane interaction (Vanderkooi *et al.*, 1973; Brown and Wüthrich, 1977b; Snel *et al.*, 1994a). Brown and Wüthrich (1977b) used horse heart cyt *c* spin-labeled at methionine-65 but the EPR spectrum of the spin label at that particular position was not sensitive enough to monitor structural changes resulting from the cyt *c*-membrane interaction. Yeast cyt *c* is another choice because it has a unique cysteine at position 102 that can be easily labeled by a thiol-specific reagent. In a recent report concerning the insertion of apocyt *c* into acidic membranes, Snel *et al.* (1994a) obtained a two-component EPR spectrum of the mem-

* To whom correspondence should be addressed.
Tel: 82-361-50-8516, Fax: 82-361-242-0459.

brane-bound yeast cyt *c*: a broader, immobile component and a narrower, mobile component. They attributed the former to a bound cyt *c* and the latter to an unbound cyt *c*. Although the chemical structure of their spin label is not the same as ours, both signals seemed to arise from the bound cyt *c* since we proved by an independent method that all the cyt *c* molecules were bound to the membrane.

In order to obtain structural information about the cyt *c*-membrane interaction, we obtained EPR spectra of spin labeled cyt *c* under various conditions. The EPR technique was sensitive enough to probe a few different states of the bound cyt *c* and a repulsive lateral interaction among the bound cyt *c* molecules. Since both the A-site (lysine-72 and -73) and the C-site (asparagine-52) proposed by the Kinnunen group (Ryt maa and Kinnunen, 1995) are far away from the spin label attached to cysteine-102, the binding at those sites is not expected to cause a significant change in the EPR spectrum of the spin label unless it accompanies a global perturbation of the protein structure. Therefore the EPR spectra of the spin-labeled yeast cyt *c* are expected to provide additional information about the model. Fortunately the binding induced a large spectral change that allowed us to propose lysine-86 and -87 for a possible binding site. Finally, the technique also provided clear-cut evidence that cyt *c* molecules compete for the binding site and that the process is reversible.

Materials and Methods

Materials

The phospholipids egg phosphatidylcholine (PC), dipalmitoyl phosphatidylglycerol (PG), and tetrastearoyl cardiolipin (CL) were purchased from Avanti Polar Lipids (Alabaster, AL). PG and CL with unsaturated fatty acid chains were avoided because oxygen-dependent degradation of the spin label occurred when liposomes were added to spin-labeled cyt *c* (Brown and Wüthrich, 1977b). (1-Oxyl-2,2',5,5'-tetramethylpyrroline-3-methyl)-methanethiosulfonate (MTSSL), a thiol-specific spin-label, was obtained from Reanal (Budapest, Hungary). All other chemicals including yeast cyt *c* were from Sigma (St. Louis, USA).

Preparation of spin-labeled cyt *c*

Thirty mg of yeast cyt *c* was dissolved in 5 ml of 10 mM potassium phosphate, pH 7.0 and treated with a 2-fold molar excess of dithiothreitol to dissociate any disulfide-bridged dimers. The solution was incubated for 1 h at room temperature under nitrogen and then excess dithiothreitol was removed by a small Sephadex G-15 column. A 1.5 fold molar excess of MTSSL (Millhauser, 1992) in ethanol (final ethanol concentration < 1%) was added to cyt *c* and the mixture was incubated for 2 h at room temperature to label the cysteine residue at position 102. Unreacted spin-label was removed by gel fitration on a Sephadex G-15 column.

Preparation of liposomes

Lipids (about 50 mg) of desired composition (1 mol cardilipin plus 8 mol egg PC for 10 mol% CL-containing liposomes and 2 mol dipalmitoyl PG plus 8 mol egg PC for 20 mol% PG-containing liposomes) were dissolved in chloroform or chloroform/methanol mixture, dried under a nitrogen stream and evacuated for 2 h. Resulting lipid film was hydrated in 1 ml of 20 mM 2-[N-morpholino]ethanesulfonic acid (MES) at pH 4 or 7, 0.1 mM EDTA at room temperature and subjected to 5 cycles of freezing and thawing. The suspension was then extruded through a polycarbonate filter (100 nm pore size) with a LiposoFast homogenizer (Avestin, Ottawa, Canada).

EPR spectroscopy

EPR measurements were performed at 25℃ on a Bruker ER-200 X-band EPR spectrometer. 30 μM of spin-labeled cyt *c* was added to a liposomal suspension and the mixture was transferred to a quartz flat cell. Spectral conditions: microwave frequency 9.76 GHz, attenuation 5 dB, modulation 100 kHz, total scan width 100 G.

Results

Yeast cyt *c* was chosen instead of horse cyt *c* because it has a free cysteine residue that can be specifically labeled by MTSSL. The position of the label is at cysteine-102 which is near the carboxy terminal (Lederer *et al.*, 1972). Optical absorption spectrum of the spin-labeled cyt *c* was identical to that of native protein. Furthermore, we were able to reproduce, with the spin labeled cyt *c*, most of the experiments performed by Kinnunen and corworkers (Rytömaa *et al.*, 1992; Rytömaa and Kinnunen, 1994). These confirm that attachment of the spin label does not significantly alter the conformation and the binding characteristics of cyt *c*.

A three-line spectrum of equal intensity was obtained for the spin label MTSSL in a buffer (data not shown). The EPR spectrum of spin-labeled cyt *c* in a buffer without liposomes indicated a motional restriction (Fig. 1a) compared to free MTSSL. Either pH (4 or 7) or ionic strength (up to 300 mM) hardly affected the EPR spectrum of unbound cyt *c*. Snel *et al.* (1994a) reported the EPR spectrum of yeast cyt *c* labeled at the same position by a maleimide spin label. As indicated

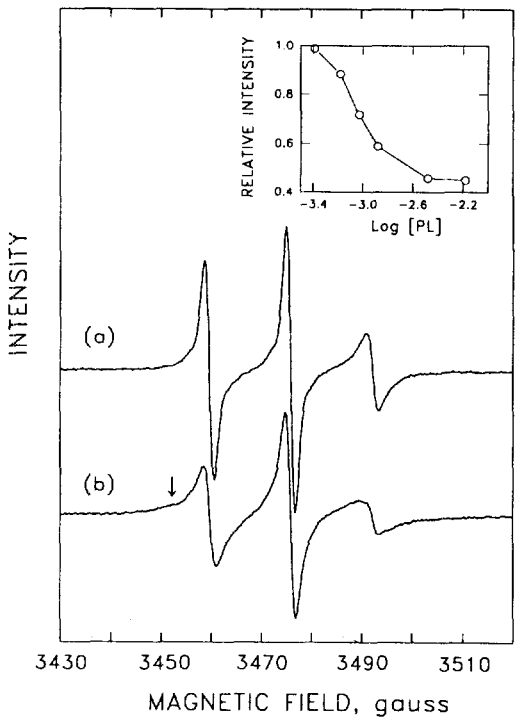

Fig. 1. The EPR spectra of spin-labeled yeast cyt *c* (30 μM) in the absence (a) and in the presence (b) of liposomes (2.2. mM in the phospholipid concentration) containing 10 mol% CL. Arrow indicates an immobilized signal. Spectral conditions: microwave frequency 9.76 GHz, power 64 mW, modulation 100 kHz. In the inset, the relative peak-to-valley height of the low-field line is plotted as a function of phospholipid concentration. The value for unbound cyt *c* was set to 1.0.

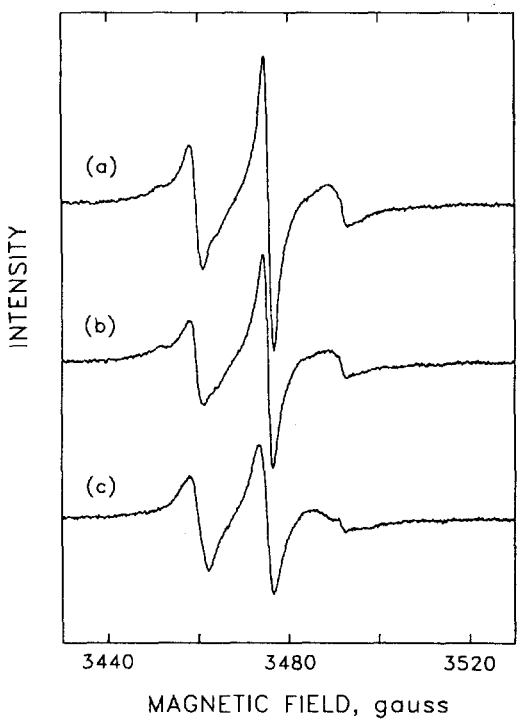

Fig. 2. The EPR spectra of cyt *c* bound to liposomes containing 10 mol% CL at pH 7 (a), pH 6 (b), and pH 5 (c). Other conditions same as Fig. 1.

by the intensity ratio of the low field line ($M_I = \pm 1$) and the high field line ($M_I = -1$), the maleimide spin label is motionally less restricted than the spin label used in this study.

EPR spectra of membrane-bound cyt *c*

The EPR spectrum of the spin-labeled cyt *c* in the presence of liposomes containing 100% PC (data not shown) was identical to that in the absence of liposomes (Fig. 1a), indicating that cyt *c* does not bind to neutral membranes. When cyt *c* was added to a large excess of liposomes containing acidic phospholipids at pH 7 (CL or PG), a significant motional restriction of the spin label was observed in the EPR spectrum (Fig. 1b). From the ratio of the low field line and high field line (Likhtenshtein, 1976), the rotational correlation times were estimated to be ~0.7 ns and ~2 ns, respectively, for the spin label on a free cyt *c* and on a membrane-bound cyt *c*. As shown in Fig. 2, the EPR spectrum was gradually converted into a broader one when pH of the cyt *c*-liposome complex was lowered. This probably corresponds to a transition from A-site binding to C-site binding. At pH 4, however, the sample contained heavy aggregates that were easily pelleted by centrifugation. The EPR spectrum of the supernatant was weaker but still the same as that before centrifugation. We do not know if aggregates were also formed in the fluorescence experiments (Rytömaa *et al.*, 1992; Rytömaa and Kinnunen, 1994; 1995) which were performed at much lower concentrations. In order to avoid the problem of aggregation at pH 4 and to obtain a result which is biologically more relevant, we studied in detail the binding of cyt *c* at neutral pH.

Effects of the cyt *c*/phospholipid ratio on the binding

Fig. 1b is the spectrum of 30 μM cyt *c* bound to 2.2 mM of total phospholipids. At this concentration of liposomes, all the cyt *c* molecules were bound to the liposomes since no cyt *c* was filtered through a membrane of molecular cut-off 100 kD. In the inset of Fig. 1, the peak-to-valley height of the low-field line was plotted as a function of phospholipid concentration. It can be seen from the plot that the first phase of spectral change was completed at a phospholipid concentration of ~2.2 mM for 30 μM of cyt *c*. This corresponds to a cyt cphospholipid ratio of ~1/70. The spectrum of bound cyt *c* contained a weak signal that can be attributed to immobilization of the spin label (see the arrow in Fig. 1b). Spectral subtraction for the estimation of the amount of immobilized species was not feasible because the EPR spectra of pure spe-

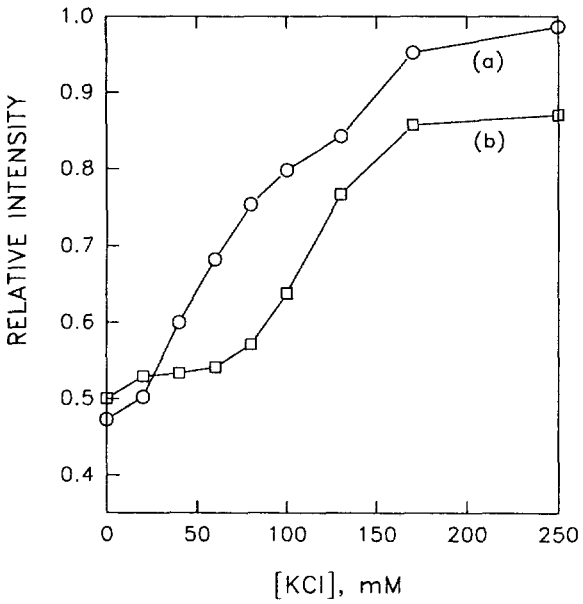

Fig. 3. The relative peak-to-valley height of the low-field line as a function of KCl concentration. The value for unbound cyt *c* was set to 1.0. For 30 μM of cyt *c*, phospholipid concentrations of the CL-containing liposomes were 2.2 mM (a) and 33 mM (b).

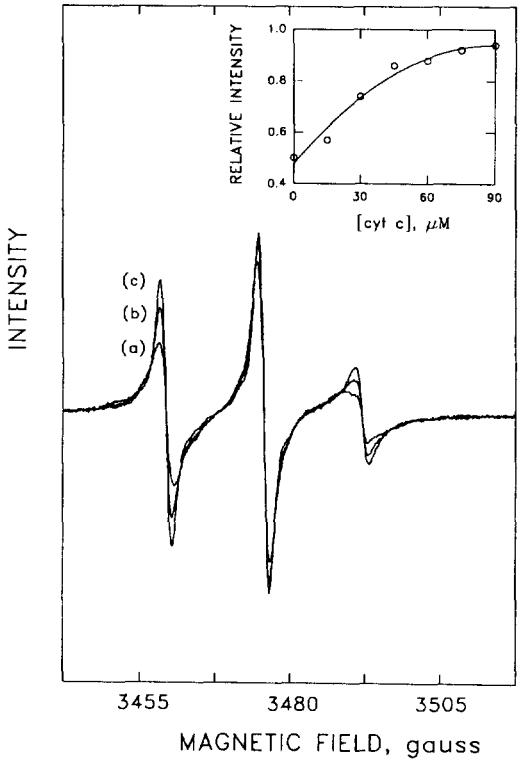

Fig. 4. Spectral changes due to dissociation of spin-labeled cyt *c* by unlabeled cyt *c* added afterwards. To a suspension of cyt *c*-liposome complex (30 μM cyt *c* and 2.2 mM phospholipids containing 10 mol% CL) was added 0 μM (a), 30 μM (b), and 90 μM (c) of unlabeled cyt *c*. Spectral conditions same as Fig. 1. In the inset, the relative peak-to-valley height of the low-field line was plotted as a function of added (unlabeled) cyt *c*.

cies were not available. Double integration of the spectra in Fig.1, however, showed that the intensity of each of the three lines was conserved. This means that the apparent decrease in the peak height was solely due to spectral broadening arising from a motional restriction.

A further change in the EPR spectrum was observed at a lower cyt cliposome ratio. The valley of the low-field line shifted slightly (~0.5 Gauss) to a higher field without a significant change in the overall intensity pattern. This small change was very reproducible in several independent measurements. Transition between the two types of EPR spectra was gradual and depended only on the cyt cphospholipid ratio. We interpret this small difference as a change in the surface coverage (see below for further evidence).

Effects of anions

When anions were present at high concentration to interfere with the electrostatic interaction between cyt *c* and liposomes, none of the spectral changes mentioned above were observed. Addition of anions to the preformed cyt *c*-liposome complex resulted in dissociation of cyt *c* from the membrane. Interestingly, anions dissociated the bound cyt *c* with different efficiency that depended on the cyt cphospholipid ratio. In Fig. 3, the peak-to-valley height of the low-field line was plotted as a function of [KCl] for the two cyt *c*/phospholipid ratios. Cyt c molecules bound to the liposomes with a high population density, i.e. a high cyt *c*/phospholipid ratio (Fig. 3a), were much easier to dissociate than those with a lower population density (Fig. 3b). This provide indirect evidence for a repulsive lateral interaction among the bound cyt *c* molecules.

Reversibility of the binding

Ryt maa and Kinnunen (1995) argued that the binding of cyt *c* to acidic membranes is not reversible so that the bound cyt *c* does not easily transfer to another liposome "molecule". Based on this observation, they proposed a unique model in which one of the two fatty acid chains of an acidic phospholipid is inserted into a hydrophobic crevice of the bound cyt *c*. Considering the importance of its biological implications, we tested reversibility of the binding by adding unlabeled cyt *c* to a suspension of spin-labeled cyt *c*-liposome complex. The EPR spectrum was monitored to see if any spin-labeled cyt *c* becomes unbound. The method is very powerful because an EPR spectrum can clearly distinguish the bound form from the unbound form. As mentioned above, we chose a cyt cphospholipid ratio of ~1/70 where all the available binding sites on the membrane were occupied by cyt

c. Since the low-field line becomes broad when cyt c binds to the membrane, increase in the peak height indicates dissociation of spin-labeled cyt c by unlabeled cyt c. As shown in Fig. 4, unlabeled cyt c added afterwards indeed displaced spin-labeled cyt c. In the inset of Fig. 4, a peak-to-valley height of the low-field line was plotted as a function of added cyt c concentration to follow dissociation of the bound cyt c. The EPR technique clearly demonstrates that the binding is competitive and reversible with respect to cyt c.

Discussion

Our data from a spin-label EPR study at various pH largely support the model proposed by the Kinnunen group and provide a spectroscopic method to identify different modes of binding. At pH 7 the mobility of the spin label is significantly reduced upon binding to a negatively charged membrane. There was no apparent difference in the spectrum of cyt c bound to CL-containing liposomes and PG-containing liposomes. The Soret and visible absorption of bound cyt c was identical to that of free cyt c. Therefore it is very likely that the structural changes due to charge interaction are confined to the interfacial region between cyt c and the surface of the membrane. The EPR spectrum of bound cyt c always contained a small immobilized signal. Snel *et al.* (1994a) attributed this immobilized signal to bound cyt c and the more mobile signal to unbound cyt c. Under the condition of full surface coverage, e.g. at a cyt cphospholipid ratio of 1/70, no cyt c was able to go through a membrane of pore size 100 nm. Therefore all the signals in our EPR spectrum arise from the bound cyt c. We tentatively assign the immobilized signal to a partial penetration of the spin-label into a hydrophobic membrane interior (Snel *et al.*, 1994b; Snel and Marsh, 1994).

The interaction between cyt c and acidic membranes is electrostatic and thus it is weakened by anions. The efficiency of anions in dissociating bound cyt c depended on the cyt cphospholipid ratio. At a lower surface coverage, it was more difficult for anions to detach cyt c from the membrane. We attribute this to a repulsive lateral interaction among the bound cyt c molecules which carry a large positive charge. Recently, Heimburg and Marsh (1995) addressed the importance of protein-protein interaction in the binding of peripheral proteins to charged lipid membranes. Ryt maa and Kinnunen (1995) found that the binding of cyt c to acidic membrane is irreversible as liposomes added to the preformed cyt c-membrane complex cannot easily dissociate bound cyt c. They invoked a model in which one of the two fatty acid chains of a phospholipid was accomodated in a hydrophobic interior of the bound cyt c, anchoring cyt c on to the membrane. We found in our experiment that unlabeled cyt c molecules readily remove the bound spin-labeled cyt c converting the EPR spectrum of bound cyt c to that of free cyt c. Our data clearly show that the binding is competitive among the cyt c molecules and is a reversible equilibrium phenomenon. Being aware of the fact that cardiolipin used in the present work has only saturated fatty acid chains, we repeated the Kinnunen's fluorescence measurements with our liposomes to find that saturation of the fatty acid chains does not make any difference. Irreversibility observed by the above authors may be explained by an extremely fast recombination that does not allow enough time for the transiently dissociated cyt c to reorient itself to bind to a new site in another liposome. The situation may be more feasible for a cyt c molecule than for a liposome to compete with the bound cyt c. Unlike the fluorescence measurement, the present technique, unfortunately, was not able to test reversibility with respect to added liposomes.

Since the spin-labeled cysteine-102 is almost on the opposite side of lysine-72 and -73 that were proposed for the A-site, it is a remote possibility for a perturbation originated at lysine-72 and -73 to propagate all the way to the carboxy terminal without causing major alterations in the heme absorption spectrum. In this respect, it is interesting that the spin-label attached to methionine-65, which is quite close to the proposed A-site and a little further away from cysteine-102, does not undergo a large spectral change upon binding. The EPR spectrum of yeast cyt c spin labeled at cysteine-102, however, clearly showed a large motional restriction caused by the binding of cyt c but the optical absorption spectrum did not. Combining the EPR and optical absorption data, we propose that lysine-86 and -87 are important in the cyt c-membrane interaction at pH 7. According to Rieder and Bosshard (1980), the lysines at 8, 13 (corresponding to 5, 11 in yeast cyt c), 86, and 87 are the most crucial residues in the binding of cyt c to cyt c oxidase and cyt c reductase. There is an α-helix extending from position 87 to the carboxy terminal where the spin label is attached. It is not surprising that the binding at lysine-86 and -87 moves the α-helix in such a way that the spin-labeled cysteine-102 is now in a sterically more hindered region. Methionine-80 is only 6 residues away from the binding site so that the heme iron-methionine bond can be disrupted. The α-helix at the amino terminal that contains lysine-5 and -11 is close to lysine-86 and -87. Therefore it is possible that these two sites together are involved in the binding. Interestingly these two a-helices form a structural motif that is conserved

in all the cyt *c* molecules from different species whose crystal structures are known.

In conclusion, the EPR spectrum of spin-labeled yeast cyt *c* clearly distinguished a bound state from an unbound state. Based on a large spectral change upon binding and the proximity of the spin-label to lysine-86 and -87, we propose these two residues (or together with the lysine residues in the α-helix of the amino terminal) to be the binding site at neutral pH. The results also indicated a possible repulsive interaction among the bound cyt *c* molecules. The binding was completely reversible in a sense that cyt *c* molecules added afterwards rapidly dissociate the bound molecules.

Acknowledgment

This work was supported by the grants from the Center for Molecular Catalysis (Korea Science and Engineering Foundation) and from Basic Science Research Institute Program (the Ministry of Education, Korea).

References

Brown, L. R. and Wüthrich, K. (1977a) *Biochim. Biophys. Acta* **468**, 356.

Brown, L. R. and Wüthrich, K. (1977b) *Biochim. Biophys. Acta* **468**, 389.

de Jongh, H. H. J., Ritsema, T. and Killian, J. A. (1995) *FEBS Lett.* **360**, 255.

Heimburg, T. and Marsh, D. (1993) *Biophys. J.* **65**, 2408.

Heimburg, T. and Marsh, D. (1995) *Biophys. J.* **68**, 536.

Hildebrandt, P., Heimburg, T. and Marsh, D. (1990) *Eur. Biophys. J.* **18**, 193.

Lederer, F., Simon, A. M. and Verdiere, J. (1972) *Biochem. Biophys. Res. Commun.* **47**, 55.

Likhtenshtein, G. I. (1976) in Spin Labeling Methods in Molecular Biology (translated by P. S. Shelnitz), Chapter 1, John Wiley and Sons, New York.

Millhauser, G. L. (1992) *Trends Biochem. Sci.* **17**, 448.

Pinheiro, T. J. T. (1994) *Biochimie* **76**, 489.

Rieder, R. and Bosshard, H. R. (1980) *J. Biol. Chem.* **255**, 4732.

Rytömaa, M., Mustonen, P. and Kinnunen, P. K. J. (1992) *J. Biol. Chem.* **267**, 22243.

Rytömaa, M. and Kinnunen, P. K. J. (1994) *J. Biol. Chem.* **269**, 1770.

Rytömaa, M. and Kinnunen, P. K. J. (1995) *J. Biol. Chem.* **270**, 3197.

Snel, M. M. E., de Kruijff, B. and Marsh, D. (1994a) *Biochemistry* **33**, 7146.

Snel, M. M. E., de Kruijff, B. and Marsh, D. (1994b) *Biochemistry* **33**, 11150.

Snel, M. M. E. and Marsh, D. (1994) *Biophys. J.* **67**, 737.

Spooner, P. J. R. and Watts, A. (1991) *Biochemistry* **30**, 3880.

Spooner, P. J. R. and Watts, A. (1992) *Biochemistry* **31**, 10129.

Vanderkooi, J., Erecinska, M. and Chance, B. (1973) *Arch. Biochem. Biophys.* **157**, 531.