THE EFFECTS OF SOME CATIONS AND ANIONS ON SPIN LABELED CYTOPLASMIC MEMBRANES OF BACILLUS SUBTILIS

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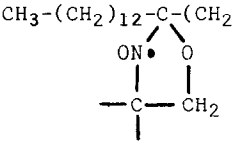
SUMMARY

The effects of $CaCl_2$, $MgCl_2$, $LaCl_3$ and some alkali halides on Bacillus subtilis cytoplasmic membranes were studied using stearic acid spin labels. The results indicate that two mechanisms are operating when these ions interact with B. subtilis membranes. At low ionic concentrations (0 to 0.1 M) there is direct cation binding to the anionic membranes which in the case of Ca^{2+}, Mg^{2+} and La^{3+}, confers rigidity on the membranes and reaches saturation when the number of cations present equals the number of anionic sites on the lipids. At high concentrations there is a further effect on the membranes that parallels the known organizing/disorganizing effects that the ions studied have on water structure.

The properties of charged biological and model membranes in aqueous solution may depend on the nature and concentration of certain ions present in solution. In this paper we have chosen to study such effects on the lipid moiety of a biological membrane. The fluidity of cytoplasmic membranes of B. subtilis strain 168 (wild-type) was probed with spin labels in the presence of various cations and anions. The chemical composition of this membrane has been well characterized (1) and an osmotically stabilized protoplast can be readily prepared. In our experiments, however, we have used shocked membranes (ghosts) with stearic acid spin label I(12,3) and its methyl ester I'(12,3) incorporated into the lipid bilayer regions of the membranes.



I(12,3): R=H
I'(12,3): R=CH_3

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The nitroxide groups of these spin labels probe the membrane environment in the vicinity of the phospholipid head groups where the effects of ions should be most marked. The spin labels have characteristic electron spin resonance (esr) spectra (Fig. 1) from which the mobility of the nitroxide group, and hence the fluidity of the membrane, may be monitored (2).

EXPERIMENTAL

B. subtilis strain 168 was grown in 3 litre batches of synthetic medium with vigorous shaking. The cells were harvested during the stationary phase (16 - 18 h) and membrane ghosts prepared by lysozyme-DNAase treatment as described elsewhere (1). The spin labels I(12,3) and I'(12,3) were purchased from SYVA, Palo Alto, Calif.. The membranes were labeled by exchange from bovine serum albumin (3) in 145 mM NaCl solution for 10 min. The mixture was then centrifuged at 30,000 g for 20 min and the pellet washed once with 5 mM NaCl and once with distilled water. The labeled membranes were then suspended in 2 ml volumes of different salt solutions prepared from analytical grade reagents and, after centrifugation, finally resuspended in 0.1 ml of the same salt solutions. The final weight of spin labels in the membranes was about 0.1% (w/w) or less than 1% of the total weight of lipids. The pH values of the suspensions were all close to 6. A pH buffer was not used in order to avoid any disturbance from competing ions. A series of separate experiments were carried out on membranes in buffers of low ionic strength where it was demonstrated that in the pH range 5 - 8 the esr spectra remained unchanged. The dry weights of the ghosts in the final suspensions were approximately 10%. Protein determination on both supernatants and pellets was made according to Lowry (4). The number of ions taken up by the membranes was tested with atomic absorption by Analytica AB, Sweden.

Esr measurements were carried out at 23 ± 0.3°C with a Varian E-9 X-band spectrometer using a rectangular cavity and quartz sample tubes of 1 mm inner bore. For accurate measurements the outermost spectral singularities were recorded separately with high gain and slow sweep time; this allowed determination of the spacings $2T_{\parallel}'$ (Fig. 1) between the high and low field extrema of the derivative spectra to within ±0.01 mT (1 mT = 10 Gauss).

RESULTS AND DISCUSSION

B. subtilis cytoplasmic membranes strain 168 prepared as described above have been reported (1) to contain protein (62%), RNA (22%) and lipids (16%). Of the lipids 75% by weight are phospholipids, mainly diphosphatidylglycerol (cardiolipin, 49%) and phosphatidylethanolamine (34%). At pH 6 only cardio-

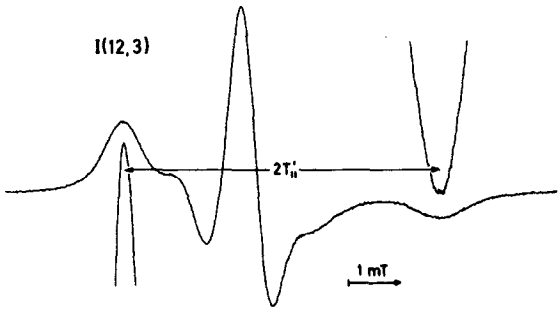

Fig. 1: Typical esr spectrum of spin label I(12,3) incorporated into B. subtilis membrane ghosts in salt solution. This particular spectrum is for membranes in 0.1 M NaCl solution: cavity microwave power 2 mW, modulation amplitude 0.2 mT. The wings were recorded separately with ten times higher gain. The spectra for I'(12,3) were similar.

lipin^{2-} is a potential cation binder. Although the lipid content of the membrane is very low there is strong experimental evidence that the spin labels reside in lipid bilayer regions in the membranes. Thus the hyperfine splitting $2T_{II}'$ is smaller when the spin labels are in the membranes than when on serum albumin; this demonstrates that there is a larger rotational freedom for the labels in the membranes. We have also observed some anisotropy in $2T_{II}'$ with membranes oriented on a flat glass surface, similar to that previously observed with oriented bilayer model membranes (2). The mobilities of various fatty acid spin labels in the membranes are also consistent with a lipid bilayer model. These observations will be reported separately.

Fig. 2 shows the variation of $2\Delta T_{II}'$ with salt concentration using I(12,3), where $2\Delta T_{II}'$ is the shift in $2T_{II}'$ relative to the salt free suspension (i.e. distilled water) for which $2T_{II}'$ was typically 6.0 mT. $2\Delta T_{II}'$ is thus a measure of the increasing ($2\Delta T_{II}' > 0$) or decreasing ($2\Delta T_{II}' < 0$) rigidity of the membranes in the presence of ions.

The results of Fig. 2 varied by as much as ±0.5 mT between different membrane batches, though the order of the ionic effects were always the same and quite reproducible for each batch studied.

Similar results were obtained with the neutral spin label I'(12,3). Since cations cannot interact directly with the uncharged head group of I'(12,3) this spin label reflects the effects of the ions on the surrounding membrane, whereas with the acid spin label I(12,3) direct interaction of cations with the probably ionized carboxyl group may also occur thus influencing the results. The values of $2\Delta T_{II}'$ with I'(12,3) were all about 30% less than with I(12,3); and in the salt free suspension the total

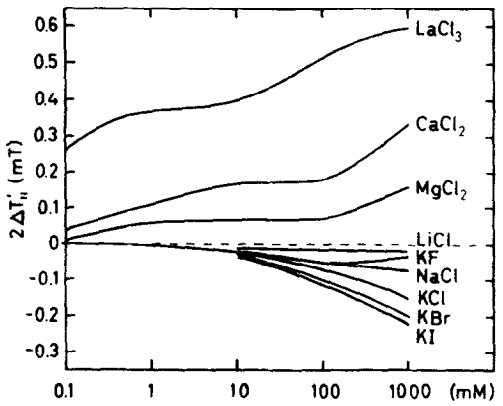

Fig. 2: Variations of the hyperfine splitting shift $2\Delta T_{\parallel}'$ of the stearic acid spin label I(12,3) in B. subtilis membrane ghosts with salt concentration of the suspending solutions. $2\Delta T_{\parallel}'$ is the value of $2T_{\parallel}'$ relative to the value in salt free solution (i.e. distilled water). Only the K-halide series is shown in full. A similar order was observed with the Na- and Li-halides.

splitting $2T_{\parallel}'$ of I'(12,3) was 0.25 mT less than that of I(12,3). The higher overall mobility and reduced sensitivity to ions of I'(12,3) may be due to its being more deeply embedded in the membrane where the mobility is higher and where the effects of ions are expected to be less marked. At present, however, we cannot exclude that in the case of I(12,3) some of the effects may be partly due to ionic interactions with the carboxyl group.

Experiments were carried out to test for reversibility. In these experiments high salt concentration suspensions were centrifuged, and the pellets washed and finally resuspended in a lower salt concentration. The esr spectra of these final suspensions were then repeatedly recorded over a period of about 4 hours. The results showed that on diluting the suspensions from 1 M to 0.1 M salt solutions the values of $2\Delta T_{\parallel}'$ returned to those of the original 0.1 M suspensions within ±0.01 mT, i.e. $2\Delta T_{\parallel}'$ is reversible in the range 1 M - 0.1 M. Such reversibility was not observed on diluting the Ca^{2+}, Mg^{2+} and La^{3+} solutions from 0.1 M to 0.001 M or from 0.001 M to distilled water.

These results indicate that two mechanisms are operating when ions interact with membranes, one at low ionic concentrations and one at high ionic concentrations.

Effects of ions at low concentrations (0 - 0.1 M)

At low ionic concentrations the cations Ca^{2+}, Mg^{2+} and La^{3+} bind tightly to the dianionic phospholipid head groups of cardiolipin^{2-} (5), and are not easily dislodged on dilution. Atomic absorption experiments were carried out

on the 2 mM solutions of $CaCl_2$, $MgCl_2$, NaCl and KCl that were used for suspending the membranes as well as on the supernatants after centrifugation. The difference in the cation concentration therefore gave the number of cations picked up by the membranes and, in the case of Na^+, K^+ and Mg^{2+}, this was found to be approximately equal to the number required to neutralize the total negative charge on the cardiolipin molecules in the membranes. In the case of Ca^{2+}, however, more than the required amount was picked up - the excess possibly taken up by membrane-bound proteins.

The binding of the cations to the anionic sites neutralizes the net negative surface charge, eliminates the repulsive forces between the lipid head groups, and induces rigidity in the membranes. The effect is seen to be strongly dependent on the cationic charge, and reaches saturation when the total number of cations equals the total number of anionic sites in the membranes (i.e. at about 1 mM concentrations in our experiments, see Fig. 2). The monovalent salts, however, appear to have a negligible ordering effect.

Effects of ions at high concentrations (0.1 - 1 M)

At high ionic concentrations another, reversible, effect seems to be operating. Here the Ca^{2+}, Mg^{2+} and La^{3+} ions induce further rigidity (in particular, high concentrations of $LaCl_3$ caused an amorphous precipitation of membranes), but the monovalent ions have an overall disorganizing effect on the membranes. Other monovalent ions than those shown in Fig. 2 were also studied, and it was found that the order in which the monovalent ions disorganized the membranes followed the Hofmeister lyotropic series:

$$K^+ > Na^+ > Li^+ \qquad\qquad I^- > Br^- > Cl^- > F^-$$

This is also the order in which these ions influence the structure of water (6). Thus I^- is a strong structure breaker, while F^- is generally considered a structure promoter; Mg^{2+} and Ca^{2+} are also structure promoters.

We suggest that the effects of the ions at high salt concentrations arise from their effects on the water structure being transmitted to the membranes' surfaces. This explanation is reasonable in view of the observed reversibility of $2\Delta T_{\parallel}'$ in the range 0.1 - 1 M; and as there are few anionic binding sites available at these high salt concentrations further direct cation binding is unlikely.

Experiments were also carried out to test how much protein was being removed from the membranes on addition of monovalent ions, as removal of membrane-bound proteins is also known to disorganize membranes. Thus Rottem et al (7) have found that removal of 75 - 90% of the proteins in mycoplasma

membranes lowered $2T_{\parallel}'$ of I(12,3) by about 0.15 mT at 23°C. The measurements on the amount of protein extracted and removed from the membranes during resuspension in the various salt solutions and distilled water showed that the 0.1 M salts and distilled water removed between 2 and 5% of the membrane-bound proteins and that the 1 M salts removed between 5 and 8%, though 1 M NaI and KI solutions removed between 10 and 20%. It is interesting to note that with KI the reversibility in $2\Delta T_{\parallel}'$ on diluting from 1 M to 0.1 M was only 70% complete, indicating an effect possibly due to protein extraction. It has been reported that the highly chaotropic ion SCN^- in 2 M concentration solubilizes 24% of the membrane-bound proteins in B. subtilis (8) and 40% in erythrocyte ghosts (9); since SCN^- is among the most effective ions for such purposes (8) it is unlikely that disruption of the membrane and significant solubilization of the membrane proteins is ocurring in our study where lower salt concentrations and less effective ions have been used. The reversibility in $2\Delta T_{\parallel}'$ (partial only in the case of KI) in the range 1 M - 0.1 M rules out any possibility of explaining these effects in terms of protein extraction, though at present we cannot exclude that some reversible ion-protein-lipid interactions are also involved.

CONCLUSION

Our results indicate that when ions interact with B. subtilis membranes two mechanisms are operating: at low concentrations cations bind tightly to the anionic membrane sites which, in the case of Ca^{2+}, Mg^{2+} and La^{3+}, confers rigidity on the membranes. At high concentrations there is an additional, reversible, effect on the membranes that parallels the organizing or disorganizing effects that the ions studied have on water structure. In particular, the disorganizing effects of the monovalent ions exceed any stabilizing effects that these ions may confer through direct binding.

Our results may be compared with those of Butler et al (10) who measured the motion of cholestane spin labels in lamellar mesophases of anionic brain white matter lipids with varying salt concentrations. These workers found that the degree of organization increased with increasing cation concentration as follows:

$$Na^+ = K^+ = Li^+ < Mg^{2+} = Ca^{2+} < La^{3+} < Th^{4+}$$

An ordering effect was observed with all these cations which was found to depend only on their charge. Further, anions were found to have no effect in the range of concentrations used (1 - 50 mM), and none of the salts decreased the degree of organization of the lipids. These observations are in qualitative agreement with ours though there are some differences, for

example, as regards the ordering effects of the monovalent salts. At present we cannot say whether this is due to the difference between the suspended biomembranes and stacked model membranes or to the dissimilar spin labels employed in the two studies.

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