Biochimica et Biophysica Acta, 596 (1980) 145—151

BBA Report

BBA 71427

LATERAL SEGREGATION OF PROTEINS INDUCED BY CHOLESTEROL IN BACTERIORHODOPSIN-PHOSPHOLIPID VESICLES

R.J. CHERRY[a], U. MÜLLER[a], C. HOLENSTEIN[a] and M.P. HEYN[b]

[a]*Eidgenössische Technische Hochschule, Laboratorium für Biochemie, ETH — Zentrum, CH-8092 Zürich (Switzerland) and* [b]*Department of Biophysical Chemistry, Biozentrum, CH-4056 Basel (Switzerland)*

(Received October 19th, 1979)

Key words: Bacteriorhodopsin; Cholesterol; Protein diffusion; Lipid membrane; Lipid-protein interaction, Phase separation

Summary

Bacteriorhodopsin in dimyristoylphosphatidylcholine vesicles is randomly distributed in the plane of the membrane and exhibits rotational diffusion above the gel to liquid-crystalline phase transition. Incorporation of cholesterol results in loss of rotational mobility of bacteriorhodopsin, which on the basis of electron microscopy and CD measurements can be assigned to the formation of protein aggregates. It is concluded that bacteriorhodopsin is soluble in the fluid phosphatidylcholine phase but segregates when cholesterol is present in the lipid bilayer.

The effects of cholesterol on the physical properties of lipid bilayers have been the subject of numerous investigations [1—16]. It is well established that cholesterol either decreases or increases 'fluidity' depending on whether the temperature is above or below the chain melting temperature (T_c) of the lipids [1]. Relatively few studies have been made of how these effects influence the diffusion of membrane components. Measurements in lipid-cholesterol bilayers of lateral diffusion of lipids [13,17,18] and of the small polypeptide Gramicidin S [19] have been reported but there are almost no studies of the effect of cholesterol on protein diffusion. Recently, rotational diffusion of band 3 proteins in the human erythrocyte membrane as a function of cholesterol content was investigated [20]. Surprisingly, no change in rotational motion was observed over the range 0.34—1.66 cholesterol : phospholipid mole ratio.

A convenient model system for studying protein diffusion consists of bacteriorhodopsin incorporated in phosphatidylcholine vesicles [21,22].

We therefore added cholesterol to this system with the aim of clarifying its effect on protein rotational motion. We report here the results of these studies which unexpectedly reveal that cholesterol induces segregation of protein molecules in the plane of the lipid bilayer.

Bacteriorhodopsin was incorporated into dimyristoylphosphatidylcholine vesicles by the Triton solubilisation and dialysis procedure described previously [22]. After formation of the bacteriorhodopsin-lipid vesicles, cholesterol was incorporated by incubation with sonicated egg phosphatidylcholine-cholesterol liposomes prepared essentially as described by Cooper et al. [23]. Incubation was for 24 h at 26°C in the dark in 0.1 M sodium acetate buffer, pH 5.0. After the incubation, bacteriorhodopsin-lipid vesicles were separated from sonicated liposomes by centrifugation through a sucrose density gradient. The phospholipid content of the vesicles was analysed according to Chen et al. [24] and cholesterol by the method of Courchaine et al. [25]. Protein was determined according to Lowry et al. [26], the retinal concentration by absorption spectroscopy using the value of 62 700 $M^{-1} \cdot cm^{-1}$ for the extinction coefficient at 568 nm [27]. Comparison of these two measurements based on the known 1-1 stoichiometry revealed that some of the bacteriorhodopsin molecules (~ 30%) lost their retinal chromophore during the incubation with liposomes. The values stated for phospholipid : protein ratios (expressed as wt./wt.) are therefore those based on the Lowry method, with a correction of 20% as indicated by recent studies [27].

Rotational diffusion of bacteriorhodopsin in the lipid vesicles was measured by observing flash-induced transient dichroism. Details of the experimental method are given elsewhere [28]. Briefly, the sample is excited by a linearly polarised light pulse of duration 1—2 μs and wavelength 540 nm. Transient absorbance changes due to ground state depletion are detected at 570 nm. The signals are analysed by calculating the absorption anisotropy $r(t)$ given by

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \qquad (1)$$

where $A_{\parallel}(t)$ and $A_{\perp}(t)$ are the absorbance changes at time t after the flash for light polarised parallel and perpendicular with respect to the polarisation of the exciting light. Measurements were made at 25°C and 37°C, i.e. above the T_c of dimyristoylphosphatidylcholine (23°C).

Fig. 1 shows the time dependence of the anisotropy before and after incorporating cholesterol into the vesicles. In the absence of cholesterol, the anisotropy exhibits an initial decay followed by a time-independent residual anisotropy. As discussed elsewhere, the curve is that expected when rotation occurs only about an axis perpendicular to the membrane [28,29]. The predicted form of $r(t)$ in this case is given by

$$r(t) = A_1 \exp(-D_{\parallel} t) + A_2 \exp(-4D_{\parallel} t) + A_3 \qquad (2)$$

where $D_{\parallel}$ is the diffusion coefficient for rotation about the membrane normal and A_1, A_2, A_3 are constants which depend on the orientation of the transition dipole moment of the 570 nm absorption band.

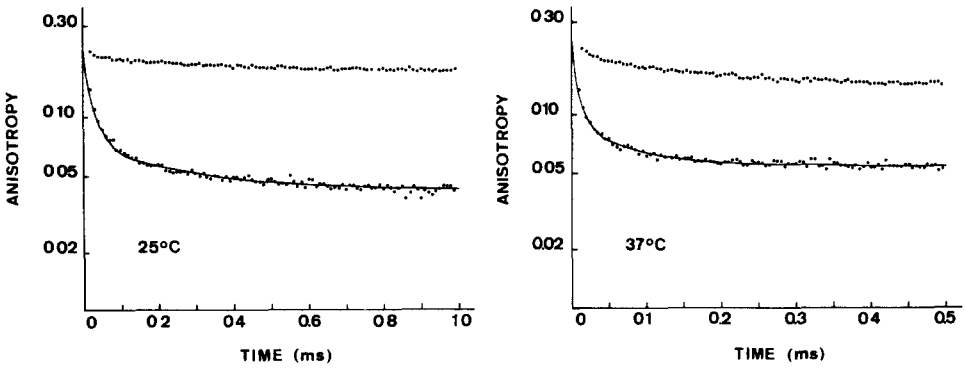

Fig. 1. Time dependence of the anisotropy r of 568 nm depletion signals at 25°C and 37°C for bacteriorhodopsin incorporated into dimyristoylphosphatidylcholine vesicles. Solid line and points, control sample (i.e. in absence of cholesterol); points only, same sample after cholesterol incorporation, X = 21 mol%, phospholipid : protein 1.2. Vesicles were in 0.1 M sodium acetate, pH 5.0.

The effect of incorporating cholesterol into the vesicles is dramatic. After cholesterol incorporation, the anisotropy decays very slowly with time, indicating that bacteriorhodopsin is almost immobilised. For the sample shown in Fig. 1, fitting Eqn. 2 to the experimental points yields a relaxation time $\phi_{\parallel}$ (defined as $1/D_{\parallel}$) of 80 μs at 25°C and 20 μs at 37°C. In the presence of cholesterol it may be estimated that $\phi_{\parallel} \sim$ 10 ms at 25°C and $\sim$ 4 ms at 37°C. These very slow relaxation times may in part be determined by vesicle tumbling so protein rotation in the membrane could be even slower. In any event, it is clear that bacteriorhodopsin rotation is slowed more than 100 times following incorporation of cholesterol.

It is likely that in addition to cholesterol, egg phosphatidylcholine also exchanges between liposomes and vesicles during the incubation [30—32]. However, immobilisation of bacteriorhodopsin is not observed when vesicles are incubated with cholesterol-free liposomes. Moreover, the rotation of bacteriorhodopsin in egg phosphatidylcholine vesicles is qualitatively similar to that in dimyristoylphosphatidylcholine vesicles above the T_c. Thus it is cholesterol, rather than egg phosphatidylcholine, which is responsible for the observed immobilisation of bacteriorhodopsin.

The large decrease in rotational mobility of bacteriorhodopsin in the presence of cholesterol is unlikely to be simply due to a change in membrane viscosity, especially as only relatively small changes in lipid lateral-diffusion coefficients above the T_c due to cholesterol have been reported [13,17,18]. In addition, recent results on the fluorescence depolarisation of small lipid soluble probes in phospholipid-cholesterol vesicles show that the effect of cholesterol on the rate of probe rotation is only minor and that the major effect is due to an increased orientational constraint [15,16]. The alternative explanation is that bacteriorhodopsin rotation is slowed by self-aggregation upon incorporation of cholesterol. Rotational diffusion measurements are particularly sensitive to aggregation since $\phi_{\parallel}$ varies as the square of the particle cross-sectional diameter in the plane of the membrane. To investigate this possibility, we carried out further experiments using electron microscopy and CD.

Fig. 2 shows freeze-fracture electron micrographs of the bacteriorho-

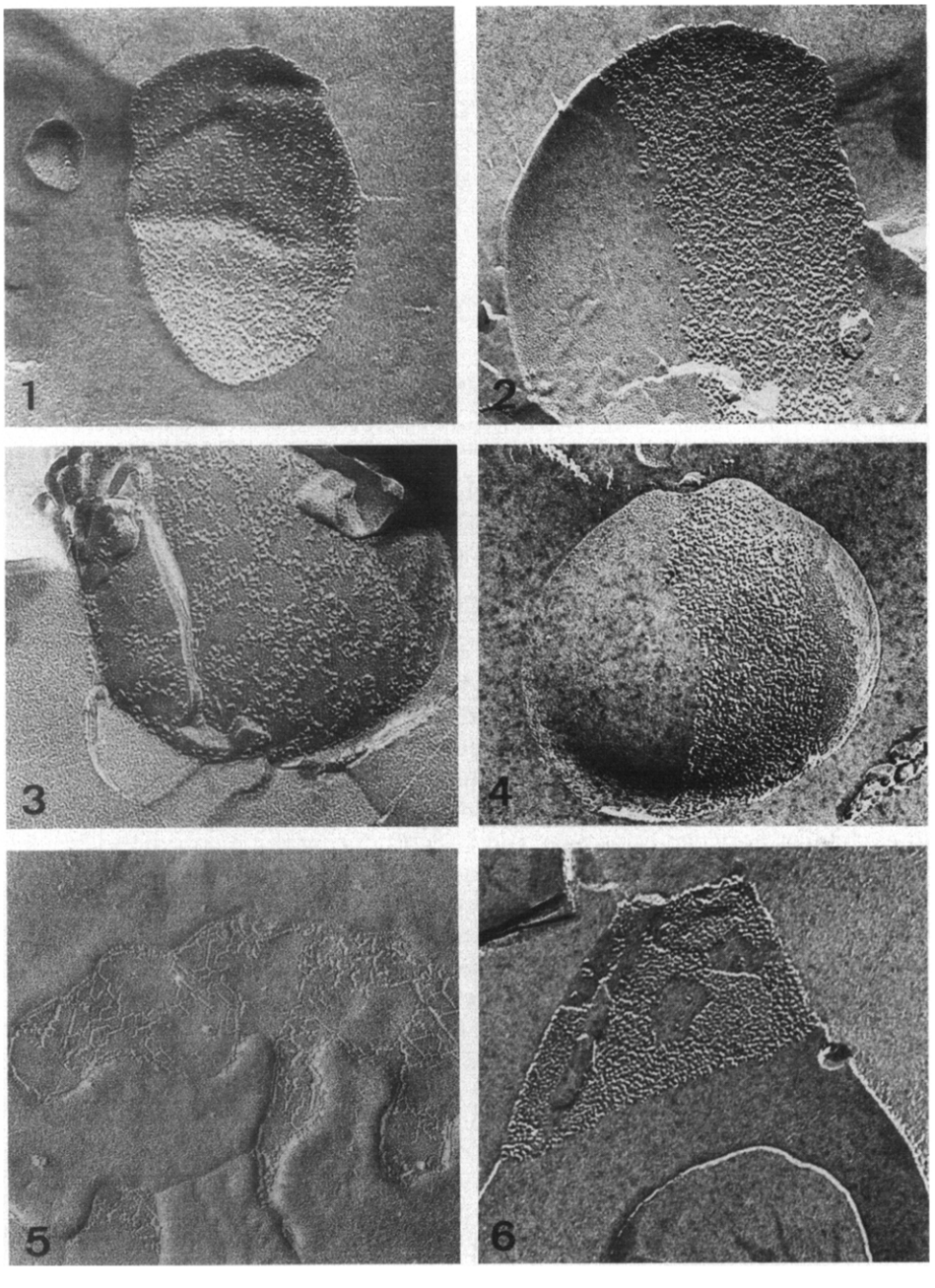

Fig. 2. Freeze-fracture electron micrographs of bacteriorhodopsin-dimyristoylphosphatidylcholine vesicles containing various amount of cholesterol. (1) X = 0 mol%, phospholipid : protein 3.2; (2) X = 10 mol%, phospholipid : protein 2.9; (3) and (4) X = 29 mol%, phospholipid : protein 1.4; (5) and (6) X = 39 mol%, phospholipid : protein 1.2. Magnification 56 000 X. Samples were frozen from ~30° C. (3) and (4) illustrate two different aggregation patterns observed in these samples.

dopsin-lipid vesicles. As previously demonstrated, in the absence of cholesterol the intra-membranous particles are randomly dispersed when the sample is frozen from a temperature above the lipid phase transition (Fig. 2 (1)). Upon incorporation of cholesterol a marked segregation takes place. The particles are clustered into defined areas leaving large smooth areas virtually devoid of particles (Fig. 2 (2—4)). At the higher cholesterol contents, the particles become densely packed (Fig. 2(6)) and 120° cracks sometimes appear on the surface of the fracture (Fig. 2(5)). This latter feature is characteristic of the hexagonal crystalline lattice of the native purple membrane [33]. We have not pursued this latter point in detail, but formation of a lattice would be reasonable in view of the known recrystallisation of bacteriorhodopsin below the T_c in dimyristoylphosphatidylcholine vesicles [22]. Both the aggregation seen in electron microscopy and immobilisation observed by rotational diffusion measurements appear to be independent of the phospholipid : protein ratio over the range investigated (4:1—1:1).

The CD spectrum of the purple membrane in the visible is characterised by a negative band at about 605 nm and a positive band at about 535 nm. This pair of so-called exciton bands was shown to be due to specifically aggregated bacteriorhodopsin [22,34,35]. Bacteriorhodopsin monomers in contrast have an entirely different CD spectrum which is positive over the whole visible wavelength range [36]. This exciton feature in the CD spectra can thus be used to distinguish monomeric from aggregated bacteriorhodopsin and to monitor its self-aggregation. On the basis of such CD measurements, it could be shown that in dimyristoylphosphatidylcholine vesicles in the absence of cholesterol, bacteriorhodopsin is monomeric above the T_c of dimyristoylphosphatidylcholine whenever the phospholipid : protein ratio is greater than about one [22]. Fig. 3 shows the CD spectrum of bacteriorhodopsin-dimyristoylphosphatidylcholine-cholesterol vesicles at 5°C and 37°C. It is clear that the exciton bands are still present at 37°C, i.e. far above the phase transition of the pure lipid. In control experiments with vesicles containing no cholesterol, no negative CD band at 605 nm was

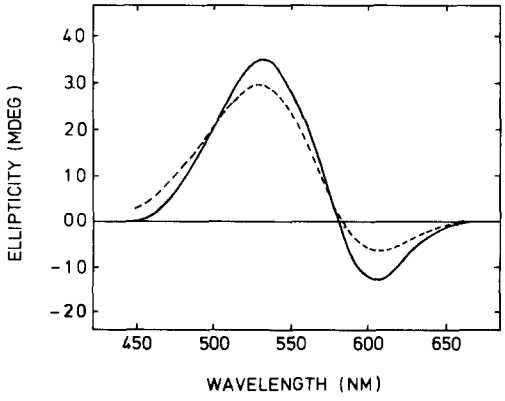

Fig. 3. Change in the CD spectrum of a suspension of bacteriorhodopsin-dimyristoylphosphatidylcholine-cholesterol vesicles when the temperature is raised from 5°C (—) to 37°C (— — —). X = 21 mol%, phospholipid : protein 1.2. The vesicles were in 0.1 M sodium acetate buffer, pH 5.0.

observed at 37°C at the same phospholipid : protein ratio of 1.2. The peak to peak amplitude of the CD effect in Fig. 3 (difference in molar ellipticity at 535 and 605 nm) is 51 000 deg cm^2 /dmol at 5°C and 36 000 deg cm^2/dmol at 37°C. For the purple membrane values between 80 000 and 100 000 have been reported [22]. The present values are in good agreement with those obtained for the purple membrane, if we take into account the 30% chromophore loss. Since the CD peak to peak amplitude depends quadratically on the fraction of retinal binding sites that are occupied [37], we expect an amplitude which is about 49% ($(0.7)^2 \times 100$) of that of the purple membrane. Although the CD amplitude increases between 37°C and 5°C, this does not necessarily indicate any further aggregation at the lower temperature. Similar temperature effects are observed in the purple membrane, where bacteriorhodopsin is aggregated at all temperatures [22]. We conclude from a comparison of the CD spectra of bacteriorhodopsin in dimyristoylphosphatidylcholine vesicles in the presence and absence of cholesterol that in the cholesterol-containing vesicles bacteriorhodopsin is aggregated and that the aggregation is induced by cholesterol.

Various other studies have indicated the existence of phase boundaries in the phosphatidylcholine-cholesterol system at cholesterol content X = 20 mol% and 33 mol% [3—9,13,14]. We have found that strong immobilisation of bacteriorhodopsin (i.e. $\phi_{\parallel}$ increases by > 100 times) occurs whenever X is greater than about 20 mol%. The experiments described above provide convincing evidence that this is a consequence of cholesterol-induced lipid-protein segregation. Protein aggregation leads to dramatic slowing of rotational motion and the appearance of exciton effects in the CD spectrum.

We have also carried out a few experiments with vesicles containing less than 20 mol% cholesterol. It was noticeable that much smaller effects on bacteriorhodopsin rotation occurred at X = 10 mol% and X = 15 mol% ($\phi_{\parallel}$ increased by up to 6 times). However, even at 10 mole %, protein segregation is still observable in freeze-fracture electron micrographs (Fig. 2(2)). These results are compatible with the previous finding that a phase transition closely corresponding to pure phosphatidylcholine is observable when $X < 20$ mol% [3,6,7,13]. Thus our results at $X < 20$ mol% can be interpreted in terms of separation of dimyristoylphosphatidylcholine and dimyristoylphosphatidylcholine-cholesterol phases with bacteriorhodopsin segregating in favour of the dimyristoylphosphatidylcholine phase. Since bacteriorhodopsin exists in a fluid dimyristoylphosphatidylcholine phase, strong aggregation is prevented, although some slowing of rotation is expected due to the effect of protein concentration [21].

The present findings may give insight into the formation of purple membrane patches in *Halobacterium halobium*. Although of course the lipid composition of the bacterial membrane differs greatly from that of the model system, the present experiments demonstrate that bacteriorhodopsin solubility in the lipid phase is sensitive to lipid composition. Thus formation of purple membrane patches may simply be a consequence of insolubility of bacteriorhodopsin in the lipid bilayer of *Halobacterium halobium*.

Kleemann and McConnell [5] previously reported protein segregation in dimyristoylphosphatidylcholine bilayers containing (Mg^{2+} + Ca^{2+})-ATPase.

Segregation, however, only occurred below the T_c of dimyristoylphosphatidylcholine, above the T_c the protein molecules were randomly distributed. The present studies appear to be the first observation of protein segregation in phosphatidylcholine-cholesterol bilayers at temperatures above the T_c of the phospholipid. Although this observation could reflect a special property of bacteriorhodopsin, it is conceivable that similar effects may occur with other membrane proteins. If so, this could have considerable implications for molecular arrangements in those cell membranes with substantial cholesterol content.

We thank Frl. Carmen Zugliani for technical assistence and the Schweizerischer Nationalfonds (grants 3.116.077 and 3.333.78) and the Fritz Hoffmann-La Roche Stiftung (grant 160) for financial support.

References

1 Oldfield, E. and Chapman, D. (1972) FEBS Lett. 23, 285—297
2 Ladbrooke, B.D., Williams, R.M. and Chapman, D. (1968) Biochim. Biophys. Acta 150, 333—340
3 Mabrey, S., Mateo, P.L. and Sturtevant, J.M. (1978) Biochemistry 17, 2464—2468
4 Shimshick, E.J. and McConnell, H.M. (1973) Biochem. Biophys. Res. Commun. 53, 446—451
5 Kleemann, W. and McConnell, H.M. (1976) Biochim. Biophys. Acta 419, 206—222
6 Verkleij, A.J., Ververgaert, P.H.J., de Kruyff, B. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 373, 495—501
7 Estep, T.N., Mountcastle, D.B., Biltonen, R.L. and Thompson, T.E. (1978) Biochemistry 17, 1984—1989
8 Engelman, D.M. and Rothman, J.E. (1972) J. Biol. Chem. 247, 3694—3697
9 Haberkorn, R.A., Griffin, R.G., Meadows, M.D. and Oldfield, E. (1977) J. Am. Chem. Soc. 99, 7353—7355
10 Gally, H.U., Seelig, A. and Seelig, J. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 1447—1450
11 Marsh, D. and Smith, I.C.P. (1973) Biochim. Biophys. Acta 298, 133—144
12 Darke, A., Finer, E.G., Flook, A.G. and Phillips, M.C. (1972) J. Mol. Biol. 63, 265—279
13 Rubenstein, J.L.R., Smith, B.A. and McConnell, H.M. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 15—18
14 Oldfield, E., Meadows, M., Rice, D. and Jacobs, R. (1978) Biochemistry 17, 2727—2740
15 Kawato, S., Kinosita, K. Jr. and Ikegami, A. (1978) Biochemistry 17, 5026—5031
16 Lakowicz, J.R., Prendergast, F.G. and Hogen, D. (1979) Biochemistry 18, 508—519
17 Fahey, P.F., Koppel, D.E., Barak, L.S., Wolf, D.E., Elson, E.L. and Webb, W.W. (1977) Science 195, 305—306
18 Wu, E.-S., Jacobson, K. and Papahadjopoulos, D. (1977) Biochemistry 16, 3936—3941
19 Wu, E.-S., Jacobson, K., Szoka, F. and Portis, A., Jr. (1978) Biochemistry 17, 5543—5550
20 Nigg, E.A. and Cherry, R.J. (1979) Biochemistry 18, 3457—3465
21 Cherry, R.J., Müller, U. and Schneider, G. (1977) FEBS Lett. 80, 465—470
22 Cherry, R.J., Müller, U., Henderson, R. and Heyn, M.P. (1978) J. Mol. Biol. 121, 283—298
23 Cooper, R.A., Leslie, M.H., Fischkoff, S., Shinitzky, M. and Shattil, S.J. (1978) Biochemistry 17, 327—331
24 Chen, P.S., Toribara, T.V. and Huber, W. (1956) Anal. Chem. 28, 1756—1758
25 Courchaine, A.J., Miller, W.H. and Stein, D.B., Jr. (1959) Clin. Chem. 5, 609—614
26 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
27 Rehorek, M. and Heyn, M.P. (1979) Biochemistry, 18, 4977—4983
28 Cherry, R.J. (1978) Methods Enzymol. 54, 47—61
29 Heyn, M.P., Cherry, R.J. and Müller, U. (1977) J. Mol. Biol. 117, 607—620
30 Martin, F.J. and Macdonald, C. (1976) Biochemistry 15, 321—327
31 Kremer, J.M.H., Kops-Werkhoven, M.M., Pathmamanoharan, C., Gijzeman, O.L.J. and Wiersema, P.H. (1977) Biochim. Biophys. Acta 471, 177—188
32 De Kruijff, B. and van Zoelen, E.J.J. (1978) Biochim. Biophys. Acta 511, 105—115
33 Blaurock, A.E. and Stoeckenius, W. (1971) Nature New Biol. 233, 152—155
34 Heyn, M.P., Bauer, P.-J. and Dencher, N.A. (1975) Biochem. Biophys. Res. Commun. 67, 897—903
35 Becher, B. and Ebrey, T. (1976) Biochem. Biophys. Res. Commun. 69, 1—6
36 Dencher, N.A. and Heyn, M.P. (1978) FEBS Lett. 96, 322—326
37 Bauer, P.-J., Dencher, N.A. and Heyn, M.P. (1976) Biophys. Struct. Mech. 2, 79—92