Circular-dichroism analyses of membrane proteins: examination of environmental effects on bacteriorhodopsin spectra

N. A. SWORDS* and B. A. WALLACE*†‡

*Department of Chemistry and Center for Biophysics, Rensselaer Polytechnic Institute, Troy, NY 12180, U.S.A., and †Department of Crystallography, Birkbeck College, University of London, London WC1E 7HX, U.K.

The secondary structure of bacteriorhodopsin is known from electron-diffraction studies, making bacteriorhodopsin a useful test system for analysing environmental influences on membrane proteins using c.d. spectroscopy. The conformational effects of detergent solubilization and incorporation into vesicles of various types were determined by comparison of the calculated secondary structures derived from c.d. spectra with the structure determined from diffraction studies. In addition, two modified forms of the native purple membrane, a shrunken form of the hexagonal lattice and an orthorhombic lattice form, were used to determine the effects of varying membrane fragment size and protein concentration within the membranes. The two different vesicle incorporation

procedures yielded bacteriorhodopsin spectra which were nearly identical with each other and very close to the structure calculated from electron-diffraction studies. Solubilization of the native protein in the non-ionic detergent n-octyl glucoside, without subsequent vesicle incorporation, resulted in a significantly altered protein conformation. Organizing the protein in different membrane lattices produced even more apparent deviations from the secondary structure determined by diffraction studies, as a consequence of optical effects caused by the high protein concentrations in the lattices. These studies show the importance of maintaining a 'native' environment, and the influence of particle geometry in interpreting c.d. studies of membrane proteins.

INTRODUCTION

Bacteriorhodopsin (BR) is the only protein found in specialized purple membrane patches in the bacterium *Halobacterium halobium* [1]. The protein consists of 248 mainly hydrophobic amino acids and a chromophore, all-*trans*-retinal, bound to lysine-216 via a protonated Schiff-base linkage [2–6]. BR functions as a proton pump to create an electrochemical gradient, allowing the bacterium to synthesize ATP under anaerobic conditions [1]. Although BR is functional as a monomer in the purple membrane [7], the protein molecules are arranged as trimers on a two-dimensional hexagonal lattice [8–10].

Membrane proteins as a class have proved difficult to crystallize, and little is known about their three-dimensional structures. It is therefore important to be able to use techniques other than X-ray crystallography to examine the relationship between the structure and function of these proteins. C.d. spectroscopy can be useful for providing information about the secondary, tertiary, and in some cases, the quaternary structures of proteins. However, because membrane proteins are associated with lipids in membranous particles, they are not readily amenable for study by spectroscopic techniques. The absorption and c.d. spectra of membrane proteins are affected by optical artifacts which are a consequence of the large size of membrane fragments in relation to the wavelength of light. The high protein concentrations within these fragments also contribute to spectral distortion. When these potential artifacts are ignored [11,12] it can be shown [13] that the calculated secondary structures derived from the c.d. spectra will be erroneous.

The large size of the membrane fragments relative to the wavelength of the incident light results in differential light scattering, a difference in the extent of scattering of left- and

right-handed circularly polarized light by an optically active sample. This difference in scattering is ordinarily detected as if it were differential absorption [14]. Differential scattering can be virtually eliminated mechanically by using a detector geometry which permits collection of the scattered light [15]. The high protein concentration within the membrane fragments is responsible for absorption-flattening effects. Absorption flattening occurs when chromophores are not uniformly dispersed in the medium, as is the case for proteins which are closely packed in membrane particles. The reduction in total cross-sectional area decreases the probability that protein molecules will encounter photons of light, and protein absorption will be correspondingly reduced. Optically active samples will also exhibit differential absorption flattening, a difference in the absorption of left- and right-handed circularly polarized light. Since the degree of flattening is directly proportional to the absorption, intense c.d. peaks will be reduced to a greater degree than weak peaks, resulting in significant distortion in the shape of the spectrum [16,17]. Differential absorption flattening cannot be eliminated instrumentally, and while theoretical correction factors can be calculated, this approach is impractical, owing to the difficulty in determining the precise physical parameters necessary for the calculations [16,35]. However, it should be possible to remove differential absorption flattening effects experimentally if the protein molecules can be uniformly dispersed. This can be accomplished either by solubilizing the proteins in non-denaturing detergents or incorporating them into phospholipid vesicles with high lipid-to-protein ratios [18]. However, detergent micelles may not represent a native-type environment, and structural changes may occur upon solubilization [13]. On the other hand, not all membrane proteins can be incorporated into vesicles in a fully active form. For instance, larger proteins may

Abbreviations used: DMPC, dimyristoyl phosphatidylcholine; NOG, n-octyl glucoside; DOC, (sodium) deoxycholate; BR, bacteriorhodopsin; CTAB, cetyltrimethylammonium bromide; DTAC, dodecyltrimethylammonium chloride; NRMSD, normalized root-mean-square deviation.

‡ To whom correspondence should be sent, at the Birkbeck College address.

be affected by the sharp radius of curvature of the vesicle, and the procedure of incorporation, which often includes solubilization as the first step, may disrupt protein structure. Because the environment of the membrane protein directly influences its structure, it is important to find a way of dispersing the protein without substantially altering its environment and thereby causing conformational changes.

The secondary structure of BR is known from electron microscopy and diffraction studies [10,19–23]. Although those studies are at relatively low resolution, they are sufficient to show that the protein consists of seven transmembrane helices, which account for about 70 % of the secondary structure, plus three small helices in the extracellular connecting loops, making the total helix content approx. 75–80 %. The remainder of the molecule consists of turn and random-coil structures; no β -sheet is present. The availability of this secondary structural information makes BR an ideal candidate for the study of environmental influences on membrane proteins. In the present study the effects of detergent solubilization and vesicle incorporation on the secondary structure were determined by comparison of the structure calculated from the c.d. spectra with the structure calculated from electron-diffraction studies. In addition, two modified forms of the native purple membrane, a shrunken hexagonal lattice form and an orthorhombic form, were studied to determine the effects of varying membrane fragment size and protein concentration and orientation within the sheets. Although the secondary structure of BR, as seen by electron diffraction, is maintained in the shrunken and orthorhombic forms [24–26], the lattice spacings in the shrunken form are reduced in size from the native purple membrane, resulting in more closely packed protein molecules. The area per protein molecule in the orthorhombic form is approximately the same as the native purple membrane, but the sizes of the membrane sheets are greatly increased and protein–protein interactions are altered [25]. Therefore, these two alternate lattice packings afford a further opportunity to examine local environmental effects on the calculated secondary structure.

MATERIALS AND METHODS

Materials

Halobacterium halobium was grown and purple membranes were isolated and purified according to the procedure of Oesterhelt and Stoerkenius [27]. Dimyristoyl phosphatidylcholine (DMPC) was obtained from Calbiochem and n-octyl glucoside (NOG) was purchased from Bachem Bioscience. Sodium deoxycholate (DOC) was from Sigma.

Vesicle preparations

Method I

Bacteriorhodopsin (BR) and DMPC vesicles were prepared in the manner of Mao and Wallace [18]: purple membranes (0.24 mg of protein/ml) and 1 mg of DMPC were sonicated to form small unilamellar vesicles. The initial lipid-to-protein mole ratio was 153:1.

Method II

Purple membranes (0.25 mg protein/ml) were solubilized in 0.1 M sodium acetate, pH 5.0, containing 1.2 % (w/v) NOG.

After solubilization, 1 mg of DMPC was added to the protein solution and vesicles were prepared according to the dialysis procedure of Cherry et al. [28]. The samples were sonicated to clarity before spectral analysis.

NOG solubilization of BR

Purple membranes (0.25 mg protein/ml) were solubilized in 0.1 M sodium acetate buffer, pH 5.0, containing 1.2 % (w/v) NOG.

Preparation of lipid-depleted purple membranes

The modified DOC extraction procedure of Glaeser et al. [24] was followed to produce 'shrunken' hexagonal membranes. The initial protein concentration was 2.0 mg/ml in 1 ml of 0.1 % DOC. The protein concentration was reduced to 0.7 mg/ml by the addition of increasing aliquots of 10 % DOC at 9 h intervals until a final concentration of 0.7 mg/ml in 6.0 % DOC was reached. The sample was washed on a 4.5–40 % (w/w) continuous sucrose gradient. The membrane samples were then collected and dialysed against 7 % DOC. The DOC concentration was gradually reduced to remove the detergent from the membranes.

Preparation of orthorhombic membranes

Method C of Michel et al. [30] was used to prepare orthorhombic purple membranes. A pellet containing 2.16 mg of purple membrane was suspended in 3 ml of 0.1 M potassium phosphate buffer, pH 5.2, containing 0.2 % Triton X-100 and 0.1 % NaN_3 . Cetyltrimethylammonium bromide (CTAB) was added to obtain a CTAB/BR molar ratio of 1.9:1. The sample suspension was then stirred for 4–5 weeks until the formation of orthorhombic rolls was observed. An alternate procedure using dodecyltrimethylammonium chloride (DTAC) was also followed: 2.6 mg of purple membranes were suspended in 1 ml of 0.1 M potassium phosphate buffer, pH 5.2, with 0.2 % Triton X-100 and 0.1 % NaN_3 . A 5.5 μl portion of 20 mM DTAC in 150 mM potassium phosphate buffer was then added to the protein sample. The sample was sonicated briefly with a probe sonicator, and then stirred for 4–5 weeks until orthorhombic rolls were formed.

Spectroscopic analyses

Absorption spectra were recorded on a Cary 2200 spectrophotometer over the wavelength range 700–190 nm. The second derivatives of the spectra were overlaid on the original spectra to identify the peak maxima. Protein concentrations of 1.0 mg/ml in 1 cm-pathlength quartz cells were used for absorption spectroscopic analyses.

C.d. spectra were obtained over the range 300–190 nm at 0.2 nm intervals with an Aviv 60DS spectropolarimeter. The variable position photomultiplier tube was placed directly adjacent to the sample cell, resulting in an average acceptance angle of approx. 90° [15]. Calibration of the instrument was performed with D-10-camphorsulphonic acid. The calibration was checked for optical rotation using sperm-whale myoglobin. Wavelength calibration was carried out using benzene vapour. Protein concentrations of 0.25 mg/ml in a 0.02 cm-pathlength quartz cell were used for the c.d. analyses. At least 40 scans were collected and averaged for each sample. The data points were analysed at 0.2 nm intervals between 240 and 190 nm by using a fitting procedure in which the sum of the fractions was not constrained to unity, but was normalized to 1.0 by dividing each fraction by

the sum of the fractions, in order to remove any variation due to concentration errors [31,32]. The reference data set of Chang et al. [32] was used. An average helix length of 26 residues was used in the data-fitting analyses reported. This length was chosen in order to approximate the length of helix necessary to span a bilayer, but other lengths were also tested; it was found that the results obtained were insensitive to variations in helix lengths greater than 20. The normalized root-mean-square deviation (NRMSD) was calculated as a measure of quality of the fit of the calculated secondary structure to the observed protein spectrum [31]. For each sample, the standard deviation was computed at all measured wavelengths to determine whether significant differences existed between different samples.

The relative protein concentrations of all samples were determined by Lowry assay in the presence of 0.1 % SDS [33]. The Lowry assay was calibrated on an absolute scale by comparison with the molar absorptivity of the native purple membrane at 568 nm. The phospholipid concentrations were analysed by using the procedure of Chen et al. [34].

X-ray-diffraction studies

Native, shrunken and orthorhombic membrane samples were prepared for X-ray diffraction by drying 1 mg of each sample on $\sim 3.05 \mu\text{m}$ (0.12 mil) Mylar film. Ni-filtered $\text{Cu K}\alpha$ radiation was used to examine the samples. The average exposure time was 48 h.

RESULTS

The c.d. spectra of the detergent-solubilized protein and the vesicle samples were compared with the spectrum of native purple membranes to examine the effects of protein solubilization and dispersion. On the basis of X-ray and electron-diffraction studies [23], which show that $\sim 80\%$ of the secondary structure of BR is α -helical, 10% turn and the remainder is random coil, a theoretical spectrum was created as a standard for comparison.

Detergent solubilization results in changes to the protein absorption maximum and c.d. spectrum relative to those of BR in the native purple membrane. In the detergent-solubilized form, the absorption maximum is shifted to 570 nm relative to the 568 nm absorption peak of the native membrane. The c.d. spectrum of the solubilized protein is somewhat closer to the theoretical spectrum than that of the native purple membrane (Figure 1), but shows differences suggestive of a non-native state. The detergent-solubilized spectrum exhibits some of the expected increases in the maxima at approximately 195 nm and 210 nm, which indicate a reduction in absorption-flattening effects. Nevertheless, the secondary structure calculated from the spectrum is not the same as that determined from the diffraction studies (Table 1).

As shown previously [13,18], incorporation of the protein into 'Method I' phospholipid vesicles results in significant changes in the c.d. spectrum relative to that of the native membrane (Figure 2). These changes reflect the decrease in differential absorption flattening effects and produce a calculated secondary structure very similar to that determined for BR by diffraction methods (Table 1). To examine the effects of different incorporation procedures, 'Method II' vesicles were prepared from detergent-solubilized protein and compared with the vesicles prepared by Method I. Both types of vesicles had an absorption maximum at 560 nm. The two types of vesicles had nearly identical c.d. spectra (Figure 2). Hence, there appears to be no residual effects due to detergent solubilization in the Method II vesicles. Thus it appears that the final environment plays a greater role in

determining the protein structure than the intermediate solubilized state. Furthermore, both types of vesicle samples were examined at both 15 °C (below the lipid phase-transition

Figure 1 C.d. spectra of native purple membranes (—), detergent-solubilized BR (---) and the theoretical spectrum determined from diffraction studies (·····)

The error bars indicate the reproducibility of the experimental data.

Table 1 Environmental effects on the calculated secondary structure of BR

Sample	Secondary structure (%)				NRMSD
	Helix	Sheet	Turn	Coil	
Native purple membranes	69	0	31	0	0.213
NOG-solubilized	73	0	21	6	0.107
Method I vesicles	74	0	23	3	0.083
Method II vesicles	75	0	22	3	0.091
'Shrunken' membranes	67	0	33	0	0.213
Orthorhombic membranes	71	0	29	0	0.276

Figure 2 C.d. spectra of Method I (—) and Method II (·····) vesicles

The error bars indicate the level of reproducibility of the data.

Figure 3 C.d. spectra of native purple membranes (—), 'shrunk' membranes (·····) and orthorhombic membranes (---)

The error bars indicate the reproducibility of the spectral data.

temperature) and 26 °C (above the phase transition). For each sample type, the far-u.v. spectra at the two temperatures were virtually indistinguishable and the calculated secondary structures differed by < 0.5 % (results not shown), although the visible c.d. spectra were very different, owing to the absence of the exciton interactions (N.A. Swords and B. A. Wallace, in preparation) at 26 °C, arising from the loss of the native trimeric packing arrangement when the protein is incorporated into vesicles comprised of fluid-phase phospholipids [28]. Hence, differences in aggregation state due to simple quaternary interactions (i.e. monomers versus trimers) are not evidenced in the region of the c.d. spectrum which is used for the analysis of secondary structures.

The effects of protein concentration within the membranes sheets on the protein spectrum were examined by comparing the spectrum of BR in native hexagonal membranes with the spectrum of 'shrunk' hexagonal membranes. The presence of native and 'shrunk' lattices were verified by X-ray diffraction (results not shown). The lattice dimensions of the membranes were 6.3 nm (63 Å) and 5.6 nm (56 Å) for the native and 'shrunk' membranes respectively, the latter being slightly smaller than that reported in the literature [24]. The unit cell size of the 'shrunk' membranes was reduced by approx. 20 % in area from the native membranes. The absorption maximum for the protein in the 'shrunk' membranes was ~ 555 nm. The c.d. spectra of the two membrane forms were slightly different from each other (Figure 3). The spectrum of the 'shrunk' membranes showed a slight shift to higher wavelength and a small increase in magnitude at around 195 nm. This was somewhat unexpected, since the tighter protein packing in this lattice form should cause an increase in absorption flattening effects which, in turn, would decrease the peak at 195 nm; but the shift suggests a change in scattering properties too. The net result is a slightly decreased calculated α -helical content.

The area/molecule in the orthorhombic unit cell is approximately the same as that of the native hexagonal form [about 11 nm² (1100 Å²)]. Like the vesicle samples, the peak maximum of the orthorhombic form is centred at 560 nm. The altered protein packing in the orthorhombic form results in a small decrease and shift to lower wavelength of the 195 nm maximum in the c.d. spectrum (Figure 3). This is not likely to be due to absorption flattening because the protein densities in the native and orthorhombic membranes are similar. The decrease in the

peak intensity could reflect an increase in differential scattering effects due to the somewhat larger size of the orthorhombic sheets compared with the native purple membrane sheets.

It should also be noted that solubilization and vesicle incorporation of BR greatly decreased the normalized NRMSD values of the calculated secondary structures relative to the native purple membrane (Table 1). The calculated secondary structures of these dispersed samples represent good fits to the measured data, since the NRMSD values are ≤ 0.1 . The calculated secondary structures of the protein in the native, 'shrunk' and orthorhombic membranes are poor fits at best, as the NRMSD values of all these are greater than 0.2. The high NRMSD values are a consequence of the absorption-flattening and differential-scattering artifacts in the measured spectra in the crystalline membranes.

DISCUSSION

This study of the effects of different environment on the secondary structure of BR yields information on the suitability of the different types of environment in which to carry out spectral analyses of membrane proteins. The structural effects on BR can be examined because, unlike most other membrane proteins, the secondary structure of the protein has been determined by independent means. Earlier c.d. studies on bacteriorhodopsin which did not account for the potential artifacts of differential scattering and absorption flattening [11,12] suggested that bacteriorhodopsin would contain a significant amount of β -sheet structure. Diffraction studies [10,19–23] now show that this is incorrect. The protein is mainly α -helical with small amounts of turn and random coil structures. Studies which did consider optical artifacts [13,35], however, produced calculated secondary structures very close to the structure estimated from diffraction studies. The disparity between the β -sheet containing secondary structure calculated by Glaeser and Jap [11] and Jap et al. [12] and the other structures is mainly due to the depression of the c.d. peak at 195 nm relative to the other peaks when optical artifacts are ignored.

Detergent solubilization results in a c.d. spectrum for BR which is closer to the theoretical spectrum than that of native membranes. However, the detergent micelle may not represent a good approximation of the native bilayer. BR is stable only for short periods of time in the detergent-solubilized form, whereas the protein retains its purple colour and conformation indefinitely in vesicles and in the native membrane fragments. The shift in the 568 nm absorption maximum to 570 nm seen for the detergent-solubilized protein also indicates a possible conformational alteration due to solubilization. There is an additional shift in the absorption of the peptide bonds in the far-u.v. region. When the protein is solubilized, the absorption band occurs at 190 nm, whereas in the membranes sheets and in the vesicles, the absorption band is measured at 193 nm [13]. Proton-pumping ability cannot be measured in the detergent-solubilized state, and this form may not be fully functional [13].

Incorporation of BR into phospholipid vesicles, either by sonication or by detergent solubilization and dialysis, disperses the protein molecules without substantially altering the environment of the protein. The area per molecule is increased to 92 nm² (9200 Å²), 8-fold increase over the three lattice forms. BR is fully functional and pumps protons after incorporation [18,37,38]. The spectra of the two vesicle forms are both similar to each other and also nearly identical with the theoretical spectrum. The low NRMSD values indicate good fits of the experimental data to the calculated secondary structures, showing that the optical artifacts in the spectra have been

significantly reduced relative to the native membrane. The close correlation of the measured spectra with the theoretical spectrum suggests that the vesicle environment maintains the protein in a native-like structural state.

For BR, the environment provided by phospholipid vesicles maintains the protein's secondary structure and proton-pumping activity. The protein can be studied by spectroscopic methods without the interference caused by the native purple membrane sheets. However, not all proteins can be successfully incorporated into vesicles. For larger membrane proteins, the conformation and/or functionality may be compromised. Spectral analyses of these proteins may thus be dependent on detergent solubilization. It should be taken into consideration, however, that detergent micelles may not maintain the protein's native structure since they differ substantially from a lipid bilayer. Hence, establishing an environment where the protein is both fully functional and in a dispersed state is important for spectral analyses.

REFERENCES

- 1 Oesterhelt, D. and Stoekenius, W. (1971) *Nature (London)* **233**, 149–152
- 2 Khorana, H. G., Gerber, G. E., Herlihy, W. C., Gray, C. P., Anderegg, R. J., Nihei, K. and Biemann, K. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5046–5050
- 3 Lemke, H. D. and Oesterhelt, D. (1981) *FEBS Lett.* **128**, 255–260
- 4 Mullen, E., Johnson, A. H. and Akhtar, M. (1981) *FEBS Lett.* **130**, 187–193
- 5 Bayley, H., Huang, K.-S., Radhakrishnan, R., Ross, A. H., Takagaki, Y. and Khorana, H. G. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2225–2229
- 6 Katre, N. V., Wolber, P. K., Stoekenius, W. and Stroud, R. M. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4068–4072
- 7 Dencher, N. A. and Heyn, M. P. (1979) *FEBS Lett.* **108**, 307–310
- 8 Oesterhelt, D. and Stoekenius, W. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2853–2857
- 9 Blaurock, A. E. and Stoekenius, W. (1971) *Nature (London)* **233**, 152–155
- 10 Henderson, R. (1975) *J. Mol. Biol.* **93**, 123–138
- 11 Glaeser, R. M. and Jap, B. K. (1985) *Biochemistry* **24**, 6398–6401
- 12 Jap, B. K., Maestre, M. F., Hayward, S. B. and Glaeser, R. M. (1983) *Biophys. J.* **43**, 81–89
- 13 Wallace, B. A. and Teeters, C. L. (1987) *Biochemistry* **26**, 65–70
- 14 Bustamante, C., Tinoco, I. and Maestre, M. F. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3568–3572
- 15 Schneider, A. S. and Harmatz, D. (1976) *Biochemistry* **15**, 4158–4162
- 16 Gordon, D. J. and Holzwarth, G. (1971) *Arch. Biochem. Biophys.* **142**, 481–488
- 17 Duysens, L. N. A. (1956) *Biochim. Biophys. Acta* **19**, 1–12
- 18 Mao, D. and Wallace, B. A. (1984) *Biochemistry* **23**, 2667–2673
- 19 Unwin, P. N. T. and Henderson, R. (1975) *J. Mol. Biol.* **94**, 425–440
- 20 Engelman, D. M., Henderson, R., McLachlan, A. D., and Wallace, B. A. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2023–2027
- 21 Henderson, R. and Unwin, P. N. T. (1975) *Nature (London)* **257**, 28–31
- 22 Baldwin, J. M., Henderson, R., Beckmann, E. and Zemlin, F. (1988) *J. Mol. Biol.* **202**, 585–591
- 23 Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E. and Downing, K. H. (1990) *J. Mol. Biol.* **213**, 899–929
- 24 Glaeser, R. M., Jubb, J. S. and Henderson, R. (1985) *Biophys. J.* **48**, 775–780
- 25 Liefer, D. and Henderson, R. (1983) *J. Mol. Biol.* **163**, 45–466
- 26 Michel, H., Oesterhelt, D. and Henderson, R. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 338–342
- 27 Oesterhelt, D. and Stoekenius, W. (1974) *Methods Enzymol.* **31**, 667–678
- 28 Cherry, R. J., Muller, U., Henderson, R. and Heyn, M. P. (1978) *J. Mol. Biol.* **121**, 283–298
- 29 Reference deleted
- 30 Michel, H., Oesterhelt, D. and Henderson, R. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 338–342
- 31 Mao, D., Wachter, E. and Wallace, B. A. (1982) *Biochemistry* **21**, 4960–4968
- 32 Chang, C. T., Wu, C.-S. C. and Yang, J. T. (1978) *Anal. Biochem.* **91**, 13–31
- 33 Lowry, O. H., Rosebrough, N. J., Farr, L. A. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 34 Chen, P. S. Jr., Toribara, T. Y. and Warner, H. (1956) *Anal. Biochem.* **28**, 1756–1758
- 35 Teeters, C. L., Eccles, J., and Wallace, B. A. (1987) *Biophysical J.* **51**, 527–532
- 36 Reference deleted
- 37 Dencher, N. A. and Heyn, M. P. (1979) *FEBS Lett.* **108**, 307–310
- 38 Grzesiek, S. and Dencher, N. A. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9509–9513