Effects of Modification of the Tyrosine Residues of Bacteriorhodopsin with Tetranitromethane

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Treatment of the purple membrane of *Halobacterium halobium* with tetranitromethane led to modification of tyrosine residues. Modification of more than 3–4 tyrosine residues per bacteriorhodopsin monomer caused a decrease in the light-induced proton-pumping ability of purple membrane in synthetic lipid vesicles, loss of the sharp X-ray-diffraction patterns characteristic of the crystal lattice, loss of the absorbance maximum at 560 nm, and change in the buoyant density of the membrane. No modification of lipid was detected. These changes were interpreted as a gradual denaturation of the protein component such that when 8–9 tyrosine residues are modified, no proton pumping is observed. Modification of less than 3–4 tyrosine residues with tetranitromethane caused an increase in light-induced proton pumping. It was possible to generate partly modified purple membrane which had completely lost the property of diffracting X-rays into the sharp pattern observed with native purple membrane, but which still retained the ability to pump protons in a vectorial manner. Retention of crystal lattice is not essential for proton pumping.

The extreme halophile *Halobacterium halobium* synthesizes distinct purple-coloured patches in the cell membrane when grown in the light and at low oxygen partial pressures (Stoeckenius & Kunau, 1968; Oesterhelt & Stoeckenius, 1974). These patches, termed the purple membrane, can occupy up to 50% of the area of the cell membrane and can be easily isolated and purified (Oesterhelt & Stoeckenius, 1974). The purple membrane contains a single species of protein, bacteriorhodopsin, which makes up 75% of the dry weight of the membrane and contains a single retinal moiety per protein molecule, bound to the protein by Schiff-base linkage to a lysine residue (Oesterhelt & Stoeckenius, 1971; Bridgen & Walker, 1976).

Bacteriorhodopsin has a relative molecular mass of 25000 (Oesterhelt & Stoeckenius, 1971; Bridgen & Walker, 1976) and is arranged within the membrane in a regular two-dimensional hexagonal lattice with three molecules per unit cell, described by the crystallographic space group P3 (Blaurock, 1975; Henderson, 1975). This unusually favourable arrangement of a membrane protein has allowed a three-dimensional structure of bacteriorhodopsin at 0.7 nm (7 Å) resolution to be prepared (Henderson & Unwin, 1975). Purple membrane therefore lends itself well to the kind of chemical analysis we have

been developing for multi-subunit enzymes and viruses (Perham, 1975). It has symmetry, it can be studied by electron microscopy and X-ray-diffraction techniques and it can be regarded as an enzyme, with light as the substrate and proton translocation as the product. In the present paper we describe the effect of treatment of the membrane with tetranitromethane. We chose this reagent because nitration of tyrosine residues can take place in hydrophobic as well as hydrophilic environments (Dickerson *et al.*, 1971; Myers & Glazer, 1971); modification of tyrosine residues might reveal their part, if any, in the biological activity of the membrane, and the formation of nitrotyrosine can be the first step in a method for preparing chemically-defined heavy-atom derivatives of proteins (Riley & Perham, 1973), which would facilitate the interpretation of electron-density maps.

Materials and Methods

Materials

The pyruvate dehydrogenase complex from *Escherichia coli* was a gift from Dr. G. Hale, and potassium cholate was a gift from Dr. J. Metcalfe. Horse heart cytochrome *c* (type III), 13-*cis*-retinal (type XV), and valinomycin were bought from Sigma (London) Chemical Co., Poole, Dorset, U.K.; tetranitromethane was bought from Aldrich Chemical Co., Wembley, Middlesex, U.K.

Asolectin (95% purified soya-bean phosphatides)

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was bought from Associated Concentrates, New York, NY, U.S.A., and further purified by exhaustively washing and stirring in dried acetone, filtering, drying under vacuum, dissolving in diethyl ether, and precipitating from dried acetone. This procedure was repeated several times. The product was dried under vacuum and stored under N_2 in small batches at $-15^\circ C$. *Halobacterium halobium*, strain R1, was grown and the purple membrane extracted and purified as described by Oesterhelt & Stoekenius (1971).

Treatment of purple membrane with tetranitromethane

The procedure was essentially that advocated for proteins by Riordan & Vallee (1972). Dark-adapted purple membrane was incubated with tetranitromethane in the dark at approx. $32^\circ C$, and the reaction stopped by passing the mixture, in the dark, down a column (25 cm $\times$ 1 cm) of Sephadex G-25 (medium) equilibrated with water at $20^\circ C$. The concentration of bacteriorhodopsin in the effluent was measured by amino acid analysis. All spectra of the purple membrane samples were recorded in water on a Cary 118 spectrophotometer at room temperature.

Lipid analysis

Lipids from native and tetranitromethane-treated purple membrane were extracted with chloroform/methanol (4:1, v/v), dried, dissolved in chloroform and run on thin-layer plates (Merck, pre-coated silica gel, F-254). Three different solvent systems were used: (1) chloroform/methanol/acetic acid/water (75:22:8:3, by vol.); (2) chloroform/methanol/water (65:30:4, by vol.); (3) chloroform/methanol/water/aq. NH_3 (sp.gr. 0.880) (65:30:3:1, by vol.). The spots were located by soaking the plates in a solution of approx. 15% (w/v) $(NH_4)_2SO_4$ saturated with ceric sulphate, drying and charring the spots.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Purple membrane samples were run in 7.5% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate by the method of Shapiro *et al.* (1969). The gels were calibrated for relative molecular mass as described by Perham & Thomas (1971) with cytochrome *c* (Margoliash & Frohwirt, 1959) and the component enzymes of the pyruvate dehydrogenase complex (Perham & Thomas, 1971) as markers.

Reconstitution in lipid vesicles

Purple membrane was incorporated into vesicles of asolectin essentially by the method of Racker & Stoekenius (1974). Potassium cholate and asolectin were added to a suspension of purple membrane in 0.15M-KCl to final proportions bacteriorhodopsin: asolectin:potassium cholate of 1:4:4 (by wt.) and a cholate concentration of 2% (w/v). This mixture was

sonicated in a Megason sonic bath (Ultrasonic Instruments International, New York, NY, U.S.A.), until clear and dialysed for 5 days against 4×9 litres of 0.15M-KCl to remove the cholate. Illumination was provided by a 650W lamp (Atlas A1/233) without the use of filters or lenses. Samples were prepared in batches with a control (unmodified purple membrane) and the pumping activity, measured as ng-atoms of protons pumped to achieve steady pH differences per nmol of protein (typical values 1.0–1.2), was expressed as a percentage of the control result.

Sucrose-density-gradient centrifugation

Samples of native or modified purple membrane in water were layered on sucrose density gradients (30–60%, w/v), and run in a Beckman L5-50 ultracentrifuge at 8500g (r_{av} , 11.8 cm) for 20 h at $4^\circ C$ using an SW27 rotor. The coloured bands were collected and a measured volume was weighed at $21^\circ C$ to give the density.

Electron microscopy

Samples were negatively stained with 1% ammonium molybdate and photographed with help from Dr. D. Ellar and Dr. T. M. A. Wilson, using an AEI electron microscope, model 801.

X-ray scattering

The scattering of X-rays by wet pelleted samples of native and modified purple membrane was kindly measured by Dr. R. Henderson of the M.R.C. Laboratory of Molecular Biology, Cambridge (see Henderson, 1975). The method was used only to detect the presence or absence of scattering from the regular lattice arrangement of bacteriorhodopsin molecules in the membrane (Blaurock & Stoekenius, 1971; Blaurock, 1975; Henderson, 1975).

Amino acid analysis

Samples were hydrolysed in sealed evacuated tubes at $105^\circ C$ in 5.7M-HCl containing 5 mM-2-mercaptoethanol for 24, 48 and 72 h respectively. The amino acids were analysed with a Rank Hilger Chromaspek Amino Acid Analyser. The amount of nitrotyrosine present was calculated by using the colour constant for aspartic acid (D. L. Bates, personal communication). The sum of tyrosine plus nitrotyrosine residues from purple membrane treated with tetranitromethane was always less than the number of tyrosine residues in the unmodified purple membrane, in agreement with results obtained by other workers with other proteins (Boesal & Carpenter, 1970; Vincent *et al.* 1970; Burleigh *et al.*, 1976). Therefore tyrosine modification was expressed in terms of the percentage of tyrosine residues that were not recovered as tyrosine after amino acid analysis. In general, only about 60% of the modified tyrosine

residues were found as nitrotyrosine after amino acid analysis.

The protein concentrations of samples were calculated by using the molecular composition values of lysine (7), arginine (7), aspartic acid (15), glutamic acid (16), valine (20) and phenylalanine (13) as published by Bridgen & Walker (1976). Our analyses indicated 10 tyrosine residues per molecule of bacteriorhodopsin, in agreement with Kushwaha *et al.* (1976) and Keefer & Bradshaw (1977), compared with 11 tyrosine residues per mol found by Bridgen & Walker (1976). We have used the value of 10 throughout this paper.

Results

Proton-pumping ability

By increasing the molar ratio of tetranitromethane to bacteriorhodopsin in the reaction mixture (from 1.0 to 100 mol/mol), preparations of purple membrane were obtained in which an increasing proportion of the tyrosine residues had been modified. Bacteriorhodopsin contains no cysteine residues (Oesterhelt & Stoekenius, 1971; Bridgen & Walker, 1976; Keefer & Bradshaw, 1977) and, by amino acid analysis, we detected no effect of tetranitromethane on methionine or histidine residues. As a result of the treatment, the colour of the membrane changed from purple (unmodified) to blue-purple (29% tyrosine residues modified) to red (61% tyrosine residues modified) and finally to orange-yellow (84% tyrosine residues modified).

Samples of the various membrane preparations were incorporated into synthetic lipid vesicles and tested for their light-dependent ability to pump protons at 22°C. Fig. 1(a) shows that in the early stages of tyrosine modification, proton-pumping ability actually rose about 20% above the control value and then, after about 35% of the tyrosine residues had been modified, steadily fell away to zero. Over 80% of the tyrosine residues in bacteriorhodopsin could be modified under the conditions we used. The linearity of the proton-pumping assay with respect to bacteriorhodopsin was checked by preparing vesicles in which the protein concentration was 162 nmol/ml, compared with the concentrations of 40–110 nmol/ml used in the nitration experiments, and showing that the extent of proton pumping in diluted samples of this stock solution was directly proportional to the protein concentration. Racker & Stoekenius (1974) and Racker & Hinkle (1974) using protein:valinomycin ratios of 4200 (w/w) and 500 (w/w) respectively showed that valinomycin increased the rate of proton uptake in the light and of proton release in the dark from purple-membrane/lipid vesicles. The extent of proton uptake was sometimes increased and sometimes decreased in

Fig. 1. Effect of tyrosine modification on light-dependent proton pumping by purple membrane in synthetic vesicles and on X-ray scattering

(a) Purple membrane was modified by treatment with tetranitromethane and the light-dependent ability to pump protons into synthetic vesicles was measured as a function of the tyrosine residues modified. For other details, see the text. (b) The horizontal line represents the percentage of tyrosine residues modified as in (a), and the vertical lines represent the presence (+) or absence (-) of X-ray scattering from the lattice in wet, pelleted samples of the membrane.

their experiments. The presence of valinomycin caused an approximate doubling of the rate of light-induced proton uptake in our vesicle preparations and a 64% increase in the extent of proton uptake when used at a protein:valinomycin ratio of 860 (w/w). These experiments with valinomycin make it clear that the activities we were measuring were due to light-induced proton-pumping rather than to some other process.

X-ray-diffraction analysis

Samples of purple membrane after treatment with tetranitromethane were examined by X-ray-diffraction analysis. Increasing modification caused loss of the crystal lattice and the correlation of loss of lattice with extent of tyrosine modification is shown in Fig. 1(b).

Fig. 2. Effect of tyrosine modification on the spectrum of purple membrane

Each line represents a sample with a different percentage of tyrosine residues modified: (a) 0%, (b) 29%, (c) 34%, (d) 37%, (e) 49% and (f) 67%. The temperature for experiments was 22°C, the pathlength of spectrophotometer cell was 1 cm and the concentration of bacteriorhodopsin was 0.53 mg/ml.

Extraction of retinaloxime

The amount of retinaloxime that could be extracted from purple membrane by the hydroxylamine treatment of Oesterhelt & Stoekenius (1971) was decreased by up to 20% after modification of the membrane with tetranitromethane. However, it is clear that this is too small to account for the observed loss of proton-pumping ability.

Spectral properties

Increasing modification of tyrosine residues was accompanied by a change in the form of the spectrum (Fig. 2), and with a loss in absorbance at 560 nm when greater than 30% of the tyrosine residues had been modified. No change in the spectrum in the region of 560 nm was produced by the addition of 13-*cis*-retinal to the modified membrane, indicating the absence of simple apomembrane (Oesterhelt & Schuhmann, 1974).

The absorbance at 360 nm, unlike that at 560 nm, increased regularly with increasing tyrosine modification, from which an apparent millimolar extinction coefficient of 2.5 per tyrosine residue modified was calculated. No special significance should be attached to this extinction coefficient since not all the modified tyrosine residues were recovered as nitrotyrosine on amino acid analysis and the chemical identity of the absorbing species is somewhat obscure. Oesterhelt *et al.* (1973) showed that the retinylidene protein formed at high concentrations of dimethyl sulphoxide (80–95%) absorbed maximally at 381 nm with an extinction coefficient approximately two-thirds of that of the unmodified purple membrane

at 560 nm. There is no evidence of such a band in the spectra shown in Fig. 2 and the change in A_{560} can best be attributed to the presence of modified tyrosine residues. Thus there is no evidence of protein denaturation sufficient to cause substantial disruption of protein-retinal interactions, and little change noted in the A_{560} , until about 30% of the tyrosine residues have been modified.

Sucrose-density-gradient centrifugation

Samples of membrane containing 0% (purple), 29% (purple-red), 61% (red) and 84% (orange-yellow) tyrosine residues modified by treatment with tetranitromethane ran as single sharp bands on sucrose density gradients. A mixture of these samples separated into a sharp purple-coloured band with a density of 1.174 g/ml (0 and 29% tyrosine residues modified) and a sharp orange-coloured band with a density of 1.192 g/ml (61 and 84% tyrosine residues modified). This is consistent with two principal physical states of the membrane.

Electron microscopy

Electron micrographs of negatively-stained membrane showed that there was a progressive decrease in the average diameter of the sheets from about 850 nm for unmodified membrane to about 150 nm for a sample with 51% of the tyrosine residues modified. As the number of modified tyrosine residues increased, the electron micrographs also showed an increased number of small membrane particles, some quite irregular in outline.

Lipid analysis

The lipid was extracted from samples of native and tetranitromethane-treated purple membrane and compared by t.l.c. When 67% of the tyrosine residues of bacteriorhodopsin were modified, no evidence was obtained for a change in the pattern of spots in any of the three solvent systems used. Similarly, 13-*cis*-retinal, and 13-*cis*-retinal that had been treated with a 35-fold molar excess of tetranitromethane at pH 8.0, appeared identical on thin-layer chromatograms in all three solvent systems.

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate

Samples of purple membrane treated with tetranitromethane were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 3). A heavily staining band corresponding to that from the unmodified membrane was observed, together with a faint band of apparent relative molecular mass 38 400. This higher-molecular-mass species was present in all samples from membrane treated with tetranitromethane that had more than 15% of the tyrosine residues modified. Quantitative

Fig. 3. Sodium dodecyl sulphate polyacrylamide-gel electrophoresis of bacteriorhodopsin after treatment of purple membrane with tetranitromethane

(a) Untreated purple membrane; (b) purple membrane with 29% of the tyrosine residues modified; (c) purple membrane with 83% of the tyrosine residues modified. For other details, see the text.

scanning of the gels showed that the intensity of stain in the higher-molecular-weight component did not increase greatly with the extent of tyrosine modification, reaching a maximal value of about 16% of the colour found in the monomer band.

In our experiments, as in those of others, bacteriorhodopsin showed an apparent relative molecular mass of about 20000 on gel electrophoresis, although its true relative molecular mass is 25000 (Bridgen & Walker, 1976). The higher-molecular-mass species is therefore running in the position of a cross-linked dimer.

Discussion

Treatment of purple membrane with tetranitromethane caused first an increase (up to 35% tyrosine residues modified) and then a gradual and complete inhibition (at 85% tyrosine residues modified) of the light-induced proton-pumping ability, measured after incorporation of the modified membrane into synthetic lipid vesicles (Fig. 1a). The chemical modification also led to loss of the initially sharp X-ray-diffraction pattern from the membrane (Fig. 1b), signifying complete loss of long-range order in the crystal lattice after about 40% of the tyrosine residues had been modified. On the other hand, the retention beyond this stage of proton-pumping ability, and of the absorbance at 560nm caused by retinal-protein interactions (Fig. 2), indicates that substantial disruption of the local structure of bacteriorhodopsin did not occur until the tyrosine residues had been extensively modified. The loss of

long-range order in the membrane revealed by X-ray-diffraction analysis can be attributed to changes in the bacteriorhodopsin since the treatment with tetranitromethane appears to have caused no change in the lipid or retinal components, as judged by t.l.c. It is not known whether the crystalline array of unmodified purple membrane is retained after incorporation of bacteriorhodopsin into the synthetic lipid vesicles described in the present paper. However, it is extremely unlikely that modified purple membrane that shows no sharp X-ray-diffraction pattern could form crystalline arrays of modified bacteriorhodopsin in artificial lipid vesicles. We infer that possession of a crystal lattice is not essential to proton pumping. This is consistent with the observations of proton pumping in various preparations of reconstituted vesicles lacking in protein crystallinity (Hwang & Stoeckenius, 1977), the demonstration of proton pumping in reconstituted vesicles above and below the lipid phase transition (Racker & Hinkle, 1974), and by the reconstitution of functional bacteriorhodopsin on the addition of retinal to cells in which the precursor bacterio-opsin molecules appear to have diffused laterally out of the characteristic two-dimensional lattice (Sumper *et al.*, 1976; Cherry *et al.*, 1977). Cherry *et al.* (1978) have reviewed and discussed much of this evidence.

The initial rise in light-induced ability to pump protons that accompanied the early stages of tyrosine modification in purple membrane (Fig. 1a) could be a direct effect. However, the increase is manifest at low extents of tyrosine modification, and could equally well be due to the presence of an increased proportion of vesicles in which the purple membrane is oriented in the same direction. The orientation of purple membrane in synthetic vesicles has been shown to be strongly dependent on the pH during reconstitution (Happe *et al.*, 1977), a factor that was not controlled carefully in the present experiments based on Racker's method (Racker, 1973; Racker & Stoeckenius, 1974). Chemical modification of the membrane could affect its propensity to orient in the vesicles.

It is interesting that a number of properties of the purple membrane were largely unaffected until about 35% of the tyrosine residues in bacteriorhodopsin had been modified. This is true of the proton-pumping activity (Fig. 1), of X-ray scattering from the crystal lattice (Fig. 1b), of the amount of retinal that could be extracted from the membrane as retinaloxime, of the absorbance in the region of 560nm (Fig. 2), and of the buoyant density of the membrane in sucrose-density-gradient centrifugation. The simplest explanation would be that about 3–4 tyrosine residues per chain can be modified with little or no effect on the bacteriorhodopsin molecule, but that further modification of tyrosine residues leads to significant changes in the properties tested.

Amino acid-sequence analysis will be required to elaborate this work and to show whether particular tyrosine residues are involved. It would also be helpful to know the chemical identity of the modified tyrosine residues that cannot be recovered as nitro-tyrosine since these comprise about 40% of the tyrosine residues affected by the treatment with tetranitromethane. If they are taking part in cross-links in the molecule, e.g. as dityrosine, such cross-links must be mostly intramolecular since sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed comparatively little bacteriorhodopsin running in the dimer position.

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References

- Blaurock, A. E. (1975) *J. Mol. Biol.* **93**, 139–158
 Blaurock, A. E. & Stoeckenius, W. (1971) *Nature (London)* **233**, 152–155
 Boesal, R. W. & Carpenter, F. H. (1970) *Biochem. Biophys. Res. Commun.* **38**, 678–682
 Bridgen, J. & Walker, I. D. (1976) *Biochemistry* **15**, 792–798
 Burleigh, B. D., Lin, W.-K. & Ward, D. N. (1976) *J. Biol. Chem.* **251**, 308–315
 Cherry, R. J., Heyn, M. P. & Oesterhelt, D. (1977) *FEBS Lett.* **78**, 25–30
 Cherry, R. J., Müller, U., Henderson, R. & Heyn, M. P. (1978) *J. Mol. Biol.* **121**, 283–298
 Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A. & Margoliash, E. (1971) *J. Biol. Chem.* **246**, 1511–1535
 Happe, M., Teather, R. M., Overath, P., Knobling, A. & Oesterhelt, D. (1977) *Biochim. Biophys. Acta* **465**, 415–420
 Henderson, R. (1975) *J. Mol. Biol.* **93**, 123–138
 Henderson, R. & Unwin, P. N. T. (1975) *Nature (London)* **257**, 28–32
 Hwang, S.-B. & Stoeckenius, W. (1977) *J. Membr. Biol.* **33**, 325–350
 Keefer, L. M. & Bradshaw, R. A. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 1799–1804
 Kushwaha, S. C., Kates, M. & Stoeckenius, W. (1976) *Biochim. Biophys. Acta* **426**, 703–710
 Margoliash, E. & Frohwirt, N. (1959) *Biochem. J.* **71**, 570–572
 Myers, B. & Glazer, A. N. (1971) *J. Biol. Chem.* **246**, 412–419
 Oesterhelt, D. & Schuhmann, L. (1974) *FEBS Lett.* **44**, 262–265
 Oesterhelt, D. & Stoeckenius, W. (1971) *Nature (London)* **233**, 149–152
 Oesterhelt, D. & Stoeckenius, W. (1974) *Methods Enzymol.* **31A**, 667–678
 Oesterhelt, D., Meentzen, M. & Schuhmann, L. (1973) *Eur. J. Biochem.* **40**, 453–463
 Perham, R. N. (1975) *Philos. Trans. R. Soc. London Ser. B* **272**, 123–136
 Perham, R. N. & Thomas, J. O. (1971) *FEBS Lett.* **15**, 8–12
 Racker, E. (1973) *Biochem. Biophys. Res. Commun.* **55**, 224–230
 Racker, E. & Hinkle, P. C. (1974) *J. Membr. Biol.* **17**, 181–188
 Racker, E. & Stoeckenius, W. (1974) *J. Biol. Chem.* **249**, 662–663
 Riley, M. & Perham, R. N. (1973) *Biochem. J.* **131**, 625–633
 Riordan, J. E. & Vallee, B. L. (1972) *Methods Enzymol.* **25B**, 515–521
 Shapiro, A. L., Vinuela, E. & Maizel, J. V., Jr. (1969) *Anal. Biochem.* **29**, 505–514
 Stoeckenius, W. & Kunau, W. H. (1968) *J. Cell Biol.* **38**, 337–357
 Sumper, M., Reitmeier, H. & Oesterhelt, D. (1976) *Angew. Chem. Int. Ed. Engl.* **15**, 187–194
 Vincent, J. P., Lazdunski, M. & Delaage, M. (1970) *Eur. J. Biochem.* **12**, 250–257