LBL-16446
c.2

Lawrence Berkeley Laboratory

UNIVERSITY OF CALIFORNIA

RECEIVED
LAWRENCE
BERKELEY LABORATORY
SEP 26 1983
LIBRARY AND
DOCUMENTS SECTION

APPLIED SCIENCE DIVISION

To be presented at the Conference on Transport of Proteins, Ions and Electrons through Mitochondrial Membranes, University College Cork, Ireland, September 27-30, 1983; and to be published in Biochemical Transactions

CARBOXYL GROUPS AND THE PROTON PUMP OF BACTERIORHODOPSIN

J.M. Herz and L. Packer

August 1983

TWO-WEEK LOAN COPY

This is a Library Circulating Copy which may be borrowed for two weeks. For a personal retention copy, call Tech. Info. Division, Ext. 6782.

APPLIED SCIENCE DIVISION

LBL-16446
c.2

Prepared for the U.S. Department of Energy under Contract DE-AC03-76SF00098

DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor the Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or the Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or the Regents of the University of California.

CARBOXYL GROUPS AND THE PROTON PUMP OF BACTERIORHODOPSIN

Jeffrey M. Herz* and Lester Packer

Membrane Bioenergetics Group and Department of Physiology-Anatomy
Lawrence Berkeley Laboratory, University of California,
Berkeley, CA 94720, U.S.A.

*Present address: Division of Pharmacology
Department of Medicine, M-013H
University of California at San Diego
La Jolla, CA, 92093

ABBREVIATIONS

EEDQ: N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline;

Tempamine: 4-amino-2,2,6,6,-tetramethyl-piperindino-N-oxyl;

TA-BR: tempamine-spin labeled bacteriorhodopsin;

AES: 2-aminoethanesulfonic acid;

5NS: 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl;

10NS: 2-(8-carboxyoctyl)-2-octyl-4,4-dimethyl-3-oxazolidinyloxyl;

15NS: 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl.

The purple membrane isolated from Halobacterium halobium contains only a single protein, bacteriorhodopsin, which functions as a light-driven proton pump. Substantial structural information has been obtained which has led to specific models of protein structure in the membrane (Engelman, et al., 1982; Huang, et al., 1982; Agard and Stroud, 1982). The retinal chromophore of bacteriorhodopsin is bound to the ε-amino group of lysine-216 by a Schiff base linkage (Katre et al., 1981, Bayley et al., 1981). Light absorption by the chromophore initiates a photochemical reaction cycle which involves configurational changes in retinal and conformational changes of the protein (for a review, see Stoeckenius et al., 1979). The role of the Schiff base linkage in the reaction mechanism is well established, but the contribution of the protein moiety to chromophore structure, the photochemical reaction cycle, and to the proton translocation mechanism is largely unknown.

The carboxyl residues of aspartic and glutamic acid have been suggested to have the following roles in bacteriorhodopsin:

(i) Structural. By forming ion-pairs within the membrane with the positively charged groups of lysine and/or arginine (Packer et al., 1979; Engelman et al., 1980); (ii) Chromophoric. By interacting with retinal and the Schiff base nitrogen to modulate absorption properties (Fisher and Oesterhelt, 1980); and (iii) Catalytic. By functioning as proton translocating groups during the photoreaction cycle. Chemical modification studies of carboxyl groups has indicated structural involvement in the photocycle (Herz and Packer, 1981). Recently, Fourier transform and kinetic infrared spectroscopy studies (Rothschild et al., 1981; Siebert et al., 1982) have demonstrated changes in the protonation state of carboxyl groups correlated with the photocycle. It was suggested that these groups reside in a hydrophobic membrane environment. In addition, current models of bacteriorhodopsin

structure place several carboxyl residues within hydrophobic membrane-protein domains. Thus, knowledge of the topography of carboxyl residues is central to the understanding of the structure and function of the light-driven proton pump.

We have used chemical modification techniques to attach reporter groups to the carboxyl residues of bacteriorhodopsin. The spin-label and chromophoric groups employed display characteristic spectra which are sensitive to changes in their microenvironment. This approach has allowed the correlation of functional properties of carboxyl groups with their local topography and mobility in distinct membrane-protein domains.

ESR Evidence for Buried Carboxyl Groups

Bacteriorhodopsin was covalently spin-labeled by reacting 4-amino-2,2,6,6-tetramethyl-piperdino-N-oxyl (Tempamine†) with protein carboxylic amino acid residues using N-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroxyquinoline (EEDQ) as a coupling agent. EEDQ is a hydrophobic, highly specific reagent for the activation of carboxyl residues (Belleau and Malek, 1968). EEDQ concentrations $\leq$ 1.0 mM produced insignificant labeling, while higher concentrations (10-30 mM) yielded progressively increased levels of nucleophile coupling (2.0-4.25 Tempamine per bacteriorhodopsin). Only a few of the 19 carboxyl residues found in bacteriorhodopsin were modified by this procedure. ESR spectra clearly revealed an increase in the immobilized spin content of samples that was correlated with higher stoichiometries of labeling (Fig. 1, left panel). Higher stoichiometries of labeling resulted in a broadening of the central line width (ΔH_0), growth of the low field shoulder of the h_{+1} peak, and the appearance of two new extrema with a maximum splitting of 68.4 G characteristic of a strongly immobilized signal. Spin-labeled 2.1 TA-BR largely retained unmodified protein spectral characteristics and proton pumping activity. However, higher stoichiometries of labeling inhibited proton

pumping activity and resulted in bleaching the 570 nm retinal-protein chromophore (Herz, 1983). The 4.25 TA-BR sample exhibited a 370 nm spectral peak characteristic of free retinal and no photocycle activity. This suggested the presence of specific buried carboxyl residues essential for proton pump activity.

Indeed, ESR spectra appear to be composed of at least two components of different mobility (Figure 1, left panel). In order to distinguish spectral components located at the protein surface from other labeled sites, the spin exchange broadening between protein bound spin labels and paramagnetic ions in solution (which requires direct contact between colliding paramagnetic species; Keith et al., 1977) was used to deconvolute the complex ESR spectrum. Titration of spin-labeled native bacteriorhodopsin with $Na_3Fe(CN)_6$ resulted in the progressive broadening of the spin signal until only a highly immobilized signal remained at high $Fe(CN)_6^{3-}$ concentrations (Fig. 1, right panel). Low concentrations of $Fe(CN)_6^{3-}$ selectively quenched the more mobile components of the spectrum, while the immobilized components were not affected. At 200 mM $Fe(CN)_6^{3-}$, only strongly immobilized spin signals remained. In addition, computer subtraction of $Fe(CN)_6^{3-}$ quenched spectra from control spectra revealed high mobility difference spectra (bottom of Fig. 1, right panel).

The presence of a large immobilized ESR signal that remained at high $Fe(CN)_6^{3-}$ concentrations suggested the existance of buried spin label residues in the native membrane-protein structure. Denaturation is expected to open the bacteriorhodopsin structure and increase the accessibility of $Fe(CN)_6^{3-}$ to previously buried protein domains. The ESR spectrum of TA-BR in high concentrations of SDS-urea appeared as a homogeneous population of spins possessing high mobility ($\tau_c = 7.61 \times 10^{-10}$ s), indicative of the release of previously immobilized labels. In addition, ESR spectra showed that $Fe(CN)_6^{3-}$ was substantially more effective in quenching the SDS-urea treated signal at

equivalent concentrations of probe.

The effects of paramagnetic broadening agents showed that two distinct protein domains were spin-labeled, and spectral characteristics demonstrated that surface groups were considerably more mobile than buried groups. The presence of the large immobilized component in 2.1 TA-BR that remained in the presence of high $Fe(CN)_6^{3-}$ concentrations but disappears upon denaturation is evidence for the existence of buried labels in protein domains.

Membrane Location of a Buried Carboxyl Group

The paramagnetic broadening of a series of stearic acid spin labels bound to purple membranes was studied in order to: (i) verify that the accessibility of $Fe(CN)_6^{3-}$ quenching agent was limited to the purple membrane surfaces, and (ii) to employ stearic acid as an empirical molecular ruler to estimate the depth of buried protein spin labels. The second goal was achieved by examining the paramagnetic broadening of the stearic acid spin labels by Cu^{2+} which is capable of acting at a distance by a through-space dipolar interaction (Hyde et al., 1979).

The effects of $Fe(CN)_6^{3-}$ and Cu^{2+} on the spectra of 5-, 10- and 16-doxylstearic acid spin labels were compared. As seen in Figure 2, the ESR spectra of 5NS bound to purple membranes exhibited a strongly immobilized spectrum plus a small contribution from an aqueous mobile component. The ESR spectral data were used to calculate the apparent order parameter ($S^{app} = 0.91$), and the half amplitude of motion ($\gamma = 21°$). These values were used to determine the nitroxide moiety distance from the membrane surface, which was found to be 5.9 Å (Herz, 1983). The amplitude and shape of the spectra were unaffected by the presence of 10-100 mM $Fe(CN)_6^{3-}$ except for the loss of the small, aqueous mobile component.

In contrast to the strongly immobilized 5NS and 10NS spectrum, the

16NS spectrum is only moderately immobilized ($2\,\bar{A}_{||}$ = 56.8 G, S^{app} = 0.59, γ = 45°). This orientation gives a calculated distance of 16.6 Å for the 16NS nitroxide from the surface. As in the case of 5NS, high concentrations of $Fe(CN)_6^{3-}$ broadened only the aqueous mobile component while leaving the immobilized component unaffected.

The dipolar broadening of 5NS, 10NS and 16NS by Cu^{2+} exhibited even greater differences when compared to spin-exchange broadening by $Fe(CN)_6^{3-}$ (Fig. 2). The 5NS spectra was almost completely broadened while the more deeply buried 10NS and 16NS nitroxides were substantially less affected. Thus, the line height reduction by Cu^{2+} was inversely correlated with the depth of the nitroxide from the membrane surface. These results indicate that $Fe(CN)_6^{3-}$ only broadens spin labels by collisional interactions and that it does not penetrate the purple membrane to the depth of the 5NS nitroxide. In contrast, the dipolar broadening effects of Cu^{2+} extend considerably beyond the membrane surface.

As a biochemical approach to corroborate the results obtained with paramagnetic quenching agents, a sequential double modification procedure was developed to selectively prereact and thereby block surface carboxyl residues so that spin-labeling of only buried residues occurred. In order to block surface residues, the first carboxyl modification used a permanently charged non-spin label nucleophile, aminoethanesulfonic acid (AES). After extensive washing, the second modification using EEDQ and Tempamine was carried out as in the single step modification. The stoichiometry of the double modification samples prepared by this procedure contained an average of 0.4 spins per bacteriorhodopsin molecule. ESR spectra of the AES/TA double modified bacteriorhodopsin showed only a strongly immobilized spin signal with a central line width of 7.6 G and a maximum hyperfine splitting of 68.7 G (Fig. 2). The strongly immobilized ESR signal was not broadened by high concentrations of $Fe(CN)_6^{3-}$

as expected for a buried spin residue. Paramagnetic interactions of the AES/TA-BR spin label with Cu^{2+} were also examined to estimate its depth from the membrane surface. The interaction of 10 mM Cu^{2+} and AES/TA-BR resulted in only a small decrease in amplitude (75% of control remained) with no apparent broadening. Comparison of the central line height reduction due to Cu^{2+} interaction with the stearic acid spin labels and AES/TA-BR showed that 16NS and AES/TA-BR are affected similarly, and hence, that the spin-labeled carboxyl group is deeply buried within the protein. Thus, we estimate the minimum distance of the buried carboxyl group(s) from the membrane surface to be 16.6 Å.

Reporter group-protein chromophore interactions in purple membranes.

Carboxyl groups on bacteriorhodopsin were labeled with nitrotyrosine methyl ester, a pH-sensitive, chromophoric reporter group. The spectral and ionization properties of the reporter group were used to monitor the protein environment in the vicinity of the retinal binding site (Herz *et al.*, 1983b). The absorption spectra (300-700 nm) for the alkaline forward titrations of modified bacteriorhodopsin in purple membranes (Fig. 3, upper half) covers the visible absorption bands of bacteriorhodopsin that arise from retinal-protein interactions, as well as the spectral region of the reporter group. Alkaline titration from pH 7.0 to pH 11.0 resulted in the formation of a new peak at 428 nm characteristic of the nitrotyrosinate ion and the simultaneous decrease in the absorbance of the 570 nm band, exhibiting an isobestic point at 480 nm. The isobestic point between the nitrotyrosine methyl ester chromophore and the bacteriorhodopsin 570 nm chromophore indicated interactions between the reporter group and retinal. The absorbance changes at 428 nm (Fig. 3, right panel) were used to obtain a pK for the spectral transition of the reporter group associated with the forward titration. The protein-bound nitrotyrosine

methyl ester residue if found to have a pK of about 10-11, at least 3 pK units higher than the model compound in solution.

The backward (acidic) titration of the same sample after 24 hr under alkaline conditions revealed a new pattern of spectral changes (Fig. 3, lower half). The decrease in absorbance at 428 nm was no longer coupled to absorbance changes at 570 nm. Instead, a new isosbestic point between a small peak at 360 nm, characteristic of the nitrotyrosyl, and the 428 nm nitrotyrosinate chromophore appeared. The backwards titration also demonstrated a new, substantially lower pK = 8.0 for the reporter group (Fig. 3, right panel). The results suggest that this reporter group is bound to a carboxyl group which resides in a hydrophobic membrane domain in the native state.

The unusual absorption spectra of the reporter group in modified purple membranes is correlated with the unusually high pK of 10-11. These spectral and ionization properties may be due to either an electrostatic interaction with a nearby group or a change in the polarity of the environment. Examples of nitrotyrosine pK changes, as well as theory, demonstrate that a change to a more hydrophobic environment will increase the pK of nitrotyrosine residues. Exposure to alkaline pH resulted in a localized configurational change that transferred the reporter group from a buried hydrophobic membrane domain into a more aqueous environment near the membrane surface. As a result of the configurational change, reporter group-retinal interactions were lost.

Summary

Chemical modification and spin-labeled studies of purple membranes have provided the first evidence for buried carboxyl residues within the hydrophobic, membrane-protein domains. These findings are consistent with

current models of bacteriorhodopsin structure (Agard and Stroud, 1982; Engelman et al., 1982; Huang et al., 1982). In addition, our studies have identified distinct functional roles for the buried carboxyl residues. Spin label data showed that modification of buried residues resulted in loss of protein activity. A pH-sensitive, chromophoric reporter group demonstrated that a carboxyl residue in a hydrophobic membrane environment interacts with the retinal chromophore of bacteriorhodopsin. The essential nature of carboxyl residues in the activity of membrane proton pumps has been demonstrated for cytochrome oxidase and the ATPase/synthetases of chloroplasts, mitochondria and bacteria. Hence, this study demonstrating essential carboxyl groups in bacteriorhodopsin appears consistent with the idea that carboxyl residues in hydrophobic environments may be a general feature required for activity of membrane proton pumps.

Acknowledgement

Research supported by the Office of Biological Energy Research, Division of Basic Energy Sciences, U.S. Department of Energy, #DE-AC03-76SF00098.

REFERENCES

Agard, D.A. and Stroud, R.M. (1982) Biophys. J. 37, 589-602.

Bayley, H., Huang, K.-S, Radhakrishman, R., Ross, A.H., Takagaki, Y. and Khorana, H.G. (1981) Proc. Natl. Acad. Sci. USA 78, 2255-2229.

Belleau, B. and Malek, G. (1968) J. Am. Chem. Soc. 90, 1651-1652.

Engelman, D.M., Henderson, R., McLachlan, A.D., and Wallace, B.A. (1980) Proc. Natl. Acad. Sci. USA 77, 2023-2027.

Engelman, D.M., Goldman, A. and Steitz, T.A. (1982) In Methods in Enzymology, Vol. 88 (Packer, L., ed.), pp. 81-88, Academic Press, New Yor,

Fisher, U., and Osterhelt, D. (1980) Biophys. J. 31, 139-146

Herz, J.M. (1983) Ph.D. Dissertation; University of California at Berkeley, U.S.A.

Herz, J.M. and Packer, L. (1981) FEBS Lett. 131, 158-164.

Herz, J.M., Mehlhorn, R.J. and Packer, L. (1983a) J. Biol. Chem. 258, 9899-9907.

Herz, J.M., Hrabeta, E. and Packer, L. (1983b) Biochem. Biophys. Res. Commun. 114, 872-881.

Huang, K.-S, Radhakrishnan, R., Bayley, H. and Khorana, H.G. (1982) J. Biol. Chem. 257, 13616-13623.

Hyde, J.S., Swartz, H.M. and Antholine, W.E. (1979) In Spin Labeling Two (Berliner, L.J., ed.), pp. 71-113, Academic Press, New York.

Katre, N.V., Wolber, P.K., Stoekenius, W. and Stroud, R.M. (1981) Proc. Natl. Acad. Sci. 78, 4068-4072.

Keith, A.D., Snipes, W., Mehlhorn, R.J., Gunter, T. (1977) Biophys. J. 19, 205-218.

Packer, L., Tristram, S., Herz, J.M., Russell, C. and Borders, C.L. (1979) FEBS Lett. 108, 243-248.

Rothschild, K.J., Zagaeski, M. and Cantore, W.A. (1981) Biochem. Biophys. Res. Comm. 103, 483-489.

Siebert, F., Mantele, W. and Kreutz, W. (1982) FEBS Lett. 141, 82-87.

Stoeckenius, W., Lozier, R. and Bogomolni, R.A. (1979) Biochim. Biophys. Acta 505, 215-278.

FIGURE LEGENDS

Fig. 1. ESR spectra of carboxyl, spin-labeled bacteriorhodopsin showing buried, strongly immobilized residues.

(left panel): Increase in immobilized spin content correlated with higher stoichiometries of labeling (2.1 - 4.25 spins/ bacteriorhodopsin molecule).

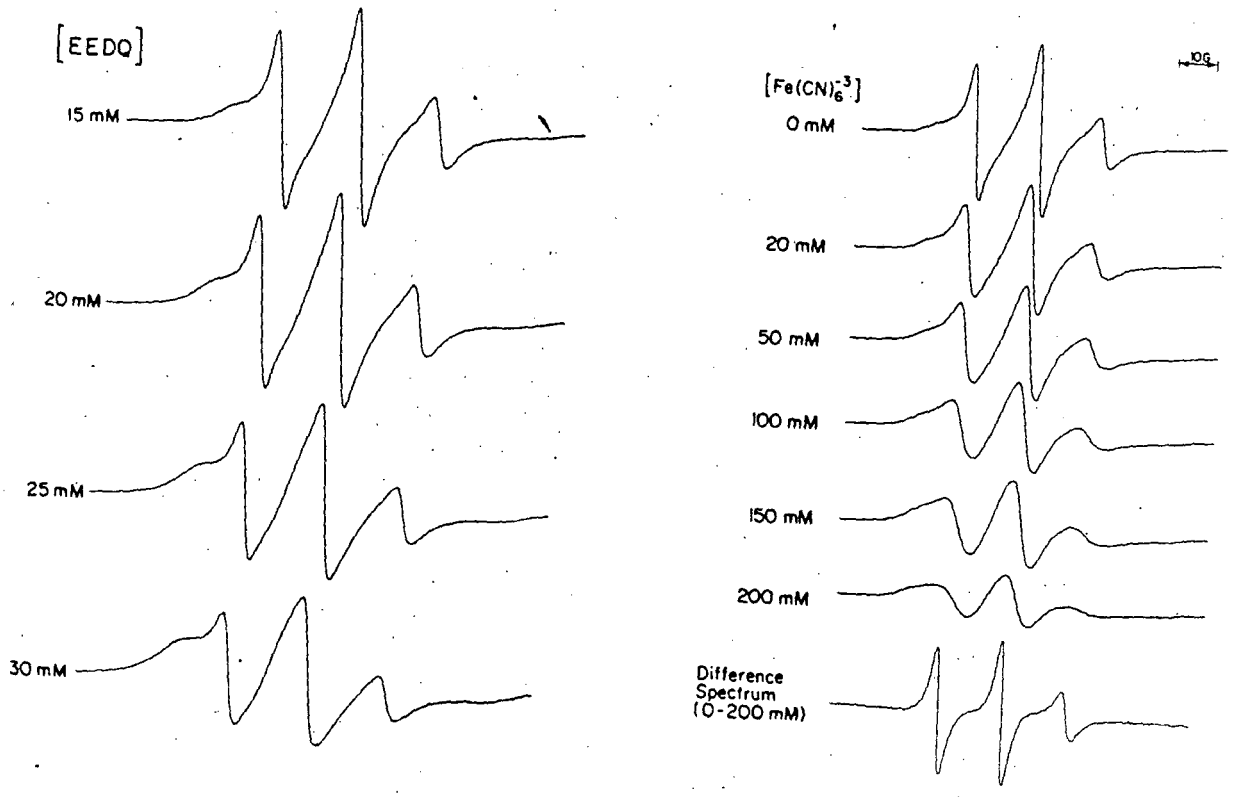
(right panel): Paramagnetic broadening of 2.1 spin-labeled bacteriorhodopsin by $Fe(CN)_6^{3-}$. For experimental details see Herz *et al.*, 1983a.

Fig. 2. Paramagetic broadening of 5NS and 16NS stearic acid spin labels bound to purple membranes and aminoethanesulfonic acid-tempamine double modified bacteriorhodopsin.

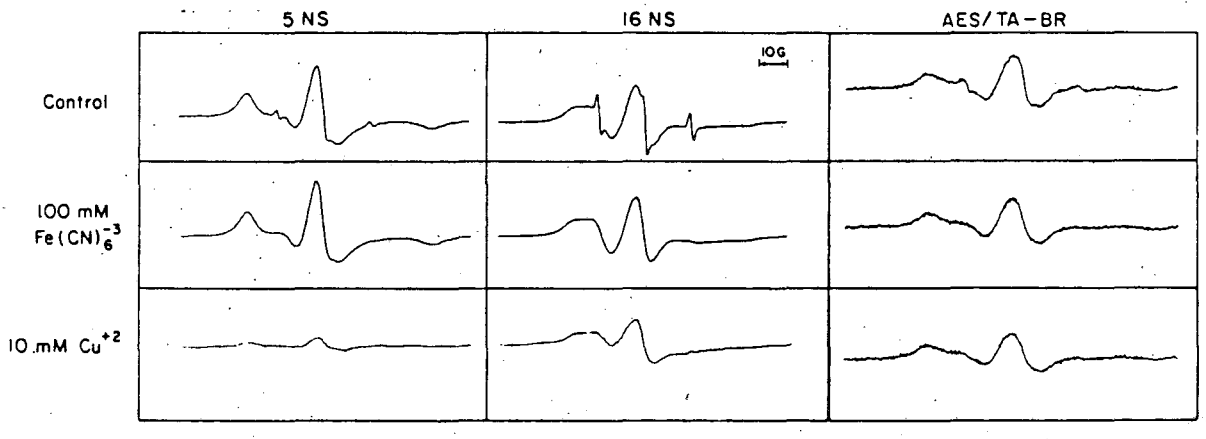
Fig. 3. Reporter group-protein chromophore interactions in purple membranes.

(left panel): Absorption spectra of nitrotyrosine methyl ester -- carboxyl modified bacteriorhodopsin during forward alkaline (pH 7-11), and backwards acidic (pH 11-5) pH titration.

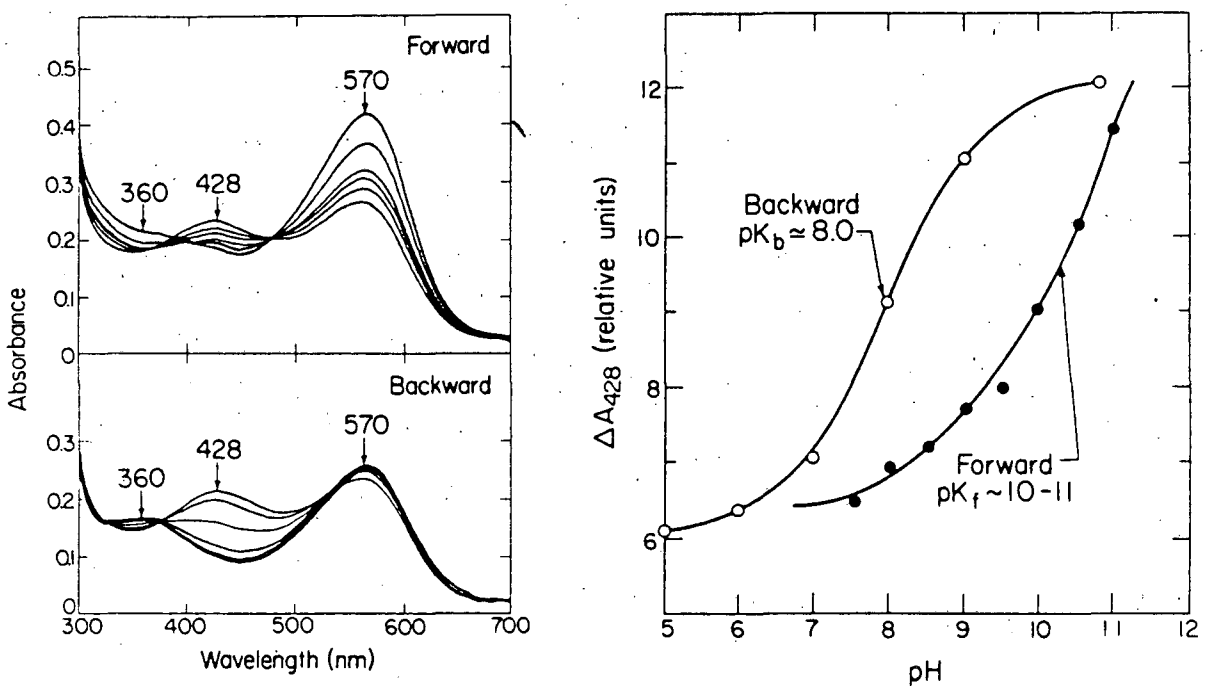
(right panel): Changes in the pK of nitrotyrosine methyl ester covalently bound to purple membranes. For experimental details see Herz *et al.*, 1983b.


[EEDQ]
15 mM
20 mM
25 mM
30 mM
[Fe(CN)$_6^{-3}$]
10G
0 mM
20 mM
50 mM
100 mM
150 mM
200 mM
Difference Spectrum (0-200 mM)

XBL838-828



XBL 838-829


Forward
570
360
428
Backward
570
428
360
Absorbance
Wavelength (nm)
Backward
$pK_b \simeq 8.0$
Forward
$pK_f \sim 10-11$
ΔA_{428} (relative units)
pH

XBL838-827

This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.

Reference to a company or product name does not imply approval or recommendation of the product by the University of California or the U.S. Department of Energy to the exclusion of others that may be suitable.

TECHNICAL INFORMATION DEPARTMENT
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720