LBL-15846

CONF-8209175--1

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Presented at the Second International Conference on Water and Ions in Biological Systems, Bucharest, Romania, September 6-11, 1982; and to be published in the Proceedings

APPLICATION OF CHEMICAL MODIFICATION AND SPIN-LABELING TECHNIQUES TO THE STUDY OF ENERGY CONVERSION BY BACTERIORHODOPSIN

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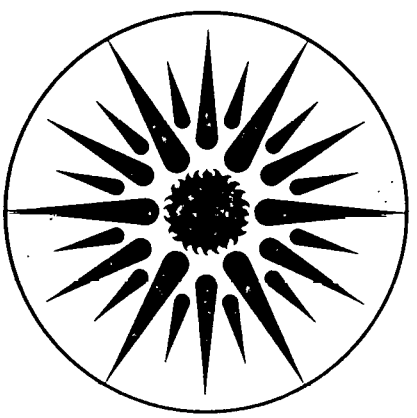

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APPLICATION OF CHEMICAL MODIFICATION AND SPIN-LABELING TECHNIQUES TO THE STUDY OF ENERGY CONVERSION BY BACTERIORHODOPSIN

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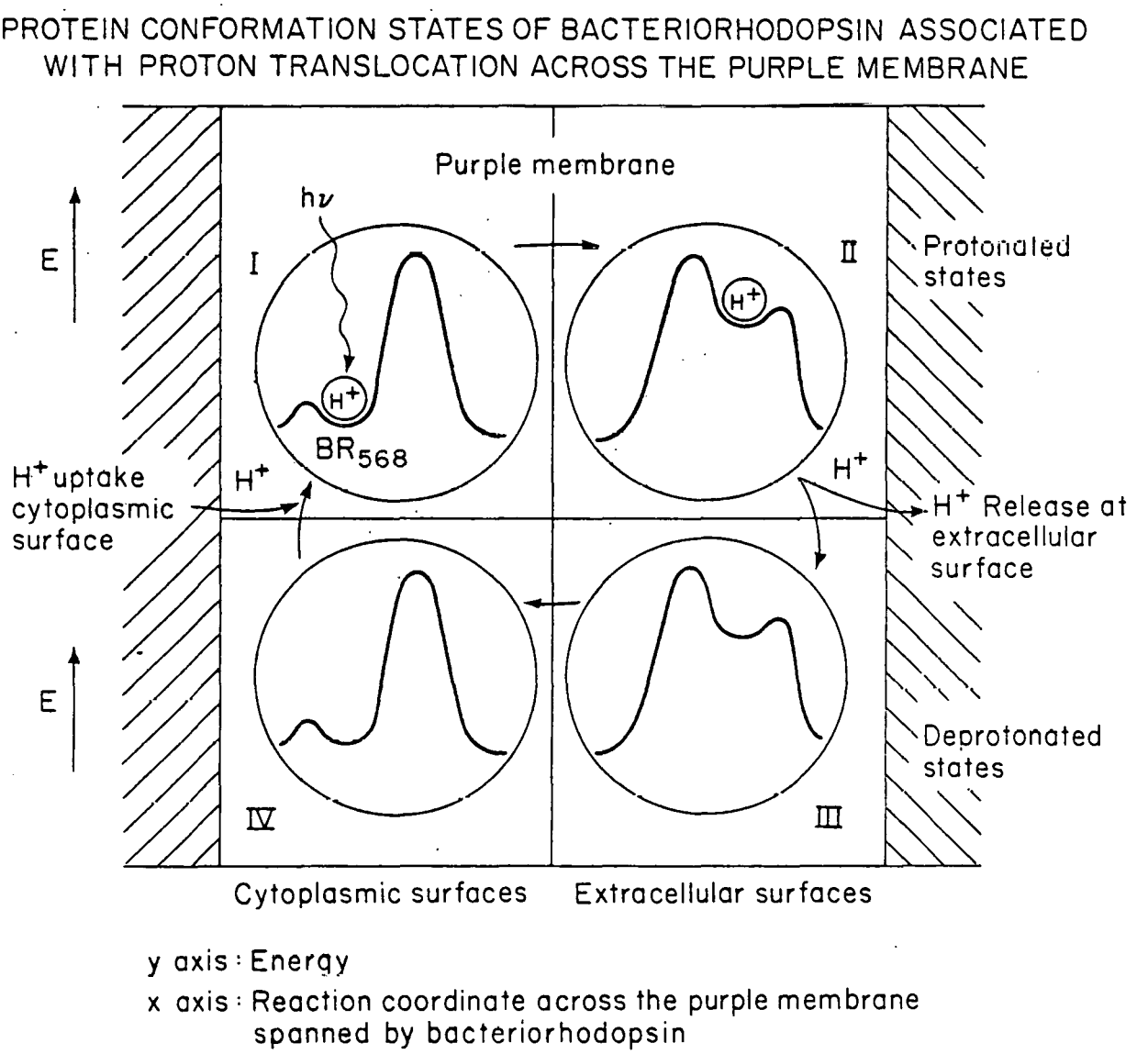
INTRODUCTION

Light generates a pH gradient and an electrical potential across the purple membrane of Halobacterium halobium. We are investigating the time-resolved changes in protonation of the side chains of specific amino-acid residues and the correlation of these changes with photon absorption and the ensuing photoreaction cycle. We seek to determine the precise molecular description of the photocycle and of the time dependent steps in the uptake, translocation, and release of protons by the retinal proton catalyst in this membrane, bacteriorhodopsin (BR).

The photocycle is initiated by absorption of visible radiation (Abs. max at 570 nm) by the all-trans retinal chromophore. The initial steps of the photocycle involve the isomerization of the chromophore to the 13-cis retinal. A deprotonated Schiff base forms with a $t_{1/2}$ of about 50 μsec. Proton release occurs in this time range (illustrated in the upper half of Fig. 1). Later, the deprotonated species decays ($t_{1/2}$ about 3-5 msec), restoring the original all-trans form of the chromophore. During this time, protons are taken up from the cytoplasmic surface of the protein. This process is shown in the bottom half of Fig. 1. The deprotonated photointermediate is referred to as the M_{412} species.

To assess significant structural features of the lipid and the protein important for proton movement, we have used chemical modification of amino acid side chains, spin labeling and deuterium isotope effects on purple membranes. Purple membranes have also been reconstituted into liposomes before and after such treatment

Fig. 1. Protein Conformation States of Bacteriorhodopsin Associated with Proton Translocation across the Purple Membrane



in order to derive information relevant to the occurrence of proton movement at the cytoplasmic surface of the purple membrane. It is hoped that this information will contribute to an understanding of the importance of the electrochemical potential developed across the closed vesicle membrane in the regulation of light energy conversion.

CHEMICAL MODIFICATION STUDIES

Modification of Positively Charged Amino Acids

Lysine. Modification of up to 80% of the ε-amino groups of lysine (5 of the 6 available lysine residues[1]) with imido-

esters (methyl acetimindate, ethyl acetimidate and methyl butyrimidate) which introduce progressively more bulky groups onto the lysyl residues but leave the charge substantially unaltered, showed little effect on the rate of M_{412} formation and decay.[2] Similar results were obtained when the charge of the amino group was changed by the use of succinic or acetic anhydride, or pyridoxyl phosphate. These studies demonstrate that the ϵ-amino groups of lysine are probably not involved in a coordinated pathway of proton translocation by the protein. However, lysine groups do play a structural role essential for activity. Using either dimethyl adipimidate (DMA) (8.3 Å) or glutaraldehyde (7.5 Å), bifunctional reagents with chemically crosslinked lysyl residues, we found a very strong inhibition of M_{412} decay[3] and a decreased proton uptake in reconstituted liposomes. Crosslinking with longer chain bifunctional reagents (like dimethyl suberimidate [11.3 Å]) of individual BR molecules to each other had no effect on activity. These effects suggest that intramolecular conformational changes and not protein-protein interactions in the membrane are essential for activity.

Arginine. With both 2,3-butanedione (BD) and phenylglyoxyl (PGO) treatments, similar effects were obtained for the modified purple membranes.[4] Modification of 3 or the 7 arginine residues resulted in a 33- and 18-fold inhibition of the $t_{1/2}$ of the first and second phases of M_{412} decay, respectively, and a 19-fold increase in the amount of M_{412} stationary state assayed at pH 8. These results indicate that the positive charges of the guanidinium groups of the unmodified residues are essential for photocycling activity.

Modification of Negatively Charged Amino Acids

Glutamate and Aspartate. Water soluble carbodiimides cause the carboxyl groups of these amino acids to become either neutral or positive. These modifications slowed down the M_{412} decay, had no effect on its formation, and caused a large increase in its photostationary state.

In the presence of the added nucleophile, glycine methyl ester (GME), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) treatment is expected to yield the amide adduct. With increasing concentrations of GME (1-250 mM), and in the presence of EDC, the degree of inhibition of M_{412} decay was partially decreased, suggesting that GME may be competing with a nucleophilic group on the protein. Evidence supporting this has come from double modification experiments. EDC treated samples previously modified by ethyl acetimidate (EA) showed M_{412} decay kinetics similar to those observed in samples modified by EDC in the presence of high GME concentrations.

The similarity suggests that EA modified lysines have become unavailable for reaction with EDC, preventing the formation of intramolecular crosslinks. Since EDC can only crosslink closely lying groups, we suspect that the ε-amino groups of lysine must be very close to the carboxyl groups.

Other important results of the carbodiimide modification are the disappearance of one of the photointermediates (O_{640}) in the photocycle and the inability to generate the acid stable form of the protein (Abs. max. at 605 nm). These results suggest that the protonation of one (or more) carboxyl residues is necessary for the generation of that photointermediate as well as for the acid stabilization of the protein.

Tyrosine. By investigating the time course of lactoperoxidase catalyzed iodination of purple membranes, we have been able to obtain evidence of an involvement of at least one tyrosyl residue in proton release and one or several tyrosyl residues involved in reprotonation.[5,6] Modification of one type of tyrosyl residue (localized on the cytoplasmic surface, according to modification studies done on intact cell envelope vesicles) markedly slows M_{412} decay but does not alter the optical and Raman spectral characteristics of the chromophore. Iodination of another tyrosyl residue alters the optical and Raman spectra of the chromophore and markedly stimulates M_{412} formation.

The modifications exhibit a strong effect on the pH dependence of the photocycle, shifting the curve to lower pH values. This suggests that the introduction of bulky groups onto the tyrosyl residues alters the pKs and sterically effects their ability to take part in proton movements. What follows provides evidence for this.

Deuterium Isotope Effects

In agreement with previous reports,[7] we find that the rise time of M_{412} is slowed by a factor of 4.7 in D_2O whereas the decay time is slowed by a factor of only 2.1. We have measured similar isotope effects for a number of chemically modified purple membranes. The similarity indicates that the basic mechanism remains similar in the modified samples (see Table 1). The activation parameters for the rates of rise and decay of M_{412} were determined from their temperature dependence.[8] For tyrosine modified samples, for example, the activation parameters are consistent with a decreased pK for a tyrosine residue (lowered E_a), an increased steric effect caused by iodine substitution (negative $S^{\ddagger}$), and an overall decrease in the rate of reprotonation (increased $G^{\ddagger}$) of the Schiff base. If the tyrosine residue is directly involved in the reprotonation of the Schiff base, the effect of its modification on the activation parameters is not surprising.

Table 1. Kinetic Parameters and Isotope Ratios for Rise and Decay of M_{412}

Sample[c]	Rise[a]			Decay[a]		
	H $t_{1/2}$	D $t_{1/2}$	H D k_1/k_1	H $t_{1/2}$	D $t_{1/2}$	H D k_2/k_2
Control	0.069	0.33	4.76	3.65	7.7	2.11
EAC (Carboxyl)	0.099	0.42	4.24	90[b]	131[b]	1.45
I_2 (Tyrosine)	0.026	0.11	4.15	420[b]	1042	2.48
NBS (Tryptophan)	0.043	0.147	3.40	19.2	67.3[b]	3.5
DMA (Lysine)	0.072	0.35	4.86	33.0	46.2[b]	1.4
EA (Lysine)	0.062	0.32	4.9	10.8	27.4[b]	2.5
BD (Arginine)	0.096	0.49	5.1	359[b]	1055	2.9
PGO(Arginine)	0.142	0.71	5.0	355[b]	724[b]	2.0

[a]Half lives in milliseconds.
[b]Decay kinetics are biphasic, only slow phase given.
[c]Assay conditions: protein 0.15-0.30 mg/ml in 100 mM KCl and 5 mM phosphate; pH 7.5-8.0

SPIN LABELING STUDIES

Nitroxide spin probe techniques have been developed for measuring membrane bioenergetic functions[9,10,11]. Electrical surface potentials at outward-facing membrane-water interfaces are measured with impermeable amphiphilic spin probes which give rise to distinct spectral lines from aqueous and membrane environments[9]. Transmembrane pH gradients are measured with spin-labeled amines and carboxylic acids by quantitating intra- and extracellular concentrations of the nitroxides. Impermeable paramagnetic line-broadening agents, e.g., ferricyanide, are used to quench extracellular signals for these quantitations.[10] Cell volumes are determined similarly, using a freely permeable spin probe, which does not respond to electrochemical potentials (TEMPONE). Transmembrane electrical potentials are estimated from the distributions of spin-labeled phosphorium ions which slowly permeate membranes and hence respond to equilibrium gradients.[11] Unfortunately, rate limitations rule out applications of these nitroxides to kinetic measurements.

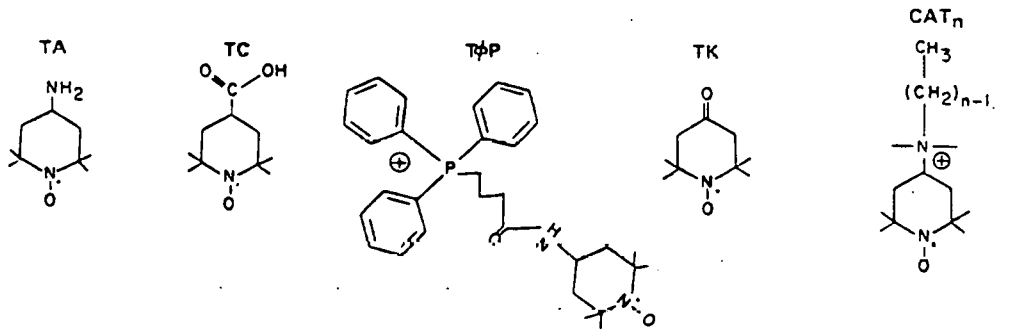
Fig. 2. Some ESR Probes of Membrane Electro-Chemical Potentials and Gradients

SOME ESR PROBES OF MEMBRANE ELECTRO-CHEMICAL POTENTIALS AND GRADIENTS

PARAMETER	PROBE	MEMBRANE PREPATION	BASIS OF MEASUREMENT	ASSAY CONDITIONS
$\Delta pH^{\cdot}$	TA TC	Sealed vesicles or cells	Increase (TA) or decrease (TC) of line height with H^+ uptake	Impermeable paramagnetic broadening agent outside
$\Delta\psi^{\cdot}$	T P	"	Increase of line height as interior becomes more negative than exterior	
Volume	TK	"	Line height	
$\Delta\psi_s^{\cdot\cdot}$	CAT_n	Vesicles, cells or membrane sheets	Relative intensities of aqueous and membrane bound signals	Low ionic strength

· Absolute gradients can be determined from simultaneous measurements with ^{14}N and ^{15}N probes E.G., TK and TΦP or TA and TC.

·· Absolute surface potentials can be determined relative to the binding of uncharged or oppositely charged probes



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Light-Induced Surface Potential Changes in Purple Membranes

The positively charged paramagnetic amphiphile 4-(dodecyl dimethyl ammonium)-1-oxyl-2,2,6,6-tetramethyl piperidine bromide (CAT_{12}) partitions between the membrane and the aqueous phase. Therefore, CAT_{12} was useful in monitoring changes in surface potential associated with the release and uptake of protons from the purple membrane.[12] Using laser flash EPR and photolysis, we showed that the uptake and release of the CAT_{12} probe

closely follows the kinetics of the rise and decay of the M_{412} intermediate.[13] This correlation indicated that the probe senses exposed negative charges at the surface from which protons are released. The number of light-induced surface charges per M_{412} in the photostationary state was calculated to be approximately 1. The value is more than 50% decreased for carboxyl and guanidinium group modification and for heavily modified tyrosine samples, suggesting that some of these groups may be located at the surface of the protein and be involved in the uptake and release of protons from the aqueous environment.

Control of the Photocycle by the Transmembrane Potential Gradient

When reconstituted into liposomes, BR will generate light-induced electrochemical gradients across the lipid bilayer. Our reconstituted systems generate pH gradients of 0.6 - 1.0 and transmembrane electrical potentials of 60-80 mV.[14] The photostationary state of M_{412} at the same light intensity and protein concentration was much higher in the reconstituted system than in the isolated purple membranes. Valinomycin (0.5 μM) decreased the photostationary state of M_{412} by one order of magnitude in the reconstituted system but had no effect in isolated purple membranes. Nigericin (0.5 μM) had no effect on either system. Because we observed that at the concentration used, valinomycin collapses the transmembrane potential and nigericin collapses the pH gradient, we assume that the electrical gradient was probably the major controlling factor in the kinetics of the photocycle.

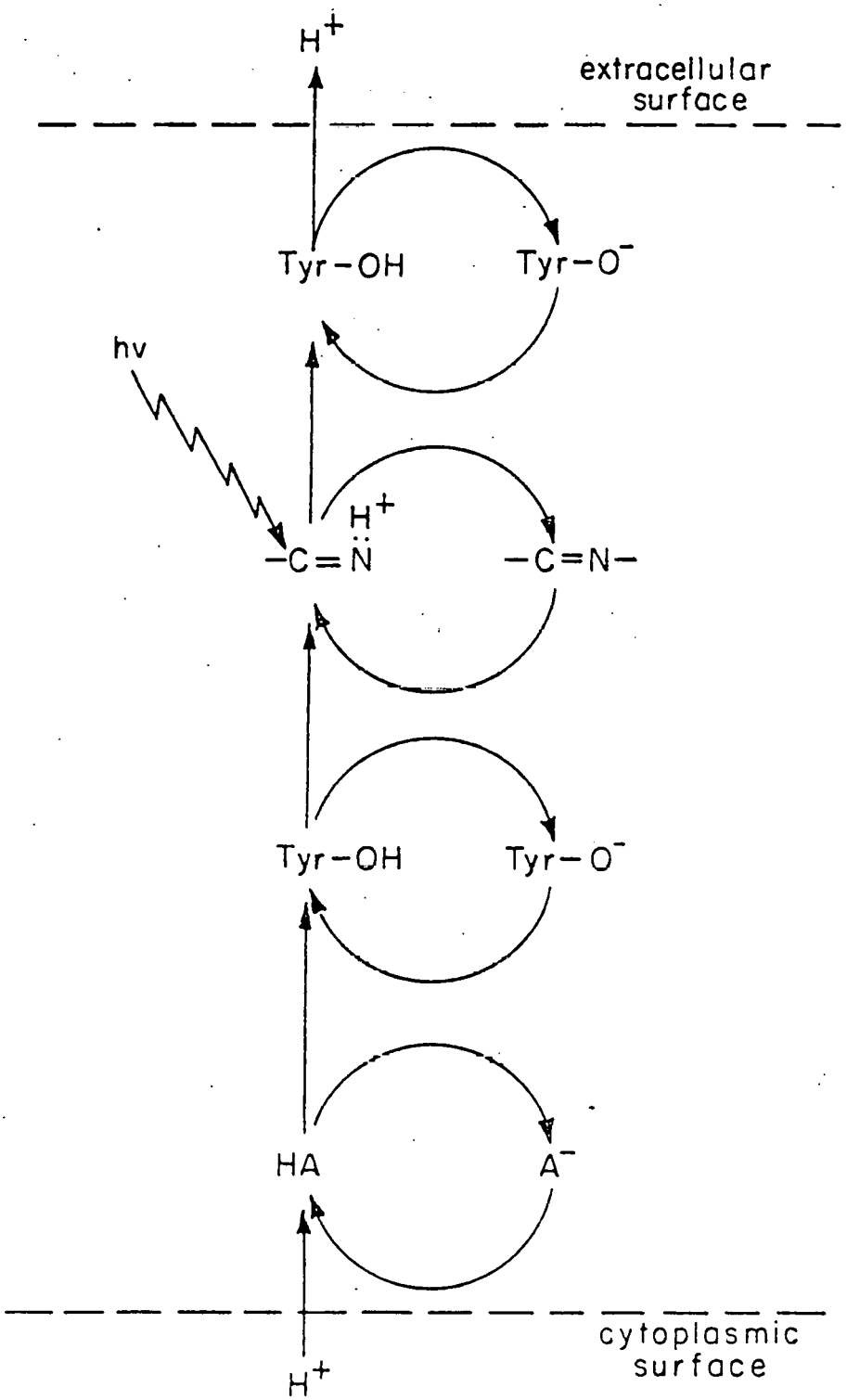
Steady state light-induced electrochemical gradients across BR-containing liposomes showed a substantial slowdown of the M_{412} decay kinetics with virtually no effect on the rise kinetics. Again, valinomycin, but not nigericin, brought the decay kinetics of M_{412} to those measured in the absence of steady state illumination.

These responses suggest that within the protein light may generate strong electric dipoles that could be very sensitive to the transmembrane electric potential gradients, which can be established across the bacterial membrane.

DISCUSSION

The sharp pH dependence seen in controls and the downward shift of the dependence in the rise and decay of the M_{412} species in tyrosine modified purple membrane preparations suggests a direct involvement of reversible tyrosine protonation in the pathway of proton movement through BR (Figure 3). Reversible dissociation of protons at both the outer and inner purple membrane surface would be expected to be pH and pK dependent, hence regulated by the electrochemical gradient. Since the pH gradients are small (<1), pK

Fig. 3. A Proposed Pathway for Uptake and Release of Protons by Bacteriorhodopsin. (We include on the pathway the reversible deprotonation of the tyrosine residues before and after the Schiff base.)



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effects on the photocycle are not to be expected, and indeed, we find that the main control parameter is the transmembrane potential.

Proton Uptake. Several structural requirements appear to be important for reprotonation of BR. Our results with modification of carboxyl and guanidinium groups suggest that a charge transfer complex essential for reprotonation may exist between the two types of groups. Moreover, the large changes in the entropy of activation for the M_{412} decay provides strong supporting evidence that interaction between these two types of groups is an essential structural feature involved in proton uptake and the reprotonation phase of the photocycle. Furthermore, as judged by double modification experiments, an interaction between carboxyl groups and at least one amino group of lysine also appears to be important for reprotonation. The above structural requirements together with the inhibitory effects of bifunctional crosslinking reagents which restrain motion of intramolecular amino groups of lysine located within 8.3 Å of one another suggests that protein conformational changes are essential to proton uptake and M_{412} reprotonation. Tyrosine may also act as a donor for the Schiff base reprotonation.

Proton Release. The specific groups involved in release of protons at the outer surface of the purple membrane probably include tyrosine residues. This conclusion is drawn from the observed effects of iodination and nitration[15] that shift the pK of the dissociation of the phenolic hydroxyl group of tyrosine residues. The pK shift may accelerate proton movement from this group on the tyrosine to a nearby basic group which may be water, OH^-, a group on the lipid of the purple membrane, or a buffer anion. The acceleration would be expected to occur in the range where proton release is pH dependent. That the tyrosine residues are involved in the early stages of the photocycle, associated with proton release, can also be argued by the pronounced deuterium isotope effect on proton release as seen before and after iodination of tyrosine.

Carboxyl groups may also play a role in the movement of protons. Modification of these groups has inhibited the generation of one of the intermediates in the photocycle and the formation of an acid state species.

Light-induced changes in the surface potential of the purple membrane suggest that protons are released from protonated groups at the surface because these surface potential changes are closely coupled to the formation of the M_{412} species. Our experiments provide evidence that the groups involved may be carboxyl and tyrosyl residues.

ACKNOWLEDGEMENTS

This work has been supported by the Office of Biological Energy Research, Division of Basic Energy Sciences, U.S. Department of Energy, under Contract Number DE-AC03-76SF00098.

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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.

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