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Reversible Inhibition of Proton Release Activity and the Anesthetic-Induced Acid-Base Equilibrium between the 480 and 570 nm Forms of Bacteriorhodopsin

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ABSTRACT In purple membrane added with general anesthetics, there exists an acid-base equilibrium between two spectral forms of the pigment: bR_{570} and bR_{480} (apparent $pK_a = 7.3$). As the purple 570 nm bacteriorhodopsin is reversibly transformed into its red 480 nm form, the proton pumping capability of the pigment reversibly decreases, as indicated by transient proton release measurements and proton translocation action spectra of mixture of both spectral forms. It happens in spite of a complete photochemical activity in bR_{480} that is mostly characterized by fast deprotonation and slow reprotonation steps and which, under continuous illumination, bleaches with a yield comparable to that of bR_{570}. This modified photochemical activity has a correlated specific photoelectrical counterpart: a faster proton extrusion current and a slower reprotonation current. The relative areas of all photocurrent phases are reduced in bR_{480}, most likely because its photochemistry is accompanied by charge movements for shorter distances than in the native pigment, reflecting a reversible inhibition of the pumping activity.

INTRODUCTION

Bacteriorhodopsin is the sole protein contained in the purple patches of halobacterial membranes, and its light-driven proton pumping activity provides electrochemical H^+ gradient fuel to the microorganism. Despite the high efficiency of this system in its direct conversion of electromagnetic to chemical energy, it is apparently structurally simple, as the functional unit consists of a single 248-amino acid polypeptidic chain, folded into seven-membrane-spanning helices, solvated by a minimal number (about seven lipids/protein) of glyco-, sulfo-, and phospholipids, and organized in a rigid two-dimensional hexagonal protein array. This structural simplicity versus high efficiency of bacteriorhodopsin offers an ideal experimental material in a variety of fields ranging from light energy conversion to artificial intelligence, including membrane transport, protein dynamics, and intra- and intermolecular interactions. This explains why purple membrane is among the most studied membraneous systems.

The efficiency of this halobacterial pigment relies on finely tuned retinal-apoprotein interactions, which determine the absorption and isomerization characteristics of the pigment, and on its competent energy dissipation through discrete events that trigger a proton release from the bacterial membrane followed by proton uptake from the cytoplasm. Besides transient absorption and vibrational spectroscopic studies (see Mathies et al., 1991, for a review) of the well-known K, L, M, N, and O optical intermediates of the photocycle of native and point-mutated bacteriorhodopsins, analysis of charge displacement currents during the photocycle (Keszthelyi and Ormos, 1983; Trissl, 1990, for a review) now provides us with a rather satisfactory picture of this proton pump (see also Balashov and Ebrey, 1994, for a comprehensive review).

Some characteristics of bacteriorhodopsin are strongly linked to its native membraneous state. For instance, its color strongly depends on the nature of amphiphilic molecules used for pigment solvatation. Earlier work in this laboratory showed that pure lipid-free, detergent-free bacteriorhodopsin is not a purple but a red pigment with a maximum absorbance at 480 nm and a cyclic photochemical activity, including a 535 nm K-like intermediate and a 385 nm deprotonated M-like intermediate (Baribeau and Boucher, 1985). A similar 480 nm spectral form with a 385 nm M intermediate was also found for purple membrane in the presence of halogenated general anesthetics (Nishimura et al., 1985; Henry et al., 1988). In fact, the equilibrium between the 570 nm and 480 nm forms of bacteriorhodopsin is an acid-base equilibrium that appears under a wide variety of experimental conditions. In purified bacteriorhodopsin, detergent microenvironmental effects shift its pK_a within a 8-pH unit range (Baribeau and Boucher, 1987). In anesthetic- or solvent-treated purple membranes, the same spectral transition occurs, between pH 4.5 and 7.5, depending on the solvent (Messaoudi et al., 1992, and references therein). It also exists in bacteriorhodopsin reconstituted from three helical fragments (Kataoka et al., 1992) and can even occur upon heating of membranes above their thermal transition temperature (Brouillette et al., 1987).

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From a structural point of view, there exists little difference between the 480 nm and 570 nm forms of bacteriorhodopsin. They both contain photoisomerizable protonated retinal Schiff bases and have identical secondary structures; however, the 480 nm form has a more relaxed tertiary structure relative to the orientation of the transmembrane helical segments with respect to each other (Pande et al., 1989; Messaoudi et al., 1993). Incidentally, a small change of helix orientation was proposed to modulate the apparent pK_a of some groups, namely Asp^{96}, in bacteriorhodopsin (Rothschild et al., 1993).

In the course of comparative studies between the native 570 nm bacteriorhodopsin and the 480 nm bacteriorhodopsin obtained by pigment purification or destabilization of purple membrane with solvents, we observed that, in spite of apparently small tertiary structural differences between these two species, the 480 nm form had a weak proton pumping activity (Harvey-Girard, 1990). In addition, Uruga et al. (1991) found that in bacteriorhodopsin-containing vesicles, stepwise addition of anesthetic induced an increase followed by a decrease of the proton pumping activity, concluded by poor pumping activity of the 480 nm pigment; and, recently, Kono et al. (1993) demonstrated that in purple membrane, light-induced proton release is a titrable phenomenon (pK_a = 8.2) that disappears under alkaline conditions. In the present study, we compare the activities of the 480 nm (bR_{480}) and 570 nm (bR_{570}) forms of bacteriorhodopsin with respect to their respective capabilities to translocate protons, photochemical activities, their charge displacement properties, and associated transient conductivity changes. We find that the equilibrium between these two photochemically and photoelectrically active chromophoric states is also an equilibrium between pumping and nonpumping states of bacteriorhodopsin.

MATERIALS AND METHODS

Sample preparation

Purple membranes were prepared from cultured cells of *Halobacterium salinarium* strain S_9, purified by the standard procedure already described elsewhere (Baribeau and Boucher, 1987) and stored in Kushner medium. Before use, they were washed free of salt by successive sedimentations in water and then suspended in the desired medium. Incorporation of purple patches into phosphatidyl choline (Soybean PC, P5638; Sigma Chemical Co., St. Louis, MO) lipid vesicles was achieved according to the instant vesicle procedure described by Racker (1973) in 150 mM unbuffered KCl. After formation, vesicles were purified by sedimentation through a 1.4–1.7 M linear sucrose gradient, collected at a buoyant density of 1.163 ± 0.003 and dialyzed against KCl to remove sucrose before use. Before action spectra measurement, the vesicle suspension pH was adjusted to the desired value with diluted NaOH or HCl.

For transient current and conductivity measurements, purple membrane fragments (final bR concentration, 19–64 μM) were oriented and immobilized in polyacrylamide gel (final concentration, 15%) by the method described by Dér et al. (1985). Gels were cast as 15 × 30 × 0.8 mm^3 sheets; they were extensively washed and soaked in water for at least 2 days before measurement and then cut into 6 × 8 × 0.8 mm^3 rectangular pieces to fit the measurement cell.

Equilibration of bR_{570} with bR_{480} was achieved by the addition of microliter amounts of enflurane (trademark Ethrane; 2-chloro-1,1,2-trifluoroethyldifluoromethyl ether; Anaquest, Pointe-Claire, QC, Canada) or halothane (trademark Somnothane; 2-bromo-2-chloro-1,1,1-trifluoroethane; Hoeschst Canada, Montréal, QC, Canada) to aqueous membrane suspensions as described by Lee et al. (1991) or to membranes immobilized in gel. In the case of gel-immobilized membranes, a higher concentration of anesthetic was needed to achieve the 480/570 nm chromophore equilibration within a reasonable period of time. Nevertheless, the transformation of bR_{570} into bR_{480}, as monitored spectrophotometrically, could be completely achieved in samples buffered above pH 7.5 but only partially (<90%) in water. Halogenated anesthetics are highly volatile; they could easily be removed by opening samples to ambient air for a while or by placing them under mild vacuum for a few minutes. Under these conditions, samples recovered their characteristic purple color. In some control experiments, anesthetics have been replaced by trace amounts of hexane or large amounts of acetone, as these solvents have been shown to produce similar chromophore equilibration (Messaoudi et al., 1992); in those cases, results were neither qualitatively nor quantitatively different.

Photochemical, H^+ pumping activity, and photoelectrical measurements

Comparison of the bleaching properties of bR_{570} and bR_{480} was made by trapping their M intermediates under continuous illumination at low temperature. For this purpose, purple membranes in 80% glycerol were added with halogenated anesthetic or trace amounts of hexane to give a mixture of both spectral forms. In most experiments, hexane was chosen because it is difficult to get samples with good optical qualities at low temperature in the presence of halogenated anesthetics. Samples were cooled to near −40°C in a cryostat fitted in a spectrophotometer and bleached by monochromatic cross-actinic illumination provided by a 500-W incandescent lamp filtered through 10-nm bandpass interference filters. Bleaching difference spectra were recorded after successive irradiation periods.

Action spectra of the proton pumping activity were obtained from the pH meter electrode response upon irradiation of purple membrane vesicles with monochromatic light provided from a 150-W Xe lamp mounted on a high-intensity monochromator (Photon Technology Int., Princeton, NJ). Measurements were performed at 10- or 20-nm intervals in a specially adapted stirred cell where the pH meter electrode was fitted to minimize anesthetic evaporation during measurements.

Transient bacteriorhodopsin absorption changes were measured with a set-up described elsewhere (Henry et al., 1988). The actinic light pulse was the 532-nm, 10-ns, 20-mJ pulse of a Q-switched Nd-YAG laser (Laser System 2000; JK Lasers, Rugby, England), and the analysis beam was detected with a 5-ns rise time photodiode protected from scattered actinic light by a high-attenuation 532-nm Raman holographic filter and a narrow interference filter. Transient proton release by bacteriorhodopsin was measured as described by Drachev et al. (1984). Samples contained 100 mM NaCl and 25 or 50 μM pH indicator paranitrophenol in unbuffered suspensions adjusted to pH 6.8. The pH indicator absorbance change was obtained by subtraction of signals obtained in its presence and in its absence. For measurements in the absence of paranitrophenol, a second cell containing an identical amount of the pH indicator was placed next to the sample cell in the analysis beam to make sure that the same light intensity fell on the detector.

Transient photocurrents were collected from oriented gel-immobilized purple membranes with platinized platinum wire electrodes in a 15 × 10 × 1 mm^3 cell filled with the desired medium. Electrode tips were mounted on the input terminals of a current voltage converter (100 kV/A, DC to 100 MHz bandwidth, model 342A; Analog Modules, Longwood, FL) contained in a shielding box whose temperature was kept at 25°C. Its output was fed to a variable-gain voltage preamplifier (DC to 2 MHz bandwidth; Stanford Research Systems, Sunnyvale, CA) to improve the signal-to-noise ratio of photocurrent measurements in the micro- and millisecond time range. The signal could then be filtered by appropriate setting of the low-pass filter before recording. Light-adapted samples were excited therein with the 532-nm pulse of the laser guided by an optic fiber. They consisted of gels pre-equilibrated with a range of anesthetic concentrations giving rise to

bR_{570}:bR_{480} ratios from 0.5:0.5 to 0:1. Because the measuring cell was airtight, anesthetic evaporation was prevented and these ratios remained the same before and after measurements, as judged from absorption spectra.

Transient conductivity change associated with the bacteriorhodopsin photocycle was measured with the same set-up (preamplifier bandwidth set at 10 kHz), except that the gel sample consisted of nonoriented purple membrane (no voltage applied during gel polymerization) and that a 7-V potential difference was maintained between the electrode tips during measurement. This voltage was switched on a few seconds before the laser flash. This set-up, although sensitive, did not allow measurements when the salt concentration was higher than 5 mM, because of amplifier saturation by the dark current.

Transient absorbance and photoelectrical signals were fed into digital storage oscilloscopes (4094 and/or 4094C; Nicolet Instrument Corp., Mississauga, ON, Canada) and recorded at sampling rates ranging from 5 ns to 20 μs per point. Data, in the form of 4 to 16 averaged traces, were transferred to a microcomputer and analyzed by means of a multiexponential curve-fitting procedure described by Martin and Maconochie (1989).

RESULTS

$bR_{570} \Leftrightarrow bR_{480}$ equilibration and the photochemical proton release activity

The reversible formation of 480 nm bacteriorhodopsin upon addition of general anesthetics to purple membrane is a pH-dependent process. Fig. 1 shows the modification of the absorption spectrum of purple membrane in the presence of

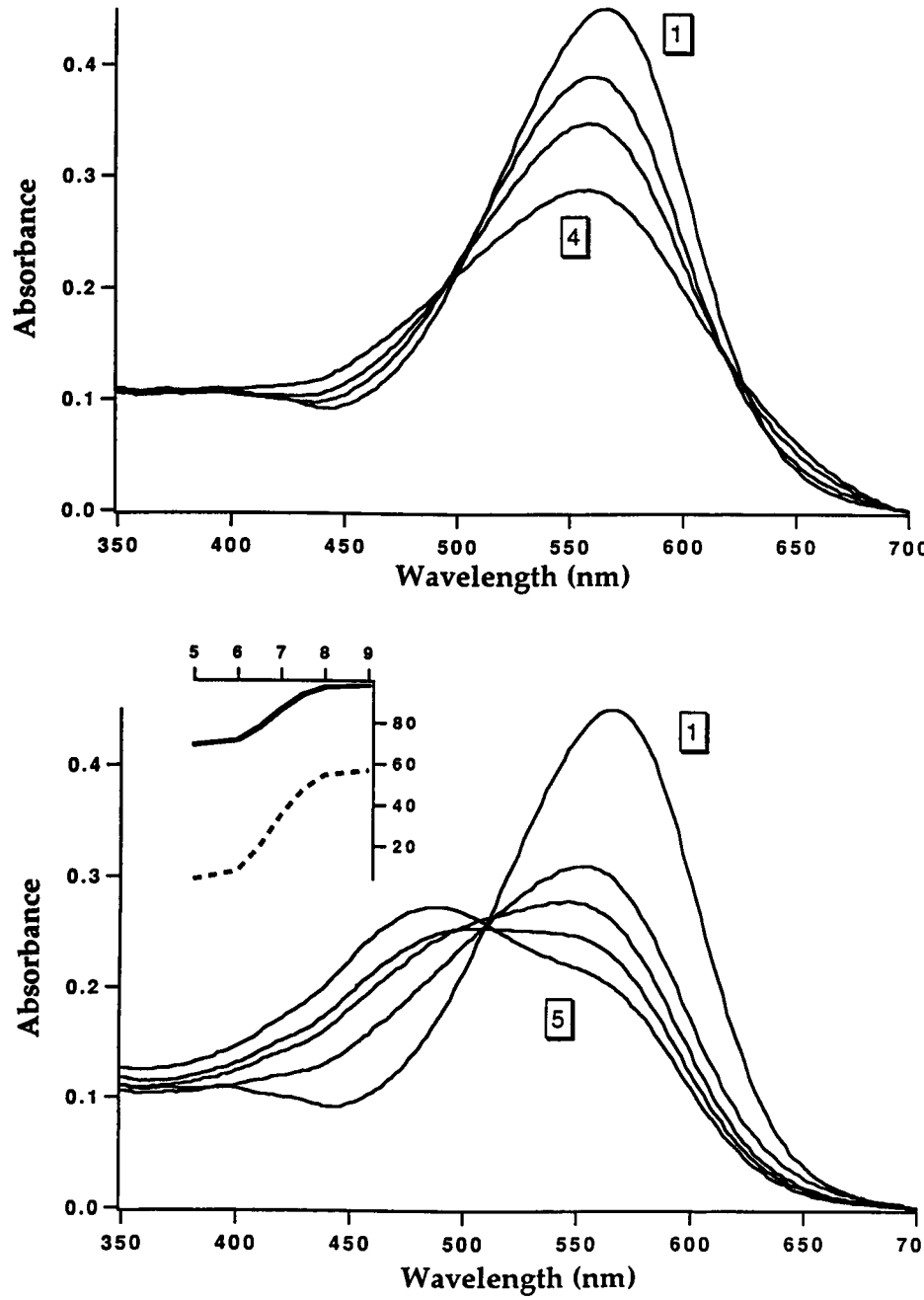

FIGURE 1 Spectral transformation of purple membrane in the presence of 0.1% (v/v) Enflurane. Sample consisted of membranes immobilized (but not oriented) in acrylamide gel to prevent eventual sample sedimentation during measurements. (*Top*) Decrease of 570 nm absorbance observed at pH = 5. Spectra 1 through 4 were measured before and 15, 30, and 60 min after the addition of Enflurane, respectively. Waiting for an additional 60 min did not show significant further spectral change. (*Bottom*) Spectrum 1 is a reminder of immobilized membranes without anesthetic. Spectra 2 through 5 are those measured for purple membrane after 60 min of incubation in the presence of 0.1% Enflurane at pH 6, 7, 8, and 9, respectively. (*Inset*) Percentage bR transformed to bR_{480} at equilibrium in the 5 to 9 pH range in the presence of 0.1% (– – –) and 0.4% (——) Enflurane.

0.1% enflurane. At pH 5, the anesthetic addition is only accompanied by a decrease in absorption intensity and maximal absorbance remains near 570 nm. At this pH, little 480 nm bacteriorhodopsin is formed, as indicated by the small absorbance increase at this wavelength, and some redshifted pigment (blue bacteriorhodopsin) appears, as indicated by the small absorbance increase in the 650 nm region. In anesthetic-treated purple membrane, the 480/570 nm chromophore equilibrium has an apparent pK_a of 7.3 (see below) and that of the 570/605 nm chromophore equilibrium is about 4 (Messaoudi et al., 1992), the same value as that observed for purified bacteriorhodopsin (Baribeau and Boucher, 1985). It is thus not surprising to find a mixture of these three species in the mildly acidic pH range. At higher pH, the purple pigment (bR570) is titrated to the 480 nm form of bacteriorhodopsin. The $bR_{570} \Leftrightarrow bR_{480}$ acid-base equilibrium has an apparent pK_a of 7.3. Obviously, at low anesthetic concentration, it is not possible to completely convert bR_{570} into bR_{480}; moreover, at high anesthetic concentration, some bR_{480} appears, even at pH 5 (see inset of Fig. 1). Titration curves obtained under conditions of low or high anesthetic concentrations give the same apparent pK_a and differ only in the amplitude of the titration curve.

The comparison between photoinduced proton release by bR_{570} and bR_{480} is shown in Fig. 2. In this experiment, the transient 400 nm absorbance change of the water-soluble pH indicator paranitrophenol was used to monitor proton release after a 10-ns, 532-nm laser flash. After subtraction of the transient signal due to the rise and decay of the M intermediate, the rapid bulk pH drops and its slower recovery associated with the photocycling of bR_{570} is clear. In the case of bR_{480} obtained by addition of enflurane to purple membranes, such a transient pH change cannot be measured. The transient proton release observed upon flashing

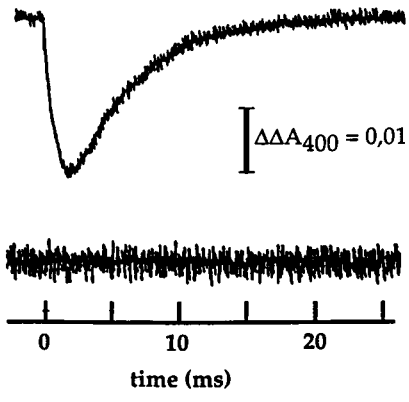

FIGURE 2 Transient proton release as measured by the 400 nm paranitrophenol absorption change upon laser excitation (time = 0). Transient absorption change due to the rise and decay of the M intermediate has been subtracted. The sample consisted of aqueous unbuffered purple membrane (7.5 μM bR) adjusted to pH 7, containing 50 mM NaCl and 25 μM paranitrophenol without anesthetic (*upper trace*) or with 0.4% (v/v) Enflurane (*lower trace*). In the lower trace, vertical scale is expanded by a factor of 3.

purple membranes completely disappeared when the pigment was quantitatively transformed to its 480 nm form in the presence of typically 0.4% (v/v) enflurane, but it was immediately re-observed when enflurane was removed and the purple color recovered after a short exposure to a mild vacuum.

Proton pumping activity and the $bR_{570} \Leftrightarrow bR_{480}$ equilibration in vesicles

In the same way as in purple membrane, fully reversible formation of bR_{480} from the original bR_{570} pigment, in the presence of halogenated anesthetics, also occurs in vesicles, irrespective of their sidedness (Uruga et al., 1991). In this case also, the level of the $bR_{570} \Leftrightarrow bR_{480}$ equilibrium in the presence of anesthetics is concentration and pH dependent. For this reason, in unbuffered samples where pH is lower than 7, it is not possible to transform bR_{570} into bR_{480} completely, even at saturating anesthetic concentration.

Asymmetrical incorporation of bacteriorhodopsin into lipid vesicles enables proton pumping action spectrum measurement from the pH gradient generated, in the light, across the vesicle membrane (Racker, 1973; Hwang and Stoeckenius, 1977). There is general agreement between the bacteriorhodopsin absorbance when it is in the form of bR_{570} and such proton pumping action spectra. However, this is not the case for the bR_{480} form. Indeed, in the presence of increasing amounts of anesthetics, it is possible to get mixtures containing varying relative concentrations of bR_{570} and bR_{480}. Under such conditions, the action spectrum for proton pumping activity always remains centered near 570 nm, showing very little, if any, proton pumping activity by the 480 nm pigment. An example of this behavior is given in Fig. 3 for a sample where more than 90% of the pigment present exists in the form of bR_{480}. This sample has its maximum absorbance at 480 nm and shows a small shoulder at 570 nm. Its proton pumping action spectrum as measured from the light-induced bulk pH change clearly shows that purple bacteriorhodopsin is the major active species, although it constitutes the minor spectral species. There is no indication therein that bR_{480} has a significant activity. In fact, from measurements in vesicle suspensions where the relative bR_{480} content varied from 0 to >0.9, we calculated that the relative efficiency of 480 nm light to elicit bulk pH change ($\Delta pH/A_{480}$ at constant light intensity) decreased from 1 to 0.08. It is also noteworthy that the maximum of the action spectrum is not located at 570 nm, but rather peaks around 550 nm. This was observed for all samples where the amount of bR_{480} exceeded that of bR_{570} and must reflect qualitative changes in the solvation of purple bacteriorhodopsin in the presence of anesthetics. After anesthetic removal, the vesicle suspension recovered its characteristic 570 nm maximal absorbance and H^+ pumping activity.

At this point, the equilibrium between the two spectral species of bacteriorhodopsin appears also as an equilibra-

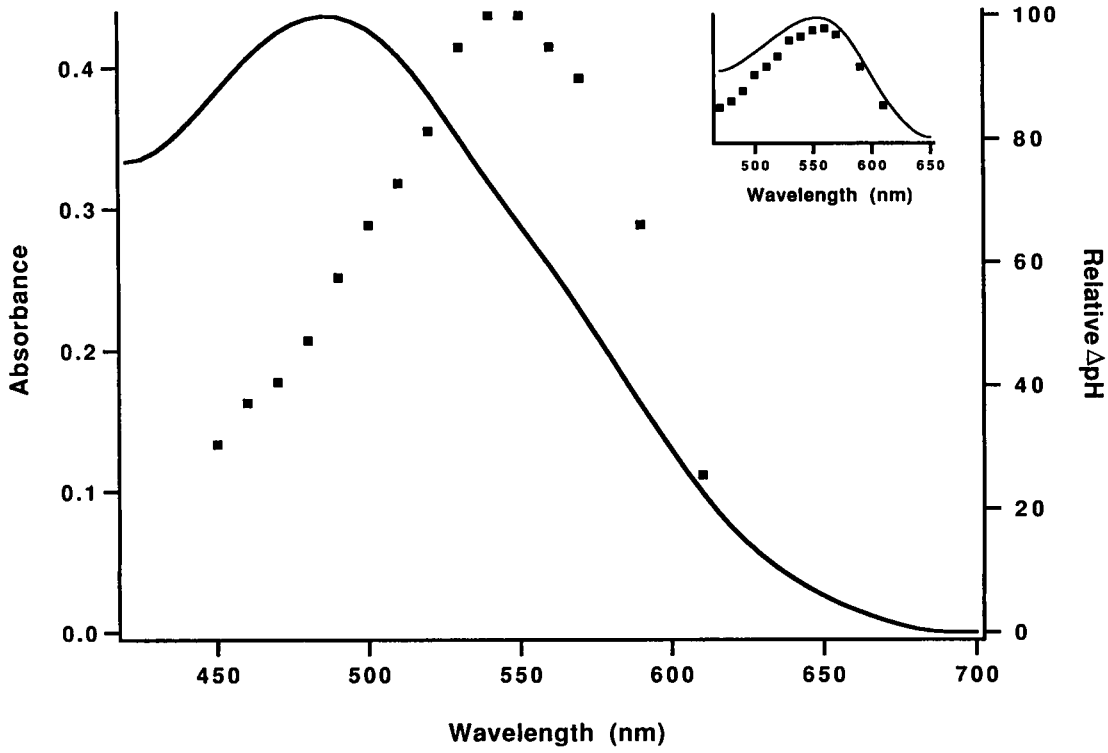

FIGURE 3 Action spectra for proton pumping across purple membrane vesicles. The solid line is the absorbance of the sample in the presence of 0.3% (v/v) Enflurane. The square black dots are the relative pH change measured as a function of excitation wavelength in the same sample under the same conditions. The inset shows the results of the same measurements on the same sample after Enflurane was removed under mild vacuum.

tion between an intact and a functionally impaired form of the pigment. Switching between these two forms may have several origins. Among others, alteration of the photochemical activity in terms of yield and/or speed might be responsible for this reversible dramatic reduction of the proton release activity.

The photochemical activity of bR_{570} and bR_{480}

The key reaction where differences might be related to the absence of significant proton pumping activity in bR_{480} is the yield with which its M intermediate can be produced under illumination. To address this point, we attempted to achieve selective photobleaching of bR_{570} and bR_{480} under low continuous light intensities of selected wavelengths in membrane suspensions containing a mixture of both spectral species at 220 K, a temperature at which the M intermediate is trapped. The results of this experiment are shown in the difference spectra of Fig. 4. In mixtures containing comparable amounts of bR_{570} and bR_{480}, irradiation at 577 nm only bleaches the 570 nm form of the pigment. The photobleaching difference spectrum shows the characteristic 570 nm minimum and 410 nm maximum of bR bleaching and M formation. When samples are irradiated with 501-nm light, bR_{570} and bR_{480} are bleached in comparable amounts. The difference spectra recorded after successive periods of irradiation clearly show the appearance of two minima at 490 nm and 570 nm and of a maximum at 370 nm. It is noteworthy that the isobestic point of the spectral transformation of bR_{570} to bR_{480} is located at 505 nm. This is to say that 501-nm light is nearly equally absorbed by both forms. Taking the amplitude of the minima in these difference spectra, we estimate a difference of less than 15% between the amounts of bR570 and bR480 bleached by 501-nm light. However, we must point out that bR_{480} has an extinction coefficient smaller than bR_{570}; in addition, the close proximity of the M λ_{max} in the former case may also make the intensity of its minimum look smaller than its actual value. It is obviously not possible to get a pure bR_{480}/M_{370} difference spectrum by irradiation of mixtures of bR_{570} and bR_{480}, as the former has nonnegligible absorption in the 480 nm range. Nevertheless, irradiation at 480 nm shows that bR_{480} bleaches 25% more than bR_{570} upon excitation at this wavelength. From these photobleaching experiments, there is thus no evidence of a large difference in the capabilities of 570 and 480 nm forms of bacteriorhodopsin to produce the deprotonated M intermediate.

The time course of absorbance changes in the M intermediate spectral region were also measured to characterize the photochemical activity of bR_{480} with respect to that of bR_{570}. Like the spectral transition from the purple to the red form, which occurs in two consecutive steps (absorbance decrease then absorbance shift), the kinetic behavior of the bacteriorhodopsin photocycle is also modified following a

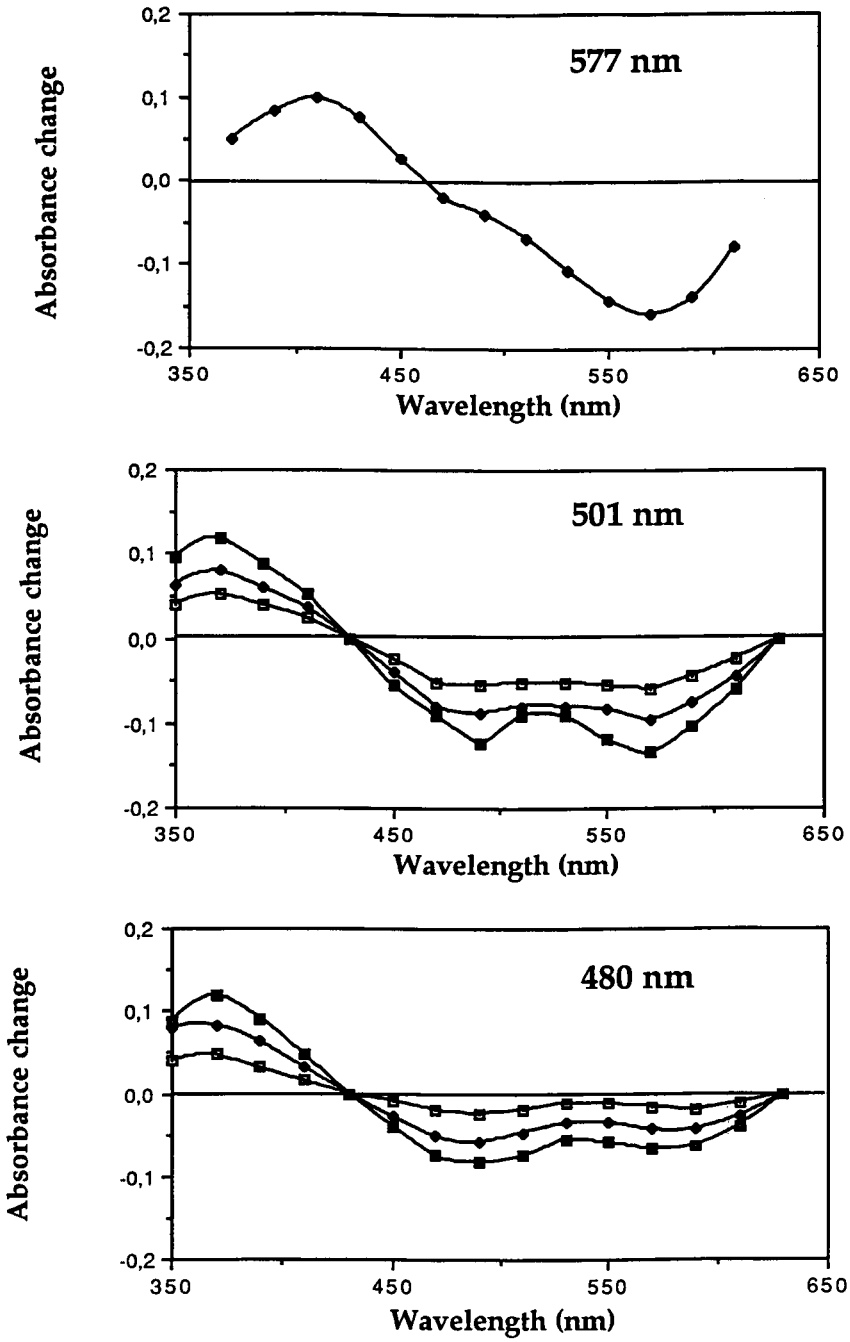

FIGURE 4 Difference spectra recorded at 220 K after irradiation of membrane suspensions containing a mixture of bR_{570} and bR_{480}. Samples consisted of 11 μM bacteriorhodopsin in 80% (v/v) aqueous glycerol added with hexane in an amount sufficient to get roughly equal quantities of bR_{570} and bR_{480} (see Materials and methods). The upper spectrum is that obtained after 60 s of irradiation with 577-nm light. Before irradiation, absorption values at 570 and 480 nm were 0.17 and 0.24, respectively. The middle spectra were measured after three successive 30-s irradiations with 501-nm light. Before irradiation, absorption values at 570 and 480 nm were 0.24 and 0.25, respectively. Lower spectra are those measured after three successive 30-s irradiations with 480-nm light. Before irradiation, absorption values at 570 and 480 nm were 0.24 and 0.22, respectively.

nonhomogeneous and nonprogressive pattern. Fig. 5 shows the time course of optical changes due to the rise and decay of the M intermediate under different anesthetic and pH conditions. The general trend is as follows. Under pH conditions where, in the presence of the anesthetic, only an absorbance decrease is observed and no bR_{480} is formed, the chromophore deprotonation and reprotonation steps (M rise and decay) both occur faster. At higher pH, where bR_{480} is formed, the deprotonation step is still more rapid but reprotonation is retarded. Details regarding lifetimes and relative amplitude of the different components of these steps are given in Table 1. It is seen that reduction of bR_{570}

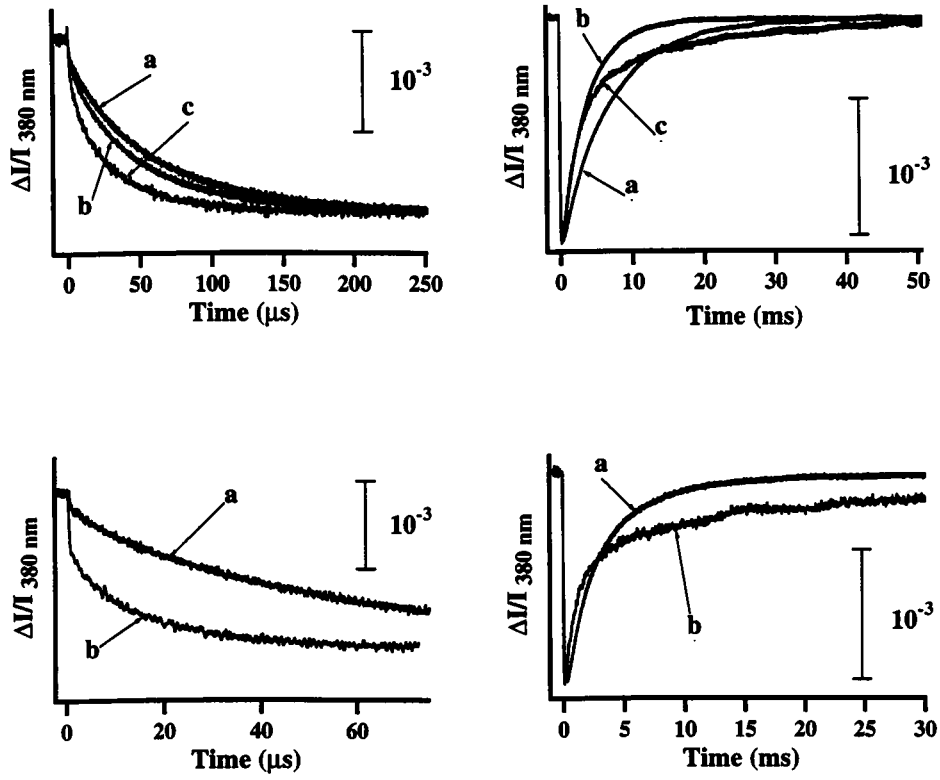

FIGURE 5 Optical signals of the rise (*left*) and decay (*right*) steps of the M intermediate in bR_{570} and bR_{480}. Purple membrane (2.9 μM bR) suspended in water (pH ≈ 5.8) (*upper set*), before (*trace a*) and after (*trace b*) the addition of 0.05 and 0.08% (*trace c*) Enflurane or suspended in 10 mM Tris-Cl buffer (pH = 8.5) (*lower set*), before (*trace a*) and after (*trace b*) the addition of 0.1% Enflurane. At low Enflurane concentration in water, bR_{570} extinction is reduced but no bR_{480} is formed. At pH 8.5, bR is quantitatively transformed into its 480 nm form by Enflurane. In the figure, traces are scaled to the same amplitude; the *y* axis value only holds for membranes without anesthetic (see text for details).

extinction upon addition of the sample with enflurane and its further transformation into bR_{480} are accompanied by an acceleration of both fast and slow M rise components. On the other hand, the M decay step, which is best fitted in many cases by the sum of three exponentials is, first, accelerated when the low extinction purple bR is formed. When bR_{480} is formed this later step is decelerated, mostly because of the appearance of a significant, very slow ~50 ms component. Such very slow components are barely observed as very minor components in the case of membranes in pure water or mildly acidic buffered water, but, never to an extent comparable to that observed for bR_{480}.

TABLE 1 Rise and decay of the M intermediate

	M rise		M decay	
Conditions	τ (amplitude)	w.a. (μs)	τ (amplitude)	w.a. (ms)
PM in water	16.4 μs (0.13)		3.94 ms (0.28)	
	63.7 μs (0.87)	**57.5**	7.84 ms (0.69)	
			63.2 ms (0.03)	**8.4**
+ 0.05%	16.7 μs (0.22)		2.39 ms (0.40)	
enflurane	56.5 μs (0.78)	**47.7**	4.41 ms (0.60)	**3.6**
+0.08%	6.67 μs (0.32)		2.26 ms (0.71)	
enflurane	34.5 μ (0.68)	**25.5**	17.7 ms (0.29)	**6.6**
PM in Tris-Cl buffer, pH 9.5	9.90 μs (0.85)		1.61 ms (0.55)	
	61.0 μs (0.15)	**17.9**	5.00 ms (0.30)	
			6.25 ms (0.15)	**3.32**
+ 0.1%	3.57 μs (0.71)		1.02 ms (0.59)	
enflurane	17.2 μs (0.29)	**7.4**	9.62 ms (0.24)	
			49.5 ms (0.17)	**11.3**

It is important to remember that in water (pH ~6), 0.05 and 0.08% enflurane only lowers the bR_{570} extinction by 15 and 25% without significant formation of bR_{480}, whereas at pH 8.5, 0.1% enflurane quantitatively transforms bR_{570} into bR_{480}.

Values in bold are the weighted average lifetime (**w.a.**), indicating the overall average speed of a given step.

Two points are worthy of mention here. First, the amplitude of the M intermediate is progressively reduced to about one-third of its original value upon progressive transformation of bR_{570} into bR_{480}. There are at least two reasons for this. One is that the absorbances changes ratio (ΔA_M:ΔA_{bR}) is smaller in bR_{480} than in bR_{570} (Henry et al., 1988), and the other resides in the fact that bR_{480} has a K intermediate with maximum absorbance near 535 nm (Baribeau and Boucher, 1985), which enables effective photostationary equilibration between bR and K during the 10-ns, 532-nm flash, resulting in the formation of less M intermediate. The second point is that the 50 ms component that we report here for the M decay step in bR_{480} is not the slowest one that we have observed. Indeed, this component may vary, depending on the nature of the anesthetic used. For instance, in the presence of halothane, another general anesthetic that induces the formation of bR_{480} at a concentration lower than that needed with enflurane (Lee et al., 1991), decay components as long as 100 ms with a relative amplitude of more than 0.5 were measured.

The kinetic behavior of the O intermediate is also affected by the presence of anesthetics in a manner that is consistent with the results observed for the M intermediate. Upon tranformation of bR_{570} into bR_{480} by enflurane, the O intermediate is formed more rapidly but in much smaller

amounts. Fig. 6 shows the optical trace recorded at 630 nm when the pigment is in the 480 nm form. Overlap of the rise and decay steps prevents precise determination of their lifetimes. Nevertheless, the 0.95 ms τ observed for the rise step obviously matches the fast 1.06 ms component observed in the M decay under identical conditions. On the other hand, the O decay phase can only be matched with the the fast component of the parent bR_{480} recovery (Fig. 6, lower trace). This is to say that if the slow components of the M decay lead to the formation of some O species, the latter might well be difficult to measure optically. In addition, when enflurane was replaced by halothane, we were unable to observe any O-like intermediate in the 560–650 nm range.

From these measurements of the photochemical activity of both spectral forms of bacteriorhodopsin, it is clear that the bR_{480} chromophore deprotonates faster than that of bR_{570}, but that its reprotonation is much slower. Although it could explain why the former appears as a poor proton pump under continuous illumination, it could hardly explain why it would not, at least transiently, release a proton. To investigate this question from another point of view, we have measured transient charge displacement currents in bR_{570} and bR_{480}.

The light-induced charge displacement in bR_{570} and bR_{480}

Fig. 7 compares the photocurrents measured in gel-oriented membranes upon $bR_{570} \rightarrow bR_{480}$ transformation by enflurane at pH 7.5, in the time domains corresponding to the B_1, B_2, and B_3 components, according to the nomenclature used by Liu (1990). B_1 is a fast negative signal (according to proton pumping direction; Keszthelyi and Ormos, 1983) whose kinetic characteristics are known to depend on the measuring system (Trissl, 1990). It corresponds to the formation of the K intermediate and its subsequent transformation into L. Under identical conditions and in the very

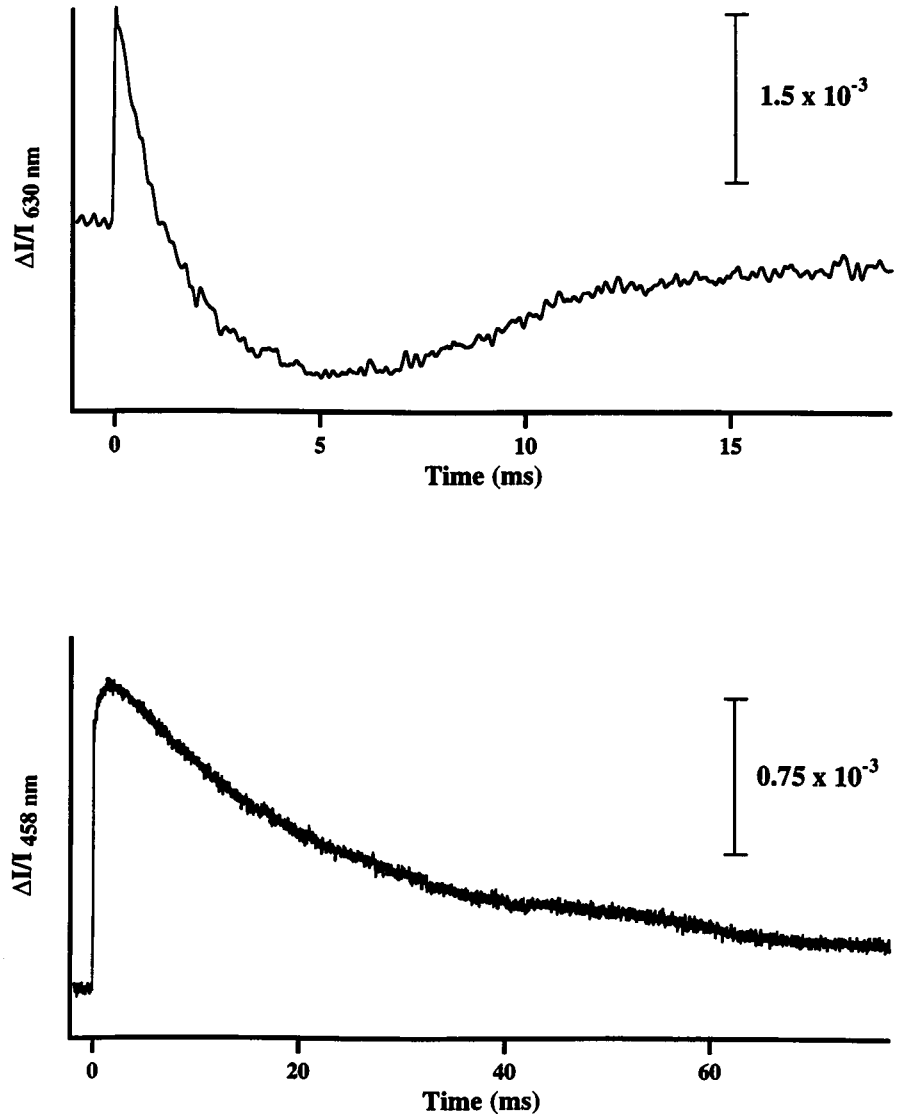

FIGURE 6 (*Upper trace*) Optical transient due to the rise ($\tau \cong 0.95$ ms) and decay ($\tau \cong 10$ ms) of the O intermediate in bR_{480} under the same experimental conditions as in Fig. 5 (*lower set, trace b*). (*Lower trace*) Recovery of bR_{480} after bleaching under the same conditions. Measurement was done at 458 nm to prevent signal contamination by any remaining bR_{570}; at this wavelength the latter form shows almost no optical transient on this time scale. The three-exponential fit of bR_{480} decay gives lifetimes of 12.2, 22.5, and 58.4 ms with relative amplitudes of 0.48, 0.26, and 0.36, respectively.

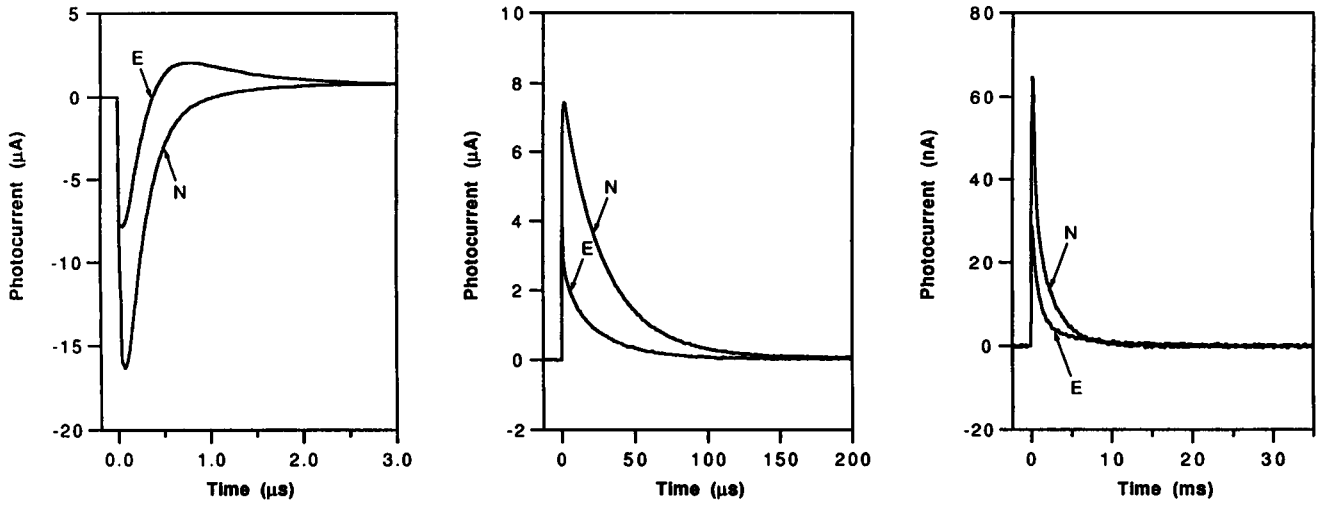

FIGURE 7 Transient photocurrent measured for gel-oriented purple membrane in the time ranges corresponding to B_1, B_2, and B_3 components. Measurements were done in 10 mM Tris-Cl buffer (pH 7.5). Curves *N* and *E* correspond to measurents on native membranes and Enflurane-treated (1% v/v) membranes, respectively.

same sample, it is noteworthy that the B_1 apparent amplitude and area are reduced in bR_{480} by comparison with bR_{570}. Taken alone, this result might indicate that less K is formed (which is certain, as discussed above) or that the charges move on a shorter distance or that a faster K $\rightarrow$ L transition prevents the detection of its real amplitude at this time resolution. Indeed, this reaction occurs faster in bR_{480}. Measurements made on different series of samples gave an average rise time of 307 ns ($\pm$15%) for this part of the signal, which dropped down to 192 ns ($\pm$6%) in the presence of enflurane or halothane.

From the transient absorbance measurements, we expected the proton extrusion current, i.e., the B_2 component, to occur faster. It is effectively the case, as fast B_2 relaxation components do appear when bR_{570} is tranformed into bR_{480}. The typical B_2 component of bR_{570} is well fitted by the sum of two exponentials with τ values close to 90 and 25 μs under mildly alkaline conditions. Under those conditions, bR_{480} is easily formed in the presence of the anesthetic. This is accompanied by acceleration of those components that are split into three with τ values of 27, 6, and 0.8 μs. The amplitude and the area of the B_2 component are greatly reduced in bR_{480}. After complete conversion of bR_{570} to bR_{480}, it is less than 20% of its original value.

The proton reuptake current, the B_3 component, is also strongly modified in bR_{480}. Its amplitude and speed are dramatically reduced. This is qualitatively consistent with the transient optical measurement. The area under B_3 is less than 15% of its original value after complete pigment transformation in the presence of enflurane or halothane. In the case of the latter, the B_3 component was even completely absent.

Buffer and salt effects on B_2 and B_3 components are well known (Liu et al., 1991, and references cited therein). Although in unbuffered pure water or salt solution, lower pH makes it more difficult to transform bacteriorhodopsin into its red spectral form, it remains possible to measure alterations of the charge displacement currents, which follow the same scheme as at higher pH. For instance, Fig. 8 shows the modification of the B_2 and B_3 components by enflurane in pure water and in 5 mM $CaCl_2$. In the former case, the B_3 component is much more evident than in the latter because of the absence of counter-ionic counter-current (Liu et al., 1990, 1991), but in both cases the current reduction effect of the anesthetic is still present, indicating its effect on the internal charge displacement.

Table 2 summarizes the general effect of anesthetics on the time constants, amplitudes, and integrated area of photocurrents of bacteriorhodopsin. Because photocurrent intensity depends on the speed and the distance of charge displacement in addition to the number of moving charges, data are not always directly comparable to optical transients, especially if pH is high (Liu, 1990). Nonetheless, like optical measurements, transient photocurrents clearly show the acceleration of deprotonation steps and deceleration of reprotonation. They are mostly characterized by the disappearance of the slow (60–100 μs) component of B_2 at the expense of a new fast submicrosecond one and by a slowing down of the B_3 components. The amplitudes of all components are reduced, but in all cases, the algebraic sum of negative and positive photocurrent components remained positive, indicating that net charge displacement occurs in bR_{480}. Nevertheless, the normalized area under the B_2 and B_3 positive components is an indication that proton pumping activity is strongly inhibited. It is noteworthy that removal of enflurane or halothane from the gel under mild vacuum or by bathing in anesthetic-free medium regenerated the original purple color of the samples and restored the typical waveform, kinetics, and amplitude of the electric response. In addition, photocurrents quantitatively comparable to those obtained in the presence of the anesthetics were obtained in the presence of trace amounts of hexane or large amounts of acetone; these experimental conditions

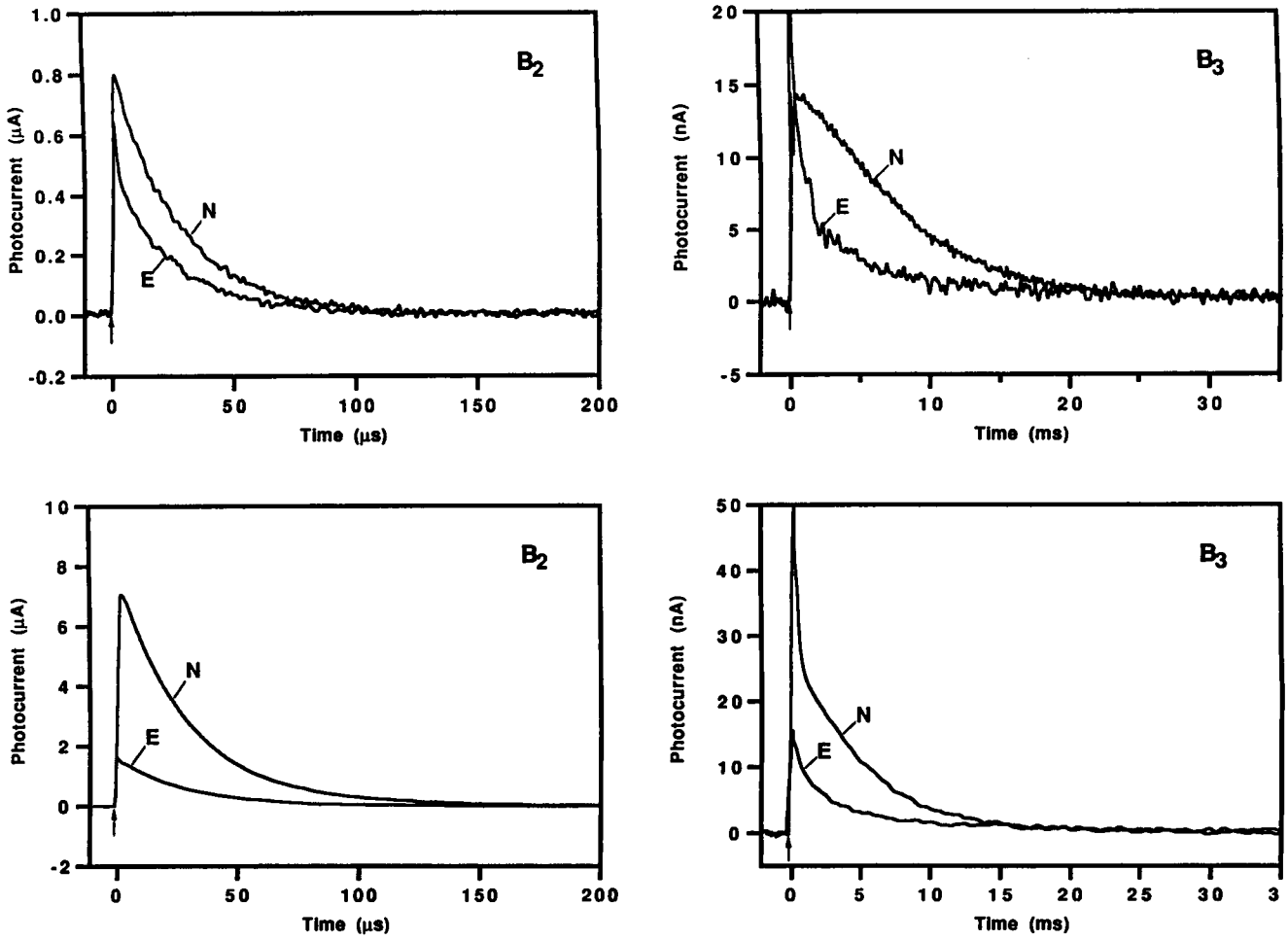

FIGURE 8 B_2 and B_3 photocurrent components measured in pure water (*upper part*) and in unbuffered 5 mM $CaCl_2$ (*lower part*), in the absence (*N*) or presence (*E*) of 1% Enflurane.

were previously found to have identical effects on the 570 and 480 nm chromophore equilibrium (Messaoudi et al., 1992).

The light-induced transient conductivity change by bR_{570} and bR_{480}

Time-resolved conductivity changes associated with the bacteriorhodopsin photocycle offers an additional means of evaluation of its proton pumping activity. The molecular origin of the transient conductivity change that occurs upon flash excitation of bacteriorhodopsin is controversial. Indeed, proton and nonproton ion movements between bulk water and the membrane hydration layer (Marinetti and Mauzerall, 1983; Marinetti, 1987) as well as change in membrane polarizability (Toth-Boconadi et al., 1994) might eventually explain the sign and size of the conductivity change observed under different experimental conditions. However, in the light of recent measurements of proton diffusion along and away from the purple membrane surface (Heberle et al., 1994; Alexiev et al., 1995), it appears clear that proton release to bulk water cannot be simultaneous with chromophore deprotonation. As a matter of fact, these measurements indicate that protons remain trapped within the membrane diffuse layer, from which they can escape only after a considerable delay. At very low ionic strength, the diffuse layer is large and makes likely the proton expulsion into that layer to trigger a counter-current of more mobile ionic species from the bulk water toward the diffuse layer and hence, a transient decrease of the bulk conductivity. This phenomenon was observed earlier under low-buffer and low-ionic-strength conditions (Marinetti and Mauzerall, 1983), and it is essentially what we observed for gel-immobilized purple membrane in 1 mM Tris buffer and 1 mM NaCl. Fig. 9 shows this behavior. Upon laser excitation, there is an abrupt decrease of conductivity followed by a slower increase and a final return to the initial level after a few hundred milliseconds.

Conversion of bR_{570} to bR_{480} upon addition of enflurane abolishes the transient conductivity signal, which is progressively recovered as enflurane is allowed to evaporate (Fig. 9). This is a clear indication that transient surface

TABLE 2 Relaxation times and areas of the positive components of bacteriorhodopsin photocurrents

Conditions	B_2 relaxation τ (amplitude)	B_3 relaxation τ (amplitude)	Relative area* (B_2 + B_3)
PM in water	59 μs (0.11)	8.8 ms (0.84)	1
	27 μs (0.89)	1.8 ms (0.16)	
+0.6% halothane	26 μs (0.22)	—	0.12
	7 μs (0.02)		
	0.7 μs (0.76)		
PM in buffer, pH 8.7	96 μs (0.12)	5.7 ms (0.87)	1
	26 μs (0.88)	1.4 ms (0.13)	
+1% enflurane	26 μs (0.27)	15.4 ms (0.20)	0.19
	4 μs (0.46)	1.14 ms (0.80)	
	0.7 μs (0.27)		
PM in water with 5 mM $CaCl_2$	84 μs (0.42)	5.7 ms (0.90)	1
	26 μs (0.58)	2.2 ms (0.10)	
+2% enflurane	28 μs (0.47)	22 ms (.09)	0.33
	7 μs (0.18)	4 ms (0.91)	
	0.8 μs (0.35)		

*Normalized to the negative component area.

charge movements are strongly inhibited in bR_{480} and might well reflect a reduced charge displacement where the proton does not reach the membrane surface.

DISCUSSION

As it is not possible to detect bulk pH change associated with the photochemical activity of the 480 nm form of bacteriorhodopsin, neither under continuous illumination nor after a short laser flash, we may infer that the 570 ↔ 480 nm spectral transition is accompanied by a switching of the pigment between pumping and nonpumping states. Whether the nonpumping state actually corresponds to a null or a dramatically reduced or slowed-down pumping activity cannot be ascertained from the pH change measurements. However, the action spectra measurements reported here clearly indicate that, by comparison with the purple bacteriorhodopsin, the proton translocation ability of bR_{480} is not significant. On the other hand, it can hardly be due to important differences in the yield of bleaching of both spectral forms, because irradiation of mixtures of bR_{480} and bR_{570} at 220 K shows that both forms bleach and produce their respective M intermediates with comparable yields, at least under continuous illumination.

Although the absence of proton release by bR_{480} cannot be explained by a reduced yield in the formation of a deprotonated M intermediate by this spectral form, the kinetic analysis of the 412 and 380 nm M intermediates can provide a partial explanation for the phenomenon. From the difference spectra of Fig. 4 and other published data (Henry et al., 1988), it is reasonable to say that the 412 and 380 nm forms of M belong to the purple and red forms of bacteriorhodopsin, respectively. Data of Table 1 show that under conditions where little, if any, bR_{480} is formed, chromophore deprotonation and reprotonation are both accelerated. As bR_{570} is transformed into bR_{480}, both fast and slow components of M rise are faster by a factor of ~3 with respect to the situation in native bR_{570}, but the M_{380} decay is ~4 times slower than that of the native M_{412} intermediate. These results fully agree with the observation by Uruga et al. (1991) that, under yellow light illumination (450–650 nm range) and pH conditions that do not favor bR_{480} formation, addition of small amounts of anesthetics to bacteriorhodopsin samples does accelerate the purple pigment cycling time and increases its H^+ pumping activity, whereas at higher anesthetic concentration, cycling time is longer and pumping activity is reduced. This is to say that in samples containing mixtures of bR_{570} and bR_{480}, the former species might translocate protons more than 12 times faster than the latter. Taking the M decay as the limiting step for H^+ pumping, one could predict that the action spectrum of a 50:50 mixture of bR_{480} and bR_{570} would be dominated by the 570 nm species but that of a 96:4 mixture should show appreciable pumping by the 480 nm species. Under the conditions of Fig. 3, bR_{570} is almost completely transformed into bR_{480}. However, the action spectrum of this sample gives no indication of proton pumping by the latter species. It is thus difficult to explain the absence of proton translocation on the basis of chromophore reprotonation rate alone.

In addition, the same kinetic analysis indicates that upon excitation by a short flash, both species deprotonate rapidly and should transiently release protons. In the presence of anesthetics, formation of the M intermediate is accelerated and occurs faster than in the absence of anesthetic. Thus, under conditions that favor high relative concentration of bR_{480} or complete transformation of bR_{570} into bR_{480}, transient proton release should be observed. Nevertheless, such transient proton release has not been detected, indicating that other factors, in addition to kinetic parameters, lower the proton pumping activity of bR_{480}.

Comparison between photocurrents due to charge displacement in native and anesthetic-treated purple membranes reveals differences comparable to those that exist in the transient absorbance analysis: in the 480 nm form, the B_2 deprotonation current occurs faster and the reprotonation B_3 current, when present, is slower. In many cases, the latter could not be resolved as the amplitude of the current produced by a much slowed down process is often too small to be measured (Butt et al., 1989; Trissl, 1990). Nonetheless, the integrated current, which is proportional to the number of cycling molecules and to the distance the charges do move, is smaller in bR_{480} (Keszthelyi and Ormos, 1983). In fact, in bR_{480}, the integrated charge displacements (current × time) are smaller than in bR_{570}. The B_1 component is two times smaller and the B_2 component is five times smaller. Although the reduction of area under B_1 can be explained by a reduced number of cycling molecules, proportionally larger reduction of other components indicates an altered charge displacement. It is likely that these area reductions reflect displacement on a shorter distance, especially if we

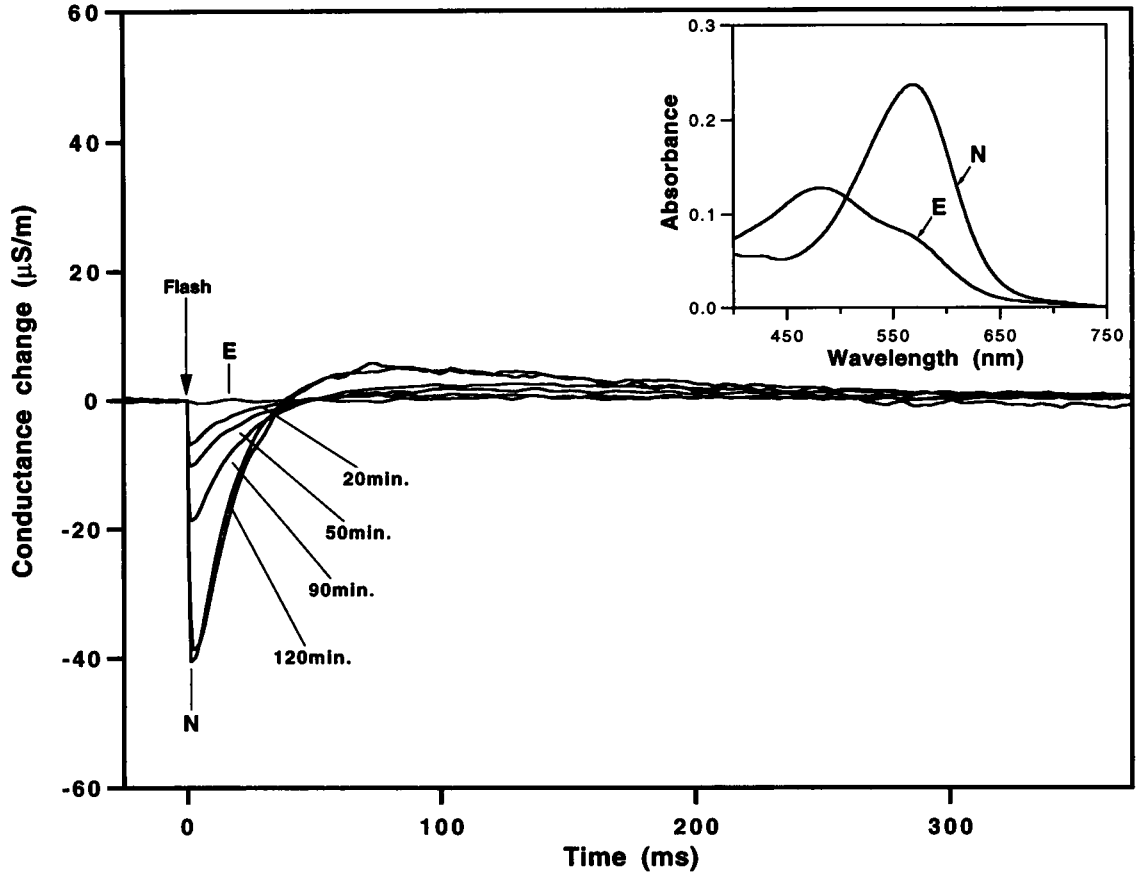

FIGURE 9 Transient conductivity change observed upon excitation of bR_{570} and bR_{480}. Sample consisted of unoriented purple membrane (64.5 μM bR) immobilized in gel (trace *N*) in 1 mM Tris-Cl buffer (pH = 8) containing 1 mM NaCl. After addition of Enflurane (2% v/v), bR_{570} rapidly converted to bR_{480} and the signal was lost (*trace E*). After opening cell to ambient atmosphere, Enflurane started to evaporate. The transient conductivity change observed after the evaporation times indicated in the figure shows the reversibility of the phenomenon. The inset gives the sample absorption before (*N*) and after (*E*) addition of Enflurane.

consider that bR_{480} possesses a relaxed tertiary structure. Should the proton displacement path length be reduced enough, the former could not reach the membrane surface and the conductivity change associated with this phenomenon would disappear, which is essentially our observation.

With respect to its charge displacement properties, bR_{480} recalls some specific features of bacteriorhodopsin under completely different experimental conditions, namely the $Asp^{85} \rightarrow$ Glu and the $Asp^{96} \rightarrow$ Asn point-mutated bacteriorhodopsins (Butt et al., 1989) and the high-pH form of bacteriorhodopsin (Liu, 1990; Kono et al., 1993). Indeed, as bR_{570} is transformed into bR_{480}, the slow (60 ~100 μs) and fast (~25 μs) B_2 current components are transformed into only fast (~0.5 μs, ~5 μs and ~25 μs) components. The ~0.5 μs and ~5 μs components are also those that dominate the B_2 signal of the high-pH form of bacteriorhodopsin and that of the $Asp^{85} \rightarrow$ Glu bacteriorhodopsin, two kinds of samples that have reduced charge displacement currents under B_2 and that, in the former case, do not pump protons (Kono et al., 1993) or, in the latter, have a delayed or decoupled pumping activity (Heberle et al., 1993). On the other hand, the slow M decay in bR_{480} and the difficulty of observing the slow B_3 component are phenomenologically comparable to what happens in $Asp^{96} \rightarrow$ Asn bacteriorhodopsin. It is also noteworthy that fast chromophore deprotonation and inhibited or delayed proton release are characteristics of the $Arg^{82} \rightarrow$ Ala (Balashov et al., 1993) and of $Tyr^{57} \rightarrow$ Phe (Govindjee et al., 1995) point-mutated bacteriorhodopsins. Both of these mutated pigments also show an acid-base chromophore equilibrium between 570 and 480 nm forms of bacteriorhodopsin, with apparent pK_a of > 11 and = 10.2, respectively.

Native bacteriorhodopsin behaves in a comparable manner when bR_{570} is titrated to bR_{480} in the presence of enflurane (pK_a = 7.3), although a similar blueshifted species only appears at pH higher than 10 in mutant pigments. Indeed, the $bR_{570} \Leftrightarrow bR_{480}$ chromophore equilibrium is an acid-base equilibrium where the 480 nm form contains an additional deprotonated group. This group must be strategically located on the proton path and close enough to the chromophore, as it has a significant influence on its absorption maximum. In the deprotonated state, it most likely acts as a proton trap that prevents charges from moving the distance they do in the native state of the pigment and

impairs the proton translocation capability of the pumping structure. The identification of Arg^{82} as a deprotonable group that would impair the proton extrusion pathway in bacteriorhodopsin has been discussed by Kono et al. (1993) and Govindjee et al. (1995). It might also well be the case in bR_{480} obtained by anesthetic treatment, although a more acidic group or cluster of groups would also be likely because Arg alone has a pK_a too high to be the one that would deprotonate near neutral pH, in the ground state, especially when the tertiary structure is relaxed, and that would remain protonated only in the native protein structure.

From the present data and their interpretation, no decisive conclusion can be drawn about a specific target site for anesthetic interaction with bacteriorhodopsin. The possibility that general anesthetics principally interact with the hydrophilic polar membrane surface is well documented, and it includes effects such as decrease of surface charge density, enhancement of counter-ion association, and destruction of hydration shell (see Ueda, 1991, for a critical review). Although a dehydrating effect of anesthetics might be an attractive explanation, as dehydrated purple membranes also have been shown to have an accelerated B_2 component and a reduced photoelectric activity (Varo and Keszthelyi, 1983), we do not think that increased counter-ion association is very likely because we observe a large effect of anesthetic on B_2 even in the presence of 5 mM divalent cations. In addition, the well-known hydrogen bond-breaking properties of anesthetics (Sandorfy, 1978) would make them excellent candidates to impair the functionality of the proton pumping channel, but this implies that anesthetics interact close to the chromophore, which does not appear to be the case, as interaction between the retinal and, at least, aromatic neighboring residues are left intact during bR_{570} transformation into bR_{480} (Lee et al., 1991). On the other hand, one must not forget that the acid-base-type chromophore equilibration considered here is also i) a property of delipidated (Baribeau and Boucher, 1987) or reconstituted (Lozier et al., 1976; Pomerleau et al., 1995) bacteriorhodopsin; ii) that, when induced with anesthetic or solvents, the phenomenon obeys the lipid solubility rule (Messaoudi et al., 1992) and occurs irrespective of membrane sidedness (Uruga et al., 1991); and iii) that only minor tertiary structural differences have been observed between both spectral forms in the purified state and in anesthetic-treated membranes (Pande et al., 1989; Messaoudi et al. 1993). In addition, general anesthetics preferentially locate near the interface and in the hydrocarbon domain of membranes in general (Barber et al., 1995) and of purple membrane in particular (Nakagawa et al., 1994). For these reasons, we believe that the switching of bacteriorhodopsin between the native purple state with high pumping activity and its red chromophoric state with strongly inhibited proton pumping is a reversible structural transition that has its origin in the hydrophobic domain of the membrane.

From this last point of view, the reversible switching between pumping and nonpumping states of bacteriorhodopsin can be sketched on the basis of destabilization of lipid-protein interactions. The efficient proton pumping channel of bacteriorhodopsin would thus appear as a fragile network of interactions whose structure is finely tuned by appropriate interactions in the native rigid membrane. Perturbation of these interactions by lipid-soluble solvents or anesthetics or substitution of native lipid-protein interactions upon delipidation or reconstitution reversibly relaxes the protein structure in such a way that a strategically located, previously hidden titrable group becomes available for deprotonation in the neutral to moderately alkaline pH range. When deprotonated, this group impairs the normal proton flow through the bacteriorhodopsin molecule, uncoupling the proton translocation activity from the photochemical activity.

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