Further Characterization of Anesthetic-Treated Purple Membranes by Time-Resolved Photoelectric Measurements

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Bacteriorhodopsin (bR) is a protein found in the purple membrane of the extreme halophilic Halobacterium Halobium.[1] There exist strong interactions between individual protein molecules and lipids, in addition to protein-protein interactions. The 570 nm absorbing pigment consists of a single polypeptide containing 248 amino acids which are folded into seven transmembrane helical segments. The pigment in the light-adapted form has an all-trans retinal chromophore bound to the ε- amino group of Lys-216 in the form of a protonated Schiff base. When it absorbs light, it undergoes a photochemical cycle during which protons are pumped from the inside to the outside of the cell resulting in a pH gradient across the cell membrane. This proton gradient drives metabolic processes such as ATP synthesis.[2]

Upon pigment formation by combination of all-trans retinal with bacterioopsin, the absorption maximum of the chromophore shifts from 360 to 570 nm. While protonation of the Schiff base can partly account for this shift (up to 440 nm), secondary interactions, mostly electrostatic in nature, must be invoked to explain the change of pigment absorption maximum.[3,4] Among blue-shifted bacteriorhodopsins, with respect to the native one, there exists a series of perturbed bacteriorhodopsin having absorption maximum near 480 nm, which can be obtained by pigment delipidation,[5] followed by dimethyl sulfoxide treatment,[6] or by addition of halogenated anesthetics.[7-9] In all these cases, resonance Raman spectroscopy indicated that they contain a protonated Schiff base having loose interactions with the protein moiety[10-12] but the nature of the protein structural change underlying the chromophore spectral change yet remains to be chracterized.

In this study, we observed the photoelectric signals in order to characterize the 480 nm bacteriorhodopsin (bR_{480}) obtained by addition of halogenated anesthetics to purple membranes with respect to the native one (bR_{570}). The photoelectric measurements could have a more general impact on the understanding of the connection between membrane structure and functions. We discussed the correlation between the photochemical cycle and photoelectric signal. Our study clearly demonstrated a great difference in conformational change of bR_{570} and bR_{480} by light absorption.

Experimental

Purple membranes (PMs) were isolated from the strain S9 Halobacterium Halobium. PMs were prepared essentially in the same manner as that described by Kates *et al.* (1982).[13] Prior to experimenation, PMs were washed to be free from salt by three successive sedimentations in bidistilled water at 17,000 g for 90 min..

Addition of volatile anesthetics (enflurane) to purple membranes was simply done by injection of the liquid product with a microliter syringe into a small quantity (1-3 mL) of PM suspension contained in a spectrophotometer cell. They were regularly equilibrated with water dissolved anesthetics, which in turn equilibrated with membrane dissolved anesthetics, by vigorous shaking of the samples every 2 min. during all experiments. Within the concentration range used, anesthetics alone showed no absorption (nor fluorescence) between 250 and 700 nm.

Absorption measurements were performed by using a Pye Unicam SP 8-100 spectrophotometer equipped with a diffuse sample holder which reduces the effects of light scattering by membrane suspensions.

For the photoelectric measurements (Figure 1), the oriented PM and the enflurane-treated PM were placed into a 0.55 cm thick cuvette and a pair of platinum electrodes were introduced into the sample solution. The sample was excited by the frequency doubled pulse (532 nm, 10 ns duration, 30 mJ/pulse) of a Q-switched Nd-YAG laser output (Laser System 2000, JK Lasers, Rugby, England). The voltage on the resister was amplified by a low-capacitance (3 pF) homemade amplifier and detected by a digital storage oscilloscope (Nicolet 4074, Nicolet Instruments Corp., Mississaga, Ont., Canada). The data were fitted with exponentials in a IBM PC/AT computer.

Results and Discussion

As previously observed by other investigators,[7,8] addition of the volatile anesthetics induces drastic change in the absorption property of purple membranes. Figure 2 shows the time progress of the change in the absorption spectrum of PM after the addition of enflurane. It is seen that the usual absorption band of PM at 570 nm gradually disappears to yield a new absorption species at 480 nm with an extinction

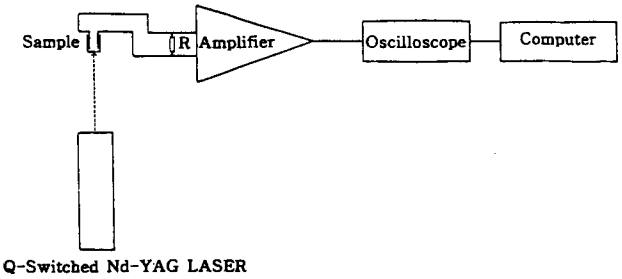

Figure 1. Scheme of the measuring apparatus.

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coefficient smaller by a 1.62 times than that of 570 nm band. Concurrently with the light-adapted PM absorption change there proceeds rapidly showing an isosbestic point at 510 nm in the presence of enflurane. The formation of the 480 nm species is completly reversible. When anesthetic-saturated PM suspension is exposed to an ambient atmosphere or a weak vaccum, the anesthetics evaporates and the suspension gradually recovers its original color. This is requisite for understanding of the molecular properties how volatile anesthetics interact with membrane lipids and/or with membrane proteins of purple membranes. Henry *et al.*[8] reported that weaker lipid-protein interactions due to increased lipid mobility might be responsible for the modification of the pigment in the presence of anesthetics. However the molecular mechanism by which volatile anesthetics might act on membranes is still controversial.

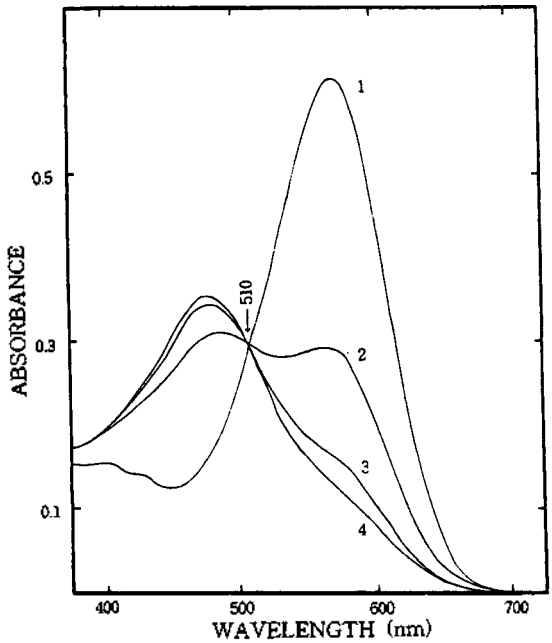

Figure 2. Absorption spectra recorded during the incubation of purple membranes in the presence of 1.0 vol % enflurane at 22 °C, in bidistilled water. 1: recorded before addition of enflurane, 2 to 4: 1, 3 and 10 min. after its addition, respectively.

Photocurrent kinetics experiments are known to provide further understanding of these different spectroscopic properties between bR_{480} and native one (bR_{570}).[14] Thus we measured the time-resolved photoelectric currents by excitation with a 532 nm laser pulse. According to this measurement, a fast downward transient photocurrent was observed corresponding to a movement of positive charge towards the cytoplasmic side of the membrane, opposite to the direction of stationary light-driven proton flow (Figure 3). This component is named B1 according to Liu.[14] This downward current is followed by a slower upward transient photocurrent (B2) in the same direction as the proton pump. B3, a millisecond range component follows in the same direction of B2. These observations qualitatively agree with previous photoelectric experiments with purple membrane sheets bound to planar bilayer membranes,[15] collodium or teflon films.[16,17] A correlation between the components of the photocurrent transient and the photochemical reaction steps inferred by flash-photometric experiments can be established on the basis of the observed time constants. The native 570 nm bacteriorhodopsin (bR_{570}) shows a photocurrent comparable to that of the 480 nm pigmement (bR_{480}) as shown in the upper and the lower parts of Figure 3A, respectively. The fast negative component of the photocurrent with a time constant of 1.3 μs (bR_{570}) as compared with a time constant of 0.86 μs (bR_{480}) is assigned to the K->L transition of the photocycle which has a spec-

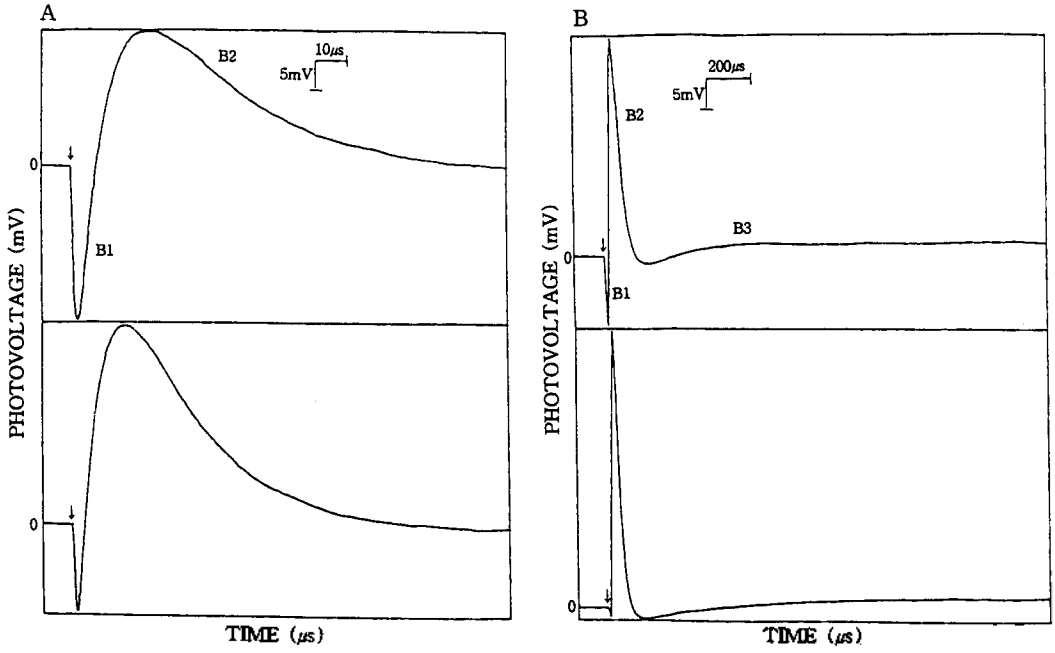

Figure 3. Photovoltage (mV) after a 10 ns laser flash at 532 nm, at two different time scales, i.e., A and B, respectively. The curves have been directly reproduced from the original recorder plot. Upper parts of A and B: Purple membrane in bidistilled water (bR_{570}). Lower parts of A and B: Enflurane-treated Purple membrane in bidistilled water (bR_{480}).

trophotometrically determined lifetime of about 1 μs.[18] However, there are a little discrepancies in the lifetimes of B1 and B2 as reported by different labs.[15,17] The B2 components of bR_{570} and bR_{480} of the transient photocurrent have time constants 24 μs and 14 μs, respectively. These values are similar to the time constant for the L-M transition which has been determined by spectrophotometric method as to be about 27 μs.[19] When the measurement was extended to the time range of the slowest component, the current from the voltage decay process could not be detected because of its small amplitude (Figure 3B).

The integration of the photocurrent over time for the B1 component is proportional to the number of the excited-bacteriorhodopsin and the moving distance of the charges during the transition.[15,20] The ratio of B1 integrated photocurrents for bR_{480} and bR_{570} is 1:3.0. This indicates that number of photoisomerized purple membrane (bR_{570}) is increased as compared with that of bR_{480}. The significant fraction of the photon energy is known to be stored in the primary photoproduct *via* photoisomerization in this step.[14] As the results, charge separation is a natural consequence of a photoisomerization. Here we could infer that the function of primary photoproduct of bacteriorhodopsin as a proton pump requires considerable photochemical energy storge. These facts lead us to conclude that the fraction of the total energy stored in the primary photoproduct of bR_{570} is larger than that of bR_{480}. On the other hand, the ratio of B2 integrated photocurrents for bR_{570} and bR_{480} is 1 : 0.90. This fact means that the proton pumping ability of bR_{480} form is decreased by about 10% because B2 component is involved in the proton pumping pathway. This is consistent with the fact reported by Girad *et al.*[21] for the 480 nm form of bacteriorhodopsin in unable to pump protons despite a complete photochemical cycle including the deprotonated M intermediate.

As for the time progress, the kinetics of B1 component of bR_{480} (0.86 μs) is faster than that of normal bR_{570} (1.3 μs). This fast time constant at the early stage of the photochemical cycle not only contains contributions from proton transfer along the pump pathway but also accounts for any movements of polar groups during conformational transition of proteins. So, B1 component means that K->L transition involves a major conformational change. The observed data indicates that charge displacement of bR_{480} is much shorter than that of bR_{570}. Therefore, it suggests that the conformational change takes place in bR_{480} through the perturbation by the anesthetics.

In conclusion, the present results show that bR_{480} is produced by the pre-conformational change of protein due to anesthetics perturbation and consequently the proton pump efficiency of the bacteriorhodopsin is reduced.

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