LOW TEMPERATURE KINETICS OF H^+ CHANGES OF BACTERIAL RHODOPSIN

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The significance of hydrogen ions in energy conservation depends upon the thermodynamic properties of the chemical reaction in which they may be involved. In many cases of bioenergetic systems the binding, release, or transport of hydrogen ions to or across membranes is readily demonstrated in an overall sense. The salient problem before bioenergeticists is to identify the chemical reactions involved.

In the case of isolated patches of membranes of *Halobacterium halobium*, the chemistry seems well-understood, as evidenced by the reports of the Workshop on Membrane Bioenergetics in this issue (1, 2), abstracts of the Annual Meeting of the Biophysical Society (3–9), as well as work elsewhere (10–12). It should therefore be possible to identify which chemical species releases H^+ in the illuminated bacterial rhodopsin.

The use of very low temperatures to isolate intermediate steps in peroxidase peroxide (13) and cytochrome oxidase oxygen reactions (14)—and, indeed, hydrogen ion uptake in cytochrome oxidase oxygen reactions—is productive of unusually clear results. We afford an example of this approach to the H^+ release problem in the "purple membranes."

EXPERIMENTAL METHOD

In this experiment we have devised an apparatus for simultaneous observation of absorption changes of bacterial rhodopsin and H^+ changes. Fig. 1 illustrates our experimental method. The bacterial rhodopsin (40–90 μM) is contained in a 3.5 mm diameter quartz EPR tube which is inserted into a brass block through which is circulated ethanol at $-50°C$ to $-20°$ (15).

Observation of pH changes is afforded by fluorescence changes of the indicator 4-Me-umbelliferon (9–30 μM) which is fluorescent in alkaline solutions. It shows low affinity for the purple membranes. The pH of the unbuffered suspension was set near the pK of the indicator (7.8) with addition of NaOH. A tungsten-iodine lamp (20% over voltage) gives adequate 366 nm excitation of the dye (Kodak Wratten 18A filter) and the fluorescence emission is measured at 450 nm (Kodak Wratten 2C and 47B). A direct coupled fluorometer is employed and is accurate to 1% for fluorescence changes and has a response speed of ~ 5 ms. Since fluorescence measurements are made from the surface of the sample, 366 nm and 450 nm are acceptable wavelengths. Suitable controls outside the range of the indicator gave no significant artifact. The quantum yield of the dye is already high at room temperature and is not greatly increased at low temperatures.

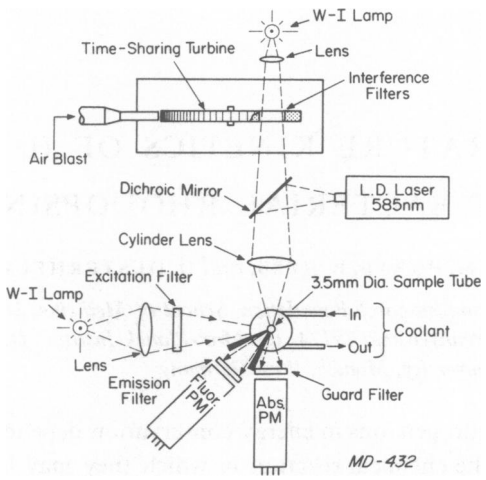

FIGURE 1 An apparatus for time-shared spectrophotometry and direct-coupled fluorometry of intermediates in the photolysis of bacterial rhodopsin. The details of the apparatus are discussed in the text.

The absorption changes of the purple membranes were measured with a turbine-driven time-sharing spectrophotometer which alternates four interference filters (5 nm half-band width) in the light path. Absorption differences at two pairs of wavelengths are provided. Filters were selected to emphasize the early steps of the light reaction (640–706 nm), and the Schiff-base deprotonation (550–540 nm). The direction of absorption increase at the first named member of a wavelength pair is indicated by the arrow in the two sets of traces of Fig. 2. Note that the recordings *a* are in the same sense and those of *b* and *c* in the opposite sense. The large scattering of frozen suspensions of purple membranes at wavelengths below 500 nm has prevented our effective use of absorbancy changes at 412 nm. The temperature was measured by a thermocouple inserted into the sample tube. The temperature along the tube was uniform to within a few degrees.

Since Schiff-base deprotonation reactions seem much more rapid than the reprotonation at −40° the data were recorded on two time scales covering minutes and seconds in order to verify the dark recovery phases as well as to time resolve the initial phases.

Flash activation was obtained with a 100 mJ rhodamine 6-G liquid dye laser tuned to 585 nm. The pulse duration was 1 μs. This illumination was joined with that of the time-sharing spectrophotometer by a dichroic mirror. The laser pulses which were about 20 mJ at the sample were not fully saturating, possibly due to absorption screening in the sample tube.

Preparations. Membrane patches of *H. halobium* were obtained from D. Oesterhelt and were prepared by his method (11). 25% ethylene glycol was added to the salt medium (3 M) and gave unusual supercooling phenomena so that separation at −40° without freezing was often possible.

RESULTS

An example of the results pertinent to the identification of the H^+ release and the intermediates of bacterial rhodopsin photolysis is afforded by Fig. 2. The top six traces are on a slow and constant time scale and the bottom six traces are on faster and different

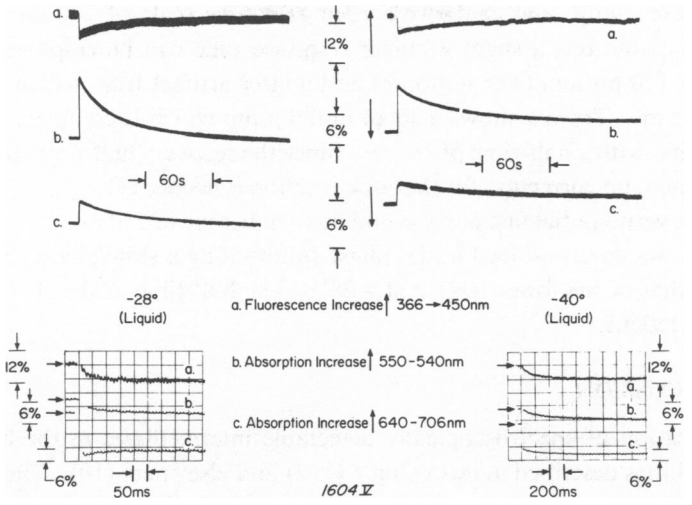

FIGURE 2 Kinetics of intermediates and of H^+ changes in a suspension of membrane patches of *Halobacterium halobium*. 90 μM bacterial rhodopsin, 9 μM 4 Me-umbelliferon, 2 M NaCl, 25% ethylene glycol, pH = 7.4. The arrows indicating absorbance changes correspond to 550 and 640 nm; 540 and 706 nm are "reference" wavelengths. Note that the arrows indicate the direction of the deflection corresponding to an absorbance increase at the first named wavelength of the pair listed. Other details are given in the text.

time scales. Two temperatures are employed, −28° at which the kinetics are not resolved and −40° at which some resolution seems possible.

The upper slow recordings show unresolved initial absorption and fluorescence changes following laser photolysis of the membranes. These traces were recorded following recovery (5–8 min) from a previous flash at the same temperature. Thus the initial state is set by those reactions that are reversible at −28° and −40°. Since the amplitudes are approximately equal at the two temperatures the initial condition prior to the flash can be considered to be identical.

Following photolysis, the recovery to the initial state has a half-time of 21 s at −28° (trace *b*) (550–540 nm). At −40°, the half-time is 29 s. The end point of the recovery is not reached and a small biphasicity can be seen. The ratio of the half-times is 1.4 and of the initial slopes is 1.8. Traces *a* and *c* show a similar slope ratio and energy of activation. Thus, it seems that the pH changes correlate well with the absorption changes in the recovery phase at these temperatures.

In the initial phase, at −28°, and on a time scale of 50 ms per division, little of the kinetics of the absorption changes is resolved due to laser artifact and amplifier bandwidth limitations. Only a slow tail of traces *b* and *c* is resolved which seems coincident with the later phases of trace *a*. In the initial phase of the pH change it appears that the reactions can be measured (5 ms amplifier response) with a $t_{1/2} \leq 25$ ms, and $k \geq 30\ s^{-1}$ at −28°.

In order to afford a more accurate correlation with absorption changes, the data at

$-40°$ are more useful, and can be recorded at a time scale of 200 ms per division. Under these conditions a slightly longer response time can be employed—affording clearer traces (10 ms amplifier response) and a laser artifact from which the amplifier recovers in 15 ms. Trace *a* shows a small initial jump which is complete in 10 ms and then a response with a half-time of 180 ms. Since the recovery half-time (trace *a* above) is about 1.5 min, no correction for the back reaction is necessary.

Trace *b* shows no initial fast portion and has a half-time of 220 ms.

Trace *c* shows an unresolved initial phase followed by a slow phase (in a direction opposite to that of the lower trace *c* at $-28°$). The half-time of the slow phase is approximately 150 ms.

DISCUSSION

The identification of spectroscopically detectable intermediates in the bacterial rhodopsin reaction is described in this volume (1, 2) and elsewhere (10). Their terminologies for the reaction sequence are $K_{610}^{LA} \rightleftharpoons L_{550}^{LA} \rightleftharpoons M_{412}^{LA}$, $R_2 \rightleftharpoons R_3 \rightleftharpoons R_4$, BR 630 $\rightleftharpoons$? $\rightleftharpoons$ BR 411, respectively. We shall discuss the absorption changes at 550–540 nm in the light of their data. It appears that neither the first nor second intermediates would be observed on our time scale, and that the third would cause a further absorbancy decrease over the first two as recorded in traces *b* and *c* at $-28°$ and $-40°$. Thus the results indicate a correlation of the deprotonation of the retinylidine-lysine bond with the formation of the M, R_4, or BR 411 intermediate, at $-40°$.

At 640–706 nm the data of refs. 1 and 2 suggest a similar sequence of changes as at 550–540 nm but of a smaller amplitude. The fast absorbance decrease at 640–706 nm appears somewhat larger than that for intermediates L or R_2. However, the slower absorption decrease of trace *c* is in accord with the spectra for M or R_4 and supports the conclusion above.

The slowness of the H^+ release at $-28°$ is an indication of another rate-limiting step in this reaction, observable at higher temperatures (1). These results may cautiously be interpreted in favor of this view and would then agree with the conclusions of Lozier that the H^+ release lags the M stage, and that of Dencher that the H^+ release agrees with formation of BR 646 following BR 411 (or M). It is possible that the energy of activation for the intermediate M differs from that of the rate-limiting step in the H^+ release.

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REFERENCES

1. LOZIER, R. H., R. A. BOGOMOLNI, and W. STOECKENIUS. 1975. Bacteriorhodopsin: a light-driven proton pump in *Halobacterium halobium*. *Biophys. J.* **15**:955.
2. KUNG, M., D. DEVAULT, B. HESS, and D. OESTERHELT. 1975. Photolysis of bacterial rhodopsin. *Biophys. J.* **15**:907.

3. MACDONALD, R. E., and J. K. LANYI. 1975. Light-driven leucine transport in *Halobacterium halobium* cell envelope vesicles. *Biophys. J.* **15**(2,Pt.2):65*a*. (Abstr.).
4. BECHER, B. M., and J. Y. CASSIM. 1975. Effects of light perturbation on the absorption spectra of purple membrane from *Halobacteria halobium*. *Biophys. J.* **15**(2,Pt.2):65*a*. (Abstr.).
5. BECHER, B. M., and J. Y. CASSIM. 1975. Effects of light perturbation on the circular dichroism of purple membrane from *Halobacterium halobium*. *Biophys. J.* **15**(2,Pt.2):66*a*. (Abstr.).
6. CHANCE, B., M. PORTE, B. HESS, and D. OESTERHELT. 1975. Low temperature kinetics of H^+ changes of bacterial rhodopsin. *Biophys. J.* **15**(2,Pt.2):66*a*. (Abstr.).
7. DEVAULT, D., M. C. KUNG, B. HESS, and D. OESTERHELT. 1975. Photolysis of bacterial rhodopsin. *Biophys. J.* **15**(2,Pt.2):67*a*. (Abstr.).
8. CHIGNELL, C. F., and D. A. CHIGNELL. 1975. A spin label study of purple membranes from *Halobacterium halobium*. *Biophys. J.* **15**(2,Pt.2):67*a*. (Abstr.).
9. RENTHAL, R., and J. K. LANYI. 1975. Light-dependent changes in electrical potential of cell envelope vesicles from *Halobacterium halobium* measured with a cyanine dye. *Biophys. J.* **15**(2,Pt.2):68*a*. (Abstr.).
10. DENCHER, N., and M. WILMS. 1975. Flash photometric experiments on the photochemical cycle of Bacteriorhodopsin. *Biophysics of Structure and Mechanism.* **1**(3). In press.
11. OESTERHELT, D., and B. HESS. 1973. Reversible photolysis of the purple complex in the purple membrane of *Halobacterium halobium*. *Eur. J. Biochem.* **37**:316.
12. HESS, B., and D. OESTERHELT. 1974. Reversible photolysis of the purple complex from *Halobacterium halobium* as a basis for the function of the purple membranes. *In* Dynamics of Energy-Transducing Membranes. L. Ernster, R. W. Estabrook, and E. C. Slater, editors. Elsevier Publishing Co., Amsterdam. 257.
13. DOUZOU, P., R. SIREIX, and F. TRAVERS. 1970. Temporal resolution of individual steps in an enzymic reaction at low temperature. *Proc. Natl. Acad. Sci. U.S.A.* **66**:787.
14. CHANCE, B. 1975. On the mechanism of the reaction of cytochrome oxidase with oxygen. *Ann. N.Y. Acad. Sci.* **244**:163.
15. CHANCE, B., N. GRAHAM, and V. LEGALLAIS. 1975. Low temperature trapping method for cytochrome oxidase oxygen intermediates. Anal. Biochem. **67**:552.