BRIEF COMMUNICATION

DYNAMICS OF pH-INDUCED SPECTRAL CHANGES IN BACTERIORHODOPSIN

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ABSTRACT The kinetics of the spectral shift induced in bacteriorhodopsin by low pH are investigated by using the rapid-mixing, stopped-flow technique. The generation of the acid form of the chromophore (A605) occurs in two distinct steps: a fast process ($t_{1/2}^{I} = 21 \pm 4$ ms) is followed by a much slower reaction ($t_{1/2}^{II} = 6 \pm 2$ s). The observations are interpreted in terms of neutralization of an acid group in the neighborhood of the retinyl chromophore, the double-staged kinetics being attributed to cooperative effects between chromophores. The method may serve as a tool for studying the kinetics of proton migration across the purple membrane.

INTRODUCTION

Bacteriorhodopsin, the pigment of protein of the purple membrane of *Halobacterium halobium* (1, 2), undergoes a photocycle during which protons are pumped from the cytoplasm across the cell membrane. This leads to a pH gradient used by the bacteria to energize ATP synthesis (3–4). It is highly probable that deprotonation of the Schiff-base linkage between the retinyl-polyene chromophore and the bacterio-opsin protein plays a central role in the proton pumping function of the pigment (1, 2, 5–7).

The 570-nm chromophore of light-adapted bacteriorhodopsin (BR_{570}^{LA}) undergoes reversible spectral shifts at low pH values (8).[1] These are characterized by an apparent pK of 2.8, associated with the generation of a red-shifted acid species absorbing $\simeq$605 nm (A605). Further acidification leads to a blue shift as a result of the formation of another acid modification (A565, pK $\simeq$ 0.5).

Such pH-induced spectral changes have recently been interpreted in terms of acid-base equilibria of protein groups in the neighborhood of the retinyl moiety.[2] In the present communication the rapid-mixing, stopped-flow method is applied for determining the kinetics of the A605 generation after fast acidification of purple membrane suspensions. To the extent that the induced spectral change is associated with the titration of an acid protein group in the neighborhood of the chromophore, the data reported may be directly relevant to the mechanism of proton migration across the membrane to the chromophore site.

[1]Mowery, P. C., R. Lozier, Q. Chae, T. W. Tseng, M. Taylor, and W. Stoeckenius. Manuscript in preparation.
[2]Warshel, A., and M. Ottolenghi. Kinetic and spectroscopic effects of protein-chromophore electrostatic interactions in bacteriorhodopsin. Manuscript in preparation.

MATERIALS AND METHODS

Unbuffered aqueous suspensions of light-adapted purple membrane fragments of M1 or S9 *H. halobium* were rapidly mixed with hydrochloric acid solutions in an American Instrument-Morrow stopped-flow apparatus (American Instrument Co., Silver Springs, Md.). Transient absorbance changes after mixing were recorded (with a ~2-ms time resolution) using a tungsten monitoring lamp, a Beckman monochromator (Beckman Instruments, Inc., Fullerton, Calif.) and RCA IP-28 or Hamamatsu R-446 photomultipliers (RCA Distributor & Special Products Div., RCA Corp., Camden, N.J.; Hamamatsu Corp., Middlesex, N.J.). The pH of the acid mixtures, in the range $2 < pH < 3$, were measured after accumulating sufficient amounts of the corresponding solutions.

RESULTS AND DISCUSSION

After acidification to a pH in the neighborhood of the apparent pK of A605, solutions of BR^{LA}_{570} undergo transient absorbance changes as shown in Fig. 1. Characteristic oscillograms at a pH of 2.5 (25°C) show a fast drop in absorbance $\simeq$570 nm (process I) followed by a much slower decay (process II). Above 610 nm, similar two-staged kinetics are observed but with opposite sign, corresponding to an absorbance growing-in. Analysis of the data indicates that both processes are closely exponential with wavelength independent half-lives of $t^{I}_{1/2} = 21 \pm 4$ ms and $t^{II}_{1/2} = 6 \pm 2$ s. No significant pH effects on $t^{I}_{1/2}$ and $t^{II}_{1/2}$, in the range $2.1 < pH < 2.7$, were observed. It appears however that the amplitude of the slow process decreases with decreasing pH.

Fig. 1 shows the two difference spectra, $\Delta D^{I}(\lambda)$ and $\Delta D^{II}(\lambda)$. Their sum, corresponding to the overall absorbance change observable within the time resolution of the instrument (2 ms to 20 s), is similar but not identical to the "static" difference spectrum between neutral and the same acid pH observed in gel membrane suspensions by Mowery et al.[1] The 560-nm minimum in the latter spectrum appears to be red-shifted by ~20 nm in respect to that of $\Delta D^{I}(\lambda)$ +

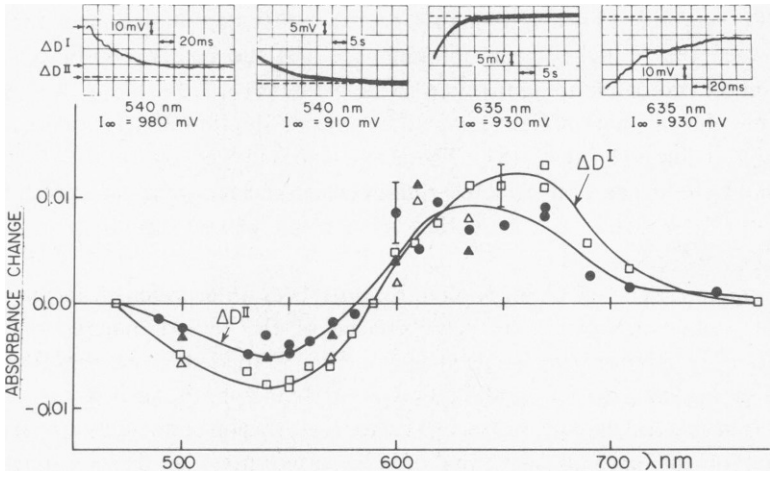

FIGURE 1 Characteristic oscillograms and difference spectra associated with the fast (ΔD^{I}) and slow (ΔD^{II}) processes, obtained after rapid acidification to 2.5 pH of a purple membrane suspension (bacteriorhodopsin = 7×10^{-5}M). I_{∞} is the final, light-to-dark, voltage difference in the acidified solution. Its level is marked by the dotted line in each oscillogram. Different point notations refer to different experimental runs.

$\Delta D^{II}(\lambda)$. At present it is still unclear if such a discrepancy is experimental in nature (sample conditions, etc.), or is a result of very fast or very slow kinetic components that remained unresolved in the present kinetic experiments.

We attempt to rationalize the nature of the double-staged kinetics of the A605 generation in terms of neutralization of an acid anionic group close to the chromophore (9).[2] In view of the presumed identity of all chromophore sites in bacteriorhodopsin (1, 2), the factor of more than two orders of magnitude between $t_{1/2}^{I}$ and $t_{1/2}^{II}$ makes it highly unlikely that the two distinct steps (which exhibit similar but not identical difference spectra) might be attributed to two different chromophore sites reacting independently with protons. A preferable alternative would be that of attributing the two-stage kinetics to a cooperative effect between chromophores. Accordingly, the fast red shift associated with process I reflects proton migration from the external medium to the chromophore site. Neutralization of the acid anionic group in a fraction of the bacteriorhodopsin molecules (possibly two out of the three members of the trimer [1, 2]) will inhibit the neutralization of the rest, as reflected by the slow process, II. Further work is in progress aiming to understand the relationship between the rates of pH-induced spectral shifts and those of the photoinduced proton uptake and release processes in bacteriorhodopsin. It appears, however, that the application of flow methods for studying the dynamics of pH effects on the spectrum may serve as a tool for following proton translocation processes in the purple membrane.

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