TIME-RESOLVED ABSORBANCE CHANGES INDUCED BY FAST ACIDIFICATION OF BACTERIORHODOPSIN IN VESICLE SYSTEMS

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ABSTRACT The direction of the accessibility to protons of the binding site in bacteriorhodopsin is of primary importance in elucidating the proton-pump mechanism. The problem is approached via the pH-dependent equilibrium $bR_{560} \rightleftharpoons bR_{605}$ in vesicles with preferentially oriented purple membranes. Fast acidification (stopped-flow) experiments with inside-out, monomeric, bR vesicles were carried out with and without a buffer enclosed in the vesicle interior. The results, showing a buffer-induced delay in the formation of bR_{605}, indicate that the binding site is accessible to protons from the inside of the vesicles. We arrive at this conclusion also by working with inside-out trimeric vesicles in the presence and in the absence of H^+ (and K^+) ionophores. The results suggest that in *Halobacterium halobium*, the binding site and thus the retinal Schiff base are exposed to the outside of the cell. This conclusion is consistent with a pumping mechanism based on a light-induced pK change.

INTRODUCTION

Accumulated evidence indicates that the retinal Schiff-base chromophore is directly involved in the photo-induced proton pump of bacteriorhodopsin, bR, (for reviews see Stoeckenius et al., 1979, and Ottolenghi, 1980). According to this assumption, a prerequisite for pumping is that the Schiff base and thus the retinal binding site are accessible to protons only from one side of the membrane (Kalisky et al., 1981). It was suggested that pumping may be due to a photo-induced change in the proton accessibility, e.g., to a change in the exposure of the Schiff base, from the cytoplasmic side to the outside. Alternatively, pumping may be initiated by a light-induced pK change of the Schiff base. A prerequisite for this mechanism is that the Schiff base is exposed to the outside. The direction of the accessibility of the binding site is thus of primary importance in elucidating the pump mechanism. Here the accessibility problem is approached via the pH-dependent equilibrium between the 560-nm chromophore of bacteriorhodopsin, bR_{560}, and the acid species, bR_{605}, (Oesterhelt and Stoeckenius, 1971; Mowery et al., 1979). The red shift associated with bR_{605} was attributed (Warshel and Ottolenghi, 1979) to the titration of a protein carboxylate moiety in close interaction with the protonated Schiff base. The kinetics of the $bR_{560} \rightarrow bR_{605}$ process were previously followed by us in purple membrane suspensions using stopped flow techniques (Druckmann et al., 1979). Here such methods are applied to vesicles with preferentially oriented purple membranes. Assuming that the Schiff base and the above carboxylate moiety have the same exposure to protons, the results are indicative of the direction of the accessibility of the Schiff base to protons.

EXPERIMENTAL

Inside-out soybean or cardiolipin vesicles containing bR were prepared based on the procedure of Racker (1973) that, according to Gerber et al. (1977), yields 85–95% orientation of the purple membrane. 10 mg of the corresponding lipid (obtained from Sigma Chemical Co., St. Louis, MO) were sonicated in a 2.5 cc, 0.15 M KCl solution for 15–20 min using a tip sonicator (W-375; Heat Systems-Ultrasonics, Inc., Plainview, NY). This was followed by pH adjustment to 6–7 and the addition of purple membrane sheets prepared from *Halobacterium halobium* strain MI. The suspensions were sonicated for 30 s after freezing twice to liquid nitrogen temperature. Casadio and Stoeckenius (1980) have shown that the lipid-bR ratio in the above suspensions determines the relative amounts of monomeric or trimeric inside-out forms of bR in the vesicles. Based on such findings, trimer or monomer systems were prepared using a molar lipid bR ratio of <70 and >160, respectively. Inside-in vesicles in which bR is oriented as in the intact cell of *Halobacterium halobium* were prepared according to Happe et al. (1977). The net orientation of bR in the vesicles was confirmed by measuring the sign of light-induced pH changes induced by continuous illumination of the preparation.

Vesicles with 0.1 M (pH = 6.5) potassium phosphate buffer enclosed in their internal volume were obtained by preparation in the buffer followed by an overnight dialysis against 0.07 M K_2SO_4 solution. The ionophores (10^{-5} M) FCCP, gramicidin, and valinomycin were obtained from Sigma Chemical Co.

Stopped-flow experiments, with a time resolution of ~2 ms, were performed using a Durrum mixing cell (Durrum Instrument Corp., Palo Alto, CA) and a Beckman spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Kinetic data were digitized using a Biomation 610 transient recorder (Gould Inc., Instruments Div., Santa Clara, CA).

Jumps to acid pH were carried out by 1:1 mixing of a bR suspension with 0.01 N HCl, yielding ≈2.3 pH.

RESULTS AND DISCUSSION

Fig. 1 *b* shows characteristic traces observed following acidification of inside-out soybean vesicles of monomeric bR. In these vesicles, the purple membranes have an exposure opposite to that of the intact bacterial cell. For a comparison the results obtained in a suspension of PM sheets are also included (Fig. 1 *a*). In the sheets the bR_{605} formation kinetics are two staged, exhibiting a fast ($\tau_{1/2}$ = 20 ± 4 ms) and a slow ($\tau_{1/2}$ = 6 ± 2 s) phase. These two components were attributed to cooperativity effects between aggregated (trimeric) chromophores in the purple membrane (Druckmann et al., 1979). As shown in Fig. 1 *b*, only a slow phase is observed in the vesicles. The above kinetic parameters, as well as others presented in the subsequent sections, are summarized in Table I. The reader is referred to this table for a comparison between the various rate parameters.

Experiments carried out above ~700 nm (where bR does not absorb) indicate that the slow absorbance change associated with the generation of bR_{605} is superimposed on a substantial acidification-induced increase in light scattering due to aggregation phenomena. Acidification-induced scattering phenomena are also observed in PM suspensions. However, not only are such changes smaller than those in the vesicles, but their kinetics are considerably slower. Consequently, the absorption changes due to the $bR_{560} \rightarrow bR_{605}$ process are easily separable from the scattering events (Druckmann et al., 1979). To separate the two processes in the vesicles, we first obtained the scattering kinetics at wavelengths where the absorbance change due to $bR_{560} \rightarrow bR_{605}$ is negligible, i.e., above 700 nm, at the isosbestic wavelength, 590 nm, and at ~450 nm. The scattering changes (e.g., Fig. 2 *b*) are found to be somewhat slower than the absorbance changes associated with the formation of bR_{605} (initial phase in Fig. 2 *a*, *c*). This enables us to estimate the net contribution of the absorbance changes by extrapolation. Accordingly, the absorbance change due to the $bR_{560} \rightarrow bR_{605}$ process (ΔA^i_{bR}) is given by the difference between the apparent initial absorbance change shown by the solid line (ΔA^i_{ap}) and the extrapolated contribution of scattering (ΔA^i_{sc}) shown by the broken line. The separate contribution of the change in scattering (ΔA^i_{sc}) is given by the traces at longer times, e.g., 20 s after termination of the initial (absorbance) phase. Complete difference spectra for ΔA^i_{bR} and ΔA^i_{sc} are given in Fig. 3. Their agreement with the known spectra for the $bR_{560} \rightarrow bR_{605}$ transition (Mowery et al.,

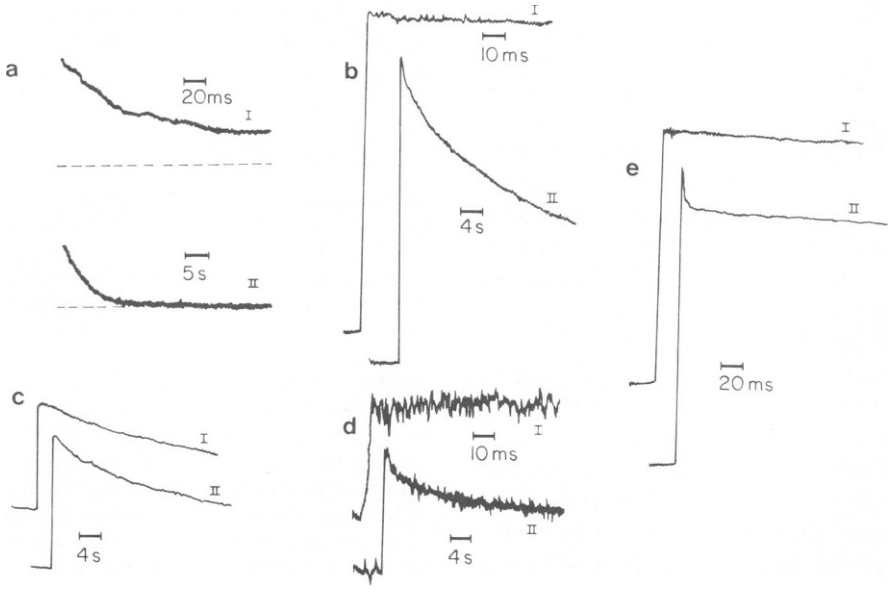

FIGURE 1 Characteristic traces of stopped-flow pH jumps in bacteriorhodopsin systems. The initial segment in traces *b–e* and the broken line in *a* refer to the final acidification products obtained in the previous jump. The vertical scale is linear with the transmittance of the solution. Traces *a–e* represent acidification of neutral systems observed at 635 nm. In such experiments a fresh (neutral) solution of bR is mixed with 0.01 N HCl to pH = 2.3 replacing the acid solution of the previous run. Consequently, a fast rise in transmittance (ΔV_i, measured in millivolts) is observed due to replacement of the acid solution containing the (bR_{570}, bR_{605}) equilibrium species by one containing only bR_{570}. The corresponding change in absorbance is given by $\Delta A_i = \log[V_o/(V_o + \Delta V_i)]$ in which V_o represents the light-to-dark deflection of the monitoring beam before mixing (initial trace segments). (*a*) Purple membrane suspensions. (*I*) ΔV_i = 32 mV, V_o = 930 mV. (*II*) ΔV_i = 16 mV, V_o = 930 mV. (*b*) Inside-out soybean vesicles. (*I*) ΔV_i = 3,496 mV, V_o = 2,490 mV. (*II*) ΔV_i = 3,456 mV, V_o = 2,345 mV. (*c*) Inside-out soybean vesicles with enclosed buffer (see text). (*I*) ΔV_i = 1,200 mV, V_o = 1,370 mV. (*II*) Same as I with FCCP (10^{-5} M) and gramicidin (10^{-5} M). ΔV_i = 1,518 mV, V_o = 1,450 mV. (*d*) Inside-in cardiolipin vesicles. (*I*) ΔV_i = 491 mV, V_o = 5,950 mV. (*II*) ΔV_i = 491 mV, V_o = 5,000 mV. (*e*) Inside-out soybean vesicles with aggregated (trimeric) bR (see text). (*I*) ΔV_i = 2,800 mV. V_o = 2,680 mV. (*II*) Same as I with FCCP (10^{-5} M) and valinomycin (10^{-5} M). ΔV_i = 3,344 mV, V_o = 2,680 mV.

TABLE I

HALF-LIVES OF PROCESSES OBSERVED FOLLOWING FAST ACIDIFICATION OF BACTERIORHODOPSIN SYSTEMS

Process	System	Enclosed buffer	Ionophores	Half-life
bR_{605} formation	PM sheets*	—	—	$\tau^{I}_{1/2} = 20 \pm 4$ ms $\tau^{II}_{1/2} = 6 \pm 2$ s
bR_{605} formation	IOM vesicles	—	—	$\tau_{1/2} = 8 \pm 3$ s
bR_{605} formation	IOM vesicles	0.1 M phosphate, pH = 6.5	—	$10\text{ s} < \tau_{1/2} < 40\text{ s}$
bR_{605} formation	IOM vesicles	0.1 M phosphate, pH = 6.5	FCCP 10^{-5} M, gramicidin 10^{-5} M	$\tau_{1/2} \sim 8$ s
bR_{605} formation	IOT vesicles	—	—	$\tau_{1/2} \sim 8$ s
bR_{605} formation	IOT vesicles	—	FCCP 10^{-5} M, valinomycin 10^{-5} M	$\tau^{I}_{1/2} \sim 3$ ms $\tau^{II}_{1/2} \sim 8$ s
bR_{605} formation	IIM vesicles	—	—	$\tau_{1/2} \sim 8$ s
Scattering	PM sheets	—	—	$\tau_{1/2} > 2$ min
Scattering	Vesicles	—	—	$10\text{ s} < \tau_{1/2} < 40\text{ s}$

IO = inside out; II = inside in; M = monomeric; T = trimeric.

*Druckmann et al. (1979).

1979) and for the scattering change validates the above analysis. A quantitative analysis of the time dependence of ΔA^{i}_{bR} yields a half-life of ~8 s for the absorbance change process.

The lack of a fast millisecond phase for the $bR_{560} \rightarrow bR_{605}$ transition in the above inside-out (monomeric) bR vesicles was rationalized by us, arguing that the retinal binding site is exposed to the inside of the vesicle. Accordingly, acidification will lead to the generation of bR_{605} only after proton penetration into the vesicle, which is a relatively slow process (of the order of seconds as estimated from the decay, in the dark, of pH gradients induced by continuous illumination). To confirm the hypothesis that the binding site is exposed to the inside, experiments were performed with inside-out monomeric vesicles containing 0.1 M phosphate buffer (pH = 6.5) enclosed in their internal volume. The results of jumps to ≃2.3 pH are shown in Fig. 2 *d*, *e*. In variance with the unbuffered systems (Fig. 2 *a–c*), the formation of bR_{605} cannot be separated from scattering as an initial faster process. Since in these solutions bR_{605} is ultimately generated (within <1 min), it is implied that the buffer has only delayed the bR_{605} generation that is now occurring on a time scale comparable with that of the scattering effects. The two processes are unseparable with the present available experimental setups. The observation that internal buffering delays, but does not prevent the bR_{605} formation, is consistent with the total buffer amount in the vesicles estimated as $\sim 10^{-6}$ mol in 2.5 ml. This value is smaller by a factor of ~10 than that of the total amount of protons added by jumping to $[H^+] = 5 \times 10^{-3}$ M (i.e., $\sim 10^{-5}$ mol in 2.5 ml). The fact that the internal buffering effect is not rapidly annihilated by the large excess of external protons is attributed to the cross membrane potential set by the initial protons diffusing into the vesicle, which will inhibit further H^+ penetration. This interpretation is in keeping with

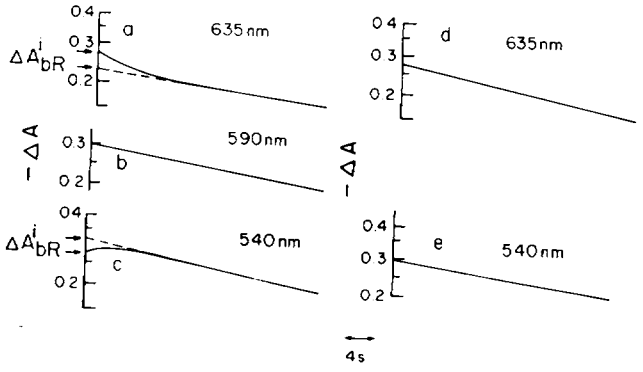

FIGURE 2 Effect of buffer on absorbance changes induced by acidification of bacteriorhodopsin in inside-out soybean vesicles (see Fig. 1 *b*, *c*); *a–c* unbuffered; *d*, *e* with enclosed buffer.

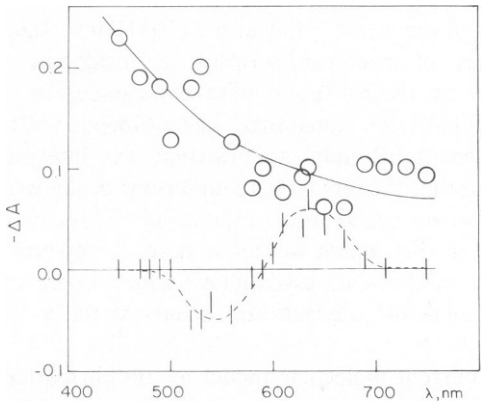

FIGURE 3 Difference spectra induced by a jump to pH ≃ 2.3 of bacteriorhodopsin incorporated in (inside-out) soybean vesicles (see Fig. 2 *a–c*). The *upper* curve, shows the effect due mostly to change in scattering obtained by extrapolation (see text). The *lower* curve shows the net changes in absorbance (ΔA^{i}_{bR}) due to the $bR_{570} \rightarrow bR_{605}$ process estimated as described in the text.

experiments carried out with internal buffering in the presence of ionophores for both K^+ (gramicidin or valinomycin) and H^+ (FCCP). Under such conditions the observed patterns were identical to those observed in unbuffered systems (compare Fig. 1 *c,I* and 1 *c,II*). Elimination of the buffering effect in this case is consistent with the fast diffusion of protons that, due to the presence of gramicidin, is not inhibited by a transmembrane potential.

To test the argument that the retinal binding site is exposed to the inside, in the case of inside-out vesicles, we performed pH jumps using inside-in cardiolipin or soybean vesicles, where the site is expected to be exposed to the outside as for intact bacterial cells. According to Hellingwerf (1979), disruption of the two-dimensional array of the purple membrane is necessary for a successful reconstitution of inside-in vesicles. Thus the inside-in bR vesicles are monomeric in nature. As shown in Fig. 1 *d* for a cardiolipin system, only a slow phase for the bR_{605} generation process (6 ± 2 s) is observed. This may at first appear as an inconsistency, since with the retinal exposed to the outside medium, the inside-in vesicles should exhibit both fast and slow bR_{605} generation components, as in the case of free purple membrane sheets. However, if the fast component is due to a cooperativity effect, and is therefore characteristic of the trimer structure (Druckmann et al., 1979), its absence in the inside-in cardiolipin vesicles may be due to the monomeric nature of bR in such vesicles. This possibility is supported by experiments (e.g., Fig. 1 *e,II*) carried out with bR trimers in inside-out soybean vesicles in the presence of FCCP and valinomycin. The role of FCCP is to accelerate the H^+ penetration into the vesicle interior so as to make binding to the site rate determining. Fig. 1 *e,II* shows the appearance of a fast millisecond component in the generation of bR_{605} in such a system, which is not observed in the absence of FCCP (Fig. 1 *e,I*) or in FCCP-doped monomeric vesicles. We thus confirm that a trimer bR structure is a prerequisite for observing the millisecond component of the bR_{605} formation.

In a recent work, Lind et al. (1981) investigated the properties of bacteriorhodopsin in inside-out vesicles obtained by reconstitution with endogenous or soybean phospholipids. An apparently instantaneous shift of the absorption of bR upon acidification was interpreted by assuming that the ionizable (counterion) group was accessible only from the exterior of the vesicle. This conclusion is opposite to that which we derive here. It appears evident that flow methods are essential even for a process that, as in the case of bR_{605} generation, occurs on the 5–10-s time scale.

The current molecular model for the formation of the acid species bR_{605} (Warshel and Ottolenghi, 1979; Fisher and Oesterhelt, 1980) is based on the protonation of the A^- counter ion in the ($=NH^+ \cdots A^- —$) retinal-protein complex of bR_{570}. In view of this interpretation, our data are indicative of an inside exposure of A^-, and thus also of the Schiff base, in inside-out vesicles. This means that in *Halobacterium halobium* cells the exposure is to the outside. Thus, pumping mechanisms based solely on a primary exposure change of the Schiff base from the inside to the outside of the cell are excluded.

The authors are grateful to Professor W. Stoeckenius and to Dr. A. Samuni for valuable discussions and to the Hebrew University School of Medicine (Jerusalem, Israel) for the use of the stopped-flow apparatus. R. Korenstein is an incumbent of the Association of the Friends of the Weizmann Institute of Science in Israel, Career Development Chair.

Assistance from grants by the U.S.-Israel Binational Science Foundation and the Fund for Basic Research (administrated by the Israeli Academy of Sciences and Humanities) are acknowledged.

Received for publication 16 March 1983 and in final form 7 June 1984.

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