Heterogeneity Effects in the Binding of *All-Trans* Retinal to Bacterio-opsin†

Noga Friedman,‡ Michael Ottolenghi,*,§ and Mordechai Sheves*,‡

Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel, and Department of Physical Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

Received June 12, 2003; Revised Manuscript Received July 23, 2003

ABSTRACT: The special trimeric structure of bacteriorhodopsin (bR) in the purple membrane of *Halobacterium salinarum*, and especially, the still controversial question as to whether the three protein components are structurally and functionally identical, have been subject to considerable work. In the present work, the problem is approached by studying the reconstitution reaction of the bR apo-protein with *all-trans* retinal, paying special attention to the effects of the apo-protein/retinal (P:R) ratio. The basic observation is that at high P:R values, the reconstitution reaction proceeds via two distinct, fast and slow, pathways associated with two different pre-pigment precursors absorbing at 430 nm (P_{430}) and 400 nm (P_{400}), respectively. These two reactions, exhibiting 2:1 (P_{430}/P_{400}) amplitude ratios, are markedly affected by the P:R value. The principal feature is the acceleration of the $P_{400} \rightarrow$ bR transition at low P:R ratios. The data are interpreted in terms of a scheme in which the added retinal first occupies two protein retinal traps, R_1 and R_2, from which it is transferred to two spectroscopically distinct binding sites corresponding to the two pre-pigments, P_{430} and P_{400}, respectively. Two noncovalently bound retinal molecules occupy two P_{430} sites of the bR trimer, while one (P_{400}) occupies the third. Binding is completed by generating the retinal−protein covalent bond. Analogous experiments were also carried out with an aromatic bR chromophore and with the D85N bR mutant. The accumulated data clearly point out the heterogeneity of the binding reaction intermediates, in which two are clearly distinct from the third. However, CD spectroscopy strongly suggests that even the two P_{430} sites are not structurally identical. The heterogeneity of the P intermediates in the binding reaction can be accounted for, either by being induced by cooperativity or by an intrinsic heterogeneity that is already present in the apoprotein. The question as to whether the final reconstituted pigment, as well as native bR, are nonhomogeneous should be the subject of future studies.

Bacteriorhodopsin (bR)[1] is the photosynthetic protein of the purple membrane (PM) of *Halobacterium salinarum.* It contains seven transmembrane α-helices and serves as a light-driven proton pump (*1*, *2*). The chromophore of the photoactive light-adapted form of bR is *all-trans* retinal bound to the ϵ-amino group of lysine-216 via a protonated Schiff base (PSB) linkage. Absorption of a photon by the chromophore induces an all-trans → 13-cis isomerization, which initiates a photocycle with several distinct spectroscopic intermediates.

bR is quite unique in its supramolecular organization. The purple membrane is organized in a two-dimensional (2-D) hexagonal crystal lattice with a unit cell dimension of ∼62 Å. Electron crystallography (*3*−*5*) indicated that bR is organized into trimers in which lipids mediate intertrimer contacts (*6*). The structural model has been refined by X-ray diffraction of bR that was crystallized from the lipidic cubic phase in the presence of endogenous lipids (*7*−*10*) and X-ray diffraction of bR that was crystallized by heterogeneous nucleation on benzamidine (*6*). Each bR trimer is associated with 30 lipid molecules, each of which is composed of various diether lipids. 70% are phospholipids, and 30% are glycosulfolipids (*11*). Six of these lipids are positioned within the bR trimer, stabilizing it by specific interactions involving the lipid headgroups (*6*), whereas 24 lipids surround the trimer. The bR lipids are essential in the formation of the 2-D crystals as well as in controlling the kinetics of the photocycle (*12*). Thus, the bR protein constitutes about 75% in weight of the purple membrane.

A fundamental point in understanding the function mechanism of bR relates to the question as to whether the three trimer components are identical. This is especially relevant when attempting to model the complex kinetics of the photocycle, which are still controversial after decades of extensive investigations (*13*). In fact, in some works a basic heterogeneity of the bacteriorhodopsin system has been invoked (*12*, *14*). However, the currently prevailing working hypothesis is that the three binding sites of the trimer members are structurally and mechanistically identical and that the complexity of the photocycle kinetics is due to its intrinsic properties (such as branching pathways, cooperativity, and pH-induced heterogeneity). In the present work,

† The research was supported by grants from the Ministry of Science Culture and Sport, the Israel National Science Foundation (Grant 659/00), Mazer Center for Structural Biology, and the Human Frontier Science Program. M.S. holds the Katzir-Makineni chair in chemistry.

* Corresponding authors. (M.O.) Phone: 972-2-6585335. Fax: 972-2-6524951. E-mail: micott@chem.ch.huji.ac.il. (M.S.) Phone: 972-8-9344320. Fax: 972-8-9344142. E-mail: mudi.sheves@weizmann.ac.il.

‡ The Weizmann Institute of Science.

§ The Hebrew University of Jerusalem.

[1] Abbreviations: bR, bacteriorhodopsin; CD, circular dichroism; PM, purple membrane; PSB, retinal protonated Schiff base.

10.1021/bi035011u CCC: $25.00
Published on Web 09/03/2003

we have approached the heterogeneity question in bR by analyzing the binding reaction of the retinal aldehyde to the apo-protein. The basic assumption was that heterogeneity, if existing, should be reflected in distinct binding pathways.

The binding of the retinal chromophore to bacterio-opsin, yielding (reconstituted) bacteriorhodopsin, has been the subject of several investigations. In this reaction, retinal displaces retinal oxime (the product of the hydroxylamine photobleaching reaction) from the binding site, generating the Schiff base bond. It is well-established that the binding process proceeds through several intermediates characterized by distinct absorption maxima (*15*, *16*). It was suggested that the first step of the reaction involves planarization of the retinal ring-chain, yielding a 400 nm species. The nature of a subsequent (430/460 nm) intermediate is still unclear, but it was shown that in this case as well the covalent chromophore-protein bond is not yet established (*17*). The decay of the latter pre-pigments to the characteristic 567 nm bR band is associated with formation of the retinal−protein covalent bond, and it is inhibited at low humidity (*18*). It was shown that the retinal binding process is associated with a proton release if binding is carried out below pH 6 and with a proton uptake above pH 8. It was also suggested that two protein residues change their pK_a during the binding process from 4.6 to 2.8 and 7.1 to 8.9, respectively (*19*). Later, these pK_a changes were ascribed to the hydrogen-bonded network connecting the protonated Schiff base with the extracellular surface, which is regulated by glutamic 204 (*17*). It was proposed that the retinal binds to bacterio-opsin in a cooperative way (*20*). Binding of retinal was also studied via the bacteriorhodopsin folding process in mixed lipid micelles. Folding is associated with two intermediate states absorbing at 380 and 440 nm. It was suggested that folded bacteriorhodopsin is formed in parallel from both intermediates. The apparent folding route is determined by the pH conditions (*21*).

In the present work, we have studied the binding of *all-trans* retinal to bacterio-opsin by optical and CD spectroscopy with special emphasis on the effects of varying the retinal/bacterio-opsin equivalent ratio. Two distinct pathways, kinetically and spectroscopically, were observed. The data are interpreted in terms of a model that requires heterogeneity in the binding intermediates. Thus, one binding site in the trimer clearly differs from the two others. Possibly, the other two, although kinetically indistinguishable, may be spectroscopically different. These findings bear, but do not solve, the question as to whether a distinction between the trimer sites is maintained in the finally generated pigment.

MATERIALS AND METHODS

Suspensions of purple membrane were prepared according to published procedures (*22*). Apo-membrane was prepared by illumination in the presence of hydroxylamine (2 M, pH 7) (*23*). All binding reactions were preformed with a fresh, sonicated, apo-membrane suspension in 20 mM phosphate buffer (pH 7, 298 K) in the dark.

Binding Reactions. Apo-membrane suspension (1 mL, 5 μM) was added to a fresh *all-trans* retinal solution (ethanol, 15−25 μL). The reaction progress was monitored by recording time-resolved absorbance spectra (Hewlett-Packard diode-array, 8543, UV−vis spectrophotometer). The kinetic component values were calculated by fitting the absorbance change at 567 nm (ΔA) as function of time, using the expression $\Delta A = 1 - a\exp(-k_1 t) - (1 - a)\exp(-k_2 t)$ where a is the relative amount of the fast component, and t is time in seconds.

The retinal equivalents were calculated according to the extinction coefficients ratio of *all-trans* retinal (42 800 M^{-1} cm^{-1}) and 55 000 M^{-1} cm^{-1} of DA bR (*24*). The expected fully reconstituted bR concentration was determined by binding the excess of *all-trans* retinal with a known amount of apo-membrane.

Binding 1.5 equiv of retinal in three consecutive stages of 0.5 equiv of each was carried out by addition of the apo-membrane suspension (1 mL, 8 μM) to a retinal solution (0.5 equiv dissolved in 10 μL of ethanol) and monitoring the absorbance spectra until complete binding was achieved (800 s). The partially bound pigment was then added to a 0.5 equiv of retinal dissolved in 10 μL of ethanol, and the absorbance spectra were monitored for an additional 800 s. A similar procedure was carried out for the third addition, and the reaction progress was monitored for 2000 s. The binding reactions of apo-membrane with the aromatic retinal analogue **1** and D85N-apo with retinal were carried out as described previously, except for different binding times.

CD Measurements. CD measurements were preformed with an Aviv circular dichroism spectrophotometer, model 202. Measurements were carried out at 283 K; at each experiment, a kinetic trace of the CD signal at one wavelength was monitored (from 340 to 460 nm, 10 nm intervals). Apo-membrane suspension (1.5 mL, 10 μM) was added to a retinal solution (0.5 equiv dissolved in 15 μL ethanol), and a kinetic trace of the CD signal was monitored until complete binding (800 s). A similar procedure was repeated twice with additional 0.5 retinal equivalents each time.

Binding as a Function of pH. The relative amount of the fast fraction as a function of pH in the binding reaction was measured using a 2:1 ratio, retinal to apo-membrane, in the dark, at 298 K. Two sets of experiments were preformed: with prewashed apo-membrane (using 2.510^{-4} M NaOH for pH adjustments) and with apo-membrane in 10 mM buffer solutions (citrate or phosphate).

RESULTS AND DISCUSSION

Retinal Binding to Native bR Apo-Membrane. Kinetic and Spectral Patterns. Binding of *all-trans* retinal to the bR apo-protein was carried out in buffered (20 mM, pH 7), dark adapted (DA) membrane suspensions that were sonicated before binding. The reaction progress was monitored by recording time-resolved difference spectra, showing the evolution of the 567 nm pigment band from parent blue-shifted precursors (Figure 1). To achieve complete binding, a minimum of a 1.5:1 retinal to apo-protein ratio is required.

This minimum ratio could only be achieved with presonicated membranes. The number of equivalents required for complete binding was found to be higher in membrane preparations that were not presonicated. The most relevant feature of the binding reaction is the dependence of the binding kinetics on the excess of added retinal. At low excess retinal (e.g., the 2:1 retinal/apo-protein ratio characterizing the system in Figure 1a), binding is biexponential with a first fast reaction ($k_f = 0.015$ s^{-1}) being followed by a slow process with $k_s = 0.003$ s^{-1} (Figure 2a).

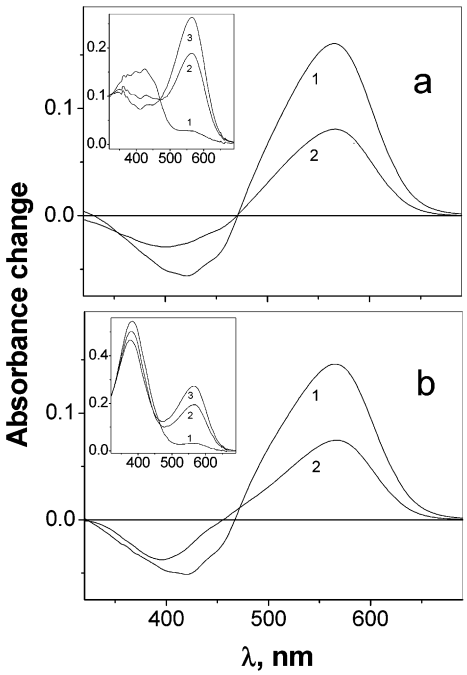


FIGURE 1: Absorbance difference spectra of retinal binding reaction to apo-membrane, pH 7, 20 mM phosphate buffer, DA, 298 K. (a) 2:1 retinal to apo-membrane ratio and (b) 5:1 ratio. (1) Spectrum of bR obtained following the fast-phase binding minus spectrum obtained 5 s after retinal addition. (2) bR spectrum after complete binding minus the spectrum obtained after fast-phase binding. Inset: bR spectrum minus apo-membrane spectrum. (1) After retinal addition. (2) After the fast-phase binding. (3) Complete binding.

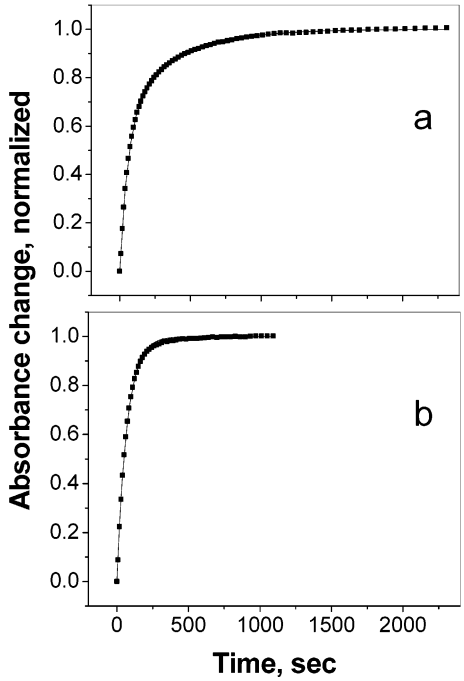


FIGURE 2: Kinetic trace showing the evolution of the 567 nm band in binding of all trans retinal to apo-membrane, pH 7, 20 mM phosphate buffer, DA, 298 K. (a) 2:1 retinal-to-apo ratio and (b) 5:1 ratio.

The two kinetic components are distinguished by different amplitudes, $A_f = 0.7$ and $A_s = 0.3$, respectively, as well as by different difference spectra. Thus, it is clearly evident from Figures 1a and 2a that in the case of the fast component, evolution of the characteristic 567 nm band of bR occurs from a precursor peaking at around 430/460 nm. On the other hand, in the slow, low amplitude, phase generation of the same bR absorption is accompanied by bleaching at around 400 nm. We note that while the two stages are characterized by spectrally different pre-pigments, no respectively distinct bR absorption bands could be resolved.

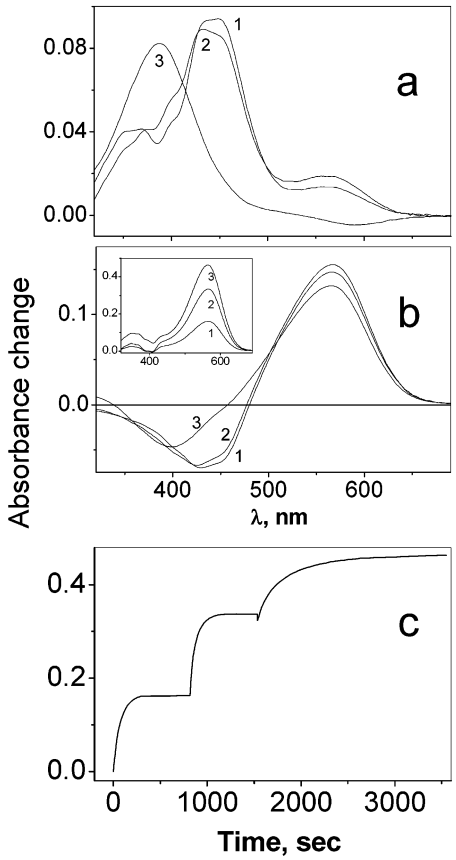


FIGURE 3: Absorbance difference spectra of 1.5 equiv of retinal binding to apo-membrane in three consecutive stages of 0.5 equiv each, pH 7, 20 mM phosphate buffer, DA, 298 K. Panel a: (1) Spectrum of the pre-pigment obtained 5 s after addition of the first 0.5 equiv minus apo-membrane spectrum. (2) Spectrum of the pre-pigment obtained 5 s after second retinal addition minus the bR spectrum monitored after complete binding of the first retinal portion. (3) Spectrum of the pre-pigment obtained 5 s after third retinal addition minus the bR spectrum monitored after complete binding of the second retinal portion. (b) bR spectrum following complete binding minus the spectrum monitored 5 s after retinal addition. (1) First, (2) second, and (3) third addition. Inset: bR spectrum of each retinal addition minus apo-membrane spectrum; (1) first, (2) second, and (3) third. (c) Kinetic trace showing the evolution of the 567 nm band.

Substantially different kinetic patterns are observed when the retinal-to-protein ratio is increased to, for example, 5:1. In this case (Figure 2b), the binding reaction exhibits only one phase with the same rate constant ($k_f = 0.015\ s^{-1}$) as that of the fast component that characterizes the 2:1 system. Interestingly, the blue shifted bR precursors are the same as in the low retinal experiment: as shown in Figure 1b, about two-thirds of the reaction originates from the 430/460 nm species (denoted here as P_{430}), while the rest is due to that absorbing at 400 nm (P_{400}).

Experiments were also carried out in which addition of the minimum retinal amount (1.5 equiv) was carried out stepwise. Figure 3 shows the behavior when a total of 1.5

retinal equivalents are added in three equal and consecutive stages, 0.5 equiv each.

The first and second stages lead to about 1/3 and 2/3 binding, respectively, both characterized by the same, $k_f = 0.015\ s^{-1}$, $P_{430} \rightarrow$ bR, transition. It is worthwhile pointing out that the difference spectra of the first two stages are similar but not identical. This may imply that two sequential spectrally distinct P430 precursors are involved in the binding reaction.

Upon addition of the last 0.5 equiv, binding was completed, with the 567 nm bR absorption being accompanied by bleaching at 400 nm. As in the 2:1 experiment described previously, such a P400 → bR reaction exhibits the slow ($k_s = 0.003\ s^{-1}$) kinetics. Moreover, when 4 rather than 0.5 equiv were added in the last stage, the same reaction took place but now exhibiting the fast, $0.015\ s^{-1}$, rate constant. We note that while a minimum of 1.5 retinal equiv is required for complete binding, 1 equiv is required for attaining 2/3 binding, followed by 0.5 equiv for the remaining 1/3. This implies that completion of the two fast binding stages requires the presence of one excess retinal outside of the binding site.

CD Difference Spectra. It is well-established that covalently bound retinal in the binding site, as well as noncovalently bound chromophores that occupy the same site, exhibit CD bands (*23*, *25*, *26*). CD spectroscopy may therefore serve as an effective tool for discriminating between in site and out of site chromophores. Thus, to obtain further information on the binding mechanism, we have followed the CD spectra of the P_{430} and P_{400} species obtained following the three consecutive additions of 0.5 retinal equivalents. Additional motivation for performing these measurements came from the indication discussed previously that the two fast binding components may not be spectroscopically identical.

To monitor the P_{430} species prior to its decay, CD measurements were carried out at 283 K immediately after retinal addition using one wavelength for each experiment, covering the complete spectral range. Figure 4a clearly indicates that the addition of 0.5 retinal equivalents leads to the displacement of retinal oxime from the binding site by the added retinal. This is evident from the depleted (negative) oxime band at ca. 370 nm and the formation of the (positive) pre-pigment band at ca. 450 nm. The subsequent addition of a second 0.5 equiv (Figure 4b) led to the formation of a pre-pigment characterized by a CD spectrum with a broad band around 420−450 nm, which is clearly different from that of the first addition. In this case, the expected negative band at ca. 370 nm was not observed, probably due to its cancellation by the positive broad band. This observation supports the suggestion made above on the basis of the optical spectra, namely, that the first and second pre-pigments are not identical. Addition of the third 0.5 equiv was not accompanied by any change in the CD spectrum (Figure 4c), indicating that in P_{400} the added retinal is trapped outside the binding site. In contrast, when the latter (third) addition is carried out with excess retinal (3 rather than 0.5 equiv), a 380 nm CD band is observed, indicating that the retinal occupied the third binding site (Figure 4d).

Reaction Mechanism. It is evident from the kinetic and spectral patterns described above that, in variance with previous suggestions, the different pre-pigment intermediates

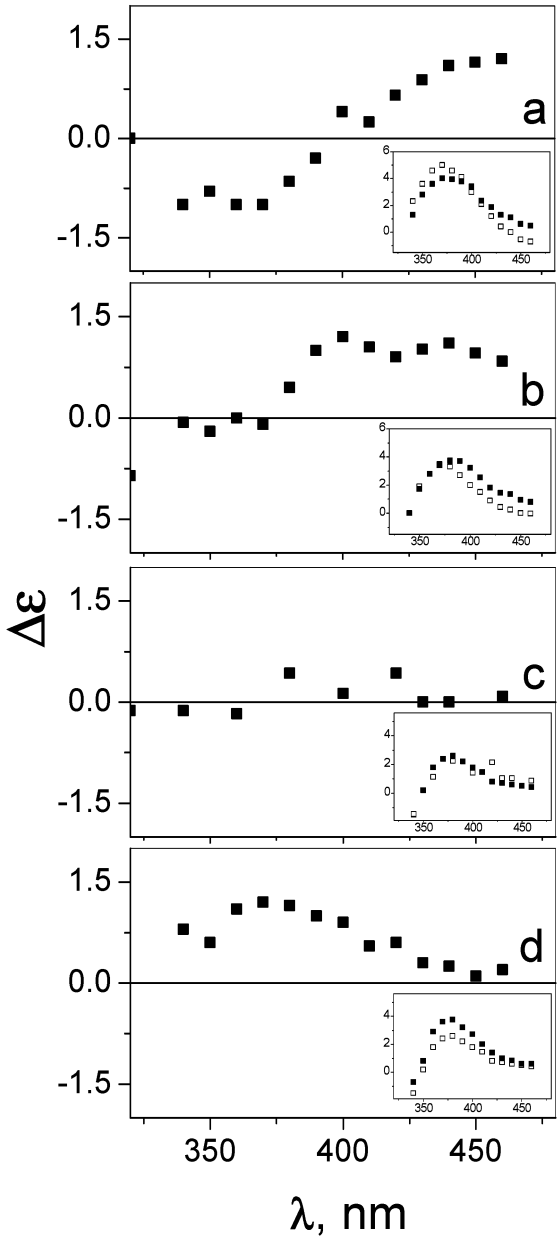
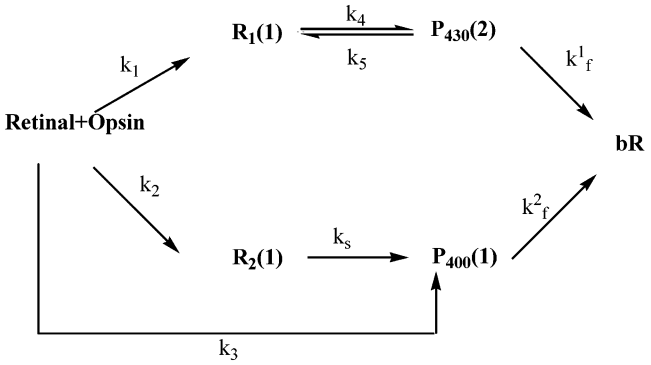


FIGURE 4: CD spectra of pre-pigments formed following addition of retinal to apo-membrane in three consecutive stages, pH 7, 20 mM phosphate buffer, DA, 283 K. (a) CD spectrum of the pre-pigment monitored 10 s after addition of the first 0.5 equiv of retinal minus the apo-membrane spectrum. (b) CD spectrum of the pre-pigment monitored 10 s after second addition of 0.5 equiv of retinal minus the bR signal after complete binding of the first retinal portion. (c) CD spectrum of the pre-pigment obtained 10 s after the third addition of 0.5 equiv of retinal minus the bR signal monitored after complete binding of the second retinal portion. (d) CD spectrum of the pre-pigment monitored 10 s after the third addition of 3 equiv of retinal minus the bR signal obtained after complete binding of the second retinal portion. Insets: CD spectra before (□) and 10 s after (■) retinal addition.

cannot be formed consecutively. A simple mechanism that is fully consistent with the present observations is shown in Scheme 1. The basic assumption is one in which the added retinal molecules undergo parallel trapping via two fast processes (k_1 and k_2) that are beyond our present temporal resolution. From such traps, denoted as R_1 and R_2, retinal is transferred to its final binding site forming the spectroscopically distinguishable pre-pigments P_{430} and P_{400}. The fol-

Scheme 1: Scheme Suggesting a Possible Mechanism for the Binding of Retinal to the Apo Protein Binding Sites[a]



[a] R_1 and R_2 denote retinal reservoirs or traps that are outside of the binding site. P_{400} and P_{430} denote retinal in distinct binding sites. Note (see text) that P_{430} may actually represent two distinct rather than identical binding sites. Numbers in parentheses represent the carrying capacity of each site. Approximate assumptions and (in parentheses) related accounting observations (see also text below): k_1, $k_4 + k_5 \gg k^1_f$ (R_1 is not accumulated). $k_1 \gg k_2$ (R_2 fills up only after filling of R_1). $k_2 \gg k_3$ (P_{400} fills up only after occupation of R_2) k_2, $k_3 \gg k_s$, k^2_f, $k_s \gg k^2_f$ (k_s is rate determining in the generation of bR at low retinal excess, while k^2_f is rate determining at high retinal excess). $k^2_f = k^1_f$ (Fast-phase rate of bR formation at low retinal equals rate at high retinal excess).

lowing are the major features of the mechanism of Scheme 1 as deduced from the relevant experimental observations:

(a) The branching scheme reflects our basic conclusion that two (or possibly all three) members of the protein trimer yield nonidentical P pre-pigments. The heterogeneity in P may be due to an initial heterogeneity in the apo-membrane or to cooperativity-induced heterogeneity during binding. The latter interpretation implies that the population of two sites, yielding two P_{430} intermediates, will affect the third site leading to a different population mechanism of the corresponding P (P_{400}) and in its blue shifted spectrum.

We note that the heterogeneity in P cannot be due to binding of different retinal isomers (i.e., all-trans and 13-cis since binding of the 13-cis isomer proceeds via a faster rate (*27*)). In addition, such a composition of isomers should give similar pathways in the three sequential additions of 0.5 equivalent each.

(b) Filling of the first two, 430/460 nm, binding sites requires filling of a reservoir (R_1) with one retinal equivalent. R_1 is a genuine reservoir in the sense that it has to be occupied for filling the two fast sites with which it is in thermodynamic equilibrium. Although kinetically undistinguishable, both optical and CD difference spectra suggest that the two P_{430} binding sites may represent two structurally different sites.

(c) Filling of the third, P_{400}, site requires the presence of at least one additional equivalent. The dependence of the rate of the $P_{400} \rightarrow$ bR transition on the excess of retinal is accounted for by assuming that the added retinal is first transiently trapped in a second reservoir (R_2), from which it is transferred to the 400 nm site with a rate $k_s = 0.003\ s^{-1}$. This (rate-determining) transition is followed by a fast ($k^2_f = 0.015\ s^{-1}$) transition to bR, with a rate comparable to that (k^1_f) of the $P_{430} \rightarrow$ bR process. We also assume that retinal in R_2 and in the 400 nm site are spectroscopically undistinguishable. Accordingly, the observation that excess retinal accelerates the reaction from 0.003 to 0.015 s^{-1} is in keeping with the assumption that the filling of R_2 allows retinal to rapidly reach (with rate k_3) the 400 nm site, so that the $P_{400} \rightarrow$ bR transition becomes the rate-determining step in the overall binding process. Thus, in variance with R_1, the second reservoir (R_2) is essentially a trap on the way to P_{400} that slows down filling of the latter site but does not have to be occupied for filling this site. In other words, R_2 is not equilibrated with the corresponding 400 nm pre-pigment, and when occupied, is circumvented so that retinal directly populates the P_{400} site.

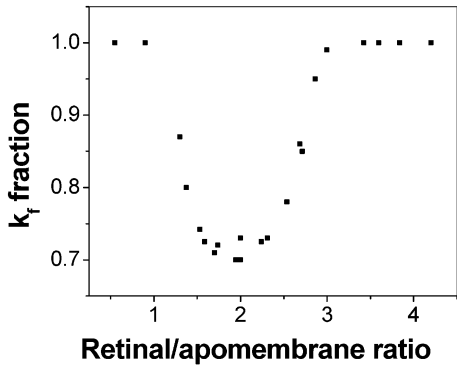


FIGURE 5: Fraction of the fast component in retinal binding reaction to the apo-membrane as a function of the retinal/apo-membrane ratio, pH 7, 20 mM phosphate buffer, DA, 298 K.

(d) According to the proposed scheme, the transition from 0.003 to 0.015 s^{-1} for the rate of the $P_{400} \rightarrow$ bR reaction should be a step function rather than a continuous change. Explicitly, upon increasing the relative retinal concentration, both k_s and k_f should retain their respective values, but their relative amplitude ratios, $A_s/(A_s + A_f)$ and $A_f/(A_f + A_s)$, should vary according to the relative weight of the branching pathways in Scheme 1. Such a predicted behavior is clearly reflected in Figure 5, in which we plot the relative amount of the fast fraction as function of the retinal/apo-membrane ratio.

At low retinal equivalents, when the R_1 branch is favored, the fast fraction predominates, namely, $A_f/(A_f + A_s) = 1$. When excess retinal is sufficient to fill up R_1 and P_{430} (with a total of $[1/3] \times 3 = 1$ equiv), the slow path, via R_2 and P_{400}, builds up reaching its maximum amount, $A_f/(A_f + A_s) = 2/3$, at about 1.5 retinal equivalents (an additional 1/3 equiv goes to P_{400} via R_2). Subsequently, R_2 becomes saturated, and consequently the fast reaction (k_3) that circumvents R_2 becomes effective. Then, the fast fraction starts to build up, attaining its original maximum value when P_{400} is exclusively populated directly from the added retinal. It should be emphasized that the recovery of the fast fraction from about 2/3 to 1 reflects the carrying capacity of R_2. Thus, a carrying capacity of, for example, one retinal equivalent in R_2 would predict a recovery over 0.33 equiv units. Since this portion of the curve extends over about one retinal equivalent (from 2 to 3), it is evident that the carrying capacity of reservoir(s) R_2 is of several retinal equivalents.

We finally note that slight deviations from the 2/3 to 1/3 stoichiometry all over our experimental observations may be within the limits of our experimental accuracy but may also be possibly due to differences in the extinction coefficients of the fast and slow species.

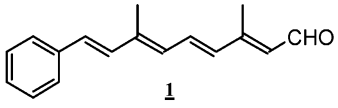
Cations and pH Effects. The phenomena discussed previously were observed in membrane suspensions at pH 7 in

the presence of 20 mM buffer. Since cation concentration and pH are known to affect the structure and function of bR, we tested the sensitivity of the retinal binding reaction to such parameters.

Binding experiments were performed with apo-membranes that were prewashed at pH 7, so as to remove part of the membrane bound cations (*28*). Such preparations are characterized by an increase of about one unit in the pK_a of Asp-85 in the reconstituted pigment (to about pK_a = 4.5) with respect to that obtained in the presence of 20 mM buffer. Two experiments analogous to those reported above were performed at pH 7: one in which 1.5 retinal equivalents were added gradually in three consecutive stages. Qualitatively, the observations were analogous to those observed for the 20 mM buffer (pH 7) experiment, namely, two fast stages with a 430 → 567 nm difference spectrum followed by a slow phase with a 400 → 567 nm difference spectrum. Similarly, the single step addition of 2 equiv yielded the same fast and slow steps with 0.7 and 0.3 amplitude fractions, respectively. While the spectral patterns are essentially identical to those of the buffer preparation, the binding reaction rate components were substantially slower, yielding k_f = 0.007 s^{-1} and k_s = 0.002 s^{-1} for the fast and slow components, respectively.

Binding experiments in both buffered (10 mM) and prewashed apo-mambrane preparations were performed over the 5−8 pH range. It was observed that neither the relative amplitude ratio nor the value of k_s is affected by pH. However, in both preparations, k_f exhibits a titration-like curve in which the k_f is replaced by k'_s characterized by a value similar to k_s. As shown in Figure 6, the pK_a values for the buffered and prewashed preparations are 4.6 and 6.6, respectively. The pH effect is attributed to the titration of a protein residue whose state of protonation affects the fast binding component. The effect of deionization that shifts the pK_a by about two pK_a units is analogous to that observed for Asp-85 (the purple ⇔ blue transition), as well as for other titrable residues in bR (*29*).

Binding of an Aromatic Retinal Analogue. Retinal analogues are known to exhibit a wide range of binding affinities and rates to native bR apo-membranes (*30*). To gain further insights into the mechanistic aspects of the binding reaction, we investigated the mechanism in the case of the synthetic aromatic analogue **1**.



1

The relevant observations (for a 2:1 retinal/apo ratio, 20 mM buffer system at pH 7) are shown in Figure 7. It is evident that binding results in the formation of two distinct products, one absorbing at 480 nm and the other at 520 nm. Displacement experiments with native, *all-trans* retinal, as well as photobleaching with hydroxylamine, exhibited features characteristic of pigments (slow displacement and light-induced bleaching) rather than to pre-pigments (fast displacement and thermal hydroxylamine bleaching). In addition, this artificial pigment exhibits characteristic photocycle patterns (*31*). Thus, it is evident that the two products correspond to genuine retinal pigments rather than to unbound pre-pigments.

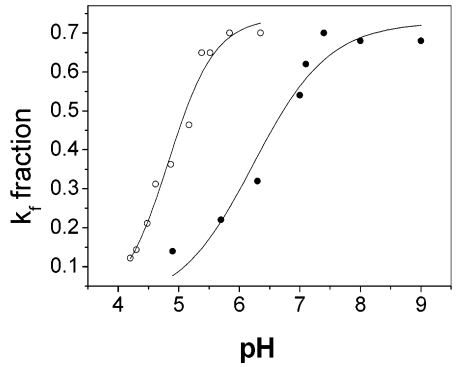


FIGURE 6: Fraction of the fast component as a function of pH in the retinal binding reaction to apo-membrane, 2:1 ratio, DA, 298 K. Washed apo-membrane (●) and 10 mM citrate buffer (○).

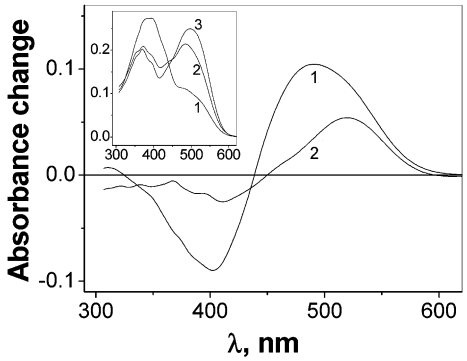
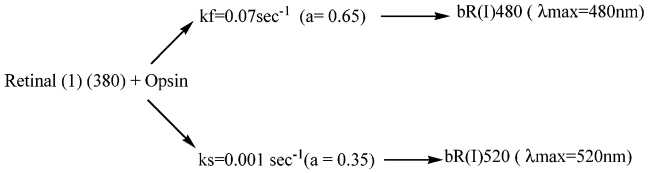


FIGURE 7: Absorbance difference spectra of aromatic retinal **1** binding to the apo-membrane, 2:1 ratio of the retinal analogue **1** to apo-membrane, pH 7, 20 mM phosphate buffer, DA, 298 K. (1) Spectrum of the pigment obtained following the fast-phase binding minus spectrum obtained 5 s after retinal analog **1** addition. (2) Spectrum of the pigment obtained after complete binding minus the spectrum obtained after fast-phase binding. Inset: Pigment spectrum minus apo-membrane spectrum. (1) 5 s after aromatic retinal **1** addition. (2) After the fast-phase binding. (3) Complete binding.

Scheme 2: Binding of Retinal Analog **1** to Bacterio-opsin



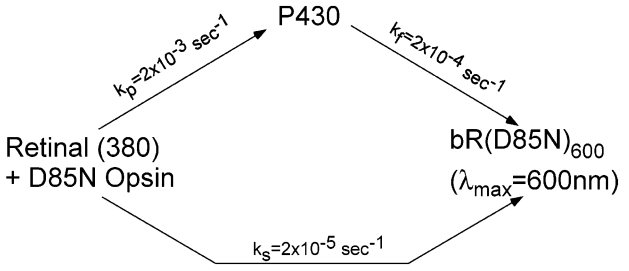
The observations are accounted for by the parallel Scheme 2.

A comparison with the reconstitution reaction of the native pigments points out at the following features: (a) The 2/3 (fast) to 1/3 (slow) binding ratios are maintained in the synthetic chromophore. (b) In variance with the native system, in this case no pre-pigments analogous to P_{430} or P_{410} are accumulated. This implies that if the basic mechanism of Scheme 1 is applicable to the aromatic pigment, then $k^1_f \gg k_4 + k_5$ and $k^2_f \gg k_3$, k_s. (c) Although no distinctive pre-pigments are observed, the heterogeneity of the final reconstitution spectrum of the aromatic system points at two different kinds of binding sites within the trimer. At present, we are unable to speculate as to whether such heterogeneity is a characteristic of this specific artificial bR or if it is also present with no spectroscopic footprints in the native pigment.

Table 1: Summary of Reconstitution Rate Parameters Values (s^{-1})

chromophore	apoprotein	chromophore apo-protein ratio	buffer/pH	k_f	k_s
all-trans retinal	native	2:1	20 mM/pH 7	0.015	0.003
all-trans retinal	native	5:1	20 mM/pH 7	0.015	
all-trans retinal	native	1.5:1 stepwise	20 mM/pH 7	0.015	0.003
all-trans retinal	native	1.5:1 stepwise	pre-washed/pH 7	0.007	0.002
aromatic retinal **1**	native	2:1	20 mM/pH 7	0.07	0.001
all-trans retinal	D85N	2:1	20 mM/pH 7	2×10^{-4}	5×10^{-5}
all-trans retinal	D85N	1.5:1 stepwise	20 mM/pH 7	2×10^{-4}	5×10^{-5}

Scheme 3: Binding of Retinal to D85N Opsin



Binding of Retinal to the Apomembrane of the D85N Mutant. As in the case of non-native chromophores, modification of the protein by selective mutations affects the binding reaction. In this work, we selected the D85N mutant, which lacks the Asp-85 residue that resides in close proximity to the retinal binding site and plays a major role in the structure and function of bR. Binding experiments of *all-trans* retinal to the D85N apo-membrane were carried out at pH 7, 20 mM buffer, both with 2 equiv of retinal and three sequential additions of 0.5 equiv each. Both experiments indicated that the predominant feature is a binding reaction that is substantially slower than that of the native apo-protein. The data, shown in Table 1, may be rationalized in terms of the branching Scheme 3.

Interestingly, in variance with the native bR systems described previously, in the case of D85N both evolution and decay of the fast track P_{430} pre-pigment are observed. With reference to Scheme 1, this implies that in the case of the mutant the $R_1 \Leftrightarrow P_{430}$ equilibrium is sufficiently slow to be detected but sufficiently fast to allow accumulation of the pre-pigment. This conclusion does not apply to the slow branch of the reaction for which no pre-pigment was accumulated.

CONCLUSION

The data presented in this paper indicate that retinal binding to apo-bR proceeds, in parallel, via (at least) two pre-pigment states, P_{430} and P_{400}. Their respective decay into bR represents covalent binding to Lys-216. The CD changes observed upon binding strongly suggest that the P_{430} pair may be composed of two states that are spectroscopically (but not kinetically) distinguishable.

Generation of the covalent bond (k_f) is rate determining for P_{430}. However, for P_{400}, it is rate determining only when the R_2 trap is filled up by relatively high excess retinal values and can therefore be circumvented. At low retinal/protein ratios, the slow transition (k_s) from R_2 to P_{400} becomes rate determining. The different nature of the two processes, covalent bond formation versus transfer from a trap to the binding site, is also reflected in their pH and salt sensitivities. Only the first is affected by the state of protonation of a (still unidentified) titrable protein residue.

The evidence clearly points out at the heterogeneity of two, and possibly three, of the trimer binding sites, when occupied by retinal prior to the formation of the Schiff base bond. Interestingly, this heterogeneity is maintained even after strongly perturbing the binding site by the Asp-85 mutation. The effect may be due to an intrinsic heterogeneity in the apo-protein or to cooperativity effects within the trimer, for example, if the occupation of the P_{430} sites affects one of the trimer members so that it exhibits a 400 nm absorption and does not accumulate a 430 nm species. At present, we cannot discriminate between these two possibilities. We note that it is also possible that both heterogeneity and cooperativity operate.

Another open question is whether the heterogeneity in the pre-pigments is also maintained in the reconstituted bR pigment. In the native system, there is no spectral distinction in the final 567 nm band that could point out at heterogeneity in bR. It is possible that the initial heterogeneity is maintained without being reflected in the optical spectrum. However, it can also be claimed that upon formation of the covalent bond, a protein structural transformation occurs so that all three members of the trimer become structurally identical. Although at present there is no clear answer to this question, the observations with the aromatic retinal (**1**), for which a 2/3 (bR(I)480) to 1/3 (bR(II)520) mixture is detected, indicate that heterogeneity is possibly maintained in the reconstituted pigment. This could suggest heterogeneity in the reconstituted bR as well. We also wish to point out that the previous conclusions as to the possible heterogeneity of the trimer strictly apply to reconstituted bR and not necessarily to the untreated native pigment. In conclusion, in view of the high relevance of heterogeneity in interpreting the photoreactions and the proton pump mechanism of bR, future studies will have to address the various questions that are still open.

REFERENCES

1. Ottolenghi, M., and Sheves, M., Eds. (1995) *Isr. J. Chem. 35*, 193−513.
2. Lanyi, J. (1999) *Int. Rev. Cytol. 187*, 161−202.
3. Henderson, R., and Unwin, P. N. T. (1975) *Nature 257*, 28−32.
4. Grigorieff, N., Ceska, T., Downing, K., Baldwin, J., and Henderson, R. (1996) *J. Mol. Biol. 259*, 393−421.
5. Kimura, Y., Vassylyev, D. G., Miyazawa, A., Kidera, A., Matsushima, M., Mitsuoka, K., Murata, K., Hirai, T., and Fujiyoshi, F. (1997) *Photochem. Photobiol. 66*, 764−767.
6. Essen, L.-O., Siegert, R., Lehmann, W. D., and Oesterhelt, D. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 11673−11678.
7. Pebay-Peyroula, E., Rummel, G., Rosenbuch, J. P., and Landau, E. M. (1997) *Science 277*, 1676−1681.
8. Luecke, H., Richter, H. T., and Lanyi, J. K. (1998) *Science 280*, 1934−1937.

9. Luecke, H., Schobert, B., Richter, H. T., Cartailler, J. P., and Lanyi, J. K. (1999) *J. Mol. Biol. 291*, 899−911.
10. Belrhali, H., Nollert, P., Royant, A., Menzel, C., Rosenbusch, J., Landau, E., and Pebay-Peyroula, E. (1999) *Structure 7*, 909−917.
11. Krebs, M., and Isenbarger, T. (2000) *Biochim. Biophys. Acta 1460*, 15−26.
12. Shrager, R., Hendler, R., and Bose, S. (1995) *Eur. J. Biochem. 229*, 589−595.
13. Shrager, R. I., and Hendler, R. W. (2003) *J. Phys. Chem. B 107*, 1708−1713.
14. Eisfeld, W., Pusch, C., Diller, R., Lohrmann, R., and Stockburger, M. (1993) *Biochemistry 32*, 7197−7215.
15. Schreckenbach, T., Walckhoff, B., and Oesterhelt, D. (1977) *Eur. J. Biochem. 76*, 499−511.
16. Schreckenbach, T., Walckhoff, B., and Oesterhelt, D. (1978) *Biochemistry 17*, 5353−5359.
17. Rudiger, M., Tittor, J., Gerwert, K., and Oesterhelt, D. (1997) *Biochemistry 36*, 4867−4874.
18. Rousso, I., Brodsky, I., Lewis, A., and Sheves, M. (1995) *J. Biol. Chem. 270*, 13860−13868.
19. Fischer, U., and Oesterhelt, D. (1980) *Biophys. J. 31*, 139−145.
20. Rehorek, M., and Heyn, M. (1979) *Biochemistry 18*, 4977−4983.
21. Lu, H. B. P. (2000) *J. Mol. Biol. 299*, 233−243.
22. Oesterhelt, D., and Stoeckenius, W. (1974) *Methods Enzymol. 31*, 667−678.
23. Aharoni, A., Ottolenghi, M., and Sheves, M. (2001) *Biochemistry 40*, 13310−13319.
24. Ebrey, T., Becher, B., Mao, B., Kilbride, P., and Honig, B. (1977) *J. Mol. Biol. 112*, 377−397.
25. Becher, B., and Cassim, J. (1977) *Biophys. J. 19*, 285−297.
26. Aharoni, A. W. L., Lewis, A., Ottolenghi, M., and Sheves, M. (2001) *J. Am. Chem. Soc. 123*, 6612−6616.
27. Gartner, W., Towner, P., Hopf, H., and Oesterhelt, D. (1983) *Biochemistry 22*, 2637−2644.
28. Yang, D., and El-Sayed, M. A. (1995.) *Biophys. J. 69*, 2056−2059.
29. Bressler, S., Friedman, N., Li, Q., Ottolenghi, M., Saha, C., and Sheves, M. (1999) *Biochemistry 38*, 2018−2025.
30. Ottolenghi, M., and Sheves, M. (1989) *J. Membr. Biol. 112*, 193−212.
31. Zadok, U. K. A., Lewis, A., Ottolenghi, M., and Sheves, M. (2002) *J. Am. Chem. Soc. 124*, 11844−11845.

BI035011U