Titration kinetics of Asp-85 in bacteriorhodopsin: exclusion of the retinal pocket as the color-controlling cation binding site

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Abstract The spectrum (the purple↔blue transition) and function of the light-driven proton pump bacteriorhodopsin are determined by the state of protonation of the Asp-85 residue located in the vicinity of the retinal chromophore. The titration of Asp-85 is controlled by the binding/unbinding of one or two divalent metal cations (Ca^{2+} or Mg^{2+}). The location of such metal binding site(s) is approached by studying the kinetics of the cation-induced titration of Asp-85 using metal ions and large molecular cations, such as quaternary ammonium ions, R_4N^+ (R = Et, Pr, a divalent 'bolaform ion' $[Et_3N^+-(CH_2)_4-N^+Et_3]$ and the 1:3 molecular complex formed between Fe^{2+} and 1,10-phenanthroline (OP). The basic multi-component kinetic features of the titration, extending from 10^{-2} to 10^4 s, are unaffected by the charge and size of the cation. This indicates that cation binding to bR triggers the blue→purple titration in a fast step, which is not rate-determining. In view of the size of the cations involved, these observations indicate that the cation binding site is in an exposed location on, or close to, the membrane surface. This excludes previous models, which placed the color-controlling Ca^{2+} ion in the retinal binding pocket.

Key words: Purple and blue bacteriorhodopsin; Titration kinetics of Asp-85; Metal cation binding site

1. Introduction

The function of bacteriorhodopsin (bR) is based on a series of light-driven proton translocation reactions (for a recent series of review articles on bR and other retinal proteins, see [1]). Such processes involve the protonated Schiff base (SBH^+), which links the retinal chromophore to the protein, as well as its neighboring Asp-85 residue located closer to the extracellular side of the membrane [2]. Proton transfer from SBH^+ to Asp-85 accounts for the generation of the blue-shifted M photocycle intermediate. This reaction is coupled to proton release to the extracellular medium, a step which initiates the proton pump cycle.

The critical role of Asp-85 in the photosynthetic function of bR is in keeping with the observation that the photocycle of the (low-pH) blue form of bR, in which Asp-85 is protonated [3–11], does not exhibit the M intermediate and is not associated with a proton pump. Interest in the purple (high-pH)↔blue (low-pH) transition, characterized by $pK_a = 2.7$ in 0.1 M NaCl, further increased when it was found that the blue form can also be generated by deionization (cation removal) [12,13]. It was also suggested that the cation (Ca^{2+} or Mg^{2+}) is directly bound to the protein residue, which controls the purple↔blue transition [12,21]. These observations are relevant to the role of metal cations in determining the structure and function of bR (for review see [14]).

The initial approach proposed to account for the pH and metal cation effects on the purple↔blue equilibrium was based on surface potential effects [12,13,15–19]. Accordingly, free (Guy Chapman) or bound metal cations on the membrane surface compete with protons and thus determine the local proton concentration around the membrane, which in turn controls the state of protonation of Asp-85. Although membrane potential effects may influence the apparent pK_a of Asp-85, it was later recognized that the purple↔blue equilibrium is determined by the competition between metal cations and protons on two specific (high-affinity) metal binding sites [20–24], which are not necessarily located on the surface. It was later suggested that the metal site, which determines the state of protonation of Asp-85 and thus the color of the pigment, is located in the retinal binding pocket [14,21,23,24], directly interacting with Asp-85 and possibly also with Asp-212 [21,23,24] and with the Schiff base [14,21]. While previous studies have only focused on equilibrium measurements, we have now approached the identification of the color-determining metal binding site in bR by studying the kinetics of the cation-induced blue↔purple transition. Kinetic experiments are combined with varying the nature and size of the cation, as recently suggested by Birge and coworkers [23,24]. We reached the conclusion that contrary to previous models, the color-determining metal binding site cannot be located in the interior of the protein, directly interacting with Asp-85, and should occupy a highly exposed position on, or close to, the membrane surface.

2. Materials and methods

Cation jump experiments were performed using a Hewlett-Packard diode array spectrophotometer or a micro volume stopped-flow reaction analyzer (Applied Photophysics Ltd., UK, SX-17MV).

Deionized blue membranes were obtained by passing bR suspensions through a Fluka Dowex 50Wx8 cation exchange column. Such suspensions (pH = 4–4.5) were exposed to ion jumps, inducing the transition to the purple form.

Large organic cations were prepared and employed as recently described by Tan et al. [24]. Out of the monovalent quaternary ammonium series, R_4N^+, we used, as characteristic examples, the propyl Pr_4N^+ and butyl Bu_4N^+ derivatives. Representative of the divalent 'bolaform' series $R_3N^+-(CH_2)_n-N^+R_3$ was ethyl derivative, R = Et, n = 4, here denoted $N_2Et_6C_4^{2+}$. OP and bathophenanthroline disulfonic acid (BP) were obtained from Aldrich. The $\{Fe^2+3OP\}$ and $\{Fe^{2+}+3BP\}$ complexes were prepared by mixing a $FeCl_2$ solution with four equivalents of the corresponding ligand. Purple bR, recomposed with two equivalents of Fe^{2+}, was prepared by adding five equivalents of $FeCl_2$ to deionized blue bR. The resulting suspension

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Abbreviations: bR, bacteriorhodopsin; OP, 1,10-phenanthroline; bP, bathophenanthroline disulfonic acid; Asp, aspartic acid; Glu, glutamic acid; Et, ethyl; Pr, propyl; Bu, butyl

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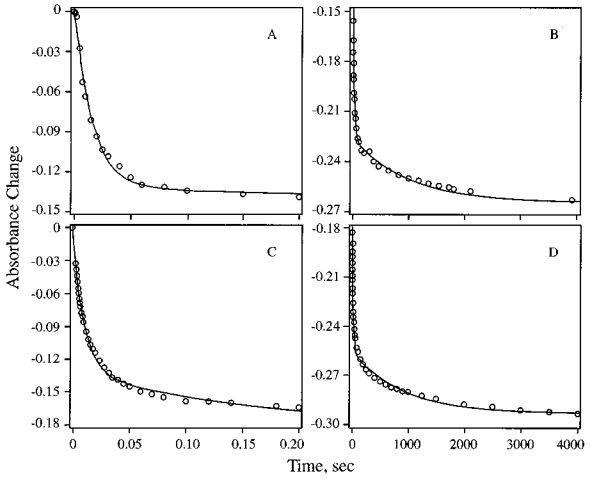


Fig. 1. Characteristic kinetic data points and 4-exponent fits (continuous lines) showing the multi-component features of the Asp-85 deprotonation reaction (the blue↔purple transition). The reaction is induced by 1:1 mixing of deionized blue bR with the corresponding salt solution. Na^+ (A and B) or $N_2Et_6C_4^{2+}$ (C and D), to final bR/cation ratios of 1:5000 and 1:10, respectively. The kinetics were monitored at the characteristic 630 nm absorption wavelength of blue bR. The fast and slow scans were recorded with the stopped-flow and spectrophotometer instruments, respectively.

was incubated for 2 h, followed by centrifugation and resuspension in water.

3. Results and discussion

Time resolved titrations of specific residues are generally difficult to perform in proteins (for a recent review see [25]). However, due to the associated changes in color, they have been carried out in bR using conventional stopped-flow techniques for both the Schiff base [26–28] and Asp-85 [12,27–31]. A characteristically unclear feature of the Asp-85 titration has been its multiphasic kinetic nature, ranging from tens of milliseconds to hours [3,12,28,29,31]. In a recent work [32], we proposed a kinetic model for the cation-induced blue↔purple transition in which the rate-determining step of the Asp-85 titration is proton transfer through an appropriate channel, rather than the binding of the metal cation. Accordingly, the multiphasic nature of the titration is interpreted in terms of several protein states, giving rise to several corresponding proton channels, with relative weights depending on the state of protonation of at least two protein residues.

The above kinetic features were shown to hold true for cations such as Na^+, Ca^{2+} and Eu^{3+}, independently of charge and size [32]. The question arises, however, as to the applicability of the suggested mechanism to larger molecular cations for which the cation binding reaction may be expected to become rate-determined. An interesting recent observation in this respect was that large organic cations, such as monovalent quaternary ammonium cations, R_4N^+, and divalent 'bolaform' cations, $N_2R_6C_n^{2+}$, can replace Mg^{2+} and Ca^{2+} in maintaining the purple form and the proton pumping ability of bR [24]. On the basis of these findings, we have carried out a series of experiments in which deionized, blue-bR, membrane suspensions were exposed to cation jumps using the characteristic cations Pr_4N^+, Bu_4N^+, and $N_2Et_6C_4^{2+}$. Representative data are shown in Fig. 1 and are summarized in Table 1. Surprisingly, these experiments indicate that the large molecular ions exhibit multi-component kinetic features of the Asp-85 titration, which are analogous to those of the smaller inorganic cations. Thus, the 4-exponential kinetic analysis [32] yields, in all three cases, rate and amplitude parameters that are essentially the same as those of, e.g., Na^+ and Ca^{2+}. This conclusion also applies to the effect of the final pH on the titration kinetics. Thus, analogously to the metal cations [32], when the final pH of the titration is raised to ~7 the contribution of the slow components, S_1 and S_2, becomes negligible. This is demonstrated in Table 1 in the case of Pr_4N^+.

With the purpose of employing ions which are even larger, and possibly structurally different from the above ammonium salts, we considered the family of strong molecular complexes formed between metal ions and bulky organic ligands. In fact, we found that the native metal cations in bR can also be replaced by the large 1:3 complex {Fe^{2+}, 3OP} formed between Fe^{2+} and OP (Scheme 1). This was shown in two kinds of experiments. First (Fig. 2A), a deionized blue-bR suspension was mixed with a {Fe^{2+}, 3OP} complex solution to a final 5:1 {Fe^{2+}, 3OP}/bR ratio, at pH 4.5, for 2 h. Centrifugation, followed by resuspension in water, regenerates the purple bR bound to two equivalents of the {Fe^{2+}, 3OP} complex, as judged by the 506 nm absorption. Moreover, acidification of this purple form to pH 2.5, followed by centrifugation and resuspension in water, resulted in the formation of

Table 1
Kinetic parameters derived from a 4-exponential analysis [32] of the blue↔purple transition, induced in deionized (blue) bR by mixing with salt solutions

	NA^+ (5000:1)	Ca^{2+} (10:1)	$N^+(Pr)_4$ (3750:1)	$N^+(Bu)_4$ (3120:1)		$N_2ET_6C_4^{2+}$ (100:1)	{Fe^{2+}, 3OP}	
				I^- salt	Br^- salt		2:1[a], 4:1[b]	10:1[a], 10:1[b]
F_1 k_1	63	64	126	80	92	85	60	83
A_1	0.48	0.25	0.61	0.54	0.55	0.45	0.38	0.57
F_2 k_1	0.65	0.8	4.0	1.5	1.9	1.8	0.5	0.20
A_2	0.16	0.16	0.49	0.12	0.14	0.19	0.11	0.14
S_1 k_3	3.6×10^{-2}	1.3×10^{-2}	–	3.5×10^{-2}	5.0×10^{-2}	3.2×10^{-2}	3.4×10^{-2}	3.4×10^{-2}
A_3	0.22	0.29	<0.05	0.17	0.19	0.22	0.38	0.16
S_2 k_4	9.0×10^{-4}	5.6×10^{-4}	–	5.3×10^{-4}	5.5×10^{-4}	5.0×10^{-4}	3.0×10^{-4}	7.0×10^{-4}
A_4	0.14	0.30	<0.05	0.16	0.12	0.14	0.13	0.13
Final pH	4.2	3.8	7.1	3.8	4.0	4.2	4.1	4.2

Ratios refer to cation:bR ratios. [bR] = 1.5×10^{-5} M. F_1, F_2, S_1, and S_2 denote the four fractions, two fast and two slow, as classified in [32]. k values are rate constants in s^{-1}. A denotes the value of the respective amplitude fraction. Initial and final (after mixing) pH values were in the ranges of 4.0–4.7 and 3.8–4.2 respectively, with the exception of $N^+(Pr)_4$ for which the final pH was 7.1.
[a]Final {Fe^{2+}, 3OP}:bR ratio. [b]OP:Fe^{2+} ratio.

the blue form and the appearance of the {Fe^{2+}, 3OP} complex in the solution. The bR-bound {Fe^{2+}, 3OP} complex is also displaced by Ca^{2+} ions, as shown in an analogous experiment, in which 10 Ca^{2+} equivalents were added to the above {Fe^{2+}, 3OP} purple bR preparation.

In a second experiment (Fig. 2B), OP was added to a purple bR suspension, obtained by binding two equivalents of Fe^{2+} to deionized blue bR (see Section 2). After mixing, the full amount of the {Fe^{2+}, 3OP} complex absorption was generated without converting any purple bR into the blue form. Furthermore, centrifugation of the bR-{Fe^{2+}, 3OP} complex, followed by resuspension in water, revealed that the {Fe^{2+}, 3OP} complex is bound to bR and is absent in the solution. These observations imply that the {Fe^{2+}, 3OP} complex effectively replaces the divalent inorganic ions with respect to the blue↔purple transition. Interestingly, analogous experiments carried out under the same conditions with the larger diphenyl-disulfonate derivative of OP, BP (MW=494), indicated that the {FE^{2+}, 3BP} complex is ineffective in restoring the purple form from a blue bR suspension.

Using quantum mechanical calculations, Tan et al. [24] were able to account for the effects of several large organic cations in bR (with the exception of Bu_4N^+) by accommodating them in the retinal binding pocket, as defined by the structural model of Henderson and coworkers [2]. However, this interpretation is not in keeping with the present observations, showing the effective binding of {Fe^{2+}, 3OP} to bR and the related effect on the blue↔purple equilibrium, as well as the displacement of the bR-bound complex by Ca^{2+} ions. Thus, the {Fe^{2+}, 3OP} complex (MW = 596) is more than twice as large as Bu_4N^+ (MW = 242) and cannot, by any means, be accommodated in the retinal binding pocket or in any other location in the interior of the protein.

Independent evidence showing that the color-controlling metal ion in bR is not in the retinal vicinity and, thus, does not interact with Asp-85 and Asp-212 directly, is provided by the titration kinetics associated with the metal-induced blue↔purple transition. As shown in Fig. 1 and Table 1, the kinetic patterns are essentially the same for all cations, including ions as different in size as Na^+ and {Fe^{2+}, 3OP}. This is in keeping with the suggestion [32] that cation binding is not a rate-determining step of the titration. Accordingly, metal binding occurs on a sub-millisecond timescale and is faster than the fastest titration component (~10 ms). Given the $\sim 2\times10^{-3}$ s time resolution of our experimental stopped-flow system and the 3×10^{-5} M concentration of {Fe^{2+}, 3OP}, we obtained a lower limit of $k > 2\times10^{8}$ s^{-1} for the metal binding rate constant. This value approaches the diffusion-controlled limit for an ion as large as the above molecular complex, excluding any cation binding site that is not highly exposed on, or close to, the membrane surface. Independently of the specific identification of the metal binding site, the accumulated cation effects on the purple↔blue equilibrium imply that metal binding/unbinding affects the apparent pK_a of the Asp-85 titration. We propose that metal binding to the suggested 'external' site affects the protein structure in the retinal binding pocket, so as to affect the pK_a of Asp-85. At present, we are unable to suggest specific protein residues as participating in the binding of the color-controlling metal cation(s). However, plausible candidates include such groups as Glu-74, Glu-9 and Glu-194, which are located on the extracellular side of the chromophore binding site [2].

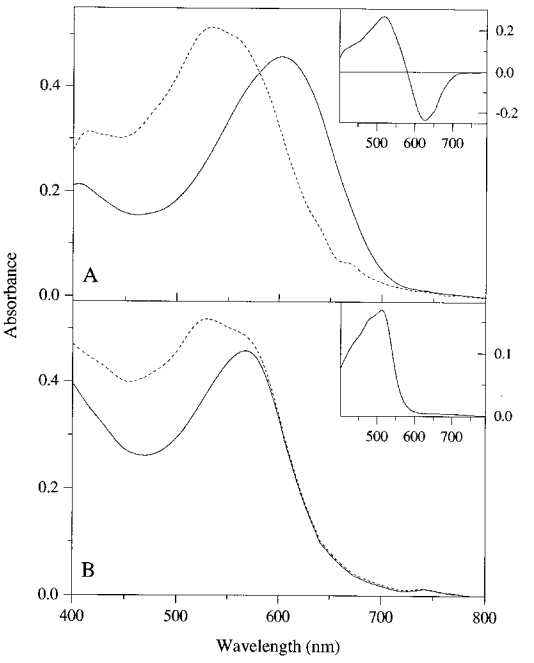
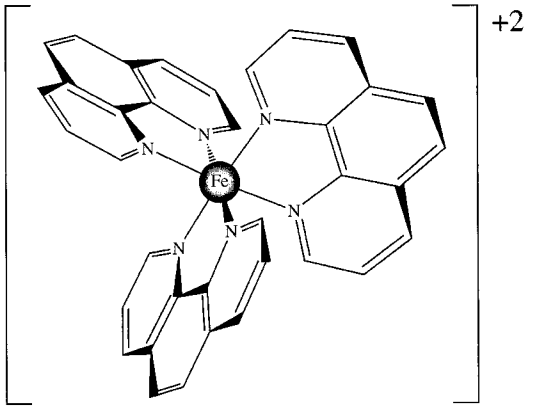


Fig. 2. A: Continuous line: Absorption of deionized blue bR. Dotted line: Absorption of this solution following the addition of {Fe^{2+}, 3OP}, centrifugation and resuspension in water (see text). Inset: Difference spectrum between the above two spectra. B: Continuous line: Absorption of a purple membrane suspension obtained by mixing five Fe^{2+} equivalents with deionized blue bR, followed by centrifugation and resuspension in water. Dotted line: Absorption of the same solution, following the addition of two OP equivalents. Inset: Difference spectrum between the above spectra, representing the characteristic spectrum of the {Fe^{2+}, 3OP} complex.



Scheme 1.

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