The titrations of Asp-85 and of the cation binding residues in bacteriorhodopsin are not coupled

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Abstract An outstanding problem relating to the structure and function of bacteriorhodopsin (bR), which is the only protein in the purple membrane of the photosynthetic microorganism *Halobacterium salinarium*, is the relation between the titration of Asp-85 and the binding/unbinding of metal cations. An extensively accepted working hypothesis has been that the two titrations are coupled, namely, protonation of Asp-85 (located in the vicinity of the retinal chromophore) and cation unbinding occur concurrently. We have carried out a series of experiments in which the purple ⇔ blue equilibrium and the binding of Mn^{2+} ions (monitored by electron spin resonance) were followed as a function of pH for several (1–4) $R = [Mn^{2+}]/[bR]$ molar ratios. Data were obtained for native bR, bR mutants, artificial bR and chemically modified bR. We find that in the native pigment the two titrations are separated by more than a pK_a unit [$\Delta pK_a = pK_a(P/B) - pK_a(Mn^{2+}) = (4.2-2.8) = 1.4$]. In the non-native systems, ΔpK_a values as high as 5 units, as well as negative ΔpK_as, are observed. We conclude that the pH titration of cation binding residues in bR is not directly related to the titration of Asp-85. This conclusion is relevant to the nature of the high affinity cation sites in bR and to their role in the photosynthetic function of the pigment.

Key words: Purple and blue bacteriorhodopsin; Metal cation binding site; Asp-85 titration

1. Introduction

The photosynthetic activity of bacteriorhodopsin (bR) is based on a series of light-driven proton translocation reactions involving its retinal polyene chromophore and several protein residues (see [1] for a recent series of review articles on bR and other retinal proteins). Of primary importance is Asp-85, located in the vicinity (on the extracellular side) of the retinal protonated Schiff base (SBH^+) [2], which links the retinal chromophore to Lys-216. Proton transfer from SBH^+ to Asp-85 accounts for the generation of the blue-shifted M intermediate in the photocycle of the neutral pH ('purple') form of bR ($\lambda_{max} = 570$ nm). This reaction is coupled to proton release to the extracellular medium, a step which initiates the trans-membrane pump cycle. The critical role that Asp-85 plays in the photosynthetic mechanism of purple bR is in keeping with the observation that the photocycle of the low pH ('blue') form of bR ($\lambda_{max} = 605$ nm), in which Asp-85 is protonated [3–11], does not exhibit the M intermediate and is not associated with proton pumping.

The purple ⇔ blue transition is closely related to the binding of 8–10 metal cations (Ca^{2+} and Mg^{2+}) to bR (see [12] for a recent review of metal cation binding to bacteriorhodopsin). The key observation in this respect is that deionization (cation removal) substantially shifts the purple ⇔ blue equilibrium from p$K_a = 2.7$ in 0.1 M NaCl to p$K_a > 5.5$ in deionized preparations [13–15]. This and other evidence show that cation binding plays a major role in determining the structure and function of bR [12].

There appear to be three main problems related to metal binding to bR and to its effects on the state of protonation of Asp-85. (a) The metal binding mechanism. The initial approach proposed to account for the pH and metal cation effects on the purple ⇔ blue equilibrium was exclusively based on surface potential effects [13,14,16–20]. Accordingly, free (Guy-Chapman) or bound metal cations on the membrane surface compete with protons, thus determining the local (membrane) proton concentration, which in turn controls the state of protonation of Asp-85. Although surface potential influences the apparent pK_a of Asp-85, it was later proposed that the purple ⇔ blue equilibrium is determined by two specific (high affinity) metal binding sites which are not necessarily located on the surface [21–27]. (b) Identification of the color determining metal binding site. It has been suggested that the high affinity metal site which determines the state of protonation of Asp-85 is located in the retinal binding pocket, directly interacting with one or more of the charged residues: Asp-85, Asp-212 and SBH^+ [12,23,26–28]. Such a location has been seriously questioned by a recent study of the kinetics of the blue ⇔ purple transition, as induced by large molecular cations [29]. (c) The third outstanding problem, which is the subject of the present work, concerns the exact relationship between the cation binding titration and the Asp-85 titration. Thus, since the pK_a of Asp-85 is influenced by the binding of one or two strongly bound metal cations, the question arises as to the coupling mechanism of these two events. A tempting approach (see, e.g. [23]) is to assume simple coupling between the two titrations, namely, a direct competition between protons and metal cations on the same color determining cation site (X^-, Y^-) according to Scheme 1, where X^- and Y^- denote two negatively charged protein residues. This approach does not exclude a modification of Scheme 1, with only two negative residues, namely with either X^- or Y^- being identified with Asp-85 itself.

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Abbreviations: bR, bacteriorhodopsin; ERS, electron spin resonance; SBH^+, retinal protonated Schiff base

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$$3H^+ + [\text{Asp-85}^-\ M^{2+} \cdots X^-, Y^-] \Leftrightarrow [\text{Asp-85H}\ \ 2H^+ \cdots X^-, Y^-] + M^{2+}$$

Purple Blue

Scheme 1.

A mechanism, such as Scheme 1, is strongly implied by any structural model in which the metal ion directly interacts with Asp-85. It is therefore clear that the above coupling problem is closely related to the identification of the metal binding site (problem (b) above). In the present work the coupling between the Asp-85 and metal site titrations is examined by carrying out a series of pH titrations of the four, strong and intermediate, Mn^{2+} binding sites in bR. The binding of Mn^{2+} as a function of pH is followed using the electron spin resonance (ESR) methodology of Duñach et al. [19]. The pK_a values of the metal cation binding titrations pK_a (Mn^{2+}) and those of the corresponding purple⇔blue titrations, pK_a (P/B), are determined in native bR and in several modified bRs and bR mutants. The data clearly indicate that the two titrations are non-coupled. The implications of this finding to cation binding in bR and to the Asp-85 titration are analyzed.

2. Materials and methods

The mutants were obtained as a generous gift from Prof. R. Needleman, Wayne State University. Deionized (DI) blue membrane was obtained by passing bR suspension through a Dowex 50 W×8 (Fluka) cation exchange column. 1–4 eq of Mn^{2+} were added to the blue membrane and the pH was adjusted to ∼7 using NaOH (∼20 eq). Spectroscopic measurements were carried out using 4×10^{-6} M bR. The purple to blue titration was carried out between pH 7 and 3. A stock solution of 8 ml of deionized bR with Mn^{2+} at pH 7 was distributed over eight samples and the desired pH was adjusted with H_2SO_4. The resulting suspension was kept at 25°C for 1.5 h to ensure complete titration, followed by absorption and pH measurements.

The ESR measurements were performed on a Bruker ER200 D-SRC. The metal ion dissociation titration was carried out using a 1×10^{-4} M pigment between pH 7 and 1.8. The ESR signal originates mostly from unbound Mn^{2+}, since the amplitude of bound Mn^{2+} is 8% of that of an identical concentration of the free ion. This was deduced from measurements of bR samples at pH 7 and 1.8 in which the Mn^{2+} is completely bound or unbound, correspondingly. The ESR of the unbound Mn^{2+} at different pH were obtained by correcting the measured ESR signal for the bound Mn^{2+} fraction.

Esterification of bR by 1-ethyl-3[3-(trimethylamino)-propyl]carbodiimide (ETC) was performed according to a previously described procedure [30]. Papain cleavage and partial delipidation were carried out according to previously described methods [31,32]. For partial delipidation, 10 mg bR was incubated at room temperature overnight in 5 ml of 20 mM CHAPS containing 5 mM acetate buffer (pH 5.4). The suspension was pelleted and the delipidation process was repeated three times. Artificial pigment derived from chromophore **1** (see Fig. 3) was prepared by incubating retinal analog **1** at 20°C with apomembrane at pH 6.8 for 6 days.

3. Results and discussion

Figs. 1 and 2 show the pH titrations of the metal binding site(s) and those of Asp-85 (the purple⇔blue transition) in the cases of 1–4 [Mn^{2+}]/[bR] molar ratios (R). As summarized in Table 1, the data of Figs. 1 and 2 clearly indicate that the pK_a (P/B) values of the four titrations do not coincide with those of the corresponding Mn^{2+} titrations. Thus, the former are all in the range between pK_a = 4 and 5, while the pK_a (Mn^{2+}) values corresponding to R = 1–3 are crowded around pK_a = 2.9. The pK_a (Mn^{2+}) value for R = 4 is shifted to 3.6, closer to the corresponding value of the color titration, pK_a (P/B) = 3.9. These observations lead to the following conclusions with relation to the metal binding sites. (a) Since for R = 1–3, pK_a (Mn^{2+}) is essentially independent of R, there appear to be at least three metal binding sites with the same relative affinity with respect to H_3O^+ ions. (b) These sites all have high or medium affinity with respect to Mn^{2+} binding, so that at neutral pH the metal is totally bound, even in the presence of one (excess) equivalent. This is in keeping with the findings of Duñach et al. [19] who reported five high and medium affinity Mn^{2+} sites in bR.

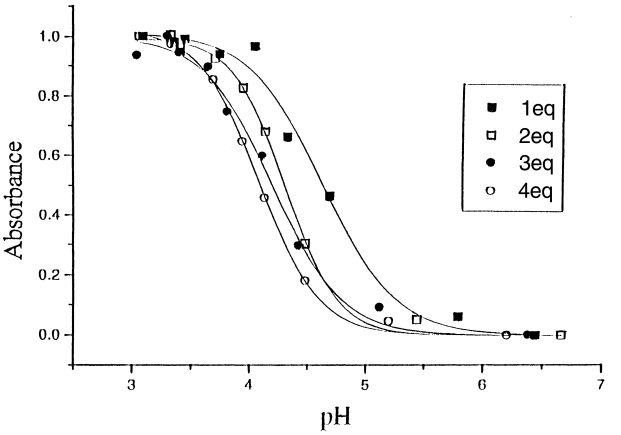

Fig. 1. Titration of the purple to blue transition of deionized bR (4×10^{-6} M) to which various equivalents of Mn^{2+} were added. Absorption was detected at 620 nm. The solid line represents the best titration fit.

The four cation binding sites described above include the two strongly bound sites which stabilize the purple form of bR above pH∼4 [12]. The present finding, namely, that pK_a (Mn^{2+}) is down-shifted relative to pK_a (P/B), implies that the two titrations are not directly coupled and therefore cannot be represented by Scheme 1. To further investigate this conclusion we extended the titrations of the two strongly bound Mn^{2+} sites, as well as the purple⇔blue titrations, to several bR mutants and modified bR. The relevant data are shown in Table 1. We first consider the two mutants, E74C and E74D, involving Glu-74 located close to the surface on the extracel-

Table 1
Titrations related to the purple and blue transitions and to Mn^{2+} binding in native bR (WT), in bR mutants and artifical bR pigments

System	pK_a (P/B)[a]	pK_a (Mn^{2+})[b]	R[b]
Wt	4.6	2.9	1
Wt	4.3	2.8	2
Wt	4.2	3.0	3
Wt	3.9	3.6	4
E74C	4.2	2.5	2
E74D	5.2[c]	2.5	2
E9D	7.2[c]	2.5	2
E204Q	4.2; 2.6 (0.1 M NaCl) [33]	1.6	2
R82Q	7.2 (0.1 M NaCl) [35,36]	2.9	2
D85E	5.4; 10.4 (two titrations)	2.8	2
D96N	3.5	3.1	2
bR 5.12	7.9	2.8	2
D212N		3.2	2

[a]Purple to blue transition in water if not otherwise indicated.
[b]R represents the equivalent number of added Mn^{2+}.
[c]Titration was carried out in the presence of ca. 40 equivalents of NaOH.

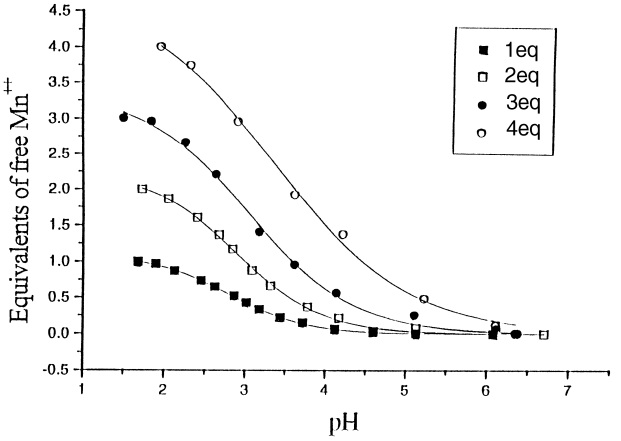

Fig. 2. pH dependence of ESR signal of the unbound Mn^{2+} of deionized bR suspension (1×10^{-4} M) with various equivalents of Mn^{2+}. The solid line represent the best titration fit.

lular side. For $R=2$, both mutants exhibit the same pK_a (Mn^{2+}) = 2.5 value which is ~0.4 units lower than that of the native pigment. While the pK_a (P/B) value of E74C is similar to that of the native pigment (4.2), E74D (5.2) is markedly different. Thus, the difference between pK_a (Mn^{2+}) and pK_a (P/B) is larger than in the native system. Substitution of Glu-9 (also located on the extracellular side) by aspartic acid reveals a behavior similar to that of E74D, with a more dramatic separation between pK_a (P/B) and pK_a (Mn^{2+}). Additional examples of complete separation between the metal and the color titrations (see Table 1) are E204Q [$\Delta pK_a = pK_a$ (P/B)−pK_a (Mn^{2+}) = 2.6 and 1.0 in 0.1 M NaCl (for P/B) [33]], D85E (ΔpK_a = 2.6 and 7.6 [34]), R82Q (ΔpK_a = 4.4 in 0.1 M NaCl (for P/B) [35,36]), D96N (ΔpK_a = 0.4 without salt) and the artificial pigment, bR 5.12, derived from chromophore **1** (Fig. 3) (ΔpK_a = 5.1 [37]). The D212N mutant does not exhibit a normal pK_a (P/B), but rather a red shift above pH 7 [38] is characterized by a pK_a (Mn^{2+}) of 3.2.

It is clearly evident that for the above pigments a wide range of pK_a (P/B) values is associated with essentially the same pK_a (Mn^{2+}) parameter, which is analogous to that of native bR. (E204Q is an exception in exhibiting a considerably lower pK_a (Mn^{2+}) value.)

Features which are indicative of the uncoupling of the two titrations are also observed with various modified bR systems. Thus, the esterification procedure of Renthal et al. [30], which was reported to involve residue E74, reduces pK_a (P/B) to below ~2 while it raises the pK_a (Mn^{2+}) to 3.5. In this

Table 2
Titrations of Mn^{2+} binding and purple to the blue transition in modified bR

Sample	pK_a (P/B)[a]	pK_a (Mn^{2+})[b]	R[c]
bR-ETC	< 2.0	3.6	1
	< 2.0	3.8	2
WT-papain treated	4.3	2.9	2
74C-ETC	2.0	3.5	2
WT-CHAPS treated	1.8	5.7	2[c]
WT-Pap-CHAPS[d]	1.8	5.3	2[c]
WT-Triton X-100	1.8	5.4	2[c]

[a]Purple to blue transition in water.
[b]R represents the equivalent number of added Mn^{2+}.
[c]Only one equivalent of Mn^{2+} was bound.
[d]Wild type was treated by papain, followed by CHAPS treatment.

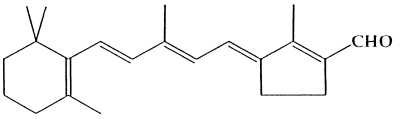

Fig. 3. Structure of all-*trans*-12,14 ethanoretinal (chromophore **1**).

case, therefore, pK_a (Mn^{2+}) > pK_a (P/B) (Table 2). This pK_a inversion means that in the esterified sample, cation removal by deionization does not affect the pK_a of the purple⇔blue equilibrium. Removal of the C-terminus by papain treatment has been reported to affect only the low affinity cation sites, without changing the purple⇔blue equilibrium [12]. In fact, as shown in Table 2, papain treatment, with or without esterification, has little effect on either pK_a value.

It has been reported that destruction of the native bR membrane by detergent treatment eliminates the cation effect on the pK_a (P/B) and reduces its value to about 1.8 [31]. As shown in Table 2, we find that in CHAPS and Triton-X solubilized bR only one Mn^{2+} is bound, with pK_a (Mn^{2+}) ≅ 5.4–5.7. This is another example of a system in which, despite cation elimination, a very low pK_a (P/B) value is maintained.

The pK_a of Asp-85 can be affected by several factors. It has been suggested that geometrical perturbation in the binding site affects the bound water structure as well as that of residues in the binding site, dramatically increasing the pK_a of Asp-85 [33,39]. This effect was revealed in several artificial pigments, which perturbed the hydrogen bonding network, and in several mutants characterized by point mutation in binding site residues like D85E or Y185F. Aspartic acids 74 and 9 are located close to the surface of the extracellular side [40–43]. Substitution of these residues by glutamic acid alters Asp-85 apparent pK_a to 5.2 and 7.2, respectively (Table 1). This effect, which might be partially attributed to perturbation of the hydrogen bonding network, involves protein residues and/or bound water molecules that connect Asp-85 to residues E74 and E9. A similar effect can be imposed by the deionization process. It was recently suggested that the color controlling cations are bound close to or on the surface [29]. Removal of these cations can induce protein conformational alterations, which perturb the hydrogen bonding network, increasing the pK_a of Asp-85. Alternatively, the effect of E74 and E9 substitution might be attributed to surface potential modification, which affects the local pH at the Asp-85 vicinity. We note that perturbation of Asp-85 pK_a by Asp-74 or Asp-9 substitution, as well as by artificial pigments, affects only slightly the pK_a of the residues responsible for the cation binding. This fact supports the possibility that Asp-85 and the cation binding residues are located in different protein domains.

4. Conclusions

We have shown that the pH titration of the high affinity, 'color determining', cation sites in bR is not directly related to the titration of Asp-85. Thus, depending on R, in native bR the two titrations may be separated by up to 2 pK_a units. Moreover, the respective pK_a values respond differently to structural changes induced by protein point mutations, chemical modification, membrane disruption and chromophore substitution. In some cases (when pK_a (Mn^{2+}) > pK_a (P/B)), the metal ions may be displaced by H_3O^+ without the proto-

nation of Asp-85, while in others (when pK_a (Mn^{2+}) < pK_a (P/B)) protonation of Asp-85 does not displace the bound metal cations. This does not mean that the two corresponding titration sites do not affect each other. The well established effect of deionization on the purple ⇔ blue equilibrium possibly indicates that structural changes are induced in bR by cation removal, affecting the pK_a of Asp-85. This effect, which characterizes native bR and other systems in which pK_a (P/B) > pK_a (Mn^{2+}), is not observed in the inverted systems where pK_a (Mn^{2+}) > pK_a (P/B). However, our data exclude Scheme 1 as a combined titration mechanism.

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