ELSEVIER

Biophysical Chemistry 78 (1999) 241–245

Biophysical Chemistry

Conformational change in bacterio-opsin on binding to retinal

Robert Renthal*, Carlos Alaniz

Division of Earth and Physical Sciences, The University of Texas at San Antonio, San Antonio, TX 78249, USA

Received 8 October 1998; received in revised form 18 February 1999; accepted 18 February 1999

Abstract

The detailed mechanism of retinal binding to bacterio-opsin is important to understanding retinal pigment formation as well as to the process of membrane protein folding. We have measured the temperature dependence of bacteriorhodopsin formation from bacterio-opsin and all-*trans* retinal. An Arrhenius plot of the apparent second-order rate constants gives an activation energy of 11.6 ± 0.7 kcal/mol and an activation entropy of -4 ± 2 cal/mol deg. Comparison of the activation entropy to model compound reactions suggests that chromophore formation in bacteriorhodopsin involves a substantial protein conformational change. Cleavage of the polypeptide chain between residues 71 and 72 has little effect on the activation energy or entropy, indicating that the connecting loop between helices B and C is not involved in this conformational change.

Keywords: Bacteriorhodopsin; Retinal binding mechanism; Protein conformational change; Entropy; Proteolysis of membrane surface loop

1. Introduction

Bacterio-opsin binds all-*trans* retinal to form the photosynthetic pigment bacteriorhodopsin in *Halobacterium salinarium* [1–3]. The detailed mechanism of this process is not known but is significant for the following reasons. (1) The retinal binding site bears a superficial resemblance to the 11-*cis* retinal binding site of the visual pigment rhodopsin [4]. Thus, understanding the mechanism of retinal binding in the bacterial system may illuminate some features of visual pigment formation, along with the enormous number of related G protein coupled receptor agonist binding interactions. (2) It has not yet been determined whether bacterio-opsin folds into a native-like conformation in the absence of retinal, or whether retinal binding is a step in the folding process [5,6]. Bacteriorhodopsin has contributed key insights into what we know of membrane protein folding [7], so it is important to clarify this point.

* Corresponding author. Tel.: +1-210-458-5452; e-mail: rrenthal@utsa.edu

0301-4622/99/$ - see front matter
PII: S0301-4622(99)00030-7

Chemical modification of bacteriorhodopsin with a water-soluble carbodiimide at glutamate 74 inhibits the rate of retinal binding [8]. This could be either a direct steric effect, or it could result from an inhibitory effect on a conformational change that is linked to retinal binding. Glu 74 is located on the surface connecting loop between helices B and C. A conformational change in the B–C loop is plausible, since the loop has been observed in two different conformations by electron cryomicroscopy [9,10] and X-ray diffraction [11,12]. We have tested the role of the B–C loop in the reaction of bacterio-opsin with retinal by measuring the entropy of activation, before and after specific proteolysis of the loop.

2. Methods

Purple membrane was prepared from *Halobacterium salinarium* by the method of Oesterhelt and Stoeckenius [13]. The retinal chromophore was bleached, and the rate of regeneration with all-*trans* retinal was measured as previously described [8]. Temperature was controlled to ± 0.1°C with a thermoelectric cuvet accessory for the Aviv/Cary 14 spectrophotometer. Bacterio-opsin was cleaved between residues 71 and 72 with chymotrypsin by the method of Gerber et al. [14].

3. Results and discussion

The rates of regeneration of bacteriorhodopsin from bacterio-opsin and retinal were measured as a function of temperature. We assume the reaction is second order:

$$\mathrm{d[bR]}/\mathrm{d}t = k_r[\mathrm{bO}][\mathrm{R}] \quad (1)$$

where, at time t, [bO] is the concentration of unregenerated bacterio-opsin, [bR] is the concentration of regenerated bacteriorhodopsin, and [R] is the concentration of free retinal. Solving Eq. (1) for [bR] gives:

$$[\mathrm{bR}] = \mathrm{R_T P_T}(\exp[k_r t(\mathrm{R_T} - \mathrm{P_T})] - 1) / (\mathrm{R_T}\exp[k_r t(\mathrm{R_T} - \mathrm{P_T})] - \mathrm{P_T}) \quad (2)$$

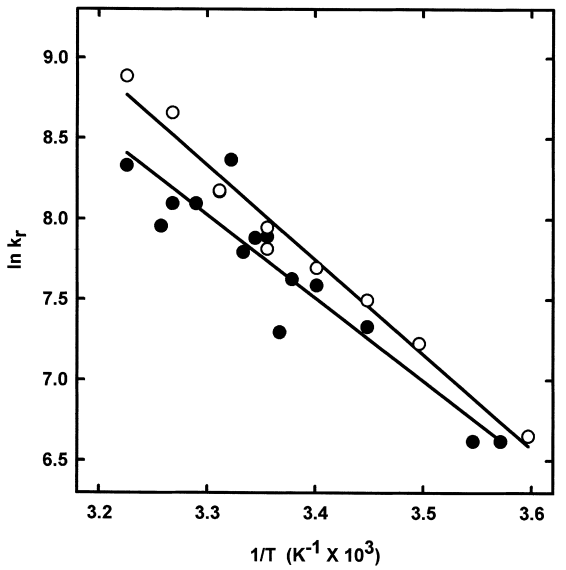

Fig. 1. Arrhenius plot of the apparent second order rate constant for reaction of all-*trans* retinal with bacterio-opsin. Absorbance increase at 565 nm as a function of time was fit to Eq. (2) by varying k_r. Open circles: hydroxylamine-bleached purple membrane. Filled circles: hydroxylamine-bleached purple membrane treated with chymotrypsin prior to reaction with retinal. Reaction conditions: 0.02 M sodium phosphate buffer, pH 7.0. Typical samples contained 5 μM bacterio-opsin and 6.5 μM retinal.

where $\mathrm{R_T}$ is total retinal and $\mathrm{P_T}$ is [bR] + [bO] (see Appendix A). We fit rate constants k_r to Eq. (2) by minimizing the residual to the experimental data at each temperature. The results are shown as Arrhenius plots in Fig. 1 for bacterio-opsin (open circles) and chymotrypsin-cleaved bacterio-opsin (filled circles). The observed activation energy, E_a, is 11.6 ± 0.7 kcal/mol for bacterio-opsin and 10.2 ± 1.1 kcal/mol for chymotrypsin-cleaved bacterio-opsin. Within the error of the measurements, there does not appear to be a significant difference between the two results.

The activation entropy measured from the intercept in Fig. 1 is -4 ± 2 cal/mol deg. This small entropy decrease is surprising because, for simple reactions, the activation entropy is often about the same magnitude as the overall entropy change for the reaction. However, in the one case where retinoid binding has been measured at

equilibrium [15], the standard entropy change is +35 cal/mol deg. Furthermore, a contribution of the activation entropy change for Schiff base formation would be expected to make the discrepancy even worse, since values are typically around +30 cal/mol deg [16]. There are many processes which might contribute to this discrepancy. In the following discussion, we suggest a protein conformational change model for regeneration of bacteriorhodopsin which is consistent with other studies and which can explain the difference between measured and expected activation entropies.

A simple mechanism for bR chromophore regeneration would consist of three different steps:

$$bO_u \overset{K_c}{\rightleftarrows} bO_f \tag{3}$$

$$bO_f + R \overset{K_{rb}}{\rightleftarrows} bO_f \cdot\cdot R \tag{4}$$

$$bO_f \cdot\cdot R \overset{k_s}{\rightarrow} bR \tag{5}$$

Reaction (3), a conformational equilibrium between two forms of bO, partially unfolded (bO_u) and nearly folded (bO_f), is suggested by the spectroscopic observations of Ludlam and Rothschild [17] and Kollbach et al. [18]. The equilibrium constant for this reaction is $K_c = [bO_f]/[bO_u]$. Reaction (4) is the binding of retinal to bO_f, with an association constant $K_{rb} = [bO_f \cdot\cdot R]/([bO_f][R])$. Reaction (5) is Schiff base formation, with a rate constant k_s. Reactions (4) and (5) have been separately observed by Schrekenbach et al. [2,19,20] and Booth et al. [5,6]. If the intermediate $bO_f \cdot\cdot R$ exists in a steady state, the rate of formation of bR will equal the rate of disappearance of bO:

$$k_r[bO_u + bO_f][R] = k_s[bO_f \cdot\cdot R] \tag{6}$$

Substitution of the equilibrium constants from (3) and (4) into Eq. (6), and assuming that equilibrium (3) lies far to the left, gives:

$$k_r = k_s K_c K_{rb} \tag{7}$$

If the activation energies and enthalpies of the reactions are temperature-independent, then it follows that:

$$\Delta S_r^{\ddagger} = \Delta S_s^{\ddagger} + \Delta S_c^{\circ} + \Delta S_{rb}^{\circ} \tag{8}$$

where $\Delta S_r^{\ddagger}$ is the activation entropy observed from the data in Fig. 1, $\Delta S_s^{\ddagger}$ is the activation entropy for Schiff base formation, ΔS_c° is the standard entropy change for the conformational change in Eq. (3), and ΔS_{rb}° is the standard entropy change for retinal binding in Eq. (4).

The quantity ΔS_{rb}° can be estimated as +35 cal/mol deg from the binding of retinol to serum retinol-binding protein [15]. This process is the transfer of retinol, which is structurally similar to retinal, from water into a hydrophobic protein cavity. There is practically no protein conformational change during retinol binding [21]. The quantity $\Delta S_s^{\ddagger}$ can be estimated as approximately +30 cal/mol deg from the activation entropies of hydrolysis for model Schiff bases [16], considering that the enthalpy changes [22] and free energy changes [23] for Schiff base formation in model compounds are less than a few kcal/mol. Substituting these values into Eq. (8), ΔS_c° is approximately −69 cal/mol deg.

The magnitude of this entropy decrease implies a substantial protein conformational change accompanies the purple chromophore formation. By comparison, the conversion of metarhodopsin I to metarhodopsin II has an entropy change from +46 to +66 cal/mol deg [24–26]. The M1 to M2 transition in the bacteriorhodopsin photocycle has an entropy change of approximately +72 cal/mol deg [27]. The quantitative similarity suggests the following possibilities: (1) bacterio-opsin undergoes a conformational change during chromophore formation; (2) this change resembles the reverse of the protein conformational change in the M1 to M2 transition that occurs during the bacteriorhodopsin photocycle; and (3) the metarhodopsin I to metarhodopsin II transition also resembles the reverse of the bacterio-opsin conformational change occurring with chromophore formation.

Some previous studies have provided evidence for a protein conformational change occurring

during chromophore formation. Rehorek and Heyn [28] showed that chromophore regeneration is cooperative. The facilitation of chromophore formation by protein–protein interactions implies that a protein conformational change occurs, analogous to the 'heme–heme' interactions when oxygen binds to hemoglobin. This point has been extended by Kollbach et al. [18], who found differences in the Schiff base pK depending on whether or not retinal was present during bacterio-opsin folding. Further evidence for a conformational difference between bacterio-opsin and bacteriorhodopsin has been presented, based on FTIR spectra [17]. However, recently Israelewitz et al. [29] described a model of retinal binding to bacterio-opsin involving a small entry window between the E and F helices. In this model, bacterio-opsin is considered to be in a nearly native structure in the absence of retinal. It was assumed that no protein conformational change occurs as part of the retinal binding process.

Our previous results [8] and those of Greenlaugh et al. [30] suggested that the protein conformational change linked to chromophore formation could involve the B–C loop. Changes in the vicinity of the B helix during the bacteriorhodopsin photocycle have been observed by diffraction methods [31–33]. Changes in this loop could contribute to the conformational entropy change in the photocycle and in chromophore formation. It is known that proteolytic cleavage of protein loops produces disorder [34]. Thus it would be expected that cleavage of the B–C loop would increase the contribution of conformational entropy change to the observed activation entropy, since the partially unfolded state would be even more disordered. However, the chymotrypsin cleavage data in Fig. 1 does not support this model. Cleavage between residues 71 and 72 has essentially no effect on the rate or activation entropy of the reaction. Therefore, the nature of the conformational change linked to chromophore formation remains to be determined.

Acknowledgements

Supported by a grant from the National Institutes of Health (GM 08194).

Appendix A:

Assume the rate of disappearance of bacterio-opsin is proportional to the molar concentrations of bacterio-opsin [bO] and retinal [R]. If there are no long-lived intermediates, then bacteriorhodopsin [bR] will form at a rate equal to the rate of disappearance of bO (this assumption is poor at 0°C, where a long-lived intermediate does form [2,19]):

$$-\mathrm{d[bO]}/\mathrm{d}t = \mathrm{d[bR]}/\mathrm{d}t = k_r[\mathrm{R}][\mathrm{bO}] \qquad \text{(A1)}$$

Since total retinal $R_T = [R] + [bR]$ and total protein $P_T = [bO] + [bR]$,

$$\mathrm{d[bR]}/\mathrm{d}t = k_r(R_T - [bR])(P_T - [bR]) \qquad \text{(A2)}$$

Integration of Eq. (A2), using the limit $[bR] = 0$ at $t = 0$ gives:

$$k_r t = (\ln((R_T - [bR])P_T) / ((P_T - [bR])R_T)))/(R_T - P_T) \qquad \text{(A3)}$$

Rearrangement of Eq. (A3) gives Eq. (2).

References

[1] D. Oesterhelt, L. Schuhmann, FEBS Lett. 44 (1974) 262–265.
[2] T. Schreckenbach, D. Oesterhelt, Fed. Proc. 36 (1977) 1810–1814.
[3] P. Towner, W. Gaertner, B. Walckhoff, D. Oesterhelt, H. Hopf, Eur. J. Biochem. 117 (1988) 353–359.
[4] J.M. Baldwin, EMBO J. 12 (1993) 1693–1703.
[5] P.J. Booth, A. Farooq, S.L. Flitsch, Biochemistry 35 (1996) 5902–5909.
[6] P.J. Booth, A. Farooq, Eur. J. Biochem. 246 (1997) 674–680.
[7] J.-L. Popot, D.M. Engelman, Biochemistry 29 (1990) 4031–4037.
[8] R. Renthal, K. McMillan, L. Guerra, M.N. Garcia, R. Rangel, C.-M. Jen, Biochemistry 34 (1995) 7869–7878.
[9] N. Grigorieff, T.A. Ceska, K.H. Downing, J.M. Baldwin, R. Henderson, J. Mol. Biol. 259 (1996) 393–421.
[10] Y. Kimura, D.G. Vassylyev, A. Miyazawa, et al., Nature 389 (1997) 206–211.
[11] E. Pebay-Peyroula, G. Rummel, J. Rosenbusch, E. Landau, Science 277 (1997) 1676–1681.
[12] H. Leucke, H.-T. Richter, J.K. Lanyi, Science 280 (1998) 1934–1937.

[13] D. Oesterhelt, W. Stoeckenius, Methods Enzymol. 31 (1974) 667–678.
[14] G. Gerber, R. Anderegg, W. Herlihy, C. Gray, K. Biemann, H.G. Khorana, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 227–231.
[15] N. Noy, Z.J. Xu, Biochemistry 29 (1990) 3888–3892.
[16] R.K. Chaturvedi, E.H. Cordes, J. Am. Chem. Soc. 89 (1967) 1230–1234.
[17] G.J. Ludlam, K.J. Rothschild, FEBS Lett. 407 (1997) 285–288.
[18] G. Kollbach, S. Steinmuller, T. Berndsen, V. Buss, W. Gartner, Biochemistry 37 (1998) 8227–8232.
[19] T. Schreckenbach, B. Walckhoff, D. Oesterhelt, Eur. J. Biochem. 76 (1977) 499–511.
[20] T. Schreckenbach, B. Walckhoff, D. Oesterhelt, Biochemistry 17 (1978) 5353–5359.
[21] G. Zanotti, S. Ottonello, R. Berni, H.L. Monaco, J. Mol. Biol. 230 (1993) 613–624.
[22] A. Giartosio, C. Salerno, F. Franchetta, C. Turano, J. Biol. Chem. 257 (1982) 8163–8170.
[23] D. Metzler, J. Am. Chem. Soc. 79 (1957) 485–490.
[24] R.G. Matthews, R. Hubbard, P.K. Brown, G. Wald, J. Gen. Physiol. 47 (1963) 215–240.
[25] H.M. Emrich, R. Reich, Z. Naturforsch. 29c (1974) 577–591.
[26] H. Imai, T. Mizukami, Y. Imamoto, Y. Shichida, Biochemistry 33 (1994) 14351–14358.
[27] G. Varo, J.K. Lanyi, Biochemistry 30 (1991) 5016–5022.
[28] M. Rehorek, M.P. Heyn, Biochemistry 18 (1979) 4977–4983.
[29] B. Isralewitz, S. Izrailev, K. Schulten, Biophys. J. 73 (1997) 2972–2979.
[30] D. Greenhalgh, C. Altenbach, W.L. Hubbell, H.G. Khorana, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 8626–8630.
[31] N. Dencher, G. Dresselhaus, G. Zaccai, G. Büldt, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 7876–7879.
[32] J. Vonck, Biochemistry 35 (1996) 5870–5878.
[33] M. Nakasako, M. Kataoka, Y. Amemiya, F. Tokunaga, FEBS Lett. 292 (1991) 73–75.
[34] H.W. Wyckoff, K.D. Hardman, N.M. Allewell, T. Inagami, L.N. Johnson, F.M. Richards, J. Biol. Chem. 242 (1967) 3984–3988.