KINETIC INTERACTION BETWEEN AROMATIC RESIDUES AND THE RETINAL CHROMOPHORE OF BACTERIORHODOPSIN DURING THE PHOTOCYCLE

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1. Introduction

In an earlier communication [1] a reaction of the protein fluorescence following 570 nm light activation of bacteriorhodopsin (BR) was described, indicating the participation of the aromatic amino acid residues in the mechanism of its photo- and protoncycle and a conformational change. Recently confirmation and extension of these data were presented suggesting a deprotonation and reprotonation of tyrosine and possibly tryptophane during the photocycle [2,3]. This notion is supported by the elucidation of the amino acid analysis of BR [4], the pH-sensitivity of its aromatic spectrum [5] as well as the results of chemical substitution experiments of tyrosine residues [6]. Here we report on the transient and steady state difference spectra of the aromatic amino acid residues, their reaction velocity constants, their approximate stoichiometry during a photocycle as well as on their interactions and time correlation with photocycle intermediates.

2. Materials and methods

Purple membrane was isolated from *Halobacterium halobium* NRL R_1M_1 [7]. Thin purple membranes layers were prepared with 95% humidity according to [8]. Purple membrane suspensions (9 μM) were analyzed in Tris–phosphate buffer (pH 7) and Tris–borate buffer (pH 9–12.5). Transient state kinetics were measured as in [9], with the following modifications: In double excitation experiments the sample was steadily excited by a Xe–Hg Hanovia lamp (200 W), whose light of 70 mW/m^2 (at 275–295 nm) and of 3 W/m^2 (at 412 nm) was filtered by a Bausch and Lomb monochromator (no. 33-86-02), and by 1.5 μs flashes (570 nm) from a rhodamin G6 dye laser (Zeiss 807101). The measuring beam of the second Hanovia lamp was filtered by two Zeiss monochromators (M4QIII) prior and after the sample, and passed through a Glan-Taylor ultraviolet prism polarizor prior to the sample for the measurement of linear dichroism. In all experiments the axis of the exciting and analyzing light formed an angle of 45° with the glass slide plane. Steady state kinetics were obtained with the equipment given in [1] operating in the single beam mode with following modifications: Light source for the ultraviolet measuring beam was the Hanovia lamp given above, filtered through a Zeiss monochromator (M4Q). Exciting light was obtained from a Zeiss 900 W Xe arc passed through a water bath with additional heat filters (Schott), 500 nm cut-off filter, a 576 nm filter (Schott) filter, and a photoshutter. Light detection and data processing methods used were as in [1,8,9].

3. Results

3.1. *Transient and steady state spectra*

The steady state light–dark difference spectrum of a purple membrane suspension and the transient difference spectrum of the maximum amplitude change obtained with a purple membrane film after a 1.5 μs (570 nm) laser flash are shown in fig.1a,b. Except for a relative more pronounced absorption change at 275 nm and the shape of the spectrum in the flash

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Fig.1a

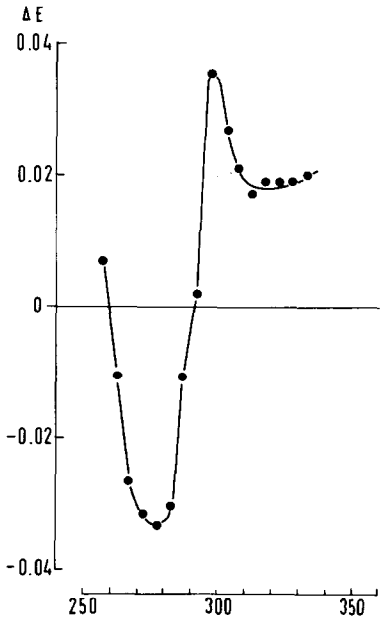

Fig.1b

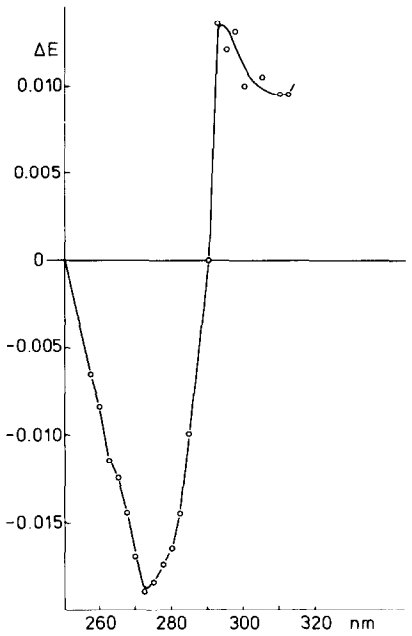

Fig.1a. Steady state light–dark amplitude difference spectrum of BR (pH 7.0, 4°C). Fig.1b. Transient state difference spectrum of the maximum amplitude change of thin layers of purple membrane (O.D. (565) ≈ 0.9, −2.5°C).

experiment, compared to the steady state spectrum, the spectra are nearly identical with respect to absorption maxima and isosbestic points, and similar to the difference amplitude spectrum of a suspension analyzed at room temperature [2]. Plots of the reciprocal steady state amplitudes versus the reciprocal light intensities give straight lines for the 275 nm absorption decrease, the 297 nm increase as well as for the M-412 nm increase (fig.2). This is in agreement with previous findings for the M-412 intermediate up to 50% light saturation [10] and demonstrates the strong correlation of the reactivity of the retinal chromophore and aromatic amino acid residues during the photocycle events. Extrapolation to light saturation yields a difference of extinction of 7000 ± 1000 ($l.cm^{-1}.mol^{-1}$), with a positive sign at 297 nm and a negative at 275 nm.

The experiments indicate that photoactivation of the retinal chromophore leads to a disappearance of tyrosine and tryptophane with concomittant appearance of a band with maximum at 295 nm, pointing to a deprotonation of aromatic residues (obviously tyrosine) correlating with a deprotonation of the

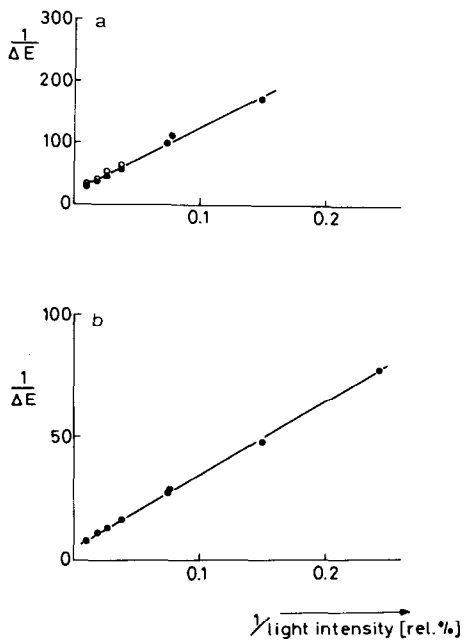

Fig.2. Relationships between the steady state amplitude and light intensity (a: (○○○) 278 nm, (●●●) 297 nm; b: 420 nm).

Table 1
Linear dichroism of 570 nm light-induced absorption changes at 275 nm and 295 nm in comparison to 412 nm

nm	LD[a]
275	− 0.23
295	− 0.078
412	− 0.24

[a] $LD = \frac{|\Delta A_v| - |\Delta A_h|}{|\Delta A_v| + |\Delta A_h|}$

(v = vertically; h = horizontally)

Table 2a
The formation (a) and decay (b) of BR-intermediates following a laser pulse

nm	k_1 [$\times 10^{-1}$][a]	k_2[a]	Sum of squares $\times 10^{-2}$
275	4.9 (0.53)	2.4 (0.47)	0.11
295	2.9 (0.46)	1.2 (0.54)	0.22
412	1.0 (0.20)	1.3 (0.80)	0.15
520	2.7 (0.29)	1.2 (0.71)	0.047
550	9.4 (0.47)	1.3 (0.53)	0.05
590	9.9 (0.75)	1.4 (0.25)	0.019

[a] Given in ms^{-1}, relative amplitudes are given in brackets

retinal chromophore. As shown in table 1 the reactions are specific with respect to the absorption changes observed with vertically and horizontally polarized measuring light illustrating the strong orientation of the residues involved as well as its change during the formation of the 295 nm component.

3.2. *Transient kinetics*

Typical formation and decay kinetics of aromatic amino acid residues measured at 275 and 295 nm in comparison with the kinetics of a M-412 intermediate are shown in fig.3, illustrating the time relationships of these components following a laser flash. The analysis of the formation kinetics give best fit when analyzed as a sum of two exponentials as summarized in table 2a. The results illustrate the inhomogeneity of the reacting aromatic amino acid residues. Both fast kinetic phases (k_1) measured at 275 nm and 295 nm with ~50% of the total amplitude are 4.9- and 2.9-times faster, respectively, than the formation rate of M-412; they are, however, slower than the disappearance rate of BR-570. This places the photochemical events involving aromatic amino acid residues of the protein in relation to the retinal chromophore intermediates between the decay of L-550 and the formation of M-412.

On the other hand the decay kinetics at 275 nm and 295 nm (table 2b) are better fitted by a single

Fig.3a

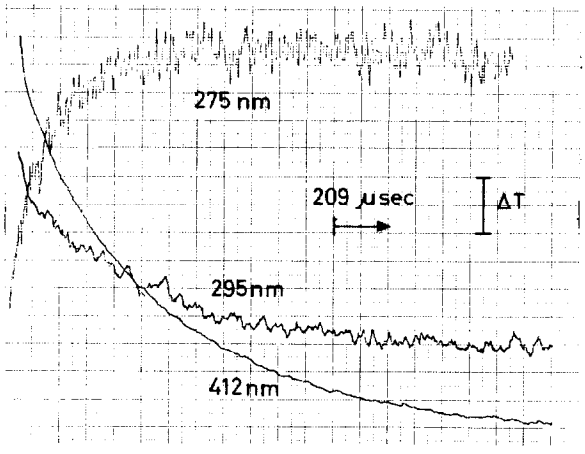

Fig.3b

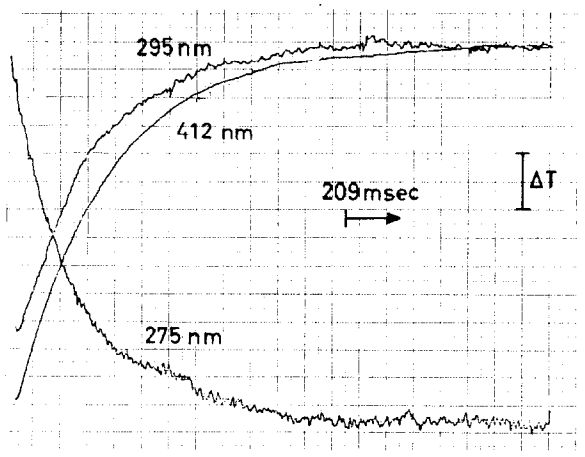

Fig.3. Formation (a) and decay (b) kinetics recorded at 3 different wavelengths in a thin-layer preparation of the purple membrane after a laser flash (O.D. (565) = 1.2, −1.5°C; ΔT (275) = 0.6%, ΔT (295) = 0.7%, ΔT (412) = 3%.

Table 2b
The formation (a) and decay (b) of BR-intermediates following a laser pulse

nm	k_1[a]	k_2[a]	Sum of squares $\times 10^{-2}$
275	3.0 (1.0)	–	1.0
295	3.5 (1.0)	–	0.42
412	15.0 (0.10)	3.35 (0.90)	0.15
520	5.0 (0.26)	3.10 (0.74)	0.28
550	4.7 (0.60)	2.63 (0.40)	0.33
590	4.7 (0.48)	2.72 (0.52)	0.019

[a] Given in s^{-1}, relative amplitudes are given in brackets

exponential in contrast to the rates of M-412 decay and BR-570 regeneration [11]. Indeed, the 275 nm amplitude spectrum shown in fig.1b decays in a homogeneous fashion over the time of observation without a change of the isosbestic point at 290 nm not allowing discrimination of the components comprising the nonhomogeneous 275 nm spectrum, as also indicated in its two exponential formation behaviour.

3.3. *Photoinduced cross reactions*

Because of the strong correlation between the reaction of the aromatic components and the retinal moiety and because of the earlier observation of the 412 nm photoactivation of the BR recovery as well as its reprotonation [1,11] double excitation experiments were carried out to test cross reactions by means of additional photoactivation of the aromatic residues and of the M-412 components [11–13]. The results show (table 3a) that upon photoexcitation of M-412 the reaction amplitude at 275 nm is diminished (17%) and its on-kinetics (especially of the fast phase) slowed down, indicating an 412 nm photoexcitation-dependent inhibition of the 275 nm state. On the other hand the appearance of the 295 nm component is not influenced by 412 nm photoexcitation.

Photoexcitation at 275 nm and 295 nm activates all tyrosine and tryptophane residues of bacteriorhodopsin. Nevertheless, a specific alteration of the retinal photocycle intermediates following a 570 nm laser flash is observed in the presence of ultraviolet light of 275 nm and 295 nm (table 3b). This demonstrates its influence on M-412 (in agreement with independent findings for steady state conditions of D. Oesterhelt et al. (personal communication)) and on absorption changes measured at 520 nm, but not at 550 nm and 590 nm. While the rates of formation are characterized mainly by an increase of the fast rate constants of the two exponentials and constant amplitudes, in the decay rates a drastic and inverse change of the amplitudes of both exponentials occurs under the influence of 275 nm photoexcitation.

3.4. *pH-dependency and humidity*

The kinetics of the aromatic amino acid residues are strongly pH-dependent as expected from the pK of the tyrosine-hydroxyl group. In experiments with purple membrane suspensions and thin-layer preparations, an increase of pH leads to an increase of the half-times of the decay rates of the 275 nm and 295 nm components. This inhibition of the decay rates effects both components in a different way depending on pH, and furthermore it does not correlate with the inhibition of the 420 nm decay rates as a function of pH as summarized in the ratio of half-times as shown in table 4. From these results it might be suggested that

Table 3a
The formation (a) and decay (b) of BR-intermediates following a laser flash in presence of additional activating light

Measuring light (nm)	Activating light (nm)	k_1 [$\times 10^{-1}$][a]	k_2[a]	Sum of squares $\times 10^{-2}$
275	412	1.7 (0.58)	2.1 (0.42)	0.016
295	412	2.9 (0.51)	1.2 (0.49)	0.07
412	275	1.6 (0.22)	1.3 (0.78)	0.13
412	295	2.5 (0.28)	1.3 (0.72)	0.058
520	275	8.1 (0.27)	1.3 (0.73)	0.07

[a] Given in ms^{-1}, relative amplitudes are given in brackets

Table 3b
The formation (a) and decay (b) of BR-intermediates following a laser flash in presence of additional activating light

Measuring light (nm)	Activating light (nm)	k_1[a]	k_2[a]	Sum of squares $\times 10^{-2}$
275	412	3.8 (1.0)	–	0.77
295	412	3.1 (1.0)	–	0.24
412	275	4.3 (0.84)	1.82 (0.16)	0.21
412	295	5.3 (0.47)	2.55 (0.53)	0.072
520	275	5.5 (1.0)	–	0.25

[a] Given in s^{-1}, relative amplitudes are given in brackets

the aromatic cycle might be rate-limiting in the overall process of the purple membrane functions. As expected from the earlier observations [8] we also observed an influence of the relative humidity on the rates of formation and decay of the 275 nm component (at $-1.5°C$): with 49 ms^{-1} and 3 s^{-1} at 94% humidity and 17.2 ms^{-1} and 1.6 s^{-1} at 85% humidity showing the dependency of the reactivity of the aromatic residues on the state of hydration.

4. Discussion

The difference spectra obtained upon pulsed and steady illumination indicate a remarkable participation of the aromatic residues in the proton translocation mechanism. This could be due to:

1. A dissociation of a phenolic tyrosine group yielding a positive peak at 295 nm upon release of a proton;
2. A charge perturbation of tryptophane yielding a positive peak at 293 nm and a broad negative peak below 280 nm;

Table 4
Ratio of decay half-times of aromatic residues and M-412 as a function of pH in purple membrane suspensions

pH	$t/2(295)$ / $t/2(420)$	$t/2(275)$ / $t/2(420)$
7	1.12	1.05
9	1.49	1.33
11	2.22	3.33

$T = 4–5$, 5°C in purple membrane suspensions

3. Solvent perturbation of both amino acid residues yielding increased polarity with negative peaks between 270–300 nm [14].

A change of the polarity of a suspension of BR by replacing water with 83% glycerol (dielectric constant = 52) decreases the positive extinction difference at 297 nm to 4600 ($l.cm^{-1}.mol^{-1}$) and the negative extinction difference at 275 nm to 3900 ($l.cm^{-1}.mol^{-1}$) clearly indicating the solvent perturbation as a contribution to the spectrum (unpublished observations). However, because of the nonlinear dependency of the difference extinction upon the dielectric constant given in glycerol–water mixtures it is not possible to extrapolate to polarities which exist at the membrane–bulk interphase or in the membrane interior. Therefore, at present time, only a rough calculation of the number of tyrosine and tryptophane residues involved in the aromatic cycle described here can be made. Based upon the differential extinction coefficient at 295 nm for the dissociated phenolic tyrosine residues (2480) and the charge perturbation of tryptophane (800–1100) [14] 1–2 tyrosine and 1–2 tryptophane residues might contribute to the positive peak at 295 nm while several tyrosine and tryptophane residues observed at 275 nm experience a higher polarity in the light compared to the dark state. In addition it should be noted that a contribution of the retinal chromophore to the difference spectrum in the 300 nm region cannot be excluded and has been observed by circular dichroism studies (unpublished) making the calculations on the stoichiometric relationships between the tyrosine molecules involved only suggestive.

The experiments lead to the observation of a dis-

sociation of at least one tyrosine hydroxyl proton in addition to the proton coming from the retinal Schiff's base. A higher stoichiometry than one has indeed been observed experimentally ([10,15,16], Parson and Ort, personal communication). Furthermore, the conclusion of a proton dissociation from a tyrosine hydroxyl group as a consequence of an internal charge perturbation induced by light corroborates with the results obtained by charge perturbation of open purple membrane sheets induced by an external electric field which yielded within a microsecond time scale an absorption increase at 300 nm of appropriate amplitude [16,17].

With respect to the general proton translocating function of bacteriorhodopsin the function of the tyrosine hydroxyl group shown here should be considered in the frame of a proton relay function being part of the function of other amino acid residues as suggested on the basis of Raman studies [18]. The kinetic analysis shows that we observe gross changes of aromatic residues with obvious deprotonation function prior to the formation of the stable photointermediate M-412. This is in agreement with recent Raman studies indicating a 12 μs time lapse between the deprotonation step and the M-412 formation [19]. Indeed, this observation might corroborate with the postulate of an up to now not observed additional photointermediate occurring between L-550 and M-412 with an absorption maximum at 460–480 nm [20]. It is interesting to note that we observed upon 275 nm light activation a cross reaction with a 3-fold change in the apparent formation kinetics at 520 nm which might be the tail of such a postulated absorption band.

The time scale of our observations does not fit to the aromatic region of BR observed in correlation with the K-590 and L-550 state at low temperature [5]. It might well be that at lower temperature the sequential steps of the complex reorientation of aromatic amino acids and perhaps others following the photocycle of the rhodopsin chromophore are severely distorted.

The exact location of the aromatic residues involved in the photocycle is difficult to evaluate at the present state. Because the linear dichroism indicates a strong specificity of an oriented reaction, on the basis of the well-known sequence data [4] the participation of the 26-residue is certainly suggested. We expect that the reacting aromatic component should be nearest-neighbour to the chromophore because of the results of the cross-activation reactions. A photoactivation of M-412 leads to a decrease of the 275 nm amplitude effecting its formation rate. This might indicate that the regeneration of BR 570 from M-412 is coupled to a pickup of a proton from a nearest-neighbour tyrosine. On the other hand the photoactivation of the aromatic region is also indicative of a donor–acceptor function in the protonation–deprotonation mechanism of the Schiff's base retinal. Here, photoexcitation of the aromatics might yield a decrease of the phenolic hydroxyl pK [21] and initiate a controlled proton release which interferes locally with the retinal cycle.

Previously we have suggested that the two exponential rates of M-412 reflect the reactions of two forms of M-412 [11], being in equilibrium [9] however decaying to BR-570 on different pathways [9,12]. Excitation with 275 nm light might either shift the equilibrium between the two forms or control rate-limiting steps in the process of BR-570 regeneration from one pathway to the other.

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