TRANS–CIS ISOMERISATION OF THE RETINAL CHROMOPHORE OF BACTERIORHODOPSIN DURING THE PHOTOCYCLE

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1. Introduction

Since the discovery of the reversible photocycle of the retinal chromophore in bacteriorhodopsin (bR) the question was asked whether the retinal chromophore undergoes an isomerisation reaction during the photocycle such as observed in rhodopsin [1,2]. Indeed, the existence of all-*trans* and 13-*cis* retinal isomers in bR, held in the dark (dark-adapted bR) [3–5] and the isomerisation of the 13-*cis* isomer into 100% all-*trans* isomer of bR by weak illumination (light adaptation) [4,5] was recognized. In [6] 13-*cis* retinal was extracted from bR kept in the M-412 state at low temperatures in the presence of guanidinium and high pH or ether and high salt, respectively. The 13-*cis* M-412 absorption spectrum with a maximum at 405 nm [6] corroborated with the action spectrum of a photoactivated M-412 component obtained under similar conditions [7]. Raman spectra had been explained in terms of 13-*cis* [8,9] and also in terms of all-*trans* M-412 [10]; in [11] Raman spectra with 15-deuteroretinal indicated that the M-412 Raman spectra originated from the 13-*cis* isomer of retinal. Furthermore, 13-*cis* retinal was extracted from both M-412 and L-550 [12], and 13-*cis* retinal extracted from M-412 could be correlated with the amount of recycling bR [13].

We have investigated the absorption changes following a 15 ns laser flash activation in the spectral range where *cis* isomer bands of the retinal chromophore are expected. We found reversible absorption changes between 300 and 375 nm as well as between 360 and 450 nm which clearly indicates the appearance of 13-*cis* and the disappearance of all-*trans* bands of the retinal chromophore reversibly during the all-*trans* photocycle*.

Abbreviations: bR, bacteriorhodopsin; M-412, L-550, K, J, intermediates of the bR photocycle according to the nomenclature of [5]

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2. Materials and methods

The purple membrane was prepared according to [15]. Absorption changes were measured with a flash photometer as in [16], modified with a double monochromator (Zeiss MM12) and additional window filters (Schott) to exclude any laser stray light. A detection electronics in combination with a transient recorder and a signal averager (Nicolet) was used which allows a time resolution up to 5 MHz. Flash excitation was obtained with a Nd/YAG pumped Rhodamin 6G dye laser (Quantel) of 15 ns pulse duration. Difference spectra between dark- and light-adapted purple membranes were recorded with a Cary 219 spectrophotometer.

3. Results

3.1. *Absorption changes over 260–450 nm following laser flash activation of light-adapted bR*

Typical formation kinetics at 1.5°C in the time range up to the formation of M-412 are shown for 4 different wavelengths in fig.1a, which demonstrates biphasic absorption changes at 276, 296 and 415 nm and a monophasic change at 335 nm. The slower phase kinetics at 276 and 296 nm follow approximately the slower phase of the absorption change observed at

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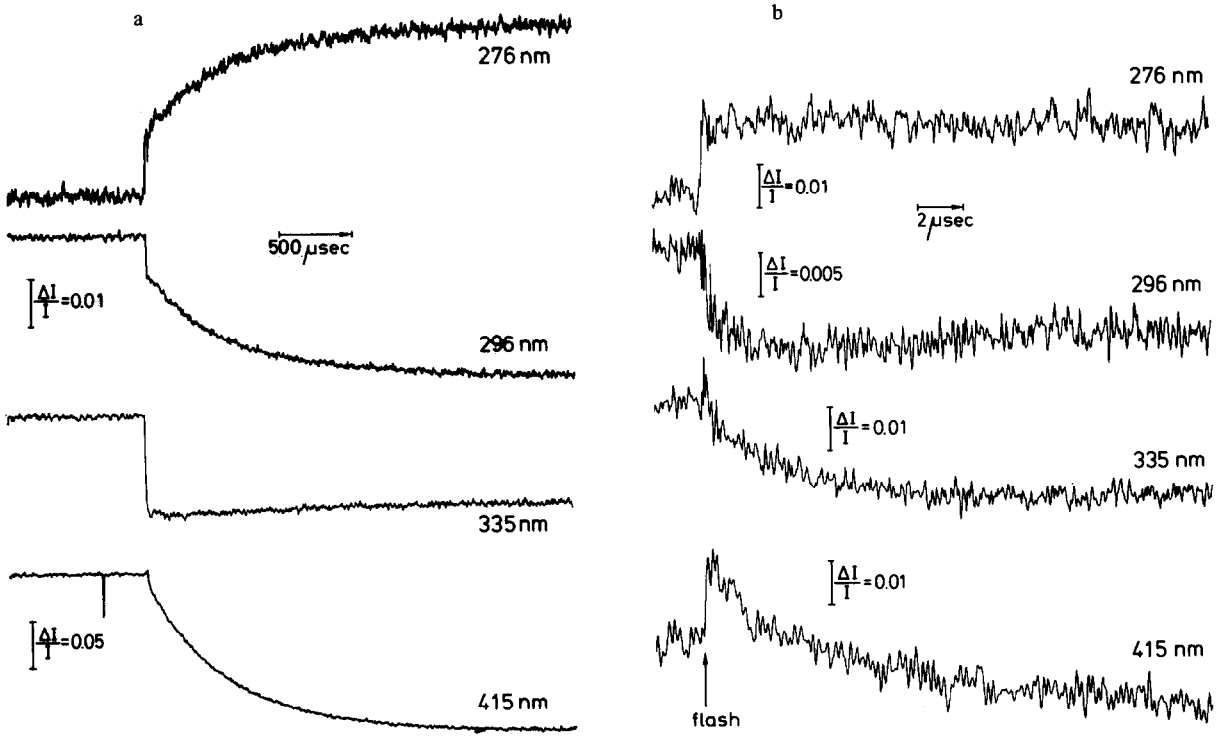
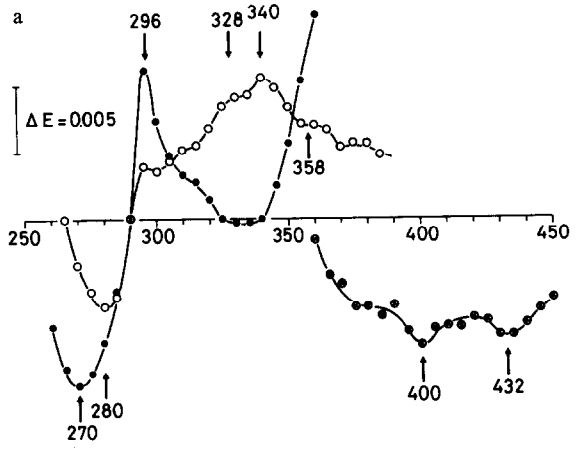


Fig.1. Formation kinetics at 276, 296, 335 and 415 nm. Light-adapted purple membrane suspension (11.5 μM bR) in 2 M NaCl, 10 mM phosphate buffer, pH 6, 1.5°C: (a) Records covering the time range up to the M-412 formation; (b) records covering the time range up to the L-550 formation.

415 nm. The amplitude difference spectrum of the slow phase components are given in fig.2a (●——●) confirming [16] with the short wavelength portion of the M-412 component indicated for simplicity only in its initial part.

The amplitude difference spectrum of the fast



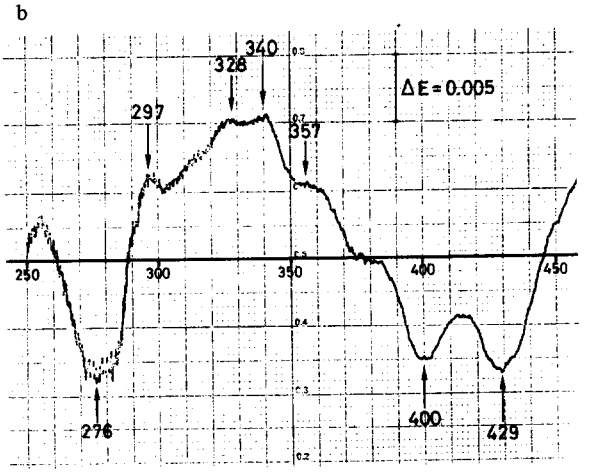


Fig.2(a) Difference spectra after flash excitation: (●——●) slow phase difference spectrum, (○——○, Ⓧ —— Ⓧ) fast phase difference spectrum (conditions as in fig.1). (b) Difference spectrum between dark-adapted (sample cuvette) and light-adapted (reference cuvette) bacteriorhodopsin. Purple membrane suspension (11 μM bR) in 10 mM phosphate buffer pH 7.0, room temperature.

phase component shows (fig.2a, ○——○) an absorption decrease below 290 nm with a minimum at 280 nm and an absorption increase above 290 nm again with maximum at 296 nm seen as pronounced shoulder (fig.2a). The following portion of the spectrum displays a structured difference spectrum with a peak at 340 nm and shoulders at 315, 328 and 358 nm, all of them reproducible ± 2–5 nm.

Resolution of the formation kinetics in the time range up to the formation of the L-550 intermediate of the photocycle are shown in fig.1b. At 415 nm a fast, non-time-resolved, absorption change towards the direction of absorption decrease (upwards) precedes the absorption increase which finally leads to the formation of M-412. This fast absorption decrease belongs to absorption changes described in the amplitude difference spectrum with minima at 400 and 432 nm (fig.2a, ⊗——⊗), both minima being reproducible with ± 2 nm.

The 276 nm absorption decrease is also non-time-resolved whereas the 335 nm absorption increase is, with a half-time of 1.8 μs (fig.1b). The reaction is not monophasic. The half-time of the fast component is 0.5–1.5 μs, that of the slow component, being 3–5 μs, corresponds to L-550 formation rate over 1–5°C [17].

Also at 296 nm two reaction phases are seen (fig.1b) with a relative contribution of 33% fast, non-time-resolved and of 66% slow phase with a half-time of 0.8 μs. The relative contribution of the 2 phases varies from 30–100% slow phase with a half-time of 0.5–0.9 μs.

3.2. *Identification of the all*-trans/*13*-cis *difference spectrum*

The nature of the 2 parts of the fast appearing difference spectrum (fig.2a, ○——○, ⊗ —— ⊗) is revealed by a comparison with the difference spectrum between the dark- and light-adapted bacteriorhodopsin which represents the difference spectrum between 50% 13-*cis*/50% all-*trans* and 100% all-*trans* retinal bacteriorhodopsin. This difference spectrum exhibits identical negative and positive absorption peaks in comparison with the fast-appearing difference spectrum after laser flash excitation (fig.2b): negative peaks at 276, 400 and 429 nm and positive peaks and shoulders at 297, 315, 328, 340 and 357 nm. This difference spectrum is not influenced by ionic strength and pH 6–9, except that the difference amplitudes at 297 and 276 nm are 1.3- and 1.5-times greater at the higher pH.

4. Discussion

Isomerisation of retinal was proposed as one possible mechanism to prevent the system from falling back into the ground state after light excitation and to store energy to perform chemical work [18–20]. On the other hand, charge stabilisation without isomerisation has been suggested [20,21]. A proton transfer step in the direct neighbourhood of the chromophore is considered as an essential part of the primary process of energy store [19–24]. Isomerisation is suggested to be coupled to the formation of the *J* [24] or *K* [18–20] intermediate of the bR photocycle, and the proton-transfer step should either occur in the excited state [23] during *J*–*K* transition [20,24] or at least within the life-time of the *K* intermediate.

The fast-appearing difference spectrum with negative absorption changes at 280, 400 and 430 nm and positive absorption changes from 290–375 nm is identical with the difference spectrum between 13-*cis* and all-*trans* retinal bacteriorhodopsin. Thus, in our experiments an all-*trans*–13-*cis* isomerisation of the retinal chromophore during the proton-pumping photocycle, is directly proven by optical measurements, supporting the postulates based on the results of extraction experiments [6,12,13] and Raman spectroscopy [8,9,11].

Our results show that all-*trans*–13-*cis* isomerisation is a complex process in time:

(1) The absorption peaks at 280, 400 and 430 nm disappear with the rate faster than 200 ns. The absorption peaks at 400 and 430 nm are attributed to weak π–π^* transitions of all-*trans* retinal in [25].

(2) Absorption peak at 296 nm appears partly faster than 200 ns, and partly in the range of a half-time of 0.5–0.9 μs and faster than the following reaction. The 296 nm absorption indicates a distraction of a positive charge from the direct neighbourhood of a tryptophan residue, as a charge perturbation acting over distance [26]. It cannot be a tyrosine deprotonation [16], because this fast absorption change is only ~50% of that expected for the deprotonation of one tyrosine [16]. However, it could be a proton-transfer step [27] leading to a negative charge in the neighbourhood of a tryptophan residue.

(3) The appearance of the absorption between 300

and 375 nm indicates a bending of the retinal into the 13-*cis* isomer [25].

Summarizing, we explain the complex time sequence with an initial step of bond rotation or bond twisting [20] leading to the disappearance of the typical all-*trans* absorptions at 400 and 430 nm. The process would start prior to [24] or simultaneous with [20] the formation of the *K* intermediate of the photocycle. It is followed by a charge-shift ahead of the retinal bending into the 13-*cis* conformation. Likewise, the 296 nm absorption change could be understood in terms of 2 charge shifts: one accompanying bond rotation and twisting; the other accompanying bending to the 13-*cis* isomer. Thus, isomerization is terminated within the life-time of the *K* intermediate and precedes the next stable state of the chromophore, namely L-550.

Within the life-time of the *K* intermediate there is a chromophore state in which the conformation of retinal is neither all-*trans* nor 13-*cis*, in agreement with the conclusions drawn from Raman experiments [28,29].

We found that the re-isomerization reaction back to all-*trans* retinal chromophore is strongly pH-dependent, being at higher pH 2-fold slower than the slow decay component of the M-412 intermediate. Kinetics as well as pH and isotope dependencies will be reported elsewhere.

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