# The N intermediate of bacteriorhodopsin at low temperatures:

## Stabilization and photoconversion

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In aqueous suspensions of purple membranes (pH 10.2, 0.4 M KCl) an intermediate having an absorption maximum at 570–575 nm (at −196°C) was produced by first heating the M intermediate up to −30°C and then stabilizing it by subsequent cooling to −60°C. We suggest that this species is the intermediate N (or P or R) found and characterized earlier near room temperature. Upon illumination at −196°C N is transformed into a bathochromically absorbing species $K_N$ which has an absorption maximum near 605 nm and an extinction 1.35 times that of N. This light reaction is photoreversible. The quantum yield ratio for the forward and back reaction is 0.18 ± 0.02. The maximum photo steady state concentration of $K_N$ is about 0.24. The N intermediate was also trapped in water suspensions of purple membranes at neutral pH and low salt concentration by illumination at $\lambda > 620$ nm during cooling. In addition to N another intermediate absorbing in the red (maximum at 610–620 nm) was accumulated in smaller amounts. It is not photoactive at −196°C and apparently is the O intermediate or a photoproduct of N.

Bacteriorhodopsin; N intermediate; O intermediate; Low temperature absorption spectroscopy

## 1. INTRODUCTION

In the work of Lozier et al. [1] it was proposed that the photochemical cycle of bacteriorhodopsin (bR) proceeds through a number of intermediates:

$$bR \longleftrightarrow K \longrightarrow L \longrightarrow M \longrightarrow N \longrightarrow O \longrightarrow bR$$

The N intermediate was poorly characterized and for some years its existence was uncertain.

Drachev et al. [2] found light-induced absorbance changes which occur more slowly than the disappearance of the M intermediate but synchronous with proton uptake in a suspension of purple membranes. Based on this finding they proposed a new intermediate, P, which follows M and is coupled to proton uptake by bR. According to their data, P has an absorption maximum at 560–570 nm and an extinction coefficient lower than the initial trans-bR.

Simultaneously, Dancshazy et al. [3] reported the existence of slowly converting intermediate 'R' having a maximum in the difference spectrum at 375 nm and linked to the recovery of bR after bleaching. Shkrob and Rodionov [4] also mentioned the existence of a very slowly converting intermediate in alkaline media. Comparison of difference absorption spectra of P and R relaxation reveals their similarity and suggests that they are the same intermediate [5,6]. Kouyama et al. [6] presented arguments for this intermediate being the N state introduced earlier [1]. Chernavskii et al. [7] obtained data suggesting that the N intermediate at neutral pH is in fast equilibrium with the O intermediate. Resonance Raman data of Fodor et al. [8] obtained under conditions of high salt and high pH where N has a long lifetime indicate that the chromophore in the N intermediate is in the 13-*cis*,14-s-*trans*,15-anti-configuration and that the Schiff base is protonated.

Kouyama et al. [6] suggested that N is photoactive and converts into L- and M-like intermediates after illumination. According to Drachev et al. [2] excitation of N with 532 nm light flashes did not cause the formation of a significant amount of M intermediate. The reason for this discrepancy is not clear.

The N (P,R) intermediate was previously studied at temperatures above 0°C. The present study was undertaken with the aim of stabilizing the N intermediate at low temperatures and observing its primary light reaction at −196°C. In the course of this work evidence for the stabilization of another intermediate having a red-shifted absorption maximum was also found.

## 2. MATERIALS AND METHODS

Purple membranes from *Halobacterium halobium* $R_1M_1$ were used. Absorption spectra were recorded on an SF-18 (Lomo) double-beam spectrophotometer. Spectra of water suspensions of purple membrane (plus 7–10 mM bicarbonate, pH 10.2 at 20°C, and 0.2–0.7 M KCl) frozen to −196°C were measured in a vertical-light-

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path cuvette in a small glass Dewar which was placed in the integrating sphere to reduce the contribution of light scattering.

Identification of different intermediates in the sample was based on the ability of these states to form primary photoproducts at −196°C which can be distinguished by selective excitation [9]. The amount of *trans*-bR or L present in the sample was calculated from the maximal absorption changes, $\Delta A$, associated with the formation of their primary photoproducts:

$$A = k \cdot \Delta A,$$

where $k$ is a coefficient determined previously [10].

The light source for actinic illumination was a slide projector with a 250 W tungsten-halogen lamp, condenser and absorption or interference filters. The intensity of the 510 nm actinic illumination was 2 W/m$^2$. The sample temperature was determined with a copper-constantan thermocouple and microvoltmeter to an accuracy of ± 4°C.

## 3. RESULTS AND DISCUSSION

### 3.1. *Stabilization of N intermediate at low temperature and the observation of its bathoproduct, $K_N$*

Fig. 1 shows the absorption spectrum of a water suspension of purple membrane which was light adapted at room temperature and cooled to −60°C in the dark (curve 1). Illumination at 570 nm caused transformation of the main portion of bR into M. The residual absorption with its maximum at 540 nm belongs apparently to L (curve 2). Subsequent heating of the sample to −30°C and cooling back to −60°C resulted in transformation of M into longwave absorbing form(s) (curve 3). To determine the concentration of different bR forms trapped in the sample it was cooled to liquid nitrogen temperature (Fig. 2, curve 1) and illuminated at 510 nm. Illumination at this wavelength does not excite M but produces bathoforms of species absorbing at this wavelength (curve 2). Subsequent illumination at $\lambda > 710$ nm caused back conversion of a part of the bathoproduct (curve 3). The difference spectrum of absorption changes induced by illumination at $\lambda > 710$ nm ('curve 2 minus curve 3', or $\Delta A_1$) has an isosbestic point near 592 nm which is characteristic of photoconversion of bathoform K$^t$ to *trans*-bR. Subsequent illumination at $\lambda > 640$ nm results in further conversion of the bathoproduct produced by 510 nm illumination (curve 4). The difference spectrum 'curve 3 minus curve 4' (or $\Delta A_2$, see Fig. 2b) has a maximum at 620 nm and a crossover point at 570 nm. It resembles the difference spectrum of 13-*cis*-bR to K$^c$ conversion [10].

In order to study the relaxation pathway of the intermediate, formed from warming M to −30°C, we heated the sample to −5°C, incubated it for 20 min at this temperature and cooled it again to −196°C. Subsequent 510 nm illumination at −196°C produced mainly absorption changes $\Delta A_1$ associated with the conversion of *trans*-bR into K. The amplitude of $\Delta A_2$ after heating to −5°C was at least 4 times smaller than in the sample warmed just to −30°C. From these data it follows that

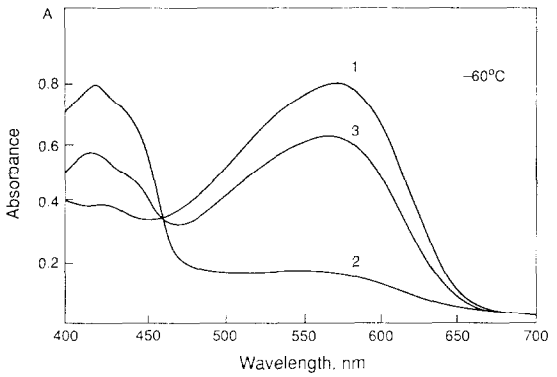


Fig. 1. Stabilization of N intermediate in water suspension of purple membrane at low temperatures: 1, absorption spectrum of light-adapted purple membrane suspension (containing 7 μM bR, 7 mM bicarbonate, pH 10.2 at 20°C and 0.4 M KCl) frozen in the dark to −60°C; 2, illuminated 5 min at 570 nm; 3, heated to −30°C and cooled again to −60°C (rate of heating ≈ 3°C/min).

the intermediate, formed at −30°C from M, converts at −5°C to bR.

Subtracting from curve 1 the absorption spectrum of *trans*-bR multiplied by an appropriate weighting factor, the spectrum of the additional species was obtained

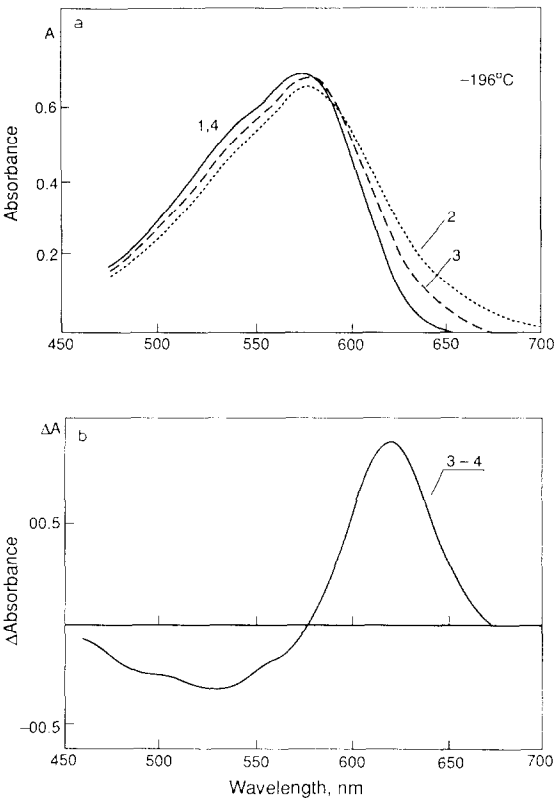


Fig. 2. Primary light reaction N ⇌ KN at −196°C. a: 1, absorption spectrum of purple membranes containing *trans*-bR, M and N (the same as 3 in Fig. 1 but cooled in the dark to −196°C); 2, after 10 min illumination at 510 nm; 3, subsequent illumination at $\lambda > 710$ nm (up to saturation); 4, after illumination at $\lambda > 640$ nm (practically coincide with 1). b: Difference spectra '2 minus 3' and '3 minus 4'.

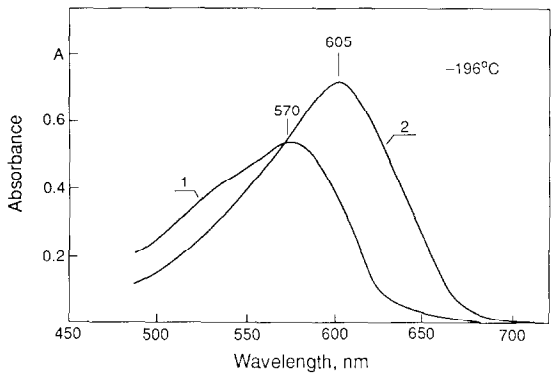


Fig. 3. Absorption spectra of intermediate N (1) and its bathoform $K_N$ (2) in water suspension of purple membrane frozen to −196°C.

(Fig. 3, curve 1). It has a maximum at 568–572 nm, 4–8 nm longer than the maximum of the 13-*cis*-bR of dark-adapted purple membrane at liquid nitrogen temperature [9]. On cooling from 20°C to −196°C the absorption maxima of *trans*-bR and 13-*cis*-bR shift to longer wavelengths by 10 and 12 nm, respectively. Supposing that the same shift occurs in the spectrum of the species we observed, one may expect the maximum for the absorption spectrum shown in Fig. 3 to be at about 560 nm at room temperature, which is characteristic for the N(P,R) intermediate [5,6]. From these observations we conclude that the species formed in alkaline suspension of purple membrane in the course of M relaxation is the N(P,R) intermediate, which we will refer to as N. The absorption change $\Delta A_2$ shown in Fig. 2b we attribute to the primary light reaction of N, which results in formation of a new state, the bathoproduct $K_N$.

The amount of N trapped in the sample by heating M to −30°C and cooling to −60°C (the whole procedure takes about 10 min) usually did not exceed 30% of total bR in the sample. It was possible to accumulate this intermediate in greater amount (up to 70%) by illumination of light-adapted water suspension of purple membrane (pH 10, 0.2 M KCl) at $\lambda > 620$ nm during

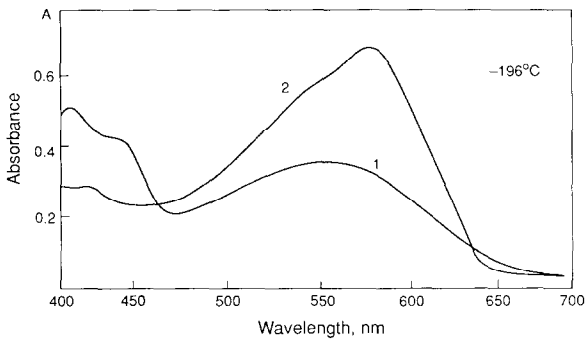


Fig. 4. Absorption spectra of: 1, suspension of light-adapted purple membrane in distilled water illuminated at $\lambda > 600$ nm during cooling to −80°C and then cooled in the dark to −196°C; 2, absorption spectrum of light-adapted purple membrane cooled in the dark (given for comparison).

cooling. By this procedure it was also possible to trap N in water-glycerol suspensions of purple membrane.

### 3.2. *Absorption spectrum and photostationary fraction of $K_N$*

To determine the absorption spectrum of $K_N$ we used the method of Fisher [11]. The relative amplitudes of the absorption changes associated with the $K_N$ formation were measured for 510, 530, 572 and 600 nm actinic illumination. The relative values of $\Delta A_2$ at 620 nm were obtained to be 1.0; 0.85; 0.70; 0.35. Based on these data the photo steady state concentration of $K_N$ and its absorption spectrum was calculated (Fig. 3). For $K_N$ absorption has a maximum at 605 nm and its extinction is 1.37 times higher than that of N. The photo steady state fraction of $K_N$ produced by 510 nm illumination was found to be 0.24 (of initial amount of N). The quantum yield ratio for the forward and back reactions $N \rightleftharpoons K_N$ is 0.18 ± 0.02, significantly lower than was found for the photoreactions of *trans*-bR (0.5 ± 0.1 [1,12]) and 13-*cis*-bR (0.4 [9]). Bathoform $K_N$ differs greatly from the bathoproduct of L [9], indicating that N and L are quite different states.

### 3.3. *Accumulation and stabilization of N and O-like intermediates in water suspensions at neutral pH and low ionic strength*

To see to what extent the accumulation of N at low temperature depends on pH and ionic strength we used a suspension of purple membrane in distilled water. Irradiation at $\lambda > 600$ nm during cooling results in a broad spectrum peaking at 570 nm and having significant absorption up to 700 nm at −196°C (see Fig. 4). Similar spectra were reported previously [13,14]. Illumination of this sample at >650 nm did not induce any absorption changes. From this we conclude that the red absorbing form is not the bathoform of any known intermediate, all of which are photoconvertible by red light. Its long-wavelength absorption tail suggests that it may be the O intermediate or a spectrally similar species produced by illumination of N. Illumination of this sample at 510 nm (at −196°C) results in formation of a bathoform with a difference spectrum which is characteristic of the $N \longrightarrow K_N$ transition, indicating that N is also present in these samples. No additional photoproduct was found for the O-like form, suggesting that it is not photoactive at −196°C. Buffering at pH 10.2 and addition of 0.7 M KCl inhibited accumulation of O.

Subtracting the corresponding amount of N, L and *trans*-bR from the mixture we find that the absorption maximum of the O-like intermediate is at 600–610 nm.

## CONCLUSION

In a frozen water suspension of purple membrane containing 0.2 M KCl, pH 10.2, the N intermediate was

produced by heating M from −60°C to −30°C and trapped by cooling to −196°C. This supports the scheme according to which N is in the same cycle as M. Upon irradiation N turns into bathoproduct $K_N$ which is similar to bathoform of 13-*cis*-bR and differs from the bathoform of L. It was possible to trap N at normal pH along with O-like intermediate with maximum at 600–610 nm.

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