N-like intermediate in the photocycle of the acid purple form of bacteriorhodopsin

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Abstract The photocycle of the acid-purple form of bacteriorhodopsin (BR) was studied by multiwavelength absorption kinetic measurements. The data were evaluated using a new method based on the differences in the kinetics of the absorption changes at different wavelengths [Tokaji, Zs. (1995) FEBS Lett. 357, 156–160]. The evaluation indicates that, in contrast to the previous suggestion, the photocycle of the acid purple BR contains an intermediate that, in its spectral and kinetic properties, resembles the N intermediate of the alkaline BR photocycle.

Key words: Kinetics; Kinetic difference; Bacteriorhodopsin intermediate; N-form

1. Introduction

Bacteriorhodopsin (BR) in *Halobacterium salinarium* at physiological pH values acts as a light-driven proton pump. With decreasing pH at about pH 3 the originally purple pigment turns blue (BR_{blue}). At even lower pH values the color of BR again becomes purple in the presence of Cl^- or some other anions (BR_{ap}).

The photocycle of BR_{ap} has been studied earlier [1,2]. It has been characterized in terms of photocycle intermediates in the study of Váró and Lanyi [2]. Their model contains only one long-lived intermediate termed O, and another, unidentified form absorbing below 500 nm and appearing with a low (15%) relative weight.

BP_{ap} is suggested to be a light-driven chloride pump [3,4]. Therefore, it was considered important to study its photocycle in more detail. From the absorbance changes we could identify an N-like intermediate in the photocycle of BR_{ap}. As control the absorbance changes were also recorded for BR_{blue}.

2. Materials and methods

The absorption kinetic measuring system and the preparation of BR incorporated into polyacrylamide gel were the same as described previously [5]. For excitation an excimer-laser-pumped dye laser containing coumarin 307 ($\lambda_e = 505$ nm, 10 ns pulse duration) was used [6].

The samples in the acid purple state contained 0.5 M Cl^- at pH 0.5. Their characteristic absorption spectra compared to that of wild-type BR (pH 7, 1 M NaCl 30 mM universal buffer [5]) and BR_{blue} (0.5 M SO_4^- at pH 0.5) at 30°C are shown in Fig. 1, indicating that the conversion into the acid purple state was close to complete (contamination with BR_{blue} less than 15% estimated from the absorption spectra at 650 and 670 nm).

The absorption changes following the excitation of BR_{ap} were recorded within the interval 324–704 nm with steps of 8 nm.

Derivation of the kinetics of the intermediates was carried out using a method based on transformations and subtractions of the signals [6] with the assumptions described below.

3. Results and discussion

The difference spectra appearing after the excitation of BR_{ap} at different time delays are shown in Fig. 2. They contain a negative (bleaching) band around 550 nm, and two positive ones around 650 and 480 nm. The positive band around 650 nm is assigned to an O-like intermediate of BR_{ap}. Its kinetics can be determined at those wavelengths where only this form contributes significantly to the absorption changes. For this, the range above 680 nm is suitable (see [2]). The kinetics of O determined this way are shown in Fig. 3 (curve O). The features of these kinetics are in good agreement with those determined in [2]. The O-like form accumulates very rapidly, decays through several time decades, and becomes negligible ($< 1\%$) at about 10 ms.

If only this intermediate were present in the photocycle of BR_{ap} in a considerable amount, the kinetics of the decay of the bleaching at about 550 nm (Fig. 3, curve B) would practically coincide with it. The difference in shape of the two kinetic curves provides the concentration change of an additional intermediate in arbitrary units (Fig. 3, curve D), if we accept that at 10 μs only the O-like intermediate (see data in [2]) is present. As shown in Fig. 3, the difference is very large (and would be even larger if the amount of O were overesti-

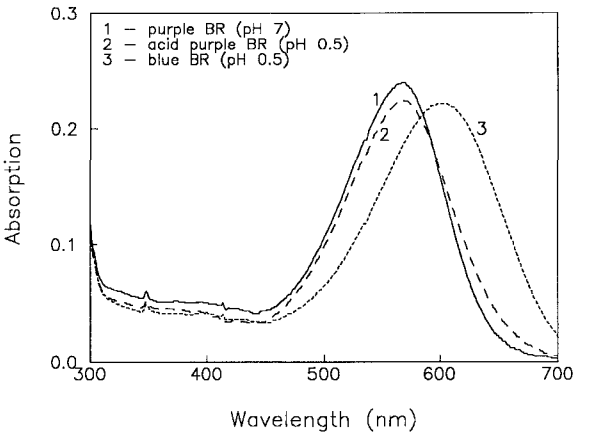

Fig. 1. Absorption spectra of BR at pH 7 and the acid purple and blue forms of bacteriorhodopsin (BR_{ap} and BR_{blue}). Conditions: pH 7, 1 M NaCl, 30 mM universal buffer; 0.5 M Cl^-, pH 0.5; 0.5 M SO_4^-, pH 0.5; 30°C.

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Abbreviations: BR, bacteriorhodopsin; BR_{ap}, BR_{blue}, acid purple and blue forms of BR; O, N, intermediates of the photocycle of BR_{ap}

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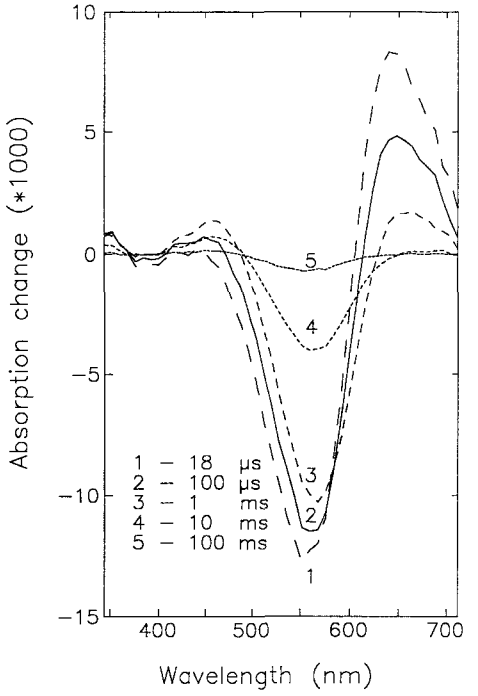

Fig. 2. Difference spectra at different times after excitation of BR_{ap}.

mated at 10 µs). Its peak amplitude is about 60% of that of the photocycling molecules, indicating that not only is a marginal species accumulated because of, e.g. the presence of residual BR_{blue}, but also another photocycle intermediate. Another argument supporting the above conclusion is that the rise of this intermediate practically coincides with the decay of O (note that there is no marked concentration change of the ground state during this process), while the photocycle of BR_{blue} lacks an 'O' form, and its blue-shifted intermediate appears faster than at 100 µs [2].

From Fig. 2 it is also obvious that at early times after the excitation of BR_{ap} an isosbestic point exists at about 570 nm (and this wavelength coincides with the peak of the absorption spectrum of the ground state). The kinetics at this wavelength are expected to be close to the real kinetics of the ground-state recovery. (This expectation is true if two intermediates exist in the sample and one of them converts into the other.) This idea is confirmed by double excitation experiments that measure the fraction of cycling molecules (for details of the method see e.g. [7]). The kinetics at the isosbestic point (curve G in Fig. 3) coincide well with the ground-state recovery measured by the bleaching at 550 nm caused by a second flash (squares in Fig. 3).

According to the result of the kinetic evaluation described above, the difference spectra of O and that of the new intermediate should be very similar to curves 1 and 5 in Fig. 2, corresponding to the earliest and latest times, respectively. Taking into account the analogies between the photocycle of BR_{ap} and that of normal BR or HR, we denote the new intermediate as 'N'. The kinetics of the O and N intermediates and the recovery of the ground state are shown in Fig. 3 by solid lines (curves O, N and G, respectively).

The kinetics of the intermediates were studied by fitting of exponential components. It transpired that 5 components are necessary, the lifetimes of which are approx. 30 µs, 200 µs, 3 ms, 25 ms and 300 ms. The first two components appear mainly in the decay of O and in the accumulation of N (with opposite signs). The third component appears in the decay of O and in the recovery of the ground state. The 3 ms component in the decay of O and the bleaching signal indicate that O may partially decay directly to the ground state. The two slowest components are negligible in the O kinetics, appearing only in the decay of N and the recovery of the ground state. The lifetimes show a good correlation with those found in the components of the photoelectric signal measured under comparable conditions on BR_{ap} [4,8]. The only exception is the slowest component (300 ms) having no observable counterpart in the electric signal, probably because of the low signal-to-noise ratio.

Although further kinetic investigations are necessary in or-

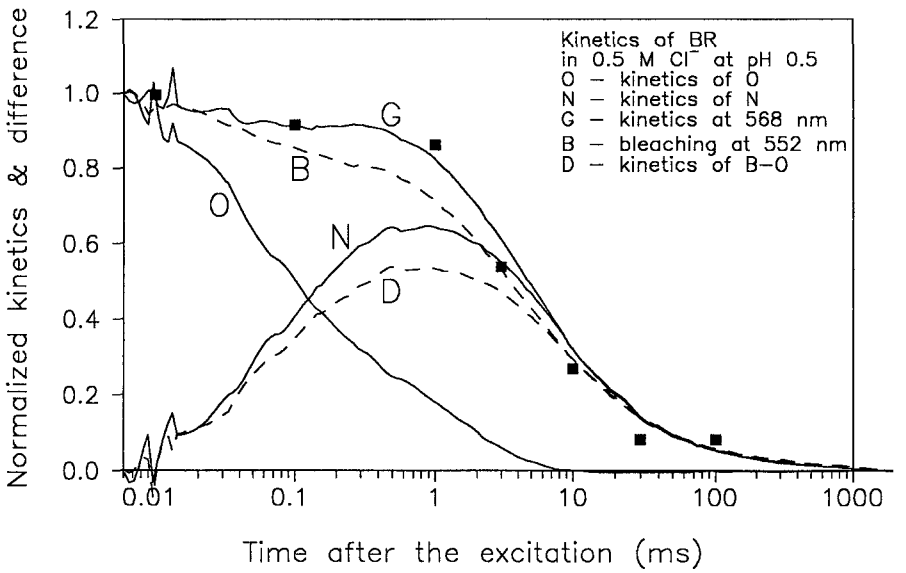

Fig. 3. Kinetics of the O intermediate measured at 680 nm (O). Kinetics of the absorption changes at the wavelength of the bleaching maximum (B) and at the isosbestic point (G). Curves D and N are the differences B–O, and G–O, respectively. The squares show the fractional concentration of the photocycling molecules measured by double excitation experiments (measuring wavelength: 550 nm).

der to provide the most probable model for the photocycle of BR_{ap}, attention should be drawn to its similarities to the photocycle of the chloride-pumping pharaeonis halorhodopsin [9].

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