

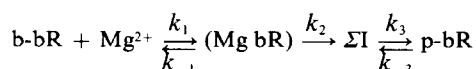
# Transition kinetics of the conversion of blue to purple bacteriorhodopsin upon magnesium binding

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Magnesium binding to cation-depleted blue bacteriorhodopsin (b-bR) was studied spectrophotometrically as well as by following stopped-flow kinetics. There exist three kinetically different steps in the binding process, yielding purple bacteriorhodopsin (p-bR). Since only the first step is dependent on the concentration of the reactants, the reaction scheme



can be proposed as the simplest model, with MgbR being the first intermediate and  $\Sigma \text{I}$  denoting a set of successive intermediates. According to this model  $k_1$ ,  $k_{-1}$  and  $k_2$  are calculated to be  $2.8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ ,  $5.0 \times 10 \text{ s}^{-1}$  and  $1 \times 10^{-2} \text{ s}^{-1}$ , respectively.

<i>Blue bacteriorhodopsin</i>	<i>Purple bacteriorhodopsin</i>	<i>Magnesium binding</i>	<i>Stopped-flow method</i>
	<i>Kinetic amplitude spectrum</i>	<i>Rate constant</i>	

## 1. INTRODUCTION

It is known that the colour of bacteriorhodopsin changes from purple to blue, when cations are depleted [1–4]. Bound cations are important not only for the colour but also for the function of bacteriorhodopsin. Blue bacteriorhodopsin does not pump protons [2]. Salt titration of blue bacteriorhodopsin suggests that there are two steps in the transition from blue to purple bacteriorhodopsin and two purple states I and II with different oscillator strengths, only the latter being the 'native' purple bacteriorhodopsin [2]. The electric field-induced absorbance change of purple bacteriorhodopsin in dried films also indicated the existence of at least one intermediate

between blue and purple bacteriorhodopsins [5]. A preliminary study of binding of monovalent, divalent and trivalent cations showed that  $\text{Mg}^{2+}$  as well as manganese ions are bound to bacteriorhodopsin with a  $K_d$  of approx.  $10^{-5} \text{ M}$  [2], and atomic absorption spectroscopy showed that either one of the two divalent cations is bound to the isolated native purple membrane [2,3]. Here, we report our studies on magnesium binding to blue bacteriorhodopsin, resulting in the formation of purple bacteriorhodopsin (state I).

## 2. EXPERIMENTAL

### 2.1. Materials

Purple membranes were isolated from *Halobacterium halobium* S9 strain according to Oesterhelt and Stoebenius [6]. Cation-depleted blue purple membranes were prepared by the cation-exchange method [1]. The concentration of

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blue bacteriorhodopsin was estimated on the basis of  $\epsilon_{605} = 52000$  [2]. Quartz-distilled water and polystyrol vessels were used for preparing blue membrane suspensions and  $MgCl_2$  solutions.  $MgCl_2$  (analytical grade) was purchased from Merck and used without further purification.

## 2.2. Rapid-mixing measurements

The stopped-flow apparatus described in [7] was used with the following modifications. (i) Silicon-coated Hamilton glass syringes were used as drive syringes to avoid the injection of cations from the glass wall. (ii) For temperature regulation the whole mixing circuit was immersed in a water bath where water was circulated by a KT40S Ultra-Kryostat (Colora Messtechnik, Lorch/Württ.). All experiments were carried out at 20°C. (iii) A trigger signal for a transient recorder (Nicolet 1090A, Nicolet, Madison, WI) was generated by the electric contact of the stop adjusting plate with the stop plate so that the triggering time was almost the same as the time when the flow stopped. The dead time was estimated by mixing of ascorbate and 2,6-dichlorophenolindophenol. The reaction started 3.5 ms before the trigger.

## 2.3. Absorbance measurements

Absorption spectra were measured with a Lambda spectrophotometer (9 UV/VIS/NIR, Perkin Elmer, England). The wavelength range 800–400 nm was scanned at 120 nm/min.

## 2.4. pH measurements

The pH of the blue purple membrane suspension,  $MgCl_2$  solution and the mixture was measured with a GK2321C pH electrode (Radiometer, Copenhagen) connected with pH meter 26 (Radiometer, Copenhagen).

Note that suspensions or solutions which were used for pH measurements were not used further because of electrolyte contamination from the pH electrode.

## 3. RESULTS

The time course of the reaction between blue bacteriorhodopsin and  $MgCl_2$  is given in the stopped-flow traces of fig.1. At 540 nm (fig.1a), a rapid increase in absorbance within a 100 ms range is observed, followed by a slower increase within

minutes and hours. Although a slight change in absorbance was observed during the following days, a baseline at 4 h after mixing was taken. At 630 nm (fig.1c) the absorbance decreased after mixing. Similar to the signal at 540 nm, 3 relaxations were observed. At 580 nm (fig.1b) the absorbance at first decreased across the baseline, then crossed the baseline again in an upward deflection and finally slowly settled right above the baseline. Although the absorption at 580 nm was originally considered as an isosbestic point, the latter trace indicates the existence of 3 different isosbestic points. These observations show that there are 3 kinetically different steps. However, it should be noted that the curves cannot be analyzed by a simple sum of 3 exponential components, suggesting that there are more intermediates involved.

To identify the reaction components we studied the kinetic amplitude spectra. The wavelength dependence of the stopped-flow signals at 150 ms, 5 min and 4 h after mixing is shown in fig.2. The isosbestic points for fast, middle and slow process were 577, 580 and 578 nm, respectively. The minimum in the difference spectra was always at 630 nm, while the maximum shifted slowly from 530 to 540 nm.

For comparison of the kinetic amplitude spectra the static difference spectra measured by the spectrophotometer 5 min after mixing of the blue bacteriorhodopsin with various concentrations of  $MgCl_2$  are shown in fig.3. For these measurements, scanning with the spectrophotometer was started exactly 4 min after mixing at a scan speed of 120 nm/min. Since  $\Delta A$  in the time range 4–7 min was less than 2% of the total change as shown in fig.1, the time factor due to the scanning is negligible. With increasing magnesium concentrations, both the maximum and isosbestic points shifted to the longer wavelength and the amplitudes at both the maximum and minimum increased. These curves obtained at different magnesium concentrations are nearly identical with those in fig.2 where  $\Delta A$  was plotted at different times and a fixed magnesium concentration, indicating that the advance of the reaction is concentration dependent. As an additional result, the shape of the minimum peak is sharper at the higher magnesium concentration. This may correspond to the transition of purple bacteriorhodopsin from state I to II observed in titration [2].

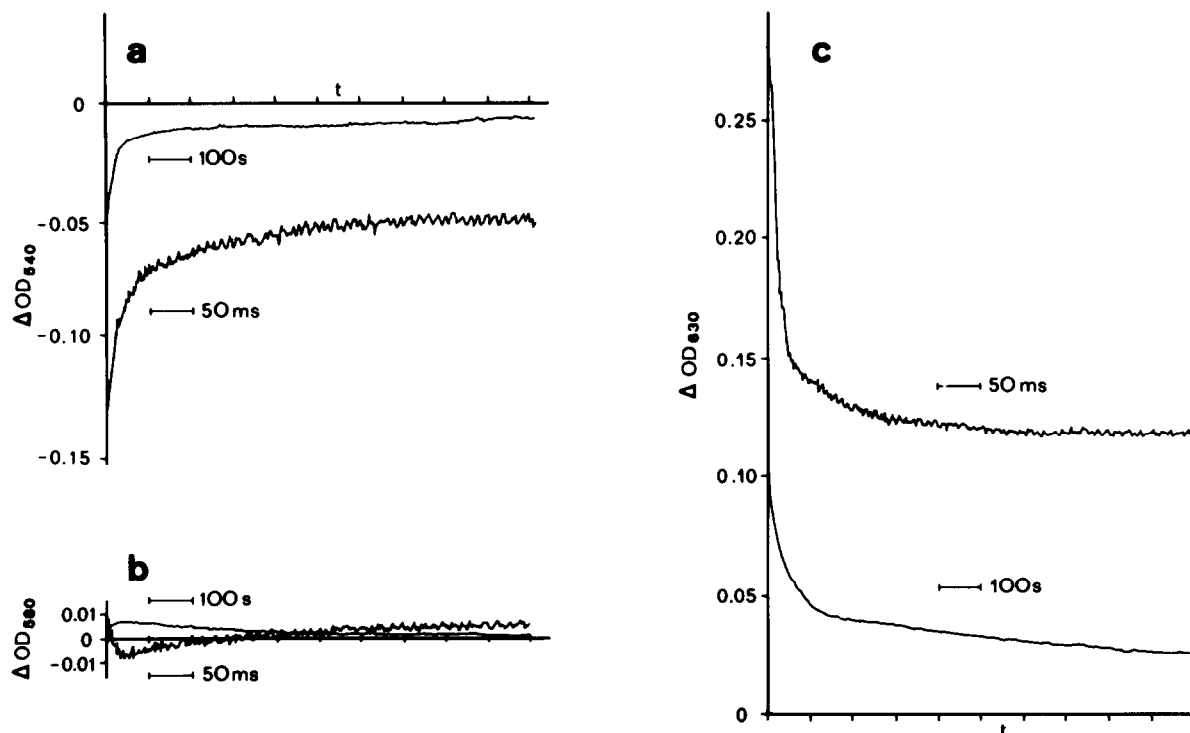


Fig.1. Typical stopped-flow signals for mixing of blue bacteriorhodopsin and  $MgCl_2$  at 540 nm (a), 580 nm (b) and 630 nm (c);  $[b\text{-bR}] = 1.73 \times 10^{-5}$  M;  $[Mg^{2+}] = 1 \times 10^{-3}$  M.

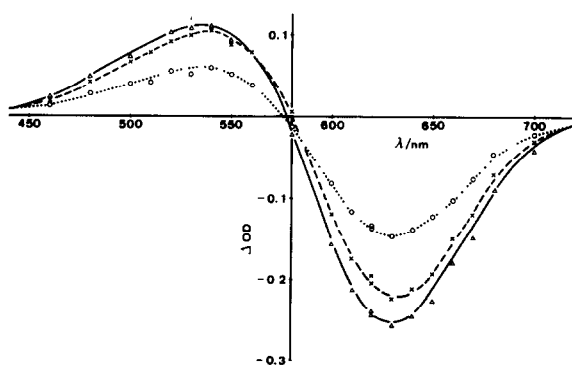


Fig.2. Wavelength dependence of the stopped-flow signals at 150 ms (O), 5 min (X) and 4 h (Δ) after mixing of blue bacteriorhodopsin and  $MgCl_2$ ;  $[b\text{-bR}] = 1.73 \times 10^{-5}$  M;  $[Mg^{2+}] = 1 \times 10^{-3}$  M.

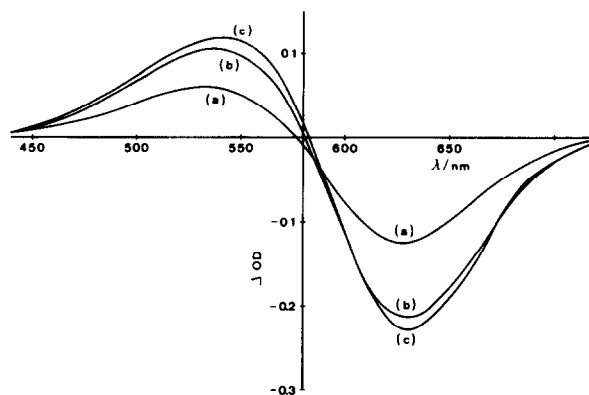


Fig.3. Difference spectra measured with the spectrophotometer at  $t = 5$  min after mixing of blue bacteriorhodopsin  $[b\text{-bR}] = 1 \times 10^{-5}$  M with various concentrations of  $MgCl_2$ ;  $[Mg^{2+}] =$  (a) 0.03 mM, (b) 0.1 mM, (c) 2 mM.

The absorbance changes at 150 ms, 5 min and 4 h after mixing were followed at different concentrations of  $MgCl_2$  and bacteriorhodopsin, as shown in fig.4. The absorbance changes are nor-

malized by the concentration of bacteriorhodopsin. Curves are drawn only for constant bacteriorhodopsin (bR) concentration ( $[b\text{-bR}] = 1 \times 10^{-5}$  M). As can be seen at both 540 and

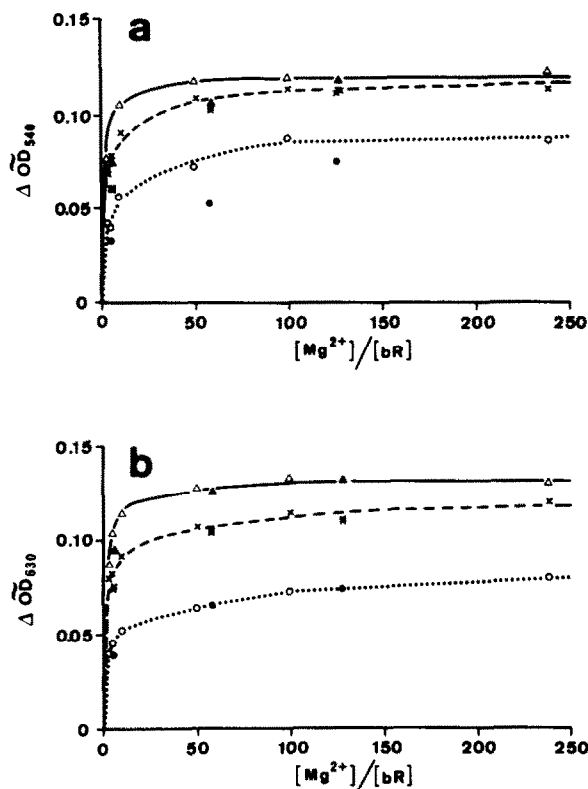


Fig.4. Dependence of  $\Delta A$  on the relative magnesium concentration to blue bacteriorhodopsin at  $t = 150$  ms ( $\circ, \bullet$ ), 5 min ( $\Delta, \blacktriangle$ ) and 4 h ( $\times, \blacktimes$ ); the first and second symbols in parentheses correspond to  $[b-bR] = 1 \times 10^{-5}$  M and the higher concentrations ( $1.5 \times 10^{-5}$  and  $3 \times 10^{-5}$  M), respectively;  $[Mg^{2+}]$  was changed in the range from  $3 \times 10^{-5}$  to  $2 \times 10^{-3}$  M; (a)  $\lambda = 540$  nm, (b)  $\lambda = 630$  nm.

630 nm, the  $\Delta A$  at 4 h is saturated at the relative magnesium concentration range 10–250, suggesting that only the first step of the reaction course depends on magnesium concentration. If the bacteriorhodopsin concentration is higher, a deviation from the curves is observed, especially at 540 nm. This may be due to the pH which is lower by 0.3 units at  $[b-bR] = 3 \times 10^{-5}$  M compared to that at  $1 \times 10^{-5}$  M. As a control, the pH of blue bacteriorhodopsin suspensions was adjusted to 4.0 and 4.3 by adding  $H_3PO_4$ . The suspension of pH 4.0 shows a smaller  $\Delta A$  at 540 nm after mixing with  $NaH_2PO_4$  than that of pH 4.3, similar to the results shown in fig.4.

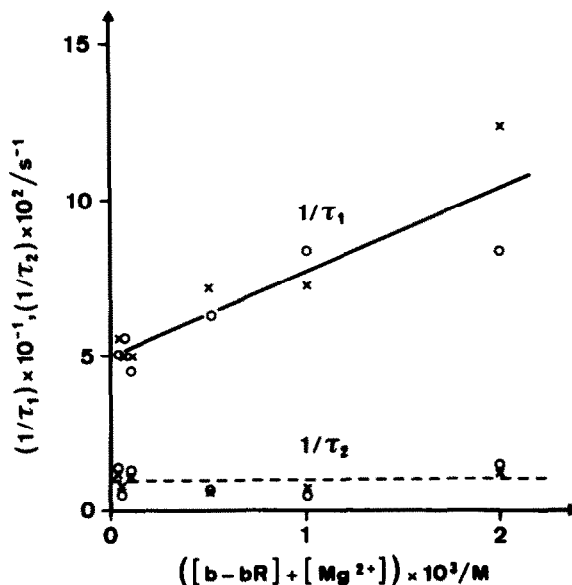
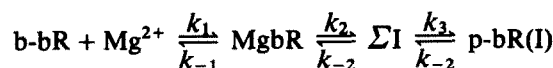


Fig.5. Concentration dependence of  $\tau_1^{-1}$  (a) and  $\tau_2^{-1}$  (b) at 540 nm ( $\circ$ ) and 630 nm ( $\times$ ).

#### 4. DISCUSSION

Our results are in accord with the global results of Kimura et al. [1] who investigated calcium binding to blue bacteriorhodopsin and in addition give us information for a reaction model. Since there are 3 kinetically different reactions in the magnesium-binding process, and since only the first step is dependent on concentrations, the following reaction scheme is proposed as the simplest model:



where b-bR and p-bR(I) denote blue and purple bacteriorhodopsin (state I), respectively, MgbR is the first intermediate of an  $Mg^{2+}$ -bR complex and  $\Sigma I$  is the sum of the following intermediates. Note that the configuration of retinal in blue bacteriorhodopsin is a mixture of 50% all-*trans*- and 50% 13-*cis*-retinal [8]. At present, it is not clear which configuration of blue bacteriorhodopsin (or both) reacts with  $Mg^{2+}$ , although a rate-determining step is not seen.

The difference in the time scale for the 3 steps allows us to assume  $k'_1, k_{-1} \gg k_2, k_{-2} \gg k'_3, k'_{-3}$

where

$$k'_1 = k_1([Mg^{2+}] + [b-bR])$$

and

$$k'_{-3} = k_{-3}[p-bR(I)].$$

Then, the relaxation times can be described by the rate constants [9]:

$$1/\tau_1 = k'_1 + k_{-1} \quad (1)$$

$$1/\tau_2 = k_2 \cdot \frac{K'_1}{1 + K'_1} + k_{-2} \quad (2)$$

$$1/\tau_3 = k_3 \cdot \frac{K'_1 K_2}{1 + K'_1 + K'_1 K_2} + k'_{-3} \quad (3)$$

where

$$K'_1 = K_1/([Mg^{2+}] + [b-bR])$$

and

$$K'_3 = K_3/[p-bR(I)].$$

$1/\tau_1$  and  $1/\tau_2$  are plotted vs  $([Mg^{2+}] + [b-bR])$  in fig.5.  $1/\tau_1$  is linearly dependent on  $([Mg^{2+}] + [b-bR])$ . The rate constants  $k_1$  and  $k_{-1}$  can then be estimated to be  $2.8 \times 10^4 M^{-1} \cdot s^{-1}$  and  $5.0 \times 10 s^{-1}$  from the slope and intercept, respectively. Since  $\tau_2$  is independent of the initial magnesium concentration in the range  $3 \times 10^{-5}$ – $2 \times 10^{-3}$  M, either  $k_2$  or  $k_{-2}$  can be set negligible according to eqn 2. Here, the latter case should apply, because the reaction continues further. Then,

$$1/\tau_2 = k_2 = 1 \times 10^{-2} s^{-1} \quad (4)$$

$$1/\tau_3 = k_3 + k_{-3}. \quad (5)$$

Because  $\tau_3$  depends on a baseline which is not known exactly, changing slightly even 20 h after mixing, it is not useful to obtain a value for  $\tau_3$ .

The figures obtained here may be compared to the  $K_d$  values obtained by static titration [2] and are of the order of  $10^{-5}$  for overall magnesium binding.

Our results indicate a multistep transition following the direction of magnesium with blue bacteriorhodopsin leading to the formation of purple bacteriorhodopsin, which is, however, different in oscillator strength from the native purple bacteriorhodopsin. If  $Mg^{2+}$  is added to cation-depleted blue bacteriorhodopsin, a complex of magnesium-bacteriorhodopsin is formed in the time scale of  $\approx 100$  ms. This complex changes in conformation, yielding purple bacteriorhodopsin through some intermediates, a process being accompanied by deprotonation. According to [1], 3 protons are released per calcium binding. Detailed kinetics of the proton release are under investigation.

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