M intermediate accumulation analysis of bacteriorhodopsin reconstituted with three partial peptides

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Abstract. Reconstituted bacteriorhodopsin containing helices C-G, A, and B has unique thermal structural change and photoreaction. Time evolution of photo reaction of bacteriorhodopsin reconstituted with three partial peptides in glycerol mixed purple membrane at around 196 K under irradiation by red light was analyzed by a proposed model. The absorption change of 560 nm and 405 nm with irradiation times was analyzed for the purpose of determining reaction rates of photo reaction of reconstituted bacteriorhodopsin and its product M intermediate. In this study it is shown that reaction rates of conversion from bacteriorhodopsin reconstituted with three partial peptides to the M intermediate can be explained by a set of linear differential equations. Although the rate of accumulation of M intermediate is as forty times smaller than that of the native bacteriorhodopsin, the accumulation was achieved, in spite of absence of peptide bond between peptides.

1. Introduction

Purple membrane is the specific membrane found in Halobacterium salinarium and related species 1. This membrane contains trimeric transmembrane protein bacteriorhodopsin $bR$ in a two dimensional crystallographical fashion. Protein $bR$ utilizes the energy of light to build up a proton electrochemical potential for ATP synthesis 2-4. So a lot of effort has been made to clear up the molecular mechanism of its unique function. Several ultrafast absorption kinetic experiments revealed the primary events of the photoreaction of $bR$, that through several excited states $bR$ changes from a light-adapted state to $K$ intermediate within five pico seconds 5-7 at room temperature. Regarding the reaction after the primary event, it was proposed recently that $K$
intermediate decays and changes into the quasi equilibrium state between $L$ intermediate and $M$ intermediate within seven micro seconds, and takes an additional 130 micro seconds to change entirely into $M$ intermediate \(^8\). And recent research on reverse photo transition $K$ intermediate to $bR$ \(^{18}\), was reported. In previous report, it was shown that a set of reaction rates can explain the time evolution of photoreaction from $bR$ to $M$ intermediate \(^9\). By the way, protein $bR$ can be reconstituted from three partial independent peptides that contain helices C-G, A, and B, respectively, \(^{10}\) or that helices A-E, F, and G, respectively, \(^{20}\) in spite of the absence of peptide bond \(^{10}\). Although the first type of reconstituted $bR$ ($recbR$), containing helices C-G, A, and B, is known that A-B peptide interaction does not influence on the stability and photo reaction \(^{19,20}\), it yields thermally, in lower temperature condition, a reversible different structural state ($P_{470}$) that has the absorption maximum at 470 nm, as is shown in the figure 1. A unique $M_{365}$ intermediate yielded from $P_{470}$ can transit directly to $bR_{560}$ by light irradiation. This phenomenon is noticeable one of the important property of the first type of $recbR$ containing helices C-G, A, and B, and the reason why this type is selected as the object of this study. However, it has not been investigated enough on the accumulation process of its $M$ intermediate. In this study, under irradiation of red light, time evolution of photoreaction from native-like state of $recbR$ that have the maximum wavelength of absorption at 560 nm to its $M$ intermediate $M_{405}$ was firstly and intensively analyzed.

![Energy diagram of recbR](image)

Fig 1 Energy diagram of recbR. The recbR shows three kinds of thermally stabilized structures, $P_{470}$, $P_{560}$, and $P_{380}$ that were identified by absorption maxima.
2. Experimental section

2.1. Materials and Method

2.1.1. Preparation of peptide of bacteriorhodopsin contain helices C-G, peptides of alpha-helix A and peptides of alpha-helix B

The purple membrane fragments were purified by sucrose step gradient methods reported by Becher and Cassim \(^{11}\). Then the peptides of the helices C-G of \(bR\) was prepared by method of Popot \(^{12}\). The peptides of alpha-helix A and B of \(bR\) which were chemically synthesized respectively were assembled with the helices C-G of \(bR\) in a dialysis process as is shown in the figure 2.

![Figure 2 Procedure of preparation of recbR](image)

Fig 2 Procedure of preparation of recbR. The reebR. Two synthetic peptide provide helix A and B. An enzymatically cleavage peptide provides helices C-G, respectively. After assembling them, spectroscopic measurement was conducted at the temperature 196 K.

2.1.2. Low temperature spectroscopy

The sample for low temperature spectroscopy \(^{13-15}\) contains 75% glycerol to prevent micro-crystallization of ice during cooling. For this preparation, the protein sample was pelleted at 35000 g for 60 min. The volume of the resultant protein pellet was measured with a pipette, and 3
times the volume of water of glycerol was added and used. The absorbance of about 560 nm was in the range of 0.1-0.8. A glass container was specially constructed to fit in a UV1200 spectrophotometer (Shimadzu, Kyoto). The spectrophotometer was controlled by a personal computer (Think Centre, IBM). A liquid acetone and dried ice (CO₂) was used as a coolant for maintaining the temperature at 196K. Temperature was monitored with a copper-constantan thermocouple. A 500W Xe lamp (Ushio, Tokyo) was used as an actinic light source. The wavelength of the actinic light was selected with interference and/or cut-off filters (Toshiba, Tokyo).

The sample was sealed in an optical cell composed of two quartz plates (1mm thickness) separated by a rubber o-ring (1 mm thickness). The cell was inserted into a copper cell holder, which was directly attached to the bottom of the container. The sample was illuminated with intense light (λ ≥ 600nm) for at least 30 min just before cooling in order to insure that it was fully light-adapted.

2.2. Characterization

2.2.1. Reaction scheme

Primary photo-reactions of recbR sequentially is estimated to occur like as that of native bR, and can be represented by the following equations:

\[ \text{recbR}_{560} + h\nu \rightarrow \text{recbR}^*_{(\text{trans})} \quad (1) \]
\[ \text{recbR}^*_{(\text{trans})} \rightarrow \text{recbR}^*_{(\text{cis})} \quad (2) \]
\[ \text{recbR}_{(\text{cis})} \rightarrow J \quad (3) \]
\[ J \rightarrow K \quad (4) \]

where J, K are intermediates and the indices indicate the absorption maxima of the corresponding forms and the asterisk denotes exited states. These events also occur reversely in photo equilibrium. So it is simplified as follows in terms of only recbR and K:
Although this state transition in room temperature is usually known as irreversible one as above, one recent study\textsuperscript{18} reported the reverse photo transition occurs. So it is also described in the photo equilibrium state as following.

\[ \text{recbR}_{560} \leftrightarrow K \]  

(5)

In the second stage, \( K \) changes to intermediate \( M_{405} \) thermally.

\[ K \rightarrow L \rightarrow M_{405} \]  

(6)

These two stages can be summarized as follows:

\[ \text{bR}_{560} \rightarrow K \rightarrow M_{405} \]  

(7)

At room temperature after the above reactions, the protein reverses back to \( \text{bR}_{560} \) through intermediates \( O \) and \( N \), as follows:

\[ M_{405} \rightarrow N \rightarrow O \rightarrow \text{bR}_{560} \]  

(8)

At the temperature around 196 K, reactions described by (8) occur at a very small rate, and the intermediate \( M_{405} \) can be accumulated. To understand the time development of \( M \) intermediate accumulation, three reaction rates \( u_1, u_2, u_3 \) can be defined for following the reactions respectively:

\[ (\text{recbR}, \text{recbR}, \text{recbR}) \xrightarrow{u_1} (\text{recbR}, \text{recbR}, M) \]

\[ (\text{recbR}, \text{recbR}, M) \xrightarrow{u_2} (\text{recbR}, M, M) \]

\[ (\text{recbR}, M, M) \xrightarrow{u_3} (M, M, M) \]

where a bracket ( ) indicates one trimeric unit. Four kinds of trimer units \( (\text{recbR}, \text{recbR} \text{recbR}), (\text{recbR}, \text{recbR} M), (\text{recbR}, M M), (M, M M) \), can be expressed as \( M_0, M_1, M_2, M_3 \) respectively.
2.2.2. Differential equations

In order to analyze the reaction rates of conversion from $bR_{560}$ to $M_{412}$ a set of linear differential equations was divided as written in (eq.9-12).

\[
\frac{dM_0(t)}{dt} = -u_1 M_0(t) \quad (9) \\
\frac{dM_1(t)}{dt} = u_1 M_0(t) - u_2 M_1(t) \quad (10) \\
\frac{dM_2(t)}{dt} = u_2 M_1(t) - u_3 M_2(t) \quad (11) \\
\frac{dM_3(t)}{dt} = u_3 M_2(t) \quad (12)
\]

Initially, $M_0$, $M_1$, $M_2$, $M_3$ can be represented as follows respectively.

\[
M_0(0) = 1, \quad M_1(0) = 0, \quad M_2(0) = 0, \quad M_3(0) = 0 \quad (13)
\]

Under above condition, the relative number of $M_{412}$ with irradiation time $t$ can be defined as $M(t)$, and be represented as follows:

\[
M(t) = M_1 + 2M_2 + 3M_3 \\
= (1 - e^{-u_1 t}) - \frac{2}{3} \left[ \frac{u_1}{(u_2 - u_1)} (e^{-u_1 t} - e^{-u_2 t}) \right] \\
- \frac{1}{3} \left[ \frac{u_1 u_2}{(u_3 - u_1)(u_2 - u_1)} (e^{-u_1 t} - e^{-u_2 t}) + \frac{u_1 u_2}{(u_3 - u_2)(u_2 - u_1)} (e^{-u_2 t} - e^{-u_3 t}) \right] \quad (14)
\]
3. Results and discussion

3.1. Dependency of formation of M intermediate with total irradiation time

Temperature was set at about 77 K. The intensity of light for irradiation was $7.56 \times 10^{12}$ photon/sec mm$^2$, which does not induced a two photon-reaction during $bR \rightarrow M$. The wavelength of light for irradiation was set greater than 600 nm, which only bR absorbs. Samples were irradiated for periods of 1 min, 1 min, 2 min, 4 min, 8 min, 16 min, 32 min, 64 min, 128 min, 256 min, 512 min, 1024 min, respectively, until totally 2048 min and the absorption spectrum was measured. The absorption spectra of recbR are shown in Fig. 3.

![Absorption spectrum change of recbR](image)

Fig 3. Absorption spectrum change of recbR. The absorbance of the M intermediate at 405 nm increased as the red light irradiation increased. On the contrary, the absorbance of the ground state of recbR at 560 nm decreased. At the total irradiation time by 2048 min, Almost amount of recbR changed to M intermediate.

Under irradiation a the M intermediate having a $\lambda$ max at around 405 nm was generated. After the irradiation (total irradiation time was 2048 min), the spectrum closed to baseline. So the relative difference of absorbance at around 405 nm was
normalized to 100% compared to the initial state before irradiation.

3.2. **Comparison of formation rate of M intermediate of recbR with native bR**

On the other hand the reaction rate of formation of $M$ intermediate was simulated computationally by the equations$^9$. The accumulation process of $recbR$ was quietly different from native $bR$. Firstly, the formation rate is as 100 times as slower than that of native $bR$. This indicates because of the absence of peptide bond assembled protein from three peptides changes step by step to $M$ intermediate. Secondly, multi-regression

![Graph](image.png)

*Fig. 4* The difference absorbance change at 405 nm. This value is corresponds to accumulation of $M$ intermediate (o).
---: calculated data, 1 is early state change, 2 is late one.

analysis method in previous report for native$bR$ could not successfully be applied on all range of time development. Then we applied the equation (14) onto an early time range and a late time range separately, and propose a model for this phenomenon to elucidate it. This is a model that there are two different modes of accumulation change.

From the early state change (indicate 1 in the figure), $recbR$ has changed with parameter
(u1, u2, u3) = (0.04, 0.0012, 0.0012). The late state change (indicate 2 in the figure), \( recbR \) has changed with

\[
\begin{align*}
(u1, u2, u3) &= (0.015, 0.00045, 0.00045) \\
\text{probability of total change mode is constant 1, as following.}
\end{align*}
\]

\[
M(t) = c_1 M_1(t) + c_2 M_2(t) , \quad c_1 + c_1 = 1
\]

These data was compared with the case of native\( bR \). The result of comparison was that the native\( bR \) changes to \( M \) intermediate, as forty times as faster than \( recbR \).

3.3. Time development of recovery from \( M \) intermediate to ground state \( bR_{560} \) under irradiation with 390 nm light

The recovery change took 256 min. Although it was faster than that of formation of
recbR, it indicates that small amount of energy is need for recovery from M intermediate to ground state.

3.4. Association of Change of Rate Constants, the Hypothesis of Energy Transfer in Purple Membrane and Transmembrane Helices

The reason why early state change and late state change occur is considered to be the helix-helix interaction from recbR to M intermediate transition in inter protein trimeric system. This consideration is also supported by Renthal et al.’s study that reported in it, in independent two transmembrane helices A and B, a förster resonance energy transfer occur between amino acid tryptophan in helix B and tyrosine in helix A. And from our previous morphological study of bR in solid film\textsuperscript{16,17}, helix-helix interaction has an essential role in the bR molecular structural change. Therefore, recbR constituted from peptides is, in late stage, also influenced to be delay from inter molecular interaction between helices C-G, A, and B, in inter protein trimeric system.

4. Conclusion

In this study, time development of absorbance at 560 nm and 405 nm of reconstituted bacteriorhodopsin (recbR) containing helices C-G, A, and B, was analyzed for the purpose of determining reaction rates of photo reaction of recbR and its product M intermediate. The result was that recbR changes as forty times as slower than native bR in spite of absence of peptide bond.
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