Note

Regeneration of Bacteriorhodopsin from Thermally Unfolded Bacterio-Opsin and All-trans Retinal at High Temperatures

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The temperature dependence of regeneration of bacteriorhodopsin (bR) from its apoprotein, bacterio-opsin (bO), and all-trans retinal was investigated using two different procedures to probe the structural properties of bO at high temperatures. Regeneration experiments performed at 25°C after incubation of bO within the temperature range of 35–75°C indicate that irreversible thermal unfolding begins at 50°C. When bO is incubated for one hour and mixed with retinal at the same elevated temperatures, however, a greater extent of regeneration to bR occurs, even at temperatures ranging from 50 to 65°C. These experimental results indicate that regeneration of bO from thermally unfolded bO and suggest dynamic structural fluctuation of bO in the unfolded state.

Key words: bacteriorhodopsin; bacterio-opsin; regeneration; retinal; structural fluctuation

Bacteriorhodopsin (bR), an integral membrane protein on the plasma membrane of Halobacterium salinarum, is composed of a retinilydene chromophore and an apoprotein, bacterio-opsin (bO), which has seven transmembrane helices as the structural motif common to all G-protein coupled receptors (GPCR), including rhodopsin.1,2 The chromophore is bound to apoprotein through a Schiff base linkage with Lys 216. While bR dissociates to retinal and bO (the inactive state of bR) by hydrolysis of the Schiff base linkage,3 the active state of the protein (the light-adapted form of bR) is recovered by spontaneous binding of all-trans retinal to bO.4–8 As pointed out by Ludlam and Rothschild,9 the all-trans retinal chromophore of bR serves as a ligand that activates its non-active form. In contrast, the retinal chromophore also stabilizes bR against heat. Cladera et al. reported that, upon removal of retinal from bR, the protein undergoes thermal unfolding at about 50°C,10,11 whereas it had been thought that bR molecules denature above 90°C after premelting of the two-dimensional crystals of the purple membrane (PM) at about 78°C.12,13 Recent extensive denaturation experiments on bR14,15 revealed a high-temperature intermediate state in the range of about 60–70°C prior to irreversible thermal bleaching above about 70°C. In the intermediate state, bR molecules in the dark undergo irreversible structural changes, while they are subject to irreversible photobleaching by continuous irradiation of visible light. Here, bR regeneration by binding of all-trans retinal to bO over a wide temperature range was examined to probe the structural properties of bO in light of this high-temperature intermediate state of bR14,15 prior to the premelting transition.

Isolation and purification of PM from Halobacterium salinarum, strain R1M1, was performed according to the standard method established by Oesterhelt and Stoeckenius.16 Purified PM was suspended in 25 mM phosphate buffer at pH 7.2. Apoprotein without retinal was prepared by irradiation with visible light in the presence of hydroxylamine at 25°C.17 Regeneration of bR from bO and all-trans retinal were performed with two different protocols (Fig. 1(A)). A temperature increase in bO ranging from 35 to 75°C was achieved by mixing 0.4 ml concentrated bO suspension with 2.3 ml preheated phosphate buffer. After incubation of bO at each temperature for one hour, they were rapidly cooled to 25°C, followed by the addition of retinal and incubation at that temperature for another hour for regeneration in protocol I. In protocol II, incubation of bR and the addition of retinal to bO were successively performed at the same elevated temperature, followed by further incubation at that temperature for another hour for regeneration and rapid quenching to 25°C. In both protocols, 0.3 ml fresh all-trans retinal solution in ethanol was added to apoprotein with a molar ratio of 2:1 (retinal:bO). The final concentration of bO was 5 μM. Spectra of regenerated bR were measured at 25°C using a Beckman Coulter DU7500 spectrophotometer. The regeneration yield was calculated by dividing absorb-
ance at 560 nm for the regenerated bR by absorbance for the native bR prior to photobleaching with hydroxylamine.

UV–Vis absorption spectra of bR regenerated by both protocols were measured, and representative spectra of bR regenerated after incubation at 55°C by (1) protocol I and (2) protocol II, and (3) spectrum of bacterio-opsin before regeneration.

Regeneration yield was nearly zero above 70°C. These results indicate that irreversible thermal unfolding of bO molecules occurs at temperatures above about 50°C, which is about 20°C lower than the heat denaturation temperature of bR; above 70°C, bO molecules are in the fully unfolded state. The destabilization of bR by removal of the retinylidene chromophore is in good agreement with calorimetric studies performed by Cladera et al.10,11)

When retinal was added to bO at incubation temperatures prior to cooling to 25°C (protocol II), significant differences in regeneration yield were observed, compared to the results using protocol I, in which retinal was added to bO after quenching at 25°C. Even at temperatures greater than 50°C, regeneration of bR using protocol II achieved a level similar to that seen at 25°C, although a small gradual decrease was observed. Upon incubation at 70°C, the regeneration yield decreased significantly, almost reaching zero at 75°C.

New findings include the following: (1) When regeneration was performed at 25°C after incubation of bO at high temperatures and quick quenching to 25°C (protocol I), significant differences in regeneration yield were observed, compared to the results using protocol I, in which retinal was added to bO after quenching at 25°C. Even at temperatures greater than 50°C, regeneration of bR using protocol II achieved a level similar to that seen at 25°C, although a small gradual decrease was observed. Upon incubation at 70°C, the regeneration yield decreased significantly, almost reaching zero at 75°C.

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New findings include the following: (1) When regeneration was performed at 25°C after incubation of bO at high temperatures and quick quenching to 25°C (protocol I), yield drastically decreased above 50°C, dropping almost to zero above 70°C. (2) The addition of retinal to bO at high incubation temperatures without cooling (protocol II) produced a much higher regeneration yield of up to 70°C in comparison to results obtained using protocol I. (3) Minimal regeneration was observed for bO incubated at 75°C, irrespective of protocol. It is interesting to note the significant discrepancies in regeneration yield between the two protocols in the temperature range of 50–70°C. Regeneration experiments with protocol I indicate that bO molecules undergo irreversible thermal unfolding by 1h above 50°C, while experiments with protocol II indicate that, even in the unfolded state, bO molecules bind all-trans retinal and are stabilized as bR in the temperature range of 50–70°C. The essential difference between the two
protocols was the temperature at which bO was mixed and incubated with retinal; structural properties of bO at high temperatures appear to explain the experimental results. One of the most plausible models for explaining the high yield of regeneration from thermally unfolded bO is dynamic structural fluctuation in the unfolded state, as shown in Fig. 3. Below 45 °C, the majority of native bO possesses an ordered structure capable of binding all-trans retinal and converting to its active form, bR. In the proposed model, however, the heat-induced unfolded bO possesses structural ensembles with various conformations that interconvert over low energy barriers. Some of these conformations cannot bind retinal, while some are regenerated to bR by retinal binding and are highly stable. Above 50 °C, the structural ensemble of bO gradually shifted to a form unable to bind retinal at elevated temperatures, leading to a dramatic decrease in regeneration yield when protocol I was employed. For protocol II, bO molecules in the unfolded state can adopt structures suitable for retinal binding by interconversion from unsuitable structures at a certain frequency during the one-hour incubation. It is likely that the bO-retinal mixture system provides opportunities for regeneration from unfolded bO by retinal binding. The lack of regeneration using either protocol at 75 °C may be attributable to destabilization of bR molecules even if bO can bind retinal, since Yokoyama et al. reported irreversible thermal denaturation of bR above about 70 °C.

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References


