Structural Changes in Bacteriorhodopsin during In Vitro Refolding from a Partially Denatured State

Venkatramanan Krishnamani and Janos K. Lanyi*
Department of Physiology and Biophysics, University of California, Irvine, California

ABSTRACT We report on the formation of the secondary and tertiary structure of bacteriorhodopsin during its in vitro refolding from an SDS-denatured state. We used the mobility of single spin labels in seven samples, attached at various locations to six of the seven helical segments to engineered cysteine residues, to follow coil-to-helix formation. Distance measurements obtained by spin dipolar quenching in six samples labeled at either the cytoplasmic or extracellular ends of pairs of helices revealed the time dependence of the recovery of the transmembrane helical bundle. The secondary structure in the majority of the helical segments refolds with a time constant of $<$100–140 ms. Recovery of the tertiary structure is achieved by sequential association of the helices and occurs in at least three distinct steps with time constants of 1), well below 1 s; 2), 3–4 s; and 3), 60–130 s (the latter depending on the helical pair). The slowest of these processes occurs in concert with recovery of the retinal chromophore.

INTRODUCTION

In this study we investigated the in vitro refolding of the α-helical bundle BR. This integral membrane protein is the simplest α-helical membrane protein and has been extensively studied. Khorana and co-workers (1–4) established a procedure for its in vitro partial unfolding and refolding Their observation that such refolding is spontaneous and independent of any helping machinery supports the idea of spontaneous helix formation in membranes (5,6). Booth and co-workers (7–14) used the SDS-denatured state of BR as a starting point for many detailed kinetic studies on the in vitro refolding of this protein. In a recent study using site-specific fluorescent labeling at several residue positions, Compton et al. (15) suggested positional differences in the kinetics of refolding in helix D, implying variable structural changes along the helix.

The diffraction structure of BR at 1.55 Å resolution revealed a trimer with each monomer consisting of a hepta-helical transmembrane bundle (16), and the structural basis of its function as a light-driven proton pump (17). The seven helices enclose a retinal whose photoisomerization from all-*trans* to 13-*cis* triggers the first step of the light-dependent proton transport (2). Here, we report on the kinetics of the refolding of the helices and the association of pairs of helices of BR, starting from the partially denatured state present in SDS micelles. We hypothesized that the kinetics of association of the individual pairs of helices would differ from the previously measured global kinetics (10,18,19), and that understanding of this kinetics would lead to a molecular-level refolding model for this transmembrane protein. Little is known about the unfolding of the protein in SDS. The only molecular-level data available for helix-helix interactions of BR in SDS micelles to date were obtained by Renthal and Alloor (20), who performed fluorescence quenching in retinal bleached BR and found that the distance between sites on helices B and F changed by only 2 Å when the protein was in SDS micelles.

The technique we used in this study, ESR spectroscopy, has been used extensively to study membrane proteins (21–27). The method is sensitive to local environmental changes of the spin label, which is covalently attached to specific locations of interest on the protein through site-directed cysteine mutations. A popular reagent used for such modifications is MTSL R1 label with a thiol-reactive end group. It has been shown that R1 substitutions cause minimal structural perturbations in T4 lysozyme (28). The use of ESR and SDSL in combination has been well established for studying membrane protein structure and dynamics (21,29). The data acquired from ESR experiments report on dynamic parameters such as the spin-label side-chain mobility, the solvent accessibility of the spin-label side chain, the distance of the side chain from a second nitroxide label, and the polarity of the environment exposed to the side chain. In this study, we primarily employed mobility changes and distance estimation.

MATERIALS AND METHODS

Spin labeling

The labeling reaction was carried out in a purple membrane suspension. Before adding the R1 reagent to label the engineered cysteines, we reduced the cysteine residues by incubating the samples in excess DTT for 30 min. The reducing agent was then removed by four sequential 50× dilutions with 100 mM sodium phosphate buffer (pH 6.5) followed by centrifugation and

Submitted November 24, 2010, and accepted for publication February 4, 2011.

*Correspondence: jklanyi@uci.edu

Abbreviations used: BR, bacteriorhodopsin; CHAPS, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate; CD, circular dichroism; CW-ESR, continuous wave electron spin resonance; DMPC, 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine; DTT, dithiothreitol; MTSL or R1, (1-oxyl-2,2,5,5-tetramethylpyrolinyl-3-methyl)-methanethiosulfonate; SDS, sodium dodecyl sulfate; SDSL, site-directed spin labeling.

Editor: Leonid S. Brown.

0006-3495/11/03/1559/9 $2.00

doi: 10.1016/j.bpj.2011.02.004

resuspension of the membranes. After the washing procedure was completed, a 10× molar excess of the R1 label was added and incubated at room temperature for 24 h. Excess label was then removed by four centrifugation and 50× dilution steps with 100 mM sodium phosphate buffer (pH 6.5). All of the labeling sites were chosen to be at the ends of the helices, where the SH group of cysteines is accessible (Fig. 1). We found that labels at several otherwise desirable locations were not accessible to the label, even in the presence of dimethylsulfoxide, which was previously used to enhance permeation of solutes across the membranes (30,31).

In vitro refolding

To increase the signal/noise ratio of the ESR spectrum, we made the initial concentration of the denatured samples 10-fold greater (100 μM) than that used in previous refolding experiments (2,3,7,10,14,19). The purple membrane was solubilized by addition of SDS in pH 6.5 phosphate buffer to maintain the 1:400 SDS/protein molar ratio of the previously established denaturing conditions (2,7). We regenerated the partially denatured BR in SDS micelles by adding an equal volume of DMPC/CHAPS micelle suspension (pH 6.5) as described elsewhere (7). The concentration of the latter was not scaled in our experiments, as we observed that the regeneration yield did not significantly change when we increased the DMPC/CHAPS micelle concentration from 2% to 5% (data not shown). This modified refolding procedure resulted in a 40–60% yield of the chromophore reconstitution, compared with a previously reported yield of 65–85% (7). It appears that a 10-fold higher SDS concentration combined with a lower DMPC+CHAPS micelle/protein ratio partially prevents chromophore regeneration and/or retinal binding. On the other hand, the rate constants for chromophore regeneration reported in previous studies (3,7,8) were reproduced in our experiments.

CW-ESR: equilibrium measurements

We obtained CW-ESR spectra of native, denatured, and regenerated equilibrium states of BR using a benchtop, high-sensitivity, X-band ESR spectrometer (Miniscope MS 300; Magnettech, Berlin, Germany) equipped with a rectangular resonator (TE102). The 12–20 μL spin-labeled samples (100 μM native BR, 90 μM denatured BR, and 45 μM regenerated BR) were placed in glass capillaries (outer diameter: ~5 mm) and inserted into the resonator such that the sample occupied the entire cavity. The instrument settings were as follows: 25 mW microwave power, 1 G amplitude modulation, ~3398–3341 G center field depending on the sample, 100 KHz modulation frequency, and 200 G sweep width at a rate of 1 G/s. A power saturation analysis verified that the 25 mW microwave power setting used is in the linear range (data not shown).

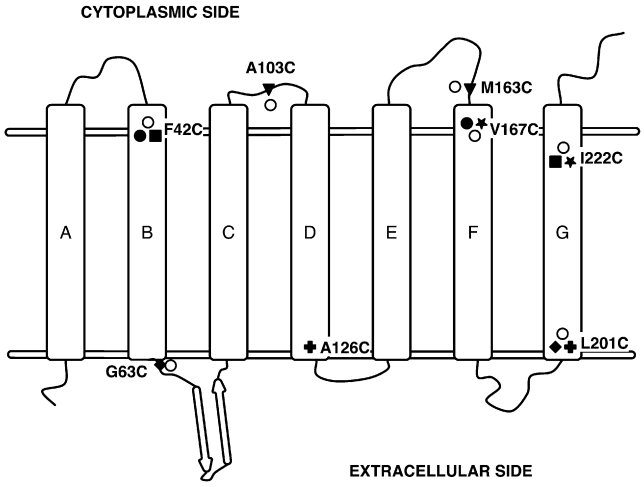

FIGURE 1 Topology diagram showing the locations of the designed cysteine mutations and labels in BR. The single mutants are shown as open circles and the double mutants are shown as shaded symbols. The residue locations for each pair of double mutants is represented with the same shaded symbol. The horizontal double lines approximate the membrane surfaces.

CW-ESR: kinetic measurements

A stopped-flow setup for the Miniscope MS300 instrument was custom-built to investigate the refolding kinetics. An ESR-signal-neutral, high-pressure-rated Teflon tube directed the sample flow from the stopped-flow mixing chamber to the resonator. The capillary cell was modified to accommodate the Teflon tube (outer diameter: ~5–6 mm), which was placed inside the stock rectangular resonator (TE102). The dead volume of this setup from mixing chamber to the ESR resonance cavity was ~0.40 mL. The sample (0.5 mL) was injected after rapid mixing into the ESR cavity for each actuation of the stopped-flow pump. The resulting dead time was 100–140 ms.

We designed single-labeled BR mutants to follow the changes in mobility of the R1 spin label during the refolding process. We used double-labeled BR mutants on the ends of the pair helices to follow the approach of the helices and the corresponding decrease in the distance between the pair of spin labels during helix bundle formation. A decrease in distance between the ends of the helices as they approach one another decreases the intensity of the central absorption peak of the R1 spin label and broadens the ESR spectrum (32). CW-ESR is sensitive to dipolar interactions between spins in a distance range of 5–20 Å. Both the single- and double-labeled samples were monitored for intensity changes at the central peak (~3347 G) during refolding.

The ESR instrument settings for the kinetic experiments were as follows: 25 mW microwave power, 1 G amplitude modulation, ~3398–3341 G center field depending on the sample, and 100 KHz modulation frequency. The time constant varied from 0.02 ms to 97 ms depending on the length of the measurement. We collected several repeated data sets for each sample for averaging and to improve the signal/noise ratio.

Light scattering

We measured the particle sizes for BR in SDS micelles and DMPC/CHAPS micelles using dynamic light scattering with a Zetasizer nano series instrument (Malvern Instruments, Worcestershire, UK). We then analyzed the time-dependent correlation of the scattering intensity obtained from the photon correlator using a built-in algorithm in the Zetasizer software to calculate the volume and number distributions.

RESULTS

Earlier kinetic studies on refolding of BR revealed mainly global structural changes during its refolding from a partially denatured state in SDS micelles to a functionally regenerated state in DMPC/CHAPS micelles (7–10,14,15,33). In this work, we explored local structural changes and how they lead to regeneration of the fully folded state. We assumed that the mutations and the attachment of R1 spin label do not affect the folding kinetics and the final folded state of the protein. Previous investigations of other proteins suggested that perturbations of the backbone fold, thermal