Supporting Material for

Structural changes in bacteriorhodopsin during in vitro refolding from a partially denatured state

Venkatramanan Krishnamani and Janos K. Lanyi

Mutagenesis and Protein Expression

Cysteines were introduced into BR, which otherwise contains no cysteine, with a well-established homologous expression system (29). The desired residue change was introduced into a synthetic oligomer primer, 20-30 nucleotide in length, custom made by Integrated DNA Technologies, Inc. The template DNA pBA2 containing the bop gene (bacterioopsin) was generously provided by M. P. Krebs. The bop gene segment was mutated at specific sites to incorporate a cysteine residue, using a commercially available PCR site-directed mutagenesis kit under the brand name Quikchange XL from Stratagene. The PCR products were digested using restriction endonuclease Dpn1 to remove traces of the original template. The resulting plasmids containing ampR (ampicillin resistance gene) were transformed and proliferated in the host *Escherichia coli* on a nutrient agar plate at 37^0C in the presence of antibiotic ampicillin. Subsequently, the plasmids were extracted, purified and sequenced to confirm the inclusion of the desired mutation.

The purified plasmid pBA2 shuttle vector having the mutated bop gene was then transformed into a modified *Halobacterium salinarum* host, MPK409, kindly provided by M. P. Krebs. In the MPK409 host cells, the native 5-fluororotic acid sensitivity gene *ura3* is deleted and substituted with an intact *ura3* gene insertion within the bop (bacterioopsin) gene segment in the *Halobacterium salinarum* chromosome (30, 31). The bop gene in the shuttle vector pBA2 contains the desired mutation, and the mevR gene is used to select for the successful integrants in MPK409 transformants (29, 32). The recombinants were selected using a two-step selection procedure using first mevinolin resistance, and then 5-fluororotic acid resistance.

The *Halobacterium* cells thus obtained were cultured in 400 ml complete medium for a day under aerobic conditions in a 2L culture flask at 38^0C to bring the cell count to a sufficient number for efficient expression of the protein. Following this, the culture flask was filled up with Rich Medium (29, 30) and cultured for an additional 4 days at 40^0C in an anaerobic condition for BR purple membrane expression. One of the planned single mutations, A126C, and two of the double mutants, E9C/G63C and S132C/L190C, failed to express in the *H. salinarum* expression system.

Protein Purification

Purple membranes were isolated using a procedure established by Oesterhelt et al. (33). Briefly, the *H. salinarum* cells were re-suspended in 4 M NaCl and treated with

DNase I for 1 hour to digest the genomic DNA. The suspension was transferred to 10,000 MW cutoff dialysis bags and dialyzed at 4^0C against distilled water overnight. All cell membranes other than purple membrane disintegrate. The purple membrane, which contains only BR and lipid, was further purified by series of centrifugation and washing steps, and recovered by centrifugation from a 40% - 60% sucrose gradient.

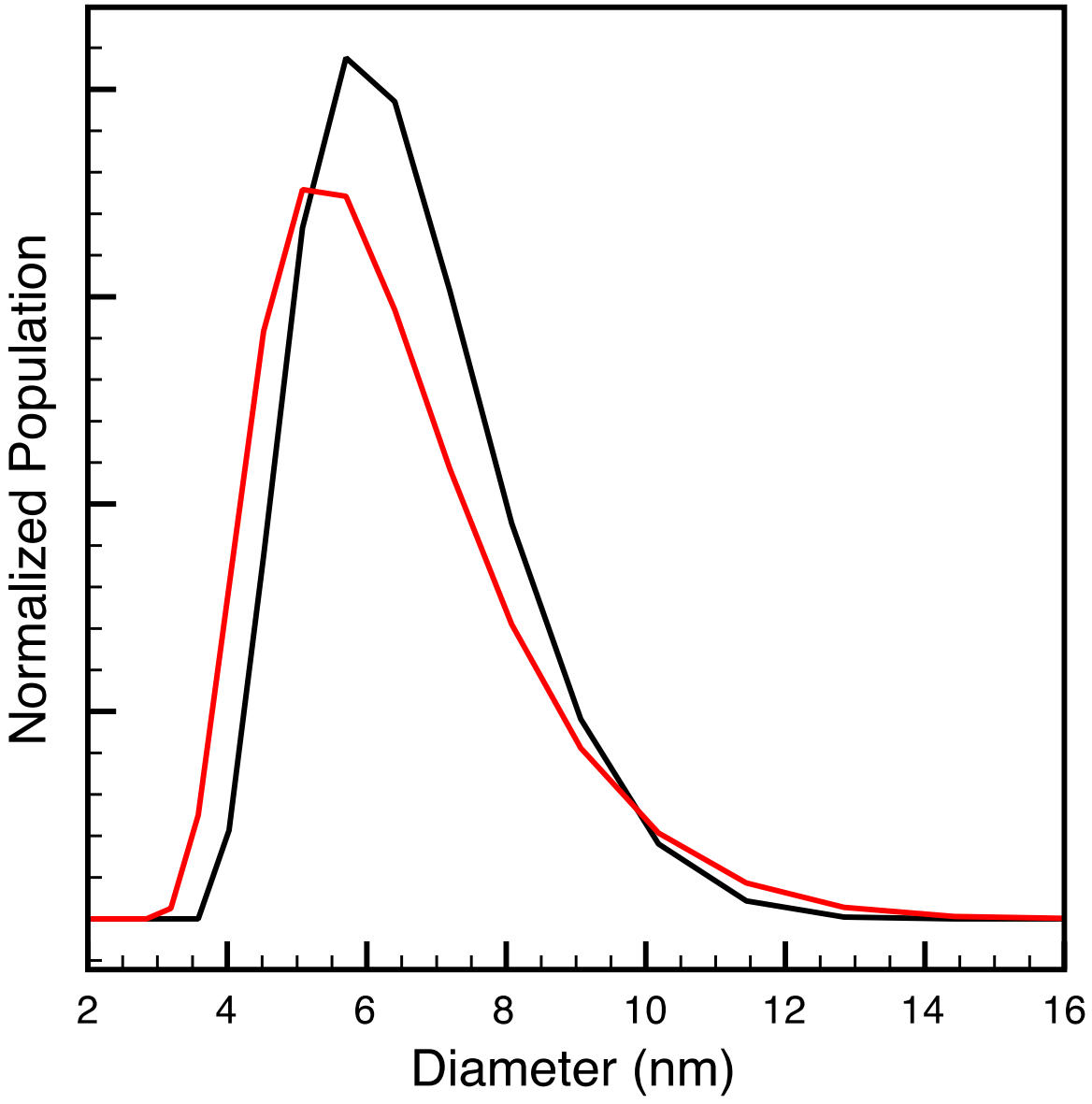

FIGURE S1. Normalized volume distributions of denatured BR in SDS micelles (black) and after regeneration in DMPC+CHAPS micelles (red), obtained from light scattering.

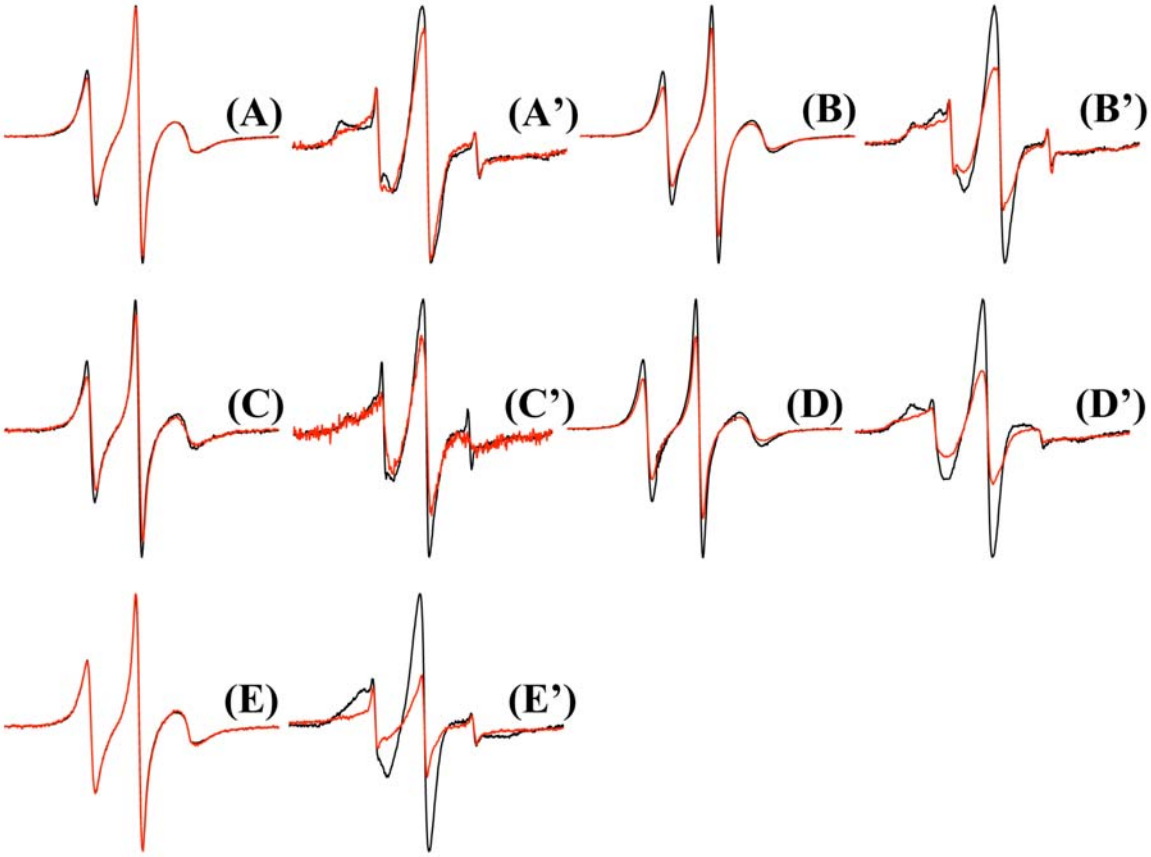

FIGURE S2. ESR spectra of dipolar interactions. Non-interacting spins (sum of single label spectra, black) and interacting spins (measured spectra for double label, red) are shown for F42CR1/V167CR1 (A and A'), F42CR1/I222CR1 (B and B'), G63CR1/L201CR1 (C and C'), A103CR1/M163CR1 (D and D') and V167CR1/I222CR1 (E and E'). Spectra in the D-state are labeled with A, B, C, D, and E, those in the R-state with A', B', C', D' and E'. The spectra are area normalized for an equal number of spins.

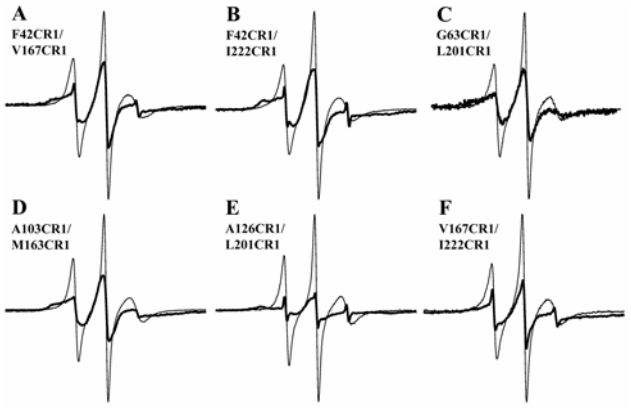

FIGURE S3. Dipolar interaction ESR spectrum of doubly labeled helical pairs of BR F42CR1/V167CR1 (A), F42CR1/I222CR1 (B), G63CR1/L201CR1 (C), A103CR1/M163CR1 (D), A126CR1/L201CR1 (E) and V167CR1/I222CR1 (F) in D-state *(thin lines)* and R-state *(bold lines)*. The central resonance peak of the ESR spectrum between the D-state and R-state was followed at ~3341 G to measure the kinetics of helix association.