

## METHODS

**SDS unfolding assays.** All bacteriorhodopsin mutants were created and purified as described<sup>9</sup>. The SDS unfolding assays were performed as described previously<sup>9</sup> for all except two of the mutant proteins by titration in 20% (w/v) SDS in 15 mM DMPC, 6 mM CHAPSO and 10 mM sodium phosphate pH 6.0 into 0.1 mg ml<sup>-1</sup> protein suspended in 15 mM DMPC, 6 mM CHAPSO and 10 mM sodium phosphate pH 6.0. For the D212A and Y185F/D212A mutants, retinal absorbance was unusually sensitive to SDS concentration, causing a large slope to the native baseline of the unfolding curves that made interpretation difficult. We therefore turned to far-ultraviolet circular dichroism. Circular dichroism was monitored at 228 nm to minimize absorbance. The mutant bacteriorhodopsins D212A and Y185F/D212A were unfolded in a 1-cm path length quartz cuvette by additions of 10% (w/v) SDS in 7.5 mM DMPC, 3 mM CHAPSO and 10 mM sodium phosphate pH 6.0 into 0.1 mg ml<sup>-1</sup> protein prepared in 7.5 mM DMPC, 3 mM CHAPSO and 10 mM sodium phosphate pH 6.0.

**X-ray crystallography.** Mutant bacteriorhodopsins D115A and T90A/D115A were crystallized by using the bicelle method<sup>27</sup>. A 4:1 protein/bicelle solution (4  $\mu$ l) of 10 mg ml<sup>-1</sup> purple membrane in water and 40% (w/v) 2.8:1 DMPC/CHAPSO was mixed with 1.5  $\mu$ l of a well solution, inverted over the well solution, and incubated at 37 °C. D115A was crystallized by using a well solution containing 1.4 M NaH<sub>2</sub>PO<sub>4</sub> pH 3.7, 0.8 M NaH<sub>2</sub>PO<sub>4</sub> pH 4.5 and 0.12 M hexanediol. The well solution for T90A/D115A was 2.4 M NaH<sub>2</sub>PO<sub>4</sub> pH 3.7, 3.5% triethylene glycerol and 0.15 M hexanediol. Crystals were transferred to a cryoprotectant solution of 4.0 M NaH<sub>2</sub>PO<sub>4</sub> pH 3.7 before being frozen under a stream of liquid nitrogen. Data were collected from D115A and T90A/D115A crystals at the Lawrence-Berkeley National Laboratory. X-ray diffraction data were processed and scaled by using the DENZO package<sup>30</sup>, and the structure was solved by molecular replacement with the use of the 1PY6 structure<sup>9</sup> and refined by using CNS with a twinning fraction of 0.50 (ref. 31) at resolutions of 2.3 Å and 2.7 Å for D115A and T90A/D115A, respectively. Five per cent of the reflections were withheld for the calculation of  $R_{\text{free}}$ . The same reflections withheld in the WT refinement were used for both mutant refinements to eliminate bias. D115A was refined to an  $R$  factor of 22.1 and an  $R_{\text{free}}$  of 27.5, and T90A/D115A was refined to an  $R$  factor of 27.3 and an  $R_{\text{free}}$  of 28.7. See Supplementary Table 2 for detailed data collection and refinement statistics.

**Hydrogen-deuterium exchange analysis of the unfolded state.** To initiate the deuterium exchange reaction, nine parts of deuterium exchange (DX) buffer (10 mM sodium phosphate in D<sub>2</sub>O (uncorrected pH 6.0)) were added to 1 part of SDS-denatured protein: 5 mg ml<sup>-1</sup> in 5.5% (w/v) SDS, 15 mM DMPC, 6 mM CHAPSO and 10 mM sodium phosphate pH 6.0. The exchange reaction was incubated at 22 °C and quenched at various time points by transferring a 40- $\mu$ l aliquot of the exchange reaction into a chilled Microfuge tube at 0 °C containing a 10- $\mu$ l droplet of 1.0 M potassium phosphate pH 2.4 and 2.5% (w/v) acid-labile surfactant, followed by rapid mixing and immediate flash freezing in liquid N<sub>2</sub>. The K<sup>+</sup> precipitates dodecylsulphate, which would otherwise inhibit the subsequent pepsinization. Acid-labile surfactant helps to solubilize bacteriorhodopsin and peptic peptides, and is degraded during subsequent low-pH chromatography. The quenched reaction was stored at -80 °C until LC-MS analyses. No significant change in the deuterium content in protein takes place at -80 °C (data not shown).

For proteolysis, the flash-frozen quenched exchange reaction was rapidly thawed to 0 °C and immediately transferred to a spin filter (SpinX, 0.22- $\mu$ m filter; Costar) chilled to 0 °C and containing 75  $\mu$ l of immobilized pepsin slurry (Pierce) prewashed in DX buffer and dried by low-speed centrifugation. After an incubation for 9 min at 0 °C, the samples were immediately centrifuged at 10,000g at 0 °C for 30 s to collect the peptic peptides in the filtrate. The filtrate was then immediately injected into an LC-MS column with a chilled syringe.

The peptide mixture was applied to a PLRP-S 100-Å column (1 mm  $\times$  50 mm; Polymer Laboratory) equilibrated in 95% solvent A (0.4% formic acid, 0.1% hexafluoroisopropanol (HFIP), 99.5% water) and 5% solvent B (0.4% formic acid, 0.1% HFIP, 99.5% acetonitrile). After washing in the same solvent mixture for 2.15 min at a flow rate of 120  $\mu$ l min<sup>-1</sup>, the flow rate was changed to 60  $\mu$ l min<sup>-1</sup> and a gradient initiated to 95% solvent B over 22.85 min. The column was cleaned at the end of each experiment by injecting 50  $\mu$ l of formic acid while eluting with 95% solvent B at 120  $\mu$ l min<sup>-1</sup> for 2 min, followed by equilibration with 5% solvent B at 120  $\mu$ l min<sup>-1</sup> for 5 min. To minimize back exchange, LC-MS was performed at pH 2.4 and all LC-MS components starting from the outlet from pump to the inlet of ion source were completely immersed in a tightly packed ice bath. ThermoFinnigan LCQ was used to collect the isotopic envelope evolution data.

The average number of amide hydrogen atoms exchanged for deuterium was determined from the measured shift in the centroid mass for each isotopic

distribution. To account for back exchange and forward exchange during analysis, the deuterium content,  $D$ , in each fragment was calculated from

$$D = N(m - m_{0\text{min}})/(m_{\text{equilibrium}} - m_{0\text{min}})$$

where  $N$  is the number of exchangeable amide sites,  $m$  is the experimentally determined centroid mass,  $m_{0\text{min}}$  is the averaged centroid mass of the 0-min exchange period control, and  $m_{\text{equilibrium}}$  is the averaged centroid mass of the equilibrium exchange control.

For 0-min exchange-period control experiments, a 4- $\mu$ l aliquot of SDS-unfolded bacteriorhodopsin prepared as described above was added to a chilled mixture of 36  $\mu$ l of DX buffer, 5  $\mu$ l of 2.0 M potassium phosphate pH 2.4 and 5  $\mu$ l of 5% (w/v) acid-labile surfactant at 0 °C, immediately vortex-mixed for 5 s and flash-frozen until proteolysis and LC-MS analysis were performed as described above.

For equilibrium exchange control experiments, peptic peptides of each protein were initially prepared, exchanged to the equilibrium in 90% D<sub>2</sub>O DX exchange buffer, and mass-analysed after adjusting the pH. In brief, peptic peptides were prepared by first incubating a 4- $\mu$ l aliquot of SDS-unfolded bacteriorhodopsin in 10 mM sodium phosphate in D<sub>2</sub>O at an uncorrected pH of 6.8, then quenching by adding 5  $\mu$ l of 2.0 M potassium phosphate pH 2.4 in 90% (v/v) D<sub>2</sub>O and 5  $\mu$ l of 5% (w/v) acid-labile surfactant in 90% (v/v) D<sub>2</sub>O, followed by proteolysis using immobilized pepsin, prewashed in 200 mM potassium phosphate pH 2.4 in 90% D<sub>2</sub>O at room temperature, in a spin-filter for 10 min. The peptic peptides in the filtrate were then collected by centrifugation and the pH was adjusted to 6.85 by the addition of 4  $\mu$ l of 2.0 M NaOH in 90% D<sub>2</sub>O to 50  $\mu$ l of filtrate, to facilitate the equilibrium exchange reaction, and incubated at 42 °C overnight. The pH of the equilibrium reaction was then readjusted to 2.5 by the addition of 2.5  $\mu$ l of 3.3 M HCl in 90% D<sub>2</sub>O at 22 °C to reproduce the minimized back exchange. An aliquot of 40  $\mu$ l of each equilibrium sample was then transferred to a vial containing 5  $\mu$ l of water and 5  $\mu$ l of 2 M potassium phosphate pH 2.4, mixed for 5 s, flash-frozen in liquid N<sub>2</sub> and kept at -80 °C until MS analysis. Although the equilibrium controls were subject to pre-proteolysis, the frozen equilibrium control samples were again incubated in immobilized pepsin before LC-MS analysis just as the deuterium exchange samples as described above, to reproduce the back-exchange process during proteolysis.

DXMS software<sup>32</sup> was used to identify the peptic peptides in the LC-MS-MS data collected before deuterium exchange experiments. MagTran software<sup>33</sup> was used to calculate the centroid mass of the isotopic envelope identified by DXMS software. Laplace software<sup>34</sup> was used to transform the observed exchange kinetics into rate constant distributions by the maximum-entropy method protocol. For visualization purposes a weighted average rate,  $k_{\text{av}}$ , was calculated for each fragment from the corresponding rate constant distribution:

$$k_{\text{av}} = \int kA(k)dk$$

in which  $k$  is the rate constant and  $A(k)$  is the normalized abundance of amide sites exchanging with rate  $k$ .

The exchange time courses were determined in triplicate, providing the mean and standard deviation for each time point. To estimate errors for  $k_{\text{av}}$  values we simulated ten time-course data sets in which the simulated data points were generated by using the mean and standard deviation for each time point as found in the experimental time-course data. The standard deviation of the  $k_{\text{av}}$  values for the simulated data were then used as error estimates.

**Analysis of unsatisfied hydrogen bonds.** All helical membrane proteins solved as of October 2007 at a resolution of 1.7 Å or better were identified. If two proteins had more than 40% sequence identity, the lower-resolution structure was rejected, leaving a set of six protein structures (PDB IDs 1C3W (ref. 35), 1KQF (ref. 36), 1U7G (ref. 37), 2A65 (ref. 38), 2B2H (ref. 39) and 2F2B (ref. 40)). The central hydrophobic region of each protein was identified as described<sup>28</sup> by finding the most hydrophobic 30-Å slice of the structure perpendicular to the membrane normal. The membrane normal was taken as the biological oligomeric symmetry axis in each structure. Hydrogen-bond satisfaction was calculated with HBPLUS, using the relaxed criteria described in ref. 29 to obtain most unlikely unsatisfied donors and acceptors<sup>34</sup>. Alternative Asn, Gln and His orientations were explored and explicit water was included in the analysis to identify all possible hydrogen bonds.

**Distance analysis of hydrogen bonds in membrane and soluble proteins.** The distribution of hydrogen-acceptor distances of  $\alpha$ -helix backbone-backbone hydrogen bonds in the hydrocarbon cores of same unique high-resolution membrane protein structures described above was compared with that of the hydrogen bonds in soluble proteins. For soluble proteins, a 30% sequence

identity cutoff returned a total of 839 structures in the same resolution range as each of the membrane proteins used in analysis. A hydrogen-acceptor distance of the  $\alpha$ -helix backbone-backbone hydrogen bond was calculated with HBPLUS by detecting the hydrogen bond mediated by carbonyl acceptor at position  $i$  and amide donor at  $i + 4$  in the helical backbone identified in the header of the PDB file by using the same relaxed criteria described above. For membrane proteins, the solvent accessibility of residues was calculated in the context of the biologically relevant oligomeric form. For buried backbone hydrogen bonds, we selected hydrogen bonds occurring between donor and acceptor residues in which both of the side chains were at least 90% buried. For surface hydrogen bonds, both donor and acceptor side-chain solvent accessibilities were 20% or higher. Student's  $t$ -test performed between the distributions of hydrogen-acceptor distances identified in the lowest-resolution soluble-protein structures (1.68 Å) and in the highest-resolution soluble-protein structures (1.40 Å) indicated that the effect of the variation in resolution on the hydrogen-acceptor distance distribution was negligible at the resolution range used in analysis (results not shown).

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