Minireview

Structural and thermodynamic consequences of *b* heme binding for monomeric apoglobins and other apoproteins

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Supplementary information

Materials and Methods

bjFixLH

The gene encoding residues 151-256 of *bj*FixL was used as provided by Dr. J. D. Satterlee (*bj*FixLH refers to this product). The vector was pET24a+ and imparted kanamycin resistance to the host cell. Additional amounts of the plasmid were prepared in *Escherichia coli* DH5α cells, and the sequence was confirmed at the Penn State Nucleic Acid Facility after purification (Qiagen Plasmid Miniprep Kit, Qiagen, Valencia, CA). For protein expression, BL21 *E. coli* cells were transformed with the plasmid and grown in Luria-Bertani medium supplemented with kanamycin (0.05 mg/mL) at 37 °C. Maximal protein expression occurred in inclusion bodies 5 – 6 h post-isopropylthio-β-D-galactoside induction (final concentration 1 mM). Cells were harvested by centrifugation (15,000 × *g* for 15 min at 4 °C) and stored at −20 °C if not immediately used.

Cells were ruptured by ultrasonication (Model 60 sonic dismembrator; Fisher Scientific, Pittsburgh, PA), and the inclusion bodies were isolated and resolubilized in 8 M urea. This procedure yielded denatured apo *bj*FixLH, which was slowly refolded by dialysis against buffer (20 mM borate, 250 mM NaCl, pH 9). After concentration (Amicon ultrafiltration unit equipped with a YM-3 membrane; Millipore Corp., Bedford, MA), the protein was purified by size-exclusion chromatography on Sephacryl S-100 resin (20 mM borate, 250 mM NaCl, pH 9.0). This was followed by passage on a DEAE Sephacel ion-exchange column (same buffer) to remove nucleic acid impurities. The pure apoprotein was recovered in the flow through (20 mM borate, 250 mM NaCl, pH 9.0). The apoprotein solution was concentrated and refrigerated until used.

Holo *bj*FixLH was prepared by addition of hemin (50 mg/mL in 0.1 M NaOH) to purified apo *bj*FixLH solution, and stirred at 4 °C for 12 – 14 hours. Excess heme was removed by centrifugation (20,000 × *g* for 45 min at 4 °C) and filtration. The solution was dialyzed against buffer (20 mM borate, 25 mM NaCl, pH 9.0), then purified by ion-exchange chromatography, using an NaCl gradient (25 – 250 mM NaCl in 20 mM borate pH 9.0) for elution. Alternatively, heme was added after the dialysis to remove urea, and the crude reconstituted protein solution was purified by passage through the ion-exchange column (25 – 250 mM NaCl in 20 mM borate pH 9.0), then the gel filtration column. The holoprotein was concentrated and refrigerated for further use. The purity of holo *bj*FixLH(151-256) was evaluated by absorbance measurement on

an AVIV (Lakewood, NJ) 14 DS spectrophotometer; a typical preparation of ferric protein exhibited a 395 nm:280 nm absorbance ratio of 3.3 to 3.5 (Suquet et al., 2005).

The extinction coefficient of met *bj*FixLH (131-residue domain) was determined by Gilles-Gonzalez *et al.* to be 126 mM^{-1} cm^{-1} at 395 nm (Gilles-Gonzalez et al., 1994) and below pH 10. This value was used without adjustment for N- and C-terminal truncation. Holoprotein concentrations are reported on a heme basis. The extinction coefficient of apo *bj*FixLH was determined by the method of Gill and von Hippel (Gill and von Hippel, 1989) as described by Pace *et al*. (Pace et al., 1995). Samples of the native protein were prepared from a stock to obtain an approximate final protein concentration of 10 μM in 10 mM borate, 50 mM NaCl, pH 8.5. Samples of denatured protein also contained 6 M Gdm-HCl (8 M stock in 10 mM borate 50 mM NaCl buffer) and were incubated for a minimum of 10 min. Absorbance spectra were recorded in triplicate from 400 nm to 250 nm. Spectra of the denatured samples were also recorded after 12 – 14 hours of incubation to verify complete denaturation. To correct for light-scattering, the double logarithmic difference plot method described by Leach and Scheraga was used (Leach and Scheraga, 1960). The extinction coefficient of denatured apo *bj*FixLH ($\varepsilon_{U,\,280}$ = 9.53 mM^{-1} cm^{-1}) was determined according to amino acid composition with ProtParam (http://us.expasy.org/tools/protparam.html). The extinction coefficient for apo *bj*FixLH was ($\varepsilon_{N,280} = 9.8 \pm 0.1$ mM^{-1} cm^{-1}) under native conditions.

apoGlbN

The apoprotein of *Synechococcus* sp. PCC 7002 (S7002) GlbN was prepared as described previously (Vuletich et al., 2006). The approximate extinction coefficient of S7002 apoGlbN was obtained according to composition (http://us.expasy.org/tools/protparam.html).

Thermal denaturation

Far UV-CD data were collected in at least triplicate on a Jasco J-810 spectrometer equipped with a Peltier thermoelectric device. Holo *bj*FixLH (≤ 25 μM, 0.2 cm path-length cell) and apo *bj*FixLH (≤ 10 μM, 1 cm path-length cell) were prepared in 20 mM borate, 250 mM NaCl, pH 9.0. Spectra (250 – 205 nm, 4-s averaging per nm) were collected every 2 °C over the range 17 °C to 75 °C with an equilibration time of 5 min for each point. The largest difference in ellipticity for holo *bj*FixLH occurred between 217 and 223 nm, and 222 nm was used. Analysis at other wavelengths produced similar results. Data at 222 nm were also used for the apoprotein, with similar results obtained between 218 and 225 nm.

Assuming a two-state situation, the signal observed throughout the experiment, Y(T), is a function of the fractional population in the native ($F_N(T)$) and unfolded ($F_U(T)$) states and the spectral properties of these states ($Y_N(T)$ and $Y_U(T)$, exhibiting a linear dependence on T):

$$Y(T) = F_N(T)Y_N(T) + F_U(T)Y_U(T) \quad (1)$$

The fractional population of the native state is given by:

$$F_N(T) = 1/(1+K_U(T)) \quad (2)$$

where $K_U(T)$ is the equilibrium constant for unfolding. This constant is related to $\Delta G°_U(T)$ via

$$\Delta G°_U(T) = -RT\ln K_U(T) \quad (3).$$

To analyze the thermal denaturation data, the following Gibbs-Helmholtz equation was applied:

$$\Delta G°_U(T) = \Delta H°_{Tm}(1 - T/T_m) - \Delta C°_p[(T_m - T) + T \ln (T/T_m)] \quad (4)$$

where ΔH°_{Tm} represents the enthalpy of denaturation at the midpoint temperature (T_m) and ΔC°_p is the change in heat capacity, assumed independent of temperature. ΔC°_p was approximated by the method described by Myers and co-workers (Myers et al., 1995). Briefly, the change in accessible surface area (ΔASA) between folded and extended-chain states was obtained with the equation

$$\Delta ASA = -907 + 93 \times N \quad (5),$$

where N is the number of residues. The ΔASA was then used to estimate ΔC°_p with

$$\Delta C^\circ_p = -119 + 0.20 \times \Delta ASA \quad (6).$$

The resulting ΔC°_p (7.0 kJ mol^{-1} K^{-1}, converted from the kcal mol^{-1} K^{-1} obtained with the equation) was applied as a constant in Equation (4).

The thermal denaturation of apo *bj*FixLH was also monitored by absorbance measurements. Experiments were run in triplicate, with a protein concentration between 5 and 10 μM (20 mM borate, 250 mM NaCl, pH 9.0) in a 1 cm path-length cell. Scans (250 – 700 nm) were collected from 19 to 85 °C (every 2 °C) and from 85 °C to 19 °C (every 3 °C) to inspect reversibility. Equilibration time was 5 min. Data at 280 nm were analyzed using Equations (1) – (4). ΔC°_p was fixed at 7.0 kJ mol^{-1} K^{-1}. This was likely an overestimate as it assumed a globular, well-folded apoprotein. For the purposes of this study, decreasing the ΔC°_p value within reasonable bounds did not affect T_m significantly.

Chemical denaturation

High concentration urea solutions (~10 M, ultrapure urea, ICN Biochemicals Inc., Aurora, OH) were purified on a mixed-bed resin column (Bio-Rad Laboratories Inc.; Hercules, CA) prior to the denaturation experiments. Stock solutions were stored at −20 °C, and, upon thawing, were used within 24 hours. The urea concentration of the stock and final solution was determined by index of refraction measurements (Leica Abbe Mark II Refractometer). Spectra were collected from 250 to 215 nm; titrations were performed with a Jasco ATS-429S titrator. Ferric *bj*FixLH (≤ 5 μM, 1-cm path-length cell) in buffer (20 mM borate, 50 mM NaCl, ~ 8.8 pH) was maintained at 20 °C, with continuous stirring of the solution in the cuvette. Apo S7002 GlbN (< 5 μM, 1-cm path-length cell) in buffer (20 mM sodium phosphate, pH 7.4) was maintained at 25 °C, with continuous stirring. Equilibration time was 5 min in all cases. Data were converted to molar residual ellipticity (MRE), and the signal, Y([U]), was processed as Y(T) above. A linear free energy two-state equation relation was used to fit the unfolding data:

$$\Delta G^\circ_U = \Delta G^\circ_U(H_2O) - m\,[\text{urea}] \quad (7)$$

where $\Delta G^\circ_U(H_2O)$ is the free energy of denaturation in the absence of urea.

The approximate value for *m* can be determined by the method described by Myers and co-workers (Myers et al., 1995) using:

$$m = 368 + 0.11\ (\Delta ASA) \quad (8)$$

NMR Spectroscopy

NMR spectra were collected at 298 K on a Bruker DRX-600 spectrometer (14.1 T, operating at a ^1H frequency of 600.18 MHz). Samples were prepared in 20 mM borate buffer (pH 8.5 – 9.2). The water signal was suppressed by presaturation. Data were analyzed using XWIN-NMR v. 3.1 and TOPSPIN v. 1.1 (Bruker BioSpin, Rheinstetten, Germany).

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