Preparation of S6803 apoHb from inclusion bodies. BL21 competent cells were transformed, grown in modified M-9 medium in the absence of heme precursors and harvested as in (1). A typical 2-L growth yielded 3 g – 5 g cell paste, which was processed as follows. The cell pellets were suspended in 10 mL chilled lysis buffer (50 mM Tris, 5 mM EDTA, pH 8.0) per 3 g cells. The mixture was then sonicated for 6 cycles (15 s on/15 s off at 70% power on a Fisher Scientific Model 60 Sonic Dismembrator) and centrifuged at 16,000 g for 15 min. This and all centrifugation steps were carried out at 4 °C. The supernatant, containing a small amount of soluble Hb, was discarded unless from isotopically labeled growths. This cycle of resuspension, sonication, and centrifugation was repeated a second time. Next, to solubilize some impurities, the pellet was treated in 40 mL wash buffer (lysis buffer containing 0.5% Triton X-100 detergent w/v) and the mixture was centrifuged as above. The wash step was repeated once. Finally, the pellet was resuspended in lysis buffer to dilute the detergent and centrifuged at 11,000g for 10 min. Apoprotein was recovered from the washed inclusion bodies by gentle stirring in 20 mL freshly prepared solubilization buffer (8 M urea, 50 mM Tris, 1 mM EDTA, pH 8.0) for at least 30 minutes at room temperature, then centrifuged for 15 min at 31,000g. All urea solutions were highly purified immediately prior to use by percolating a concentrated stock solution through an AG 501-X8(D) mixed bed ion exchange column (1 x 25 cm, Bio-Rad, Hercules, CA) before diluting to the appropriate concentration and buffer composition.

The chilled, clarified supernatant was applied to a G-50 sizing column (2.5 x 85 cm, 4 °C) to remove remaining impurities and denaturant, and to refold the target apoprotein. Elution was performed with 50 mM Tris, 1 mM EDTA, pH 8.0, and monitored by UV-absorbance spectroscopy. Fractions were analyzed by SDS-PAGE and those containing purified apoprotein were pooled and extensively dialyzed against dd (18 MΩ cm) H2O or buffer. If in dd H2O, the pH of the dialysate was adjusted to 7.6, and the protein yield was determined by UV-absorbance spectroscopy. The protein solution was concentrated by ultrafiltration in a 200-mL Amicon cell equipped with a YM3 Diaflo® membrane (MW cut-off 3,000 Da) and either used directly or lyophilized from water.

Apoprotein Extinction Coefficient. Apoprotein solutions were prepared in 50 mM Tris/1 mM EDTA, pH 8.0 (folded) or 8 M urea in the same buffer (unfolded) to final concentrations yielding a 280-nm absorbance between 0.15 and 0.30. Absorbance spectra between 260 nm and 320 nm were collected for duplicate folded and unfolded samples of identical concentration and averaged, respectively, before correcting for background absorption. The values from different trials were reproducible and well within instrumental error and the reliability of the unfolded estimate.

Reconstitution of S6803 ApoHb with Hematin. Compared to the published procedure (1), gradient elution was unnecessary when reconstituting pure apoprotein; 0.15 M NaCl in equilibration buffer was used instead.

Heme:Protein Stoichiometry. Bovine hemin chloride was dissolved in a minimal amount of 0.1 M NaOH, diluted with water to a final concentration of approximately 1.9 mM, and filtered through a 0.2-μ pore-size membrane. The concentration was determined with subsequent hemochromogen assay (2). A 2.5 mL volume of 17.5 μM apoprotein solution in 100 mM phosphate buffer at pH 7.1 was placed in a 3-mL quartz cuvette; the reference cuvette contained the same volume of buffer only. The absorption spectrum of the solution was obtained from 250 nm to 450 nm. Increments of the hematin solution were added to both sample and reference cells. After each addition, absorbance spectra were collected until the trace showed no further
changes. The differential Soret absorbance obtained by this method is proportional to the concentration of holoprotein in the sample cell. Ferric heme addition proceeded until the peak Soret absorption no longer increased for several additions, indicating that the reconstitution of the protein was complete. The optical spectrum of the adduct at equivalence measured against buffer plus ferric heme excess is identical to that of the ferric holoprotein reported previously (1).

Figure S1: Hemin titration of S6803 apoHb. Protein concentration was 17.5 µM in 100 mM phosphate buffer, pH 7.1, 298 K.

Figure S2: $^1$H-$^{15}$N HSQC spectrum of oxidized S6803 rHb at pH 7.2, 25 °C.
Figure S3: CD spectrum of ferric S6803 rHb at 25 °C in the Soret region. The trace is the average of two spectra collected on 65 μM and 75 μM protein (per heme basis) in 20 mM phosphate buffer, pH 7.2.

Figure S4: Heme loss from oxidized S6803 rHb to equine apoMb. The absorbance at 634 nm is measured as a function of time at pH 7.2, 298 K. The solid line represents the fit to a sum of two exponential terms describing a fast process ($t_{1/2} \approx 2$ hrs) and a slower process ($t_{1/2} > 17$ hrs).