Supporting Information

The folding mechanism of an extremely thermostable $(\beta\alpha)_8$-barrel enzyme: a high kinetic barrier protects the protein from denaturation

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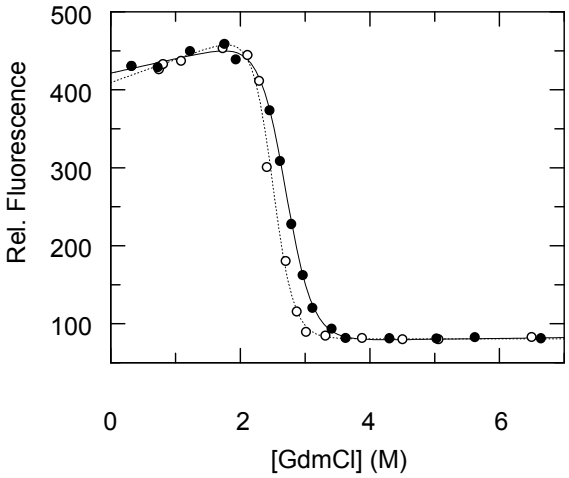

Figure S1. GdmCl-induced equilibrium unfolding and refolding transitions of HisF at 45 °C after an incubation time of 10 days. Closed symbols represent unfolding experiments started with folded protein, and open symbols represent refolding experiments started with protein that was previously unfolded in 6.0 M GdmCl. The transitions were followed by Trp/Tyr fluorescence (excitation at 280 nm; emission at 320 nm) in 50 mM Tris/HCl buffer (pH 7.5). The discrepancy between the traces is caused by the fact that unfolding has not yet reach equilibrium.

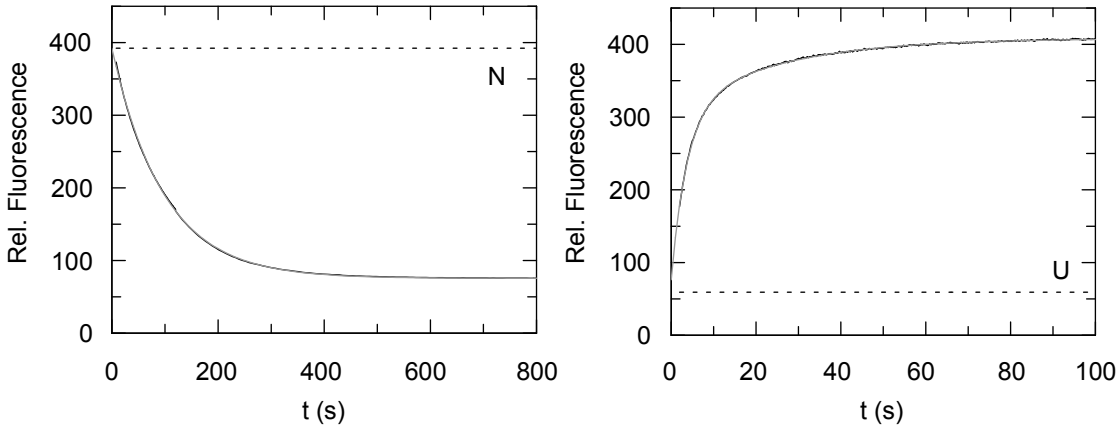

Figure S2. (a) Unfolding kinetics of HisF at 6.1 M GdmCl and (b) refolding kinetics at 0.96 M GdmCl. The traces of the Trp/Tyr fluorescence signal (excitation at 280 nm; emission at 320 nm) were monitored after manual mixing in 50 mM Tris/HCl buffer (pH 7.5) at 25 °C. Gray solid lines represent fits of monoexponential and biexponential functions to the unfolding (τ = 98 s) and refolding curves (τ_1 = 3.7 s and τ_2 = 24 s), respectively. Dashed lines indicate the initial fluorescence values of the native (N) and unfolded (U) state.

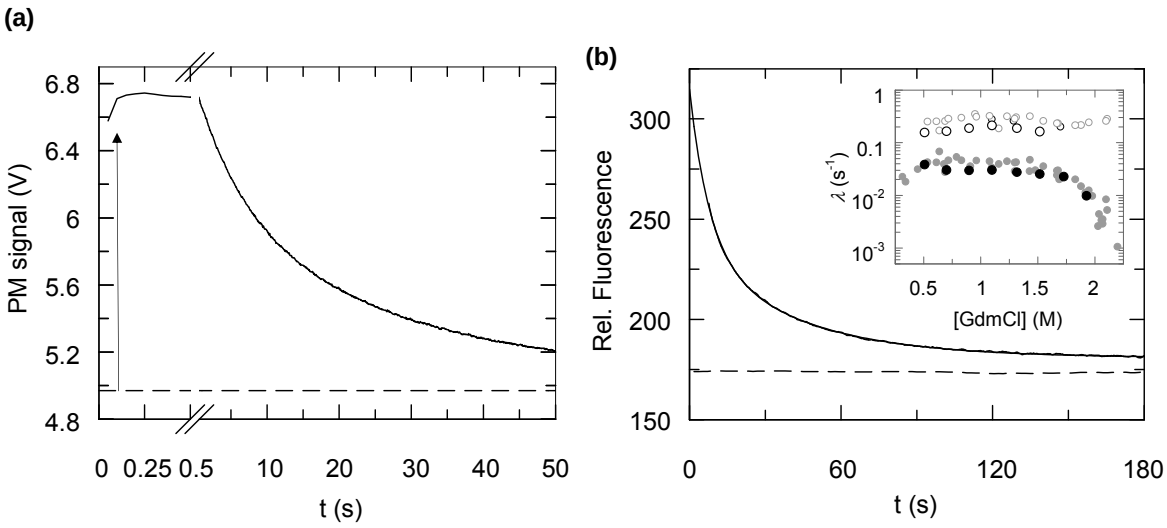

Figure S3. Refolding kinetics of HisF followed by the fluorescence signal of bound ANS. Unfolded HisF was refolded in 0.6 M GdmCl in the presence of 400 µM ANS. The ANS fluorescence in the absence of HisF is indicated as dashed lines. (a) Fluorescence traces (excitation at 350 nm; emission at > 395 nm) after stopped-flow mixing. The rapid increase of the signal (arrow) within the dead time of mixing (5 ms) demonstrates binding of ANS to hydrophobic regions of the burst-phase intermediate I_{BP}. (b) Fluorescence traces (excitation at 350 nm; emission at 479 nm) after manual-mixing. The decrease of the signal demonstrates that ANS is displaced as folding proceeds to species with less hydrophobic surfaces. Inset: rate constants of folding of HisF determined as observed by ANS fluorescence (black circles) and by CD and Trp/Tyr fluorescence (gray circles, data taken from **Figure 5a**).

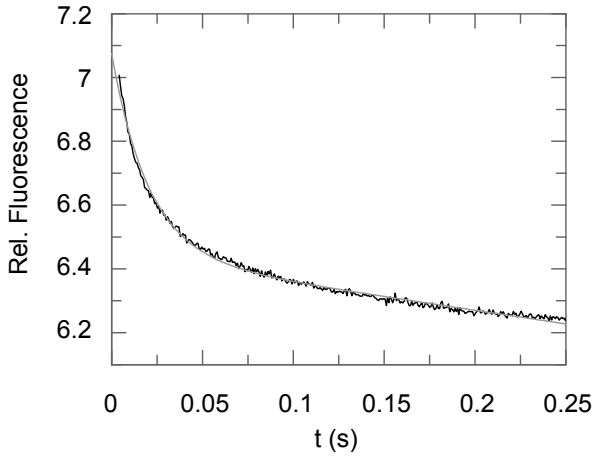

Figure S4. Analysis of the putative folding intermediate I of HisF by interrupted refolding experiments. Unfolded protein was refolded in 1.0 M GdmCl for 10 s to allow for the conversion of U to I. Then the protein was diluted to 6.0 M GdmCl, and the decay of Trp/Tyr fluorescence (excitation at 280 nm; emission at > 320 nm) was followed. The gray solid line represents a mono-exponential function and a linear term fitted to the experimental curve. With a time constant of 0.02 s the unfolding reaction is three orders of magnitude faster than unfolding of N (**Figure 5a**).

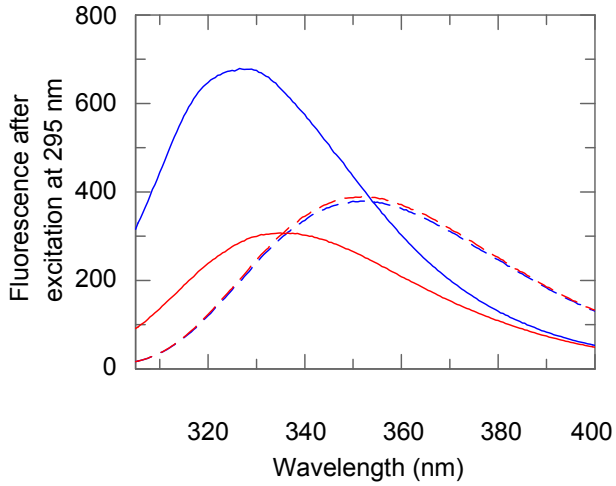

Figure S5. Fluorescence emission spectra of wild-type W156-HisF (blue) and W35-HisF (red). Spectra were recorded in 50 mM Tris/HCl buffer (pH 7.5) in absence (solid line) and presence of 6.0 M GdmCl (broken line). The fluorescence emission maxima of the native proteins (maxima: 327 and 335 nm) are red-shifted upon unfolding (maxima: 352 and 351 nm).

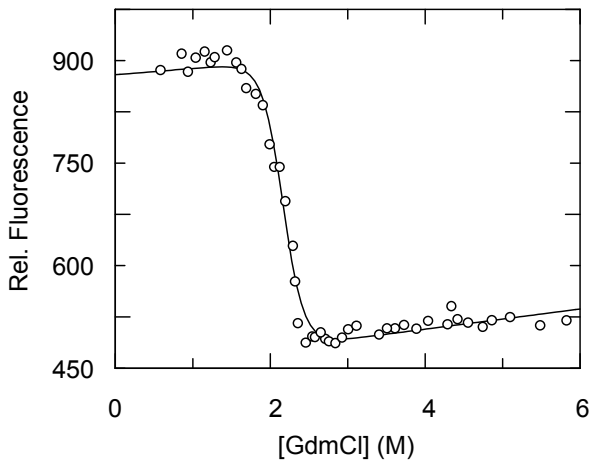

Figure S6. Equilibrium refolding transition of W35-HisF at 45 °C. The transition was followed by Tyr/Trp fluorescence (excitation at 280 nm; emission at 320 nm) in 50 mM Tris/HCl buffer (pH 7.5). The continuous line represents a fit of the two-state model to the data, yielding the following thermodynamic parameters: m = 19.7 kJ mol^{-1} M^{-1}, $\Delta G_D°$ = 42.6 kJ mol^{-1}. The corresponding values for wild-type HisF are m = 18.0 kJ mol^{-1} M^{-1} and $\Delta G_D°$ = 47.9 kJ mol^{-1} (see **Table 1**).

Table S1. Rate constants and relative amplitudes of refolding kinetics at 0.6 M GdmCl followed by fluorescence of single Trp residues at different positions in HisF.

	λ_1 (s-1)	λ_2 (s^{-1})	A_1	A_2	Position
W156-HisF	0.20	-	-1.31	0	α5
L35W-HisF	0.46	0.026	-0.28	1.97	α1
F49W-HisF	0.19	0.015	-0.52	1.68	β2
F77W-HisF	0.15	0.059	-0,26	-2.01	β3
F210W-HisF	0.33	0.057	-0,10	-0.11	α7
F214W-HisF	0.23	0.027	0.55	0.38	α7
F240W-HisF	0.17	0.015	0.16	0.34	α8

Wild-type HisF contains the single Trp156 (W156-HisF). The variants contain the indicated single Trp residues (**Figure 11a**), together with the W156Y exchange. Kinetic traces were monitored by fluorescence above 320 nm after excitation at 290 nm in 50 mM Tris/HCl buffer (pH 7.5) at 25 °C.