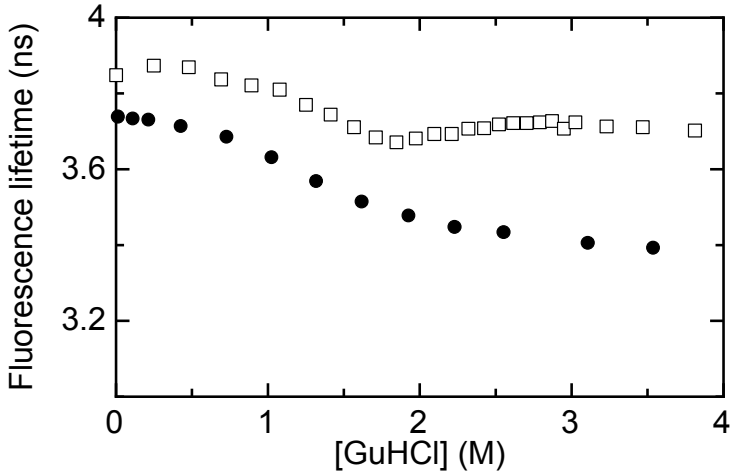
Supplementary Information

SI1. Fluorescence lifetimes of directly excited acceptor and those obtained from sensitized acceptor fluorescence in double-labeled apoflavodoxin as function of guanidinium hydrochloride (GuHCl) concentration (Figure S1).

Figure S1. Open squares represent the long fluorescence lifetime recovered from excitation of double-labeled apoflavodoxin at 450 nm and detection at 603 nm, as described in our manuscript. Black dots represent the fluorescence lifetimes recovered from excitation of apoflavodoxin that was covalently labeled with Alexa Fluor 568 on position 178. In the latter case, Alexa Fluor 568 was directly excited at 575 nm and fluorescence emission was filtered through a combination of a Balzers 635 (FWHM ~13 nm) and a Schott 610 Long Pass filter, using 4096 channels (5 ps/channel), at a temperature of 25 °C. The difference of 0.2–0.3 ns between the fluorescence lifetimes can be attributed to thermal quenching [36] of the fluorophore, because the FRET experiments were conducted at 20 °C.



SI2. A three state-model for apoflavodoxin folding describes the denaturant-dependence of the donor fluorescence lifetime without acceptor (τ_d).

Amplitude-averaged decay times τ_d data are taken from [8]. The following linear three-state model for protein folding and associated equations are used to describe τ_d as function of [GuHCl] [24]:

$$I \rightleftharpoons U \rightleftharpoons N \tag{S1}$$

$$K_{UI} = [I]/[U],\ K_{IN} = [N]/[I] \tag{S2}$$

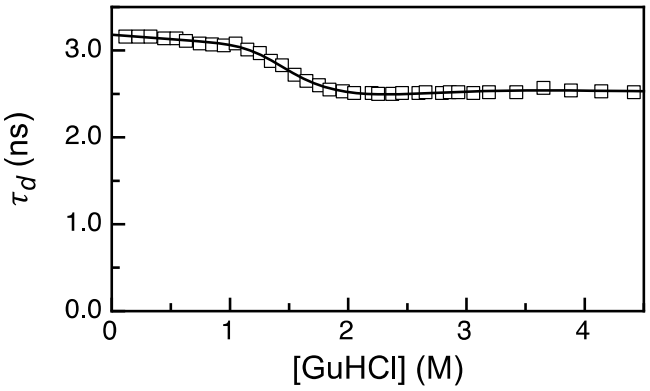
$$K_{ij}([D]) = K^{0}_{ij}\exp(m_{ij}[D]) \tag{S3}$$

$$\begin{aligned} f_U &= 1/(1 + K_{UI} + K_{UI}K_{IN}) \\ f_I &= K_{UI}/(1 + K_{UI} + K_{UI}K_{IN}) \\ f_N &= K_{UI}K_{IN}/(1 + K_{UI} + K_{UI}K_{IN}) \end{aligned} \tag{S4}$$

$$Y^{obs} = (a_U + b_U[D])f_U + (a_I + b_I[D])f_I + (a_N + b_N[D])f_N \tag{S5}$$

in which *I*, *U* and *N* represent off-pathway folding intermediate, unfolded and native apoflavodoxin, respectively, K_{ij} is the equilibrium constant of the i–j equilibrium, m_{ij} is the constant that describes the denaturant concentration-dependence of K_{ij}, superscript 0 designates the parameter at zero denaturant concentration, [*D*] is the concentration of denaturant, f_i is the fractional population of state I at a certain denaturant concentration, Y^{obs} is the observed spectroscopic signal parameter, a_I is the spectroscopic property of state I at zero denaturant concentration, and b_i describes the denaturant concentration-dependence of a_I. In order describe the amplitude-averaged decay times of the donor (τ_d) as function of GuHCl concentration, parameters ΔG_{ij}^0 and m_{ij}^0 as published in [8] were fixed in the analysis to extract the values of a_I and b_I of each folding state. The result of this fit is shown in Figure S2.

Figure S2. Denaturant-dependence of amplitude-averaged decay time τ_d. The model of Equations (S1)–(S5) is fitted to the average fluorescence lifetime of Alexa Fluor 488 (open squares; [8]) and describes the dependence of τ_d on [GuHCl] (black line).



SI3. Estimation of Förster distances of dye-labeled apoflavodoxin along its unfolding trajectory.

Let us summarize the four contributing factors to the Förster distance, R_0, between donor and acceptor dye molecules during unfolding. These factors have been discussed previously [11,16], and are summarized below. R_0 (in Å) is given by:

$$R_0 = 0.2108\left(\kappa^2 n^{-4} J Q_d\right)^{1/6} \tag{S6}$$

where κ^2 is the orientation factor between transition dipole moments, which can take values between 0 and 4, *n* is the refractive index of the medium between both dye molecules in the wavelength range of the spectral overlap integral *J* (in units of $M^{-1}\cdot cm^{-1}\cdot nm^4$) and Q_d is the fluorescence quantum yield of the donor in the absence of energy transfer.

As first approximation the orientation factor can be set to the dynamic averaging limit of 2/3 for the whole denaturation trajectory, since both donor and acceptor dyes are flexibly bound to apoflavodoxin, exhibiting reorientation rates in the range of 0.1–0.2 $(ns)^{-1}$ [22]. Although the reorientation rates are somewhat smaller than the transfer rates of 1.3–0.12 $(ns)^{-1}$, $\kappa^2 = 2/3$ is a reasonable approximation.

The refractive indices are more difficult to assess. Knox and van Amerongen show that the refractive index used in the Förster equation corresponds to the refractive index through which energy is transferred [38]. This would imply for folded protein that both Alexa labels sense a combination of refractive index of buffer (n = 1.337) and of folded protein (n = 1.60, [37]). Hence, the medium

between the Alexa dyes is heterogeneous with respect to refractive index. During unfolding the refractive index of buffer increases, for instance n = 1.389 at 3 M GuHCl and n = 1.451 at 6.9 M GuHCl [22]. Thus, upon adding denaturant the refractive index of the medium between the dye labels attached to the protein will decrease and finally adopts the value of the buffer. Previously, we reasoned that a value of ~1.5 would be a good estimate [22] of the refractive index at all denaturant concentrations. We used n = 1.45 for the calculation of R_0.

The spectral overlap integral (J) of labeled apoflavodoxin in buffer compared to protein in 6.9 M GuHCl shows a 14% increase, leading to a negligible increase of 2.3% in R_0 (from 53.1 to 54.3 Å).

The fluorescence quantum yield of the donor (Q_d) fluorescence is an important determinant of R_0 that is easy to assess. It has been found previously that fluorescence of A488-apoflavodoxin quenches upon unfolding of the protein [8]. This quenching has static and dynamic components, and is probably due to interaction of the donor dye with Trp74. The tertiary structure of the native protein prevents this interaction. However, upon unfolding Trp74 becomes exposed to transient interactions with A488 that is attached to Cys69. Dynamic quenching of the fluorophore introduces another non-radiative decay path of the excited state of the fluorophore that competes with the FRET process. Hence, this dynamic quenching results in a decrease of Q_d. The fluorescence lifetime of A488-apoflavodoxin is directly proportional to the quantum yield of fluorescence, for which Q_d = 0.84 for the protein in buffer [25]. This enables us to evaluate all quantum yields and thus all Förster distances for doubly labeled apoflavodoxin in the denaturation trajectory. The Förster distances in the denaturation trajectory are presented in Figure 4 of the main text. The first point in Figure 4 gives a value R_0 = 53 Å, whereas the last point yields R_0 = 51 Å.