Fluorescence resonance energy transfer analysis of the folding pathway of Engrailed Homeodomain

Fang Huang, Giovanni Settanni and Alan R. Fersht

Medical Research Council Centre for Protein Engineering and Cambridge University Chemical Laboratory, Hills Road, Cambridge CB2 0QH, UK

The Engrailed Homeodomain folds on the microsecond time scale via an intermediate that is experimentally well characterised using structural Engrailed–Homeodomain mimics. Here, we analysed directly the changes in distance between key residues during the kinetics of unfolding and at equilibrium using fluorescence resonance energy transfer (FRET). Trp was the donor and 5-(((acetylamino)ethyl)amino) naphthalene-1-sulphate, the acceptor, substituted in positions that caused little change in stability. Distances calculated for the native state were in good agreement with those derived from the NMR structure. The distances between the N- and C-termini of Helix I and of Helix III increased, then decreased and finally increased again with increasing GdmCl concentration on equilibrium denaturation. This behaviour implied that there was a folding intermediate on the folding pathway and that this intermediate was populated at low concentrations of GdmCl (~1 M). We analysed the changes in distance during temperature-jump relaxation kinetics, using a qualitative and very conservative procedure that drew conclusions only when changes in fluorescence of mutants containing either the donor or the acceptor alone would not obscure the change in the FRET signal when both donor and acceptor were present. The distance changes obtained under equilibrium and kinetic measurements were self-consistent and also consistent with the known high-resolution structures of the mimics of the folding intermediates. We showed that for analysing distances in disordered ensembles, it is important to use FRET probes with a critical distance close to the average separation in the ensemble. Otherwise, average distances could be over or underestimated.

Keywords: EnHD/folding/FRET/protein

Our strategy for solving the pathways of protein folding is to characterise the structures of all the ground and transition states as far as possible experimentally, determine the rate constants for their interconversion and combine the experimental data with atomistic simulation. The Engrailed Homeodomain (EnHD) has proved to be a very tractable system for such studies (Mayor et al., 2000, 2003a, 2003b; Religa et al., 2005, 2007; DeMarco et al., 2004; Hubner et al., 2006). This DNA binding protein is a three-helix bundle with 61 residues (Clarke et al., 1994). Most importantly, it folds via a compact intermediate that has been well characterised experimentally. Its structure was possible to solve because its L16A mutant exists at physiological ionic strength as the denatured state, which is, in fact, a well-structured folding intermediate as the more unfolded states are at higher energy (Religa et al., 2005). The NMR structures of EnHD L16A mutant and a fragment containing Helix II, turn and Helix III (an HTH motif) have been solved, and the HTH motif is an ultrafast independently folding domain (Religa et al., 2005, 2007). In L16A, Helix II (residues 28–37) and Helix III (residues 42–56) are similar to those in the native state but Helix I (residues 10–22) moves away. Here, we have used an additional series of lower resolution experiments to probe the folding pathway, based on fluorescence resonance energy transfer (FRET). These experiments were designed to measure changes in distance between key residues during the kinetics of folding and at equilibrium.

FRET is an invaluable tool for the assessment of distances in biomolecules (Stryer, 1978; Lakowicz, 1999). Although intrinsic fluorescent amino acids, such as Trp and Tyr, may be applied as probes to investigate protein folding kinetics and thermodynamics, they are sensitive only to the local environment rather than the global structural change. FRET has its distinct strength in providing structural information because of the strong dependence of FRET efficiency on distance, which allows measurement of the distance between two residues in a protein monitoring of conformational changes upon denaturation and intramolecular fluctuations (Magg and Schmid, 2004; Haas, 2005; Magg et al., 2006). In principle, FRET could be applied to investigate the distance between any residues in a protein and accordingly can provide a distance map for a protein; it can also monitor the distance change during relaxation. For these reasons, FRET has been widely applied in the studies of protein folding and related fields (Nishimura et al., 2000; Weiss, 2000; Schuler et al., 2002; Teilmann et al., 2002; Magg and Schmid, 2004; Sridevi et al., 2004; Haas, 2005; Magg et al., 2006; Huang et al., 2007).

To investigate protein folding with FRET, particular attention should be paid to the effects of the introduced fluorophore. In general, the selection of FRET donor and acceptor is subject to the following criteria: (i) the introduced fluorophores should impose minimum effects on the structure, stability and folding kinetics of the target protein, which

Abbreviations: AEDANS, 5-(((acetylamino)ethyl)amino) naphthalene-1-sulphate; CD, circular dichroism; [D]$_{20s}$, the denaturant concentration at which the protein is 50% denatured; GdmCl, guanidinium chloride; IAEDANS, 5-(((2-iodoacetyl)amino)ethyl)amino) naphthalene-1-sulphate; $l_{ex}$, the fluorescence intensity with the excitation and emission polarizer at vertical and horizontal orientation, respectively; $l_{em}$, the fluorescence intensity with both the excitation and emission polarizer at vertical orientation; LED, light emitting diode; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; $T_m$, Förster critical distance, T-jump, temperature-jump; $T_m$, melting temperature.

© 2008 The Author(s)

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
requires the fluorophores either to be natural amino acids or their analogues, or extrinsic fluorophores with small size and weak hydrophobicity; (ii) the fluorophores can be introduced into a protein conveniently and site-specifically; (iii) the critical distance ($R_0$) for the donor/acceptor pair should be compatible with the distance ($R$) to be measured, i.e. $R/2 < R_0 < 2R$. In this work, to fulfil the criteria, the natural amino acid Trp was selected as energy donor and an extrinsic fluorophore with small size, 5-(((2-iodoacetyl)amino)ethyl)amino) naphthalene-1-sulphonic acid (1,5-IAEDANS), was introduced as the energy acceptor through Cys. This donor/acceptor pair has proved to be a very good FRET system for small proteins (Magg, 2004; Magg et al., 2006). Its critical distance of $\sim 22$ Å suits very well the dimensions of Drosophila melanogaster EnHD.

We applied FRET measurements in equilibrium and fast kinetic studies on the folding and unfolding of EnHD. Eight mutants were designed, where the FRET donor and acceptor were separated by Helix I, Helix II, Helix III, Helices I and II, Helices II and III or Helices I, II and III as shown in Fig. 1. This allowed us to monitor changes of all the key distances determining the framework of the protein. The influence of introduced AEDANS on the protein’s structure, stability as well folding kinetics was checked with different techniques. Significant interaction between AEDANS and the labelled protein was not observed, which renders it unlikely that the labelled mutants have different folding pathways from that of the unlabelled protein. Structural changes of the proteins were also investigated under different conditions. We found that the distance change in EnHD was very anisotropic. The distance between residues separated by two or three helices increased upon denaturation; however, the distance between residues separated by only one helix could give shorter distances upon denaturation. This inconsistency of distance change indicated the existence of an intermediate on the folding pathway. Studies on thermostability suggested that the folding intermediate was stabilized at low concentration of GdmCl relative to the native state.

**Materials and methods**

**Protein expression, purification and labelling**

Expression and purification of EnHD have been described elsewhere (Ades and Sauer, 1994; Mayor et al., 2000). In brief, the EnHD mutants were overexpressed in a pSEA100 vector in C41(DE3) Escherichia coli cells at 37°C and purified on ion-exchange columns followed by HPLC. All the mutants were based on the pseudo-wild-type EnHD K52A W48F. Tyr at position 25 was mutated with Phe if it was not replaced with Trp or Cys.

Trp acts as fluorescence donor. AEDANS acts as acceptor, and was covalently labelled to proteins through Cys by forming an S-C bond. Protein labelling was carried out in 50 mM Tris buffer (pH 7.4), where protein concentration was kept at 50–100 μM and 4-fold tris(2-carboxyethyl)phosphine (TCEP) was added and incubated for 10 min. Ten-fold IAEDANS was added and the mixture was shaken at room temperature for $\sim 2$ h. The progress of labelling reaction was followed by MALDI-TOF MS. After labelling, the reactive dye was quenched with 20-fold $\beta$-mercaptoethanol and separated from the labelled protein on HPLC.

**Fluorescence and absorption spectroscopy**

Steady-state fluorescence spectroscopy and anisotropy data were acquired on a fluorometer (PTI QM-7, Photon Technology International, Birmingham, NJ, USA). The typical concentration for steady-state fluorescence was 10 μM. Fluorescence anisotropy was measured at 360 nm with excitation wavelength of 295 nm. Time-resolved fluorescence measurements were carried out on a single photon counting setup (LifeSpec-ps, Edinburgh Instruments, Edinrburgh, UK) with a LED featuring $\sim 500$ ps pulse width at 282 nm. The typical concentration for time-resolved fluorescence experiments was 50 μM. The $I_{VV}$ and $I_{HL}$ were acquired at the same period of time; the corresponding instrument response function was obtained by measuring scattering light of Ludox solution excited at 282 nm in the presence of polarizer. The time-resolved fluorescence anisotropy signal was then calculated from deconvoluted $I_{||}$ and $I_{\perp}$, which are the fluorescence intensities with the excitation and emission polarisers in parallel and perpendicular positions, respectively, and corrected with the ratio of the sensitivities of the detection system for vertically and horizontally polarized light (G-factor), and fit with exponential equation (cf. Data analysis). Absorption spectroscopy was collected on Cary 4000 UV–Vis spectrophotometer (Varian, Inc., Palo Alto, CA, USA).

**Equilibrium titration**

In chemical denaturation experiments, both fluorescence and CD signals of 5 μM protein were acquired at 25°C on an Aviv spectrometer (Aviv Associates, Lakewood, USA). CD signals were collected at 222 nm. The donor was excited at 280 nm and fluorescence from the donor and acceptor was selectively collected by using a 360 nm bandpass filter (UG1, Comar) and a 495 nm long-pass filter (GG495, Comar), respectively. The distance change during chemical denaturation was also confirmed by measuring fluorescence intensity of Trp on a PTI QM-7 fluorometer and Fluormax (Horiba Jobin Yvon, North Edison, NJ, USA). Experiments on the temperature dependence of CD signal were carried out on a J-815 CD Spectrometer (JASCO, Tokyo, Japan).

**Kinetics**

Kinetics was measured on a modified Hi-Tech PTJ-64 temperature-jump apparatus with a $5 \times 5$ mm cell with 280 nm (for the excitation of the donor) or 336 nm (for the
excitation of the acceptor) band-pass filters for excitation light and 360 nm band-pass filter and 495 nm long-pass filter for fluorescence from the donor and acceptor, respectively. Protein concentration was in the range of 20–150 μM in NaAc buffer (50 mM pH 5.7) plus 100 mM of NaCl. EnHD mutants were either measured in NaAc buffer (pH 5.7, 50 mM NaAc and 100 mM NaCl) at 50 °C or in 2 M GdmCl (buffered to pH 5.7 with 50 mM NaAc and 100 mM NaCl) at 25 °C. One millimole of 1,4-dithiothreitol (DTT) was added in all the experiments for unlabelled proteins.

**Data analysis**

In chemical denaturation experiments, distances between donor and acceptor were calculated at each GdmCl concentration. The distances were obtained via the following steps: (1) the quantum yield of Trp in each mutant in buffer was determined by fluorescence lifetime according to \( \Phi_{\text{Trp}} = \frac{i_{\text{Trp}}}{i_{\text{NATA}}} \), where \( i \) is the average lifetime of Trp and \( \Sigma \alpha_i \tau_i \) for multiple exponential decay; (2) the fluorescence quantum yield of the unlabelled protein (i.e. without AEDANS) in GdmCl solution was calculated according to the fluorescence intensity in GdmCl solution, the fluorescence quantum yield of this protein in buffer and the concentration of GdmCl; (3) energy transfer efficiency was calculated according to the Trp fluorescence intensity of the acceptor-labelled and -unlabelled protein at the same concentration with equation \( E = \frac{i_{\text{unlabelled}} - i_{\text{labelled}}}{i_{\text{unlabelled}}} \) or Trp fluorescence lifetime with equation \( E = \frac{i_{\text{unlabelled}} - i_{\text{labelled}}}{\tau_{\text{unlabelled}}} \); (4) the critical distance for Trp/AEDANS at different conditions was calculated with \( R_0 = 22(1.3346^2 \rho / (0.16n^2))^{1/6} \) and (6) the distance between donor and acceptor was calculated according to the energy transfer efficiency and critical distance \( R = (1/E - 1)^{1/6} R_0 \).

To get the rotation rate of Trp, the time-resolved polarized fluorescence \( I_{\text{CV}}(t) \) and \( I_{\text{VH}}(t) \) signals were globally fitted to Eq. (6a and b), respectively.

\[
I_{\text{CV}}(t) = G \cdot I_1(t) \tag{6a}
\]

\[
I_{\text{VH}}(t) = I_1(t) \tag{6b}
\]

where \( G = I_{\text{VH}}/I_{\text{HH}} \) is the G factor, \( I_1 \parallel \) and \( I_1 \perp \) can be described with Eqs. (7) and (8).

\[
I_1(t) = L_\parallel \ast i_1 \perp \quad \tag{7a}
\]

\[
I_1(t) = L_\parallel \ast i_1 \perp \quad \tag{7b}
\]

\[
i_1(t) = \frac{1}{3} i(t)(1 + 2r(t)) \tag{8a}
\]

\[
i_1(t) = \frac{1}{3} i(t)(1 - r(t)) \tag{8b}
\]

where \( i(t) \) is the total intensity; \( r(t) \), the anisotropy; \( L_\parallel \) (t) and \( L_\perp \) (t) are the instrument response functions with perpendicular and parallel polarization direction, respectively. The asterisk symbol indicates the convolution operation. \( i(t) \) has been approximated as a triple exponential function, and \( r(t) \) as a single exponential function. Fits were obtained by minimising the sum of squared errors using the Levenberg–Marquardt algorithm.

**Molecular dynamics simulation**

MD simulations of the EnHD D10A25 have been performed based on a model of the NMR structure of EnHD WT (T. Religa, personal communication) for the initial conformation. Simulations have been carried out using the GROMACS package (Lindahl et al., 2001) and the OPLSAA force field (Jorgensen et al., 1996). TIP4P model (Jorgensen et al., 1983) has been used for the water. The molecule has been immersed in a periodic box of 5278 water molecules and 7 Cl- ions have been added to neutralise the overall charge of the system. The dimensions of the initial box (5.25 nm × 5.41 nm × 5.99 nm) have allowed for at least 1 nm distance between the protein and the box boundaries. A cut off of 1.0 nm has been used for the calculation of the non-bonded interactions and PME has been used for the treatment of the long range electrostatic interactions. A time step of 1 fs for the integration of the equations of motion has been used. Temperature and pressure have been controlled by the Berendsen algorithm (Berendsen et al., 1984) with 0.1 and 0.5 ps coupling constants, respectively. The pressure has been kept constant at 1 atm. Before the production run, the system has been minimised using steepest descent for 1000 steps and conjugate gradient for other 1000 steps. Then, relaxation of the solvent molecules has been performed for 10 ps. The latter has been followed by an equilibration of the whole system carried on for 0.5 ns letting the temperature gradually increase from 3–300 K. Eventually, atom coordinates and velocities from the last equilibration snapshot have been used to start the production run. The latter consists of 50 ns of trajectory at 300 K and 1 atm where atom coordinates and velocities have been saved every 10 ps.

The anisotropy decay \( r(t) \) of a fluorophore is given by:

\[
r(t) = r_0 (P_2(\mu_a(0) \cdot \mu_2(t)))
\]

\[
\approx 2/5 P_2(\cos \beta)(P_2(\mu_a(0) \cdot \mu_2(t)))
\tag{9}
\]

Where \( \mu_a(0) \) is the normalized absorption dipole moment at time 0 and \( \mu_2(t) \) is the normalized emission dipole moment at time \( t \). \( P_2(\beta) = (3\beta^2 - 1)/2 \) is the second-order Legendre polynomial and \( \beta \) is the angle between the emission and absorption dipole moments on the molecule. The \( \langle \cdot \rangle \) symbol indicates the average over the ensemble of molecules, which, in the present case, has been approximated by a time average along the simulation. The fluorescent moieties in the construct are the Trp side chains at positions 10 and 25 along the sequence. The absorption of Trp is assumed to mainly occur along the \( 1^{\text{En}} \) transition dipole, whose direction, in the present case, has been approximated by the vector connecting the NE1 atom and the midpoint between the CZ3 and CE3 atoms on the Tryptophan side chains (Callis, 1997). The wobbling-in-a-cone model used to fit protein-frame anisotropy decays provides a relation between the maximal angle \( \theta_{\text{max}} \) spanned by the transition dipole and the residual
anisotropy at large times $A_\infty$.

$$A_\infty = \frac{(1 + \cos \theta_{\max}) \cos \theta_{\max}}{2}$$ (10)

**Results**

To avoid the interference of fluorescence from Tyr in the experiments, Tyr was replaced with Phe where it was appropriate. Trp at position 48 in the wild-type EnHD was not used as FRET donor, since its fluorescence is significantly quenched. To ensure the mutants were sufficiently stable, all were based on the K52A pseudo-wild-type, a stabilized mutant (Mayor et al., 2003). The positions of donor and acceptor and the distances investigated are schematically shown in Fig. 1 and the codes and mutations of the mutants are as follows:

- D58A25 Y25C W48F K52A K58W
- D25A10 S10C Y25W W48F K52A
- D10A25 S10W Y25C W48F K52A
- D25A41 Y25W N41C W48F K52A
- D58A10 S10C Y25F W48F K52A K58W
- D58A41 Y25F N41C W48F K52A
- D25A41 Y25F N41C W48F K52A
- D10A41 S10C Y25W W48F K52A
- D8A41 F8W Y25F N41C W48F K52A

D and A represent donor and acceptor, respectively. To check the position dependence of the FRET results, the donor/acceptor-exchanged mutant (D25A10→D10A25) was also investigated. The donor/acceptor-exchanged mutant gave very similar results, which suggests that the results measure the distance between donor and acceptor rather than the effects of local environment on the donor and acceptor. The stability and folding kinetics of each mutant were also studied with CD and fluorescence to check for perturbation by the introduced fluorophore.

**Fluorescence spectroscopy**

The donors and acceptors were introduced at flexible and solvent-exposed regions, either in the loops between helices or at the unstructured N- or C-terminus (Fig. 1). This design facilitated the exposure of fluorophores to solvent, which not only minimizes the possibility of strong interactions between fluorophore and protein and reduces the influence of the fluorophore on the structure and stability of labelled proteins, but also prevents significant local changes in environment or shifts of spectra upon denaturation. All the mutants under conditions favouring folding had a fluorescence emission peak at ~345 nm in the absence of FRET (spectra not shown). This peak shifts only up to 5 nm from ~345 to 350 nm upon the transition from native state to denatured state (from 0 to 6 M GdmCl). This very small change of fluorescence spectrum does not affect much the overlap of the emission spectrum of Trp and the absorption spectrum of AEDANS or the critical distance of Trp/AEDANS donor/acceptor pair. For example, the spectral overlap ($J$) for Trp in D25A10 and AEDANS in 0 and 6 M GdmCl is 5.65 $\times$ 10$^{-13}$ and 5.48 $\times$ 10$^{-13}$ M$^{-1}$ cm$^{-1}$ nm$^{-2}$, respectively, which results in only a 0.5% decrease of the critical distance over the whole range of GdmCl concentration. Since the change of spectral overlap affects the critical distance only negligibly, the spectral overlap was not recalculated in chemical denaturation experiments.

The fluorescence quantum yield of Trp in each mutant was determined with the average fluorescence lifetime. The determination of fluorescence quantum yield with fluorescence lifetime can avoid the error introduced by measurements of concentration since fluorescence lifetime is independent of concentration. For all the donor-only mutants, the time-resolved fluorescence decay of Trp in native state can be fitted to a bi-exponential equation. The average fluorescence lifetimes (cf. Data analysis) of Trp in donor-only mutants are listed in Table I. With the fluorescence lifetime and quantum yield of N-acetyl-tryptophanamide (NATA) as reference, we calculated the fluorescence quantum yield of Trp in all the mutants (Table I). The different fluorescence lifetime and quantum yield indicates the different local environment of Trp. Its longer fluorescence lifetime than that of NATA also suggests that the Trp is partially protected by the protein, which does not mean that the introduced Trp affects the structure or stability of the mutant significantly, as can be seen below. Owing to the change of fluorescence quantum yield, the critical distance is longer than that for NATA and AEDANS (Table I). The critical distances in present work were calculated with the assumption $\kappa^2$=2/3 (see below for detailed discussion) and calculated with Eq. (1).

$$R_0 = 0.211(\kappa^2n^{-4}Q_DJ(\lambda))^{1/6}$$ (1)

where $\kappa^2$ is the orientation factor; $n$, the refractive index; $Q_D$, the fluorescence quantum yield in the absence of FRET and $J$, the spectral overlap. As shown in Table I, the critical distance for all the mutants in native state in buffer is in the range of 23–24 Å, a little longer than the $R_0$ for NATA/AEDANS. The distances to be measured range between 11 and 26 Å in protein native state, which are in the very sensitive distance range for the Trp/AEDANS FRET pair.

**Tryptophan dynamics**

The orientation factor ($\kappa^2$) is normally assumed to be 2/3, which is the value for a system with donor and acceptor randomly rotating before energy transfer, in the calculation of critical distances (Lakowicz 1999). Significant error might be introduced, if the orientation of the transition dipoles is fixed. The selection of $\kappa^2$ value is therefore a major concern in the application of FRET to measure donor/acceptor distance (Haas et al., 1978). To check that 2/3 can be applied here, we measured steady-state fluorescence anisotropy of Trp in each mutant in its native state. The anisotropy values of Trp in all the mutants were in the range of 0.039–0.052 (Table I). Assuming that the local rotation of Trp can be neglected, with the known global rotation rate of EnHD obtained from NMR backbone dynamics measurement ($1.5.0$ ns$^{-1}$ at 25°C, T Religa, personal communication), the fundamental anisotropy of Trp (0.26, excited at 295 nm) (Lakowicz et al., 1983) and the fluorescence lifetime of Trp ($\sim5$ ns), an anisotropy of 0.13 is expected according to the Perrin equation (Lakowicz, 1999). Smaller anisotropy values of 0.039–0.052 indicated a fast local rotation of Trp during its excited lifetime and support the assumption of 2/3 for $\kappa^2$ for folded proteins. In a denatured protein, the free-rotation assumption is true in general because the flexible polypeptide linking the donor and acceptor can further facilitate the orientation freedom of the donor and acceptor.
Table I. Fluorescence properties of Trp and donor–acceptor distance

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fluorescence lifetime of Trp</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unlabelled (ns)</td>
<td>4.4</td>
<td>5.1</td>
<td>2.8</td>
<td>2.1</td>
<td>1.9</td>
<td>1.9</td>
<td>1.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Labelled (ns)</td>
<td>5.1</td>
<td>2.1</td>
<td>1.9</td>
<td>1.9</td>
<td>1.6</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fluorescence quantum yield</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of Trp in unlabelled mutant&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.26 ± 0.01</td>
<td>0.23 ± 0.01</td>
<td>0.27 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.23 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td>0.25 ± 0.01</td>
<td></td>
</tr>
<tr>
<td><strong>Fluorescence anisotropy of unlabelled mutant</strong></td>
<td>0.052 ± 0.005</td>
<td>0.045 ± 0.005</td>
<td>0.039 ± 0.005</td>
<td>0.039 ± 0.005</td>
<td>0.048 ± 0.005</td>
<td>0.063 ± 0.005</td>
<td>0.049 ± 0.005</td>
<td></td>
</tr>
<tr>
<td><strong>Critical distance in buffer R&lt;sub&gt;c&lt;/sub&gt;(Å)</strong></td>
<td>23.0 ± 1</td>
<td>23.9 ± 1</td>
<td>24.0 ± 1</td>
<td>23.0 ± 1</td>
<td>23.4 ± 1</td>
<td>23.5 ± 1</td>
<td>23.8 ± 1</td>
<td></td>
</tr>
<tr>
<td>WT NMR D/A distance (Å)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.4</td>
<td>20.4</td>
<td>16.3</td>
<td>25.8</td>
<td>26.0</td>
<td>16.6</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td>L16A NMR D/A distance (Å)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23.4</td>
<td>22.8</td>
<td>18.0</td>
<td>23.0</td>
<td>24.6</td>
<td>37.0</td>
<td>37.2</td>
<td></td>
</tr>
<tr>
<td><strong>FRET D/A distance (Å)</strong>&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steady-state fluorescence</td>
<td>19 ± 1</td>
<td>22 ± 1</td>
<td>22 ± 1</td>
<td>18 ± 1</td>
<td>22 ± 1</td>
<td>25 ± 1</td>
<td>20 ± 1</td>
<td>19.0 ± 1</td>
</tr>
<tr>
<td>Time-resolve fluorescence</td>
<td>21 ± 1</td>
<td>23 ± 1</td>
<td>26 ± 1</td>
<td>22 ± 1</td>
<td>23 ± 1</td>
<td>22 ± 1</td>
<td>21 ± 1</td>
<td>17.3 ± 1</td>
</tr>
<tr>
<td>Kinetics 50°C</td>
<td>Increase</td>
<td>Increase–decrease–increase</td>
<td>Increase–decrease–increase</td>
<td>Decrease–increase</td>
<td>Increase</td>
<td>Increase–decrease–increase</td>
<td>Increase</td>
<td>Increase</td>
</tr>
<tr>
<td>Kinetics 25°C 2M GdmCl</td>
<td>Increase</td>
<td>NA</td>
<td>NA</td>
<td>Increase</td>
<td>NA</td>
<td>NA</td>
<td>Increase</td>
<td>Increase</td>
</tr>
</tbody>
</table>

<sup>a</sup>Average lifetimes were calculated with equation \( \tau = (B_1 \tau_1 + B_2 \tau_2)/(B_1 + B_2) \), where \( B_1 \) and \( B_2 \) are the amplitude of \( \tau_1 \) and \( \tau_2 \), respectively. The numbers contain 5% error.

<sup>b</sup>Fluorescence quantum yield was calculated with NATA as reference, which has lifetime of 3.0 ns and fluorescence quantum yield of 0.16 in NaAc pH 5.7 buffer (This work, measured with quinine sulphate as standard).

<sup>c</sup>Measured by steady state fluorescence.

<sup>d</sup>They have poor fitting quality with \( \chi^2 > 2.3 \) and are not considered.

<sup>e</sup>The distances are those between the Cα of labelled residues in the NMR structure.
Tryptophan dynamics was investigated with both fluorescence anisotropy measurements and molecular dynamics (MD) simulation. An example of fitting the time-resolved fluorescence anisotropy is shown in Fig. 2. The time-resolved fluorescence anisotropy provides rotational relaxation rates and the limit of anisotropy \( r(0) \), the anisotropy at time zero (the maximal value). Faster relaxation processes were not directly observed. However, the fit of the time-resolved fluorescence anisotropy gave the limit of anisotropy of 0.045–0.065, which was much smaller than the fundamental anisotropy, which is 0.18 for NATA when excited at 287 nm and is likely to be greater than 0.15 when excited at 282 nm (Pierce and Boxer, 1995). The small values \( r(0) \) are strong evidence for the existence of a fast process that could not be resolved because of the time resolution of the system (Lakowicz, 1999). These results indicated that, apart from the tumbling of the whole protein on a few nanosecond time scale, the Trp could rotate at a much faster rate. This is in very good agreement with the MD simulation results.

Fluorescence anisotropy decay obtained from MD simulation for mutant D10A25 with Cys25 mutated to Trp is shown in Fig. 3. The anisotropy decay associated with the two tryptophan residues present in the constructs was computed from the simulation data. In addition, in order to separate the contribution to the anisotropy decay due to intra-molecular relaxation of the tryptophan side chains, another decay curve was computed where the component due to the tumbling of the whole protein had been removed. These data showed that the flexibility of the Trp 25 side chain relative to the protein frame accounted for 15% of the total expected anisotropy decay, although most of it was governed by tumbling motions of the protein in solution. The internal fluctuations in this case provided a stretched exponential relaxation of the anisotropy in the picosecond time scale and fitted a wobbling-in-a-cone model (Schröder et al., 2005) with a cone angle of 19°. In the case of the Trp10, instead, the internal fluctuations of the side chain had a more complex relaxation profile that could be fitted with a cone-in-a-cone model (Schröder et al., 2005). The faster fluctuations provide 33% of the total anisotropy decay and occur on the 20 ps time scale, corresponding to a cone of 26° spanned by Trp10 transition dipole. The fast protein-frame relaxations, multiplied by the relaxations due to the tumbling of the protein provided the total lab-frame anisotropy decay (Lakowicz, 1999). Thus, it was possible to separate the anisotropy decay contribution due to the tumbling of the protein by dividing the total anisotropy decay by the protein-frame relaxation. An exponential fit of the protein tumbling anisotropy decay from simulations provided two relaxation times for both Trp transition dipole moments (Table II). The fast phase was on the 1 ns time scale for both the Trp side chains. The slower phase was faster for Trp 10 (3.9 ns) than for Trp 25 (7.8 ns). The different tumbling relaxation time scales were probably related to the anisotropic rotational diffusion constants around the moments of inertia of the protein.

The isotropic parameter \( \kappa^2 \) that governs the efficiency of the FRET depends on the relative orientation of the emission and absorption dipoles of the donor and acceptor (Lakowicz, 1999) and can be described as \( \kappa^2 = (\cos \theta_D - \cos \theta_A)^2 \), where \( \theta_T \) is the angle between the emission transition dipole of the donor and the absorption transition dipole of the acceptor, \( \theta_D \) and \( \theta_A \) are the angle between these dipoles and the vector joining the donor and the acceptor. In the present case, the \( \kappa^2 \) has been computed along the simulated trajectory, using the \( 1L_a \) transition dipoles of Trp 10 and Trp 25. The simulated average value of \( \kappa^2 \) is 0.75, which is slightly larger than the expectation value for the freely rotating fluorophores (i.e. \( \kappa^2 = 2/3 \)). In the FRET experiment, either of the two Trp was replaced by AEDANS. It is expected that the dipole of AEDANS, which was attached to the protein by a linker, experiences larger fluctuations than the corresponding Trp dipole. Thus, the \( \kappa^2 \) value measured in the simulations should be considered as an upper limit for the experimental \( \kappa^2 \). With \( \kappa^2 = 0.75 \) for the native state and \( \kappa^2 = 2/3 \) for the denatured state, the critical distance and the D/A distance for D25A10 were recalculated. The distances are very similar to those obtained with \( \kappa^2 = 2/3 \) for both native and denatured states (Fig. 4). This supports the assumption of \( \kappa^2 = 2/3 \) does not significantly affect the distances and their changes observed in the experiments.

![Fig. 2. Time-resolved anisotropy fluorescence data and fit for D25A41. The measured \( I_{\chi_2}(t) \) (black) and \( I_{\chi_3}(t) \) (red) signals have been plotted along with the global fit (green and blue, respectively). The instrument response for the parallel direction \( I_L \) is plotted in grey. Residuals of the fit for \( I_{\chi_2}(t) \) (black) and \( I_{\chi_3}(t) \) (red) signals are plotted below the data. The \( \chi^2 \) of the fit was 1.11.](image-url)
Fluorescence resonance energy transfer analysis

Thermodynamics and kinetics

The thermodynamics and kinetics of all the mutants were investigated by CD and fluorescence spectroscopy. In thermal denaturation experiments, we preferred to use CD to characterize the proteins since fluorescence signals significantly with temperature. The thermal denaturation curves for the labelled and unlabelled mutants differed slightly but gave quite similar melting points $T_m$, which ranges from 52.8°C to 59.9°C (Table III). The change of stability upon labelling ($\Delta \Delta G$) was estimated from $\Delta H_{T_m}$, $T_m$ and $\Delta T_m$ (Table III). Among all the mutants, D58A25 gave the largest stability change upon labelling with $\Delta \Delta G=0.26 \text{kcal mol}^{-1}$, which is small. Very good overlap was obtained for two successive thermal denaturation scans on the same sample of protein, which shows that there was not irreversible denaturation during the experiments. The different mutants having a similar melting point as the wild type (54.7°C) indicate similar stability, with $\Delta \Delta G_{\text{mutant-WT}}$ in the range of $-0.19\sim0.52 \text{kcal mol}^{-1}$.

Protein stability was also studied by chemical denaturation, using both CD and fluorescence signals. The denaturation midpoint $[D]_{50\%}$ and m-value were obtained by fitting the CD signal as a function of GdmCl concentration (Table III). Consistent with the thermal denaturation results, the denaturation midpoints for the mutants do vary slightly. But, the midpoint is the same within error for the same mutant with and without the AEDANS labelled. The fluorescence signal obtained from donor-only proteins was also used to find the $[D]_{50\%}$ and m-value (Table III). Some of the thermodynamic parameters obtained from fluorescence differ from those obtained from CD experiments, which is a signature of multi-state folding (Mayor et al., 2003). The dependence of fluorescence intensity on GdmCl concentration when FRET is occurring is very complex and not quantitatively predictable owing to the strong distance dependence of fluorescence intensity. The fluorescence signal from the mutants with both donor and acceptor was not used, therefore, for the calculation of thermodynamic parameters. As can be seen in Table III, $[D]_{50\%}$ ranges from 1.8 to 2.4 M, which is quite similar to the value for the wild-type EnHD ($[D]_{50\%}=2.36 \text{M}$, $m=1.43 \text{kcal mol}^{-1} \text{M}^{-1}$ from CD and $[D]_{50\%}=2.29 \text{M}$, $m=1.45 \text{kcal mol}^{-1} \text{M}^{-1}$ from fluorescence, this work).

The relaxation rate constants at 50°C for engineered EnHD mutants were in the range of $6 \times 10^2$ to $1 \times 10^3 \text{s}^{-1}$ (Table III), which are compatible with the relaxation rate constant of the wild-type EnHD, $9.3 \times 10^4 \text{s}^{-1}$ at 52°C. Some of the mutants had significant differences in kinetics, e.g. the unlabelled EnHD D25A41 has a rate constant of $8.5 \times 10^3 \text{s}^{-1}$ at 50°C whereas its labelled analogue has $5.6 \times 10^4 \text{s}^{-1}$. The reason for such a large difference is unlikely to be due to the change of folding pathway upon labelling since the other two mutants with acceptor at the same site residue 41 (EnHD D58A41 and D10A41) give very similar relaxation rate constants for the labelled and unlabelled mutants. The kinetics constants may have large errors due to the very small amplitudes since we deliberately introduced Trp into solvent exposed regions with consequently very small local environment changes upon denaturation.

Distance change upon equilibrium denaturation

The distances between donor and acceptor in the native state were calculated from fluorescence intensity (Table I). The distances obtained from FRET are generally quite similar to those obtained from NMR experiments. D8A41 has a very large discrepancy of 6–8 Å. One possible explanation is that Trp8 is in an unstructured region in the protein and the distance obtained from the NMR structure is just the distance for one of the possible conformations. An alternative reason for this difference is that the distance obtained from NMR is the distance between C$\beta$ whereas the FRET results give the distance between the donor (Trp) and acceptor labelled to the Cys through a linker with a six-covalent-bond length.

As can be seen from Fig. 5, the critical distance ($R_0$) always decreased upon denaturation, because of the decrease of fluorescence quantum yield of Trp upon denaturation and the increase of refractive index with GdmCl concentration [cf. Eq. (1)]. The change of distance between donor and acceptor is not the same as the change of $R_0$. Although the distances between donor and acceptor increase very differently (Fig. 6). The distance between donor and acceptor separated by two or three helices always increased with GdmCl concentration, including the distances for mutants D58A25, D10A41 and D58A10. But, the distance between fluorophores separated by a single helix changed with increase of GdmCl concentration in a complex way. The distance between donor and acceptor in mutant D25A10

---

**Table II.** Trp fluorescence anisotropy decay rate from MD simulation

<table>
<thead>
<tr>
<th></th>
<th>Trp10</th>
<th>Trp25</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$</td>
<td>0.77</td>
<td>0.59</td>
</tr>
<tr>
<td>$t_1$ (ns)</td>
<td>3.9</td>
<td>7.8</td>
</tr>
<tr>
<td>$\tau_2$ (ns)</td>
<td>0.94</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The data were obtained by fitting the curves in Fig. 3 with equation $y=A \exp(-t/t_1)+(1-A)\exp(-t/\tau_2)$.

---

**Fig. 4.** Critical distance and donor/acceptor distance with different $k^2$ assumption. The critical distance and donor/acceptor distance were either calculated by assuming that $k^2=2/3$ for proteins in both native state and denatured state or assuming that $k^2=0.75$ for protein in native state, giving $R_{\text{pop}}$, and $k^2=2/3$ for protein in denatured state, giving $R_{\text{gdn}}$, and the critical distance ($R_0$) is the population weighted average of the critical distances $R_0=\langle |N| R_{\text{pop}}(D) R_{\text{gdn}}(D) \rangle (\langle |N| + |D|)$ where $|N|$ and $|D|$ are the concentrations of protein in native state and denatured state, respectively.
Kinetics at 50°C

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Unlabelled (s⁻¹×10⁸)</td>
<td>7.0 ± 0.1</td>
<td>10.1 ± 0.1</td>
<td>10.0 ± 0.1</td>
<td>8.5 ± 0.1</td>
<td>10.4 ± 0.1</td>
<td>6.0 ± 0.1</td>
<td>9.4 ± 0.1</td>
<td>9.1 ± 0.1</td>
</tr>
<tr>
<td>Labelled (s⁻¹×10⁸)</td>
<td>7.1 ± 0.1</td>
<td>10.0 ± 0.1</td>
<td>9.5 ± 0.1</td>
<td>7.6 ± 0.1</td>
<td>11.0 ± 0.1</td>
<td>7.4 ± 0.1</td>
<td>10.2 ± 0.1</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>Kinetics in 2 M GdmCl at 25°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unlabelled (s⁻¹×10⁸)</td>
<td>5.0 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>9.1 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>4.4 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td>Labelled (s⁻¹×10⁸)</td>
<td>5.4 ± 0.1</td>
<td>3.0 ± 0.1</td>
<td>8.4 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>4.5 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>4.2 ± 0.1</td>
</tr>
</tbody>
</table>

| Chemical denaturation from CD | | | | | | | | |
| Unlabelled [D]₅₀° (M) | 1.81 ± 0.1 | 2.21 ± 0.1 | 1.81 ± 0.1 | 2.36 ± 0.1 | 2.23 ± 0.1 | 2.39 ± 0.1 | 2.32 ± 0.1 | 1.95 ± 0.1 |
| Unlabelled m (kcal mol⁻¹ M⁻¹) | 1.50 ± 0.05 | 1.51 ± 0.05 | 1.51 ± 0.05 | 1.36 ± 0.05 | 1.29 ± 0.05 | 1.40 ± 0.05 | 1.52 ± 0.05 | 1.47 ± 0.05 |
| Labelled [D]₅₀° (M) | 1.79 ± 0.1 | 2.24 ± 0.1 | 1.84 ± 0.1 | 2.33 ± 0.1 | 2.38 ± 0.1 | 2.37 ± 0.1 | 2.40 ± 0.1 | 2.02 ± 0.1 |
| Labelled m (kcal mol⁻¹ M⁻¹) | 1.43 ± 0.05 | 1.47 ± 0.05 | 1.42 ± 0.05 | 1.48 ± 0.05 | 1.30 ± 0.05 | 1.38 ± 0.05 | 1.36 ± 0.05 | 1.52 ± 0.05 |

| Chemical denaturation from Fluorescence | | | | | | | | |
| Unlabelled [D]₅₀° (M) | 1.70 ± 0.2 | 2.18 ± 0.2 | 1.73 ± 0.2 | 1.89 ± 0.2 | 2.33 ± 0.2 | 2.60 ± 0.2 | 2.16 ± 0.2 | 1.97 ± 0.2 |
| Unlabelled m (kcal mol⁻¹ M⁻¹) | 1.06 ± 0.1 | 1.44 ± 0.1 | 1.50 ± 0.1 | 1.22 ± 0.1 | 2.65 ± 0.1 | 2.12 ± 0.1 | 1.34 ± 0.1 | 1.56 ± 0.1 |

| Thermodenaturation | | | | | | | | |
| Unlabelled T₅₀° (°C) | 55.7 ± 0.5 | 53.9 ± 0.5 | 54.3 ± 0.5 | 55.6 ± 0.5 | 57.3 ± 0.5 | 58.9 ± 0.5 | 57.4 ± 0.5 | 53.5 ± 0.5 |
| Labelled T₅₀° (°C) | 52.8 ± 0.5 | 55.9 ± 0.5 | 54.9 ± 0.5 | 55.1 ± 0.5 | 58.9 ± 0.5 | 59.9 ± 0.5 | 57.3 ± 0.5 | 54.1 ± 0.5 |
| ΔΔG (kcal mol⁻¹)ᵇ | 0.26 ± 0.05 | 0.20 ± 0.05 | 0.05 ± 0.05 | 0.05 ± 0.05 | 0.14 ± 0.05 | 0.10 ± 0.05 | 0.09 ± 0.05 | 0.05 ± 0.05 |

ᵇErrors were obtained from fitting standard deviation rather than that derived from several parallel experiments and may be larger than the latter.

Fig. 5. Critical distance at different GdmCl concentration.

Distance changes during kinetic Tjump experiments

We performed qualitative experiments on a series of mutants based simply on whether the amplitude of fluorescence emission decreased or increased during the kinetics after a T-jump. The T-jump experiments cause a decrease in the population of the native state and an increase in the population of denatured states, which would include the folding intermediate of known structure and any other denatured states. The changes in fluorescence of donors and acceptors have a series of complications that render a quantitative interpretation too difficult for the experimental set up. During the kinetic process of denaturation, the inherent fluorescence of the donor and acceptor can change because of their changes in environment in different states, which will affect the measured FRET emission. For example, if the emission of the donor increases on denaturation, then this will lead to an intrinsic increase in FRET emission that will be added to any change caused by a change of distance between donor and acceptor. The increase could be larger than the decrease in FRET efficiency caused by the donor and acceptor moving apart. Similarly, if the intrinsic fluorescence of the acceptor increases on denaturation, it will also cause an apparent increase in FRET efficiency, which could negate a decrease caused by donor and acceptor moving apart. Accordingly, we measured the fluorescence change on a T-jump for a construct containing the donor tryptophan but without the AEDANS label in order to gauge the contribution of the inherent fluorescence of the donor. We then measured the change in fluorescence of acceptor of that construct which was labelled with AEDANS but without FRET to gauge the inherent change in fluorescence of the acceptor. We finally measured the FRET for the construct containing both donor and acceptor. We consider that the only results that are interpretable are for those mutants where the algebraic sign of the FRET amplitude is opposite to that expected from the changes in intrinsic fluorescence of the isolated donor and acceptor.

The changes of fluorescence intensity of the donor and acceptor after T-jump in the presence and absence of FRET for all the mutants are shown in Fig. 7. The fluorescence emission from the donor (Trp) in D58A10 in 2 M GdmCl at 25°C decreased after T-jump in the absence of FRET, whereas it increased in the presence of FRET. This suggests the distance between donor and acceptor increased upon denaturation so that the FRET efficiency decreases. Very weak increase in the fluorescence from the acceptor was observed.
in the absence of FRET, which was also reversed in the presence of FRET, corresponding to an increase of donor/acceptor distance. The opposite amplitude for the donor in the absence and presence of FRET also suggested that the increase of fluorescence from the donor caused by the increase of donor/acceptor distance is more than enough to compensate for the decrease of fluorescence caused by the environment change upon denaturation. The same case was observed for this mutant in buffer at 50°C. The other two mutants, D8A41 and D58A25, behaved in the same way as D58A10 in both 2 M GdmCl at 25°C and buffer at 50°C. D10A41 behaves in a slightly different way; it has the same behaviour as D8A41 in 2 M GdmCl at 25°C, but opposite amplitude was not observed in the presence and in the absence of FRET in buffer at 50°C. This is likely due to a smaller change of donor/acceptor distance at 50°C in buffer than that in 2 M GdmCl at 25°C. Different from D58A10, D10A41 and D8A41, mutants D58A41, D25A10 and D25A41 had another pattern of behaviour. All of them had the same fluorescence change for the donor in the presence and absence of FRET in both 2 M GdmCl and buffer. But, the fluorescence intensity from the acceptor reversed in 2 M GdmCl: it decreased upon T-jump in the presence of FRET whereas it did not change or slightly increased in the absence of FRET, which indicated an increase of the donor/acceptor distance. The increase of the donor/acceptor distance may not be significant so that the increase of fluorescence intensity gained from decrease of FRET efficiency could compensate its decrease caused by environment change for the donor. A decrease of fluorescence intensity was, therefore, observed after T-jump in the presence of FRET for the donor. In these mutants, the donor/acceptor distance may either increase very slightly or decrease in buffer at 50°C upon T-jump so that the opposite amplitude was not observed in fluorescence change for both donor and acceptor. D10A25 behaved the same as D25A10 in buffer at 50°C, but gave increased acceptor fluorescence signal upon T-jump in 2 M GdmCl at 25°C, suggesting a smaller increase in distance or even a decrease. D10A25 and D25A10 are expected to have the same behaviour since only the positions of the donor and acceptor were exchanged. The observed difference was likely due to the different environment at positions 10 and 25. The fluorescence of the acceptor at position 10 did not change upon denaturation, whereas the one at position 25 had a significant increase in fluorescence intensity. The decrease in fluorescence intensity for the acceptor at position 25 caused by increase of distance could not have negated the intrinsic increase in fluorescence intensity (Fig. 7).

The change of distance between donor and acceptor upon T-jump is summarised in Table I, according to this simple picture that only a decrease in FRET is qualitatively interpretable. The results show that in 2 M GdmCl at 25°C all the distances increase upon T-jump, whereas the distance change in buffer at 50°C is not clear for D25A10, D10A25, D25A41, D58A41 and D10A41, although the distance increases for the other mutants. Apart from D10A41, all the other four mutants that showed non-clear-cut distance change were the mutants where the donor and acceptor were separated by only one helix. These distance changes agreed with the equilibrium experiments very well, where decreased distance was observed under certain conditions for these mutants. The only odd mutant was D10A41, which showed a significant increase of distance in equilibrium experiment, but an insignificant distance change in the kinetic experiment. To check the change of the distance between the N-terminus of Helix I and the C-terminus of Helix II, we investigated another mutant (D8A41). This mutant is expected to give a more significant distance change since the distance between residues 8 and 41 is very small in the native state and it is large in the denatured state if the

Fig. 6. Distance changes for EnHD mutants in chemical denaturation.
denatured protein is a random coil chain. D8A41 showed a distance increase in both chemical denaturation and T-jump experiments. Since there is only a difference of two residues for D10A41 and D8A41, a similar distance change would have been expected for these two mutants. The experiment for D8A41 implied that the distance between donor and acceptor in D10A41 also increased in a T-jump. But, it did not increase significantly so that the fluorescence intensity change cannot be reversed.

Discussion

Multi-state folding and further proof of a folding intermediate

Wild-type EnHD folds through a compact intermediate, where helix I moves apart from the helices II and III, whereas helices II and III retain near native state structure (Religa et al., 2005). A mimic of this intermediate EnHD(L16A) has been shown to be equivalent to the folding intermediate from kinetic experiments at physiological ionic strength, and its solution structure was solved by NMR spectroscopy (Religa et al., 2005). Subsequently, it was found that the helix-turn-helix motif formed by helices II and III is a stable entity per se and is formed and reacts fast enough to be on the reaction pathway (Religa et al., 2007). The formation of the helix-turn-helix motif corresponds to the microsecond phase in the folding kinetics, and the docking of helix I to the 10 μs phase (Religa et al., 2007). The intermediate unfolds further under denaturating conditions (Religa et al., 2005). The evidence for the intermediate is by inference, using close structural analogues of the intermediate. In the present study, we have now shown...
directly that there is an intermediate during the kinetics and at equilibrium, where it becomes populated at low concentrations of GdmCl. The changes in geometry of this intermediate were consistent with those expected from the known structure of the putative intermediates, previously solved by NMR spectroscopy.

CD spectroscopy measures secondary structure, whereas fluorescence from Trp in the absence of FRET measures the local environment. In three-state folding, an inconsistency between CD and fluorescence is often found since they measure different structural processes. In this work, a systematic and significant deviation between CD and fluorescence was not observed. This is a consequence of the fluorescent probes having been inserted into solvent-exposed positions that are insensitive to the folding state of the protein. For FRET measurements, however, the change of distance between the donor and the acceptor provides additional information on the conformational change of the protein upon denaturation. As can be seen from Fig. 6, the GdmCl-dependence of donor–acceptor distance was very different from mutant to mutant, where distance between different key residues was measured. The non-cooperativity of distance change was a clear indication of multi-state folding, consistent with previous results (Mayor et al., 2003).

The experiments also indicated that this intermediate could be stabilized or populated at low concentration of GdmCl. The observed distance is the average distance of the native state, intermediate and denatured state. The observed change in distance, therefore, depends on the relative amount of each species and the donor–acceptor distance in each species. The magnitude of this change will vary from case to case. If the distances between donor and acceptor in the

---

**Fig. 7. continued**
intermediate and in denatured state are both greater than that in the native state, then an increasing distance is expected to be observed with increasing GdmCl concentration since the population of the native state always decreases. But, if the distance in the denatured state is smaller and the distance in the intermediate is greater than that in the native state, a complex distance change may be observed. In this case, a distance increase followed by a decrease may be observed if the intermediate is populated under certain conditions. The denaturation curve of D25A10 suggested that the intermediate was significantly populated at ~1 M GdmCl and donor–acceptor distance in the intermediate was greater than in native state at ~1 M GdmCl and the intermediate was further denatured when the concentration of GdmCl was higher. It also implied that the distance between residues 25 and 10 in the denatured state was less than the distance in the intermediate at ~3 M GdmCl. But, the distance in the denatured state increased with GdmCl concentration. The expansion of denatured state with denaturant concentration is also observed for other proteins (Kuzmenkina et al., 2006; Huang et al., 2007). The assumption that the intermediate is populated at low GdmCl concentration was also supported by the denaturation curve of the other mutants. For example, the distance between residues 58 and 25 did not change when GdmCl concentration was below 1 M, which is expected because the population of the intermediate does not influence the distance between 58 and 25 much since Helix II is similar in both the native and intermediates, as is Helix III (Religa et al., 2005). The distance began to increase after the GdmCl concentration was higher than 1 M, where the denatured state began to be populated. In the mutant D58A10, the donor–acceptor distance increased rapidly at...
low concentration of GdmCl, which is also consistent with the above assumption. The rapid distance increase was because the first helix moves away from helices II and III to form the intermediate, where the distance between residues 58 and 10 is large.

**Distance change upon denaturation**

The change in distance on a T-jump varied with conditions. The donor/acceptor distance either decreased or increased insignificantly upon T-jump for some mutants at 50°C, which agreed with the chemical denaturation results. But, the distance between donor and acceptor increased upon T-jump for all the mutants at 25°C in 2 M GdmCl. A reasonable explanation is that loosely folded proteins, especially poorly structured polypeptides, are more expanded in GdmCl. This agreed with the positive slope of the denatured baseline in the equilibrium titration.

Figure 6 shows that the distance changes at equilibrium were significantly anisotropic. Some mutants gave very flat baselines for the native and denatured states, whereas others had very steep baselines; some had significant distance increases, whereas others appeared to be more compact due to the unfolding transition. It must be emphasised that a flat baseline for the denatured state does not necessarily mean the distances do not increase with the concentration of GdmCl, because of insensitivity of distance measurement and systematic deviation in the denatured state (discussed later). Except for the donor/acceptor distance in D10A25, D25A10 and D58A41, all the other distances increased upon denaturation even at low GdmCl concentration, indicating more expanded denatured structures. The distance between

![Fluorescence resonance energy transfer analysis](image)
residues 10 and 25 increased when the concentration of GdmCl was lower than 1.5 M and reached a plateau. With further increase of GdmCl concentration, the distance decreased and reached the minimum at ~3 M GdmCl, and then increased again. The distance change did not synchronise with the CD signal, which has only one transition in GdmCl-mediated denaturation. The CD signal of a protein measures the secondary structure change, whereas the distance change measures the global conformational change between the donor and acceptor. The difference between the CD signal and the distance change from FRET is evidence for the non-cooperative denaturation transition of EnHD. To exclude the possibility that the increase--decrease--increase distance change resulted from the local effect of external probes, another mutant with exchanged donor/acceptor positions was prepared. It gave a very similar distance change with a plateau shifting to ~0.7 M GdmCl. This control experiment suggested that the increase--decrease--increase distance change behaviour was a property of the protein itself and not the introduction of external probe. From the distance changes for 10–25 and 41–58, it can be seen that the distance between the two termini of a helix could get shorter upon denaturation. But, this change did not happen at all the helices, as shown by the distance of 25–41. The distance change between the two termini of a helix is strongly sequence-dependent.

It should be emphasised that small distance changes found in FRET experiments may not be due to a conformational change but can also result from a change of donor or acceptor orientation. For example, in the native state, the fluorophores may not be able to rotate freely and the orientation factor \( \kappa^2 \) therefore does not equal 2/3, whereas in the denatured state, the donor and acceptor can rotate freely and \( \kappa^2 \) is 2/3. In this case, with the assumption of \( \kappa^2 \) being the same, different distances may be obtained from FRET, even without conformational changes. To confirm the observed distance change in the denaturation experiments was due to the conformational change rather than the change of \( \kappa^2 \), the dynamics of Trp was investigated carefully. Both anisotropy experiments and MD simulation suggested that Trp has a very fast rotation over the whole protein tumbling. This fast rotation and the multiple conformations of indole group of Trp strongly supported the assumption of \( \kappa^2 \) being 2/3 for the native state of the protein. For the denatured state, this assumption is true in general because of the fast rotation of the protein backbone. We conclude, therefore, that the observed distance change in the denaturation of EnHD results from the conformational change rather than the change of FRET critical distance. The \( \kappa^2 \) value of 0.75 obtained from simulation was also applied to check the effect of selection of the value of \( \kappa^2 \). No significant effect was observed (Fig. 4), which is because of the sixth-root dependence of the critical distance on \( \kappa^2 \).

**Meaning of distances obtained from FRET**

One question in the application of FRET is what do the derived distances mean? The distance obtained from steady-state FRET experiments is normally considered as an average distance or mean distance, although its exact meaning is not generally addressed. For a system with a unique donor/acceptor distance (i.e. where the ensemble of distances has a very small spread), the distance obtained from FRET is just the distance between donor and acceptor, such as in the native state of proteins, where one can assume a single conformation and a closely defined distance. However, in a system with a broad distance distribution \( f(x) \), such as denatured protein, the question is much more complex. First, in a denatured protein, the observed distance is shorter than the real distance because of intramolecular diffusion during the fluorescence lifetime of the donor (Sahoo et al., 2006). When a donor has a very short fluorescence lifetime and the diffusion is slow so that the fluorophores cannot diffuse much during the fluorescence lifetime, the measured distance may be very close to the real distance (Beechem, 1989). The lifetime of excited Trp is very short, ~3 ns. When Trp acts as donor the obtained distance in folded protein is close to the real distance, but the intramolecular diffusion of a random-coiled peptide cannot be completely neglected on the grounds of fast diffusion, and the observed distance from steady-state fluorescence measurement is expected to be shorter than the real distance. The difference between the measured distance and the real distance depends on both of the flexibility of the peptide and the lifetime of Trp or the efficiency of the energy transfer.

The other factor influencing the measured distance is more complex. To simplify this question, we can assume that there is no diffusion during the lifetime of the donor. In the absence of diffusion, the apparent energy transfer efficiency \( (E) \) for ensemble molecules with average donor–acceptor distance of \( R \) can be expressed by Eq. (2) and the energy transfer efficiency for an individual molecule \( (E_i) \) with donor–acceptor distance of \( x \) can be expressed by Eq. (3).

\[
E = \frac{R_0^6}{R_0^6 + R^6} \tag{2}
\]

\[
E_i = \frac{R_0^6}{R_0^6 + x^6} \tag{3}
\]

Assuming that (i) the total energy absorbed and total energy transferred in the system is \( I \) and \( I_{ET} \), respectively; (ii) the absorbed energy and transferred energy by an individual molecule with distance of \( x \) are \( I_i \) and \( I_{ET,i} \) respectively; (iii) each molecule has the same probability to be excited, i.e. \( I_i \) is the same for all the molecules, and the donor–acceptor distance distribution is \( f(x) \), Eq. (4) can be obtained.

\[
E = \frac{I_{ET}}{I} = \int_0^\infty f(x) I_{ET}^i dx = \int_0^\infty f(x) (I_{ET}^i / I_i) dx = \int_0^\infty f(x) E dx = \int_0^\infty f(x) E_i dx \]

\[
= \int_0^\infty f(x) dE \]

\[
= \int_0^\infty f(x) \frac{R_0^6}{R_0^6 + x^6} dx \]

\[
= \int_0^\infty f(x) R_0^6 dx \]

\[
= \frac{\int_0^\infty f(x) dx}{\int_0^\infty f(x) 1 / R_0^6 + x^6} dx - R_0^6 \tag{4}
\]

which means the apparent energy transfer efficiency is the distance-distribution weighted average of the energy transfer efficiency of individual molecules. The combination of Eqs. (2), (3) and (4) gives Eq. (5).

\[
R_0^6 = \int_0^\infty f(x) dx \int_0^\infty f(x) 1 / R_0^6 + x^6 dx \]

Equation (5) shows that the distances observed in FRET experiments are not the average of the distances of each
individual molecule, but a value determined by the distance distribution and critical distance.

Figure 8 is a simulation showing the dependence of FRET distance on the critical distance and distribution of formers in the ensemble. As can be seen, for a system with a narrow distribution (with small standard deviation), the average distance can be measured very accurately with FRET with a critical distance in the range of \( R/2 < R_0 < 2R \). But, the observed FRET distance can be quite different from the average distance when the distance distribution is broad. The deviation from the true average distance depends on how different is the critical distance from the average distance and the broadness of the distribution. We can expect, therefore, that distances obtained for native states of proteins are reasonably accurate, but distances for denatured states may have large errors. Figure 8 also suggests that the absolute value of the deviation increase with the average distance, although the relative deviation decreases slightly. To get an accurate distance, it is essential to select a FRET donor/acceptor pair with a critical distance similar to the distances to be measured, particularly for systems with a broad distribution of states.

The above discussion is very pertinent for understanding the distance change in the chemical denaturation in the current work. Most of the mutants give a positive slope for the denatured baseline, which should be due to the expansion of the denatured protein with the increase of GdmCl concentration. However, D58A10 gives a very flat denatured baseline. This is quite likely due to the long distance and broad distance distribution, i.e. although the distance increases with GdmCl concentration, the observed distance does not change due to the negative deviation. As shown in Fig. 8, when the critical distance is far from the real distance, the observed distance has significant deviation; when the standard deviation is large (broader distribution), the observed distance is also further away from the real one. To overcome the systematic deviation, time-resolved FRET is a good technique, which directly gives the distance distribution without deviation (Beechem and Hass, 1989).

![Fig. 8.](image)

**Fig. 8.** Dependence of observed FRET distance on critical distance. Curves were obtained according to Eq. (5) by simulating with Maple (version 8, Maplesoft, Waterloo, Canada), where a Gaussian distribution for the distance between donor and acceptor was assumed, i.e. \( y = \exp(-2(x - x_c)^2/w^2) \), where \( x_c \) is the distance of separation between donor and acceptor at the peak of the Gaussian distribution. The black, blue and red curves correspond to distributions where \( w \), the width of the distribution, is equal to \( x_c/10 \), \( x_c/2 \) and \( x_c \), respectively. (a) \( x_c = 20 \) A and (b) \( x_c = 40 \) A. There are increasing systematic errors as the critical distance, \( R_0 \), moves away from \( x_c \), which increases also with \( w \). It is thus crucial to match \( R_0 \) and \( x_c \).

### The folding pathway of EnHD

FRET distance measurements suggested that: the distances 8–41, 10–41, 10–58 and 25–58 increased during denaturation; the distances 10–25 and 41–58 increased and then decreased and increased again and distance 25–41 decreased and then increased. The change of distance between the key residues provided essential information on the folding of EnHD. First, the complex distance change immediately suggested the existence of a folding intermediate. The titration curves shown in Fig. 6 further suggested that the intermediate was stabilized at \( \sim 1 \) M GdmCl. Secondly, FRET measurements also indicated that the distances 10–25 and 41–58 for the intermediate were longer than the corresponding distances for the native state and the denatured state at low GdmCl concentration. It also suggested that at low GdmCl concentration, the denatured state was quite compact. Previous work has proved that the intermediate on the folding pathway of EnHD is native-like but with Helix I moving away from the other two helices. The NMR structure also shows that the second helix becomes extended in the intermediate. According to the NMR structure, the donor–acceptor distance in the intermediate is longer than that in the native state except the distances 10–58 and 41–58. It is, therefore, reasonable to see an increase of the distance in the native state to intermediate state transition. On the other hand, it is also possible to see a decreased distance for the helix-coil transition for the single helix motif at low denaturant concentration, where the coil is more compact than in high denaturant concentration. A decrease in distance was not observed for 10–58 and 41–58, although the distance in the intermediate is shorter. This may result from two reasons. First, the distance between residues 10 and 58 could be quite long in the denatured state. Since the observed distance is the average distance of the native state, intermediate and denatured state, the observed distance may not decrease if the distance decrease cannot compensate for the distance increase. On the other hand, if residues 57 and 58 are not structured, the distance between residue 58 and other residues obtained from NMR structure may have quite large error, which could be the case for 41–58. Further, the distance between Helix I and Helix III measured by NMR is just the distance in one of the possible conformations and may be far from the average distance of the intermediate ensemble.

Although the FRET results cannot provide the structure of the folding intermediate, they are consistent with previous NMR results and strongly support a three-state folding model. The FRET experiments further suggest that in GdmCl denaturation, EnHD first becomes more expanded at low denaturant concentration and then shrinks, and expands again at higher denaturant concentration. The congruence of results from different types of experiments using different techniques and of different resolution helps to establish the mechanism of folding of EnHD.

### Funding

This work was funded by the Medical Research Council.

### References


Received October 17, 2007; revised October 17, 2007;
accepted November 5, 2007

Edited by Valerie Daggett