Chapter 6

An Unfolding Intermediate of Cytochrome c₅₅₂ Revealed with trFRET¹

6.1 Introduction

Cytochrome c_{552} belongs to the class-I *c*-type cytochrome family, which includes yeast cytochrome *c*. Cytochromes in this family share the common structural feature where the N- and C-terminal helices cross each other (α I and α IV in Fig. 1A). However, cytochrome c_{552} has the distinct structural components of a β -sheet around the heme propionate and extra helices in the C-terminal region (Figure 6.1). The role of these extra structures has never been fully elucidated.

We are interested in the contribution of these distinct structural features to the folding kinetics and thermodynamic stability of this thermostable protein. We have probed the unfolding of cytochrome c_{552} under equilibrium unfolding conditions. The structural conformations are monitored using trFRET with

¹Adapted from Yamada, S.*; Bouley Ford, N.*; Ford, W.; Keller, G.; Gray, H.; Winkler, J. "Snapshots of a Protein Folding Intermediate" *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 1606-1610 [51].



Figure 6.1: Crystal structure of WT cytochrome c_{552} at pH 5.44 (PDB code: 3VNW). Dns-labeled sites are shown as spheres: group I (cyan) and group II (yellow). Helix α VI and β -sheets I and II are distinct structural features of cytochrome c_{552} that exhibit group II folding behavior (green). For reference, Trp91 is also highlighted in cyan.

fluorescent probes distributed throughout the protein, including the region distinct

to cytochrome c_{552} (Figure 6.1).

6.2 Stability of Labeled Mutants to Denaturation

Solvent-exposed residues that form no intramolecular bonds were chosen for trFRET studies to minimize structural perturbations upon labeling. Seven single-mutation variants of cytochrome c_{552} were prepared; in each mutant, a residue



Figure 6.2: CD spectra of Dns cytochrome c_{552} variants. The spectra were measured with 20 μ M protein in 10 mM sodium citrate buffer (pH 3.0) at 25°C: WT (black), Dns126 (magenta), Dns110 (blue), Dns95 (cyan), Dns76 (green), Dns59 (yellow), Dns40 (orange), and Dns6 (red).

(Lys6, Glu40, Glu59, Lys76, Lys95, Lys110 or Lys126) was mutated to cysteine and further modified by covalent Dns attachment.

Circular dichroism (CD) spectra of all Dns variants were virtually identical with that of WT (Figure 6.2), indicating that the native secondary structure was preserved in the Dns-labeled proteins. Denaturation curves of the Dns variants, obtained from Dns fluorescence, UV-visible heme absorption, and CD measurements, showed midpoints ([Gdn]_{1/2}) close to 4 M guanidine hydrochloride (Gdn), consistent with that of WT (Table 6.1 and Figure 6.3). Standard denaturation parameters *m* and $-\Delta G_w$ [59], determined from these curves, also showed no substantial deviation from those of WT (Table 6.1). We conclude from these results that Dns labeling does not perturb the stability or secondary structure of any of the



Figure 6.3: Denaturation curve of Dns cytochrome c_{552} variants. Normalized signal of steady state fluorescence (red), heme absorbance (blue), and circular dichroism (green) versus Gdn concentration. Fluorescence data for Dns6 is not provided because there was negligible difference between native and unfolded states.

variants.

6.3 Steady-State trFRET Measurements

We employed trFRET to acquire site-specific D-A (Dns-heme) distance distributions ($P(r_{DA})$) upon denaturation with Gdn as a method to detect unfolding intermediates. A Tikhonov regularization (TR) method was used to extract distance distributions (Figure 6.5) from the fluorescence decays shown in Figure 6.4. Measurements were performed at pH 3 to prevent misligation of the heme by histidine residues or the N-terminal amino group. In most cases, Dns fluorescence decays could not be fit to single exponential functions, as r_{DA} components exist with various probabilities, consistent with the presence of multiple conformations.

At 0 M Gdn, r_{DA} for all Dns variants obtained from TR fitting are consistent with the WT crystal structure (Figure 6.5 and Table 6.2). The most probable D-A distances extracted from the fluorescence decays (r_{mode}) are slightly longer (1-4 Å) than the C γ -Fe distances in the crystal structure, likely owing to the length of linker between the Dns fluorophore and the C γ atom.

Denaturation with Gdn slowed the fluorescence decay in all Dns variants (Figure 6.4), consistent with an increase in the average distance between D and A. Extended populations were observed in all but one of the Dns variants upon the addition of Gdn (Figure 6.5). Since Dns6 is located close to the heme and thus exhibited only a modest broadening of the D-A distribution when denatured (Figure 6.4), it will not be considered further.

$\operatorname{orption}^{\mp}$	m $-\Delta G$	mol/M) (kJ/mo	$.9\pm0.8$ 49 ± 4	1±0.3 31±1	4±0.6 27±3	8±0.5 25±3	6±0.7 38±3	7 ± 0.8 41 ± 4	7±0.6 32±3	7±0.6 32±3	ne absorption).	3 nm, respectively.	respectively.			
Abso	$[Gdn]_{1/2}$	(M) (kJ/s)	4. 3±0.02 11	4. 3±0.02 7.	4.2±0.05 6.4	4.3±0.06 5.	4.4±0.03 8.	4.2±0.03 9.	4.1±0.03 7 .	4.2±0.03 7.	uorescence and hen	151 are 355 and 513	re 290 and 360 nm, 1			
${ m m}^{\ddagger}$	$-\Delta G$	(kJ/mol)	$54{\pm}7$	37 ± 8	$31{\pm}4$	43 ± 8	47 ± 9	39 ± 5	51 ± 8	42 ± 6	100 mM for fl	ssion waveler	wavelength a	nm.	391 nm.	
Circular dichroisr	ш	(kJ/mol/M)	13.2 ± 1.6	$9.3{\pm}1.8$	$7.6 {\pm} 0.8$	11.1 ± 1.9	11.0 ± 2.0	$9.6{\pm}1.1$	13.2 ± 2.0	$10.7{\pm}1.5$	r dichroism and	intensities. Excitation and emis tence. Excitation and emission v	on and emission	surements at 222	neasurements at (neasuremenus at
	$[Gdn]_{1/2}$	(M)	$4.1{\pm}0.03$	$4.0 {\pm} 0.06$	$4.1{\pm}0.04$	$3.9{\pm}0.05$	$4.3 {\pm} 0.05$	4.1 ± 0.04	$3.9{\pm}0.03$	$3.9{\pm}0.04$	nM for circula		ence. Excitati	llipticity meas	e absorbance r	
	$-\Delta G$	(kJ/mol)	$49{\pm}4^{\dagger}$	N/A	39 ± 6	32 ± 5	48 ± 3	$51{\pm}4$	$51{\pm}6$	48 ± 2	e buffer (10 n	fluorescence	Irp91 fluoresc	ŧΕ	∓Heme	
Fluorescence*	ш	(kJ/mol/M)	$11.9{\pm}0.8^{\dagger}$	N/A	$9.1{\pm}1.2$	$7.9{\pm}1.1$	11.0 ± 0.7	$12.2 {\pm} 0.9$	$12.8{\pm}1.5$	$12.0 {\pm} 0.5$	C in pH 3.0 citrat	surements of Dns	vere taken from []]			
	$[Gdn]_{1/2}$	(M)	$4.1{\pm}0.02^{\dagger}$	N/A	$4.3 {\pm} 0.05$	$4.1{\pm}0.05$	$4.4{\pm}0.02$	$4.2 {\pm} 0.02$	$4.2 {\pm} 0.02$	$4.0 {\pm} 0.01$	easured at 25°	ady-state meas	†Ďata of WT v			
		Variant	ΜT	Dns6	Dns40	Dns59	Dns76	Dns95	Dns110	Dns126	M	**Ste				

Table 6.1: Denaturation parameters for Dns cytochrome *c*₅₅₂ variants.



Figure 6.4: Fluorescence decay curves of Dns cytochrome c_{552} variants upon Gdndenaturation: Gdn 0 M (black), 3 M (red), 3.5 M (yellow), 4 M (green), 4.5 M (cyan), 5 M (blue), and 7 M (magenta).

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Figure 6.5: Gdn-induced changes in the Dns-heme distance distributions $[(P(r_{DA}))]$ for Dns cytochrome c_{552} variants at pH 3.0, extracted from TR fitting of fluorescence decay curves (Figure 6.4): Gdn 0 M (black), 3 M (red), 3.5 M (yellow), 4 M (green), 4.5 M (cyan), 5 M (blue), and 7 M (magenta). The area of each bar reflects the probability amplitude over the corresponding distance range. Group I and group II variants display three-state and two-state folding transitions, respectively.

Variant	Distance C_{γ} -Fe (Å)*	$r_{mode} \ (\text{\AA})^{\dagger}$	$r_{mean}~(\text{\AA})^{\dagger}$
6	17.5	21	22
40	22.7	22	25
59	16.3	21	22
76	18.9	19	22
95	21.7	22	25
110	21.6	21	22
126	16.7	21	23

Table 6.2: Native-state donor-acceptor distances for dansyl-labeled cytochrome c_{552} variants.

*Measured from the crystal structure (PDB 3VNW). †Extracted from the Dns-heme distance distributions.

We defined three categories of structural conformations from the distance distributions: native/compact (C: $18 \le r_{DA} \le 22$ Å); intermediate (I: $25 \le r_{DA} \le 35$ Å); and extended (E: $r_{DA} \ge 35$ Å), and we plotted their relative populations as functions of [Gdn] (Figure 6.6). Population of E conformations in all variants occurs at Gdn concentrations greater than 4 M, which is consistent with the denaturation curves determined using absorption, CD, and steady-state fluorescence spectroscopic methods (Figure 6.3). Interestingly, the Dns variants can be divided into two groups that differ in how C, I, and E populations change with Gdn concentration; Dns40, 76, 95, and 110 are in group I, and Dns59 and 126 are in group II (Figures 6.5 and 6.6).

C populations in group I transform into I components when denatured by 3 to 4 M Gdn, which then disappear at higher concentrations (\geq 5 M, Figure 6.6). The findings for group I variants are consistent with a three-state transition among the C, I, and E populations. The appearance and disappearance of I structures indicate that they arise from an unfolding intermediate state and are not simply



Figure 6.6: Population changes of structural conformations as a function of the Gdn concentration: native/compact (N), blue triangle; intermediate (I), red square; and extended (E), black circle. Group I and group II variants display three-state and two-state folding transitions, respectively.

constituents of the denatured protein ensemble. The I state has a folding stability similar to that of the native structure as I appears at relatively low [Gdn] in the presence of C (Figure 6.5). Absorption spectroscopy indicates that the heme environment in the intermediate is structurally similar to the native state; the heme Soret peak maxima are comparable at 0 and 3 M Gdn, indicating that the heme is low-spin with an intact, native Fe-S(Met) ligation (Figure 6.7). It is possible that I corresponds to a local minimum on the native protein folding energy landscape. In the absence of denaturant, its population is too small to be detected, but the introduction of a low concentration of denaturant shifts the equilibrium to populate this extended conformation rather than the compact native structure.

With fluorescent probes at Dns59 and Dns126, we found no evidence for

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Figure 6.7: Absorption spectra of Dns110-cytochrome c_{552} in Gdn: Gdn 0 M (blue), 3 M (red), and 7 M (black). The spectra of 4.9 μ M protein were collected in 10 mM sodium citrate buffer (pH 3) at 25°C.

the existence of I components during Gdn denaturation; these two derivatives form group II (Figure 6.6). Extended structures appear in both groups around 4 M. If we exclude the possibility that mutation and labeling at residues 59 and 126 have altered the unfolding pathway, then we can conclude that the unfolding intermediate formed at low Gdn concentrations involves little structural rearrangement from the native structure in the vicinity of Dns59 and Dns129 with slight expansion in the rest of the protein (Figure 6.8).



Figure 6.8: Schematic of proposed equilibrium unfolding intermediate with group II variants at native distances and group I variants at intermediate distances from the heme.

6.4 Cytochrome *c*₅₅₂'s Distinct Structural Features

Interestingly, the two group II residues are located in the distinct protein structural region that differentiates cytochrome c_{552} from other class-I *c*-type cytochromes (position 59 on β -sheet β I and 126 on the extra helix α VI). The extra helix α VI and the β -sheet β I of cytochrome c_{552} form a network of interactions (via residues 59, 125, and 131) linking them to the heme propionate (Figure 6.9). The WT crystal structure reveals that the amide nitrogen and carbonyl oxygen of Glu59 can hydrogen bond with the Lys131 carbonyl oxygen and the Arg125 side chain, respectively. Additionally, the Arg125 side chain forms salt bridges with the heme propionate and the carbonyl oxygen of Lys131. It is likely that these interactions stabilize this region of the polypeptide chain near the heme, making it more resistant to denaturation than the parts of the protein containing group I residues. By way of contrast, the other distinct helix in cytochrome c_{552} (α V) does not exhibit enhanced stability, insofar as the Dns110 label, located in α V, displays a three-state folding transition (Figures 6.5 and 6.6).

In the original report of the *T. thermophilus* cytochrome c_{552} x-ray crystal structure, it was proposed that extra structural elements in the protein were responsible for its stability at high temperatures [75]. The inherent stability of the cytochrome c_{552} fold is reflected by the finding that the apoprotein retains the same secondary structure as the holoprotein [76]. In marked contrast, the apoprotein of mitochondrial cytochrome *c* is unstructured; in this case, the heme, which acts as a hydrophobic nucleation site, is needed to stabilize the native fold [77]. Our



Figure 6.9: Crystal structure of WT cytochrome c_{552} obtained at pH 5.44 (PDB code: 3VNW). Red dashed lines indicate electrostatic interactions in the region of distinctive structures of cytochrome c_{552} .

cytochrome c_{552} unfolding data confirm that the extra C-terminal helices and β -sheet enhance the stability of the native structure by stabilizing the heme region.

Analogous features in yeast cytochrome c behave similarly under equilibrium unfolding conditions. In our work on yeast cytochrome c, I components were observed at moderate-to-high Gdn concentrations (2.7–5.9 M Gdn) for Dns39 and 50 near the heme propionate site and for Dns66 near the Met80 axial ligand site [21], indicating that compact structure is preserved in the heme region when unfolded. Our work on cytochrome c_{552} reveals that heme region variants (Dns59 and 126) do not develop I or E populations at mild Gdn concentrations where intermediate structures are found in other regions of the protein (3–4 M Gdn, Figures 6.5 and 6.6). We conclude that β -sheet β I (Dns59) and helix α VI (Dns126) are substantially more resistant to denaturant-induced unfolding.

6.5 Conclusions

Our trFRET experiments have shed new light on region-specific conformational changes that occur in cytochrome c_{552} upon increasing denaturant concentration. Dansyl probes at seven different residues in the protein reveal the existence of a loosely packed unfolding intermediate structure. We have also revealed that two sites associated with the distinct structural features of this class-I cytochrome are more resistant to unfolding than the rest of the protein.

6.6 Acknowledgments

This work was carried out in collaboration with Seiji Yamada, a visiting scientist from Sony Corporation [51].

Chapter 7

Characterization of Cytochrome c_{552} 's **Folding Intermediate**¹

7.1 Introduction

Folding intermediates can lead to misfolding; however, certain intermediates can actually help the protein find its native structure by avoiding traps in the folding energy landscape [8, 9, 78, 21]. These states are referred to as on-pathway intermediates [79].

An on-pathway folding intermediate of cytochrome c_{552} was identified by Brunori and coworkers [49]. The native Trp91 fluorescence quenching during refolding was biphasic. Rate constants for refolding and unfolding were measured by stopped-flow mixing and double-mixing experiments at different Gdn concentrations. Rate constants for the fast unfolded \leftrightarrow intermediate and slow intermediate \leftrightarrow native transitions in the absence of denaturant were extracted from chevron plots: $k_{UI} = 1100 \text{ s}^{-1}$, $k_{IU} = 0.2 \text{ s}^{-1}$, $k_{IN} = 2 \text{ s}^{-1}$, and $k_{NI} = 4 \times 10^{-6} \text{ s}^{-1}$, where U,

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I, and N are the unfolded, intermediate, and native states, respectively.

We have further characterized the refolding kinetics for the fast formation of this intermediate by microfluidic continuous flow mixing and trFRET. A strength of trFRET is that it allows us to characterize heterogeneity in ensembles of protein conformations [80].

7.2 Folding Kinetics

We triggered refolding using a continuous flow mixer to dilute denatured protein ([Gdn]= 6 M) with folding buffer ([Gdn]= 0 M) on a sub-millisecond timescale ([Gdn]_{final}= 1 M) and monitored the reaction progress using trFRET. To monitor the kinetics of cytochrome c_{552} refolding, we selected the Dns110 derivative (located at helix α V) as representative of the three-state transition folding variants from group I. We also measured the refolding of Dns59 (data not shown); denaturation of this variant resulted in partial aggregation.

The Dns110 fluorescence decay rate increases as the protein folds, indicating more efficient energy transfer to the heme (Figure 7.1A). Very little compaction of the protein occurs on timescales shorter than 200 μ s. The distance distributions (P(r_{DA}), Figure 7.1B) reveal intermediate (25 \leq $r_{DA} \leq$ 35 Å) and compact (18 \leq $r_{DA} \leq$ 22 Å) components developing in the first millisecond of folding. Compact populations gradually increase with time, but some extended ($r_{DA} \geq$ 35 Å) and intermediate structures still remain at 3.8 ms, the longest measurable reaction time.



Figure 7.1: Dns110-cytochrome c_{552} folding triggered by Gdn jump from 6 to 1 M in a continuous flow mixer (pH 3.0, ambient temperature). (A) Fluorescence decay curves. For clarity, only 7 of the 23 observed decays are displayed: unfolded in 6 M Gdn (red), 0.2 (magenta), 0.6 (yellow), 1.0 (green), 2.0 (cyan), 3.4 ms (blue), and folded in 1 M Gdn (>30 min after initiation of folding reaction, black). (B) P(r_{DA}) distributions for refolding extracted from fitting the fluorescence decay curves.

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Figure 7.2: Folding kinetics of Dns110-cytochrome c_{552} triggered by Gdn jump from 6 to 1 M in a continuous flow mixer (pH 3.0, ambient temperature). Integrated Dns-cytochrome c_{552} fluorescence intensity (M_0) is shown as a function of folding time. The solid line is a monoexponential fit with a rate constant of 1230 s⁻¹.

The kinetics of $P(r_{DA})$ evolution were evaluated by moment analysis (see Methods 2.5). The time course of the normalized integrated fluorescence intensity (M_0) can be fit to a monoexponential function $(k_{obs} = 1230 \text{ s}^{-1}, \text{ Figure 7.2})$. For a two-state process, the time course of the first (M_1) and second (M_2) moments will be exponential, with a rate constant corresponding to k_{obs} for the reaction [29]; we find k_{obs} of 1060 and 1140 s⁻¹ from the time courses of M_1 and M_2 , respectively (Figure 7.3A/B). The time course of the variance (V) in a two-state transition is biphasic, with a growth rate constant equal to $2k_{obs}$, and a decay equal to k_{obs} [29]; we find that V agrees with this model ($k_{obs} = 1100 \text{ s}^{-1}$, Figure 7.3C). These results indicate that Dns110 exhibits a two-state transition with a rate constant of 1100 s⁻¹ from the unfolded conformations.

Interestingly, the millisecond folding process does not appear to produce the

native state, as roughly one fourth (24%) of the normalized integrated fluorescence intensity persists at the end of this kinetics phase (Figure 7.2). Since the extrapolated endpoints to the fits of the moments ($M_1 = 31.5$ Å, $M_2 = 1200$ Å², and V = 200Å²) are not in accord with values for the native protein ($M_1 = 23$ Å, $M_2 = 560$ Å², and V = 31 Å², Figure 7.3), the millisecond process must correspond to formation of an intermediate folding ensemble. Notably, this ensemble has a greater population of extended components (Figure 7.1B, 3.4 ms) than that observed under equilibrium unfolding conditions. The early appearance of structures with 30 to 35 Å-Dns110heme distances suggests partial collapse involving the heme and helix α V, but the scarcity of 20-Å structures indicates little native-state formation.

This refolding intermediate is distinct from the unfolding intermediate reported in the previous chapter. They two intermediates have different structural ensembles. The refolding intermediate forms rapidly and is partially comprised of extended structures, whereas the compact unfolding intermediate is structurally and energetically similar to the native state. The equilibrium unfolding intermediate, which contains native heme ligation, is not observed during refolding and thus occurs after the rate-determining step.

Brunori found evidence for an on-pathway folding intermediate in cytochrome c_{552} using native Trp91 fluorescence as a probe [49]. The difference between the previously reported rate constant for intermediate formation ($k_{UI} = 400 \text{ s}^{-1}$, [Gdn]= 1 M) and ours can be rationalized by the differing pH conditions and temperatures employed (pH 2.1, 10°C versus pH 3.0, 18°C). The final step in

cytochrome c_{552} folding, the transition from intermediate to native structure, was reported to have a one-second time constant ([Gdn] = 1 M) [49]. The native protein contains five proline residues, all in the more stable trans configuration in the crystal structure. Brunori and coworkers observed a third refolding phase (k \approx 10^{-2} s⁻¹) that, on the basis of interrupted unfolding experiments, they attributed to proline isomerization steps [49]. Hence, the early folding intermediate that we and Brunori have observed is not likely due to the presence of cis-Xaa-Pro traps.

Although the folding intermediate was proposed to be a single compact structure in which 60% of the exposed surface in the unfolded protein had been buried [49], our trFRET data suggest instead that it is a heterogeneous ensemble of compact and extended structures. Moment analysis of the Dns110 folding kinetics indicates that this ensemble has developed about 75% of native structure (Figure 7.2). For comparison, the burst phase ensemble of yeast cytochrome *c* contains a comparable amount of native character, 60-75%, depending on residue [78].

The remarkable difference between the two proteins is that, in yeast cytochrome *c*, this structure develops in less than 150 μ s [25], whereas comparable structure development in cytochrome c_{552} is almost ten times slower. The sluggish refolding of cytochrome c_{552} carries over into the final phase as well, as a full second is required for formation of the native state; in yeast cytochrome *c*, the folded state develops in tens to hundreds of milliseconds in the absence of misligation [78]. This finding presents a challenge to theoretical models that account for contact order [81], as well as hydrophobic and electrostatic forces [82].



Figure 7.3: Analysis of the $P(r_{DA})$ fits of the folding kinetics of Dns110-cytochrome c_{552} . (A) Mean distance between Dns110 and the heme (M_1) as a function of folding time. The solid line is a monoexponential fit with a rate constant of 1060 s⁻¹. This fit extrapolates to 31.5 Å, in contrast to M_1 for the folded protein of 23 Å. (B) Time course of the second moment (M_2) . The solid line is a monoexponential fit with a rate constant to M_2 for the folded protein of 560 Å². (C) Time course of the variance (V). The solid line is a double exponential fit with rate constants 2070 and 1100 s⁻¹. This fit extrapolates to 200 Å², in contrast to V for the folded protein of 31 Å².

7.3 Conclusions

Microfluidic mixing by a continuous flow mixer with a 150- μ s dead time has enabled us to resolve the fast formation (~ 1 ms) of the on-pathway intermediate of cytochrome c_{552} . We determined by time-resolved FRET that the intermediate is a heterogeneous ensemble of states with Dns110-heme distances ranging from 15 to \geq 50 Å. This supports the proposal from energy landscape theory that intermediates are a build-up of states due to a kinetic barrier [7, 8], not a discrete structure along a single folding pathway. Our finding likely extends to intermediates in other proteins, emphasizing the importance of using techniques that allow for characterization of heterogeneous ensembles [80].

7.4 Acknowledgments

This work was carried out in collaboration with Seiji Yamada, a visiting scientist from Sony Corporation. We would also like to acknowledge William Ford for his contribution in writing code to aid in the analysis of mixer data.

Chapter 8 Protein Folding Summary

We have investigated the denaturant-induced unfolding of thermostable cytochrome c_{552} by time-resolved fluorescence energy transfer (FRET) between the heme and each of seven site-specific fluorescent probes. The protein exhibits twostate or three-state equilibrium unfolding transitions, depending on the protein region monitored. Interestingly, the observed unfolding intermediate contains native contacts in the unique structural features that differentiate cytochrome c_{552} from other class-I *c*-type cytochromes. This suggests that the extra structural features provide additional stabilization for this thermostable protein.

We then resolved the formation of cytochrome c_{552} 's refolding intermediate by coupling time-resolved FRET to a continuous flow microfluidic mixer. Fitting the rates of energy transfer between a dansyl fluorophore, attached to singlecysteine variants (residue 110 for cytochrome c_{552}), and the cytochrome heme provides us with time-dependent distance distributions, a measure of the reaction progress. The intermediate is comprised of a heterogeneous ensemble of compact and extended polypeptides, an interesting finding that likely extends to other proteins. We have also characterized the fast folding of cytochrome cb_{562} by timeresolved FRET and microfluidic mixing. Cytochrome cb_{562} belongs to an interesting family of four-helix bundle cytochromes that have nearly identical structures, but widely divergent folding pathways. This rare characteristic creates a unique opportunity to study the relationship between amino acid sequence and folding pathways in proteins with similar structural topologies. The folding rates for two variants that probe folding between the C-terminal heme and either the first helix or the third helix were essentially identical. This suggests that cytochrome $c-b_{562}$ folds by a two-state mechanism in which the entire protein folds cooperatively and rapidly over several milliseconds.

In addition, we have investigated intrachain contact dynamics in unfolded cytochrome cb_{562} by monitoring electron transfer, which occurs as the heme collides with a ruthenium photosensitizer covalently bound to residues along the polypeptide. Intrachain diffusion for chemically denatured proteins proceeds on the microsecond timescale with an upper limit of 0.1 μ s. The rate constants exhibit a power-law dependence on the number of peptide bonds between the heme and Ru complex. Adherence of our data to a slope of -1.5, consistent with theoretical models for ideal polymers, demonstrates that cytochrome cb_{562} is minimally frustrated. Importantly, as predicted for a funneled energy landscape, our work shows that making tertiary contacts can enhance the formation of partially folded structures.