New light on protein folding: Unraveling folding and unfolding mechanisms using time-resolved and two-dimensional vibrational spectroscopy
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Small-sized, structurally simple proteins generally provide excellent model systems for studying the fundamental forces that control protein folding. A suitable testing-ground for proteins exhibiting a mixed $\alpha/\beta$ fold is the 28-residue zinc-finger mutant FSD-1, which is designed to form a metal-independent folded $\beta\beta\alpha$-motif. Although the folding pathway of FSD-1 has been actively studied, the folding mechanism remains largely unclear. In particular, it is unclear in what stage of folding the $\alpha$-helix is formed. In this chapter, we investigate the folding mechanism of FSD-1 using temperature-dependent UV circular dichroism (UV-CD), Fourier transform infrared (FTIR) spectroscopy, and two-dimensional infrared (2D-IR) spectroscopy. Our UV-CD and FTIR data show different thermal melting transitions, indicating multistate folding behavior. Based on our equilibrium 2D-IR results, we find the $\alpha$-helix to be the thermodynamically most stable structural element of FSD-1. To investigate the folding and unfolding dynamics of FSD-1, we use time-resolved temperature-jump ($T$-jump) spectroscopy in the amide I’ spectral region. The conformational changes induced by the nanosecond $T$-jump are probed with transient IR absorption experiments as well as with transient IR dispersed pump-probe (DPP) spectroscopy, resulting in a structurally and temporally resolved picture of the (un)folding process. We find bi-exponential $T$-jump relaxation kinetics with time constants of $80 \pm 13$ ns and $1300 \pm 100$ ns at 322 K, implying that the folding process of FSD-1 involves an intermediate conformation. We observe a different kinetic response for different structure-sensitive amide I’ frequencies, determined by the structural nature of the intermediate state. Our equilibrium and transient results suggest that the folding of FSD-1 involves the early formation of $\alpha$-helical contacts, followed by the formation of the $\beta$-hairpin and hydrophobic contacts.
8.1 Introduction

Zinc finger domains of the Cys$_2$His$_2$ class are relatively small protein motifs that are abundantly found in various types of mammalian cells, and play an essential role in regulating gene expression as well as in mediating protein-protein interactions by acting as target-specific binding domains [1–5]. To specifically bind to nucleic-acid and protein target sites, Cys$_2$His$_2$-type zinc fingers are often arranged in tandem repeats. Each individual domain typically consists of 30 amino-acid residues and folds autonomously around a central zinc ion coordinated by two Cys and His residues, forming a $\beta\alpha$-motif which is characterized by an N-terminal $\beta$-hairpin and a C-terminal $\alpha$-helix [6, 7]. The $\alpha$-helix (often named the “recognition helix”) of an individual zinc finger determines its sequence-specific binding to RNA and DNA bases and other protein target sites [1, 5, 8, 9]. Gaining knowledge about the fundamental forces that determine the stability and folding pathway of zinc finger motifs becomes increasingly relevant, as a growing number of proteins in this family have been connected to human diseases, including cancer and neurological disorders [10, 11].

An attractive target molecule for such protein folding investigations is the 28-residue zinc finger miniprotein FSD-1 (“full sequence design 1”), which was designed by Dahiyat and Mayo [12] to mimic the three-dimensional $\beta\alpha$-fold of the natural zinc finger Zif268. It consists entirely of naturally occurring amino-acid residues and is one of the smallest model proteins to exhibit structural properties more typical of larger proteins. Unlike natural zinc-finger proteins, FSD-1 forms a folded $\beta\alpha$-motif without zinc binding. It folds into a well-ordered globular structure that contains an N-terminal $\beta$-hairpin (residues 3-13), a $\beta$-turn (residues 7-9), a C-terminal $\alpha$-helix (residues 14-25), and a compact hydrophobic core structure which is formed by packing of the hydrophobic side-chain groups of Tyr3, Ala5, Ile7, Phe12, Leu18, Phe21, Ile22, and Phe25 (see Fig. 8.1) [12]. Enhanced stabilization of the folded $\beta\alpha$-structure is provided through hydrogen bonding of the backbone amide proton of residue Glu15 to the side-chain carbonyl oxygen of residue Asn14 [12, 13]. On the hydrophilic side of the $\alpha$-helix, three pairs of hydrogen-bonding interactions are formed between different charged side-chain groups, [12] and a mutation study has identified these electrostatic interactions as important stabilizing features of the miniprotein [13].

From its short sequence, FSD-1 is expected to fold rapidly into its native structure according to simple folding kinetics. While the folding and unfolding times of FSD-1 have not yet been experimentally characterized, the $T$-jump-induced relaxation kinetics of the close analog FSD-1ss (involving the substitution of the Lys residues at positions 6 and 26 with two non-natural fluorescent aromatic amino acids) monitored using Förster resonance energy transfer (FRET) showed bimodal relaxation kinetics with time constants of 150 ns and 4.5 $\mu$s near its melting temperature ($T_m$) [14]. The observation of two kinetic timescales indicates a more complicated folding scenario involving the formation of at least one effective folding intermediate, suggesting a possible three-state folding mechanism ($U \rightleftharpoons I \rightleftharpoons F$), see section 1.3.2. However, the nature of the folding and unfolding pathways of
both FSD-1ss and FSD-1 is still under ongoing discussion, and as yet, the folding kinetics of FSD-1 have not been resolved experimentally.

Previous experimental [12, 13, 16] and theoretical [16–26] investigations of the equilibrium and kinetic properties of FSD-1 have reached different conclusions regarding its folding and unfolding mechanism. In particular, the nature of the melting transition and the stages of folding in which the different secondary and tertiary structural elements are formed are subject of debate. Both experimental [12, 16] and simulation-based [16, 19, 21, 22] melting curves show that FSD-1 exhibits an unusually broad thermal unfolding profile, indicating a relatively weak folding cooperativity between the α-helix and β-hairpin. The breadth of the folding-unfolding transition has been assigned to a spread in melting temperatures ($T_m$) of the individual structural elements of the miniprotein, viz., the β-hairpin, the hydrophobic core structure, the α-helix, and the tertiary fold [21]. The α-helical segment has been identified as the thermodynamically most stable structural component [16, 18–22], and several computational studies have provided evidence that the early formation of the α-helical structure initiates the folding process of FSD-1, after which the folding proceeds with the concurrent formation of the β-hairpin and tertiary contacts [17–21]. In contrast, Monte Carlo folding simulations have revealed a pathway for FSD-1 folding that begins with the collapse of the hydrophobic side groups, followed by the folding of individual secondary structural elements, of which the formation of the β-hairpin moiety occurs prior to the folding of the α-helix [27]. On the other hand, from all-atom ab initio folding simulations it has been concluded that the folding of secondary structures precedes the formation of hydrophobic contacts [22, 24], and that the folding process of FSD-1 involves two distinctive pathways [22]: these simulations predict that the majority of the folding occurs through the formation of the α-helical structure.

Figure 8.1: (a) Stick and (b) ribbon representation of the main-chain fold of FSD-1 showing the β-hairpin (blue), α-helix (green), and the aromatic side-groups forming the hydrophobic core structure. Structure optimized and rendered with Chimera [15].
contacts prior to that of the β-hairpin and the globular fold, while a minor route exists in which the β-hairpin moiety is formed first [22]. Additional evidence for the existence of multiple folding pathways was provided by all-atom action-derived molecular dynamics (ADMD) [25]. However, this computational study predicts a folding scenario in which the hydrophobic collapse occurs first, after which either the α-helix or the β-hairpin is formed [25] (i.e., there are two independent folding pathways, in contrast to Ref. [27]).

As summarized above, several very different scenarios exist for the folding of the zinc finger mutant FSD-1. In particular, it remains unclear in what stage of folding the different secondary and tertiary structural elements are formed. Existing computational studies provide a variety of possible folding scenarios, while experimental kinetic data of FSD-1’s folding transition has not yet been reported. Here, we investigate the temperature-induced structural changes of FSD-1 in thermal equilibrium using UV circular dichroism (UV-CD), Fourier transform infrared (FTIR) spectroscopy, and two-dimensional infrared (2D-IR) spectroscopy. In order to study FSD-1’s folding dynamics, we use a nanosecond temperature-jump ($T$-jump) to perturb its folding-unfolding equilibrium, and the subsequent re-equilibration is tracked by probing the time-dependence of the conformationally sensitive amide I transitions using transient linear IR absorption experiments as well as transient dispersed pump-probe (DPP) IR spectroscopy (see chapter 7). We find that the $T$-jump relaxation kinetics at a final $T$-jump temperature close to FSD-1’s melting temperature can be well described by a bi-exponential function. Accordingly, our findings show that the folding mechanism of FSD-1 involves the occurrence of an intermediate state, the structural details of which can be determined based on the time- and frequency-resolved $T$-jump data.

### 8.2 Equilibrium properties

#### 8.2.1 Linear infrared spectrum

Fig. 8.2a shows equilibrium FTIR spectra of FSD-1 in the amide I’ spectral region for a range of temperatures between 274 and 372 K. The major peak centered at 1644 cm$^{-1}$ in the low-temperature spectrum arises from amide I’ backbone vibrations of amino-acid residues involved in an α-helical and β-hairpin conformation [28]. We observe a small shoulder at the high-frequency side of the amide I’ band ($\sim$1680 cm$^{-1}$), characteristic of proteins that exhibit a mixed α/β fold [28]. The minor spectral features occurring on the low-frequency side of the amide I’ peak observed at 1585 and 1607 cm$^{-1}$ originate from the symmetric and antisymmetric CN-stretch vibrations of the side-chain guanidinium (CN$_3$H$_5^+$) group of Arg [28]. The former spectrally overlaps with contributions of the CO-stretching mode of the side-chain carboxylic group (COO$^-$) of Asp and the carboxyl terminus that appears at 1586 cm$^{-1}$ [28]. The minor component observed at 1565 cm$^{-1}$ is attributed to the COO$^-$ group of Glu [28]. At increasing temperature, the center frequency of the amide I’ peak shifts from 1644 to 1648 cm$^{-1}$, primarily due to the loss of secondary and tertiary structural contacts upon thermal unfolding of
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Figure 8.2: (a) Temperature-dependence of the FTIR absorbance of FSD-1 in the amide I’ region collected between 274 and 353 K. (b) Equilibrium FTIR difference spectra obtained by subtracting the FTIR spectrum at 274 K from those at higher temperatures. All spectra were corrected for the contribution of the solvent.

The miniprotein. In addition, we observe several temperature-induced changes in IR absorbance at frequencies where the charged residues Arg, Asp, and Glu contribute. Possibly, these changes are indicative of breaking hydrogen bonds formed between oppositely charged side chains (salt bridges) and/or changes in the degree of solvent exposure of the charged residues as the miniprotein unfolds.

To study the frequency-dependent absorbance changes caused by thermal unfolding of FSD-1 in more detail, equilibrium FTIR difference spectra were obtained by subtraction of the low-temperature spectrum (274 K) from those at higher temperatures (see Fig. 8.2b). The temperature-induced blue shift of the amide I’ mode is reflected in broad negative and positive features centered at 1631 and 1664 cm\(^{-1}\), respectively. This spectral signature is indicative of the differences in relative \(\alpha\)-helical and \(\beta\)-hairpin populations between the folded and unfolded state ensembles, thereby providing an excellent IR marker to probe the conformational changes of the miniprotein in response to thermal unfolding. More specifically, the low frequency amide I’ mode (1631 cm\(^{-1}\)) follows the loss of \(\alpha\)-helical and \(\beta\)-hairpin populations, and the high frequency amide I’ mode (1664 cm\(^{-1}\)) results from the concomitant increase in the unfolded-state ensemble [28]. In addition, the small negative and positive features at 1556, 1575, and 1589 cm\(^{-1}\) arise from the absorbance change of the charged side chains of the Arg, Asp, and Glu residues upon
thermal unfolding [28]. These spectral features can potentially provide information about the loss of tertiary contacts and/or the unfolding of the hydrophobic cage structure due to the disruption of favorable electrostatic interactions and/or changes in the degree of solvent exposure involving the side groups of residues Asp, Glu and/or Arg as the entire miniprotein unfolds.

### 8.2.2 Unfolding in thermal equilibrium

The temperature-induced unfolding transition of FSD-1 was analyzed in thermal equilibrium using UV-CD and FTIR spectroscopy. Temperature-dependent UV-CD spectra were measured in the 190–265 nm wavelength range between 267–368 K, while FTIR spectra were collected between 274 and 372 K with incremental steps of 5 K. The low-temperature UV-CD spectrum of FSD-1 (see the inset of Fig. 8.3a) shows a negative intensity extremum at 207 nm with a shoulder at 220 nm, indicating a mix of β-hairpin, α-helix, and coil structure (see section 2.1.1).

In Fig. 8.3 we compare the dependence on temperature of the ellipticity at 222 nm ($\theta_{222}$) and the temperature-dependent FTIR response at the frequencies of two different structure-sensitive vibrational modes, viz., 1575 cm$^{-1}$ ($\nu_{\text{COO}^-}$ of Glu, Fig. 8.3a) and 1631 cm$^{-1}$ (main amide I’ mode, Fig. 8.3b). All UV-CD and FTIR thermal denaturation curves show a broad sigmoidal transition, reflecting the temperature-induced conformational changes of FSD-1 upon unfolding in thermal equilibrium. Singular value decomposition (SVD) of the temperature-
dependent UV-CD spectra and global fitting analysis indicates that the folding is well described by a two-state transition with a thermal melting point \( T_m \) of 311.0 ± 1.3 K, in close agreement with the transition midpoint of 315.0 K reported previously for FSD-1 [12]. Interestingly, from least-squares fits of the temperature-dependent FTIR data probed at 1575 cm\(^{-1}\) and 1630 cm\(^{-1}\) to a two-state model [29], we obtain two distinct melting temperatures of \( T_m = 306.0 \pm 1.1 \) K (1575 cm\(^{-1}\)) and \( T_m = 350.5 \pm 2.8 \) K (1630 cm\(^{-1}\)). The observation of IR-frequency dependent melting points is indicative of multiple thermal unfolding transitions [30], and is probably caused by different stabilities of the different structural elements of the miniprotein upon temperature-induced unfolding. This suggests that the folding pathway of FSD-1 proceeds through the occurrence of at least one effective intermediate conformation, as is confirmed by SVD analysis of the temperature-dependent FTIR data (see section 8.5.2 for details). The individual transitions probably involve the loss of secondary structure due to the unfolding of the entire polypeptide backbone (1630 cm\(^{-1}\)) and the melting of the hydrophobic cage and/or the loss of tertiary contacts probed by the absorption changes involving the side-chain COO\(^-\) group of Glu (1575 cm\(^{-1}\)). As the hydrophobic core of FSD-1 is formed by an unusually large number of buried aromatic side chains which give rise to a positive CD signal at 230 nm that interferes with the negative bands at 218 nm and 222 nm (originating from the \( \beta \)-hairpin and the \( \alpha \)-helix, respectively), it was concluded previously by Wu et al. [31] that the thermal denaturation transition measured using UV-CD spectroscopy mainly reflects the melting of the hydrophobic core structure. This would be a plausible explanation for the close agreement between the melting points probed at 222 nm using UV-CD and at 1575 cm\(^{-1}\) using FTIR.

### 8.2.3 Equilibrium 2D-IR spectroscopy

Improved resolution of the amide I’ spectral features is achieved by spreading the amide I’ absorption data over a second frequency dimension using polarization-sensitive 2D-IR experiments, which give rise to characteristic cross-peaks resulting from the interaction (or coupling) between different amide I’ normal modes. Studying the vibrational coupling between different amide I’ normal modes at varying temperatures can provide additional information about the conformational changes of FSD-1’s backbone during thermal denaturation. In view of the global melting temperature of FSD-1 of 311.0 ± 1.3 K, we measured parallel and perpendicular polarization 2D-IR spectra at 275 K such that the folding equilibrium favors the native state (Fig. 8.4b,c), and at 333 K where the folding equilibrium is dominated by the unfolded conformation (Fig. 8.4g,h). The low-temperature 2D-IR spectra shown in Fig. 8.4b,c reveal a broad absorption band along the diagonal produced by spectrally overlapping contributions arising from the \( \alpha \)-helical and \( \beta \)-hairpin structural features of FSD-1. The main amide I’ peak is centered at approximately 1644 cm\(^{-1}\), with small shoulders around 1630 cm\(^{-1}\) and 1680 cm\(^{-1}\). At the low-frequency side of the diagonal response, we find two separated bands due to symmetric and asymmetric combinations of the side-chain guanidinium (CN\(_3\)H\(_5\)\(^+\))
group of Arg, appearing at 1585 cm\(^{-1}\) and 1607 cm\(^{-1}\), respectively. With increasing temperature, the amide I’ diagonal peak shifts to slightly higher frequency while decreasing in intensity (see Fig. 8.4g and h).

The diagonal contribution to the 2D-IR response can be eliminated by constructing a polarization-weighted difference 2D-IR spectrum \((\Delta A = 3 \times \Delta A_\perp - \Delta A_\parallel)\), in which only cross peaks contribute to the observed signal (see chapter 7) [32–34]. The polarization-weighted difference spectra of FSD-1 at 275 K and 333 K are shown in Fig. 8.4d and i, respectively, and display distinct signatures typical of an \(\alpha\)-helical and \(\beta\)-hairpin conformation [35–39]. At low temperature (Fig. 8.4d), we observe two characteristic cross peaks centered at approximately \((\nu_{\text{probe}}, \nu_{\text{pump}}) = (1638, 1660)\) and \((1650, 1635)\) cm\(^{-1}\), arising from the delocalized \(A^- (\parallel)\) and \(E_1^- (\perp)\) symmetry modes of amide I’ oscillators involved in an \(\alpha\)-helical backbone conformation (see chapter 7) [35, 36, 40].

By elevating the temperature, we find that the \(\alpha\)-helix amide I’ cross peak signature decreases in intensity, but does not disappear completely, indicating that a considerable amount of \(\alpha\)-helical structure remains present in the thermally denatured state of FSD-1 at 333 K, when only \(\sim 25\%\) of the peptide is in the native state (Fig. 8.3a). This suggests that the \(\alpha\)-helix is thermally more stable compared to the \(\beta\)-hairpin of FSD-1, in agreement with previous studies [16, 18–22]. We find that at 333 K, the fraction of the folded population has decreased to \(25\%\) of its value at 275 K (see Fig. 8.3a), while the \(\alpha\)-helix intensity at 333 K has decreased to only \(60\%\) of its value at 275 K (see Fig. 8.4d and i). This implies that the transition midpoint of \(350.5 \pm 2.8\) K as derived from the temperature-dependent FTIR response probed at 1630 cm\(^{-1}\), mainly reflects the unfolding transition of the \(\alpha\)-helical structure, whereas the transition midpoints monitored at 222 nm using UV-CD and at 1575 cm\(^{-1}\) using FTIR probably mainly arises from contributions of the \(\beta\)-hairpin moiety, hydrophobic contacts, and tertiary structure elements. These findings are in agreement with those reported previously [21].

Fig. 8.4e and j depict the projections of the polarization-weighted difference 2D-IR spectra \((\int \Delta A_{3\perp-\parallel} d\nu_{\text{pump}})\) onto the probe axis at 275 K and 333 K, respectively. As discussed in chapter 7, such projection spectra \((\int \Delta A d\nu_{\text{pump}})\) are obtained by integration of the 2D-IR response over \(\nu_{\text{pump}}\), which results in a transient spectrum acquired over one frequency dimension that is equivalent to the dispersed pump-probe (DPP) spectrum [34, 38, 41]. Indeed, the polarization-weighted integral of the 2D-IR \((\int \Delta A_{3\perp-\parallel} d\nu_{\text{pump}})\) and the polarization-weighted DPP \((\Delta A_{3\perp-\parallel}^{\text{DPP}})\) spectra of FSD-1 are in qualitative agreement (see Fig. 8.4e). The small discrepancy observed between the \(\int \Delta A_{3\perp-\parallel} d\nu_{\text{pump}}\) and \(\Delta A_{3\perp-\parallel}^{\text{DPP}}(\nu_{\text{probe}})\) spectra in the frequency region where the coupling between the Arg\(^+\) modes participates is caused by the difference in time delay between the IR-pump and IR-probe pulses, which are 1 ps and 200 fs for the 2D-IR and DPP spectra, respectively [41]. At 1 ps, more intramolecular energy transfer (IVR) [42, 43] will have occurred than at 200 fs, resulting in more intense integrated Arg\(^+\) cross peaks in the \(\int \Delta A_{3\perp-\parallel} d\nu_{\text{pump}}\) spectrum relative to the \(\Delta A_{3\perp-\parallel}^{\text{DPP}}(\nu_{\text{probe}})\) spectrum [41]. As expected based on the temperature-dependent FTIR and 2D-IR spectra, with
Figure 8.4: (a, f) Solvent-corrected infrared absorption spectrum of FSD-1 at 275 K and 333 K respectively. (b, g) 2D-IR spectrum, with parallel polarization of the pump and probe pulses, of FSD-1 at 275 K and 333 K, respectively. (c, h) 2D-IR spectrum, with perpendicular polarization of the pump and probe pulses, of FSD-1 at 275 K and 333 K, respectively. (d, i) Polarization difference 2D-IR spectrum (2.5ΔA⊥ − ΔA||) of FSD-1 at 275 K and 333 K, respectively. (e) Projection of the 2D-IR difference spectrum in panel d onto the probe axis and the DPP spectrum at 322 K. (j) Projection of the 2D-IR difference spectrum in panel i onto the probe axis. For the 2D-IR spectra: blue indicates negative absorption change, red positive absorption change; the contour intervals are 40 mOD; the delay between pump and probe pulses is 1 ps. The delay between pump and probe pulses for the DPP and t-DPP spectra is 200 fs.
increasing temperature the 2D-IR projection spectrum shifts to higher frequency and loses intensity (Fig. 8.4e and j).

Equilibrium difference 2D-IR spectra were obtained by subtraction of the 2D-IR spectra measured at 275 K from the spectra collected at 333 K (Fig. 8.5). As can be seen from Fig. 8.5b, the resulting temperature-dependent spectral 2D-IR changes for parallel polarization show different overlapping and interfering positive and negative features. Note that the loss of intensity in the thermal difference spectrum appears as a negative feature if the corresponding area is positive in the 2D-IR spectrum, and vice versa. The observed spectral changes are mainly a reduced intensity and blue shift of the diagonal peaks due to the loss of secondary structure when the miniprotein unfolds upon thermal denaturation. We observe only a minor intensity loss of the cross peaks due to the vanishing of the $\alpha$- and $E$-symmetry modes of the $\alpha$-helix with temperature.

$$
\Delta \int \Delta A \, d\nu_{\text{pump}} = \int \Delta A_{T=275K}^{275K}(\nu_{\text{pump}}, \nu_{\text{probe}}) d\nu_{\text{pump}} - \int \Delta A_{T=333K}^{333K}(\nu_{\text{pump}}, \nu_{\text{probe}}) d\nu_{\text{pump}}.
$$

As can be seen, the $\Delta \int \Delta A_{\parallel} \, d\nu_{\text{pump}}$ signal displays an intensity loss in the low- and high-frequency regions of the spectrum and an intensity gain in the center region. The positive spectral feature centered at 1647 cm$^{-1}$ can be attributed to...
overlapping contributions arising from the loss of \(\alpha\)-helical and \(\beta\)-hairpin structure, while the positive shoulder observed at 1633 cm\(^{-1}\) probably mainly follows the loss of \(\beta\)-hairpin configurations in the ensemble [39]. The negative features on the high-frequency side of the spectrum originate from overlapping contributions due to the loss of \(\alpha\)-helical and \(\beta\)-hairpin conformations, and the appearance of disordered configurations.

### 8.3 Temperature-jump dynamics

We study the folding and unfolding dynamics of FSD-1 using \(T\)-jump spectroscopy. A nanosecond \(T\)-jump initiates a rapid shift of the thermodynamic folding equilibrium, thereby initiating a conformational re-distribution process between the folded and unfolded state ensembles. The subsequent structural changes that take place as the miniprotein proceeds to its new equilibrium are observed using both transient linear IR absorption experiments and transient dispersed IR pump-probe (DPP) spectroscopy in the amide I' spectral region (1600–1700 cm\(^{-1}\)). In both cases, the initial temperature of the system was \(T_i = 315\) K, and was raised by 7 K within approximately 5 ns. Considering the global melting temperature of FSD-1 of 311.0 ± 1.3 K, the relative contributions of the effective folding and unfolding rate constants to the observed \(T\)-jump relaxation rate constant are comparable.

#### 8.3.1 \(T\)-jump amide I' IR-probe experiments

The time-dependent linear IR absorbance changes following a \(T\)-jump are shown in Fig. 8.6a for nanosecond to microsecond delay times. We observe a broad spectral change with negative and positive contributions centered at approximately 1631 cm\(^{-1}\) and 1664 cm\(^{-1}\), respectively, which reflects the blue-shift of the amide I' mode during the conformational re-distribution process. We find that the time-resolved spectra at long delay times after the \(T\)-jump resemble the fully equilibrated FTIR difference spectrum (see Fig. 8.6a).

Fig. 8.6b shows the \(T\)-jump-induced relaxation kinetics monitored at the frequency where the transient absorption changes associated with the \(\alpha\)-helical and \(\beta\)-hairpin conformations (1631 cm\(^{-1}\)) and the increase of the unfolded state ensemble (1664 cm\(^{-1}\)) of FSD-1 occurs. Both \(T\)-jump relaxation traces show an instantaneous absorption change followed by a well-resolved kinetic phase. The former arises primarily from the intrinsic response of amide I' oscillators to the increased temperature [44], leading to a blue-shift of the amide I' absorption band and resulting in an absorption change that rises concomitantly with the time-resolution of the \(T\)-jump pulse (\(\sim 5\) ns). The kinetic phase observed in the amide I' spectral region results from the structural rearrangements of the miniprotein during the \(T\)-jump re-equilibration process. We find that the \(T\)-jump relaxation dynamics monitored at different frequencies can be well described by a bi-exponential function, and a global fit of the time- and frequency-resolved data reveals \(T\)-jump relaxation time constants of \(\tau_1 = 90 \pm 9\) ns and \(\tau_2 = 1290 \pm 66\) ns, indicating that
the folding of FSD-1 proceeds through the occurrence of at least one effective intermediate state (see section 1.3.2). The fast $T$-jump relaxation time constant likely reflects fast fluctuations due to a rapid pre-equilibration in the folded or unfolded free-energy basin, whereas the slower process can be attributed to crossing of the rate-limiting free-energy barrier and represents the global (un)folding process of the entire miniprotein. From the globally fitted relaxation dynamics monitored at 1631 cm$^{-1}$ and 1664 cm$^{-1}$, we find that the relative amplitudes of the kinetic responses is frequency-dependent (see Fig. 8.6b), which is determined by the structural nature of the intermediate state involved in the folding process of FSD-1. The transient absorption change at 1664 cm$^{-1}$ (disordered configurations) is dominated by the slow kinetic phase, whereas the $T$-jump response at 1631 cm$^{-1}$ ($\alpha$-helix and $\beta$-hairpin) shows a significant amplitude of both the fast and slow phases. Thus, the fast kinetic time scale likely follows rapid conformational rearrangements associated with the formation of the $\alpha$-helical and/or $\beta$-hairpin configurations. However, because of the overlapping contributions of the $\alpha$-helix and the $\beta$-hairpin, a more specific structural assignment of the folding intermediate is difficult based on these $T$-jump IR-probe experiments only. Furthermore, the $T$-jump response of the C=O-stretch mode of the side-chain carboxylic groups (1575 cm$^{-1}$) is dominated by the intrinsic temperature dependence of this mode, and our signal-to-noise ratio is not sufficient to measure the kinetic phase of the transient signals at 1575 cm$^{-1}$.
8.3 Temperature-jump dynamics

8.3.2 Transient IR dispersed pump-probe spectroscopy

In order to achieve additional structural resolution in the amide I’ region, we extend the T-jump IR-probe experiment to include a broad-band mid-IR pump (BB-IR) in a similar manner as in chapter 7, to measure transient dispersed pump-probe (t-DPP) spectra [41]. Time-resolving the DPP relaxation in response to a T-jump can provide additional insights into the structural evolution of the protein during the re-equilibration process due to its enhanced conformational sensitivity relative to linear IR spectroscopy. In addition, the contribution of the solvent to the DPP signal is negligible. This is because the transition dipole moment of the solvent is typically much smaller than that of the solute (and the intensity of the DPP signals is proportional to the fourth power of the transition dipole of the vibrational oscillator $|\mu|^4$, as opposed to $|\mu|^2$ in the linear IR response) [34]. In Fig. 8.7a, we present the transient DPP spectra in the amide I’ region at different delay times after the T-jump pulse for parallel polarization of the IR pump and probe pulses ($\Delta A_{\parallel}$), which is dominated by the diagonal response of the system. The time-dependent DPP spectral changes in the nano- to microsecond T-jump window consist of negative and positive spectral features that at long time delays (> 6000 ns) closely resemble the equilibrium 2D-IR projection spectrum ($\Delta \Delta A_{\parallel}$) (see Fig. 8.7a).

Fig. 8.7b shows the T-jump-induced DPP relaxation kinetics probed at 1633 cm$^{-1}$ ($\beta$-hairpin), 1647 cm$^{-1}$ ($\alpha$-helix and $\beta$-hairpin), and 1670 cm$^{-1}$ ($\alpha$-helix, $\beta$-hairpin, and disordered configurations). From a least-squares global fit of the transient DPP data to a bi-exponential function, we find relaxation time-constants of $\tau_1 = 80 \pm 13$ ns and $\tau_2 = 1300 \pm 100$ ns.
Figure 8.8: Decay-associated spectra (DAS) of FSD-1, showing the pre-exponential factors of the fast (blue) and slow (cyan) phase derived from the global bi-exponential fit of (a) the $T$-jump/IR-probe data, and (b) the $T$-jump/DPP-probe data.

constants of $\tau_1 = 80 \pm 13$ ns and $\tau_2 = 1300 \pm 100$ ns, which are in quantitative agreement with the time scales obtained from the conventional $T$-jump IR-probe experiments. Again, we find that the relative amplitudes of the kinetic responses are strongly frequency-dependent: the time trace probed at 1670 cm$^{-1}$ can be effectively described by the single slow kinetic phase, while the $T$-jump kinetics monitored at 1633 cm$^{-1}$ and 1647 cm$^{-1}$ exhibit significant contributions of both time scales.

To quantify the frequency-dependence of the relative contribution of the two kinetic time scales, decay-associated spectra (DAS) were constructed by plotting the relative amplitudes derived from the global bi-exponential fit as a function of frequency, for both the conventional $T$-jump/IR-probe data and the transient DPP data (see Fig. 8.8). We find that the DAS of the $T$-jump/IR-probe data are in agreement with those of the $T$-jump/DPP-probe data (Fig 8.8a and b, respectively): comparing the shapes of the two DAS in Fig 8.8 to the schematic spectral changes in Fig 8.9, we see that the slow and fast components are in both measurements caused by a blue shift and an intensity loss of the amide I' peak, respectively. Comparison of the spectral shapes of the slow DAS components (Fig 8.8a and b, cyan) to the schematic spectral changes shown in Fig 8.9a, indicates that the slow kinetic phase corresponds to a blue-shift of the amide I' mode. The slow component of the $T$-jump/IR-probe DAS (Fig 8.8a, cyan) is an intensity loss in the low-frequency region of the spectrum and an equal intensity gain in the high-frequency region similar to that of the schematic FTIR difference spectrum in Fig 8.9a (dark-green). The corresponding slow phase of the transient DPP DAS (Fig 8.8b, cyan) displays an intensity loss in the low- and high-frequency regions of the spectrum and an intensity gain in the center region which can be understood from the schematic $\Delta \Delta A$ spectrum in Fig 8.9a (magenta). Therefore, we conclude that the slow kinetic time scale is primarily due to blue-shifting of the amide I’ mode. The similarity of Fig 8.8a to Fig 3.2b and 7.7A, and of Fig 8.8b to
Fig 7.5F suggests that this blue shift is probably dominated by contributions of the α-helix. On the other hand, the fast DAS components show a similar spectral shape as the spectral changes in Fig 8.9b caused by a loss in intensity. This intensity loss is probably related to a rapid change in solvent-exposure of the β-hairpin. These observations, combined with our equilibrium 2D-IR data that identified the α-helix as the thermodynamically most stable structural component, suggest that the fast fluctuations involve mainly rapid structural rearrangements in the conformational state of the β-hairpin that is related to a transition close to the folded state. Although the connection between the abovementioned spectral and structural features requires further investigation, our findings indicate a folding

Figure 8.9: Schematic FTIR, 2D-IR, and 2D-IR projection spectra illustrating the spectral changes in case of (a) a blue shift and (b) an intensity loss. FTIR and 2D-IR spectrum at high (left panel) and low (center panel) temperature, respectively, the corresponding FTIR (ΔA, dark-green) and 2D-IR difference spectrum, and the projection of the 2D-IR difference spectrum (ΔΔA, magenta) onto the probe axis (right panel). The latter is equivalent to the T-jump DPP-probe response. Difference FTIR and 2D-IR spectra are obtained by subtraction of the low-temperature spectrum from the high-temperature spectrum. For the 2D-IR spectra: blue indicates negative absorption change, red positive absorption change.
scenario of FSD-1 that proceeds through the occurrence of an intermediate state that contains well-defined α-helical structural elements, and mainly differs from the native structure in the structural arrangement of the β-hairpin.

8.4 Conclusions

Our equilibrium UV-CD and FTIR data reveals probe-dependent thermal unfolding transitions of FSD-1, indicating multiple structural transitions when the mini-protein unfolds upon temperature-induced denaturation. From our temperature-dependent UV-CD, FTIR, and equilibrium 2D-IR measurements, we find that the thermodynamic stability of the α-helix of FSD-1 is greater than that of the β-hairpin, the hydrophobic core structure, and the tertiary structural contacts, in agreement with previously reported MD results [21]. Our $T^*$-jump IR-pump-probe measurements show bimodal relaxation dynamics with time constants of 80 ± 13 ns and 1300 ± 100 ns, providing kinetic evidence that the folding proceeds via an intermediate conformation. The combined analysis of our steady-state and time-resolved data suggest that the folding pathway proceeds through the formation of an intermediate conformation with well-defined α-helical structural elements. This would indicate a folding scenario in which the formation of α-helix precedes the formation of the β-hairpin, the hydrophobic core, and the tertiary structure.

8.5 Additional methods and data

8.5.1 Materials and methods

The 28-residue zinc finger miniprotein FSD-1 (“full sequence design 1”) consisting of the sequence QQYTA KIKGR TFRNE KELRD FIEKF KGR is used in all experiments [12]. The FSD-1 miniprotein (≥ 95 % purity) was purchased from GL Biochem (Shanghai) and used without further purification. The peptide was lyophilized against a 35% DCl/D$_2$O solution to remove residual trifluoroacetic acid (TFA) and to achieve H/D-exchange. FSD-1 stock solutions of 7–10 mM were prepared by directly dissolving dry peptide in a 50 mM KD$_2$PO$_4$/K$_2$DPO$_4$ buffer (pH$^*$ = 7.0), resulting in peptide solutions at pH$^*$ = 2.5. The pH$^*$ of the peptide solution was adjusted to neutral pH level (pH$^*$ = 7.0) by addition of NaOD solution (pH$^*$ = uncorrected pH meter reading in D$_2$O and the NaOD volume added was less than 1% of the peptide solution).

UV-CD spectra were measured using an Olis DSM-1000 CD Spectrophotometer equipped with a Julabo (CF31) temperature controller. The peptide concentration used for the CD measurements was 40 µM in a 20 mM phosphate buffer (pH = 6.9) and all measurements were performed using 2-mm quartz cuvettes. The temperature-dependency of the ellipticity was monitored between 257.5–190 nm with incremental steps of 0.75 nm and averaging over 5 s of data acquisition time. Singular value decomposition (SVD) and global fitting and of
the temperature-dependent dataset was performed using the Olis GlobalWorks software package.

Temperature-dependent equilibrium Fourier-transform infrared (FTIR) spectra were collected using a Bruker Vertex 70 spectrometer. The peptide was kept at 7–10 mM concentration between 2 mm thick CaF$_2$ windows separated by a 50 µm Teflon spacer. Temperature control was achieved by a circulating water bath. Typically, 75 scans were signal averaged at a resolution of 2 cm$^{-1}$ to generate one spectrum.

A detailed description of the optical setups as used for the equilibrium 2D-IR, the $T$-jump IR-probe, and the transient DPP experiments can be found in sections 2.1.2, 2.2.1, and 2.2.2, respectively.

8.5.2 SVD analysis of the temperature-dependent FTIR data

SVD analysis of our temperature-dependent FTIR data (Fig. 8.2a) were performed to confirm the presence of an intermediate state. As can be seen in Fig. 8.10, four principal components were required to describe the temperature-dependent FTIR data. The fifth (cyan) and higher vectors of the SVD all represent uncorrelated noise contributions, and their weights were negligible relative to those of the first components (< 0.5%). For a two-state folder, one would expect that the data could be described by three principal spectral components, viz. an average spectrum that remains invariant with temperature, a shift caused by the intrinsic temperature-dependence of the amide I’ mode, and a shift caused by the global (un)folding transition. As we find that our data can be well described by four vectors, we conclude that the fourth target vector accounts for the absorption changes induced by the formation of a folding intermediate, and thus that the folding transition of FSD-1 involves the formation of an effective intermediate state. A more pre-

![Figure 8.10: Frequency-dependence (a) and temperature-dependence (b) of the first five target vectors resulting from singular-value decomposition (SVD) of the FTIR data as shown in Fig. 8.2a. The fifth target vector (cyan) represents uncorrelated noise contributions.](image-url)
cise interpretation can be based on the linear combination of the different target vectors \[45\].
References

