Probing the folding intermediate of Rd-apocyt b_{562} by protein engineering and infrared T -jump

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(RECEIVED August 17, 2006; FINAL REVISION March 5, 2007; ACCEPTED March 13, 2007)

Abstract

Small proteins often fold in an apparent two-state manner with the absence of detectable early-folding intermediates. Recently, using native-state hydrogen exchange, intermediates that exist after the rate-limiting transition state have been identified for several proteins. However, little is known about the folding kinetics from these post-transition intermediates to their corresponding native states. Herein, we have used protein engineering and a laser-induced temperature-jump (T -jump) technique to investigate this issue and have applied it to Rd-apocyt b_{562} , a four-helix bundle protein. Previously, it has been shown that Rd-apocyt b_{562} folds via an on-pathway hidden intermediate, which has only the N-terminal helix unfolded. In the present study, a double mutation (V16G/I17A) in the N-terminal helix of Rd-apocyt b_{562} was made to further increase the relative population of this intermediate state at high temperature by selectively destabilizing the native state. In the circular dichroism thermal melting experiment, this mutant showed apparent two-state folding behavior. However, in the T -jump experiment, two kinetic phases were observed. Therefore, these results are in agreement with the idea that a folding intermediate is populated on the folding pathway of Rd-apocyt b_{562} . Moreover, it was found that the exponential growth rate of the native state from this intermediate state is roughly $(25 \mu\text{sec})^{-1}$ at 65°C.

Keywords: T -jump; infrared; Rd-apocyt b_{562} ; intermediate; protein folding

Understanding the folding mechanism of a protein requires a detailed characterization of its folding energy landscape. However, providing a truly quantitative description of the folding energy landscape of a protein remains challenging because most of the experimental techniques employed in protein folding studies lack either the necessary time resolution or sufficient structural resolution. For example, it has been difficult to measure the growth rate of the native structure from the rate-

limiting transition state ensemble because of the downhill nature of this process. Nonetheless, if the consolidation of the final, native conformation involves a large enough free energy barrier or a post-transition intermediate, it is possible to experimentally examine this process in detail. Herein, we have employed a protein-engineering technique (Chu et al. 2002b; Takei et al. 2002), in conjunction with a temperature-jump (T -jump) infrared method (Huang et al. 2002; Wang et al. 2004), to study the major post-transition kinetic events of a four-helix bundle protein, Rd-apocyt b_{562} (Chu et al. 2002b; Takei et al. 2002).

Recently, a native-state hydrogen exchange method (Bai et al. 1995) has been applied to several small proteins. Partially unfolded forms (PUFs) were identified for some systems (Roder et al. 1988; Jeng and Englander 1991), including cyt c (Maity et al. 2005), Rd-apocyt b_{562} (Takei et al. 2002), and barnase (Bai 2003; Vu et al.

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Article published online ahead of print. Article and publication date are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.062505607>.

2004). Since these intermediates were not observed in the conventional kinetic mixing/folding experiments, they have been suggested to exist after the rate-limiting transition state and were referred to as “hidden intermediates” (Bai 2003). These observations are rather intriguing because they raise an important question concerning the mechanism of protein folding. Specifically, is folding after the rate-limiting step a downhill process that encounters only diffusive barriers (Sabelko et al. 1999; Garcia-Mira et al. 2002; Yang and Gruebele 2004), or does it involve distinct steps (Maity et al. 2005)? While conventional mixing/folding experiments are quite useful in the study of the kinetics of early-folding intermediates, they fail to report intermediates existing after the rate-limiting transition state. In principle, such an intermediate can be detected in mixing/unfolding experiments. However, the denaturant may destabilize the intermediate more than the native state and, as a result, significantly decrease its population, making the detection of such post-transition intermediates difficult. On the other hand, it has recently been shown that the *T*-jump technique, when combined with an appropriate conformational reporter, can also be effective in detecting such hidden intermediates (Wang et al. 2005), since a *T*-jump-induced relaxation contains information for both folding and unfolding kinetics, can also reach a nano-second time resolution, and does not require the use of chemical denaturants (Huang et al. 2002).

To validate the utility of the *T*-jump IR technique in the study of post-transition folding intermediates and also to provide a better understanding of the folding mechanism of Rd-apocytochrome *b*₅₆₂, we studied the folding kinetics of the wild type and two mutants of Rd-apocytochrome *b*₅₆₂. Rd-apocytochrome *b*₅₆₂ is a redesigned four-helix bundle protein based on apocytochrome *b*₅₆₂, and its structure has been solved to a high resolution (Feng et al. 2003). An intermediate with the N-terminal helix unfolded has been identified in a native-state hydrogen exchange experiment but was not

observed in the stop-flow folding experiment (Takei et al. 2002). Furthermore, the intermediate has been shown to reside after the transition state on the folding pathway (Chu et al. 2002a; Zhou et al. 2005). Therefore, Rd-apocytochrome *b*₅₆₂ constitutes a good model system for further *T*-jump studies. The first mutant (Mutant-1) contains double destabilizing mutations in the N-terminal helix with the substitution of two large hydrophobic residues in the core by Gly and Ala (V16G/I17A). The second mutant (Mutant-2) involves mutations of several large hydrophobic residues in the N-terminal helix to Gly or Asp (W7D/L10G/L14G/V16G/I17G/Y101W).

The rationale for designing these mutants is illustrated in Figure 1 with a cartoon representation of the structure of the native and intermediate states (Fig. 1D). Mutant-1 was designed to moderately destabilize the native state of the wild type (Fig. 1A) but still remain folded at relatively low temperature (Fig. 1B). At higher temperature, however, the intermediate state becomes significantly populated, and thus allows the relaxation between the intermediate and the native states to be measured along with that between the unfolded and the intermediate states. In contrast, Mutant-2 (Fig. 1C) was designed to mimic the post-transition intermediate and should behave as a two-state folder between the unfolded state and the intermediate state in the temperature range of the experiment. Indeed, NMR studies at room temperature have confirmed that Mutant-1 is fully folded, whereas Mutant-2, the mimic of the post-transition intermediate, has its N-terminal helix unfolded (H. Feng, Z. Zhou, and Y. Bai, unpubl.).

We found that Mutant-2 exhibits single-exponential relaxation kinetics in response to a *T*-jump over the entire temperature range in which the data were obtained. In contrast, Mutant-1 exhibits double-exponential relaxation kinetics in response to a *T*-jump, indicative of population conversions among at least three species and the existence of a folding intermediate. These results are therefore consistent with our design and also the earlier demonstration

Figure 1. A qualitative illustration of the protein-engineering approach used in the present study, wherein destabilizing mutations were introduced to enhance the population of the intermediate state at high temperature. The curves represent the temperature-dependent free energies of the unfolded and intermediate states, relative to that of the folded state. (A) Wild-type Rd-apocytochrome *b*₅₆₂, (B) Mutant-1, (C) Mutant-2 (i.e., the mimic of the folding intermediate). In C, the dashed line represents the hypothetical native state, which is significantly destabilized and not populated under the conditions of these experiments. (D) Cartoon representations of the structures of the native (N) and intermediate (I) states of Rd-apocytochrome *b*₅₆₂, where the red helix is at the N terminus.

that there is an on-pathway folding intermediate for Rd-apocyt b_{562} (Chu et al. 2002a; Zhou et al. 2005). Furthermore, the relaxation rate of the fast phase, which represents the transition between the intermediate and the native state, is $\sim(25 \mu\text{sec})^{-1}$ around 65°C , suggesting that this process encounters a free energy barrier rather than proceeding via a downhill manner.

Results

Equilibrium CD study

The far UV CD spectra of the wild type, Mutant-1, and Mutant-2 at 25°C exhibited typical characteristics of helical proteins (data not shown), consistent with their helix bundle structure. The thermal denaturation of these proteins was further monitored by probing the ellipticity at 222 nm as a function of temperature. As shown (Fig. 2), these proteins unfold cooperatively with increasing temperature. To further quantify the unfolding thermodynamics of Mutant-1 and Mutant-2, we followed the common practice and fit their thermal denaturation CD curves to an apparent two-state model, i.e.,

$$\theta(T) = \frac{\theta_F(T) + K_{eq}(T) \times \theta_U(T)}{1 + K_{eq}(T)}, \quad (1)$$

$$K_{eq}(T) = \exp(-\Delta G(T)/RT), \quad (2)$$

$$\Delta G(T) = \Delta H_m + \Delta C_p \cdot (T - T_m) - T \cdot [\Delta S_m + \Delta C_p \cdot \ln(T/T_m)], \quad (3)$$

where $\theta_F(T)$ is the folded CD baseline, $\theta_U(T)$ is the unfolded CD baseline, $K_{eq}(T)$ is the equilibrium constant for unfolding, $T_m = \Delta H_m/\Delta S_m$ is the thermal melting

Figure 2. Temperature-dependent mean residue ellipticities at 222 nm of Rd-apocyt b_{562} (+), Mutant-1 (Δ), and Mutant-2 ($\circ$). Solid lines are fits to the corresponding experimental data according to the method described in the text.

temperature, ΔH_m and ΔS_m are the enthalpy and entropy changes at T_m , respectively, and ΔC_p is the heat capacity change associated with unfolding, which has been assumed here to be temperature independent. In the fit, both $\theta_F(T)$ and $\theta_U(T)$ were treated as a linear function of temperature (i.e., $\theta_F[T] = a + bT$, $\theta_U[T] = c + dT$, where a , b , c , and d are constants). The corresponding thermodynamic parameters that resulted from the best fit are listed in Table 1. We did not attempt to fit the CD thermal unfolding curve of the wild type due to a lack of unfolded baseline. As expected, Mutant-1 is less stable than the parent (Fig. 2), and Mutant-2, which is designed to mimic the folding intermediate, has a lower T_m than Mutant-1. This is due primarily to the loss of the stabilizing contribution from the N-terminal helix, which was eliminated in Mutant-2. Interestingly, the thermal unfolding transition of Mutant-1 can also be adequately described by a simple two-state model, suggesting that one should take special precaution when interpreting the folding mechanism of a protein based solely on equilibrium measurements. As a matter of fact, a number of proteins that appear as apparent two-state folders in equilibrium thermodynamic studies actually possess one or more intermediates along the folding pathway toward the native state (Daggett and Fersht 2003; Sanchez and Kiefhaber 2003; Kamagata et al. 2004).

T-jump relaxation kinetics

The T-jump-induced relaxation kinetics of the wild type, Mutant-1, and Mutant-2 were studied by an infrared technique, the details of which have been described elsewhere (Huang et al. 2002). Briefly, a 1.9- μm T-jump pulse was used to quickly increase the temperature of the protein solution of interest, typically by 8°C – 10°C , and the resulting relaxation kinetics were probed by monitoring the optical density change of the amide I' band of the polypeptide. The amide I' band of proteins mainly arises from the backbone C=O stretching vibration and has been shown to be a sensitive conformational reporter (Huang et al. 2002; Zhu et al. 2004). In the current study, a probing frequency of 1632 cm^{-1} was used because it has been shown that hydrated helical amides absorb at this frequency (Zhu et al. 2003). As shown (Figs. 3, 4),

Table 1. Unfolding thermodynamic parameters determined from CD spectroscopy for Mutant-1 and Mutant-2 in D_2O ($\text{pH}^* = 4$)

Protein	Mutant-1	Mutant-2
ΔH_m (kcal mol^{-1})	52.4 ± 5.1	37.3 ± 5.6
ΔS_m (cal $\text{mol}^{-1} \text{K}^{-1}$)	158 ± 13	117 ± 17
ΔC_p (cal $\text{mol}^{-1} \text{K}^{-1}$)	1400 ± 200	528 ± 150
T_m ($^\circ\text{C}$)	57.8 ± 2.2	44.7 ± 1.2

Figure 3. (Bottom panel) A representative T -jump-induced relaxation trace of Rd-apocyt b_{562} measured at 1632 cm^{-1} . The T -jump was from 72.1°C to 83.9°C . The smooth line is the fit to the following single-exponential function: $\Delta\text{OD}(t) = A \times [1 - B \times \exp(-t/\tau)]$, with $A = -0.025$, $B = 0.53$, and $\tau = 79.3\text{ }\mu\text{sec}$. (B) Shown are the residuals of the fit. (A) Also shows the residuals of the fit to the following double-exponential function: $\Delta\text{OD}(t) = A \times [1 - B_1 \times \exp(-t/\tau_1) - B_2 \times \exp(-t/\tau_2)]$, with $A = -0.025$, $B_1 = 0.48$, $B_2 = 0.08$, $\tau_1 = 86.1\text{ }\mu\text{sec}$, and $\tau_2 = 17.3\text{ }\mu\text{sec}$.

the T -jump-induced relaxation traces contain two distinct phases. According to our early interpretation (Huang et al. 2001, 2002; Zhu et al. 2003, 2004), the instantaneous phase, whose decay time is not resolved due to the 10- to 15-nsec rise time of the infrared detector, is largely due to temperature-induced spectral shift (Bredenbeck et al. 2005; Xu et al. 2005), although other factors, such as end fraying of the helices as well as imperfect subtraction of the T -jump-induced D_2O absorbance change can also contribute to this fast phase. On the other hand, the slow phase is well resolved and is attributed to conformational changes associated with the folding and unfolding processes. For this reason, only the slow kinetic phase will be discussed below. Interestingly, the slow relaxation phase can be modeled by a single- or double-exponential function, depending on the protein as well as the final temperature. For example, Mutant-2 exhibits first-order relaxation kinetics over the entire experimental temperature range (Fig. 5), suggesting that kinetically it folds in a two-state manner. On the other hand, the relaxation kinetics of Mutant-1 show rather complicated temperature dependence. At relatively low temperatures the relaxation kinetics can be adequately described by a single-exponential function, whereas at higher temperatures (near and above the thermal melting temperature)

the relaxation deviates significantly from a single-exponential function and can be best fit by a double-exponential function (Fig. 4). Fitting the relaxation data of the wild type to a double-exponential function only slightly improves the fit (Fig. 3). Therefore, these results corroborate the expectation that the mutations leading to Mutant-2 increase the population of the intermediate state.

It is easy to show that for a two-state folding scenario the observed relaxation rate constant (k_R) is exactly the sum of the folding (k_f) and unfolding (k_u) rate constants, while the ratio of k_f and k_u is the equilibrium constant of the system. Therefore, using the equilibrium constant obtained from the CD experiment and the measured relaxation rate constant, we were able to determine the folding and unfolding rate constants for Mutant-2 at each temperature. As indicated (Fig. 6B), the folding rate of this protein exhibits non-Arrhenius behavior. However, its unfolding rate shows Arrhenius temperature dependence, which is typical to protein unfolding. Since a quantitative description of the relaxation kinetics of Mutant-1 requires knowledge of the thermodynamics of two coupled equilibria, no attempt was made to extract its folding and unfolding rate constants for each individual step. Instead, only the measured relaxation rate constants were presented for this mutant (Fig. 6A).

Figure 4. (Bottom panel) A representative T -jump-induced relaxation trace of Mutant-1 measured at 1632 cm^{-1} . The T -jump was from 55.7°C to 64.8°C . The smooth line is the fit to the following double-exponential function: $\Delta\text{OD}(t) = A \times [1 - B_1 \times \exp(-t/\tau_1) - B_2 \times \exp(-t/\tau_2)]$, with $A = -0.033$, $B_1 = 0.62$, $B_2 = 0.22$, $\tau_1 = 75.8\text{ }\mu\text{sec}$, and $\tau_2 = 25.1\text{ }\mu\text{sec}$. (B) The residuals of the fit. (A) The residuals of a single-exponential fit.

Figure 5. (Bottom panel) A representative T -jump-induced relaxation trace of Mutant-2 measured at 1632 cm^{-1} . The T -jump was from 40.3°C to 49.0°C . The smooth line is the fit to the following single-exponential function: $\Delta\text{OD}(t) = A \times [1 - \exp(-t/\tau)]$, with $A = -0.019$ and $\tau = 282\text{ }\mu\text{sec}$. (Top panel) The residuals of the fit. (Note: The unresolved fast kinetic phase was subtracted.)

Discussion

Relaxation kinetics of Mutant-2

As expected, Mutant-2, which is designed to mimic the post-transition folding intermediate of Rd-apocytochrome b_{562} , exhibits the characteristics of a two-state folding mechanism, both thermodynamically and kinetically. Our kinetic results suggest that for this protein there is only one observable free energy barrier separating the unfolded and folded states. Therefore, these results are consistent with the stopped-flow kinetics of Rd-apocytochrome b_{562} (Chu et al. 2002a) and also corroborate the design principle. Moreover, similar to those observed for many other proteins, the folding rate of Mutant-2 exhibits a concave downward temperature dependence with a maximum folding rate of $\sim(680 \pm 75\text{ }\mu\text{sec})^{-1}$.

While several models have been proposed to interpret such non-Arrhenius behaviors often observed for protein folding kinetics (Bryngelson et al. 1995; Galzitskaya and Finkelstein 1995; Oliveberg et al. 1995; Scalley and Baker 1997; Kuhlman et al. 1998), here we simply attribute the nonlinear temperature dependence of the folding rate of Mutant-2 to the heat capacity change associated with its folding/unfolding process (Oliveberg et al. 1995). An earlier NMR study revealed that a similar mutant 4GD7 (W7D/L10G/L14G/V16G/I17G) has a well-defined hydrophobic core in the folded region (Feng et al. 2003). Therefore, it is not surprising that the folding/unfolding process of Mutant-2 was found to yield a relatively large heat capacity change (Table 1). Similarly, it is

also possible that a high degree of hydrophobic burial has already occurred when folding reaches the transition state, resulting in a heat capacity change between the transition and the thermally denatured states. In fact, the latter (i.e., $\Delta C_p^\ddagger$) has been used as a semiquantitative measure of the degree of hydrophobic burial (Gomez et al. 1995) corresponding to the formation of the transition state ensemble and, thus, how similar the structure of the transition state is to that of the folded state.

To help further understand the nature of the transition state of Mutant-2, we have globally fit its temperature-dependent folding and unfolding rate constants to a transition-state model of protein folding, i.e., Equations (4) and (5). The best fits yielded the following parameters of activation: (1) for folding, $\Delta H^\ddagger(T_m) = -5.9 \pm 2.6\text{ kcal mol}^{-1}$, $\Delta S^\ddagger(T_m) = -50 \pm 10\text{ cal mol}^{-1}\text{ K}^{-1}$, $\Delta C_p^\ddagger = -452 \pm 80\text{ cal mol}^{-1}\text{ K}^{-1}$; (2) for unfolding, $\Delta H^\ddagger(T_m) = 31.3 \pm 2.6\text{ kcal mol}^{-1}$, $\Delta S^\ddagger(T_m) = 67 \pm 10\text{ cal mol}^{-1}\text{ K}^{-1}$, $\Delta C_p^\ddagger = 76 \pm 50\text{ cal mol}^{-1}\text{ K}^{-1}$.

$$k_f = A \cdot \exp(-\Delta G^\ddagger/RT), \quad (4)$$

$$\Delta G^\ddagger = \Delta H^\ddagger + \Delta C_p^\ddagger \cdot (T - T_m) - T \cdot [\Delta S^\ddagger + \Delta C_p^\ddagger \cdot \ln(T/T_m)]. \quad (5)$$

Figure 6. (A) Arrhenius plot of the observed relaxation rate constants of Mutant-1. The symbols represent the slow, dominant rate constant (Δ) as well as the fast rate constant ($\square$), which is observed only at higher temperatures. (B) Arrhenius plot of the observed relaxation rate constant (Δ) as well as folding ($\circ$) and unfolding ($\diamond$) rate constants of Mutant-2. Solid lines are fits to Equations (4) and (5).

Since the pre-exponential factor (*A*) was arbitrarily set to 10^{10} sec^{-1} , the recovered $\Delta S^\ddagger$ should not be interpreted as the absolute change in entropy. It has long been realized that it is difficult to accurately determine the heat capacity change of protein folding using spectroscopic methods, and the interpretation of folding/unfolding kinetics using a temperature-independent heat capacity of activation may be problematic (Plaxco et al. 1998). Nevertheless, the resultant $\Delta C_p^\ddagger$ for folding of Mutant-2 is nearly 85% of the equilibrium value, suggestive of a late folding transition state, with most of its hydrophobic surfaces buried.

Relaxation kinetics of Mutant-1

While Mutant-1 also showed apparent two-state equilibrium thermal unfolding behavior, its *T*-jump-induced relaxation kinetics suggested that at least one folding intermediate is populated on the folding pathway. Since a PUF, which has the N-terminal helix unfolded, was observed for Rd-apocyt *b*₅₆₂ in a native-state hydrogen exchange experiment and has been demonstrated to be an on-pathway folding intermediate (Zhou et al. 2005), the folding mechanism of Mutant-1 may be described by the following, albeit minimum, three-state model:

where U, I, and F represent the unfolded, intermediate, and folded states, respectively, and k_i stands for the rate constant of individual microscopic steps, with k_1 being the rate-limiting rate constant for folding. It is easy to show that in response to a *T*-jump, such a three-state model gives rise to double-exponential relaxation kinetics, and the two characteristic rate constants are nonlinear functions of the microscopic rate constants (Nolting 1999). The amplitude of each exponential component is also a complicated function of several parameters, such as all k_i s as well as the initial concentration of each species. Therefore, depending on the microscopic rate constants, which are determined by the final temperature, the initial populations of U, I, and F, which are determined by the initial temperature, the optical probe used in the experiment, as well as the signal to noise ratio, it is possible that the amplitude of one of the components is too small to be detected. Thus, under appropriate experimental conditions, the measured relaxation kinetics of a three-state system can be modeled by a single-exponential function. We believe that this is most likely the explanation for the observation that the *T*-jump-induced relaxation kinetics of Mutant-1 at relatively low temperatures could be described by first-order kinetics. While other interpretations are also possible, aggregate formation can be ruled out because all of the protein samples used in the experiment did not show the charac-

teristic infrared signatures of aggregates, even at the highest temperature. Interestingly and also consistently, the *T*-jump-induced relaxations of the wild-type Rd-apocyt *b*₅₆₂ do not show significant, if any, deviation from single-exponential kinetics, indicating that the mutations associated with Mutant-1 indeed make the intermediate state more visible.

Because of the lack of some critical information, such as the temperature-dependent free energy change for each microscopic step as well as the helicity of the intermediate, a rigorous analysis of the *T*-jump results of Mutant-1 is rather difficult to attain. However, it can be shown that under certain assumptions the three-state model presented above gives rise to a trend in the amplitude of the fast/slow kinetic component similar to that observed for Mutant-1 over the temperature range of the data obtained (Fig. 7). To do so, we assumed (1) the helicity of the intermediate is 50% of that of the folded state, and (2) at T_m , $\Delta H_{U-I} = 8.5 \text{ kcal mol}^{-1}$, $\Delta S_{U-I} = 26.0 \text{ cal K}^{-1} \text{ mol}^{-1}$, $\Delta C_{pU-I} = 0.1 \text{ kcal K}^{-1} \text{ mol}^{-1}$, $\Delta H_{I-F} = 43.9 \text{ kcal mol}^{-1}$, $\Delta S_{I-F} = 132.0 \text{ cal K}^{-1} \text{ mol}^{-1}$, and $\Delta C_{pI-F} = 1.3 \text{ kcal K}^{-1} \text{ mol}^{-1}$, respectively. In addition, the rate constant of each individual microscopic step was estimated according to the relaxation rate constants observed in the *T*-jump experiment. Under these conditions, the percentage of the slow (and fast) component of the double-exponential relaxation kinetics in response to a *T*-jump of 10°C, solved based on the three-state scheme discussed above, shows a similar trend as that observed in the experiment (Fig. 7). Despite this qualitative agreement, however, it is worth pointing out that this analysis is simply used to show that a three-state model can describe the *T*-jump-induced relaxation data, and the thermodynamic parameters described above are by no means quantitative measures of the folding energy landscape. Furthermore, this analysis alone does not exclude the possibility that the observed double-exponential relaxation of Mutant-1,

Figure 7. Relative percentages of the slow kinetic phase of Mutant-1 corresponding to different final temperatures, obtained from experiment (○) and calculation (●).

in response to a *T*-jump, is not caused by a pre-transition intermediate.

Nature of the kinetic barrier

Taken together, the kinetic results of the wild type, Mutant-1, and Mutant-2 are consistent with the earlier results of Bai and coworkers (2003), which indicated that an intermediate exists on the folding pathway of Rd-apocyt *b*₅₆₂, and it is likely populated at the native side of the major folding or rate-limiting free energy barrier (Takei et al. 2002; Feng et al. 2003; Wang et al. 2005). Moreover, the relaxation time (~ 25 μ sec at 65°C) observed for the transition between the intermediate state and the native state, which involves the formation and docking of the native N-terminal helix, suggests that a kinetic barrier exists between these two conformations. According to the NMR structure of the intermediate (Takei et al. 2002), which shows that the N-terminal helix is unfolded and that the overall fold of the protein contains multiple nonnative interactions among side chains in the folded region, we speculate that this free energy barrier arises from breaking such nonnative hydrophobic interactions during the folding of the intermediate state to the native state. However, it should be pointed out that this barrier is rather small compared with the rate-limiting free energy barrier encountered in the folding of Rd-apocyt *b*₅₆₂. Therefore, it is likely that the folding rates of small proteins are dominantly determined by the topology of backbones, and the side chain reorganization plays only a minor role. This hypothesis is consistent with the recent structural studies on the hidden and early folding intermediates. For example, hidden intermediates of Rd-apocyt *b*₅₆₂ have been shown to have native-like backbone topology (Feng et al. 2005). On the other hand, the early folding intermediate of the engrailed homeo-domain, which populates before the rate-limiting transition state, has a nonnative backbone topology (Religa et al. 2005). It remains unclear whether all early folding intermediates have misfolded backbone topology.

It should be noted that two PUFs have been observed in the native-state hydrogen exchange study for Rd-apocyt *b*₅₆₂ (Chu et al. 2002a). The reason that we did not observe the other PUF in the *T*-jump experiment, which involves the further unfolding of the C-terminal region of the C-terminal helix in addition to the unfolding of the N-terminal helix, could be that it is less stable than the present intermediate (Feng et al. 2005). Alternatively, the formation of this intermediate may be too rapid to be resolved with our current setup, or it has a helical content similar to the transition state. Recently, Chu et al. (2002a) have performed Φ value analysis on the wild-type Rd-apocyt *b*₅₆₂ to map out the rate-limiting transition state. Small Φ values were obtained for the sites in the N-terminal helix and the

C-terminal region of the C-terminal helix. On the other hand, large Φ values occurred in the two middle helices and the N-terminal region of the C-terminal helix, indicating that the transition state is partially folded with the middle helices and the N-terminal region of the C-terminal helix formed. This evidence seems to indicate that the secondary structure of the transition state is similar to the structure of PUF-1. Since our infrared probe is only sensitive to helical content, any kinetic event that does not involve a net helical content change would evade detection, even though the tertiary packing of the protein is changing.

Conclusions

Based on prior knowledge from the native-state hydrogen exchange study, we have used a general procedure to measure the folding kinetics of the intermediates that occur after the rate-limiting transition state. The method relies on the combination of protein engineering and a laser-induced *T*-jump relaxation method. The central idea is to use rational mutations to dissect the folding mechanism of a protein by making its post-transition intermediates visible. The initial success in the study of the folding free energy landscape of two mutants of Rd-apocyt *b*₅₆₂ suggests that this approach should be easily applicable to other systems. In addition, we found that the folding of the N-terminal helix after the rate-limiting transition state of Rd-apocyt *b*₅₆₂ involves a free energy barrier rather than a downhill process. The exponential growth rate of the native state from the intermediate state is roughly $(25 \mu\text{sec})^{-1}$ at 65°C.

Materials and Methods

Protein expression and purification

The protocols used for the mutation, expression, and purification of the proteins studied here have been described in detail elsewhere (Feng et al. 2003).

Circular dichroism

Thermal denaturation experiments of the two mutants were carried out on an AVIV 62DS spectropolarimeter using a 1-mm quartz cell. CD samples were prepared by dissolving deuterated proteins directly into D₂O. Final concentrations were ~ 15 μ M for all samples. Mean residue ellipticity was calculated using the equation $[\theta] = (\theta_{\text{obs}}/10lc)/N$, where θ_{obs} is the ellipticity measured at 222 nm in millidegrees, l is the optical path length (cm), c is the concentration of the protein (M), and N is the number of residues.

T-jump IR setup

The *T*-jump-coupled transient IR apparatus has been described in detail elsewhere (Huang et al. 2002). Briefly, the 3-nsec,

10-mJ, 1.9- μ m, and 10-Hz *T*-jump pulse was generated via Raman shifting the Nd:YAG fundamental, 1064 nm, in a mixture of H₂ and Ar pressurized at 750 psi. A CW lead salts IR diode laser was used as the probe. Transient absorbance changes of the probe (1632 cm⁻¹) induced by the *T*-jump pulses were detected by a 50-MHz MCT detector. Digitization of the signal was accomplished by a digital oscilloscope.

Acknowledgments

This research was supported in part by the NSF (CHE-0094077) and the intramural research program of the NIH, National Cancer Institute, Center for Cancer Research.

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