Folding Kinetics of the All-β-sheet Protein Human Basic Fibroblast Growth Factor, a Structural Homolog of Interleukin-1β*

(Received for publication, April 20, 1999, and in revised form, August 16, 1999)

David Estapé‡ and Ursula Rinas§

From the GBF National Research Center for Biotechnology, Biochemical Engineering Division, Mascheroder Weg 1, 38124 Braunschweig, Germany

The refolding and unfolding kinetics of the all-β-sheet protein human basic fibroblast growth factor (hFGF-2) were studied by fluorescence spectroscopy. The kinetics of the unfolding transition are monophasic. The refolding reaction at high and low guanidinium chloride (GdmCl) concentrations is best described by mono- and biphasic folding, respectively. Refolding and unfolding of hFGF-2 (155 amino acids) is very slow compared with other non-disulfide-bonded monomeric proteins of similar size. For example, the rate constant for unfolding at 4.5 mol liter⁻¹ GdmCl is 0.006 s⁻¹, and the refolding rate constants at 0.4 mol liter⁻¹ GdmCl are 0.001 s⁻¹ and 0.0009 s⁻¹ (15°C, pH 7.0). A characterization of the thermodynamic nature of the folding process using transition state theory revealed that the slow refolding is almost exclusively controlled by entropic factors, namely the strong loss of conformational freedom during refolding. The rate of the slow unfolding kinetics is mainly (and at low denaturant concentrations exclusively) controlled by the large positive change in enthalpy. hFGF-2 shows similar slow folding kinetics to that of its structural homolog interleukin-1β. Since both proteins show very little sequence identity, it is suggested that their slow folding kinetics are determined by the complex β-sheet arrangement of the native molecules.

Human basic fibroblast growth factor (hFGF-2)² is a globular single chain heparin-binding polypeptide synthesized by different cell types and is involved in processes associated with proliferation and differentiation of cells (1). The wound healing activity of hFGF-2 renders it to a potential therapeutic agent of industrial importance (2).

The determination of the three-dimensional structure of hFGF-2 by X-ray crystallography revealed a complex all-β-sheet protein without any disulfide bond (3–5). The overall structure shows strong homology with the fold of interleukin-1β and can be described as a trigonal pyramid with three topological units consisting each of four anti-parallel β-sheets. Both proteins share only 10–13% sequence identity, but superposition of their carbon backbone involves 9 of the 12 β-sheet strands, including the anti-parallel β-sheet incorporating the N- and C-terminal regions of the molecules (50 C° of the two proteins could be superimposed with a root mean square discrepancy of 0.52 Å; see Ref. 3).

Electron density was not observed for the first 28 and the last 3 amino acids of hFGF-2 (155 amino acid form) suggesting that these parts of the molecule are disordered and/or are very flexible. hFGF-2 contains nine proline residues (6), a relatively high content compared with other molecules of similar size. Five of the Xaa-Pro peptide bonds are localized in the flexible N-terminal part of the protein, and they probably adopt conformations that are usually adopted by Xaa-Pro peptide bonds in unfolded proteins or short peptides (~70–90% trans; see Ref. 7). The remaining four other Xaa-Pro peptide bonds are in cis conformations in native hFGF-2 (3–5).

Folding studies with the hFGF-2 structural homolog interleukin-1β revealed the very slow folding kinetics of this protein (8–11). Slow folding kinetics result very often from peptidyl-prolyl trans/cis isomerization processes (7). Interleukin-1β (153 amino acids) contains eight prolines (3–5, 12). One Xaa-Pro peptide bond is localized in the flexible N-terminal part of the protein, and six of the other Xaa-Pro peptide bonds are in trans conformations, and the Tyr³⁹⁰-Pro⁹¹ peptide bond adopts the cis conformation (12). The molecular origin of the slow folding kinetics of interleukin-1β has not yet been identified and, despite the presence of a cis proline in the native protein, could not be attributed to prolyl isomerization processes (8–10).

It was shown that the formation of unstable β-sheets in interleukin-1β occurred rapidly on the millisecond time scale (9). However, the stabilization of the three-dimensional structure of the protein with the establishment of native hydrogen bonds began only after 1 s with the protein passing through several folding intermediates containing an increasing content of stable hydrogen bonds within the β-sheets of the three topological units (9, 10). Experimental results from folding studies using pulse-labeling hydrogen exchange and electrospray ionization mass spectrometry suggested that the folding of interleukin-1β proceeds through an obligatory, defined intermediate on the folding pathway (10, 11). The final stabilization of the native structure of interleukin-1β was observed on a time scale of minutes and could be monitored by tryptophan fluorescence emission spectroscopy (8, 9).

hFGF-2 and interleukin-1β both have a single tryptophan localized at a conserved position in an anti-parallel β-sheet segment whose α-carbon atoms are superimposable with a root mean square discrepancy of 0.52 Å in both proteins (3). The tryptophan is partly exposed to the solvent and localized at the end of a very tight loop connecting two anti-parallel β-strands (3–5, 13). In contrast to interleukin-1β, the fluorescence emission of the single tryptophan present in hFGF-2 is completely quenched in the native molecule (13). Unfolding of hFGF-2 leads to a tryptophan-dominated fluorescence spectrum with
an increase in the intensity of the emission and an accompanying shift of the maximum wavelength from 306 to 355 nm. The unusual total quenching of the tryptophan fluorescence in hFGF-2 is also abolished in response to marginal perturbations within the native structure of hFGF-2. Therefore, the disappearance of the tryptophan emission during refolding of hFGF-2 can be used as a sensitive probe for the attainment of the correct interactions within the complex \( \beta \)-sheet arrangement of the native molecule.

In this study, we describe the refolding and unfolding kinetics of hFGF-2 at different temperatures and varying concentrations of guanidinium chloride (GdmCl) using fluorescence emission spectroscopy. In addition, we have characterized the thermodynamics of the unfolding and refolding process using transition state theory.

**EXPERIMENTAL PROCEDURES**

Unfolding and Refolding of hFGF-2—The preparation and storage of the hFGF-2 stock solution (10 \( \mu \)mol liter\(^{-1} \)) hFGF-2 in 0.1 mol liter\(^{-1} \) sodium phosphate buffer, pH 7.0) and fluorescence emission spectroscopy were performed as described previously (15). Unfolding of hFGF-2 was initiated by diluting hFGF-2 stock solutions into 0.1 mol liter\(^{-1} \) sodium phosphate buffer, pH 7.0, in the presence of varying concentrations of GdmCl. Refolding of unfolded hFGF-2 (in 2 mol liter\(^{-1} \) GdmCl) was initiated by dilution into 0.1 mol liter\(^{-1} \) sodium phosphate buffer, pH 7.0, in the presence of varying concentrations of GdmCl. The reversibility of the unfolding and refolding processes were guaranteed by the presence of 0.1 mol liter\(^{-1} \) \( \beta \)-mercaptoethanol in all solutions. Unfold-
RESULTS AND DISCUSSION

hFGF-2 is a very unstable protein and exhibits a strong tendency toward aggregation during refolding (13). However, it can be reversibly unfolded by urea or GdmCl at low protein concentrations (0.5 \( \mu \)mol·l\(^{-1}\) \( \approx 9 \) \( \mu \)g·ml\(^{-1}\)) and in the presence of reducing agents.

Kinetics of Unfolding and Refolding—The results of typical unfolding and refolding kinetics of hFGF-2 as determined by fluorescence spectroscopy and using a manual mixing technique are shown in Fig. 1. The unfolding reaction of hFGF-2 is described by a simple single exponential decay at all GdmCl concentrations investigated (e.g. Fig. 1A). The refolding kinetics at intermediate GdmCl concentrations are best described by monophasic folding (Fig. 1B). At low GdmCl concentrations the refolding can be described by a biphase reaction, and the rate constants for both phases can be determined with sufficient accuracy (Fig. 1C).

The GdmCl-dependent rate constants of refolding and unfolding experiments were performed in 2.0 mol·l\(^{-1}\) (asterisks), 2.5 mol·l\(^{-1}\) (triangles), 3.0 mol·l\(^{-1}\) (crosses), 3.5 mol·l\(^{-1}\) (circles), 4.0 mol·l\(^{-1}\) (plus signs), and 4.5 mol·l\(^{-1}\) GdmCl (squares) at a protein concentration of 0.5 \( \mu \)mol·l\(^{-1}\) in 0.1 mol·l\(^{-1}\) sodium phosphate buffer, pH 7.0, 0.1 mol·l\(^{-1}\) BME, and varying concentrations of denaturant. Please note that for clarity the rate constants of the very slow phase of refolding are omitted from this figure.

Unfolding experiments were performed at 15 \( ^\circ \)C (triangles), 20 (diamonds), 25 (circles), 30 (squares), and 35 \( ^\circ \)C (triangles) at a protein concentration of 0.5 \( \mu \)mol·l\(^{-1}\) in 0.1 mol·l\(^{-1}\) sodium phosphate buffer, pH 7.0, 0.1 mol·l\(^{-1}\) BME, and varying concentrations of denaturant. Please note that for clarity the rate constants of the very slow phase of unfolding are omitted from this figure.

By combining Equations 2 and 3, the enthalpic \( \Delta H^\ddagger \) and the entropic components of the activated state \( \Delta S^\ddagger \) can be determined from the Eyring plot (ln\( k_\text{T}/T \) versus \( T^{-1} \)) according to Equation 4.

\[
\ln \frac{k_\text{T}}{T} = \ln \frac{k_B}{R} - \frac{\Delta H^\ddagger}{R} - \frac{\Delta S^\ddagger}{R T} \quad \text{(Eq. 4)}
\]

If there is linearity for the temperature dependence of the folding rate constants, the enthalpy and entropy of activation can be determined from the slope and the \( T^{-1} \) intercept of the Eyring plot, respectively. With the determination of the enthalpic \( \Delta H^\ddagger \) and entropic components of the activated state \( \Delta S^\ddagger \), the activation free energy \( \Delta G^\ddagger \) is then calculated according to Equation 3.

\[
\Delta G^\ddagger = \Delta H^\ddagger - T \Delta S^\ddagger
\]

The amplitude associated with the major phase of refolding represents 75–85% of the total amplitude observed at low GdmCl concentrations and decreases with increasing denaturant concentrations (data not shown). The amplitude or in the case of biphasic folding the sum of the two amplitudes associated with the refolding and unfolding transitions correspond to the entire amplitude expected from equilibrium unfolding experiments. This shows that the very fast and early phases of refolding that cannot be resolved by using manual mixing techniques are not associated with any or only with an insignificant change in tryptophan emission.

Temperature Dependence of Unfolding Kinetics and Kinetics of the Major Refolding Phase—The GdmCl-dependent refolding and unfolding kinetics of hFGF-2 were studied at temperatures ranging from 15 to 35 \( ^\circ \)C (Fig. 3). The unfolding rate constant shows a strong dependence on the denaturant concentration.
and is also affected considerably by the temperature. Unfolding of hFGF-2 is slow at all the temperatures studied. The slopes of the denaturant dependence of $\ln k_f$ at a given temperature are linear at all temperatures investigated indicating monophasic unfolding of hFGF-2. Linear denaturant dependences of $\ln k_f$ are in general taken as an indication of a two-state folding transition (17); however, it should be noted that they can also be associated with multiple state folding and cannot be taken as an unambiguous proof of a two-state folding process (18).

The refolding of hFGF-2 is also a slow process within the entire temperature range investigated. But in contrast to unfolding, there is not a pronounced effect of the temperature on the rate constants of refolding. Particularly, the rate constant of the major phase of refolding does not vary at a given temperature but is strongly affected by the denaturant concentration (Fig. 3). The small temperature effect on the rate constant of refolding, there is not a pronounced effect of the temperature on the rate constants of refolding. Particularly, the rate constant of the major phase of refolding does not vary at a given temperature but is strongly affected by the denaturant concentration. Unfolding of hFGF-2 is slow at all the temperatures studied. The slopes of the denaturant dependence of $\ln k_f$ at a given temperature are linear at all temperatures investigated indicating monophasic unfolding of hFGF-2. Linear denaturant dependences of $\ln k_f$ are in general taken as an indication of a two-state folding transition (17); however, it should be noted that they can also be associated with multiple state folding and cannot be taken as an unambiguous proof of a two-state folding process (18).

The refolding of hFGF-2 is also a slow process within the entire temperature range investigated. But in contrast to unfolding, there is not a pronounced effect of the temperature on the rate constants of refolding. Particularly, the rate constant of the major phase of refolding does not vary at a given temperature but is strongly affected by the denaturant concentration (Fig. 3). The small temperature effect on the rate constant of the very slow phase of refolding is discussed below. Thus, the kinetics of the major refolding phase are mainly determined by the residual denaturant concentration and not by the temperature. The denaturant dependence of $\ln k_f$ of the major phase of refolding is linear suggesting that this phase is monophasic and not a composite of several folding transitions. However, other techniques such as circular dichroism spectroscopy or calorimetry which are not applicable, due to the low solubility of hFGF-2 folding intermediates, might reveal more intermediates on the folding pathway.

**Transition State Analysis of Unfolding**—The dependence of the unfolding rate constant on temperature and denaturant concentration was utilized to characterize the thermodynamic nature of the transition state during unfolding (15) (see “Experimental Procedures”).

An Eyring plot ($\ln(k_fT^{-1})$ versus $T^{-1}$) of the temperature dependence of the unfolding rate constant revealed a linear dependence at all denaturant concentrations investigated (Fig. 4) indicating that there is no significant change in the heat capacity between the native and the transition state (19). Therefore, the free energy of activation and the corresponding enthalpic and entropic components of unfolding of hFGF-2 as function of the denaturant concentration were calculated assuming that $\Delta H^\ddagger$ and $\Delta S^\ddagger$ are independent of the temperature within the temperature range investigated.

These calculations revealed that the enthalpy, the entropy, and the free energy of activation are linearly dependent on the denaturant concentration (Fig. 5). The activation free energy of unfolding of hFGF-2 is positive at all denaturant concentrations and decreases with increasing concentrations of GdmCl. Decreasing free energies of activation of unfolding with increasing denaturant concentration have been observed before (19, 20). They indicate that the transition state interacts more strongly with the denaturant compared with the native state of the protein. The enthalpy of activation is also positive at all denaturant concentrations and decreases with increasing concentrations of GdmCl. A positive activation enthalpy of unfolding reveals that internal noncovalent interactions that are stabilizing the native protein are disrupted during the transition from the folded to the activated state. The decrease of the activation enthalpy with increasing concentrations of GdmCl indicates stronger solvation of the transition state compared with the native state of the protein. The activation entropy also decreases with increasing denaturant concentrations. The entropic term is positive at low denaturant concentrations but becomes negative above 2.4 mol·liter$^{-1}$ GdmCl. Negative entropic values for the activation entropy for unfolding appear unexpected, but they have been reported previously (20, 21). They reflect the overcompensation of the loss of the conformational order by the concomitant ordering of solvent and cosolvent components caused by the disruption of hydrophobic interactions.
interactions during the unfolding of the protein molecule. The high enthalpic barrier for unfolding at low denaturant concentrations is reduced by the gain in (conformational) entropy during the transition from the folded to the activated state. At high denaturant concentrations both the positive change in enthalpy and the negative change in entropy caused by the ordering of solvent molecules during the transition from the folded to the activated state contribute to the energy barrier for unfolding. Altogether, the slow unfolding kinetics of hFGF-2 are mainly (and at low denaturant concentrations exclusively) determined by the large positive change in enthalpy.

**Transition State Analysis of the Major Phase of Refolding**—The Eyring plot of the rate constant of the major phase of refolding again emphasizes its temperature independence at a given denaturant concentration within the temperature range investigated (Fig. 6). Chemical reaction rates generally increase with increasing temperature, but the opposite has been observed for protein folding reactions (22, 23). For example, it has been shown that the refolding rate of chymotrypsin inhibitor 2 and barnase initially increases and then goes through a broad maximum and finally decreases with increasing temperature. This affords into an Eyring plot of refolding with strong curvature resulting in positive activation enthalpies of refolding at low and negative values at high temperatures (22, 23). The studies of the refolding of hFGF-2 have been in the temperature range where the rate constant of the major phase of refolding has reached its apparent highest value, thereby showing only a broad maximum in the Eyring plot (Fig. 6) where the enthalpy of activation for the major phase of refolding is approximately zero (Fig. 7).

The enthalpy, the entropy, and the free energy of activation of the major phase of refolding as a function of denaturant concentration were calculated from the data shown in Figs. 3 and 6 with the simplification that the enthalpy of activation of the major refolding phase is set to zero and the assumption that the enthalpic and entropic terms do not show a significant change at a given denaturant concentration within the temperature range investigated (Fig. 7). The calculations revealed that the free energy of activation of the major refolding phase is positive at all denaturant concentrations and increases linearly with increasing concentrations of GdmCl indicating that the unfolded state interacts more strongly with the denaturant than does the transition state. The entropy of activation for the major refolding phase has negative values at all denaturant concentrations which decrease with increasing concentrations of GdmCl. Thus, the kinetics of the major refolding phase are exclusively controlled by entropic factors. The slow refolding kinetics must result from the loss of conformational freedom during refolding causing a strong decrease in the entropy during the transition from the unfolded to the activated state. The gain in entropy during refolding through the release of solvent and co-solvent components bound to the unfolded protein is largely overcompensated by the massive loss in conformational entropy.

**Transition State Analysis of the Very Slow Phase of Refolding**—In contrast to the rate constant of the major refolding phase, the rate constant of the very slow phase of refolding (only observable at low denaturant concentration, e.g. below 0.8 to 0.9 mol-liter\(^{-1}\) GdmCl) does not show a pronounced dependence on the denaturant concentration (see also Fig. 2). The Eyring plot revealed a slight temperature effect reflecting the modest increase of the refolding rate of the very slow phase with increasing temperature (Fig. 8). The data shown in Fig. 8 were utilized to calculate the enthalpy, the entropy, and the free energy of activation for the very slow phase of refolding. These calculations revealed values for \(\Delta H_f^{\#} \), \(\Delta S_f^{\#} \), and \(\Delta G_f^{\#} \) of 33 kJ-mol\(^{-1}\), \(-190\) J-mol\(^{-1}\)-K\(^{-1}\), and \(89\) kJ-mol\(^{-1}\), respectively. Therefore, neither the major nor the very slow phase of refolding of hFGF-2 has properties consistent with proline
isomerization, particularly, the activation enthalpies of refolding are low (∼0 and 33 kJ mol⁻¹, respectively). The activation enthalpies for proline isomerization are in general high and of the order of 80 kJ mol⁻¹ (7, 24, 25).

**Similar Slow Folding Kinetics of Structural Homologs hFGF-2 and Interleukin-1β—**Folding of hFGF-2 is very slow compared with other non-disulfide-bonded monomeric proteins of similar size. Folding studies with the hFGF-2 structural homolog interleukin-1β revealed that it also refolds in a slow and a very slow phase to its native structure at low denaturant concentrations (10). Since both proteins show very little sequence identity and there are no indications for rate-limiting prolyl isomerization processes, it is suggested that their slow folding kinetics are determined by their complex β-sheet arrangement. In both proteins, the slow folding kinetics can be monitored by fluorescence emission of the single conserved tryptophan suggesting that they may share a common folding pathway and that the final stabilization of the native structures occurs in the vicinity of this tryptophan.

**Acknowledgments—**The assistance of Michael Schmidt in preparing the figures is gratefully acknowledged. We are grateful to Victor Wray for English language editing.

**REFERENCES**
1. Burgess, W. H., and Maciag, T. (1989) *Annu. Rev. Biochem.* 58, 575–606
2. Meyer-Ingold, W. (1993) *Trends Biotechnol.* 11, 387–392
3. Eriksson, A. E., Cousens, L. S., Weaver, L. H., and Matthews, B. W. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 3441–3445
4. Eriksson, A. E., Cousens, L. S., and Matthews, B. W. (1993) *Protein Sci.* 2, 1274–1284
5. Zhang, J., Cousens, L. S., Barr, P. J., and Sprang, S. R. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 3446–3450
6. Abraham, J. A., Whang, J. L., Tumolo, A., Mergia, A., Friedman, J., Gospodarovice, D., and Fiddes, J. C. (1986) *EMBO J.* 5, 2523–2529
7. Schmid, F. X. (1992) in *Protein Folding* (Creighton, T. E., ed) pp. 197–241, W. H. Freeman & Company, New York
8. Craig, S., Schmeissner, U., Wingfield, P., and Pain, R. H. (1987) *Biochemistry* 26, 3570–3576
9. Varley, P., Gronenborn, A. M., Christensen, H., Wingfield, P. T., Pain, R. H., and Clore, G. M. (1993) *Science* 260, 1110–1113
10. Heidary, D. K., Gross, L. A., Roy, M., and Jennings, P. A. (1997) *Nat. Struct. Biol.* 4, 725–731
11. Jennings, P., Roy, M., Heidary, D., and Gross, L. (1998) *Nat. Struct. Biol.* 5, 11
12. Clore, G. M., Wingfield, P. T., and Gronenborn, A. M. (1991) *Biochemistry* 30, 2315–2323
13. Estappe, D., van den Heuvel, J., and Rinas, U. (1998) *Biochem. J.* 335, 343–349
14. Uijtewaal, H., and Baldwin, R. L. (1986) *Methods Enzymol.* 131, 51–70
15. Eyring, H. (1935) *J. Chem. Phys.* 3, 107–115
16. Matthews, C. R. (1987) *Methods Enzymol.* 154, 498–511
17. Jackson, S. E., and Fersht, A. R. (1991) *Biochemistry* 30, 10428–10435
18. Mizuguchi, M., Arai, M., Ke, Y., Nitta, K., and Kuwajima, K. (1998) *J. Mol. Biol.* 283, 265–277
19. Jackson, S. E., and Fersht, A. R. (1991) *Biochemistry* 30, 10436–10443
20. Chen, B.-L., Baase, W. A., and Schellman, J. A. (1989) *Biochemistry* 28, 691–699
21. Chen, X., and Matthews, C. R. (1994) *Biochemistry* 33, 6356–6362
22. Oliveberg, M., Tan, Y.-J., and Fersht, A. R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 92, 8926–8929
23. Tan, Y.-J., Oliveberg, M., and Fersht, A. F. (1996) *J. Mol. Biol.* 264, 377–389
24. Brandts, J. F., Halvorson, H. R., and Brennan, M. (1975) *Biochemistry* 14, 4953–4963
25. Schmid, F. X. (1986) *Methods Enzymol.* 131, 70–82