Effect of Polyanions on the Refolding of Human Acidic Fibroblast Growth Factor*

(Received for publication, July 5, 1991)

Jonathan M. Dabora, Gautam Sanyal, and C. Russell Middaugh†
From the Pharmaceutical Biophysics Group, Department of Pharmaceutical Research, Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486

Acidic fibroblast growth factor (aFGF) is unstable at physiological temperatures in the absence of polyanions such as heparin. Therefore, the effect of temperature on the kinetics of refolding of aFGF has been examined in the presence and absence of several polyanions. The protein folds into its native state at temperatures up to 30 °C without polyanions with an activation energy of ~14 kcal/mol, but does not acquire native structure above this temperature. When heparin, inositol hexosulfate, or sulfate ion are present, aFGF refolds below 30 °C with a slightly reduced activation energy (10–11 kcal/mol). In addition, the protein now also renatures between 30 and 50 °C with activation energies of 1–2 (heparin), 16 (inositol hexosulfate), and 7 (sulfate) kcal/mol. Trace heavy metals appear to inhibit the refolding process, but a molecular chaperone (bovine 70-kDa heat shock cognate protein) and a peptidylprolyl isomerase (the FK506-binding protein) have no effect. It is concluded that the rate of refolding of aFGF at physiological temperatures is probably controlled by the interaction of a native-like state of the protein with an unknown polyanionic species.

Acidic fibroblast growth factor (aFGF) is a 15.9-kDa protein with a broad spectrum of mitogenic, angiogenic, and chemotactic activities. 1 The protein contains 12 antiparallel β strands arranged with an approximate 3-fold symmetry and has significant homology to a number of other growth factors such as basic FGF and interleukin 1α and β. Despite possessing extracellular receptor mediated activity, aFGF lacks a signal sequence and contains 3 cysteine residues in a reduced state which are apparently not required for biological activity. 2

One of the distinguishing characteristics of this growth factor is its dependence on the presence of sulfated polyanions such as heparin for its stability and biological activity. For example, the presence of heparin protects aFGF from thermal, proteolytic, and pH-dependent degradation. 3,4,5 It has recently been demonstrated that these effects arise from the preferential binding of polyanions to the native (folded) state of the protein (9) as previously suspected from the binding of aFGF to heparin affinity columns. 6 Surprisingly, the protein is very unstable at physiological temperatures and is presumably stable in vivo only because it is found complexed to a variety of extracellular matrix heparin-like molecules, such as heparan sulfate. 7 It has been postulated that this interaction may provide the growth factor in a form which can both locally and rapidly be activated through the action of enzymes such as heparanase. 8,9

This lack of stability in the absence of cofactors raises the intriguing question of how this protein folds into its native state (which has been shown to be necessary for biological activity) under physiological conditions. An obvious possibility is that polyanions are absolutely necessary for the folding of aFGF. We have, therefore, investigated the effects of the presence of anions on the kinetics of refolding of aFGF as a function of temperature. In the absence of polyanions, we also examined the effects of sulfhydryl reagents, a molecular chaperone (hsc70), and a peptidylprolyl isomerase (FKBP) upon folding to see if any of these other agents known to sometimes assist in folding might have significant effects on the acquisition of native structure in aFGF.

MATERIALS AND METHODS

Recombinant human aFGF was isolated from Escherichia coli cells as described previously. 9 This protein is greater than 99% homogeneous as examined by various electrophoretic techniques. Purified bovine hsc 70 was provided by Dr. L. Highpower (University of Connecticut), and its isolation and characterization are described in Sadis et al. (14). Recombinant human FKBP was obtained from Dr. A. Marcy of Merck & Co. (Rahway, Nj) and its preparation and purification will be described elsewhere. 7 Heparin was obtained from Hepar, inositol hexosulfate and EDTA from Sigma, sodium sulfate from Baker, dithiothreitol and guanidine hydrochloride (Gdn-HCl) from Bethesda Research Laboratories; and ATP from Boeringer Mannheim.

The refolding of aFGF was monitored by fluorescence intensity changes employing either a Hitachi F-2000 or Perkin-Elmer MPF-44B spectrofluorometer interfaced to computers. Sample temperature was controlled by a circulating water bath. Fluorescence intensity was measured at 350 nm with 280 nm excitation employing a band pass of 6–10 nm. The protein was unfolded in 2 M Gdn-HCl for 2 h at 4 °C. Kinetic refolding experiments were performed by preincubating 2 ml of 6.2 mM sodium phosphate, 120 mM sodium chloride (pH 7.2) containing the desired polyanion or other accessory molecule in a 1 X 1-cm quartz cuvette at the desired temperature for approximately 5 min. Refolding was initiated by diluting 100 µl of the unfolded aFGF at the desired concentration into the cuvette and refolding monitored for 200 s. A lag time of approximately 5 s occurred between sample injection and initiation of data recording. The refolding mixture was gently stirred during the refolding process. In experiments involving hsc 70 and FKBP, the accessory proteins were present at a 1:1 molar ratio with aFGF. A 10-fold molar excess of ATP and MgCl₂ was employed in some of the hsc 70 experiments.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 215-661-3438.

1 The abbreviations used are: aFGF, recombinant human acidic fibroblast growth factor; hsc70, bovine 70-kDa heat shock cognate protein; FKBP, FK-506-binding protein; Gdn-HCl, guanidine hydrochloride.

Kinetic fluorescence intensity data \( F \) were fit to an exponential rate equation,

\[
F = \sum_{i=1}^{n} A_i \exp(-k_i t)
\]

where fits employing \( i = 1, 2, \) and 3 exponentials were routinely examined. Values of the rate constants \((k_i)\) were determined from averages of three to six experiments at each temperature.

**RESULTS AND DISCUSSION**

The unfolding and refolding of aFGF can be easily monitored by changes in its circular dichroism or fluorescence emission spectrum (9). Fluorescence was chosen for these studies because of the large change in the spectrum which occurs during refolding. The native protein contains a single tryptophan residue which appears to be quenched by the presence of proximate positive charges (9). As a consequence, the fluorescence emission spectrum of folded aFGF is dominated by a tyrosine emission peak near 306 nm. When the protein’s structure is disrupted, this quenching is relieved and characteristic tryptophan emission is seen at 350 nm. Thus, folding can be conveniently monitored by the loss of fluorescence at 350 nm as quenching of the single tryptophan is induced by the formation of native structure.

This change in fluorescence spectrum can be produced by structural disruption with Gdn-HCl among other perturbants. Denaturation of aFGF occurs between 0.5 and 2 M Gdn-HCl with a midpoint at approximately 1.1 M and \( \Delta G_r \approx -2.8 \) kcal/mol (25 °C) (results not illustrated). Therefore, in these experiments, refolding was initiated by diluting a solution of aFGF in 2 M Gdn-HCl into buffer and refolding was allowed to proceed in ~0.1 M Gdn-HCl. Both the loss and return of the native state were found to be complete as monitored by CD and fluorescence under these conditions. The result of a typical refolding experiment is illustrated in Fig. 1. The time-dependent decrease in fluorescence associated with aFGF refolding is accurately fit by a single exponential. Folding is essentially complete after 1 min at 25 °C. This rate of refolding was, surprisingly, markedly dependent on protein concentration, with the rate doubling as the protein concentration was raised from 1 to 20 pg/ml at 25 °C (Fig. 2). This concentration dependence of refolding was abolished, however, in the presence of EDTA with the time of refolding substantially enhanced. In addition, the presence of the chelating agent caused much more complex refolding kinetics to occur, with both rapid and slow phases now evident when compared to refolding in the absence of EDTA (Fig. 1). Three exponential terms are, in fact, necessary to reasonably fit this data, but it is difficult to obtain reproducible values of the three rate constants due to the noise in the original data.

It thus appears that the refolding of aFGF is inhibited by the presence of trace heavy metals in solution and that this inhibition can be overridden by either the presence of EDTA or an increase in protein concentration at which the ratio of protein to metal ions becomes more favorable. This sensitivity to heavy metals presumably reflects the presence of the three free thiols and possibly methionine in aFGF (4).

For further experiments, we chose solution conditions in which trace heavy metals were present because of the simpler kinetics and to simulate the more natural environment provided by their presence. When the refolding of aFGF was measured as a function of temperature and the rate constants obtained from a single exponential fit of the data were plotted in the form of an Arrhenius plot, a straight line was obtained with an activation energy of ~14 kcal/mol (Fig. 3). No refolding was observed, however, at temperatures above 30 °C.

In the presence of a 3-fold weight excess of heparin (Mw ~ 16,000), the rate at which aFGF refolds between 10 and 30 °C is not dramatically altered, although the activation energy is slightly decreased to ~10 kcal/mol (Fig. 4A). In contrast to the results in the absence of heparin, however, the presence of the sulfated polysaccharide allows folding to proceed between 30 and 50 °C. Furthermore, the activation energy in this region is found to be only 1–2 kcal/mol, a value characteristic of a diffusion controlled process in water.
Because of the presence of the three free thiols in aFGF and the partial inhibition of folding by heavy metals, we tested the effect of 1 mM dithiorthreitol on aFGF folding. This reagent abolished the effect of protein concentration on refolding but had no effect in the presence of EDTA and was unable to induce folding above 30 °C. The presence of 8 proline residues in aFGF suggested the possibility that cis/trans isomerization of one or more of these residues might constitute a rate-limiting step in the folding of aFGF (15). The presence of peptidylprolyl isomerase activity in the form of the FK506-binding protein (16, 17), however, also had no effect. Finally, we examined the ability of the bovine hsc 70 protein both in the presence and absence of ATP to enhance refolding (18). Again, however, there was no detectable effect of this protein on refolding, and it was not able to induce any renaturation of aFGF above 30 °C. The effects of dithiorthreitol, hsc70, and FKBP on the complex refolding kinetics of aFGF in the presence of EDTA have not been quantitatively examined due to our inability to reproduce model fits to the more complex experimental data, but they appear to have little effect under this condition as well.

Thus, at lower temperatures, aFGF refolds rapidly into its native state like many small compact proteins. In the absence of polyanion, it displays linear Arrhenius plots over the limited temperature range available for kinetic measurements. Although the folding process of most proteins produce nonlinear plots over a wider temperature range (19–21), they also often display near linearity over the temperature investigated in this work. Over the wider temperature range available in the presence of stabilizing anions, the data seems best fit by two straight lines, although noise in the data cannot exclude the possibility that it might also be well fit by a curved function.

Surprisingly, at low temperature (10–30 °C), the presence of polyanions seems to only slightly decrease the activation energy of refolding. This suggests that at these temperatures polyanions do not substantially accelerate the refolding process, but rather bind to aFGF after the formation of native-like tertiary structure. Some precedent for this idea comes from the fact that ligands have little effect on refolding of alcohol dehydrogenase with the exception of Zn²⁺ which seems to inhibit the formation of a nonproductive folding intermediate (22). In contrast, at temperatures above 30 °C where aFGF is unfolded in the absence of polyanions, the rate of refolding is dependent on the nature of the stabilizing entity. For example, in the case of heparin very little temperature dependence of folding is observed. It is thus clear that the rate-limiting step is now due to the interaction of aFGF with heparin. Note that the rate of refolding above 30 °C differs from that predicted by a simple extrapolation of the lower portion of the plot to higher temperatures. Greater temperature dependence is observed for the smaller inositol hexasulfate and sulfate anions at high temperature compared to heparin mediated refolding. This suggests that the energetics of these complexes differ significantly from those observed with heparin. The smaller temperature dependence observed in the presence of heparin may reflect partial interaction of the heterogeneous sulfated polysaccharide with states of the protein other than native ones resulting in some inhibition of the folding process.

The hypothesis that the interaction of aFGF with polyan-
ions controls the rate of folding of the growth factor is consistent with the lack of effect of the other agents tested. Although aFGF is a proline-rich peptide, the presence of a peptidylprolyl isomerase activity does not appear to affect refolding. In this regard, the low temperature activation energies observed in these studies are below those (16–20 kcal/mol) usually observed for proteins in which the cis/trans isomerization of proline residues provides the rate-limiting step in folding. It is perhaps less surprising that a molecular chaperone has no effect since these proteins are thought to operate through inhibition of aggregation of folding intermediates (18), a phenomenon not observed in the refolding of aFGF, at least under the dilute Gdn-HCl conditions employed in these studies. The lack of effect of these various agents suggests that the refolding of aFGF is dominated by the rapid formation of an early compact intermediate such as the molten globule state (23, 24). In support of this idea, the temperature at which aFGF unfolds is found to be very different when detected by far ultraviolet CD and fluorescence measurements (9). We have, however, not yet examined shorter times at which this might occur, but these current results suggest that formation of any early compact intermediate is probably not significantly affected by the presence of polyanions.

We are thus left with the question of the identity of the natural stabilizing ligand that must be present during the intracellular folding of aFGF. One possibility is that some form of proteoglycan (e.g. heparan sulfate) plays this role (1, 13). In addition, we have found that a wide variety of both large and small polyanions are quite efficient at stabilizing aFGF (9). These include compounds such as nucleic acids, ATP, inositol polyphosphates, and various highly negatively charged polypeptides, all of which occur naturally inside cells. Stabilization by nucleic acids is an especially provocative finding in conjunction with recent observations of the presence of aFGF in the nucleus of various cells (25, 26), but actual isolation of intracellular aFGF-polynion complexes will be necessary to establish the identity of the natural ligand. It may be especially noteworthy that removal of stabilizing ligands causes protein unfolding, thereby providing a unique method of control of the protein's biological activity.

REFERENCES