Analysis of the extent of unfolding of denatured insulin-like growth factor

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Abstract

Insulin-like growth factor (IGF-1) contains three disulfide bonds. In the presence of denaturant and thiol catalyst, IGF-1 shuffles its native disulfide bonds and denatures to form a mixture of scrambled isomers. The composition of scrambled IGF varies under different denaturing conditions. Among the 14 possible scrambled IGF isomers, the yield of the beads-form isomer is shown to be directly proportional to the strength of the denaturing condition. This paper demonstrates a new approach to quantify the extent of unfolding of the denatured protein.

Keywords: denaturation; GdmCl; GdmSCN; insulin-like growth factor; protein folding; scrambled proteins; unfolding; unfolding intermediates; urea

Denaturation and unfolding (denatured and unfolded) are commonly used to describe the process of inactivation of native proteins. There is a distinction between denaturation and unfolding (Dill & Shortle, 1991). A denatured protein may adopt varied degrees of unfolding. They can be partially unfolded, such as molten globule (Ptitsyn, 1987; Kuwajima, 1989), or extensively unfolded, such as random coil (Tanford, 1968, 1970). The problem is that it is inherently difficult to analyze the structure and extent of unfolding of a denatured protein. Unlike the native protein that adopts a defined conformation, a denatured protein consists of conformational isomers that exist in a state of equilibrium (Dill & Shortle, 1991; Tanford, 1968, 1970). The number of these isomers is staggering, and there is no effective method available for their isolation and characterization. As a consequence, the structural data of a denatured protein are usually derived from measurement of the average physico-chemical property of the collective conformational isomers (Tanford et al., 1967; Kuwajima et al., 1979; Pace, 1986; Shortle & Meeker, 1989; Goto et al., 1990; Timasheff & Arakawa, 1990; Shirley, 1995). To gain further insight into the structure of denatured proteins, novel methods will be required.

Denaturation of a disulfide containing protein in the presence of mild concentration of thiol reagent and denaturant may provide a useful approach to analyze the structure of denatured proteins

(Chang, 1995). This process allows the denatured protein to be trapped as a mixture of scrambled isomers. Scrambled proteins are fully oxidized species (disulfides formed quantitatively) that comprise at least two non-native disulfide bonds (Chang et al., 1995a). For a protein including three disulfides, there are 14 possible scramble isomers. They are stable in both acidic and alkaline solutions. Their composition can be analyzed by HPLC and pure species of scrambled isomers can be purified for further structural characterization (Chang, 1995, 1996). Most importantly, scrambled isomers are intracross-linked by different size disulfide loops and adopt varied degree of unfolding. Their composition thus may signal and represent the extent of unfolding of the denatured protein.

This technique is applied here to analyze the structure of IGF-1 denatured in the presence of urea, GdmCl, and GdmSCN. IGF-1 is a globular protein of 70 amino acids with three disulfides (Humbel, 1990). It is an unique case of one sequence encoding two structures. Oxidative folding of IGF-1 at physiological pH generates two isomers, the native IGF-1 (N-IGF), and a scrambled species IGF-swap (IGF-S), with a molar ratio of 60:40 (see Fig. 1 for their disulfide structures) (Raschdorf et al., 1988; Hober et al., 1992; Miller et al., 1993; Meng et al., 1998). In vitro, these two IGF isomers exist in a state of equilibrium and are readily interconvertible as trace concentration of thiol reagent is introduced (Hober et al., 1992; Miller et al., 1993). Circular dichroism analysis revealed that both IGF species adopt similar secondary structures, but one-dimensional ¹H-NMR spectra indicate that their tertiary folds are as different as two unrelated proteins (Miller et al., 1993). IGF-S also retains only 10% of the receptor binding affinity of that exhibited by the N-IGF (Narhi et al., 1993). It is demonstrated here that the extent of unfolding of denatured IGF-1 can be analyzed quantitatively based on the composition of denatured scrambled isomers.

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Abbreviations: CHAPS, (3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate); IGF-1, insulin-like growth factor 1; GdmCl, guanidine hydrochloride; GdmSCN, guanidine thiocyanate; HPLC, high-performance liquid chromatography.

Fig. 1. Disulfide structures of the native and scrambled IGF-1. The structures of N-IGF and IGF-S were reported earlier (Raschdorf et al., 1988; Humbel, 1990). The structures of IGF-a, IGF-b1 and IGF-b2 are presented for the first time. Their structures were derived from the Edman sequencing and mass analysis of disulfide containing peptides of thermolysin and trypsin digested samples.

Results and discussion

Denaturation and unfolding of the native IGF-1 in the presence of urea, GdmCl, and GdmSCN

In this study, the purified N-IGF was allowed to denature and unfold in the Tris-HCl buffer (pH 8.4) containing 2-mercaptoethanol (0.2 mM), and varied concentrations of urea, GdmCl, and GdmSCN. Two control experiments were performed (see Materials and methods). Observation with one control experiment indicated that under 6 M GdmCl without 2-mercaptoethanol, the disulfide structure of N-IGF remained intact (see top chromatogram at the left column of Fig. 2). In the second control experiment, it was demonstrated that in the presence of 2-mercaptoethanol without denaturant, 40% of N-IGF converted spontaneously to IGF-S, results consistent with those observed by other laboratories (Hober et al., 1992; Miller et al., 1993). Therefore, the recovery of IGF-S is counted as the native species during the calculation of denaturation and unfolding curves (Figs. 3, 4). Figure 2 shows the results of denaturation of N-IGF carried out in the presence of denaturant and 2-mercaptoethanol. The extent of the denaturation of N-IGF and the composition of the denatured scrambled IGF vary substantially under different denaturing conditions. Aside from N-IGF and IGF-S, about seven fractions of scrambled IGF were observed (see the sample denatured under 6 M urea, Fig. 2). All these fractions contain IGF isomers that possess three disulfide bonds. This conclusion is supported by two pieces of evidence: (1) The HPLC profiles of the denatured IGF remain indistinguishable regardless of whether the sample is quenched by acidification (4% trifluoroacetic acid) or by alkylation (iodoacetic acid). (2) Denatured samples quenched by alkylation were analyzed for their content of disulfide bond and free cysteine by the dabsyl chloride method (Chang & Knecht, 1991) and MALDI mass spectrometry. Both methods confirm that the denatured samples contain three disulfides.

The disulfide structures of scrambled IGF-a and IGF-b

The two fractions of scrambled IGF that predominate under strong denaturing conditions are designated as a and b (Fig. 2). They were isolated and digested by thermolysin and trypsin. Thermolytic and tryptic peptides were then purified by HPLC (Fig. 5) and characterized by amino acid sequencing and mass spectrometry (Table 1). Fraction a was shown to contain a single isomer (IGF-a) adopting the beads-form disulfide pairing [Cys6–Cys18, Cys47–Cys48, Cys52–Cys61] (Fig. 1). Fraction b consists of either IGF-b1 [Cys6–Cys18, Cys47–Cys52, Cys48–Cys61] or IGF-b2 [Cys6–Cys18, Cys47–Cys61, Cys48–Cys52] or a mixture of these two isomers (Fig. 1). Conventional methods of peptide mapping and Edman sequencing were unable to distinguish these two isomers because enzymatic cleavage between Cys47 and Cys48 was not possible (data not shown). It is interesting to notice that IGF-a accommodates one disulfide bond that is formed by two adjacent Cys [Cys47–Cys48]. This type of disulfide pattern requires substantial steric constraint. Such unusual disulfide pattern has been reported before (Henschen, 1986; Marti et al., 1987; Lengweiler et al., 1996).

The denaturation curve and the unfolding curve of IGF-1

The results presented in Figure 2, together with the structural information (Fig. 1), reveal two important characteristics of the state of denatured IGF. (1) Each denaturing condition appears to generate a unique composition of the native and scrambled IGF. This is demonstrated by the evolution of the pattern of scrambled IGF under different denaturation conditions (Fig. 2). Although IGF-a and IGF-b constitute the major portion of the scrambled isomers under strong denaturing conditions, at least five additional fractions of IGF isomers well populate under mild denaturing conditions (e.g., at 6 M urea, 4 M GdmCl, or 2 M GdmSCN). (2) The

Fig. 2. Denatured states of IGF-1 under different concentrations of urea, GdmCl, and GdmSCN. N-IGF (0.5 mg/mL) was allowed to denature in the Tris-HCl buffer (0.1 M, pH 8.4) containing 2-mercaptoethanol (0.2 mM) and indicated concentrations of the denaturant. After the reaction reached equilibrium (16 h incubation at 23 °C), the samples were acidified with 4% trifluoroacetic acid and analyzed by HPLC. The first control experiment demonstrated that 6 M GdmCl alone did not affect the disulfide structure of N-IGF (see top chromatogram at the left column). The second control experiment showed that 40% of N-IGF was converted to IGF-S (data not shown). "N" indicates the native species. The isomer "S" coexists with "N" under physiological conditions. The two major fractions of scrambled isomer are marked "a" and "b." Fraction "a" contains one pure scrambled isomer IGF-a. Fraction "b" consists of either IGF-b1 or IGF-b2, or a mixture of these two isomers (see Fig. 1). Solvent A was water containing 0.1% trifluoroacetic acid. Solvent B was acetonitrile/water (9:1, by volume) containing 0.1% trifluoroacetic acid. The gradient was 15%B to 55%B in 50 min. The flow rate was 0.5 mL/min. Column was Vydac C-18 for peptides and proteins, 4.6 mm, 10 μ m. Column temperature was 23 °C.

recovery of the beads-form isomer (IGF-a) is shown to be directly dependent upon the strength of the denaturing condition. These data thus permit separate construction of the denaturation curve of the native IGF-1 and the unfolding curve of the denatured IGF-1. The extent of denaturation is defined by the amount of N-IGF (and IGF-S) in percentages converted to the scrambled species. Figure 3 shows the denaturation curve of the native IGF in the presence of increasing concentrations of urea, GdmCl, and GdmSCN. In this case, GdmSCN is about two- and fourfold more potent than GdmCl and urea as a denaturant. Plotting of the unfolding curve (Fig. 4) is based on the composition of the scrambled species and is determined quantitatively by the amount of IGF-a detected as a fraction of the total denatured scrambled IGF (excluding IGF-S). IGF-a has been identified as an indicator to evaluate the extent of unfolding for two apparent reasons: (1) Among the 14 possible scrambled IGF isomers, IGF-a contains the smallest disulfide loops and appears to represent the most extensively unfolded species. (2) Among the seven fractions of scrambled IGF observed, IGF-a is the only one whose yield is directly proportional to the strength of the denaturing conditions.

Fig. 3. The denaturation curves of N-IGF. Denaturation is measured by the conversion of N-IGF (plus IGF-S) to the scrambled isomers. The denaturants are GdmSCN (■), GdmCl (●), and urea (▲). Denaturation was carried out at 23 °C for 16 h in the Tris-HCl buffer (0.1 M, pH 8.4) containing 2-mercaptoethanol (0.2 mM) and increasing concentration of the indicated denaturant.

In addition, studies have also been performed to investigate the combined effect of denaturant and temperature. In all cases, the addition of high temperature has increased the extent of unfolding of the denatured IGF. For instance, in the presence of 6 M GdmSCN alone, 96% of the native IGF was denatured to the state of scrambled species and 29.5% of the denatured species adopt the structure of IGF-a. A sequential denaturation by 6 M GdmSCN (16 h at 23 °C) and elevated temperature (69 °C, 15 min) denatured

Fig. 4. The unfolding curves of denatured IGF-1. Plotting of the unfolding curves was based on the yield of IGF-a as a fraction of the total denatured scrambled isomers (excluding IGF-S). The denaturants are GdmSCN (■), GdmCl (●), and urea (▲). The denaturing conditions are described in the caption of Figure 2. At room temperature, GdmSCN exhibits a maximum solubility of around 6 M in the Tris-HCl buffer. The data should be allowed a standard deviation of $\pm 5\%$.

Fig. 5. Peptide maps of IGF-a derived from the trypsin (top) and thermolysin (bottom) digestions. All fractions were isolated and analyzed by Edman sequencing and MALDI mass spectrometry. Those that contain disulfide peptides are marked and their sequences are summarized in Table 1. Peptides were separated under the HPLC conditions described in Figure 2, except for using a different gradient consisting of 5–22% B, linear, over 30 min, 22–60% B from 30 to 31 min, 60% B for 5 min, and then returned to 5% B within 2 min.

more than 97% of the native IGF, with 36% of the denatured species adopting the disulfide structure of IGF-a.

The properties of scrambled IGF

IGF-a and IGF-b (IGF-b1 and IGF-b2) are two scrambled IGF that are well populated under strong denaturing conditions. Despite their similarity (both contain the disulfide pairing of Cys6–Cys18), they exhibit limited and sharply different solubility. Both IGF-a and IGF-b are well solubilized at the range of 0.2–0.5 mg/mL in the acidic solution or in the alkaline buffer containing denaturant. However, in the alkaline solution alone (e.g., Tris-HCl buffer, pH 8.4), the solubility of IGF-a and IGF-b is lower than 5 μ g/mL and 0.2 mg/mL, respectively. For instance, HPLC purified IGF-a, after lyophilization, can no longer be brought into the Tris-HCl buffer even in the presence of detergent (e.g., 1% CHAPS). This poor solubility has prevented us from performing any reliable refolding experiments using IGF-a as the starting material. On the other hand, at low sample concentration (50 μ g/mL) and in the presence of thiol catalyst (0.2 mM of 2-mercaptoethanol), it was possible to show that IGF-b refolded spontaneously to form N-IGF and IGF-S (Fig. 6) with a final molar ratio of 60:40. The rate of

Table 1. Disulfide containing peptides derived from thermolysin (Th) and trypsin (T) digestion of IGF-a

Peptide	Sequence (sequence position)	Disulfide linkage
Th-1 ^a	LCGAE (5–9) VCGDRG (17–22)	6–18
Th-2	FRSCD (49–53) YCAP (60–63)	52–61
Th-3	IVDECCFR (43–50)	47–48
T-1 ^b	SCDLR RLEMYCAPLKPAK (51–55 + 56–68)	52–61
T-2	RAPQ TGIVDECCFR (37–50)	47–48
T-3	GPETLCGAELVDALQFVCGDR (1–21)	6–18

^aThis fraction also contains a noncysteine peptide, LVDA (residues 10–13).

^bTrypsin cleavage at RR (residues 55–56). This fraction also contains a noncysteine peptide encompassing residues 22–36.

formation of N-IGF and IGF-S are very similar, additional evidence that these two IGF isomers possess comparable thermodynamic stability (Hober et al., 1992; Miller et al., 1993). The poor solubility of IGF-a and IGF-b may account for their absence along the folding pathway of fully reduced IGF (Hober et al., 1992, 1997; Miller et al., 1993; Hua et al., 1996; Rosenfeld et al., 1997). Unlike the cases of IGF and bovine pancreatic trypsin inhibitor (BPTI) (Creighton & Goldenberg, 1984; Creighton, 1990; Weissman & Kim, 1991), scrambled isomers of hirudin (Chatrenet & Chang, 1993), tick anticoagulant peptide (Chang, 1996), potato carboxypeptidase inhibitor (Chang et al., 1994), and epidermal

Fig. 6. Renaturation (refolding) of scrambled IGF-b to form the native IGF-1. To initiate the folding, IGF-b (50 μ g/mL) was reconstituted in Tris-HCl buffer (0.1 M, pH 8.4) containing 2-mercaptoethanol (0.2 mM). The folding intermediates were trapped in a time-course manner by removing aliquot of the sample and acidified with 4% trifluoroacetic acid. Samples were analyzed by HPLC using the conditions described in the caption of Figure 2.

growth factor (Chang et al., 1995b) were found to exist as folding intermediates along the pathways of their oxidative folding.

Our results also demonstrate that IGF-1 is intrinsically plastic and may potentially adopt unlimited compositions of conformational isomers. Plasticity is nonetheless a common property for most proteins and denatured proteins like scrambled IGF usually become less soluble as unfolding exposes the hydrophobic segments of the core structure (Dill & Shortle, 1991).

Materials and methods

Materials

Human insulin-like growth factor 1 (IGF-1) is a recombinant protein provided by Protein Institute Inc. (Broomall, Pennsylvania). The protein is fully biologically active with a specific activity of 1×10^6 units/mg and is more than 95% pure as judged by HPLC and N-terminal sequence analysis. GdmCl, GdmSCN, urea, acetonitrile, ethanol, and 2-mercaptoethanol were products of Merck (Darmstadt, Germany) with purity of greater than 99%.

Denaturation of the native IGF-1 (N-IGF)

The protein (0.5 mg/mL) was dissolved in the Tris-HCl buffer (0.1 M, pH 8.4) containing 0.2 mM of 2-mercaptoethanol and selected concentrations of denaturants (urea, GdmCl, and GdmSCN) or subjected to additional denaturing conditions (elevated temperature). Two control experiments were simultaneously performed. One was to place the N-IGF (0.5 mg/mL) in the Tris-HCl buffer containing 6 M GdmCl without thiol reagent. The second was carried out in the same buffer including 2-mercaptoethanol (0.2 mM) without denaturant. The denaturation and unfolding were performed at 23 °C for 16 h (overnight). Indeed, the reaction typically reached an equilibrium within 4 to 6 h. This state of equilibrium includes both the molar ratio between the native and scrambled species, as well as that among scrambled isomers. The denatured samples were subsequently acidified with an equal volume of 4% trifluoroacetic acid, stored at -20 °C, or analyzed by HPLC.

Structural analysis of scrambled IGF

Isolated fractions of scrambled IGF-1 (~10 µg) were treated with 1 µg of thermolysin (Sigma, P-1512) in 30 µL of N-ethylmorpholine/acetate buffer (50 mM, pH 6.4), or digested with 1 µg of trypsin in 30 µL of ammonium bicarbonate buffer (50 mM, pH 7.9). Digestions were carried out at 23 °C for 16 h. Peptides were then isolated by HPLC and analyzed by amino acid sequencing and mass spectrometry to identify the disulfide containing peptides.

Protein analytical methods

The disulfide content of scrambled proteins was determined by the dansyl chloride precolumn derivatization method (Chang & Knecht, 1991), which permits direct quantification of the disulfide bonds of proteins. Amino acid sequence analysis was performed with a Hewlett-Packard G-1000A sequencer. The MALDI mass spectrometer was a homebuilt time of flight (TOF) instrument with a nitrogen laser of 337 nm wavelength and 3 ns pulse width. The

calibration was performed either externally or internally, by using standard proteins (Hypertensin, M.W. 1031.19 and Calcitonin, 3418.91).

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