Probing the Folding Pathways of Long R³ Insulin-like Growth Factor-I (LR³IGF-I) and IGF-I via Capture and Identification of Disulfide Intermediates by Cyanylation Methodology and Mass Spectrometry*

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Ying Yang‡§, Jiang Wu‡¶, and J. Throck Watson‡**

From the Departments of ¶Chemistry and §§Biochemistry, Michigan State University, East Lansing, Michigan 48824

This report describes an integrated investigation of the refolding and reductive unfolding of insulin-like growth factor (IGF-I) and its variant, long R³ IGF-I (LR³IGF-I), which has a Glu³ to Arg³ substitution and a hydrophobic 13-amino acid N-terminal extension. The refolding performed in glutathione redox buffer was quenched at different time points by adjusting the pH to 2.0–3.0 with a 1 N HCl solution of 1-cyano-4-dimethylaminopyridinium tetrafluoroborate, which trapped intermediates via cyanylation of free sulfhydryl groups. The disulfide structure of the intermediates was determined by chemical cleavage followed by mass mapping with mass spectrometry. Six refolding intermediates of IGF-I and three refolding intermediates of LR³IGF-I were isolated and characterized. Folding pathways of IGF-I and LR³IGF-I are proposed based on the time-dependent distribution and disulfide structure of the corresponding trapped intermediates. Similarities and differences in the refolding behavior of IGF-I and LR³IGF-I are discussed.

Considerable insight into the folding and unfolding pathways of a protein can be obtained from trapping and characterizing intermediates involved in the dynamic process (1–4). Disulfide-containing proteins provide an opportunity to capture thiol-containing intermediates by chemical reaction during the time course of folding or unfolding (5–8). The detailed folding pathways of several proteins (9–11) have been studied in this way, including bovine pancreatic trypsin inhibitor (6, 8, 12, 13) and ribonuclease A (14–18).

To isolate and characterize intermediates that are involved in folding or unfolding of proteins containing disulfide bonds, it is necessary to stop thiol/disulfide exchange reactions that convert one intermediate to another. One traditional approach involves trapping of thiol groups irreversibly by alkylation with iodoacetate, iodoacetamide, or vinylpyridine under alkaline conditions. However, rearrangement of intermediates during the trapping procedure with iodoacetate has been observed for both bovine pancreatic trypsin inhibitor (8) and ribonuclease A (19, 20). Another traditional method quenches thiol/disulfide exchange by lowering the pH to ≤2 by acidification. An advantage of acid quenching is its reversibility; intermediates trapped at low pH can be isolated and allowed to undergo further rearrangement or refolding after readjusting the pH in experiments designed to more completely characterize particular pathways (8, 10). While quenching by acidification occurs at a diffusion-controlled rate, it does not completely stop thiol/disulfide exchange (21); further chemical modification is also necessary for the structural characterization of an acid-trapped intermediate.

We recently developed a methodology to trap folding intermediates based on the cyanylation of thiol groups by 1-cyano-4-dimethylaminopyridinium (CDAP)¹ tetrafluoroborate under acidic conditions (22). This approach has several unique advantages. First, cyanation of thiol groups in acidic solution quenches the refolding process and minimizes thiol/disulfide exchange. Second, cyanation of thiol is already part of our procedure for structural elucidation of the intermediates, which involves partial reduction, cyanation, chemical cleavage, and mass mapping (22, 23). Third, this methodology is fast, simple, and even applicable to disulfide structural analysis of proteins containing adjacent cysteines (24).

Insulin-like growth factor I (IGF-I) (25) is a single-chain polypeptide of 70 residues containing three intramolecular disulfide bonds, two of which involve adjacent cysteines (Fig. 1). IGF-I is postulated to be the mediator of growth hormone action on skeletal tissue as well as of mitogenic activity on several cell types (26, 27). The refolding of IGF-I has been studied by several groups (28–32). Hobert et al. (28) proposed a folding pathway for IGF-I, based on the structural analysis of refolding intermediates trapped by pyridylethylation at pH 8.7. Four intermediates were identified, including a native-like one-disulfide intermediate, two two-disulfide intermediates, and a mismatched three-disulfide intermediate. A different refolding pattern was obtained by acidic quenching (29, 30); in addition to the four intermediates detected by pyridylethylation, a non-native two-disulfide species and a mixed two-disulfide intermediate with GSH were also detected. Furthermore, a different equilibrium distribution of intermediates was obtained by each of the two trapping procedures. It has been documented that the oxidative refolding of IGF-I follows a pathway governed by thermodynamic rather than kinetic principles, resulting in two folding isomers of similar thermodynamic stability but different disulfide conformations (native and mismatched isomers) (28–32). Milner et al. (33)
0.1 M citrate buffer at pH 3.0 was prepared prior to use. IGF-I. \textit{boldface R} in LR3IGF-I indicates the replacement of Glu at position 3 in IGF-I. An overall scheme proposed that a salt bridge between Glu\textsuperscript{3} and Arg\textsuperscript{56} in IGF-I might stabilize the mismatched isomer, accounting for the observation of more than one folding outcome of IGF-I.

Recombinant human long R\textsuperscript{3} insulin-like growth factor-I (LR3IGF-I) is a variant of human insulin-like growth factor-I (IGF-I) in which glutamate 3 is replaced by arginine, and a 13-residue extension appears at the N terminus (Fig. 1). LR3IGF-I is substantially more potent than IGF-I in affecting carbohydrate metabolism and in stimulating the growth of fetal tissue in animals (34). The refolding behavior of LR3IGF-I has been described as being significantly different from that of IGF-I (33); however, in that preliminary report, neither the disulfide structure of the intermediates nor the folding pathway of LR3IGF-I was determined.

We report here on important aspects of the folding pathway of both LR3IGF-I and IGF-I by structural elucidation of disulfide intermediates involved in the folding and unfolding processes. Our results were obtained using cyanation chemistry for trapping intermediates and specific cleavage/mass mapping for subsequent structural elucidation of the intermediates (22–24). Similarities and differences in the folding patterns of LR3IGF-I and IGF-I are highlighted and rationalized on the basis of their structures.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human long R\textsuperscript{3} insulin-like growth factor-I (LR3IGF-I) was purchased from Sigma. IGF-I was obtained from Austral Biologicals (San Ramon, CA). The proteins were purified prior to use by reversed phase HPLC as described below. Tris(2-carboxyethyl)phosphine hydrochloride was purchased from Pierce. Guanidine hydrochloride was obtained from Roche Molecular Biochemicals. GSSG, GSH, citric acid, sodium citrate, hydrochloric acid, and CDAP were purchased from Sigma and used without further purification. Acetonitrile and trifluoroacetic acid were of HPLC grade. Tris(2-carboxyethyl)phosphine hydrochloride was purified prior to use by reversed phase HPLC as described below. Tris-HCl buffer (pH 8.7), containing 1 mM GSSG, 10 mM GSH, 0.2 M KCl, and 1 mM EDTA, to a final protein concentration of 0.1 mg/ml. The refolding intermediates were trapped at different times by the method described below. After the reductive unfolding reached the equilibrium, cysteine was added to a final concentration of 100 mM to drive the reductive unfolding reaction to completion.

**Refolding of Isolated Specific Folding Intermediates of LR3IGF-I**—Refolding of the reduced, unfolded LR3IGF-I was quenched at 30 min by adding 1.0 mM HCl to pH 2. After HPLC separation, the fractions containing a two-disulfide intermediate and a mismatched (three-disulfide) protein isomer were dried; reconstituted in 0.10 M Tris-HCl buffer (pH 8.7) containing 1 mM GSSG, 10 mM GSH, 0.2 M KCl, and 1.0 mM EDTA to a final protein concentration of 0.1 mg/ml; and incubated for up to 30 min at room temperature. At designated time points, aliquots were removed, and refolding intermediates were trapped by the method described below. After the reductive unfolding reached the equilibrium, cysteine was added to a final concentration of 100 mM to drive the reductive unfolding reaction to completion.

**Refolding or reductive unfolding intermediates were trapped in a time course manner by removing aliquots (0.1 ml) of protein solution and mixing with 1.0 mM HCl containing freshly prepared 0.2 mM CDAP to give a solution of pH 2–3. Cyanation of free thiol groups by the CDAP proceeded at room temperature for 10 min. The trapped intermediates were immediately separated by HPLC under the conditions described below. The HPLC fractions were collected manually and analyzed by MALDI-time-of-flight mass spectrometry. Those with 0-, 52-, 104-, or 156-Da increases over the mass of the intact protein correspond to three-disulfide (native or nonnative), two-disulfide, one-disulfide, and the completely reduced species, respectively.**
the presence of two mixed disulfide bonds with two glutathiones.

**Structural Elucidation of Disulfide Intermediates**—The di-sulfide structure of purified refolding or reductive unfolding intermediates was determined by the partial reduction/cyanyla-tion/chemical cleavage/mass mapping approach, as described previously (22, 23).

**HPLC Separation**—The separation of folding/unfolding intermediates was carried out by reversed phase HPLC with a linear gradient elution using Waters model 6000 pumps controlled by a PC. The UV detection was at 215 nm. The column was a Vydac C18 (catalog no. 218TP54; 10-µm particle size, 300-Å pore, 4.6 × 250 mm). Solvent A was 0.1% aqueous trifluoroacetic acid. Solvent B was acetonitrile/water (9:1, v/v) containing 0.1% trifluoroacetic acid. The linear gradient was 30–50% solvent B in 45 min at a flow rate of 1 ml/min. The HPLC fractions were collected manually, and the contents were then dried under reduced pressure for further use.

**Mass Spectrometry**—MALDI mass spectra were obtained on a Voyager Elite time-of-flight mass spectrometer (PerSeptive Biosystems Inc., Framingham, MA) equipped with delayed extraction and a model VSL-337ND nitrogen laser (Laser Science, Newton, MA). The accelerating voltage in the ion source was set to 20 kV. Grid and guide wire voltages were 93.6 and 0.2% of the accelerating voltage, respectively. Data were acquired in the positive linear DE mode of operation. Time-to-mass conversion was achieved by external and/or internal calibration using standards of bradykinin (m/z 1061.2), bovine pancreatic insulin (m/z 5734.5), and horse skeletal myoglobin (m/z 16952) obtained from Sigma. All experiments were performed using α-cyano-4-hydroxycinnamic acid (Aldrich) as the matrix. Saturated matrix solutions were prepared in a 50% (v/v) solution of acetonitrile/aqueous 1% trifluoroacetic acid, mixed in equal volumes with peptide or protein samples, and applied to a stainless steel sample plate. The mixture was allowed to air-dry before being introduced into the mass spectrometer.

**RESULTS**

**Temporal Distribution of Refolding Intermediates of LR3IGF-I and IGF-I**—The distribution of intermediates during the refolding process is represented by HPLC chromatograms of cyanlated species trapped at designated times. Fig. 3, a and b, shows an array of chromatograms of LR3IGF-I and IGF-I intermediates trapped by reaction with CDAP under acidic conditions at various times after initiating refolding in the GSSG/GSH buffer. In addition to native LR3IGF-I (N) and completely reduced LR3IGF-I (R), three well populated species were observed at different time points during the refolding of LR3IGF-I (Fig. 3a). The oxidative state of the trapped intermediates was determined by MALDI mass spectrometry based on the mass shift between intermediate and the intact protein. Thus, peaks I’ and II’ in Fig. 3a correspond to intermediates containing one and two disulfide bonds, respectively, as evidenced by 104- and 52-Da shifts from the mass of the intact protein. Peak III’ represents a mixture of a mismatched three-disulfide intermediate (the same mass as intact protein) and a mixed two-disulfide intermediate in which two SH groups each formed a mixed disulfide bond with a glutathione (as indicated by a shift of +1612 Da from the mass of the intact protein).

Reduced/unfolded LR3IGF-I was first converted to a one-disulfide intermediate (peak I’) and then a two-disulfide intermediate (peak II’) within 1 min in the presence of 10 mM GSH, 1 mM GSSG. The three-disulfide intermediate and mixed two-disulfide intermediate coeluted as peak III’ by 2 min. Considerable native protein (N) was formed by 30 min, a time at which equilibrium had been reached, and the ratio of the mismatched and native protein remained constant thereafter.

Fig. 3b shows the HPLC trace of refolding intermediates of IGF-I trapped at 10 s, 1 min, 2.5 min, and 30 min, respectively.

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**FIG. 3.** HPLC chromatogram showing the time-dependent distribution of CDAP-trapped intermediates during the refolding of LR3IGF-I (a) and IGF-I (b). See “Experimental Procedures” for HPLC conditions.
During the course of refolding, a total of six well populated intermediates were trapped and identified. Mass analysis by MALDI showed that peak IIIc represents a mismatched protein isomer containing three disulfide bonds; peaks IIc, IIc, and IIc are two-disulfide intermediates; peak IImu represents a mixed two-disulfide species with two glutathiones; and peak I is a one-disulfide species. The intermediate distribution at equilibrium (see bottom panel) is very similar to that previously reported from a study using acidic quenching (29, 30).

**Temporal Distribution of Reductive Unfolding Intermediates of LR3IGF-I and IGF-I**—In order to prevent the formation of mixed disulfide intermediates as would occur with GSH/GSSG in the refolding process, the reductive unfolding of both proteins was performed in cysteine solution. The time-dependent distribution of the CDAP-trapped intermediates during reductive unfolding of LR3IGF-I is shown in Fig. 4a. The pattern of intermediates was the same (but inverted) as that observed during the refolding of LR3IGF-I. The mismatched three-disulfide isomer was observed as the earliest intermediate, accounting for \(-10\%\) of the total protein at 3 h. Prolonged incubation of up to 71 h did not alter the ratio of the native and mismatched protein isoforms, suggesting that the native and mismatched species had reached a state of equilibrium. However, the two-disulfide intermediate II\textsuperscript{c} did accumulate during the incubation. After 71 h of incubation, thermodynamic equilibrium among the three species had been reached. When the concentration of cysteine was increased to 100 mM and the mixture was incubated for another 30 min after reaching the 71-h equilibrium point under the initial conditions, the completely reduced/unfolded protein (R) became the predominant species, accompanied by only minor amounts of two- and one-disulfide intermediates.

The reductive unfolding of IGF-I under the same conditions (Fig. 4b) showed a similar pattern to that of LR3IGF-I, but the ratio of the mismatched isomer at equilibrium was significantly higher (35%) than that (10%) observed during the unfolding of LR3IGF-I. Unlike the refolding of IGF-I in which many more intermediates were observed at thermodynamic equilibrium, during reductive unfolding of IGF-I the mismatched three-disulfide species appeared as the only early intermediate to reach an equilibrium with the native protein within 2 h. Further incubation up to 20 h did not significantly change the pattern of unfolding intermediates. Apparently, the trace of cysteine (0.25 mM) in the solution was insufficient to effect further reduction of the disulfide bonds. Increasing the cysteine concentration to 100 mM and incubating for another 1 min shifted the equilibrium distribution of intermediates of IGF-I to the pattern shown in the bottom panel of Fig. 4b; both the one-disulfide intermediate (I) and the two-disulfide intermediate (II\textsubscript{c}) (but not II\textsubscript{b} and II\textsubscript{c}) were formed within 1 min. After 30 min, II\textsubscript{c} and I were almost completely converted to reduced/unfolded IGF-I (R) (data not shown).

**Refolding from Intermediates II\textsuperscript{c} and III\textsuperscript{c} of LR3IGF-I**—The rejoining of the complete native sequence from the native two-disulfide intermediate (II\textsubscript{c}) was not observed in the refolding experiments. Formation of the two-disulfide intermediate containing the stable Cys\textsuperscript{18Cys\textsuperscript{61}} structure (I\textsuperscript{c}) was not observed in the refolding experiments starting with purified II\textsuperscript{c} or III\textsuperscript{c}. The refolding experiments initiated from the two-disulfide intermediate (II\textsuperscript{c}) did not increase the yield of native LR3IGF-I. Analysis by HPLC (Fig. 5a) showed formation of approximately \(90\%\) of native LR3IGF-I at 30 min. On the other hand, refolding from the two-disulfide intermediate (II\textsubscript{c}) of IGF-I yielded all of the intermediates (I, II\textsubscript{a}, II\textsubscript{b}, II\textsubscript{c}, III, and N) (31). Furthermore, the
The intermediates IIB and IImix were not observed by vinylpyridine trapping of intermediate III (with the acid-trapped intermediate II pathway of LR3IGF-I and IGF-I, the refolding of both LR3IGF-I as reported elsewhere (29, 30).

Structural Elucidation of Disulfide Intermediates—The methodology developed in our laboratory (22–24), based upon chemical cleavage at cyanylated cysteine residues and subsequent mass mapping of the fragments, was employed to determine the disulfide structure of the trapped intermediates. The disulfide structures of the respective folding intermediates are summarized in Table I.

DISCUSSION

Trapping and Structural Identification of Folding Intermediates—Proper trapping of folding intermediates and subsequent structural determination are critical steps in the elucidation of a folding pathway and in studies of the associated kinetics. The trapping method should stop thiol/disulfide exchange, which may occur on the microsecond time scale in refolding buffer. The cyanylation of thiol groups by CDAP in acidic solution effectively quenches the refolding process by blocking reactive thiol groups. The folding intermediates of IGF-I trapped by CDAP and their distribution pattern are similar to those obtained by acid trapping under the same refolding conditions (29, 30). By mass mapping the cleavage products resulting from cyanylated intermediates, the disulfide structures of six well populated intermediates were identified, including a native one-disulfide intermediate (I, Cys18–Cys61), a native two-disulfide intermediate (II, Cys18–Cys61/Cys6–Cys47), two nonnative two-disulfide intermediates (IIb, Cys18–Cys61/Cys6–Cys47; IIc, Cys18–Cys61/Cys6–Cys52); a mismatched intact protein (III, Cys18–Cys61/Cys6–Cys47/Cys48–Cys52); a mixed two-disulfide intermediate with glutathione (II mix) (nonnative disulfide residues underlined). The intermediates IIb and IIc were not observed by vinylpyridine trapping (28). Furthermore, intermediate I was the major form captured by pyridylethylation (28), while it was the minor form in the distribution of the folding intermediates trapped by the CDAP approach described herein and also by acid trapping as reported elsewhere (29, 30).

In order to compare the flow of intermediates in the folding pathway of LR3IGF-I and IGF-I, the refolding of both LR3IGF-I and IGF-I was performed under identical conditions in a GSSG/GSH buffer. Comparison of the HPLC pattern of folding intermediates for LR3IGF-I and IGF-I exhibited some similarities and discrepancies. All the trapped intermediates contain a native Cys18–Cys61 structure, which is the first disulfide bond formed in the refolding process (Fig. 3, a and b) and the last disulfide bond reduced in the unfolding process (Fig. 4, a and b). The most abundant two-disulfide intermediates (IIa in IGF-I and II in LR3IGF-I) and mismatched three-disulfide intermediates (III in IGF-I and III in LR3IGF-I) also have a homologous disulfide structure (Table I). Refolding kinetics indicate that formation of the native Cys18–Cys61 disulfide bond is very fast in both proteins, as is the subsequent formation of the native Cys18–Cys61/Cys6–Cys48 intermediate. However, the formation of the last disulfide bond, Cys47–Cys52, is slower, supporting the conclusion that its formation is energetically unfavorable (29, 31, 32).

The folding result of LR3IGF-I is different from that for IGF-I in that the native three-disulfide isomer (N) is the predominant species at equilibrium, and much less mismatched disulfide bond formation is observed. Oxidation of the native intermediate, Cys18–Cys61, results only in the formation of the native Cys18–Cys61/Cys6–Cys48 intermediate in the refolding of LR3IGF-I, while as many as four two-disulfide intermediates are observed in the refolding of IGF-I, each containing a Cys18–Cys61 bond (Table I). Furthermore, the formation of early intermediates in IGF-I is much faster; at 2.5 min, ~90% of the reduced IGF-I is oxidized, whereas only ~50% of the reduced LR3IGF-I is oxidized in the same time period. Nevertheless, an equilibrium distribution of intermediates can be reached for both proteins within 30 min.

Rosenfeld et al. (31) observed that the oxidative refolding from the native two-disulfide intermediate of IGF-I (IIa in Fig. 3b, with Cys18–Cys61/Cys6–Cys48 linkage) formed an equilibrium mixture of all of the disulfide intermediates observed from the refolding of reduced/unfolded IGF-I. The refolding experiment starting with the homologous intermediate of LR3IGF-I (II in Fig. 3a) resulted in an equilibrium mixture of starting intermediate, native protein, and mismatched protein isomer (coeluting with the glutathione adduct). The one-disulfide intermediate, I, was not observed. The absence of I in the refolding from II could be attributable to the greater stability of the Cys6–Cys48 pair in LR3IGF-I, as compared with the homologous pair in IGF-I. Nevertheless, comparison of a and b in Fig. 5 reveals that the refolding courses from the isolated two-disulfide intermediate and the mismatched isomer, respectively, resulted in an identical distribution of intermediates, suggesting that the refolding of LR3IGF-I is also thermodynamically controlled and all of the trapped intermediates are thus interconvertible.

Three distinct features are recognized after comparing the unfolding processes of LR3IGF-I and IGF-I. First, the conversion of native IGF-I to the three-disulfide intermediate III (Fig. 4b) is faster than the conversion of native LR3IGF-I to the three-disulfide intermediate III (Fig. 4a). Second, no appreci-
13 hydrophobic amino acids. Milner variant contains two unrelated structural changes: a substitution of native and mismatched protein isomers; however, the two proteins share a similar mechanism, leading to the formation of the native protein isomer. The LR 3IGF-I isomer at equilibrium implicates the involvement of nonnative intermediates (I or II A). The nonnative disulfide linkage is underlined. The numbers in parentheses indicate the corresponding HPLC peaks in Fig. 3, a and b. The dashed arrows show the pathway of interconversions that probably occurs in the folding of IGF-I.

The observed differences in the folding of IGF-I and LR3IGF-I can be rationalized to the variation in the thermodynamic stability of the folding intermediates. The LR3IGF-I variant contains two unrelated structural changes: a substitution of Glu by Arg at position 3 and an N-terminal extension of 13 hydrophobic amino acids. Milner et al. (33) proposed that a salt bridge between Glu9 and Arg56 in IGF-I accounted for the high proportion of mismatched protein isomer during the folding of IGF-I. After the charge substitution, Arg at position 3 in LR3IGF-I, the salt bridge between residues 3 and 56 was replaced by charge repulsion. Thus, the mismatched folding intermediates may be destabilized, leading to predominant formation of the native protein isomer. The N-terminal extension apparently causes steric hindrance, decreases the flexibility of the molecule, and thus limits the number of conformational changes leading to disulfide bond formation. As a result, the refolding kinetics is much slower for LR3IGF-I, and native folding intermediates with minimum conformational energies are preferentially formed. Both structural changes may account for the difference in the folding pathway, and it is not possible to assign which change is dominant in determining the folding process.

Based on the disulfide structure and kinetic analysis of folding intermediates, proposed folding pathways for both IGF-I and LR3IGF-I are shown in Fig. 6. Overall, the folding of the two proteins is a thermodynamically controlled process. The two proteins share a similar mechanism, leading to the formation of native and mismatched protein isomers; however, the most productive routes may be slightly different. According to our results, the folding of IGF-I is more flexible in the formation of nonnative two-disulfide intermediates and the mismatched protein isomer via rearrangement of disulfide bonds or thiol/disulfide exchange. The nonnative two-disulfide intermediates (IIb and IIc) can be formed by either direct oxidation of the native one-disulfide intermediate (I) or disulfide rearrangement of IIa. While IIa can lead directly to the mismatched protein isomer (III), both IIb and IIc revert to productive intermediates (I or IIa).

Although only native one- and two-disulfide intermediates were trapped for LR3IGF-I, the presence of the mismatched isomer at equilibrium implicates the involvement of nonnative disulfide intermediates, because neither II′ nor native protein can spontaneously convert to the mismatched isomer without breaking/reforming disulfide bonds. Although these intermediates were so short lived as not to be detected, they represent productive intermediates for the formation of mismatched species (35). We speculate that these intermediates may have a more native-like structure but are thermodynamically less stable (than the native two-disulfide intermediate II′). Therefore, they can rapidly convert to the mismatched isomer. The glutathione intermediates detected during the refolding experiment from II′ strongly support the participation of glutathione in the disulfide rearrangement, largely accounting for the formation of Cys18–Cys61/Cys8–Cys47 and/or Cys18–Cys61/Cys48–Cys52. The involvement of glutathione in the rearrangement of the two-disulfide species was further supported by the energetics of late folding intermediates. The refolding of an IGF-I mutant protein showed that the formation of Cys8–Cys52 is energetically unfavorable (32). Therefore, the conversion of the native two-disulfide intermediate (II′ in LR3IGF-I and II′ in IGF-I) to other intermediates, such as glutathione adducts, was as unfavorable as the conversion to native protein. Another route leading to the formation of precursor intermediates of the mismatched isomer is the direct oxidation of the Cys18–Cys61 intermediate, which is obviously a route of choice for the refolding of IGF-I, as observed in Fig. 3b. However, the absence of I′ during the refolding from II′ and III′ at least suggests that the direct oxidation of I′ is not essential for the formation of the nonnative two-disulfide intermediates, although we cannot exclude the possibility. It seems that a disulfide rearrangement between the two-disulfide intermediates is a more favorable route for the formation of the precursor intermediates of the mismatched three-disulfide species.

The availability of chemical and mass spectrometric evidence for the disulfide structure of trapped intermediates provides a great advantage in studies of the folding process. Furthermore, investigations of both the folding and unfolding processes by the same methodology for a given protein and its mutant provides the basis for integrating time-dependent “snapshot” observations for insight into the dynamic phenomenon. Despite the great experimental challenges associated with providing concrete solutions to problems in protein folding, the data presented here should help provide an integrated view of the folding patterns for the family of insulin-like proteins.

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Probing the Folding Pathways of Long R^3Insulin-like Growth Factor-I (LR^3IGF-I) and IGF-I via Capture and Identification of Disulfide Intermediates by Cyanylation Methodology and Mass Spectrometry
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