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Oxidative Folding of Leech-Derived Tryptase Inhibitor *Via* Native Disulfide-Bonded Intermediates

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ABSTRACT

Leech-derived tryptase inhibitor (LDTI), comprising 46 residues and a fold stabilized by three disulfide bonds, is the only protein known to inhibit human β-tryptase with high affinity. The present work examines its oxidative folding and reductive unfolding with chromatographic and disulfide analysis of the trapped intermediates. LDTI folds and unfolds through a sequential oxidation of its cysteine residues that give rise to the accumulation of a few one- and two-disulfide intermediates. Three species containing two native disulfide bonds (IIa, IIb, and IIc) are detected in LDTI folding, but only one (IIb) seems to be productive and oxidizes into the native structure. Stop/go experiments indicate that the intermediates IIa and IIc must reduce or rearrange their disulfide bonds to reach the productive route. The acquisition of the native structure is extremely fast and efficient, probably influenced by the low levels of non-native three-disulfide (scrambled) isomers occurring along the reaction. Finally, the Cys14–Cys40 disulfide bond, buried in native LDTI and formed in IIa and IIb intermediates, appears to be a key factor for both the initiation of folding and the stability of this molecule. Together, the derived data provide a molecular basis for development of new LDTI variants with altered properties. *Antioxid. Redox Signal.* 10, 77–85.

INTRODUCTION

Unveiling how a string of amino acid residues folds into a biologically active three-dimensional structure is still a major challenge in structural and molecular biology (15, 25). Although protein-folding studies were initially hampered by the transient nature of kinetic folding intermediates, in proteins containing cysteine residues the trapping of disulfide-bonded intermediates through alkylation or acidification of the folding reaction made it feasible to circumvent this problem (4, 30). Pioneering work on bovine pancreatic trypsin inhibitor (BPTI), ribonuclease A, lyzozyme and hirudin, performed in the 1980s and early 1990s (5, 14, 27, 35, 36), established a basis for studies of oxidative folding, that is, the composite process by which a reduced and unfolded protein gains both its native disulfide bonds and its native structure. This and subsequent work took advantage of modern chromatographic techniques to isolate the intermediates trapped along the folding process. The heterogeneity of the intermediates together with their subsequent structural-characterization and disulfide-pairing determination helped to define the folding pathways of numerous small, disulfide-rich proteins (1).

Surprisingly, a great diversity of folding mechanisms was observed among these proteins, even those containing similar structures and disulfide patterns. Thus, BPTI folds through a limited number of disulfide intermediates that adopt native disulfide pairings and native-like substructures (17), funneling the folding reaction toward the native state and preventing the

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accumulation of scrambled isomers (*i.e.*, fully oxidized species containing at least two non-native disulfide bonds). By contrast, the folding of tick anticoagulant peptide, a protein with the same disulfide pattern and an extremely similar structure, shows a high heterogeneity of intermediates, including an important population of scrambled isomers that act as major kinetic traps of the reaction (7, 13). Other proteins, such as epidermal growth factor or leech carboxypeptidase inhibitor, show both similarities and dissimilarities to these two opposite folding models, owing to the presence of both native-like intermediates and scrambled isomers populating their folding reactions (2, 11). It is still unclear which features of the primary structure of a small, disulfide-rich protein influence its type of oxidative folding. This fact highlights the need for new and detailed studies to gain insights into the molecular rules that govern the folding process.

Leech-derived tryptase inhibitor (LDTI) is the only protein identified so far that tightly inhibits human β-tryptase (K_i = 1.4 n*M*), a trypsin-like serine protease stored in the granules of mast cells and implicated in the pathogenesis of allergic and inflammatory disorders such as asthma and rheumatoid arthritis (20, 31, 32). LDTI also binds to trypsin and chymotrypsin with nanomolar affinity but does not inhibit any of the serine proteases of the blood-coagulation cascade (3, 33). This small protein of 46 residues and three disulfide bonds was isolated from the medicinal leech *Hirudo medicinalis* and belongs to a subgroup of "nonclassic" Kazal-type inhibitors. This subgroup, which also contains other inhibitors identified in blood-sucking parasites such as the plasmin inhibitor bdellin B-3 or the thrombin inhibitor rhodniin, differs slightly from other Kazal proteins in respect to the cysteine spacing. The structure of the recombinant form of LDTI expressed in *Saccharomyces cerevisiae* was solved both in solution by NMR spectroscopy and in complex with trypsin by x-ray crystallography (18, 24, 34), showing a protein core formed by a short central α-helix and a small triple-stranded antiparallel β-sheet (Fig. 1). The potential of this molecule as a template for the development of new inhibitors with therapeutic interest was confirmed by the generation of chimeric variants able to inhibit strongly both tryptase and thrombin (23).

The current study examines the oxidative folding and reductive unfolding of LDTI. The characterization of the acid-trapped intermediates that accumulate along these two processes reveals the predominance of a few native disulfide-bonded intermediates that efficiently guide this protein toward its native conformation, avoiding the accumulation of significant amounts of scrambled isomers that would otherwise trap the folding reaction and limit its efficiency. These results identify LDTI as a protein with a BPTI-like oxidative folding and as a model to decipher how the hierarchic establishment of native contacts funnels folding toward functional conformations.

MATERIALS AND METHODS

Protein expression and purification

LDTI was obtained by heterologous expression in the yeast *Saccharomyces cerevisiae,* as previously described (3, 26). In brief, the plasmid containing the synthetic gene for LDTI was used to transform the *S. cerevisiae* strain S-78. Shake-flask fermentations of yeast transformants were carried out in YED medium. The protein was subsequently purified from the culture supernatant by cross-flow filtration (Minisette, Pall, Germany) followed by cation exchange (Fractogel SO_3^-; Merck, Darmstadt, Germany) and reverse-phase (C18 column; Vydac,

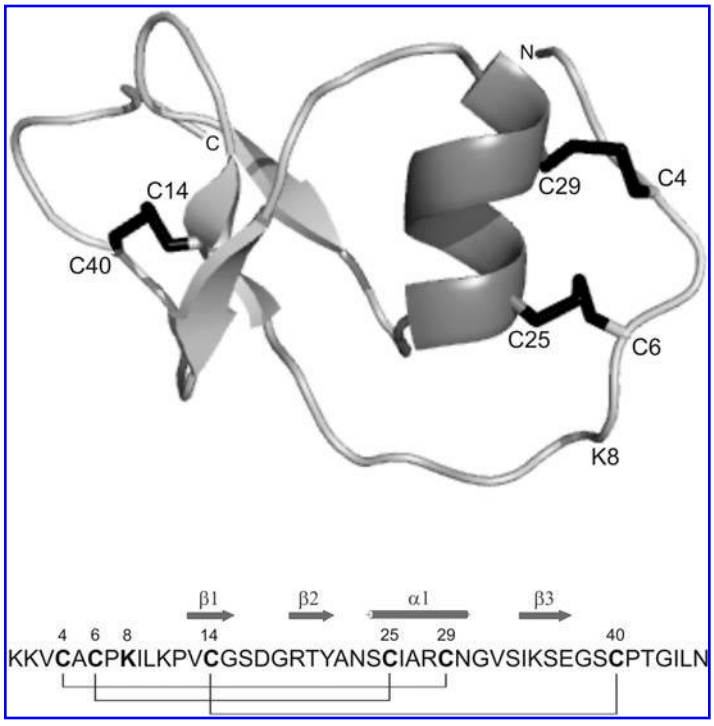

FIG. 1. Three-dimensional structure of LDTI. The Protein Data Bank accession number for the structure is 1LDT. The disulfide bonds are shown in a stick representation. The N-/C-termini and the reactive Lys8 residue are labeled. The amino acid sequence of LDTI and its secondary structure elements and disulfide pairings are schematically shown at the bottom. Figure prepared with PyMOL.

Hesperia, CA) chromatography. Protein identity and purity were confirmed by mass spectrometry (MS) and automated Edman degradation. The concentration of LDTI in solution was determined by measuring the absorbance at 280 nm and by using the calculated absorption coefficient $E_{0.1\%} = 3.46$. Recombinant LDTI was fully active (>95%) as determined by titration with bovine trypsin, assuming an equimolar interaction between inhibitor and enzyme.

Oxidative folding

Native LDTI (1 mg) was reduced and unfolded in 0.1 *M* Tris-HCl buffer, pH 8.5, containing 6 *M* guanidine hydrochloride (GdnHCl) and 100 m*M* dithiothreitol (DTT) for 2 h at 23°C (chemical reagents purchased from Sigma, Buchs, Switzerland). To initiate the refolding, the protein was passed through a PD-10 column (Sephadex G-25; GE Healthcare, Uppsala, Sweden), previously equilibrated with 0.1 *M* Tris-HCl, pH 8.5. After elution in a volume of 1.2 ml, the protein was immediately diluted to a final concentration of 0.5 mg/ml in the same buffer, both in the absence (Control−) and presence of redox agents: 0.25 m*M* 2-mercaptoethanol (Control+), 1 m*M* oxidized glutathione (GSSG), or 0.5 m*M*/1 m*M* oxidized/reduced glutathione (GSSG/GSH). To monitor the refolding reaction, aliquots were removed at various intervals and quenched with either 4% aqueous trifluoroacetic acid (TFA) (kept frozen) or 0.1 *M* Tris-HCl, pH 8.5, containing 0.2 *M* vinylpyridine (for 45 min at 23°C). Acid-trapped intermediates were subsequently analyzed with reversed-phase high-performance liquid chromatography (RP-HPLC) by using a linear 10–40% gradient of acetonitrile with 0.1% TFA during 50 min in a 4.6-mm Jupiter C4 column (Phenomenex, Torrance, CA) at a flow rate of 0.75 ml/min. Aliquots derivatized with vinylpyridine were diluted 1:10 in 0.1% aqueous TFA and analyzed with matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) MS by using an Ultraflex spectrometer (Bruker, Bremen, Germany). Samples were prepared by mixing equal volumes of the protein and matrix solutions [10 mg/ml of 2,6-dihydroxyacetophenone (Sigma) dissolved in 30% acetonitrile containing 20 m*M* ammonium citrate, pH 5.5].

Stop/Go folding

Selected acid-trapped intermediates occurring during the oxidative folding of LDTI were isolated by RP-HPLC as detailed earlier. After purification, the intermediates were freeze-dried, and folding was reinitiated at 23°C by dissolving samples in 0.1 *M* Tris-HCl buffer, pH 8.5, at a final protein concentration of 0.5 mg/ml. Folding intermediates were then similarly trapped by acidification and analyzed with RP-HPLC.

Reductive unfolding and disulfide scrambling

Native LDTI (0.5 mg/ml) was dissolved at 23°C in either 0.1 *M* Tris-HCl, pH 8.5, or 0.1 *M* phosphate buffer, pH 4.5, containing varying concentrations (0.1–300 m*M*) of DTT or Tris(2-carboxyethyl)phosphine (TCEP), respectively. To monitor the unfolding reaction, time-course aliquots of the samples were trapped with 4% aqueous TFA and analyzed with RP-HPLC, as detailed in "Oxidative folding." For disulfide-scrambling experiments, native LDTI was dissolved to a final concentration of 0.5 mg/ml in 0.1 *M* Tris-HCl, pH 8.5, containing 0.25 m*M* 2-mercaptoethanol and different concentrations of denaturants (0–8 *M* urea, 0–8 *M* GdnHCl, or 0–6 *M* guanidine thiocyanate; GdnSCN). The reaction of disulfide scrambling was allowed to reach equilibrium for 20 h at 23°C. The samples were then quenched by acidification and analyzed with RP-HPLC.

Disulfide-pairing determination

Selected acid-trapped intermediates that populate the oxidative folding and reductive unfolding of LDTI were purified with RP-HPLC by using the conditions described in "Oxidative folding" and freeze-dried. Each sample (~50 μg) was alkylated for 45 min at 23°C with 1 ml of 0.1 *M* Tris-HCl buffer, pH 8.5, containing 0.2 *M* vinylpyridine. The derivatized samples were then separated from reagents with RP-HPLC in a 4.6-mm Protein C4 column (Vydac), and analyzed with MS to verify the complete derivatization of the SH groups. The samples were then dried and treated with trypsin and endoproteinase Glu-C (both sequencing grade, from Roche Applied Science, Basel, Switzerland), at 1:20 (wt/wt) protease/substrate ratio, in 50 m*M* ammonium bicarbonate, pH 7.5. After incubation for 15 h at 37°C, the fragments resulting from digestion were analyzed with MALDI-TOF MS in reflector mode by mixing equal volumes of sample and matrix solution (10 mg/ml of α-cyano-4-hydroxycinamic acid (Bruker) dissolved in 30% acetonitrile containing 0.1% TFA). A mixture of peptides from Bruker (mass range of 1,000–4,000 Da) was used as mass calibration standard. The obtained peptide mass fingerprint was compared with the expected proteolytic digest of residues 1–46 (form C) of the UniProtKB/TREMBL entry P80424 (available at http://ca.expasy.org/sprot/) by using the program SequenceEditor (Bruker).

RESULTS

Oxidative folding of LDTI

The oxidative folding of LDTI was examined with acid-trapping and RP-HPLC analysis of the disulfide intermediates that accumulate along the folding reaction. The reduced and unfolded protein was allowed to refold in Tris-HCl buffer at pH 8.5 in the absence and presence of different redox agents (for details, see Materials and Methods). As shown in Fig. 2a, only a few intermediates populate the folding process of LDTI, which is almost completed after 2 h of reaction, even in the absence of any thiol catalyst or oxidizing agent (Control−). Similar RP-HPLC profiles were observed regardless of the absence and presence of 2-mercaptoethanol (see Control− and Control+), with an outstanding accumulation of the fractions (IIa, IIc, and I) and a minimal occurrence of three-disulfide scrambled isomers. The presence of oxidizing agent accelerates the folding rate of LDTI ~20 times without a significant alteration of the overall chromatographic pattern (Fig. 2b). Thus, the addition of 1 m*M* GSSG promotes only a slight increase of the IIc intermediate but, surprisingly, does not lead to a higher accumulation of scrambled isomers.

To evaluate the disulfide bond content of the intermediates that occur along the folding reaction, samples were quenched

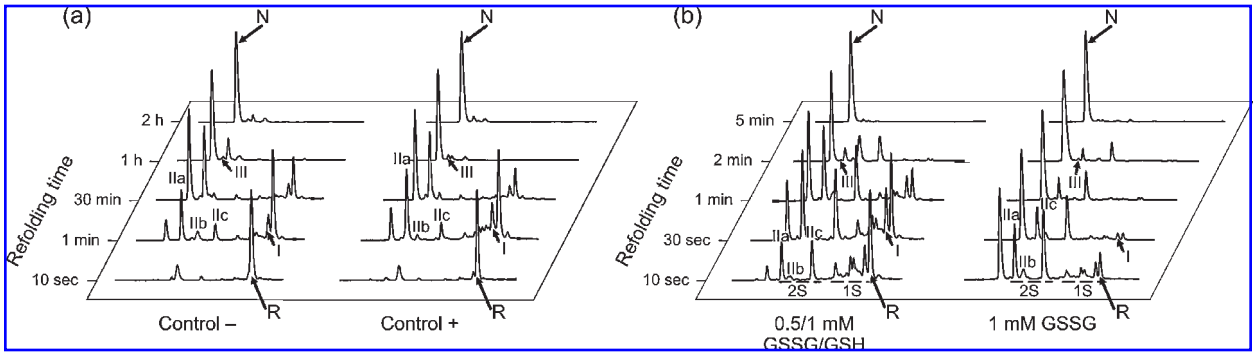

FIG. 2. Oxidative folding of LDTI. (**a**) RP-HPLC traces of the acid-trapped intermediates that occur along the oxidative folding of LDTI. The reactions were performed in Tris-HCl buffer (pH 8.5) in the absence (Control−) and presence (Control+) of 0.25 m*M* 2-mercaptoethanol as detailed in Materials and Methods. The retention times of the native (N) and reduced/unfolded (R) protein are labeled. I accounts for the predominant fraction of one-disulfide intermediates. IIa, IIb, and IIc constitute the major two-disulfide folding intermediates. Fraction III contains scrambled isomers. 1S and 2S are ensembles of species with the corresponding number of disulfide bonds. (**b**) RP-HPLC traces of the folding reactions carried out in Tris-HCl buffer (pH 8.5) in the presence of either GSSG/GSH (0.5 m*M*/1 m*M*) or GSSG (1 m*M*) alone.

at selected time points by alkylation of the SH groups with vinylpyridine and subsequently analyzed with MS. The mass spectra show a rapid formation of one- and two-disulfide intermediates (1S and 2S ensembles) that quickly diminish to give rise to the formation of the 3S ensemble (Fig. 3). The amount of three-disulfide species detected with MS nearly coincides with that of native protein (N) found with RP-HPLC, thus confirming the extremely rare occurrence of scrambled isomers. Based on vinylpyridine derivatization coupled with RP-HPLC and MS analysis, among the 2S ensemble, only three intermediates accumulate significantly [*i.e.*, IIa, IIb, and IIc (data not shown)]. By contrast, a higher degree of heterogeneity is found among the 1S ensemble, with a major accumulation of the peak eluting close to the reduced and denatured LDTI (R). This fraction, I, seems to contain at least two species (see later in "Disulfide pairing analysis"). Finally, the alkylation and subsequent MS analysis of the fraction eluting between N and IIa (III) confirms the presence of scrambled isomers.

To assess the kinetic role of the two-disulfide intermediates, the predominating species IIa and IIc were isolated with RP-HPLC and allowed to resume their folding in Tris-HCl buffer (pH 8.5). Along both stop/go reactions, the formation of R, various one-disulfide species (including I), and all two-disulfide intermediates was detected, indicating that neither IIa nor IIc is capable of forming native LDTI by a direct oxidation of their free thiol groups, but rather that these intermediates reduce or reshuffle (rearrange) their disulfide bonds to reach the productive pathway (Fig. 4). In particular, the IIc intermediate first must undergo a disulfide reshuffling to render IIa, which will in turn lead to the productive route toward native LDTI. In both reactions, the process is extremely fast, with ~25% of native LDTI formed after 1 min. The findings that both intermediates must either reduce or reshuffle before proceeding to the native form, together with the low accumulation of the IIb intermediate in the stop/go reactions, might indicate that IIb acts as the only productive form capable of oxidizing into native LDTI directly.

Reductive unfolding of LDTI

The reductive unfolding of native LDTI was examined by using both DTT and TCEP as reducing agents. The unfold-

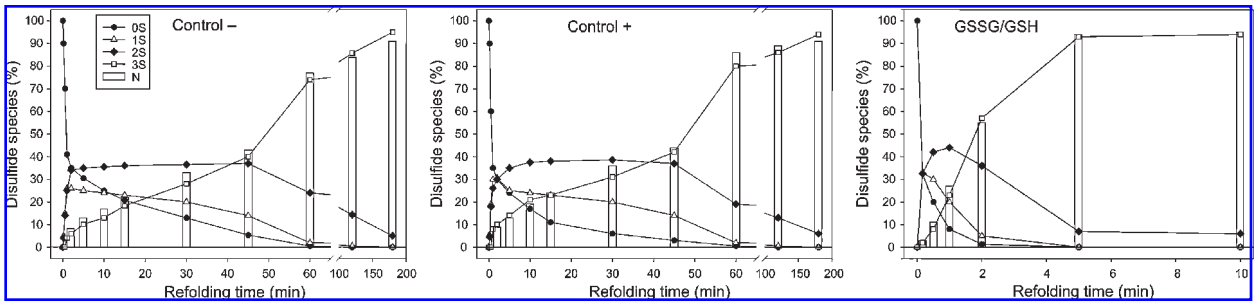

FIG. 3. Disulfide species in the oxidative folding of LDTI. Folding was performed in Tris-HCl buffer (pH 8.5) in the absence (Control−) and presence of redox agents: 0.25 m*M* 2-mercaptoethanol (Control+) or GSSG/GSH (0.5 m*M*/1 m*M*). The percentages of disulfide species were determined by derivatization with vinylpyridine and MALDI-TOF MS analysis (data not shown). XS, an ensemble of species with X disulfide bonds. The recovery of native LDTI is represented by bars and was calculated from the peak areas in the corresponding RP-HPLC chromatograms.

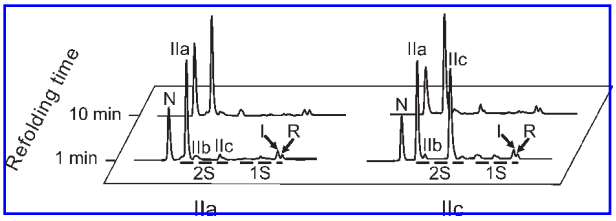

FIG. 4. Stop/Go folding of the IIa and IIc intermediates of LDTI. Both intermediates were trapped by acidification, purified with RP-HPLC, freeze-dried, and dissolved in Tris-HCl buffer (pH 8.5) to reinitiate the folding reaction. Folding intermediates were subsequently trapped with acid and analyzed with RP-HPLC, as described. N and R, the native and reduced/unfolded forms. 1S and 2S are ensembles of intermediates with one and two disulfide bonds, respectively. The intermediates IIa, IIb, IIc, and I are also labeled.

ing intermediates were trapped by acidification at various times and subsequently analyzed with RP-HPLC. Primarily, partially reduced species with chromatographic elution times equivalent to those of the intermediates occurring during oxidative folding accumulate on addition of DTT (Fig. 5a). Low concentrations of DTT (0.5–2 m*M*) break one of the three native disulfide bonds of LDTI and cause the accumulation of IIa, which subsequently reduces to I. However, other minor species with two (IIb and IIc) or one (I) disulfide bonds can be detected along the reaction. The use of TCEP as reductant (16, 19) significantly alters the pattern of intermediates that accumulate; reducing the pH to 4.5 causes the reductive reactions to prevail over the disulfide reshuffling, thus preventing the accumulation of intermediates containing non-native disulfide bonds that may appear at pH 8.5. As shown in Fig. 5b, under these conditions, only three peaks occur along the unfolding reaction (*i.e.*, IIa, IIb, and I). In addition, the extent of accumulation of these forms differs from that obtained in the presence of DTT, with a higher and lower predominance of IIb/I and IIa, respectively.

Disulfide scrambling of LDTI

It has been well established that, in the presence of a denaturant and a thiol initiator, unfolding of a native disulfide-containing protein results in reshuffling of its native disulfide bonds, a process called disulfide scrambling (8, 10). On this basis, unfolding of native LDTI was performed in the presence of increasing concentrations of different denaturants (urea, GdnHCl, or GdnSCN) and 0.25 m*M* 2-mercaptoethanol at pH 8.5. After reaching equilibrium, the reactions were analyzed with RP-HPLC (Fig. 5c). In the presence of the thiol initiator and 0–8 *M* urea, only a minor fraction of scrambled isomers is detectable, representing $<2\%$ of the total protein (data not shown). It is worth mentioning that the HPLC peak corresponding to this fraction correlates with that of the intermediate III observed in the folding process (see also Fig. 2a). The use of 1–6 *M* GdnHCl increases the accumulation of this fraction up to $\sim 5\%$ of the total protein. By contrast, high concentrations of GdnHCl or GdnSCN destabilize the scrambled forms and, surprisingly, cause a minor accumulation of IIb.

Disulfide-pairing analysis

After examination of the oxidative folding and reductive unfolding of LDTI, it was essential to determine the disulfide pairings of the common intermediates that accumulate. Samples of IIa and IIc were isolated by partial reduction or oxidation of native or reduced LDTI, respectively, purified with RP-HPLC and alkylated with vinylpyridine. The derivatized intermediates were subsequently digested with trypsin and endoproteinase Glu-C, and analyzed with MALDI-TOF MS (Table 1). Mass fingerprint analysis of the resulting fragments revealed that these two species comprise native disulfide bonds (IIa: Cys6–Cys25 and Cys14–Cys40; IIc: Cys4–Cys29 and

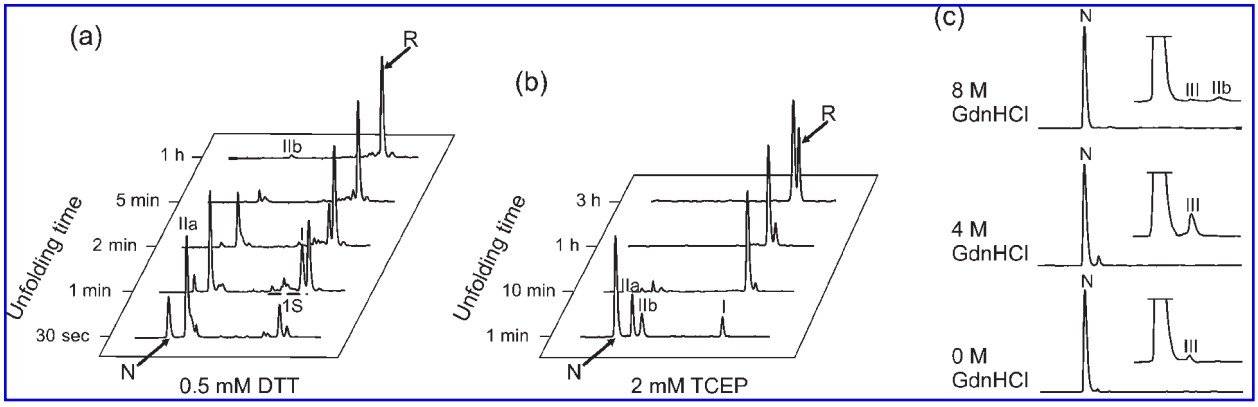

FIG. 5. Reductive unfolding and disulfide scrambling of LDTI. (**a**) RP-HPLC traces of the acid-trapped intermediates that occur along the reductive unfolding of LDTI. The native protein was treated with 0.5 m*M* DTT in Tris-HCl buffer (pH 8.5). N and R, the native and reduced/unfolded forms. IIa, IIb, IIc, and I are indicated. 1S is an ensemble of one-disulfide intermediates. (**b**) RP-HPLC traces of the reductive unfolding of LDTI by using 2 m*M* TCEP in phosphate buffer (pH 4.5). (**c**) RP-HPLC traces of the acid-trapped scrambled isomers generated by disulfide scrambling. The native form of LDTI was denatured in Tris-HCl buffer (pH 8.5) containing 0.25 m*M* 2-mercaptoethanol as thiol initiator and the indicated concentration of denaturant. The peaks corresponding to N, III, and IIb are augmented by fourfold inside each RP-HPLC profile.

Table 1. Assignment of Peptide Fragments of the Folding Intermediates IIa, IIc, and I

	Theoretic mass, MH (Da)	*Experimental mass, MH (Da)*	*Fragment sequence*
IIa	725.3	725.5[a]	V3-K8 (C4-PE, C6-SH)
	824.4	825.3	C29-K35 (C29-PE)
	998.5	998.3[a]	T20-R28 (C25-SH)
	1,077.5	1,077.3[a]	S36-N46 (C40-SH)
	1,144.6	1,144.4[a]	I9-R19 (C14-SH)
	1,719.7	1,720.5	V3-K8 + T20-R28 (C4-PE, C6-C25)
	2,003.0	2,003.8	I9-R19 + G38-N46 (C14-C40)
	2,219.1	2,219.7	I9-R19 + S36-N46 (C14-C40)
IIc	965.5	966.1	G38-N46 (C40-PE)
	1,181.5	1,181.9	S36-N46 (C40-PE)
	1,248.6	1,249.3	I9-R19 (C14-PE)
	2,336.1	2,334.5	V3-K8 + T20-R28 + C29-K35 (C4-C29, C6-C25)
I	824.4	825.4	C29-K35 (C29-PE)
	829.3	830.4	V3-K8 (C4-PE, C6-PE)
	1,102.5	1,103.5	T20-R28 (C25-PE)
	1,144.6	1,144.4[a]	I9-R19 (C14-SH)
	1,248.6	1,249.7	I9-R19 (C14-PE)
	1,719.7	1,720.8[a]	V3-K8 + T20-R28 (C4-PE, C6-C25)
	2,003.0	2,003.7	I9-R19 + G38-N46 (C14-C40)
	2,219.1	2,220.1	I9-R19 + S36-N46 (C14-C40)

Peptides were generated by incubation of samples with vinylpyridine and subsequent digestion with trypsin and endoproteinase Glu-C. Fragments containing pyridylethyl (PE-) cysteines, sulfhydryl groups (C-SH), and disulfide bonds (C-C) were analyzed with MALDI-TOF MS. Fragment limits refer to the amino acid sequence numbering of LDTI (UniProtKB/TREMBL entry P80424).

[a]Minor species.

Cys6–Cys25). Based on the TCEP-reduction analysis, IIb also has native disulfide bonds, which therefore should be Cys4–Cys29 and Cys14–Cys40.

Partial reduction of intermediate IIa with TCEP allowed the isolation of two species that co-elute within fraction I. IIa had been previously derivatized with vinylpyridine and repurified with RP-HPLC. The disulfide bonds Cys14–Cys40 and Cys6–Cys25 were confirmed by alkylation, trypsin and Glu-C digestion, and subsequent MS analysis. However, the MS data showed a higher intensity of the ionic peptides corresponding to the Cys14–Cys40 disulfide bond (data not shown). This higher prevalence, together with the fact that both IIa and IIb intermediates contain this disulfide bond, might indicate a higher stability of the Cys14–Cys40 disulfide pairing. Direct formation of fraction I from N is likely to comprise other species, but this has not yet been confirmed because of the mixture of partially derived species that were obtained after alkylation of I.

DISCUSSION

The Kazal-type family of serine protease inhibitors (Merops family I1) includes a large number of proteins that are characterized by their amino acid sequence homology, their particular disulfide-bond pattern, a common overall fold, and the reactive-site position. These proteins represent an attractive system for studies of oxidative folding because of their small size and high number of disulfide bonds. However, few in-depth studies of their folding have been done to date. LDTI, a nonclassic Kazal-type inhibitor, comprises a particular cysteine pattern strongly cross-linked by a cystine-stabilized α-helical motif (CSH) that is widely found in bioactive peptides, such as endothelins or toxins from insects and snakes (21, 22), and makes it an interesting model for folding analyses.

The current work investigates the oxidative folding and reductive unfolding of LDTI by acid-trapping and RP-HPLC/disulfide-pairing analysis of its intermediates. LDTI folds from the reduced and unfolded protein to the native state through a sequential oxidation of cysteine residues. Although 75 different disulfide-bonded intermediates are theoretically possible, only a few species populate the oxidative folding pathway of this molecule. Importantly, all detected intermediates contain one or two native disulfide bonds, suggesting a strong bias toward the establishment of native interactions during the folding pathway. Therefore, its folding reaction comprises the sampling of a reduced number of specific metastable states rather than a large conformational search among species of similar free energy. The presence of a discrete number of native-like intermediates, together with the minor accumulation of scrambled isomers, seems to account for the high efficiency of LDTI folding. By contrast, scrambled forms act as kinetic traps in the folding reactions of numerous other small, disulfide-rich proteins such as hirudin, tick-anticoagulant peptide, and potato carboxypeptidase inhibitor (2, 9, 37). In the absence of redox agents, LDTI is able to recover its native state quantitatively in <2 h of refolding. This extraordinary speed of disulfide folding has been reported before only for BPTI. The predominant folding pathway followed by LDTI at pH 8.5 is summarized in

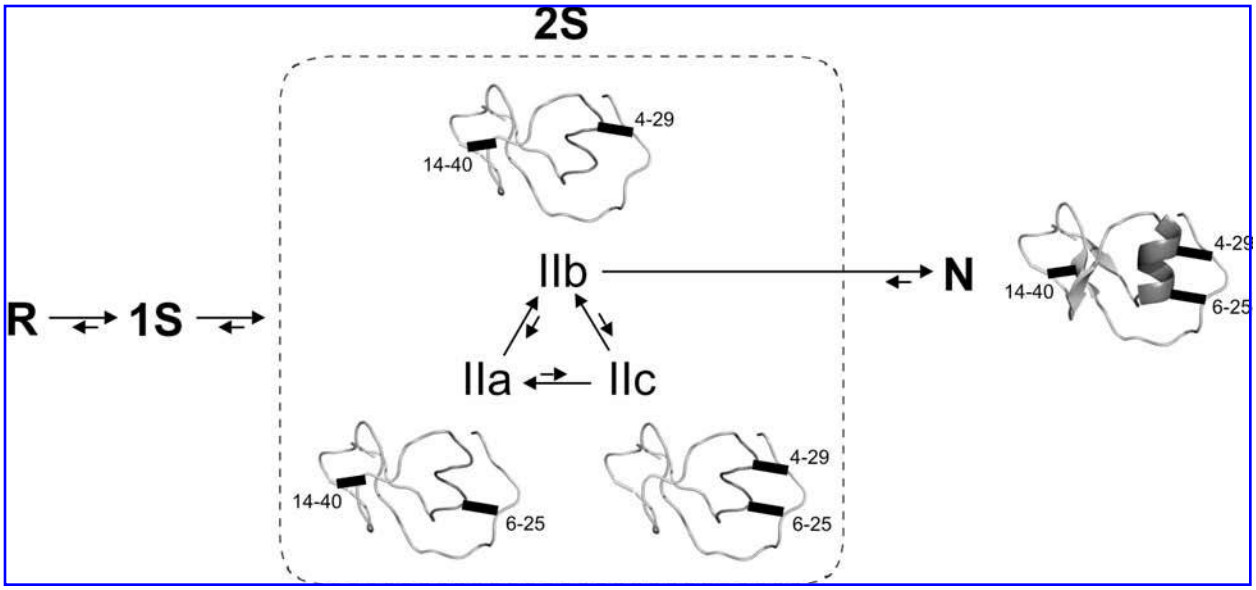

FIG. 6. Schematic diagram of LDTI folding. The predominant oxidative folding pathway of LDTI is outlined by arrows. R and N, the reduced/unfolded and native forms of this protein. XS are ensembles of molecules with X number of disulfide bonds. The native disulfide bonds of IIa, IIb, IIc, and N are shown inside the structures.

Fig. 6. Initially, R is oxidized to a mixture of one-disulfide species, which significantly accumulate as fraction I. The relative concentration of disulfide bonds of this ensemble deviates from a random distribution calculated based on loop entropies, with a high prevalence of the [14–40] species that comprise the longest loop closed by native disulfides. Thus, the entropic contribution to the stability of this one-disulfide intermediate is the lowest among those of native disulfide bonds, and its abundance can be explained only by the establishment of preferential noncovalent interactions between residues surrounding both cysteines. The interactions that stabilize this intermediate may play an important role in the initial steps of LDTI folding. Cys14 is located in the β-strand 1, and Cys40, in the loop located at the end of the β-strand 3, both involved in the triple-stranded β-sheet that may be the first structural element to fold. The two cysteine residues that are cross-linked by the Cys14–Cys40 disulfide bond are buried in the native structure of LDTI, with $<10\%$ of accessible surface. Their preferential burying relative to other cysteines in the structure of this intermediate hinders their reduction and rearrangement, and thus may cause its accumulation as the major species of the 1S ensemble.

Next, one-disulfide intermediates oxidize a second pair of cysteines to form two-disulfide species that accumulate mainly as IIa, IIb, and IIc, all of them containing native disulfide bonds. Although both IIa and IIb intermediates compose the Cys14–Cys40 disulfide bond, only the former accumulates strongly, constituting the major kinetic trap of the folding reaction. From a thermodynamic point of view, the lack of accumulation of IIb in all tested conditions is surprising, because the second disulfide in this species (Cys4–Cys29) closes a longer loop than that in IIa (Cys6–Cys25) and is expected to promote a higher reduction of entropy in the unfolded state and a higher stability in the IIb intermediate. Thus, the highly unequal accumulation of IIa and IIb may be caused only by kinetic mechanisms. In IIb, the formation of the Cys4–Cys29 disulfide bond would strongly restrict the conformational motion around the free Cys6 and Cys25 residues, allowing a rapid oxidation of the third pair of cysteines to generate N and avoiding its accumulation during the folding reaction. Therefore, IIb most likely is the productive intermediate of LDTI folding. IIc, containing the two disulfide bonds that link the α-helix to the N-terminal loop (Cys4–Cys29, Cys6–Cys25), also accumulates significantly during the folding process, but much less than IIa, probably because its higher disulfide exposition results in lower disulfide stability. This is further supported by the reductive unfolding data. Only in the presence of GSSG, which promotes disulfide bond formation, does this intermediate accumulate to similar levels than IIa. As expected, IIb does not accumulate significantly under these oxidizing conditions, but rather proceeds rapidly into the native form.

The identification of IIb and IIa/IIc as productive and nonproductive intermediates, respectively, is confirmed by the stop/go experiments. The two latter species cannot proceed directly to the native form, probably because of the native-like structure of their β-sheet and α-helix, respectively, and need either reduction or rearrangements of their disulfide bonds to reach the productive route *via* IIb. Although non-native disulfide-bonded intermediates necessarily must occur during LDTI folding, these species were not detectable, indicating that they are highly disfavored thermodynamically. In addition, their non-native disulfide bonds probably are solvent exposed, resulting in a fast reduction, which would explain the detection of both 1S and reduced forms in the stop/go reactions of the IIa and IIc intermediates. Differences in solvent accessibility of the cysteine residues in the native structure of LDTI are also likely to determine the population of intermediates occurring during its reductive unfolding. As inferred from the reductive unfolding experiments, LDTI unfolds in a sequential way through two-

and one-disulfide intermediates to reach R. At pH 8.5, both the oxidative-folding and reductive-unfolding pathways are virtually identical and share a common pattern of disulfide intermediates. However, the unfolding reaction changes significantly when reducing the pH of the reaction to 4.5, which minimizes disulfide reshuffling and thereby prevents the interconversion of intermediates. Under this condition, only the intermediates containing the Cys14–Cys40 disulfide bond (*i.e.*, IIa, IIb and subsequently I) accumulate to a detectable extent. Although the two disulfide bonds that stabilize the α-helix in the native structure (Cys 4–Cys29 and Cys6–Cys25) are solvent exposed, the presence of a thiol initiator and increasing concentrations of denaturant reshuffle only a low fraction of the native protein into scrambled isomers. Thus, increasing concentrations of urea and GdnHCl only lead to the formation of $<5\%$ scrambled isomers. So far, BPTI is the most stable protein in terms of disulfide scrambling, with a total resistance against 8 *M* urea and 0.5% denatured protein against 7.5 *M* GdnHCl (6). This highly unusual conformational stability was ascribed to its folding process that is characterized by the presence of intermediates comprising mainly native disulfide bonds. As discussed earlier, the LDTI intermediates probably also adopt native-like conformations, indicating the existence of stable secondary structure elements that govern the folding reaction and avoid the formation of conformationally unstable scrambled isomers. Conversely, in nondenaturing conditions, the presence of a thiol initiator reshuffles the native disulfides of LDTI to form $\sim$2% scrambled isomers, a phenomenon already described for other proteins such as hirudin, epidermal growth factor, and potato carboxypeptidase inhibitor (12). The coexistence of scrambled forms in equilibrium with the native protein is of interest in the field of protein aggregation (28), because the aggregation process that ultimately leads to many neurodegenerative diseases is often caused by partial unfolding of the polypeptide chain under mild conditions (29).

The data reported here indicate the presence of a strong native-like bias in both the intermediates that funnel the reaction toward the native structure and those that act as kinetic traps, freezing it. The same interactions that stabilize the final folded structure seem to guide LDTI toward its native state. However, the importance of non-native disulfide species, including the scrambled isomers, should not be disregarded, and further experiments in this direction are necessary to determine their role in LDTI folding. It is worth remembering that in BPTI folding, populated predominantly by native-like intermediates, the minor non-native disulfide population plays a relevant kinetic role in the early stages of disulfide formation (17). Although LDTI and BPTI are very different in respect to sequence and structure, the similarity of their folding processes is very great. BPTI folds through two nonproductive intermediates, [14–38; 30–51] and [5–55; 14–38] (36), which are analogues to IIa and IIc, respectively, in LDTI folding. In addition, the productive intermediate [5–55; 30–51] is mimicked by IIb.

As is usual for small serine protease inhibitors, LDTI interacts with target enzymes through its binding loop (residues 3–10), with Lys8 as the primary determinant of inhibition (18, 34). Although tryptases are far more selective than trypsin in substrate specificity and are highly resistant to inhibition, LDTI has favorably adapted to this type of proteases. Unlike the other Kazal-type inhibitors, LDTI has a truncated central α-helix and a particular disulfide bond conformation that allow this molecule to fit into the central pore of the enzymatically active tryptase tetramer. In addition, the basic amino-terminal residues of LDTI favorably interact with an acidic surface patch (around residue 148) exhibited by the tryptase. Preliminary results from tryptic digestions indicate that the IIa intermediate significantly inhibits trypsin (data not shown). IIa and IIb are likely to display highly native-like structures, owing to the presence of either Cys6–Cys25 or Cys4–Cys29 and Cys14–Cys40, which would stabilize the two segments of secondary structure in the native protein (*i.e.*, the α-helix and the β-sheet, respectively). In IIa, the Cys6–Cys25 bond probably locks the reactive binding loop in a fashion similar to native LDTI, thereby causing native-like inhibitory properties, whereas in IIb, a longer and more-flexible loop might prevent tryptase inhibition. Additional experiments directed to evaluate the structure and activity of these major LDTI folding intermediates will help to understand its native-like nature and gain insights into the oxidative folding of this molecule.

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ABBREVIATIONS

BPTI, bovine pancreatic trypsin inhibitor; DTT, dithiothreitol; GdnHCl, guanidine hydrochloride; GdnSCN, guanidine thiocyanate; GSH, reduced glutathione; GSSG, oxidized glutathione; K_i, inhibition constant; LDTI, leech-derived tryptase inhibitor; MALDI-TOF MS, matrix-assisted laser desorption/ionization–time of flight mass spectrometry; N, native protein; R, reduced and unfolded protein; RP-HPLC, reversed-phase high-performance liquid chromatography; TCEP, Tris(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid.

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