The Unfolding Pathway and Conformational Stability of Potato Carboxypeptidase Inhibitor*

Received for publication, January 10, 2000, and in revised form, February 16, 2000

Jui-Yoa Chang‡§, Li Li‡, Francesc Canals¶, and Francesc X. Aviles¶

From the ‡Research Center for Protein Chemistry, Institute of Molecular Medicine, University of Texas, Houston, Texas 77030 and the ¶Institut de Biologia Fonamental and Departament de Bioquimica, Universitat Autonoma de Barcelona, 08193 Bellaterra, Spain

The unfolding and denaturation curves of potato carboxypeptidase inhibitor (PCI) were investigated using the technique of disulfide scrambling. In the presence of denaturant and thiolinitiator, the native PCI denatures by shuffling its native disulfide bonds and converts to form a mixture of scrambled PCI that consists of 9 out of a possible 14 isomers. The denaturation curve is determined by the fraction of native PCI converted to scrambled isomers under increasing concentrations of denaturant. The concentration of guanidine thiocyanate, guanidine hydrochloride, and urea required to denature 50% of the native PCI was found to be 0.7, 1.45, and 8 M, respectively. The PCI unfolding curve was constructed through the analysis of structures of scrambled isomers that were denatured under increasing concentrations of denaturant. These results reveal the existence of structurally defined unfolding intermediates and a progressive expansion of the polypeptide chain. The yield of the beads-form isomer (Cys8–Cys12, Cys18–Cys24 and Cys27–Cys34) as a fraction of total denatured PCI was shown to be directly proportional to the strength of the denaturant condition. Furthermore, the PCI sequence was unable to fold quantitatively into a single native structure. Under physiological conditions, the scrambled isomers of PCI that constitute about 4% of the protein were in equilibrium with native PCI.

The conformational stability of proteins has been regularly studied with denaturation curves, where changes in parameters that can be correlated with the three-dimensional structure of the protein are measured as a function of denaturant concentration in the protein solution. The measured parameter is usually a physicochemical property such as UV absorbance, fluorescence, or circular dichroism of the protein of interest (1, 2). Whereas denaturation of a protein can be assessed by many different parameters, most conventional approaches are not able to determine the degree of unfolding of a protein in a given denatured state, the presence and concentration of intermediates, or the characteristics of the denatured state. There is growing evidence that within the ensemble of conformations that constitute the denatured states of a protein, there is a population of molecules that still possess residual structure (3–7), i.e. the denatured state does not imply a completely unfolded conformation. Characterizing the denatured state of proteins is a subject of increasing interest for the understanding of protein folding and stability (8–10).

Recently, we have proposed a new methodology for studying stability toward denaturants and the unfolding pathway of disulfide-containing proteins (11, 12). This approach is based on the observation that when disulfide-containing proteins are treated with denaturants in the presence of a trace amount of a thiol initiator, proteins can reversibly shuffle their disulfide bonds, leading to a mixture of native and disulfide-scrambled species (fully oxidized species that have at least two non-native disulfide bridges) (13). The composition of this mixture depends on the type of denaturant and its concentration. The trapping of such scrambled species and its analysis by chromatographic methods permit not only an evaluation of the extent of protein denaturation by measuring the amount of native protein converted into disulfide-scrambled form, but also gives insight into the degree of unfolding of the protein through the relative composition of the different scrambled species. This novel methodology has been used to characterize the unfolding mechanism of several disulfide-containing proteins such as hirudin, tick anticoagulant peptide, RNase A (11), and cardiotatin III (14) and to characterize the denatured states of tick anticoagulant peptide (12) and insulin-like growth factor (15) under different denaturant conditions. These studies demonstrate the potential of this approach for comparing the stability of disulfide-containing proteins and characterizing the extent of unfolding of the denatured proteins.

The present work describes a study of the conformational stability and the unfolding pathway of the potato carboxypeptidase inhibitor (PCI),1 using disulfide-scrambling methodology. This 39-residue protein inhibitor has a central globular structure stabilized by three disulfide bridges (16, 17). PCI was recovered from Escherichia coli by expression of a synthetic gene constructed from the sequence of the mature protein (18), and the inhibitory determinants at the C-terminal tail were mapped by protein engineering (19). PCI shares a peculiar disulfide-stabilized loop scaffold, known as the cystine knot or T-knot (20), with other plant protease inhibitors and with animal peptidic growth factors. In particular, we have recently shown that PCI displays structural homology with epidermal growth factor, which makes this molecule an epidermal growth factor antagonist of potential interest as an antitumoral agent (21, 22).

The disulfide-folding pathway of PCI, which was described in a previous work (23), resembles that of hirudin (24) or tick

---

1 The abbreviations used are: PCI, potato carboxypeptidase inhibitor; GdnHCl, guanidine hydrochloride; GdnSCN, guanidine thiocyanate; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight mass spectrometry.
anticoagulant peptide (25). The common mechanism for the refolding of these proteins consists of the initial stage of non-specific packing that leads to the formation of scrambled species followed by the final stage, where scrambled species re-shuffle disulfide bridges and consolidate to form the native structure. Some peculiar features were observed in the case of PCI. On the one hand, a rather slow consolidation stage was observed, which may be attributed to the lower number of noncovalent interactions guiding the folding when compared with other proteins. Moreover, the refolding of PCI cannot be driven to completion even under optimized redox conditions or in the presence of protein-disulfide isomerase; some scrambled species inevitably remain in the refolding mixture. The present study of the stability and unfolding of PCI using the technique of disulfide scrambling facilitates a greater understanding of these features and redresses the question of whether native proteins have a unique conformational state.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant PCI was obtained by heterologous expression in E. coli and purified by ion-exchange chromatography on a TSK-DEAE column (Amersham Pharmacia Biotech), followed by reverse-phase HPLC (18). The protein was more than 99% pure, as judged by HPLC and N-terminal sequence analysis. Urea, guanidine hydrochloride (GdnHCl), and guanidine thiocyanate (GdnSCN), with purities of greater than 99%, were obtained from Merck.

**Preparation of Standard Scrambled PCI**—Standard scrambled PCI refers to those forms that exist as folding intermediates along the folding pathway of the fully reduced PCI (23). These are prepared by allowing the folding to proceed in alkaline buffer without the addition of reductant. The native PCI (0.5 mg/ml) was first reduced with dithiothreitol (30 mM) in Tris-HCl buffer (0.1 M, pH 8.4) containing 0.25 mM 2-mercaptoethanol for 24 h. The end product consisted of 20% native PCI and 80% scrambled isomers that were detected in nine fractions. The HPLC profiles are shown in Fig. 1. Chromatogram Std. X-PCI. Equilibrium Between Native and Scrambled PCI Under Physiological pH—Native PCI (0.5 mg/ml) was dissolved in Tris-HCl buffer (0.1 M, pH 8.4) or sodium phosphate buffer (50 mM, pH 7.4) containing varying concentrations of 2-mercaptoethanol (0.05–0.3 M). The incubation was carried out at 23 °C for 20 h. The end products were then quenched with equal volumes of 4% aqueous trifluoroacetic acid and analyzed by HPLC. A parallel control experiment was performed with incubation of native PCI in Tris-HCl buffer without thiol agent.

**Denaturation and Unfolding of PCI in the Presence of Denaturant**—Native PCI (0.5 mg/ml) was dissolved in Tris-HCl buffer (0.1 M, pH 8.4) containing 0.25 mM 2-mercaptoethanol and selected concentrations of denaturants (urea, GdnHCl, and GdnSCN). The reaction was allowed to reach equilibrium and was typically performed at 23 °C for 20 h. 2-Mercaptoethanol (0.15 mM) was replenished twice at 4 and 8 h. To monitor the kinetics of unfolding, aliquots of the sample were removed at defined time intervals, quenched with 4% trifluoroacetic acid, and analyzed by HPLC. The denatured sample was subsequently acidified with an equal volume of 4% trifluoroacetic acid and stored at −20 °C. The GdnSCN-denatured samples were further purified by gel filtration (NAP-5 columns) prior to HPLC analysis.

**Fluorescence Measurements of the Denatured Scrambled PCI**—The fluorescence spectrum of PCI was measured with an LS-50B spectrophotometer (Perkin-Elmer) scanning from 300 to 450 nm, with excitation at 278 nm. PCI (0.5 mg/ml) was dissolved in Tris-HCl buffer (0.1 M, pH 8.4) containing 0.25 mM 2-mercaptoethanol and selected concentrations of denaturants (urea and GdnHCl). Denaturation of PCI was performed at 23 °C for 24 h. Each sample was then diluted 50 times with the same concentration of the denaturant to a final concentration of 10 μg/ml shown for measurement. Blank samples containing equivalent concentrations of the denaturant were also analyzed. Fluorescence intensities of the blank samples were subtracted from readings of the PCI samples. Each measurement was repeated, and fluorescence intensity was monitored at 354.5 nm (see Fig. 4). The PCI denaturation curve was constructed according to conventional formulas (1, 2). The fraction denatured (FD) was determined byFd = (yn - yd)/(yn - yd), where yn is the fluorescence reading of the native protein, yd is the fluorescence reading of the sample, and yd is the fluorescence reading of the fully denatured protein.

**Reductive Unfolding of the Native and Scrambled PCI**—The native and scrambled PCI (0.5 mg/ml) were treated with selected concentrations of dithiothreitol in Tris-HCl buffer (0.1 M, pH 8.4). The reaction was analyzed either in a time course with fixed concentrations of dithiothreitol, or at fixed time points with varying concentrations of dithiothreitol (0.2–100 mM). The reaction was quenched by mixing the sample with an equal volume of 4% aqueous trifluoroacetic acid.

**Structural Analysis of Scrambled PCI—Isolated fractions of scrambled PCI (~20 μg) were treated with 2 μg of thermolysin (Sigma, P-1512) in 30 μl of N-ethylmorpholine/acetate buffer (50 mM, pH 6.4), or digested with 2 μg of Lys-C endopeptidase in 30 μl of ammonium bicarbonate buffer (50 mM, pH 7.9). Digestions were carried out at 37 °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.

**Amino Acid Sequencing and Mass Spectrometry**—The amino acid sequence of disulfide-containing peptides was analyzed by automated Edman degradation using a Perkin-Elmer Procise sequencer (model 494) equipped with an on-line phenylthiohydantoin-derivative analyzer. The molecular mass of disulfide-containing peptides was determined by MALDI-TOP mass spectrometry (Perkin-Elmer, Voyager-DE STR).
RESULTS

The PCI Sequence Adopts Multiple Conformations at Physiological pH—After native PCI was incubated in buffer (pH 7.4–8.4) containing trace amounts of thiol agent, a minute fraction of the protein shuffled its native disulfide bonds and isomerized to form scrambled structures. This spontaneous reaction eventually reached a state of equilibrium with a $K_{eq}$ of 0.04 ± 0.004, which corresponds to a standard free energy change of 1.9 kcal/mol. Both 2-mercaptoethanol (0.05–0.3 mM) and reduced glutathione (0.2–1 mM) were effective as thiol initiators to “jump start” this spontaneous conversion. In the absence of thiol agent (control), the native disulfides of PCI remained stable (see Fig. 1, chromatograms A and Ax15). The scrambled isomers were detectable by HPLC in nine distinct fractions (Fig. 1, chromatograms B and Bx15). The compositions were indistinguishable from those observed along the folding pathway of fully reduced PCI (Fig. 1, chromatogram Std. X-PCI).

These experiments were also performed with $^{15}$N-PCI, and similar results were observed. About 3–4% of scrambled isomers of $^{15}$N-PCI exist in equilibrium with the native structure under the pH range of 7.4–8.4 in the presence of thiol agent. These results therefore suggest that $^{15}$N- and $^{14}$N-PCI possess comparable conformational stability. In both cases, about 4% of the protein was unable to form the native conformation.

The Denaturation Curve of PCI Examined by Conversion of Native to Scrambled Structures—In the presence of denaturant and thiol initiator, native PCI was denatured to form scrambled isomers. Fig. 2 displays HPLC chromatograms of PCI denatured under increasing concentrations of urea, GdnHCl, and GdnSCN. The denatured PCI was composed of at least nine scrambled isomers marked alphabetically in Fig. 2. The denaturation curve was clearly dependent upon the strength of denaturant. The denaturation curves, calculated from the fraction percent of PCI converted to the scrambled isomers, are shown in Fig. 3. Based on the concentration that is required to achieve the same extent of PCI denaturation, GdnSCN is about 2- and 12-fold more potent than GdnHCl and urea, respectively. Indeed, urea is unable to fully denature the native PCI. Even at 8 mM urea, only 44% of the native PCI was denatured. The significant difference of potency between urea and GdnHCl, while expected (1), contrasts sharply to that observed for the tick anticoagulant peptide (12), where urea and GdnHCl exhibit nearly indistinguishable denaturing potency.

The Denaturation Curve Determined by Fluorescence Measurement—PCI contains two tryptophan residues at sequence positions 22 and 28. The extent of denaturation was clearly dependent upon the strength of denaturant. The denaturation curves, calculated from the fraction percent of PCI converted to the scrambled isomers, are shown in Fig. 3. Based on the concentration that is required to achieve the same extent of PCI denaturation, GdnSCN is about 2- and 12-fold more potent than GdnHCl and urea, respectively. Indeed, urea is unable to fully denature the native PCI. Even at 8 mM urea, only 44% of the native PCI was denatured. The significant difference of potency between urea and GdnHCl, while expected (1), contrasts sharply to that observed for the tick anticoagulant peptide (12), where urea and GdnHCl exhibit nearly indistinguishable denaturing potency.

The PCI Denaturation Curve Determined by Fluorescence Measurement—PCI contains two tryptophan residues at sequence positions 22 and 28. The native and scrambled (denatured) PCI exhibit distinct fluorescence intensities. These signals were used to plot the PCI denaturation curves in the conventional manner, where the signal of the native protein and that of the denatured sample (5 mM GdnHCl, representing the fully denatured structure) are required. Fig. 4 displays the fluorescence spectra of native PCI and urea- and GdnHCl-denatured PCI. The fraction of PCI denatured with increasing concentrations of urea was calculated from the differential intensities monitored at 354.5 nm. The urea denaturation...
The disulfide structures of scrambled isomers of PCI—Scrambled PCI isomers were isolated in nine fractions (Fig. 2) from reverse-phase HPLC with a linear acetonitrile gradient. Two of the fractions, X-PCI-a and X-PCI-h (X refers to scrambled; a and h to fractions marked in Fig. 2), were further isolated for structural characterization. These two fractions were selected because the relative concentrations correlate with the strength of the denaturing conditions. These two fractions were digested by thermolysin and Lys-C endopeptidase. Peptides were isolated by HPLC (Fig. 5) and characterized by Edman sequencing and mass spectrometry. The results, summarized in Table 1, unambiguously demonstrate that X-PCI-h contains Cys8–Cys27, Cys12–Cys18, and Cys24–Cys34 bonds and X-PCI-a adopts the disulfide pairings of Cys8–Cys12, Cys18–Cys24, and Cys27–Cys34 (the beads-form isomer) (Fig. 6). The structure of X-PCI-h was deduced from the identification of two major thermolytic peptides, h-4 and h-10 (Table I). Peptide h-10 contains Cys24–Cys34. Peptide h-4 contains two disulfides which can be either (Cys8–Cys27, Cys12–Cys18), (Cys8–Cys12, Cys18–Cys27), or (Cys8–Cys12, Cys18–Cys27). The first disulfide structure was assigned because further digestion of h-4 by Lys-C endopeptidase generates two distinct fragments, one with molecular mass of 1077 Da (1059 plus 18 because of cleavage at Lys–Thr) corresponding to residues 11–20 (Cys12–Cys18). The other structure (Mr, 666) is consistent with Ala–Cys and Ile–Cys–Asn–Lys linked by Cys8–Cys27. In the case of X-PCI-a, the presence of Cys18–Cys24 and Cys27–Cys34 was confirmed by the structures of thermolytic peptides a-5, a-6, and a-11 (Table I). The third disulfide, Cys8–Cys12, was found in the peptide a-1 generated by Lys-C digestion. Two minor peptides (a-7 and a-8) containing Cys27–Cys34 were also found within the Lys-C digest of X-PCI-a. This is because of nonspecific cleavage, possibly by a contaminant in thermolysin.

The deduced structure of X-PCI-h is identical to that found for the chromatographic equivalent scrambled form isolated during refolding of fully reduced PCI2 (23). However, the beads-form isomer X-PCI-a could not be identified in the mixture of scrambled forms generated upon refolding of PCI in the absence of denaturants,2 presumably because of its low relative concentration under these conditions.

The unfolding curve (pathway) of PCI—Denatured PCI may adopt different degrees of unfolding with its structure characterized by the composition of scrambled species. The plotting of the PCI unfolding curve was based on the relative concentration among scrambled isomers of denatured PCI. The results, observed in the presence of increasing concentrations of urea, GdnHCl, and GdnSCN, are presented in Fig. 7. The relative concentrations of eight scrambled PCI isoforms are shown here. These curves display two important features concerning the unfolding mechanism of PCI. 1) With low concentrations of denaturant, two species of scrambled PCI, X-PCI-f and X-PCI-h, were well populated and constitute about 28 and 26% of the total scrambled PCI, respectively. The predominance of X-PCI-f persisted along the unfolding pathway, but the concentration of X-PCI-h diminished precipitously as the strength of the denaturing condition increased. This phenomenon was observed with the three different denaturants. Under 8 mM GdnHCl and 6 mM GdnSCN, X-PCI-h accounted for only 4 and 5%, respectively, of the total scrambled PCI. 2) The concentration of X-PCI-a was proportional to the strength of the denaturing condition. This phenomenon was most evident with samples denatured with GdnHCl and GdnSCN. As the concent...
The mechanism of “all-or-none” disulfide reduction has been shown in numerous single domain proteins (11). The dramatic difference in stability between the native and non-native (scrambled) disulfide bonds has also been demonstrated in other proteins. Unlike the native disulfide bonds that are stabilized by noncovalent interactions, non-native disulfide bonds of scrambled proteins are considerably more fragile and can be readily reduced by 0.5–1 mM dithiothreitol within 5–10 min of incubation at room temperature. This phenomenon has been shown with scrambled isomers of RNase A (26), hirudin, and tick anticoagulant peptide (11).

**DISCUSSION**

The unfolding mechanism and conformational stability of PCI were investigated using the technique of disulfide scrambling between the native and non-native (scrambled) structures (13, 27). This method permits distinction between denaturation and unfolding and enables analysis of the extent of unfolding of the denatured protein (12, 15).

The conformational stability of PCI is represented by its denaturation curves. These curves were determined by the fraction of the native PCI converted to the scrambled species under increasing concentrations of denaturants. GdnHCl and GdnSCN were shown to denature PCI quantitatively. The denaturation curves exhibit a conventional two-stage mechanism in which the phase of rapid denaturation occurs at 1–2 M GdnHCl and 0.5–1 M GdnSCN, respectively. Urea is a much weaker denaturant. Less than 50% of the native PCI was denatured with 8 M urea, and at concentrations of 0–8 M urea, the denaturation curve was close to linear. The stability of PCI can be compared with a number of disulfide-containing proteins that were analyzed by the same method. These proteins include hirudin (28), insulin-like growth factor (15), tick anticoagulant peptide (12), and RNase A (26). Their stabilities are defined by the concentration of denaturants that are required to achieve 50% denaturation of the protein (Table II). Two crucial aspects of these data need to be emphasized. 1) The relative stability of these five proteins, as characterized by their ability to resist denaturation, is dependent upon the nature of denaturant. Based on the denaturation curves of GdnHCl and GdnSCN, PCI appears to possess the least stable structure because it requires the lowest concentration of GdnHCl (or GdnSCN) to reach 50% denaturation. However, the order of stability differs in the case of urea denaturation, which indicates that PCI is far more stable against this denaturant.
than insulin-like growth factor, tick anticoagulant peptide, and RNase A (Table II). 2) As a denaturant, GdnHCl is generally more potent than urea, although the relative potencies vary from protein to protein. For instance, GdnHCl is about 6- and 2-fold more potent than urea in denaturing PCI and RNase A, respectively. However, in the case of tick anticoagulant peptide, GdnHCl is actually less effective than urea. These discrepancies are intriguing and most likely attributed to the differential mode of action between GdnHCl and urea (1, 29–31), a long standing issue that is not yet fully understood.

The denatured PCI was found to comprise at least nine scrambled isomers (Fig. 2). (PCI may adopt 14 possible scrambled species.) The unfolding pathway of PCI was determined by the composition of its scrambled isomers generated under increasing concentrations of a denaturant. There are two distinct features of the PCI unfolding pathway. One is the accumulation of X-PCI-h as an intermediate under mild denaturing conditions. As the strength of the denaturing conditions increased and unfolding progressed, the yield of X-PCI-h decreased precipitously. It is likely that X-PCI-h was stabilized by noncovalent interactions, either native or non-native, which were sensitive to denaturant. In this respect, it is relevant to mention that X-PCI-h was cross-linked by Cys8–Cys27, Cys12–Cys18, and Cys24–Cys34, none of them native disulfide bonds. Another unique feature was the increased recovery of X-PCI-a (beads-form isomer). Along the pathway of unfolding, the yield of X-PCI-a as a fraction of the total scrambled PCI increased from 5 to 32% (Fig. 7). Among the 14 possible scrambled isomers of PCI, X-PCI-a contained the smallest disulfide loops and presumably was one of the most extensively unfolded isomers. PCI is not alone in displaying this property. The predominance of beads-form scrambled isomer under strong denaturing conditions is similarly observed with the unfolding behavior of tick anticoagulant peptide (12) and insulin-like growth factor (15). For example, in the presence of 6 M GdnSCN, more than 63% of

![Fig. 7. The unfolding curves (pathway) of PCI. The recoveries of eight scrambled isomers of the denatured PCI are plotted against increasing concentrations of three denaturants; urea, GdnHCl, and GdnSCN. X-PCI-a (△), X-PCI-b (○), X-PCI-c (●), X-PCI-d (▲), X-PCI-e (□), X-PCI-f (◁), X-PCI-g (○), and X-PCI-h (●). S.D. (not shown) is ±5%.

![Fig. 8. Reductive unfolding of native and scrambled PCI. Left, reductive unfolding of native PCI. The native PCI was treated with indicated concentrations of dithiothreitol (DTT) in Tris-HCl buffer (0.1 M, pH 8.4) at 23 °C for 20 min, quenched with an equal volume of 4% trifluoroacetic acid and analyzed by HPLC using the conditions described in the legend to Fig. 2. Reduction of the three native disulfide bonds of PCI undergoes an "all or none" mechanism to form the fully reduced PCI (R) with little accumulation of partially reduced species. N, native species. Right, reduction of denatured PCI with dithiothreitol. A mixture of native and scrambled PCI denatured by 8 M GdnHCl was treated with 1 mM dithiothreitol at 23 °C for 5 min. Reduced samples were quenched with 4% trifluoroacetic acid and analyzed by HPLC. Under these conditions, the native PCI remains intact, whereas scrambled isomers of PCI convert quantitatively to the fully reduced PCI.]

TABLE II

The concentration of denaturant required to denature 50% of the protein

This was determined by the method of disulfide scrambling between the native and scrambled isomers. The data has a standard deviation of ±5%.

| Proteins             | GdnSCN | GdnHCl | Urea |
|----------------------|--------|--------|------|
| PCI                  | 0.7    | 1.45   | >8   |
| RNase A              | 0.75   | 2.25   | 5.75 |
| Tick anticoagulant peptide | 1.0    | 4.2    | 4.0  |
| Insulin-like growth factor | 1.5    | 3.2    | 5.5  |
| Hirudin              | 2      | 5      | >8   |

* The current study.  
* Ref. 26.  
* Ref. 12.  
* Ref. 15.  
* A. Bulychev and J-Y. Chang, unpublished data.
the denatured tick anticoagulant peptide was found to be the beads-form isomer. In the case of insulin-like growth factor-I, the content of the beads-form isomer also rises from 5 to 30% as the concentration of GdnSCN increases from 1–6 M. These data taken together indicate a common pathway for the unfolding of small disulfide-containing proteins that encompasses a gradual expansion and relaxation of the polypeptide conformation toward the shape of linear structure.

This study also demonstrates that the sequence of PCI folds into multiple structures under physiological pH. This property was first observed during our previous folding experiments of the fully reduced PCI (23). The kinetics and efficiency of PCI folding can be accelerated by the presence of selected redox buffers that includes reduced/oxidized glutathione and protein-disulfide isomerase (32). However, the end products of PCI folding have always been found to comprise 4–5% of scrambled isomers that are unable to convert to the native conformation even under optimized folding conditions. Our data here confirm that these non-native structures indeed exist in a state of equilibrium with the native structure. There are similar examples. The insulin-like growth factor-I folds into two distinct equilibrium with the native structure. There are similar examples.

The oligomerization behavior or the proteolytic turnover of the prion exists thermodynamically in equilibrium with the benign proregions (37). The presence of these could play a role in facilitating the in vivo folding of the protein (38).

Whereas the mature form of PCI and insulin-like growth factor-I may represent unique cases, the phenomenon of "one-sequence multiple structures" is probably far more common that we thought. As a matter of fact, in the view of thermodynamic considerations, one can probably speculate that no sequence will fold mathematically 100% into one single structure (native). Whether or not non-native scrambled isomers are noticeable or detectable will depend on the relative concentration of the isomer with respect to the native (predominant) species and on the sensitivity of methods available for detection. For the majority of proteins that do fold into multiple structures, the non-native species may constitute less than 0.1% of the total protein under physiological conditions. Most chromatographic systems simply do not pick up fractions that are less than 0.1–0.5% of the predominant one unless the sample is heavily overloaded. This finding with the PCI study may have far reaching implications. For instance, one of the major models (39–41) explaining the infectivity of prion disease speculates that trace concentration of the scrapie form of prion exists thermodynamically in equilibrium with the benign cellular prion under physiological conditions. The aggregation of monomer leads to infectivity and the formation of amyloid. The oligomerization behavior or the proteolytic turnover of many proteins (with or without disulfides) could be affected by an equilibrium between multiple structures.

Acknowledgments—We thank Dr. C. Nick Pace for stimulating discussions and Dr. Salvador Ventura for providing 15N-labeled potato carboxypeptidase inhibitor.

REFERENCES
1. Pace, C. N. (1986) Methods Enzymol. 131, 266–280
2. Pace, C. N., Shirley, B. A., and Thomson, J. A. (1989) in Protein Structure (Creighton, T. E., ed) pp. 311–329, IRL Press, Oxford, United Kingdom.
3. Dill, K. A., and Shortle, D. (1991) Annu. Rev. Biochem. 60, 795–825
4. Neri, D., Billeter, M., Wider, G., and Wu¨trich, K. (1992) Science 257, 1559–1563
5. Pan, H., Barber, E., Barany, G., and Woodward, C. (1995) Biochemistry 34, 13974–13981
6. Alexandrescu, A. T., Abeygunawardana, C., and Shortle, D. (1995) Biochemistry 33, 1036–1072
7. Smith, L. J., Piebig, K. M., Schwalbe, H., and Dobson, C. M. (1996) Fold. Des. 1, 95–106
8. Shortle, D. (1993) Curr. Opin. Struct. Biol. 3, 66–74
9. Wu¨trich, K. (1994) Curr. Opin. Struct. Biol. 4, 93–99
10. Shortle, D. (1996) PASEB J. 19, 27–34
11. Chang, J-Y. (1997) J. Biol. Chem. 272, 69–75
12. Chang, J-Y. (1999) J. Biol. Chem. 274, 123–128
13. Chang, J-Y. (1985) J. Biol. Chem. 260, 25661–25666
14. Chang, J-Y., Kumar, T. K. S., and Yu, C. (1998) Biochemistry 37, 6745–6751
15. Chang, J-Y., Maerki, W., and Lai, P-H. (1999) Protein Sci. 8, 1463–1468
16. Clore, G. M., Gronenborn, A. M., Nilges, M., and Ryan, C. A. (1987) Biochemistry 26, 8012–8023
17. Rees, D. C., and Lipscomb, W. N. (1982) J. Mol. Biol. 160, 475–498
18. Molina, M. A., Aviles, F. X., and Querol, E. (1992) Gene (Amst.) 116, 129–138
19. Molina, M. A., Marfing, C., Oliva, B., Aviles, F. X., and Querol, E. (1994) J. Biol. Chem. 269, 21467–21472
20. Lin, S. L., and Nussinov, R. (1995) Nat. Struct. Biol. 2, 835–837
21. Blanco-Aparicio, C., Molina, M. A., Fernández-Salas, E., Frazier, M. L., Mas, J. M., Querol, E., Aviles, F. X., and de Llorens, R. (1998) J. Biol. Chem. 273, 12370–12377
22. Mas, J. M., Aloy, P., Martí-Renom, M. A., Oliva, B., Blanco-Aparicio, C., Molina, M. A., de Llorens, R., Querol, E., and Aviles, F. X. (1998) J. Mol. Biol. 284, 541–548
23. Chang, J-Y., Canals, F., Schindler, P., Querol, E., and Aviles, F. X. (1994) J. Biol. Chem. 269, 22087–22094
24. Chatrenet, B., and Chang, J-Y. (1993) J. Biol. Chem. 268, 20988–20996
25. Chang, J-Y. (1996) Biochemistry 35, 11792–11799
26. Chang, J-Y. (1999) Anal. Biochem. 268, 147–150
27. Jensen, E. V. (1959) Science 130, 1319–1323
28. Emsley, P., and Teller, P. (1990) J. Protein Chem. 18, 771–777
29. Tansford, C. (1998) Adv. Protein Chem. 23, 121–282
30. Pace, C. N., Laurents, D. V., and Thomson, J. A. (1990) Biochemistry 29, 2564–2572
31. Liepinsh, E., and Otting, G. (1994) J. Am. Chem. Soc. 116, 9670–9674
32. Chang, J-Y. (1994) Biochem. J. 300, 643–650
33. Raschdorf, F., Dahinden, R., Maerki, W., Richter, W. J., and Merryweather, J. P. (1988) Biomed. Mass Spectrom. 16, 3–8
34. Hober, S., Forsberg, G., Palm, G., Hartmanis, M., and Nilsson, B. (1992) Biochemistry 31, 1749–1756
35. Miller, J. A., Nair, L. A., Hua, X., Rosenfeld, R., Arakawa, T., Rohde, M., Prestrelski, S., Lauren, S., Stoney, K. S., Tesi, L., and Weiss, M. A. (1995) Biochemistry 34, 5203–5213
36. Meng, H., Burleigh, B. D., and Kelly, G. M. (1998) J. Chromatogr. 443, 183–192
37. Villanneuva, J., Canals, F., Prat, S., Ludevid, D., Querol, E., and Aviles, F. X. (1998) FEBS Lett. 440, 175–182
38. Shinde, U., and Insuey, M. (1993) Trends Biochem. Sci. 18, 25–29
39. Brown, P., Goldfarb, L. G., and Gajdusek, D. C. (1993) Lancet 337, 1019–1022
40. Jarrett, J. T., and Lansbury, P. T. (1993) Cell 73, 1055–1058
41. Aguzzi, A., and Brandner, S. (1999) Lancet 354, 22–25
The Unfolding Pathway and Conformational Stability of Potato Carboxypeptidase Inhibitor
Jui-Yoa Chang, Li Li, Francesc Canals and Francesc X. Aviles

J. Biol. Chem. 2000, 275:14205-14211.
doi: 10.1074/jbc.275.19.14205

Access the most updated version of this article at http://www.jbc.org/content/275/19/14205

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 10 of which can be accessed free at http://www.jbc.org/content/275/19/14205.full.html#ref-list-1