Size-exclusion High Performance Liquid Chromatography of Native Trypsinogen, the Denatured Protein, and Partially Refolded Molecules

FURTHER EVIDENCE THAT NON-NATIVE DISULFIDE BONDS ARE DOMINANT IN REFOLDING THE COMPLETELY REDUCED PROTEIN*

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Size-exclusion high performance liquid chromatography was used to compare the Stokes radius of the mixed disulfide of trypsinogen refolded for 10 min with the Stokes radius of denatured trypsinogen in high concentrations of urea. After folding for 10 min, rechromatography of a collection of sequential fractions of an initial separation showed that the fractions display microheterogeneity as seen in the value of the Stokes radius of each fraction. These intermediate species differed in their Stokes radius, and each had a globular structure cross-linked by disulfide bonds. In contrast, when trypsinogen with the native disulfides intact was equilibrated at different concentrations of urea (0–8 M), a progressive increase in Stokes radius was observed with extent of unfolding. Rechromatography of a series of fractions collected at a specific urea concentration showed that each had the same Stokes radius as the fraction in the initial separation. Urea-denatured trypsinogen and partially refolded trypsinogen must therefore differ in the disulfide pairing that links regions of the polypeptide chain. These observations support the suggestion that non-native disulfide bonds are responsible for the many stable conformations that form early in the folding of the mixed disulfide of trypsinogen (Light, A., and Higaki, J. N. (1987) Biochemistry 26, 5556–5564). These intermediates initially are loose structures (large Stokes radius) that become more compact with time (decreasing Stokes radius). The intermediates must therefore undergo a continuing disulfide interchange until native disulfides form late in the process when the stable conformation of the native molecule is reached.

Folding studies of disulfide-containing proteins were pioneered by Anfinsen and his co-workers when they successfully refolded fully reduced and denatured bovine ribonuclease and regenerated the properties of the native protein (White, 1966; Anfinsen and Haber, 1961; Anfinsen, 1973). In addition to ribonuclease, examples of other proteins that were successfully refolded are: egg-white lysozyme (Anderson and Wetlauber, 1976), bovine pancreatic trypsin inhibitor (Creighton, 1978), bovine trypsinogen (O dorzynski and Light, 1979; O dorzynski, 1978), bovine chymotrypsinogen (Moore, 1981, Duda and Light, 1982), bovine serum albumin (Teale and Benjamin, 1976, a and b), and immunoglobulin molecules (Goto and Hamaguchi, 1982). The stabilizing effect of disulfide bonds on intermediate structures and the identification of native disulfides in regenerated molecules are two major advantages gained in the selection of disulfide-containing proteins.

In attempting to refold bovine trypsinogen and chymotrypsinogen by the Anfinsen procedure, we found that the fully reduced proteins rapidly aggregate. We successfully refolded the proteins as the mixed disulfide derivative of the protein and glutathione (O dorzynski and Light, 1979, Light, 1985). Recently, we reported that size-exclusion HPLC may be used to detect stable intermediates as a function of the refolding time (Light and Higaki, 1987; Light et al., 1987). The separation was based on differences in the hydrodynamic volumes (Stokes radius) of the unfolded and refolded molecules. The mixed-disulfide derivative of the unfolded protein occupies a large hydrodynamic volume; the native molecule is compact and has a much smaller hydrodynamic volume. Partly folded molecules are present with intermediate hydrodynamic volumes (Light and Higaki, 1987). These structures are stabilized by disulfide bonds and do not change in hydrodynamic volume because disulfide interchange is inhibited. We concluded that the mechanism of folding initially follows a multiple pathway process (Harrison and Durbin, 1985; Ptitsyn, 1987) rather than an ordered pathway (Levinthal, 1968).

We continue to investigate the size-exclusion HPLC of intermediate species and now present additional evidence showing that globular intermediates are stabilized by non-native disulfide bonds. Although we suspect that native disulfide pairing occurs late in the folding process, the direct identification of disulfide pairing by peptide mapping is extremely difficult with trypsinogen which contains six disulfides. We therefore used SE-HPLC to examine partly folded molecules and partly denatured molecules, and have compared the hydrodynamic volumes of fractions of urea-denatured trypsinogen containing only native disulfides with early folded samples of trypsinogen.

EXPERIMENTAL PROCEDURES

Materials—Bovine trypsinogen, trypsinogen-(S-S), (1X crystallized) was purchased from Worthington. Iodoacetic acid, guanidine hydrochloride, diithioerythritol, glutathione, and urea were obtained from Sigma. Glutathione disulfide was prepared by oxidizing glutathione to the disulfide form.

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1 The abbreviations used are: HPLC, high performance liquid chromatography; trypsinogen-(S-S), native trypsinogen with intact disulfides; trypsinogen-(S-G), trypsinogen-glutathione mixed-disulfide derivative; Rₙ, Stokes radius in Angstroms; SE-HPLC, size-exclusion HPLC.
Ribbons (Light and Higaki, 1987). Other biochemicals were of the highest purity available. The Toya Soda TSK G-2000 SW column and GSWP guard column were from Varian.

Denaturation of Trypsinogen—Trypsinogen-(S-S) (1–2 mg/ml) was dissolved in 0.2 M Tris, pH 8.6, containing 0.2 M NaCl at different concentrations of urea, and equilibrated 24 h at room temperature before analysis by SE-HPLC. Although trypsinogen is rapidly unfolded in urea (Harris, 1956), we chose a 24-h equilibration to equal the time used with refolded samples. Since refolded samples may contain intermediate species that would be difficult to dissolve, 24 h increased the probability that all intermediates were soluble. The elution times of trypsinogen at all concentrations of urea used in these studies however were the same after 24 h as after 8 min.

The fraction of the denatured molecule (FD) was calculated from the ratio (YN − YD)/(YN − Y0), where YN and YD are the Stokes radius for the native and denatured state of the protein, and Y0 is the apparent molecular weight at intermediate concentrations of urea (Tanford, 1968). The denatured state for these calculations was assumed to be 8 M urea, and higher concentrations were not used. The Stokes radius was estimated from the behavior of the molecules on SE-HPLC (see below).

Refolding of Trypsinogen-(S-SG)—Trypsinogen-(S-SG) was obtained in the procedure of Light and Higaki (1987), and refolding was as described by Odonzynski and Light (1979). The final protein concentration was 20 to 25 μg/ml and the guanidine HCl was 0.01 M or lower. The iodoacetate-treated sample was dialyzed exhaustively at 4°C against 1 mm HCl for 7 days and then lyophilized. The removal of glutathione disulfide required the extensive dialysis conditions. The sample was then dissolved in 2 ml of 0.2 M ammonium formate, pH 4.0, containing 2 M urea, and left to equilibrate at least 24 h at room temperature before use. Cystine was removed by filtration through a 0.65-μm Millipore filter.

SE-HPLC—A Varian Model 5020 HPLC unit was used with a Gilson Model HM Holochrome detector at 280 nm. The elution times were normalized for a flow rate of 1 ml/min and for two TSK G-2000 SW columns connected in series.

Trypsinogen and protein standards (Light and Higaki, 1987) were separated on a single TSK G2000 SW column (0.75 x 7.5 cm) protected by a GSWP guard column at 290 nm. The elution times were normalized for a flow rate of 1 ml/min and for two TSK G-2000 SW columns in series. The buffer was 0.2 M NaCl, pH 8.6. The flow rate was 1 ml/min, and two TSK G-2000 SW columns were used in series. Two samples of trypsinogen in 4.0 M urea gave different elution times and Stokes radii. Fig. 2 shows that one point is below the transition zone and the other above.

RESULTS

SE-HPLC of Native Trypsinogen Denatured in Urea—The profiles of native trypsinogen in increasing concentrations of urea are shown in Fig. 1. A single symmetrical peak was observed at the different concentrations of urea. As the urea concentration increased, the elution time decreased from 31.2 min for the native protein until it reached the value of 23.6 min at 6 and 8 M urea (Table I). The parental peaks of trypsinogen in 2, 4, 6, and 8 M urea were collected as a set of sequential fractions. Each fraction was chromatographed under the same conditions as in the initial separation. The rechromatographed fractions had the same elution times within experimental error as their parental peaks. At each concentration of urea, the molecules expanded to a specific hydrodynamic volume and fractions of the peak were identical with one another.

The increase in Stokes radius with increasing concentrations of urea was used to calculate the fraction of molecules in the denatured state, FD (Tanford, 1968). Fig. 2 shows that

\[ \text{SE-HPLC provided an acceptable measure of the transition of native trypsinogen to denatured molecules. Indeed, the point of equal amounts of native and denatured molecules was at 4.0 M urea, in good agreement with the known stability of trypsin (Harris, 1966). The figure also shows that the transition is highly cooperative and agrees with a two-state transition (Lumry et al., 1966). Intermediate species could not be detected (Fig. 1), and duplicate separations at 4 M urea showed that one sample had a Stokes radius of 25.5 Å ("native" molecules) and the second a Stokes radius of 41.4 Å ("denatured" molecules) (Fig. 2). The unfolding process is limited in the conformations possible because of the presence of six native disulfide bonds.} \]

SE-HPLC of Refolded Trypsinogen-(S-SG)—A sample of trypsinogen-(S-SG) folded for 10 min was separated into a set of sequential fractions. On rechromatography of the frac-
Understanding the mechanism of protein folding is still one of the outstanding problems in molecular biology. A major difficulty in understanding how proteins fold is to identify intermediate species that form as the polypeptide changes from an unfolded molecule to that of a stable globular structure. In the urea or guanidine-induced unfolding of sperm whale myoglobin (Corbett and Roche, 1984), bovine growth hormone (Brems et al., 1985), bovine trypsinogen (Higaki, 1986; Light and Higaki, 1987), and bacterial thioredoxin (Shlongo et al., 1987), SE-HPLC showed that denatured molecules and partly folded structures elute early compared to the elution time found for native proteins.

SE-HPLC follows the change in protein conformation during the folding process by measuring the hydrodynamic volume of individual components (Corbett and Roche, 1984). Previous studies (Light and Higaki, 1987) on the folding of trypsinogen-(S-SG) identified intermediate species that are stabilized by intrachain disulfide bonds. Since SE-HPLC separates molecules on the basis of size and shape, the procedure is ideal for a comparison of the Stokes radius of partially refolded samples of trypsinogen-(S-SG) with those of denatured trypsinogen with intact disulfide bonds.

Native trypsinogen dissolved in different concentrations of urea has a Stokes radius varying from 19.6 Å (native) to 40.9 Å (unfolded) (Table 1). The initial change in the Stokes radius from 19.6 to 24.4 Å represents only a small expansion of trypsinogen. Presumably, the structures are compact and resemble the conformation of the native protein. At higher urea concentrations (greater than 4 M), the value of 40.9 Å is that of the unfolded molecule. Rechromatography of fractions collected during the initial separation showed the same elution time for each fraction, and these were identical with the elution time of the parental peak (data not shown). Trypsinogen at different urea concentrations (intact disulfide bonds) behaves as if it has a homogeneous conformation (Stokes radius). Therefore, urea denaturation produces one dominant conformation at a specific urea concentration. Furthermore, the denaturation is highly cooperative and a two-state transition (Fig. 2). This behavior is typical of a process in which intermediate species cannot be detected (Shortle, 1987).

In contrast, a sample of trypsinogen-(S-SG) after a 10-min folding contained more than one species and behaved as a microheterogeneous sample of differing conformations (Fig. 3). The intermediates varied between 38.2 Å and 21.6 Å. These intermediates decreased in amount with increases in the time of refolding (Light and Higaki, 1987). The species that were separated chromatographically at early time periods were precursors of the molecules that accumulated later in
the folding process (Light et al., 1987). The degree of micro-
heterogeneity of the folded sample decreased as the folding
time increased.

The intermediates in the refolded samples differed from
those found after denaturation of trypsinogen with intact
disulfide bonds. Since the intermediates had been trapped at
a given time in the folding process by inhibiting further
disulfide interchange, the observed hydrodynamic volumes
suggested that the large number of intermediate species in
the refolding of trypsinogen-(S-SG) represent conformations
that differ from one another because of the large number of
possible disulfide pairings. Since the protein has six disulfides,
the only way such a large number of conformations could be
produced is if the intermediates have non-native disulfide
bonds.

Experimental as well as theoretical arguments have been
presented for an ordered pathway of protein folding (Anfinsen
and Scheraga, 1975; Creighton, 1978; Levinthal, 1968; Wet-
laufer, 1973). The large number of intermediate species de-
tected in the folding of trypsinogen argues against an ordered
pathway and for multiple pathways in the initial folding
process (Harrison and Durbin (1985), Ptitsyn (1987), Dill
(1987), Shortle (1987)). This suggestion is in agreement with
the finding by Light and Odorzynski (1979) and Odorzynski
and Light (1979) that if one or two disulfides are selectively
reduced and carboxymethylated, alternative pathways have
to be followed to regain native structure: one or two modified
disulfides no longer stabilize intermediate structures and a
different pathway is used to obtain the same native confor-
mation.

In conclusion, comparing the refolding of a completely
reduced sample and the unfolding of the native molecule
shows that the pathway of urea-induced unfolding of trypsin-
ogen does not involve all the intermediate species found in
the refolding of trypsinogen-(S-SG). Moreover, SE-HPLC
makes it possible to obtain a clearer picture and a more
detailed characterization of partially refolded intermediates.

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