Refolding of the Mixed Disulfide of Bovine Trypsinogen and Glutathione*

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The mixed disulfide of bovine trypsinogen and glutathione refolded with high yields at protein concentrations of 20 μg/ml or less, at 4-25°C, pH 8.0 to 8.7, in the presence of 3 to 6 mM cysteine under anaerobic conditions. The regenerated protein behaved as native trypsinogen as judged by gel exclusion chromatography, isoelectric focusing, and activation with bovine enterokinase or trypsin. However, refolded samples that were quenched with iodoacetate and analyzed by disc gel electrophoresis formed two components corresponding to trypsinogen and S-(carboxymethylcysteine)2-(179-203)-trypsinogen. The use of cysteine as a disulfide interchange catalyst caused reduction of the methylated derivative. The overall yield of the regenerated product was 70% and the half-time at 4°C was 55 min.

Recent studies of the mechanism of protein biosynthesis have clarified the individual steps leading from amino acids to the completed polypeptide chain (2-5). The final steps when the completed polypeptide chain leaves the ribosome complex and folds to a globular structure are still poorly understood. The pioneering studies of Anfinsen and co-workers (6-11) with ribonuclease, lysozyme, and other disulfide-containing proteins showed that the amino acid sequence alone dictated the three-dimensional structure. The refolding of disulfide-containing proteins produced a unique pairing of half-cystine residues and the order of disulfide pairing provided valuable information on the folding pathway (11). Trypsinogen is homologous with other pancreatic serine proteinases and, therefore, knowledge gained from the refolding of trypsinogen should be applicable to other members of this group.

Anfinsen (6) failed because the denatured and fully reduced protein had a strong tendency to aggregate (15). Initially, we bound trypsinogen to agarose and found that the immobilized protein refolded with high yields since intermolecular aggregation was prevented (15). Next, we modified trypsinogen with citraconic anhydride (2-methyl maleic anhydride). Fully reduced citraconylated trypsinogen remained soluble and refolded with a yield of 60%, but only at low protein concentrations (16). Apparently the change in charge of trypsinogen increased solubility, but the low protein concentrations that were used created technical difficulties. A third method was tried that took advantage of an observation of Bradshaw et al. (17) that a mixed disulfide derivative of reduced lysozyme and cysteine refolded in the presence of a mercaptan. We used a mixed disulfide of trypsinogen and glutathione (trypsinogen-S-SG1) in our studies because it introduced multiple charges at the 12 half-cystine residues. The refolding of this derivative of trypsinogen is the subject of this report.

RESULTS

Preparation and Properties of Trypsinogen-S-SG—Fully reduced trypsinogen formed a mixed disulfide when treated with GSSG at pH 8.7 to 9.3. Performic acid oxidation and amino acid analysis showed that 16.8 to 12.4 glutathione moieties were introduced based on the cysteic acid, glutamic acid, and glycine values. All other residues were found in theoretical amounts. Free sulfhydryl groups were absent as determined by the method of Ellman (26).

A mixture of trypsinogen and trypsinogen-S-SG was separated on a column of Sephadex G-100 SF (Fig. 1A). The mixed disulfide derivative had the larger hydrodynamic volume, corresponding to a molecular weight of 46,000. Only monomeric species were present since SDS-gel electrophoresis gave a single band with mobility equivalent to a molecular weight of 27,000. Clearly trypsinogen-S-SG behaved as an unfolded structure on gel chromatography.

Isoelectric focusing of trypsinogen and trypsinogen-S-SG showed that they possessed isoelectric points of 9.3 and 5.4, respectively. The lower isoelectric point of the mixed disulfide derivative resulted from the introduction of 12 negative charges from the residues of glutathione. The value for the

1 The abbreviations used are: trypsinogen-S-SG, trypsinogen-glutathione mixed disulfide; PTT, basic pancreatic trypsin inhibitor (Kunitz); (Cm)2-trypsinogen, S-(carboxymethylcysteine)2-(179-203)-trypsinogen; (Cm)2-trypsin, S-(carboxymethylcysteine)2-(179-203)-trypsin; SDS, sodium dodecyl sulfate; Tos-Arg-OMe, N-tosyl-L-arginine methyl ester.

2 Portions of this paper (including "Experimental Procedures," Figs. 1 to 4, and Tables I to IV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 850 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78M-1584, cite author(s), and include a check or money order for $1.65 per set of photocopies.
Refolding of the Trypsinogen-Glutathione Mixed Disulfide—Optimal conditions for the refolding of the mixed disulfide were determined from variations of protein and mercapto- 

tan concentration, pH, and temperature. Cysteine at concentrations of 3 to 6 mM resulted in the highest yield (Table I). Mercaptoethanol was less effective and dithioerythritol was 

the poorest catalyst. GSH gave lower yields than cysteine by about 10%. A mixture of GSH (3 mM) and GSSG (0.3 mM), as used by Saxena and Welauffer (27) in the refolding of reduced 

lysozyme, did not improve the yield over that of GSH alone. In all cases, 70% of the mercaptan remained in the reduced state after 18 h with the refolding conditions used, based on 

analyses with the Ellman reagent. 

The effect of pH on the refolding of trypsinogen-S-SG is shown in Table II. High yields were observed in a pH range of 8.0 to 8.9 with a maximum at 8.5 (28). The yield of refolding as a function of temperature is given in Table III. The maximum yield was obtained at 4°C and 22°C. Table IV gives the yield at 4°C as the protein concentration varied from 0.002 to 0.20 mg/ml. The protein remained soluble after 18 h; however, on activation at 39°C, precipitation occurred at protein concentrations greater than 0.02 mg/ml. The highest yield was found if the protein concentration was 0.02 mg/ml or less. 

Trypsinogen-S-SG was used within 2 weeks from the time that it was prepared since the refolding yield decreased on aging even when the sample was stored at -20°C in a desiccator. 

The refolding of trypsinogen-S-SG at 4°C and 22°C is presented as progress curves in Fig. 2. Since the rate of refolding was slower at 4°C, the values, particularly at early times, were easily obtained and with high precision. Accordingly, the data at 4°C were examined to see if refolding followed first order kinetics. The inset in Fig. 2 shows an excellent fit of the data with first order behavior. Calculations of $k_{1/2}$ and $t_{1/2}$ were made using a least squares analysis and a computer fit of the data to a theoretical curve. A $t_{1/2}$ of 55 min was found. Refolding was followed for better than 20 half-lives in order to obtain a reliable infinite time value. 

In Fig. 2, the dashed curve shows the rapid loss of GSH from trypsinogen-S-SG as a function of refolding time. The time for a 50% decrease at 4°C was approximately 2 min. With the loss of the mixed disulfide, protein disulfides formed rapidly. This exchange occurred before any appreciable degree of correct refolding of the protein was observed, since the level of labile cysteine was low at 2 min (7). 

Properties of the Refolded Protein—Several methods were used to show that the refolded product had the same properties as native trypsinogen. The refolded material could be activated with trypsin or enterokinase with an activation rate which was identical with the activation rate of native trypsinogen. The limited proteolysis observed in the activation process served as a probe of structure since the site of cleavage was dependent on the conformation of the protein (29). 

Isoelectric focusing of the refolded material gave an isoelectric point of 9.3, which was the same as that found for trypsinogen. Gel exclusion chromatography was performed on a sample which was refolded for 18 h and quenched with iodoacetic acid (Fig 1B). The percentage of each component was determined with a DuPont model 310 Curve Resolver (dashed lines). The major component, which corresponded to trypsinogen (Fig. 1A), accounted for 79% of the total protein. The small peak at 43 ml, which corresponded to the mixed disulfide (Fig 1A) was 6% of the total, and another peak at 49 ml between the two known components comprised 15% of the total. The percentage of refolded trypsinogen, namely, 79%, did not agree with the yield of 40% found from activity measurements; the nature of this discrepancy is discussed below. 

Disc gel electrophoresis was carried out on the above sample (Fig. 3, Slot C). The refolded sample gave two main bands, which were nearly equal in intensity, and also weaker, more diffuse bands. The major bands corresponded closely in their mobility to trypsinogen (Fig. 3, Slot A) and (Cm)$^2$-trypsinogen (Fig. 3, Slot B). Quenching with iodoacetamide (gel not shown) resulted in only one major band with the mobility of trypsinogen and the weaker diffuse bands also observed in Fig. 3, Slot C. 

Refolded trypsinogen-S-SG was activated with enterokinase and submitted to affinity chromatography on PTI-Sepharose (Fig. 4C). Both (Cm)$^2$-trypsin (Fig. 4A) at 15 ml and trypsin (Fig. 4B) at 43 ml were present in the refolded sample and the sum of the two corresponded to 70% of the total protein applied to the column. The component corresponding in volume to (Cm)$^2$-trypsin had one-tenth the specific enzymatic activity toward Tos-Arg-OMe as trypsin, which agreed with previous results for (Cm)$^2$-trypsin toward synthetic trypsin substrates (18). The first component on amino acid analysis contained 1.7 carboxymethylcysteine residues/molecule of trypsinogen while the second component lacked carboxymethylcysteine. The refolding of trypsinogen-S-SG formed native trypsinogen and (SH)$^2$-(179,203)-trypsinogen, which was then converted to (Cm)$^2$-trypsinogen when iodoacetate was added. The origin of (SH)$^2$-trypsin was established by reacting native trypsinogen at a concentration of 0.02 mg/ml with 3 mM cysteine for 18 h at 4°C under anaerobic conditions. The mixture was treated with iodoacetic acid, activated with enterokinase, and subjected to affinity chromatography on PTI-Sepharose. The results were identical with those found earlier (Fig. 4C). Therefore, (SH)$^2$-trypsinogen was formed from the refolded trypsinogen as a result of the reducing medium used for the refolding. Disulfide 179 to 203 of trypsinogen is known to be the only easily reducible disulfide of the native molecule and the high ratio of mercaptoan to protein produced a selective reduction (30). 

If refolded trypsinogen was made mercaptan-free by dialysis at pH 3, the (SH)$^2$-trypsinogen was converted to native trypsinogen on air oxidation for 2 h at pH 8.1, at room temperature. The activated sample was separated on PTI-Sepharose and the elution profile was identical with that shown in Fig. 4B. (Cm)$^2$-trypsin was absent while trypsin was the only active component present, corresponding to 65% of the total protein. 

The refolding conditions produced an equilibrium mixture of trypsinogen and (SH)$^2$-trypsinogen. The quenching of the mixture with iodoacetic acid trapped the (SH)$^2$-trypsinogen as (Cm)$^2$-trypsinogen. Therefore, only a 40% yield of active molecules was found when the calculations were based on activity measurements toward Tos-Arg-OMe. Since (Cm)$^2$-trypsin has a specific activity only 10% that of trypsin, the presence of (Cm)$^2$-trypsin in a 30% yield contributed no more than 3% to the final activity. Therefore, the refolding yield calculated from activity measurements must be corrected to 70% which is very close to the yield estimated from gel chromatography. 

DISCUSSION

As a substrate for the study of the refolding process, the mixed disulfide of trypsinogen and GSH has a number of desirable features. The derivative is readily prepared from the reaction of fully reduced trypsinogen and GSSG in 8 M urea. The content of GSH is easily determined from amino acid analysis of a performic acid-oxidized sample. Refolding of the soluble protein begins on adding a mercaptan which serves as
a disulfide interchange catalyst; refolding is stopped on the addition of iodoacetate, iodoacetamide, or other alkylating agents, or simply by lowering the pH to 3. Higher protein concentrations of the mixed disulfide can be used in refolding compared with citraconylated trypsinogen. The soluble regenerated trypsinogen can be activated rapidly to trypsin and further characterizations can be made of enzymatic properties. Finally, the use of a soluble protein makes it relatively simple to characterize the refolded product by physical probes while a Sepharose-bound protein was difficult to study with spectral and hydrodynamic methods (15).

The half-time of refolding trypsinogen-S-SG at 4°C was 55 min. The rate is equal or faster than other proteins which were refolded with similar conditions. Lysozyme as a mixed disulfide of cysteine (17) or as a fully reduced protein in the presence of the GSH/GSSG mixture (27) refolded with a half-time of approximately 5 to 7 min at 35°C. If we calculate the rate of refolding of trypsinogen-S-SG at 35°C from our data at 4°C, using a doubling of the rate for a 10°C increase in temperature, we find a similar half-time as lysozyme. Trypsinogen-S-SG refolded faster than bovine serum albumin (the 17 disulfides are the same per weight of protein as trypsinogen). At 24°C, serum albumin had a half-time of 60 min (31) which is the same as trypsinogen-S-SG at 4°C. The half-time of the refolding of fully reduced ribonuclease A at 25°C is 99 min in the presence of GSH/GSSG (32). The same half-time was found when the mixed disulfide of ribonuclease and GSH was quenched by lowering the pH to 3 with 2 M HCl. Ribonuclease activity was completely lost by this treatment (18). Recently, reduced chymotrypsinogen had been refolded from a Sepharose-bound protein was difficult to study with spectral agents or simply by lowering the pH to 3. Higher protein concentrations of the mixed disulfide can be used in refolding compared with citraconylated trypsinogen. The soluble regenerated trypsinogen can be activated rapidly to trypsin and further characterizations can be made of enzymatic properties. Finally, the use of a soluble protein makes it relatively simple to characterize the refolded product by physical probes while a Sepharose-bound protein was difficult to study with spectral and hydrodynamic methods (15).

The early formation of protein disulfides produced few native molecules since the yield of refoldable trypsinogen was very low (Fig. 2). Therefore, the process of folding continued with changes occurring in the conformation of the globular structure at rates limited by disulfide interchange and polypeptide isomerization (36). Finally, the structure of the native molecule was produced, disulfide bonds no longer interchanged, and further conformational changes did not take place.

Bradshaw et al. (17) suggested that a mixed disulfide protected half-cystine residues during refolding of the polypeptide chain in vivo. The mixed disulfide of trypsinogen and GSH, but not of the protein and cysteine, eliminated aggregation and allowed the molecule to refold rapidly. The intracellular concentration of GSH is approximately 10 mM (37).

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REFOLDING OF BOVINE TRYPsinogen

EXPERIMENTAL PROCEDURES

Materials

Trypsinogen (7.5 mg/ml) and trypsin (25 mM) were obtained from Worthington Biochemical Corp. (free-trypsinogen was prepared as described previously.) Bovine pancreatic trypsin was obtained from Calbiochem-Behring Corp. Trypsinogen was isolated by precipitation of trypsinogen in diethyl ether as described previously. Trypsin was isolated by precipitation of trypsin in diethyl ether. E. coli trypsin was obtained from Sigma Chemical Co. No. T-10. 

Reagents and chemicals were obtained from the following sources: sigmoid acids, sodium hydroxide, hydrochloric acid, and malonic acid from Sigma Chemical Co., St. Louis, Mo. 

Methods

Preparation of a Mixed Disulfide Derivative

Trypsinogen was prepared from trypsinogen (75 mg/ml) in 25 mM sodium acetate buffer at pH 5.5, by treatment with 2.5 mM disulfide and 2.5 mM dithiothreitol, followed by precipitation by acetone. The precipitate was dissolved in 25 mM sodium acetate buffer at pH 5.5, and the disulfide form was isolated by precipitation with acetone and dialyzed against 25 mM sodium acetate buffer at pH 5.5. 

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Preparation of a Mixed Disulfide Derivative

Trypsinogen was prepared from trypsinogen (75 mg/ml) in 25 mM sodium acetate buffer at pH 5.5, by treatment with 2.5 mM disulfide and 2.5 mM dithiothreitol, followed by precipitation by acetone. The precipitate was dissolved in 25 mM sodium acetate buffer at pH 5.5, and the disulfide form was isolated by precipitation with acetone and dialyzed against 25 mM sodium acetate buffer at pH 5.5. 

Effect of Refolding on the Refolding of Trypsinogen

The refolding of trypsinogen was studied by the method of refolding.
Refolding of Bovine Trypsinogen

Figure 1 A) Gel exclusion chromatography of trypsinogen and trypsinogen-S-56. Trypsinogen (2 mg) and trypsinogen-S-56 (2 mg) were dissolved in 400 μl of 7 M urea, pH 3.5. Chromatography was in a column (0.9 × 160 cm) of Sephadex G-100 Super Fine equilibrated and eluted with 0.1 M acetic acid at 1.5 ml/hr at room temperature. B) Gel exclusion chromatography of trypsinogen-S-56 refolded for 18 h. Refolding conditions: 10 μg/ml of trypsinogen-S-56, 3 mM cysteine, 0.05 M triis, 0.05 M CaCl₂, pH 6.1. The reaction was quenched with iodoacetic acid. The refolded sample (4 mg) was chromatographed as in A. The solid line represents the absorbance at 280 nm. The dashed lines represent the components of the elution profile determined by curve resolver.

Figure 2 - Rate of refolding of trypsinogen-S-56 at 4°C (O) and 22°C (●). Refolding conditions: 10 μg/ml of trypsinogen-S-56, 3 mM cysteine, 0.05 M triis, 0.05 M CaCl₂, pH 6.1, at 4°C. Samples were quenched with iodoacetic acid, diluted, activated with enterokinase and the trypsin activity measured toward Tos-Arg-MCA. Inset is the first-order plot of refolding at 4°C. Dashed line is the loss at 4°C of GSH from trypsinogen-S-56. The GSH content was estimated from amino acid analyses of performic acid oxidized samples.

Figure 3 - Disc gel electrophoresis of A) trypsinogen, B) [His]₆-trypsinogen, C) trypsinogen-S-56 refolded for 18 h at 33 μg/ml, 4°C, pH 8.1, in the presence of 3 M guanidine, and quenched with iodoacetic acid. D) Activated refolded trypsinogen-S-56 obtained from an elution at pH 3.0 from PTH-Sepharose. E) Activated refolded trypsinogen-S-56 obtained from an elution at pH 3.0 from PTH-Sepharose.

Figure 4 - Affinity chromatography on PTH-Sepharose of A) [His]₆-trypsin, B) trypsin, and C) trypsinogen-S-56 refolded for 18 h at pH 8.1, 4°C, in the presence of 3 M guanidine, and quenched with iodoacetic acid. The dashed lines represent specific activity (μmol/min/mg) toward Tos-Arg-MCA.
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