Nonreversibility in the Reductive Denaturation of Chymotrypsinogen A[1] (36694)

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The hypothesis that achievement of tertiary structure and concomitant appearance of biological activity of proteins is determined by the inherent thermodynamics of the linear array of amino acids comprising their polypeptide chains has been supported by a number of investigations (1–9). However, various difficulties have occasionally been reported regarding the reoxidation of trypsin (3, 4) and more recently, pepsin and pepsinogen (7, 8), following their denaturation and reduction of disulfide bonds. The tendency of denatured, reduced trypsin to rapidly precipitate at neutral or alkaline pH led to the conclusion that polymerization of the protein via intermolecular disulfide bonds had occurred (4). In the case of pepsin, no recovery of enzymatic activity could be obtained by reoxidation of fully reduced enzyme or zymogen, and extensive dimerization of the proteins occurred (7).

Both the pancreatic enzyme chymotrypsin and the pancreatic hormone insulin exist as multichain proteins in their biologically functional states. Reduced insulin chains do not combine appreciably under conditions which are optimal for reoxidation of sulfhydryl groups to disulfide bonds (10). Denatured α-chymotrypsin, upon dilution into a reducing medium in the presence of a disulfide-exchange enzyme, rapidly loses all enzymatic activity (11), which implies its conversion to an altered molecular form. In each of these instances, once reduction of certain disulfide bridges occurs, complete separation of polypeptide chains comprising the active protein molecules can occur.

Since both these proteins are products of single-chain precursors (chymotrypsinogen and proinsulin) and thus have already lost portions of their original structures as synthesized by their respective ribosomal systems, it is possible that they have lost the requisite thermodynamic "information" to reconstitute biologically active three-dimensional structures on reoxidation. The fact that proinsulin can be reoxidized "properly" supports this hypothesis (10).

To further examine the validity of this hypothesis, an investigation of the reoxidation of completely reduced, denatured chymotrypsinogen A was made.

Materials and Methods. Bovine chymotrypsinogen A, six times crystallized, was purchased from Pentex Corp. Twice crystallized bovine trypsin and chicken egg-white ovalbumin were obtained from Worthington Biochemical Corp. Dextran, bovine serum albumin, *N*-benzoyl-L-tyrosine ethyl ester, *N*-acetyl-L-tyrosine ethyl ester, and N-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester were obtained from Sigma Chemical Co.; *p*-chloromercuribenzoate from Nutritional Biochemicals Co.; and dehydroascorbic acid from Mann Research Laboratories. β-Mercaptoethanol was purchased from Matheson, Coleman and Bell. The mixed-bed ion-exchange resin, Amberlite MB-3, was obtained from Mallinckrodt Chemicals; Sephadex G-25 and G-75 (fine) from Pharmacia; triethylaminoethyl-cellulose (Cellex-T) from Bio-Rad; and carboxymethyl cellulose from Schleicher and Schuell. Reagent grade urea was ob-

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tained from the J. T. Baker Company and was further purified by passage through Amberlite MB-3 and by recrystallization from ethanol.

Denaturation and Reduction. Following the general procedure of Anfinsen and Haber (1), 20 mg of chyrotrypsinogen A were dissolved in a solution 8 *M* in urea; and the pH was adjusted to 8.6 with 5% (v/v) methyl amine. To this was added β-mercaptoethanol to a final concentration of 0.1 *M*. The container was then thoroughly flushed with nitrogen, stoppered, and allowed to stand at room temperature for 4.5 hr. After this reduction period, the pH was adjusted to 3.0 with acetic acid; and the entire solution was passed through a 2.5 × 30-cm column of G-25 Sephadex equilibrated and eluted with 0.1 *M* acetic acid. Thus freed from urea and mercaptoethanol, the reduced protein was stored under nitrogen in the cold (0–5°). The degree of reduction was ascertained by titration of the protein's sulfhydryl groups with PCMB[2] by the method of Boyer (12). Lower concentrations of mercaptoethanol in the reductive denaturation step did not yield full reduction of all five disulfide bridges in chymotrypsinogen.

Oxidation of the reduced zymogen. Solutions for the air-reoxidation of the reduced zymogen were prepared at protein concentrations ranging from 0.01 to 0.1 mg/ml based on an extinction determined in this laboratory for the denatured, reduced protein of 20.7 at 282 nm and 1% (w/v) concentration. In most experiments, an aliquot of the reduced zymogen in 0.1 *M* acetic acid was diluted into a Nalgene, glass, or ovalbumin-coated glass beaker which contained only 0.1 *M* acetic acid, or a 0.1 *M* acetic acid solution of ovalbumin (1 mg/ml), bovine serum albumin (1 mg/ml), dextran (1–2 mg/ml), or glycerol (up to 30%, v/v). In some cases, EDTA, dehydroascorbic acid, and β-mercaptoethanol at 10^{-3} *M* and calcium chloride at 0.1 *M* (separately and in combination) were present in the reoxidizing solutions.

Other solutions were similarly prepared, with the exception that aliquots of denatured, reduced zymogen still in the presence of urea and mercaptoethanol were diluted directly without prior gel filtration.

In all cases, the pH was then adjusted with sodium hydroxide to 5.0 or to 7.5–9.8. (The reason for pH adjustment in this manner was to avoid the rapid precipitation which occurred when the reduced material was diluted using any buffer system tested of pH > 5.0.) Adjustment of pH as described resulted in either a longer period of solubility, or in the presence of ovalbumin or at pH 5.0, complete solubility throughout the air-reoxidation period. The solutions were allowed to stand at room temperature (both 37° and 0° were found inhibitory) for 20–22 hr, and up to 7 days when at pH 5.0.

In some cases, reoxidation was attempted by dialysis. After 4.5 hr in a solution 8 *M* in urea and 10^{-3} *M* in mercaptoethanol, 0.02 mg/ml chymotrypsinogen at pH 8.6 was dialyzed 24 hr against 3 changes of 0.01 *M* Tris–maleate buffer (pH 7.1) at 25°.

Control solutions of chymotrypsinogen never exposed to denaturant or reductant, hereafter referred to as "native chymotrypsinogen," and chymotrypsinogen denatured in 8 *M* urea in the absence of reducing agent were also prepared and treated according to the above procedures.

Activation of zymogen. After the oxidation period, trypsin was added at a zymogen to trypsin ratio of 5:1. Based on the results of such activation of control solutions containing native chymotrypsinogen, activation of reoxidized chymotrypsinogen was allowed to proceed at room temperature for at least 4 hr at pH 8–9, or 8 hr at pH near 7, or 12 hr at pH 5.0.

Assays. In all cases, a solution containing all materials with the exception of the zymogen was assayed, and any esterolytic activity recorded was subtracted from that of the activated preparation. Enzymatic activity toward BTEE at pH 7.8 was determined by the method of Hummel (13); ATEE at pH 7.8 as described by Cunningham and Brown

[2] The abbreviations used are: PCMB, *p*-chloromercuribenzoate; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; ATEE, *N*-acetyl-L-tyrosine ethyl ester; ZTNE, *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester; EDTA, ethylenediaminetetraacetate, disodium salt; TEAE, triethylaminoethyl; CM, carboxymethyl.

(14); ZTNE at pH 5.0 by the method of Kézdy, Thomson and Bender (15), and at pH 7.1 by a modification of that described by Martin, Golubow and Axelrod (16). The potentiometric assay using ATEE contained no buffer. Spectrophotometric assays with ZTNE contained peroxide-free dimethoxyethane as solvent for ZTNE, and were performed at pH 7.1 in 0.05 *M* Tris–maleate buffer. An extinction coefficient was obtained for *p*-nitrophenol at 400 nm by allowing complete enzymatic esterolysis; this value was confirmed by separate measurement based on the relative concentrations of protonated and ionized species of *p*-nitrophenol at pH 7.1 and the concomitant adjustment of the extinction coefficient determined at pH 8.0. The two values thus obtained agreed within a total range of 3%; the value used at pH 7.1 was $9.05 \times 10^3\ M^{-1}cm^{-1}$ at 400 nm.

Results. The reduced protein, when isolated from urea and mercaptoethanol and stored in 0.1 *M* acetic acid, was found to be very susceptible to precipitation during air-oxidation. Numerous attempts with a variety of buffers at different pH values failed to improve this condition. Knowing that protein in dilute solutions binds to glass surfaces, we employed glass beakers coated with ovalbumin (10), and subsequently Nalgene beakers, pipets, etc. Also, attempts were made to lower the probability of intermolecular disulfide bond formation by utilizing various agents as molecular separators in the reoxidizing solutions, *viz.*, ovalbumin, serum albumin, glycerol, and dextran. The presence of dextran, serum albumin, and/or glycerol failed to provide detectable activatable chymotrypsinogen from denatured, reduced zymogen, whether simple dilution, gel filtration, or dialysis was employed.

The presence of ovalbumin in the Nalgene system completely eliminated the rapid or eventual precipitation which otherwise occurred when pH was raised for air-oxidation. If such solutions were allowed to stand at pH 5.0, no turbidity or precipitation could be detected even after 7 days, at which time less than 1 sulfhydryl group was titratable. However, only 0.6% chymotryptic activity relative to that of native zymogen could be recovered on exposure to trypsin (see Table I). Maximum recoverable activity was achieved by isolating the reduced chymotrypsinogen and allowing it to oxidize at a concentration of ca. 0.02 mg/ml for 20–22 hr at pH 8–9 in the presence of 1 mg/ml ovalbumin. Under these conditions, 1.0–1.4% relative enzymatic activity was obtained by activation with trypsin (Table I). The addition of calcium chloride, mercaptoethanol, and/or dehydroascorbic acid had no measurable effect on the potential recovery of chymotryptic activity. The use of dialysis procedures for reoxidation also failed to yield increased recovery of biological activity; turbidity formed during dialysis, and after centrifugation, only 12% of the protein remained in solution. Its activation with trypsin yielded only 0.4% relative enzymatic activity (Table I).

TABLE I. Oxidation of Reduced Chymotrypsinogen A.

Expt[a]	pH during oxidation	Ovalbumin (mg/ml)	Recovery of soluble protein (%)	Assay	Sp act[b] (%)	Activity recovered (%)
Gel filtered	8.8	1	100	ZTNE, pH 7.1	1.4	1.4
	5.0	0	100	ZTNE, pH 5.0	0.6	0.6
Dialyzed	7.1	0	12	ZTNE, pH 7.1	3	0.4
Diluted	8.8	0	16	ATEE, pH 7.8	2.5	0.4

[a] These represent typical experiments, in which the reduced protein was either separated from urea and mercaptoethanol by gel filtration through a G-25 Sephadex column in 0.1 *M* acetic acid, or by dialysis against 0.01 *M* Tris–maleate buffer at pH 7.1, or was not separated but simply diluted into 0.1 *M* acetic acid.

[b] Activity expressed as percentage of that exhibited by an equivalent concentration of native chymotrypsinogen A subjected to identical conditions of activation.

Since activition of native chymotrypsinogen by trypsin in the presence of ovalbumin required several hours and resulted in considerably lower chymotryptic activity than such activation in the absence of ovalbumin, attempts were made to separate the zymogen from ovalbumin following reoxidation. After dialysis and prior to the addition of trypsin, solutions were chromatographed on TEAE-cellulose, CM-cellulose, and G-75 Sephadex. Native chymotrypsinogen (0.02 mg/ml) could be isolated in 80% yield from ovalbumin (1 mg/ml) by such chromatographic procedures and thus isolated, was fully activated by trypsin, as assayed with BTEE. By contrast, reoxidized chymotrypsinogen in the presence of ovalbumin, brought to pH 7.3, when chromatographed under identical conditions, eluted as a peak representing 30% recovery of protein, but possessed no esterolytic activity towards BTEE or ATEE after incubation with trypsin.

Discussion. The results indicate that reduction of chymotrypsinogen A by mercaptoethanol in the presence of 8 *M* urea leads to changes in the zymogen which are not reversed by air reoxidation under the conditions tested, whereas chymotrypsinogen denatured in 8 *M* urea in the absence of reductant is fully activatable. Since, upon complete reduction, the zymogen contains 10 sulfhydryl groups, one might anticipate some difficulty in controlling the formation of intermolecular disulfide bridges which could lead to extensive aggregation of polypeptide chains. This was, indeed, found to be the case. However, one could also anticipate that completed nascent polypeptide chains would be subject to the same complications *in vivo* unless factors other than simple thermodynamic interactions of the 245 amino acid side chains exert control in the biosynthesis of chymotrypsinogen. In the experiments described, we attempted to minimize aggregation through adjustment of pH, dilution of the reduced material, the addition of molecular separators, and the use of Nalgene containers to eliminate protein association on glass surfaces, since analogous interactions could serve to prevent aggregation *in vivo*.

Interestingly, the difficulties encountered in attempts to reoxidize trypsin (3, 4) and pepsinogen and pepsin (7, 8) suggest that there may be a more rigidly controlled environment *in vivo*. Both disulfide exchange and a physiological system which governs such exchange have been demonstrated (17–19). However, as described in this communication, addition of catalytic amounts of mercaptoethanol after reoxidation of reduced chymotrypsinogen failed to yield any increase in recovery of potential enzymatic activity. Givol *et al.* (11) have demonstrated that chymotrypsinogen is stable toward the disulfide exchange enzyme, whereas chymotrypsin is not, thereby implying that the zymogen contains the necessary "information" in its primary sequence for proper refolding. Using conditions similar to those in which Martin and Frazier (20) had demonstrated reversible pH-dependent denaturation of chymotrypsin, Givol *et al.* (11) did not reduce denatured chymotrypsin and chymotrypsinogen prior to dilution into the disulfide exchange medium (10^{-3} *M* in mercaptoethanol). In the present study, we found the nonreductive denaturation of chymotrypsinogen, under conditions otherwise identical to those employed for complete reduction, to be completely reversible, so that full chymotryptic activity was attained by trypsin activation. However, attempts to reoxidize chymotrypsinogen, once it was completely reduced, were unsuccessful, thus implying that upon complete reduction, the molecule loses its ability to refold to its biologically functional conformation. If the polypeptide chain of chymotrypsinogen A does contain the "information" for proper folding in its primary sequence, then we must look further to account for the low recovery of activity. Alternately, it is possible that the zymogen, once it has been reduced and diluted, forms a stable tertiary structure which is not activatable by trypsin; in other words, the biological form of chymotypsinogen A is not that of greatest thermodynamic stability.

Summary. Investigation of the air-reoxidation of denatured, fully reduced chymotrypsinogen A resulted in a maximum recovery of 1.4% of enzymatically active chymotrypsin upon treatment with trypsin. Un-

der the same conditions, urea-denatured chymotrypsinogen, which had not been reduced, yielded 100% recovery of enzymatic activity. Thus the biologically functional conformation of the polypeptide chain comprising chymotrypsinogen may not be that of greatest thermodynamic stability.

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