SELECTIVE REDUCTION OF A DISULFIDE BOND IN CHYMOTRYPSIN A_α *

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Treatment of chymotrypsin with dithiothreitol in the presence of phenylpropionate results in the selective reduction of the interdisulfide linkage connecting the segment comprising the A- and B- chains with the C-chain of the protein molecule, with concomitant loss of enzymatic activity. Oxidation of the reduced protein in the presence of phenylmethane sulfonyl fluoride results in the regeneration of enzymatically active protein.

INTRODUCTION

The role of disulfide linkages in the maintenance of the appropriate three-dimensional conformation essential for the biological activity of the protein has received considerable attention in recent years (1-8). In a recent report from our laboratory (9), treatment of chymotripsin with DTT at pH 9.2 in the presence of phenylpropionate was shown to result in the reduction of one of the disulfide linkages with concomitant loss of catalytic activity. The present report concerns the identification of the disulfide bond cleaved under the above mentioned conditions.

MATERIALS AND METHODS

Three times recrystallized chymotrypsin was obtained from Worthington Biochemicals, Freehold, New Jersey. DTT was obtained from Calbiochem, Los Angeles, California. PCMB and ATEE were products of Schwarz-Mann, Orangesburg, New York. ^{14}C-Iodoacetamide was obtained from

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DTT, dithiothreitol.
PCMB, p-chloromercuribenzoicacid.
ATEE, α-N-acetyl-Tyrosine ethyl ester
DFP, diisopropylfluorophosphate.
GSH, glutathione;
GSSG, Oxidized glutathione;
PMSF, phenylmethane sulfonyl fluoride
DIP, diisopropylphosphoryl.

New England Nuclear, Boston, Massachusetts.

The protein concentration was determined spectrophotometrically (10). PMS-chymotrypsin (11) and monosuccinyl-chymotrypsin (12) were prepared according to published procedures. Selective reduction of chymotrypsin was accomplished as described earlier (9): protein (25 mg) was dissolved in 100 mM tris buffer containing phenylpropionate (100 mM) at pH 9.2 and stirred for 4 minutes prior to the addition of 100 µl of DTT (1M in 10 mM HCl). The reaction was terminated after one minute by either of the following procedures: a) the reaction mixture was adjusted to pH 3.5 and was treated with 200 ml cold ethanol-acetic acid mixture (39:1). The precipitate formed was collected by centrifugation and was washed thrice with 3 x 30 ml aliquots of the above acidified ethanol. Appropriate aliquots of the solution of this precipitate in 100 mM acetic acid were used for sulfhydryl determination; or b) the reaction mixture was treated with ^{14}C-iodoacetamide (185 mg in 2 ml H_2O) and 50 µl of DFP (400 mM in isopropanol). After 20 minutes at pH 8.0, the alkylated protein was isolated by precipitation with cold ethanol-acetic acid mixture.

Chromatography: Prior to chromatography, the alkylated protein (approximately 100 mg, pooled from four individual experiments as above) dissolved in 10 ml of guanidine hydrochloride was treated with succinic anhydride (240 mg) in six equal portions over a period of 60 minutes, with the pH kept constant at 8.0. Following dialysis against cold 100 µM sodium bicarbonate solution, the succinylated protein was applied to a 2 x 100 cm Sephadex G-100 column equilibrated with 10 mM ammonium acetate, pH 6.0. Elution was accomplished with the same ammonium acetate solution. Fractions corresponding to distinct components (based on absorbance at 280 nm) were pooled and lyophilized. Each of the isolated components was purified by rechromatography under conditions mentioned above.

Alternatively, the dialysed solution of the succinylated protein

was further reduced with DTT (13) prior to chromatography. Following the bubbling of N_2 through the solution of protein, DTT (31 mg) was added and the pH was adjusted to 9.5. After the reaction had proceeded for 12 hours in the sealed vessel at 25°C, unlabelled iodacetamide (0.77 g) in 10 ml of tris-HCl buffer (3M, pH 8.0) was added. The reaction was terminated after 10 minutes by the addition of 2-mercaptoethanol (0.45 ml). The reaction mixture was dialysed extensively against 100 μM sodium bicarbonate and subjected to chromatography on Sephadex G-100 as described above.

Oxidation of the reduced protein (14): Following the limited reduction of protein, phenylpropionate and the excess of DTT were removed by chromatography on a 2 x 25 cm column of Sephadex G-25 with water as eluant. The fractions containing the protein were pooled and diluted with appropriate amounts of phosphate buffer (50 mM, pH 7.0) to obtain a final concentration of 0.2 mg protein per ml. GSH and GSSG (to achieve a final concentration of 5 mM and 0.5 mM respectively) and PMSF (10 μmoles in 1 ml isopropanol) a reversible inhibitor (10) were added and the reaction allowed to proceed at 25°C. At desired intervals, aliquots were removed and adjusted to pH 2.0 and kept at 37°C for 3.5 hours. The aliquots were then adjusted to pH 7.0 and allowed to stand for 2 hours at 25°C prior to the determination of catalytic activity. Reactivation of unreduced PMS-chymotrypsin under identical conditions served as a control.

Chymotryptic activity was determined by the spectrophotometric method (15) with ATEE as substrate. The determination of sulfhydryl groups was accomplished by titration with PCMB (16). Amino acid analyses were performed by ion-exchange chromatography (17) with a Beckman Model 120-B amino acid analyzer. Radioactivity measurements were performed using Packard model 3008 Tri-Carb liquid scintillation counter.

RESULTS AND DISCUSSION

In agreement with the earlier report (9) treatment of chymotrypsin with DTT at pH 9.2 in the presence of phenylpropionate resulted in the

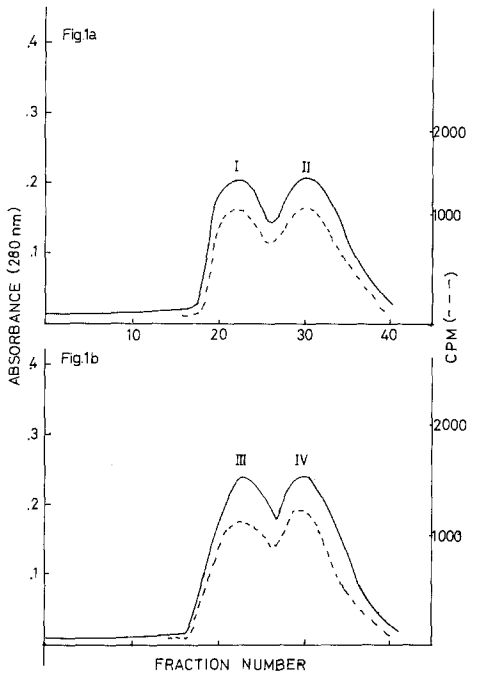

Fig.1. Chromatography of selectively reduced chymotrypsin.

a) chymotrypsin was selectively reduced with DTT at pH 9.5 in the presence of phenylpropionate. Following alkylation with ^{14}C-iodoacetamide, the material was succinylated prior to chromatography column, 2 x 100 cm Sephadex G-100; eluant, 10 mM ammonium acetate pH 6.0; flow rate, 16 ml/hr. (6 ml per fraction).

b) The succinylated material (above) was treated with DTT to achieve reduction of all the remaining disulfide linkages and the sulfhydryl formed were alkylated with unlabelled iodacetamides prior to chromatography under conditions mentioned above.

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appearance of approximately 1.8 to 2.4 SH groups with concomitant loss of catalytic activity. Phenylpropionate, which is obligatory for the rapid reduction of the disulfide bond, was found to exert a transient inactivation of the enzyme, with the initial activity being restored after four minutes. However, since the reduction with DTT is initiated only after four minutes of standing in the tris-phenylpropionate buffer, this phenomenon is unrelated

to the loss of catalytic activity accompanying the reduction of the protein.

Chromatography of the succinyl derivative of the alkylated (with ^{14}C-iodoacetamide) protein subjected to limited reduction, yielded two components I and II (Fig.1a), each possessing approximately the same specific radioactivity, (counts per minute per absorbance unit at 280 nm). Analyses for the amino acid content of these components showed II to correspond to that of the segment comprising A- and B- chains while that of I agreed with that of the C- chain of the protein (Table 1). To achieve a more reproducible resolution of the components, the succinyl derivative of the partially reduced and alkylated protein was fully reduced and subsequently alkylated with unlabelled iodoacetamide. Chromatography of this preparation yielded two components III and IV (Fig.1b) each with similar specific radioactivity as before. Analyses of these components showed that the amino acid composition of components of III and IV (Fig.1b) corresponded closely to that of B- and C- chains of the protein respectively (Table 1). These observations are consistent with the selective reduction of the interdisulfide bond linking the segment comprising A- and B- chains with that of the C- chain (or disulfide bond #3 according to the nomenclature (18)) of the protein.

Such partially reduced inactive protein could be renatured to an active enzyme provided the oxidation was performed in the presence of PMSF to prevent autocatalytic degradation. Nearly 60% of the initial activity could be regenerated with PMSF present during the oxidation procedure. These results are comparable to the recovery of approximately 75% of the activity from the unreduced PMS-chymotrypsin under identical conditions of treatment. Omission of PMSF during the oxidation of reduced protein resulted in less than 10% recovery of the active protein.

The inability of DTT to effect such rapid and selective cleavage of disulfide bonds in indoleacryloyl-chymotrypsin was recorded earlier (9). In the current study, monosuccinyl chymotrypsin was found to be likewise resistant to DTT. No cleavage of disulfide bonds could be detected even after

TABLE 1.

AMINO ACID ANALYSES OF THE COMPONENTS FROM SUCCINYL CARBOXYAMIDOMETHYL DERIVATIVES OF REDUCED CHYMOTRYPSIN.[a]

	SELECTIVE REDUCTION.[b]				SELECTIVE FOLLOWED BY FULL REDUCTION.[c]			
	I[d] Found	Expected for C- chain	II Found	Expected for A-B segment	III Found	Expected for B- chain	IV Found	Expected for C segment
Lysine	6.0	6	9.0	8	9.1	8	6.3	6
Histidine	-	0	2.1	2	2.1	2	0.1	0
Arginine	1.9	2	1.2	1	1.0	1	1.9	2
CM-cysteine	0.8	1	0.9	1	2.8	4	4.2	5
Aspartic acid	9.7	9	14.4	13	12.8	13	8.8	9
Threonine	11.7	9	15.8	13	12.5	13	8.5	9
Serine	10.7	11	16.6	16	13.9	15	9.9	11
Glutamic acid	5.0	4	11.6	11	9.6	10	3.9	4
Proline	4.7	4	4.3	5	3.0	3	3.7	4
Glycine	11.1	10	12.5	13	10.7	11	10.3	10
Alanine	11.1	10	12.4	12	10.9	11	10.7	10
½ Cystine	3.8	4	3.7	4	-	0	-	0
Valine	8.6	8	13.7	15	12.8	13	7.8	8
Methionine	1.9	2	-	0	-	0	1.7	2
Isoleucine	3.1	3	6.4	7	5.7	6	3.1	3
Leucine	9.0	8	10.0	11	9.2	9	7.6	8
Tyrosine	2.0	2	2.0	2	1.8	2	2.0	2
Phenylalanine	Trace	0	6.0	6	6.1	6	-	0

a. The preparations were hydrolyzed with 5.7N HCl at 105°C for 36 hours in sealed evacuated tubes prior to analysis.

b. After selective reduction, the protein was alkylated with ^{14}C-Iodoacetamide. The succinylated material was chromatographed to obtain components I and II (Fig.1a).

c. The succinylated carboxyamidomethyl derivative was further reduced and alkylated with unlabelled iodoacetamide. Chromatography (Fig.1b) yielded components III and IV.

d. Component I (C- chain) emerges earlier than II (A-B fragment) presumably due to aggregation. This phenomenon has been noted often.

5 minutes of exposure of the protein to DTT at pH 9.5. These observations suggest that conformational changes accompanying the binding of a competitive inhibitor (phenylpropionate) are distinct from that occurring upon acylation of the enzyme. Only the former type of changes render the interchain disulfide (between residues 136 and 201) readily accessible to reduction by DTT. Such an interpretation derives additional support from the recently recorded differences in the conformations of trypsin-benzamidine complex and DIP-trypsin (19).

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