IMPLICATIONS OF THE CHEMICAL MODIFICATION OF CHYMOTRYPSIN

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SUMMARY

The short N-terminal chain of δ-chymotrypsin, a two-chain member of the chymotrypsin family, was removed by reduction. The residual reduced major chain exhibited significant enzymic activity after refolding at low pH had occurred, and most of the native activity was regained upon subsequent oxidation. The catalytic capacity of the chymotrypsins therefore appears to be determined largely by the major chain, the primary structure of which shows extensive homology with that of elastase. Although α-chymotrypsin was found to be irreversibly inactivated by the elastase inhibitor dinitrophenylbromobutanone, a much faster rate of modification of a histidine, rather than a glutamate, residue was observed.

The two pancreatic proteases chymotrypsin and elastase are thought to have evolved in divergent fashion from a common ancestor after gene duplication events. Thus, while the two enzymes have various features of structure and mechanism of action in common, each has developed a distinctive substrate specificity (1,2). The results of inhibition and reductive cleavage studies reported here have a further bearing on the specific relationship between chymotrypsin and elastase, as well as on the general phenomenon of renaturation of reduced proteins.

MATERIALS AND METHODS

Bovine α-chymotrypsin (3 x recrystallized) and chymotrypsinogen (6 x recrystallized) from Miles-Seravac Laboratories (Cape Town) were purified by chromatography on Sephadex G-100 (2.5 x 90cm column; 0.1M-ammonium acetate-8M-urea, pH 6.0), dialysis against 0.001M-HCl and freezedrying. δ-Chymotrypsin was prepared from purified chymotrypsinogen by rapid activation with trypsin (3) and separation from other reaction components by chromatography on CM-cellulose (2.5 x 60cm column, 0.05M-sodium acetate, pH 4.5). Amino acid analyses (4) were carried out on a Beckman Model 120B

analyzer, and spectral measurements were taken on a Zeiss DMR 21 recording spectrophotometer.

Elastase (Whatman, Maidstone, Kent) and chymotrypsin assays were carried out with the ester substrates p-nitrophenyl t-butyloxycarbonyl-L-alaninate (NBA) and ethyl benzoyl-L-tyrosinate (BTEE) respectively (5,6). The inactivation experiments on α-chymotrypsin with 1-bromo-4-(2,4-dinitrophenyl)-butan-2-one (DPBB) were conducted at 25°C according to published procedures for elastase (7).

Reduction of α- and δ-chymotrypsin was accomplished by incubating 2 x 10^{-4}M solutions of the enzymes (50 mg) in 0.1M-Tris-8M-urea, pH 8.6 with excess mercaptoethanol (1µL/mg protein) under a nitrogen atmosphere for 4h at 37°C. The samples were subsequently dialyzed (3 x 2L, 24h, 4°C) against HCl (10^{-3}M)-mercaptoethanol (10^{-4}M)-$CaCl_2$(10^{-2}M) and oxidized by diluting appropriate aliquots to a concentration of 0.3 mg protein/ml with 0.05M-Tris-0.01M-$CaCl_2$ buffer, pH 8.5, and leaving them exposed to air for 24 hours.

Some samples of α-chymotrypsin were alkylated immediately after reduction by adding a hundred-fold excess, over the available thiol content, of iodoacetamide and quenching the reaction after 10 minutes with a five-fold excess of mercaptoethanol. After dialysis (0.02M-Tris-8M-urea, pH 8.0), the alkylated B- and C-chains were separated on a DEAE anion exchange column (8).

RESULTS AND DISCUSSION

The inhibition of α-chymotrypsin by DPBB

The pH-rate profile in Fig. 1 and the inhibitor concentration dependence (Table 1) illustrate that α-chymotrypsin was readily inhibited at near-neutral pH values by moderate excesses of DPBB. Exhaustive dialysis of inhibited preparations yielded yellow-coloured solutions with no regain of enzymic activity, and calculations based on the known extinction coefficients of α-chymotrypsin (9) and DPBB (7) showed that the irreversibly inactivated enzyme contained one dinitrophenyl moiety per molecule. The decrease in the

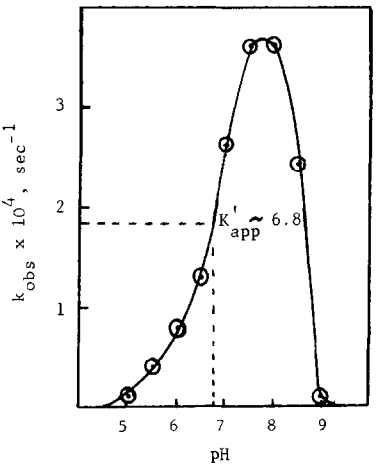

Figure 1: The pH-rate profile for the inactivation of α-chymotrypsin by DPBB
$[DPBB] = 5 \times 10^{-4}$M; $[Enzyme] = 1.8 \times 10^{-6}$M; $[CH_3CN] = 10\%$

Table 1: The effect of DPBB concentration on the rate of inactivation of α-chymotrypsin at 25°C

$[DPBB]$, M	Molar excess DPBB:enzyme	$k_{obs} \times 10^4$, sec^{-1}	$t_{1/2}$	k^a_{calc}, $M^{-1}sec^{-1}$
1×10^{-5}	6	0.16	10.5 h	1.61
1×10^{-4}	60	1.30	100 min	1.30
5×10^{-4}	300	4.68	23 min	0.94
1×10^{-3}	600	4.70	20 min	0.47

a: $k_{calc} = k_{obs} / [DPBB]$

calculated second-order rate constant ($k_{calc} = k_{obs}/[DPBB]$) with increasing inhibitor concentration apparent in the last column of Table 1 suggests that the irreversible reaction between α-chymotrypsin and DPBB was preceded by the reversible formation of a non-covalently linked enzyme-inhibitor complex. While the general features of the inhibition of α-chymotrypsin by DPBB are therefore very similar to those reported earlier for elastase (7), significant

differences were observed in the detailed nature of the process. Not only was the rate of inactivation of α-chymotrypsin (at 25°C) found to be about three times faster than that of elastase (at 37°C), but the involvement of different ionizable groups on the enzymes was indicated by the respective pH-dependence graphs for inhibition. Whereas ionization of a group with a $pK_a \sim 6.8$ was essential for the inhibition of α-chymotrypsin (cf. Fig. 1), a more acidic group ($pK_a \sim 4.5$) contributed to the inactivation of elastase.

Conventional amino acid analysis of control and inhibited α-chymotrypsin preparations as well as their respective B- and C-polypeptide chains after reduction and alkylation showed that one histidine residue of the B-chain was lost after treatment with DPBB (cf. Table 2). Taken together with the pH-

Table 2: Amino acid composition of α-chymotrypsin and its S-amidocarboxymethylated B- and C-chains before and after inactivation by DPBB

Amino acid[a]	Number of residues per molecule[b]								
	Intact molecule			B-chain			C-chain		
	N	T	I	N	T	I	N	T	I
Lys	14.0	14	13.6	8.0	8	8.0	6.0	6	6.0
His	2.2	2	1.2	2.4	2	1.3	0.0	0	0.3
Arg	2.9	3	2.6	1.6	1	1.2	1.7	2	1.8
CMCys	-	-	-	3.0	4	3.0	4.6	5	4.9
Asp	21.5	22	22.4	11.1	13	12.2	9.0	9	9.6
Thr	20.9	22	21.7	12.7	11	11.1	8.2	9	8.8
Ser	23.9	28	25.1	15.1	12	13.7	11.4	12	11.0
Glu	15.9	16	16.4	11.4	11	11.1	5.0	4	5.6
Pro	9.1	9	9.6	3.2	3	2.7	3.9	4	3.6
Gly	23.5	23	25.0	13.2	11	12.7	10.7	10	11.0
Ala	22.2	21	22.1	13.3	11	11.1	8.9	9	10.0
½ Cys	10.2	10	10.4	-	-	-	-	-	-
Val	22.0	23	22.4	12.6	11	10.4	5.8	8	7.1
Met	1.8	2	2.0	0.0	0	0.0	1.0	2	1.4
Ile	9.0	10	9.4	4.3	5	5.3	2.7	3	2.9
Leu	19.0	19	19.0	9.0	9	9.0	8.0	8	8.0
Tyr	4.1	4	4.5	1.2	2	1.8	1.9	2	1.7
Phe	6.1	6	6.2	5.8	6	5.6	0.7	0	0.8

[a]: Tryptophan was not determined.
[b]: N = Native; T = Theoretical; I = Inhibited. Values are calculated on the basis of lysine or leucine content of the basic and acidic/neutral amino acids, resp., and represent the means of duplicate 24-hour hydrolysates. Theoretical values are taken from reference 2.

dependence, these results represent persuasive evidence that chymotrypsin is inactivated due to the covalent interaction of DPBB with a histidine essential for enzymic activity. In view of the comparable structures and reactivities of DPBB and TPCK, a well-studied substrate analogue inhibitor of chymotrypsin (10), it seems reasonable to assume that DPBB, like TPCK, modifies the functionally important histidine-57 in the α-chymotrypsin sequence (1). Elastase, by contrast, had been found to be inactivated through the covalent modification of the γ-carboxyl group of glutamic acid-6 (7).

In another comparative experiment, addition of DPBB to the assay systems for α-chymotrypsin and elastase, respectively, revealed that DPBB also acted as a reversible competitive inhibitor ($K_i \sim 2 \times 10^{-5}$M) for the hydrolysis of BTEE, but had no effect on the hydrolysis of NBA by elastase (The limited amount of irreversible inactivation that took place during the short assay period could be ignored). The different behaviour of the inhibitor towards the two enzymes under these conditions can be explained on the one hand by the structural analogy of DPBB to aromatic substrates of chymotrypsin which would give rise to competitive binding effects, and its unrelatedness, on the other hand, to substrates of elastase, coupled with the suggestion put forward earlier (7) that the irreversible inactivation of elastase by DPBB is due to an unfavourable conformational change after covalent interaction.

Reduction and reoxidation of δ-chymotrypsin

Reduction of the five disulphide bonds of δ-chymotrypsin, a two-chain member of this family of proteases, followed by dialysis against 0.001M-HCl (with or without prior alkylation of the cysteines) was found to be a satisfactory procedure for the removal of the N-terminal thirteen-residue chain from δ-chymotrypsin. The remaining single-chain fragment comprising residues 16 to 245 had an amino acid composition reflecting the loss of the minor chain, residues 1 - 13, as judged by the content of the two amino acids proline and leucine which are expected to show the greatest change (cf. Table 3). When a reduced sample of the major chain was allowed to become oxidized

by exposing it at an alkaline pH to air, it recovered as much as 88% of the activity of a control δ-chymotrypsin towards BTEE (Table 3). The near-ultra-

Table 3: Properties of δ-chymotrypsin and its major reductive fragment (residues 16 - 245)

Component	Indicator amino acids, residues/molecule[a]		Relative activity against BTEE, %
	Proline	Leucine	
Native δ-ChT	9.1 (9)	19.2 (19)	100
Reduced fragment[b]	7.3 (7)	17.3 (17)	26 - 33
Reoxidized fragment[c]	7.5 (7)	16.1 (17)	68 - 88

a: Values are calculated on the basis of the expected alanine content, and represent the means of duplicate 24-h hydrolysates; those in brackets represent the theoretical content.

b: After dialysis under a nitrogen atmosphere against 10^{-3}M-HCl - 10^{-2}M-$CaCl_2$

c: Dialyzed, after oxidation, against 10^{-3}M-HCl

violet circular dichroism spectra presented in Fig. 2 illustrate the loss by the reduced chain of the optically active band at ~265nm exhibited by the intact disulphide bonds of native δ-chymotrypsin, and the recovery of the band to an extent of about 75% after oxidation.

The fate of cycteine-122, derived from the interchain disulphide bond linking ½-cystine-122 to the minor chain, has not been established after oxidation of the reduced major chain. It is unlikely to have remained unoxidized, though, in view of the exposed position of the parent disulphide in the crystal structure of chymotrypsin (11). A molecular weight determination by sedimentation equilibrium analysis demonstrated size heterogeneity, but the limiting value of 30,100 daltons (vs. 25,400 obtained for a homogeneous preparation of native δ-chymotrypsin) suggested that partial dimerization could have taken place,

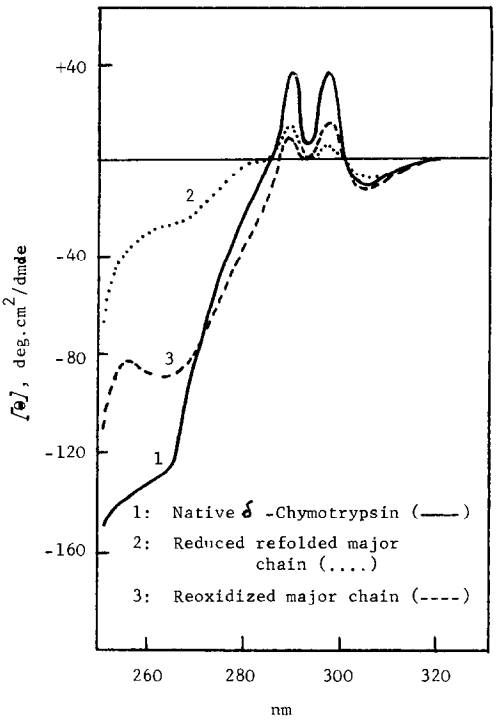

Figure 2: Circular dichroism spectra of δ-chymotrypsin and its major reductive fragment (residues 16 - 245). Cell length = 1 cm; Concentration = 0.1% protein in 10^{-3}M-HCl; Jasco Model J20 dichrograph

possibly through the formation of a new inter-major chain disulphide bond from two cysteine-122 residues.

These results on the oxidation of the reduced major chain demonstrated that the correct folding and disulphide pairing into an active conformation could be achieved for δ-chymotrypsin without the assistance of the N-terminal thirteen residues. It would thus appear that the latter part of the chymotrypsin molecule has as sole function the protective sequestration of the enzyme precursor chymotrypsinogen in inactive form until after secretion into the digestive tract. This interpretation is strengthened by the absence in the literature of reports which implicate residues of the N-terminal chain in the maintenance of either the structural integrity or the catalytic ability of α- and δ-chymotrypsins. It is relevant that in comparisons of the sequence

homology between chymotrypsin and elastase it is customary to equate the sixteenth residue of chymotrypsin with the first residue of elastase (2); the results reported here could be regarded as direct experimental verification that the sequence comprised of residues 16 - 245 indeed represents the active entity of the chymotrypsins.

Finally, we have obtained preliminary evidence that the reduced major chain of δ-chymotrypsin possessed significant activity prior to oxidation (c.f. Table 3), provided it had been allowed to refold under conditions known to stabilize the native conformation of the enzyme, whilst maintaining the reduced state of the cysteine sulphydryl groups (low pH, calcium ions, nitrogen atmosphere). It is tempting to speculate that similar results might also be obtained with other disulphide-containing single-chain proteins of moderate size (12), especially in the light of the work of Anfinsen and his collaborators who convincingly showed that disulphide bonds per se are not required for the correct refolding of denatured proteins (13). Furthermore, a recent report (14) would appear to suggest that reduced lysozyme directly refolds into the correct conformation, to the virtual exclusion of all others, since only a very limited number of incorrectly formed disulphide bonds could be detected in a time-dependent reoxidation study. Because of the additional conformational stability conferred on entropy grounds by disulphide bonds to the native structure of proteins (15), one would expect that a reduced, refolded molecule should be a somewhat less efficient biological species.

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