MODIFICATION OF THE AMINO GROUP OF ISOLEUCINE-16 IN CHYMOTRYPSIN WITH RETENTION OF ACTIVITY

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SUMMARY

The N-terminal isoleucine-16 residue of δ-chymotrypsin has been modified to the corresponding amidine by reaction with either ethyl acetimidate or methyl picolinimidate. The modified δ-enzymes show no change in specific activity or in the active site concentration but have one less isoleucine amino group as determined by both end-group analysis and potentiometric titration. Despite the fact that the amidinated amino group has a pK' about 12, k_{cat}/K'_m versus pH profiles (acetyl-L-tryptophan ethyl ester as substrate) are bell-shaped with the upper pK' 9.3. Thus, the Ile-16 amino group is neither essential for chymotrypsin activity nor responsible for the kinetically seen group with pK' 9.

The amino acid residues Asp-102, His-57, and Ser-195 have been proposed to participate as a coupled "charge-relay" system in the mechanism of chymotrypsin catalyses (1). Evidence for the requirement of another group has been based upon the dependence of k_{cat}/K'_m on pH for various substrates (2) and of K_s on pH for amide hydrolysis (3,4), proton uptake as a function of the binding of various substances (5-7), and kinetics of the acylation reaction involving nonspecific substrates (2,3). It has been proposed that this group, with a pK' 9, is the protonated α-amino group of Ile-16 (8-12). Functionally, this group has been postulated to regulate the concentration of the requisite active site conformation necessary for the formation of enzyme-substrate complexes; a concept that has received support from crystallographic (13-15) and circular dichroism studies (16). Conversion of chymotrypsinogen to the active enzyme is believed to result in the formation of an internal ion pair involving Ile-16 and the carboxylate anion of Asp-194 (13,14).

Amino group modification studies have played a significant role in the development of this concept. Treatment of the amino group of Ile-16 with either acetic anhydride (8-11) or nitrous acid (12) results in considerable diminution of chymotrypsin activity toward specific substrates. However, careful potentiometric titration studies of the reaction between chymotrypsin and either diisopropylphosphofluoridate (17) or formaldehyde (18,19) have indicated that the α-amino group of Ile-16 is not perturbed in its ionization characteristics nor essential for enzyme activity. The recent results of Valenzuela and Bender (20,21) lead to a similar conclusion.

In this paper we wish to report that we have succeeded in blocking the amino group of Ile-16 using two different amidinating agents without any effect on the specific activity of chymotrypsin.

EXPERIMENTAL

Acetylated chymotrypsinogen was prepared by the addition of 140 µl (2550 µmoles) acetic anhydride over a 60 min. period to 1.0 g (40 µmoles) chymotrypsinogen in a 15 ml vol. (0.15 M KCl plus 0.02 M $CaCl_2$) at 4° and maintained at pH 7.9 $\pm$ 0.1 with a Radiometer pH-stat. Excess acetic anhydride was hydrolyzed at pH 6.5. Before reaction with acetic anhydride, the chymotrypsinogen had been reacted with a 1.1 molar excess diphenylcarbamyl chloride over the residual chymotrypsin activity at pH 7.5, 20° for 1 hr.

Amidinated chymotrypsinogen was prepared by the addition of 2.25 g ethyl acetimidate·HCl (18,220 µmoles) in 4 ml cold 4 M KOH (solution pH, 7.8) to 1.0 g diphenylcarbamyl chloride-treated chymotrypsinogen at 4° in a 11.5 ml vol. After 4 hrs incubation at pH 9.3, the pH was adjusted to 6.5 and the solution dialyzed against cold 0.005 M acetate.

Activation of chymotrypsinogen or of the modified chymotrypsinogens to the δ-enzyme state was effected with one part trypsin to 50 parts zymogen at pH 7.5 and 4° for 210 min. Upon gel filtration through Sephadex G-50 at pH 6.5 in the cold, the initial emerging material comprising the major peak was lyophilized. Yields were about 70% of the starting material.

Reamidination of amidinated-δ-chymotrypsin (10.2 μmoles/35 ml vol.) was done at pH 9.3 and 4° under a nitrogen atmosphere by the addition of 188 mg (1522 μmoles) ethyl acetimidate·HCl previously neutralized with KOH. After incubation for 3 hr, reagent addition was repeated and incubation continued for another 3 hr period. The solution was then taken to pH 6.5 and dialyzed against cold 0.005 M acetate.

In other experiments, 200 mg (8 μmoles) acetylated-δ-chymotrypsin in 25 ml water at 20° was treated with 116 mg (852 μmoles) methyl picolinimidate at pH 9.5. After 2 and 4 hrs, reagent addition was repeated. The solution was then adjusted to pH 6.5 and dialyzed against cold water.

Potentiometric titrations were done as previously described (17,18,22). Rate assays were done at 20° and pH 8.0 using acetyl-L-tyrosine ethyl ester (0.01 M) in a solution of 0.15 M KCl. The dependence of k_{cat} and K'_m (the steady-state parameters) on pH were evaluated with the nonionizable substrate, acetyl-L-tryptophan ethyl ester, by procedures described elsewhere (19). Trans-cinnamoylimidazole was used as the reagent for active site assays (23). End group assays were performed using either the Sanger (24) or the Edman (25) procedure.

Protein concentrations were calculated from A_{280} measurements with A_M equal to 50,000. However, if the protein had been treated with methyl picolinimidate, the absorbance was measured at 290 nm with A_M equal to 42,550.

RESULTS AND DISCUSSION

Data obtained with δ-chymotrypsin and the amino group-modified enzymes are presented in Table I. Within reasonable limits, the enzyme activity and active site concentration of all enzyme preparations are similar. Modification of the Ile-16 amino group by treatment with either ethyl acetimidate or methyl picoliimidate has no effect on either parameter. End-group analyses are in substantial agreement with titration results. δ-Chymotrypsin has 4.8 groups titrating between pH 6 to 9 representing two imidazoles, two α-amino groups and overlapping ionizations of the carboxyl and phenolic groups.

TABLE I - Effect of Amino Group Modification on Chymotrypsin

Enzyme Preparation	Expt.	Activity (sec^{-1})	Active Sites (%)	Ionizable Groups[a] (pH 6-9)	Isoleucine Equivalents
δ-Chymotrypsin		147	92	4.8	1.0
Amidinated-δ-Chymotrypsin	1	150	91	3.8	1.0
	2	136	82		0.95
Reamidinated-δ-Chymotrypsin	1	142	88	2.8	0.16
	2	130	80		0.20
Acetylated-δ-Chymotrypsin	1	131			0.95
	2	129			0.98
Same, treated with methyl picolinimidate	1	125			0.30
	2	123			0.35

[a] From several experiments with a variation of ± 0.03 groups.

The one less titratable group in amidinated-δ-chymotrypsin is due to the amidination of the α-amino groups of Cys-1 (the amidine group has a pK' of about 12 (26)). In the reamidinated enzyme, with most of the isoleucine amino group modified, only 2.8 groups can now be titrated in the pH region 6 to 9.

Considering the proposed function of the amino group of Ile-16, it may be argued that amidination merely substitutes one positively charged group for another. However, the reagents used are of greater bulk and the resulting amidines have pK's of about 12 (26). Therefore, since the proposed function of the Ile-16 amino group in its protonated state is said to maintain, through ion pair bonding with the carboxyl group of Asp-194, a catalytically active conformation of the enzyme, it would then follow that the kinetic behavior of the reamidinated enzyme should be considerably different from that of δ-chymotrypsin. However, this was not observed. Using acetyl-L-tryptophan ethyl ester as substrate, reamidinated-δ-chymotrypsin shows a dependence on pH for k_{cat}/K'_m which is bell-shaped and with pK'_1 equal to 6.7 and pK'_2

TABLE II - The pH-Dependence of the Hydrolysis of Acetyl-L-Tryptophan Ethyl Ester by Reamidinated-δ-Chymotrypsin at 20°

pH	k_{cat} (sec^{-1})	$K'_m \times 10^4$ (Molar)	$k_{cat}/K'_m \times 10^{-4}$ (M^{-1}-sec^{-1})
6.5	22.6	1.5	15.1
7.0	36.5	1.6	22.8
7.5	38.8	1.2	32.3
8.0	42.9	1.4	30.6
8.8	42.2	1.6	25.6
9.0	42.9	1.6	26.8
9.5	41.9	2.9	14.4
10.0	42.9	5.1	8.4
10.5	35.3	7.2	4.9

equal to 9.3 (Table II). This is very similar to that observed for δ-chymotrypsin (27) and amidinated-δ-chymotrypsin.

We thus conclude that the amino group of Ile-16 is not essential for chymotrypsin activity and it is not the group with pK' about 9 seen on acylation (2,3) or on binding of substrate (3-7).

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