THE CATALYTIC ACTIVITY OF THE INACTIVE CONFORMATION OF δ-CHYMOTRYPSIN

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1. Introduction

Chymotrypsin has two main conformations. At neutral pH the active conformation, stabilised by a salt bridge between the α-ammonium ion of Ile-16 and the carboxylate ion of Asp-194, predominates. At high pH values the salt bridge is deprotonated and a conformation thought to resemble chymotrypsinogen is the major species [1–3]. Acylation of Ile-16 anchors the enzyme in this "inactive" conformation. It has been shown that acylation of all the amino groups of the zymogen followed by activation by trypsin to δ-chymotrypsin leads to a fully active enzyme [1, 2, 4]. Acylation of the Ile-16 amino group, which is released on activation, then abolishes most of the activity. However, in these studies about 10% of activity remains towards specific ester substrates as further acylation of the enzyme leads to concomitant irreversible denaturation.

Pursuing our interest in the specificity of chymotrypsin and the inactivity of chymotrypsinogen, it became important to know the upper limit of the activity of the "inactive" conformation of chymotrypsin. A previous study [3] based on rapid rate measurements suggested this is low.

2. Materials and methods

The inactive conformation of δ-chymotrypsin was prepared by a modification of known procedures [1, 2, 5].

2.1. *The amino groups of chymotrypsinogen* (Worthington Lot CGC 8HA) *were blocked with the readily removable dimethylmaleic anhydride*; 125 mg of chymotrypsinogen was dissolved in 12.5 ml of 0.12 M pH 9 carbonate buffer in a Radiometer autotitrator assembly and 0.5 g of dimethylmaleic anhydride in acetonitrile slowly added at 0°, the pH being maintained at 9.0 by the addition of sodium hydroxide.

2.2. *The modified zymogen was activated*; 3 mg of trypsin was added to the cold mixture which was then dialysed at 4° against 5 ℓ of 10 mM borate and 10 mM calcium chloride at pH 8 for 2 hr.

2.3. *The modified δ-chymotrypsin*, which had an activity of ~ 140% towards 1 mM acetyl-L-tryptophan ethyl ester, *was then maleylated* on Ile-16 after removal of the trypsin by shaking with CM-50 Sephadex equilibrated with the borate buffer; the protein solution was transferred to the autotitrator at 0°, the pH adjusted to 9.0 and 100 mg of maleic anhydride added whilst maintaining the pH. (Note: succinylation of chymotrypsin also increases k_{cat} [4]).

2.4. *The dimethylmaleyl groups were removed* by lowering the pH to 6 for 10 min, then further lowering to 5.0 and leaving at room temp. for 30 min.

2.5. *The active enzyme was removed by affinity chromatography*; the pH was increased to 7.8 by the addition of sodium hydroxide and concentrated pH 7.8 Tris added until 0.05 M. The material was affinity chromatographed at 4° [5], the active material remaining on the column. The inactive fraction was dialysed overnight against two changes

Table 1
Activity of δ-chymotrypsin maleylated on Ile-16, chymotrypsinogen and imidazole[a].

Sample	Activity towards ATEE[b] (%)	Activity towards PNPA[c] (%)	Activity towards ATEE after incubation at pH 3[d] (%)
δ-Chymotrypsin	100	100	–
Ile-16 maleylated -δ-chymotrypsin	0.086	1.14	88
Chymotrypsinogen[e]	0.43	4.15	–
Imidazole[f]	10^{-9}[g]	3.4	–

[a] 25°, pH 7.8. [b] 1 mM acetyl-L-tryptophan ethyl ester. [c] 1 mM *p*-nitrophenyl acetate. [d] The maleyl group on Ile-16 is hydrolysed at low pH. [e] Commercial sample (Worthington Lot CGC 8HA). [f] Initial rate of hydrolysis of 1 mM substrate compared with the enzyme velocity. [g] Estimated from linear free energy plots.

of 0.05 M Tris. (The dialysis buffer was saved for use as the buffer for the blanks in the activity assays and the eluant for the chromatography .) The material was then subjected to further affinity chromatography, concentrated and chromatographed a third time.

The resultant enzyme modified on Ile-16 was assayed against acetyl-tryptophan ethylester and *p*-nitrophenylacetate and compared with an authentic sample of δ-chymotrypsin. The conditions and results are given in the table.

The pH of the enzyme solution was lowered to 3 and after dialysis against 10^{-3} M HCl at room temp. left and periodically assayed. After 2–3 weeks up to to 88% of activity was regained as the maleyl group was eliminated. The rate of reactivation was 50% lower at pH 4 as expected for the loss of a maleyl group [7].

It is estimated from the rates of hydrolysis of maleic and dimethylmaleic half amides [7] that about 5% of the dimethylmaleyl groups were lost during the activation process and then replaced by maleyl groups during the reaction with maleic anhydride. This is immaterial to this study. The amino groups of the zymogen were reversibly blocked only to prevent precipitation of the enzyme when lowered to pH 3. A zymogen control which had been reacted with maleic anhydride could be fully activated but precipitated on the pH being lowered to 3 as the enzyme became uncharged. The reversibly blocked material was soluble at low pH indicating the majority of amino groups are free as described in sect. 2.5.

3. Results and discussion

The inactive conformation has less than 0.1% activity towards acetyl-tryptophan ethyl ester. This residual activity is probably due to active enzyme remaining in the sample. There is an activity of ~ 1% towards *p*-nitrophenyl acetate. This cannot be accounted for by unremoved active enzyme – but this is somewhat less than the activity of imidazole under the same conditions. Insulin has been shown to have a similar reactivity [8].

Evidence has been presented [2] that δ-chymotrypsin which has been acetylated on all the amino groups may be acetylated on Ser-195 by *p*-nitrophenyl acetate giving an observable "burst" of *p*-nitrophenol release. No burst is seen with the enzyme which is maleylated on Ile-16. I have shown that the salt bridge is not essential for δ-chymotrypsin to take up the active conformation; 12% remains in the active conformation at high pH where the salt bridge is deprotonated [3]. Blocking Ile-16 with the small acetyl group presumably allows sufficient conformational flexibility to allow the enzyme to be acylated by *p*-nitrophenyl acetate. The bulkier maleyl group imposes greater conformational rigidity and Ser-195 is less reactive.

The following conclusions may be drawn. The "inactive" conformation of the enzyme is as catalytically inactive as the zymogen. The equilibrium between the inactive and active conformations of chymotrypsin has been studied as a function of pH [3] and these results are sufficient to describe the events necessary for activity. The pH dependence indicates that the "charge relay system" [9] has the same ionisation state in both conformations; the only major ionisation seen between pH 3 and 13 is that of Ile-16 [3]. (If the pK_a of Asp 102 altered significantly during the conformational change then this would cause a large perturbation in the equilibrium constant). This is consistent with the finding of Fersht and Sperling [10] that Asp-102 is negatively charged at pH values greater than 3 in both the enzyme and the zymogen.

In the absence of correct substrate binding the enzyme is no more reactive than a solution of imidazole. The presence of catalytic groups which are reactive in the absence of correct substrate binding is incompatible with the notion of enzyme specificity.

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