HYDROLYSIS OF L-3-BENZYL-2,5-DIKETOMORPHOLINE BY CHYMOTRYPSIN*

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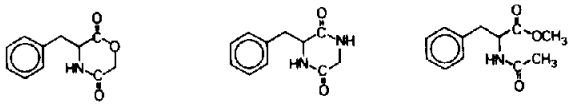
1. Introduction

It is well known that the stereospecificity of the α-chymotrypsin-catalyzed hydrolysis of *N*-acylamino acid esters and amides is due to interaction of the substrate's aromatic group with the hydrophobic moiety of the enzyme's active site and to hydrogen bonding of the α-acylamide nitrogen to the corresponding acceptor in the chymotrypsin molecule. These two interactions orientate the substrate group undergoing cleavage in the direction of the catalytic groups of the enzyme. However, analysis of the K_m values for a number of similar substrates [1] has shown this parameter to be practically independent of the presence or absence in the substrate of a hydrogen donating >NH group. Modification of this group affects mainly the catalytic constant k_{cat}. Since hydrogen bond formation is accompanied by a change of about 4 kcal/mole in the free energy of the system, it is difficult to understand why the hydrogen bonding does not show up in the dissociation constants of the enzyme-substrate complexes.

A possible explanation could be that the substrate binds to the enzyme with the *N*-acylamide group in the energetically less favourable *cis*-conformation [1, 2]. The difference in free energies of the *cis* and *trans* amide groups could thereby nullify the energy effect of the hydrogen bond.

In order to ascertain whether a *cis* amide group could take part in hydrogen bond formation with the enzyme, we investigated the substrate properties of L- and D-3-benzyl-2,5-diketomorpholines, (I), and L-3-benzyl-2,5-diketopiperazine (II). These compounds can be regarded as conformationally restricted analogous of the typical chymotrypsin substrate, methyl *N*-acetyl-L-phenylalaninate (III).



(I) (II) (III)

Chymotrypsin was found to catalyze the hydrolysis of L-(I), the rate of the reaction being higher than with (III). Compounds D-(I) and (II) did not undergo chymotryptic hydrolysis.

2. Materials and methods

L-3-Benzyl-2,5-diketomorpholine, L-(I), was prepared by treating *N*-(bromoacetyl)-L-phenylalanine [3] with Ag_2O in dioxan for 2 days at 20°. M.P. 115–116°; $[\alpha]_D^{20}$ −21.2°; $C_{11}H_{11}NO_3$, found %: C 64.23, H 5.50, N 6.88. Compound D-(I) was prepared likewise. L-3-Benzyl-2,5-diketopiperazine, (II), was prepared according to [4], m.p. 270–274°, $[\alpha]_D^{20}$ + 110° (in CH_3COOH). α-Chymotrypsin was a crystalline preparation of the Leningrad meat-packing factory with a 67–70% active enzyme content. The enzymic hydrolysis of the morpholine derivatives was studied with an automatic Radiometer TTT Ic pH-stat at pH 7.2 and 25° in an aqueous 0.1 M KCl solution containing 20% (v/v) of dimethylformamide under the condi-

* Part III of a series on "Conformationally Restricted Substrates of Chymotrypsin"; Part II, ref. [11]; part I, ref. [12].

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tions where $[S]_o \gg [E]_o$. The inhibition of hydrolysis of (III) by D-(I) was studied under the above conditions at $[I]_o$ = 0.048 and 0.25 mM. Compound (II) was hydrolysed by incubation for 3.5–24 hr with 1.6×10^{-5} moles of chymotrypsin ($[S]_o$ 1×10^{-3} M) in phosphate buffer pH 7.88. The amino nitrogen content was determined in aliquots according to [5]. The alkaline hydrolysis of L-(I) and (III) was studied titrimetrically at pH 9.5 (35°) in 0.1 M KCl solution containing 20% (v/v) of dimethylformamide.

3. Results and discussion

Table 1 presents the kinetic constants of the enzymatic hydrolysis of L-3-benzyl-2,5-diketomorpholine, L-(I), and methyl *N*-acetyl-L-phenylalaninate, (III), obtained under identical conditions. It is seen that the specificity of chymotrypsin towards L-(I) exceeds (in terms of $k_{cat}/K_{m(app)}$) that of the typical substrate (III). However, since the reactivity of diketomorpholine derivatives is considerably higher than that of acetylphenylalanine ester under non-enzymic conditions, it is more correct to compare the normalized values of the second order catalytic constants*. But in this case too chymotrypsin was found to have similar specificity towards L-(I) and (III). The stereospecificity index $\frac{[k_{cat}/K_{m(app)}(L)]}{[k_{cat}/K_{m(app)}(D)]}$ differing less than threefold for the two substrates.

A peculiarity of the enzymic hydrolysis of diketomorpholines is that the amide group does not necessarily retain its *cis*-conformation at the acyl-enzyme deacylation stage, since in this case the acyl moiety, which is an *N*-(glycolyl)-phenylalanine residue, is capable of undergoing *cis* – *trans* isomerization. It is therefore possible that D-(I) can acylate the enzyme as effectively as L-(I) (i.e. that there is no stereospecificity at acylation stage) but that the deacylation rate of *N*-(glycolyl)-D-phenylalanine-chymotrypsin is very low. Then, providing $[D\text{-}(I)]_o \gg [E]_o$, irreversible (or pseudoirreversible) inhibition of the enzymic activity of chymotrypsin would take place. However, a study of the inhibition of the enzymic hydrolysis of (III) by D-(I) has shown that it is a purely competitive inhibitor. Hence, the difference in substrate properties of L-(I) and D-(I) must be due to different orientation at the stage of enzyme acylation of the ester group undergoing hydrolysis. Such orientation can take place only during bidentate binding of both enantiomers in the active site; hence one may say that the *cis*-amide of diketomorpholines interacts as effectively with the acceptor in hydrogen bonding with the enzyme as does the amide group in (III).

Noteworthy is the very low K_i value for D-(I) (0.065 mM). Assuming that K_s in the enzymic hydrolysis of L-(I) is similar in value to K_i of D-(I), the difference in the free energy of binding of L-(I) and L-(III) by the enzyme would be about 2.2 kcal/mole, i.e. close to the difference in the free energies of *cis* and *trans* amides [8]. Further support for the *cis*-conformation of the amide group in the enzyme active site, comes from the three-dimensional chymotrypsin model constructed on the basis of crystallographic data [9]. Chymotrypsin is known (see e.g. [10]) to be less capable of catalyzing the hydrolysis of peptides X-Tyr-Y, where X and Y are amino acid residues, if X is of the D-configuration. Thus it was shown in our laboratory that the rate of hydrolysis of L-Val-L-Tyr–$GlyNH_2$ was considerably higher than that of D-Val–L-Tyr–$GlyNH_2$*. Analysis of the threedimensional enzyme model shows that this phenomenon on can be only rationalized in terms of the *cis*-conformation of the amide group linking the Val and Tyr residues. Only then will there be steric hindrance arising from interaction between the valine and enzyme Met^{192} side chains.

The inability of chymotrypsin to catalyse the hydrolysis of L-3-benzyl-2,5-diketopiperazine, (II), is rather difficult to explain in the light of our data. It may be possible that the equilibrium of the reaction: enzyme + (II) $\rightleftarrows$ aminoacyl-enzyme is sharply shifted to the left owing to the preferential attack of the ester carbonyl in the acyl-enzyme at the stage of acylation by the amino group. We are now attempting to elucidate this question.

* The normalization, however, is only nominal for compounds with different conformation (see [6]).

* The experimental details will be reported elsewhere.

Table 1
Enzymic* and alkaline hydrolysis of compounds (I) and (III).

Compound	$[S]_o$ (mM)	$[E]_o$ ($M \times 10^7$)	k_{cat} (sec^{-1})	K_m(app) (mM)	k_{cat}/K_m(app) ($M^{-1}\ sec^{-1}$)	k^{OH} ($M^{-1}\ sec^{-1}$)	$\left[\frac{k_{cat}}{K_m(app)}\right]^{**}$	Stereospecific index
L-(I)	0.14–1.0	0.25	124	1.25	1×10^5	179	1.9×10^3	1.9×10^4
D-(I)	does not react (K_i = 0.065)						0.1	
L-(III)	0.3 –2.0	1.0	19.3	3.45	5.6×10^3	3.3	5.6×10^3	5.6×10^4
D-(III)	does not react						0.1	

* Conditions: aqueous 0.1 M KCl solution containing 20% (v/v) dimethylformamide, 25°, pH 7.2.
** Corrected value [7]. Reactivity of L-(III) under non-enzymic conditions is taken as a unit.

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