THE IMPORTANCE OF THE CONFORMATION OF THE TETRAHEDRAL INTERMEDIATE FOR THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF PEPTIDE SUBSTRATES

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1. Introduction

Peptide bonds involving the carbonyl group of amino acid residues with large hydrophobic side chains are known to be easily hydrolyzed by α-chymotrypsin. However, if the amido nitrogen is contributed by a proline residue the peptide bond is resistant to chymotryptic cleavage. This finding, which also applies to trypsin and elastase, has emerged from the extensive use of these endopeptidases in the study of the amino acid sequence of polypeptides and proteins [1].

Assuming that the unreactivity of these peptide bonds results from an unfavorable interaction of the methylene groups of the proline ring with the enzyme active site, it would be interesting to know whether the steric hindrance occurs upon formation of the enzyme–substrate complex or during the subsequent bond-change steps, and whether this steric hindrance is related to the ring structure of proline or simply to substitution of the amido nitrogen. In order to answer these questions the dipeptides *N*-acetyl-L-phenylalanyl-L-proline amide (I) and *N*-acetyl-L-phenylalanyl-sarcosine amide (II) were synthesized and their behaviour as model substrates of α-chymotrypsin studied.

The results of the kinetic analysis can be interpreted by considering the interactions occuring between enzyme and dipeptide during the formation of the enzyme-substrate complex and the subsequent nucleophilic attack of Ser-195 to form a tetrahedral adduct. This interpretation focusses attention on the conformation of the tetrahedral intermediate and leads to an interesting new aspect of the steric course of the α-chymotrypsin catalyzed hydrolysis of peptide substrates.

2. Materials and methods

N-acetyl-L-phenylalanyl-L-proline amide (I) was synthesized by coupling H-Pro-OMe·HCl* and Boc-Phe-OH with 1-isobutoxycarbonyl-2-isobutoxy-1,2-dihydroquinoline [2,3] and replacing the end groups using conventional methods as described for previous peptide syntheses [4]. m.p. 87–89°C. $[\alpha]_D^{26} = -22.0°$ (c=1, ethanol)..

$C_{16}H_{21}N_3O_3 \cdot ½\ H_2O$	calc. C 61.53, H 7.09, N 13.45
	found C 61.01, H 6.82, N 13.10

N-acetyl-L-phenylalanyl-sarcosine amide (II) was synthesized by coupling H-Sar-OEt·HCl and Ac-Phe-NH-NH_2, using the azide method, and replacing the C-terminal ester group by ammonolysis. m.p. 151–153°C. $[\alpha]_D^{25} = +26.5°$ (c=1, dimethylformamide).

$C_{14}H_{19}N_3O_3$	calc. C 60.63, H 6.91, N 15.15
	found C 60.54, H 6.85, N 15.17

Details of these syntheses are described elsewhere [5].

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* IUPAC/IUB rules for peptides are followed, see Eur. J. Biochem. (1972) 27, 201–207.

The kinetic measurements were carried out with a pH-stat as described previously [4,6]. The operational normality of α-chymotrypsin (three times crystallized, salt free, Sigma Lot 51C-8050) was determined by the *N*-cinnamoylimidazole method [7].

3. Results and discussion

Both dipeptides I and II were tested as substrates of α-chymotrypsin at pH 7.90 and 25°C in 0.2 M sodium chloride solution as described previously for other peptide substrates [4,8]. No hydrolysis of I and II could be observed at an enzyme concentration of 40 μM, at which the hydrolysis of Ac-Phe-Gly-NH_2, the least reactive dipeptide studied so far [8], can easily be followed. At an enzyme concentration of 80 μM a reaction was observed whose recorder trace was identical to that obtained for a blank experiment in absence of substrates and represents therefore only enzyme autolysis. It is thus concluded that the dipeptides I and II are unreactive under these conditions. Dipeptides I and II were then investigated as inhibitors of the hydrolysis of a specific substrate, Ac-Phe-OMe. They proved to be competitive inhibitors with inhibition constants K_I three to five times larger than the dissociation constants K_{EA} of the enzyme–substrate complexes of typical dipeptides (see table 1). This indicates that I and II form enzyme–substrate complexes of normal stability and that the reason for their unreactivity has to be sought in the nature of the enzyme–substrate interactions occurring during the subsequent bond-change steps.

A skeletal model of the enzyme, constructed on the basis of the co-ordinates given by Birktoft and Blow [9], was used to verify that this conclusion is consistent with the active-site structure. The orientation within the active site of good dipeptide substrates like Ac-Phe-Gly-NH_2 (III) and Ac-Phe-Ala-NH_2 (IV) can be deduced with confidence from structural data on complexes of α-chymotrypsin with *N*-formyl-L-tryptophan [10] and of trypsin with pancreatic trypsin inhibitor [11,12] and soybean trypsin inhibitor [13]. From the models of the enzyme–substrate complexes for III and IV, models for I and II can be generated by introducing the necessary methyl or methylene groups. It can thus be inferred that these additional groups are directed towards the solvent and do not interfere with any group of the enzyme and hence that I and II are able to form productive enzyme–substrate complexes. Their unreactivity can be understood by considering the stereoelectronic course of the transformation leading to the acyl-enzyme intermediate.

There is considerable evidence that the formation of the acyl enzyme proceeds via a tetrahedral adduct [14–17] formed by attack of the hydroxyl group of Ser-195 on the reactive carbonyl of the substrate. The conformation of this tetrahedral intermediate can be predicted by taking into account the steric restrictions imposed by the enzyme–substrate interactions and on the basis of the stereoelectronic theory recently proposed by Deslongchamps et al. [18–20]. These authors have shown that the tetrahedral intermediate formed during the hydrolysis of esters and *N*-disubstituited imidate salts breaks down in a specific way that is controlled by the orientation of the lone pair orbitals of the heteroatoms bound to the central carbon: 'specific cleavage of a carbon–oxygen or a carbon–nitrogen bond being allowed only if the other two heteroatoms of the tetrahedral intermediate have each an orbital oriented antiperiplanar to the leaving *O*-alkyl or *N*-alkyl group' [19]. From the principle of microscopic reversibility this must also be valid for the reverse reaction, the formation of the tetrahedral intermediate, i.e. the developing lone pair orbitals on the heteroatoms must be antiperiplanar to the new carbon–oxygen or carbon–nitrogen bond. Application to the case of the chymotrypsin-catalyzed hydrolysis of a peptide substrate gives the steric situation depicted in fig.1a. The important point for this discussion is that the lone pair orbital developing on the nitrogen points towards the solvent and the

Table 1
Dissociation constants of enzyme–inhibitor and enzyme–substrate complexes mesured at pH 7.90 and 25°C in 0.2 M sodium chloride

Substrate	K_I (mM)	K_{EA} (mM)
Ac-Phe-Pro-NH_2 (I)	75 ± 5	
Ac-Phe-Sar-NH_2 (II)	64 ± 3	
Ac-Phe-Gly-NH_2 (III)[a]		14.6 ± 0.3
Ac-Phe-Ala-NH_2 (IV)[a]		25.0 ± 1.2

[a] Reported in a previous communication [8].

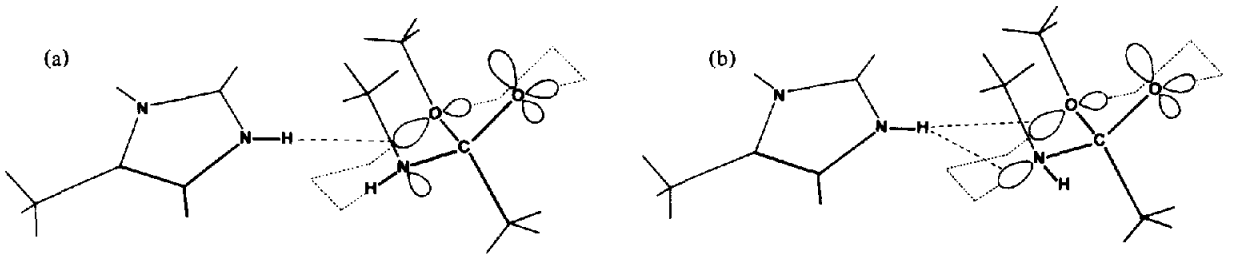

Fig.1. (a) Conformation of the tetrahedral intermediate formed by attack of the hydroxyl of Ser-195 on the reactive carbonyl of a peptide substrate. (b) Conformation of the tetrahedral intermediate after inversion at the nitrogen atom. L_{x1} and L_{y1} denote the aminoacid residues of a peptide substrate linked by the peptide bond to be split. To facilitate the spatial view, the relevant atoms are superimposed to a trans-decalin frame (dotted lines). Possible hydrogen bonds are indicated by dashed lines.

N–H bond towards the inside of the enzyme molecule. When the hydrogen is replaced by an alkyl group, as in I and II, the alkyl substituent would come too close to the imidazole ring of His-57. We therefore conclude that the dipeptides I and II are inactive because steric hindrance prevents formation of the tetrahedral intermediate. Normal substrates have a hydrogen atom instead of an alkyl group pointing towards His-57; there is no steric hindrance and formation of the tetrahedral intermediate is possible.

With regard to the mechanism of the acylation step, the proposed conformation of the tetrahedral intermediate has a further interesting consequence. The stereoelectronic requirements for cleavage of the carbon-nitrogen bond to form the acyl enzyme are fulfilled, but the cleavage step still requires protonation of the nitrogen atom. It is generally accepted that the imidazole of His-57 is the protonating agent. However, in the situation shown in fig.1a, direct proton transfer from the Nε2 atom of His-57 could not occur since the lone pair orbital of the leaving nitrogen points in the wrong direction. Protonation of the leaving nitrogen could, in principle, occur in two ways: (a) The necessary proton transfers (protonation of the leaving nitrogen and deprotonation of Nε2 of His-57) are mediated by the solvent. (b) Inversion at the leaving nitrogen produces a second intermediate in which the orientations of the N–H bond and of the lone pair orbital are interchanged (fig.1b). Such an inversion would not only make direct protonation of the leaving nitrogen by His-57 possible; it would also stabilize the C–Oγ (Ser-195) bond since this would now be antiperiplanar to the N–H bond rather than to the lone pair orbital. Reasons which seem to favor the second alternative, as well as other aspects of the reaction mechanism connected with the stereochemistry of the tetrahedral intermediate, will be discussed in a further publication.

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