THE INTERACTION OF D-AMINO ACID RESIDUES WITH THE AROMATIC BINDING SITE OF α-CHYMOTRYPSIN

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1. Introduction

Chymotrypsin is highly stereospecific in its catalytic task cleaving almost exclusively peptide bonds formed by L-amino acids. With the three-dimensional structure of the substrate binding site known, this specificity has now been characterized in terms of molecular interactions [1]. D-Amino acid residues (e.g. acetyl-D-tryptophan amide), though rejected as parts of substrate molecules, can form strongly reversible interactions with the enzyme's active site [2–4]. It has therefore to be assumed that chymotrypsin, and probably other proteolytic enzymes as well, achieves its stereospecificity not by preferential binding of L-residues but by selectively positioning configurational isomers with respect to the catalytic functionalities of the enzyme. In order to get information about the fit or rather misfit of D-amino acid residues to the active site of chymotrypsin, binding of a series of diastereomeric peptide inhibitors (virtual substrates) of the general structure Z-L-Ala-L-Ala-L (or D)-P_1** and of acetyl-L (or D)-amino acids has been investigated by inhibition studies. The results presented indicate that a D-residue in position P_1 occupies the specificity determining subsite S_1 of the enzyme. Two distinct binding modes for a D-residue to S_1 emerged depending on whether the D-residue is part of a tripeptide inhibitor or in the form of an acetyl-amino acid.

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** Abbreviations according to IUPAC-IUB rules, 'Symbols for Amino Acid Derivatives and Peptides, Recommendations (1971)', see, e.g. J. Biol. Chem. 247 (1972) 977. Additional abbreviations are: Phe(3F) etc., ring-substituted phenylalanines, nature and position of the substituent are given in the parentheses; Ala(1Nph), β-(1-naphthyl) alanine; Ala(6Qui), β-(6-quinolyl)-alanine. The nomenclature of the substrate–enzyme interactions (S_1, S_2,... P_1, P_2,...) is according to Schechter and Berger [5].

2. Materials and methods

α-Chymotrypsin, three times crystallized, salt free and lyophylized, was from Worthington (Freehold, N.J.), batch CDI 2DC. Preparation of enzyme solutions, and active site titrations have been reported elsewhere [6]. Amino acids and derivatives were prepared according to known procedures [7, 8]. Ac-(L-Ala)$_3$-OMe and Ac-L-Tyr-OEt were obtained from Miles Yeda, Rehovot. Succinyl-L-Phe-OMe was prepared by succinylation of Phe-OMe with succinic anhydride at pH 9 in aqueous solution (pH stat). Peptide inhibitors were synthesized by coupling the peptide succinimido-oxy ester Z-L-Ala-L-Ala-ONSu [9] with the appropriate L or D-amino acid. Details of the synthesis have been reported [6]. Initial rates of enzymic hydrolysis were determined by the pH stat method in an assembly by Radiometer, Copenhagen. For further details see ref. [6]. Straight lines in double reciprocal plots were fitted by linear least squares analysis of data points. On the basis of duplicate to quadruplicate measurements $\bar{K}_i$-values are estimated to be reproducible within ± 20%.

3. Results

Reversible inhibition of the α-chymotrypsin-catalized hydrolysis of Ac-L-Tyr-OEt and Succ-L-Phe-OMe by all inhibitors listed in tables 1 and 2 was of the

Table 1
Inhibition constants, $\bar{K}_i$ (= $1/K_i$), and differences in standard free energies of binding, $\Delta\Delta F^\circ$, for pairs of diastereomeric tripeptides Z-L-Ala-L-Ala-L (or D)-P_1*.

Residue P_1	$\bar{K}_i$ (mM^{-1})	$-\Delta\Delta F^\circ$ (kcal/mole)
L-Ala(6Qui)	2.6	0.6
D-Ala(6Qui)	1.0	
L-Phe	4.5	0.7
D-Phe	1.4	
L-Phe(3F)	9.0	0.5
D-Phe(3F)	3.8	
L-Phe($4CH_3$)	13.0	0.7
D-Phe($4CH_3$)	4.1	
L-Ala(1NpH)	16.0	0.8
D-Ala(1NpH)	4.4	
L-Trp	36.0	0.6
D-Trp	13.0	
Z-$(Ala)_2$-Trp LLL	36.0	1.7
Z-$(Ala)_2$-Trp LDL	2.7	

* Inhibition determined at pH 6.5, 25°C and 0.15 μ (KCl) by assaying the esterase activity towards 0.35 mM Ac-L-Tyr-OEt or 1 mM Succ-L-Phe-OMe in presence of increasing amounts of inhibitor. $[E_0]$ was 1.6×10^{-8} M and 5.5×10^{-8} M, respectively. $\bar{K}_i$ values are means of three independent determinations, two of which were against Ac-L-Tyr-OEt.

Table 2
Inhibition constants, $\bar{K}_i$ (= $1/K_i$), and differences in standard free energies of binding, $\Delta\Delta F^\circ$, for pairs of enantiomeric acetylamino acids*.

	$\bar{K}_i$ (mM^{-1})	$-\Delta\Delta F^\circ$ (kcal/mole)
Ac-L-Ala(1Nph)	0.33	−0.5
Ac-D-Ala(1Nph)	0.81	
Ac-L-Ala(2Nph)	1.0	−0.4
Ac-D-Ala(2Nph)	2.1	
Ac-L-Trp**,†	0.37	−0.3
Ac-D-Trp**,††	0.62	
Tfa-L-Trp‡	1.6	−0.4
Tfa-D-Trp‡	3.3	

* See footnote of table 1.
** Data by Johnson and Knowles [3], from equilibrium dialysis.
† pH 6.6, 0.1 M phosphate, 4°C.
†† pH 6.72, 0.1 M phosphate, 4°C.
‡ Data from Smallcombe et al. [13], pH 6.5, 0.1 M citrate, 21° C.

competitive mode. Inhibition constants, $\bar{K}_i$, were obtained from plots of v/v_i vs. $[I]$ at fixed $[S]$ according to the equation $v/v_i = 1 + \bar{K}_i [I]/(1 + \bar{K}_{M(app)} [S])$, where

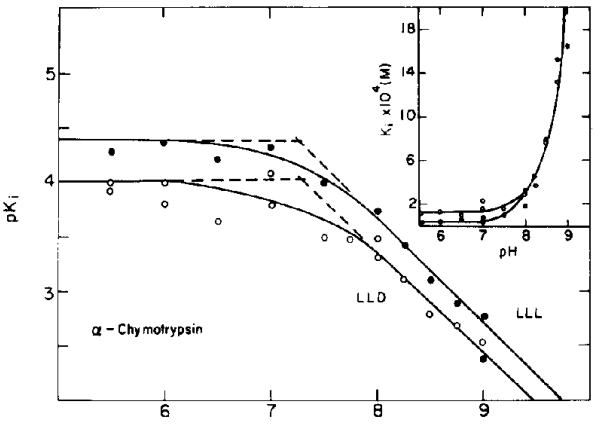

Fig. 1. pH-Dependence of the inhibition by Z-L-Ala-L-Ala-L (or D)-Ala(1NpH) represented according to Dixon [14]. A single protonatable group is determined from the intersection of the tangents (dashed lines).

v is the reaction rate in absence and v_i in presence of a competitive inhibitor. $[S]$ and $[I]$ are the concentrations of free substrate and inhibitor, respectively, which were taken as equal to their total concentrations. $[I]$ was varied as to give 6 to 10 values for v/v_i between about 1.2 and 4. $\bar{K}_{M(app)}$ used in calculating $\bar{K}_i$ was obtained from the usual double reciprocal plots. The stoichiometry of inhibitor binding was tested by plotting $\log(v/v_i - 1)$ vs. $[I]$ according to the equation $\log(v/v_i - 1) = \log\{\bar{K}_i/(1 + \bar{K}_{M(app)}[S])\} + \log[I]$. A one to one interaction of all inhibitors with the active site was indicated by slopes of 1. Values for $\bar{K}_i$ at pH 6.5 and 25°C and $\Delta\Delta F^\circ$, the difference in free energy of binding for diastereomeric inhibitor pairs, are compiled in tables 1 and 2. The pH-dependence of the inhibition was studied in detail for the pair Z-L-Ala-L-Ala-L (or D)-Ala(1Nph). Results are given in fig. 1. Inhibition for both peptides was equally dependent on a single protonatable group of $pK_a = 7.3$. The K_i values for the LLL inhibitor tended to increase more steeply with increasing pH than did those of the LLD inhibitor (inset of fig. 1).

4. Discussion

There is by now ample evidence rom X-ray data [1, 10] that in case of an L-amino acid residue bound to the aromatic pocket of chymotrypsin (S_1 subunit)

the S_1-P_1 interaction may schematically be subdivided into four regions of contact: the interaction of the P_1 side chain (S) with the S_1 pocket (s), the binding of the carbonyl moiety (carboxyl in the case of inhibitors, C) to the catalytic locus (c), the fit of the C_α-proton (H) to a very limited space of the enzyme (h) and the contact area between the acylamido portion (N) and, depending on the size of the acyl group, subsites S_2, S_3 etc. (n). An LLD inhibitor may be bound to the enzyme with either an S_1-P_1 interaction or without. In the following, strong evidence is presented for a preserved S_1-P_1 interaction for tripeptide inhibitors and acetyl-D-amino acids.

Assuming a D-P_1 residue binds to subsite S_1 then two exchanged interactions among the four contact regions are formally needed. The six possibilities can be presented as follows:

L-residue	*Possibilities for D-residue*					
	1	2	3	4	5	6
S:s	H:s	S:s	C:s	N:s	S:s	S:s
C:c	C:c	C:c	S:c	C:s	H:c	N:c
H:h	S:h	N:h	H:h	H:h	C:h	H:h
N:n	N:n	H:n	N:n	S:n	N:n	C:n

Possibilities 1 and 2 must be excluded for steric reasons since they both contain forbiddedn contacts of bulky groups (S and N) with the spatially restricted region h. Possibilities 3 and 4 are characterized by the loss of the hydrophobic S:s contact. Since the series of tripeptide inhibitors varies only in the side chain S of P_1, 3 and 4 would lead to $\Delta\Delta F^\circ$-values which must depend on the structure of P_1. Finally in modes 5 and 6 groups are exchanged (H vs. C and N vs. C) which have a common structure in either one of the two series of inhibitors. $\Delta\Delta F^\circ$ should therefore not depend on the structure of P_1 and should take a constant value for all inhibitor pairs.

In case there is no contact between a D-residue and S_1 neither constant $\Delta\Delta F^\circ$ nor $\Delta\Delta F^\circ$-values are expected which depend on the structure of the P_1-side chain in a similar way as is observed for LLL inhibitors or acetyl-L-amino acids (table 1 and [6]). In addition the pH-dependency of the inhibition might be altered due to removal of the carboxyl group from the catalytic locus. However, direct electrostatic interactions are long range forces [11], and therefore only a grossly altered binding mode would be detected by a change in pH-dependency of $\bar{K}_i$ as, e.g., 'reverse' binding with interactions S_1-P_3, S_2-P_2 and S_3-P_1.

Table 1 shows that $\Delta\Delta F^\circ$ for LLL vs. LLD inhibitors is constant almost within the experimental error which was about ± 0.2 kcal/mole. Binding of LLL peptides is favored by −0.5 to −0.8 kcal/mole. In contrast, a D-residue in P_2 diminishes binding by 1.7 kcal/mole in case of the peptide Z-L-Ala-D-Ala-L-Trp. This correlates well with the known stereospecificity in subsite S_2 [12]. Moreover, inhibition constants are equally dependent on a pK_a of about 7.3 for both diastereoisomers (fig. 1). The slightly steeper increase with pH of K_i's of LLL inhibitors may be interpreted by a longer distance between the carboxyl group of the LLD inhibitor and the charge of the catalytic site. All these observations are best accounted for by an intact S_1-P_1 interaction in mode 5. In this mode the carboxyl group is in sterically unfavorable interaction with h which explains the lowered binding energy. Binding mode 6 is much less plausible for the following reason. Interaction in S_2-P_2, S_3-P_3 and possibly S_4-P_4 (Z-group) contributes at least 2 kcal/mole to the overall binding energy. This value originates from the difference in binding between corresponding inhibitors of the structure Ac-L-P_1 and Z-L-Ala-L-Ala-L-P_1 [6]. It is difficult to imagine how the loss of all the contacts with S_2, S_3 and S_4 with their minimum of at least 2 hydrogen bonds [10] would be so well compensated for by mode 6 where the Z-L-Ala-L-Ala-portion (N) must protrude over and out of the catalytic locus.

Binding of acetyl-D-amino acids (table 2) is again either in mode 5 or 6 for the same reasons as outlined for the tripeptides. Mode 6 is in this case much more likely since binding of the D-enantiomers is favored over that of the L-enantiomers which would disagree with a C:h interaction but would be well explained by a favorable fit of the acetyl group to the catalytic site (N:c). There is additional evidence for mode 6 from ^{19}F-NMR data [4]. The trifluoroacetyl group of Tfa-D-Phe(4F) experiences a different microenvironment than does the Tfa group of the L-isomer. The change in chemical shift has been explained as due to the nearby positive charge of the catalytic locus [4]. Fig. 2 summarizes schematically the proposed binding to α-chymotrypsin of LLD and LLL inhibitors and acetyl-D-amino acids.

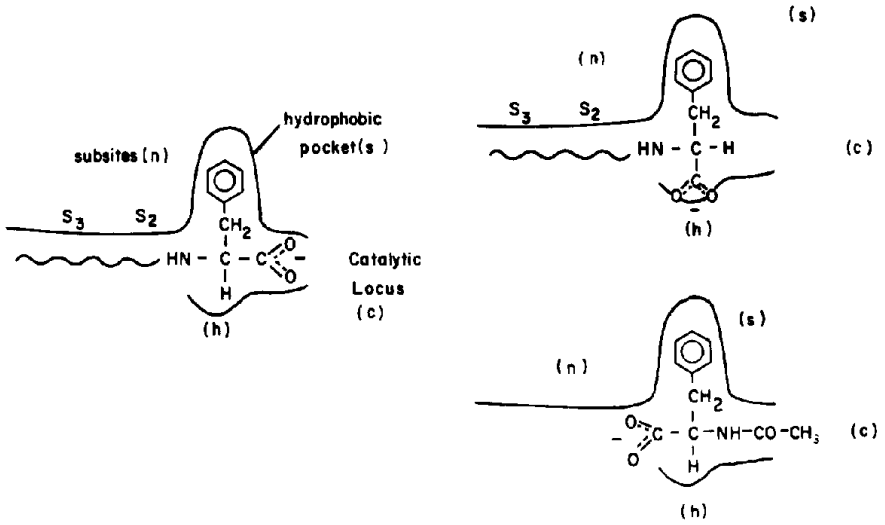

Fig. 2. Schematic representation of the proposed binding of D-amino acid residues of tripeptide inhibitors and acetylamino acids to subsite S_1. Binding of an L-residue is shown on the left.

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