THE BINDING OF SPECIFIC AND NON-SPECIFIC ALDEHYDE SUBSTRATE ANALOGS TO α-CHYMOTRYPSIN

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1. Introduction

Aldehyde analogs of specific substrates have been found to be very effective reversible inhibitors for hydrolytic enzymes [1–3]. For example, the aldehyde analogs of papain [1], elastase [2], and asparaginase substrates [3], have been observed to bind better than the analogous substrates by several orders of magnitude. This tight binding has been postulated to be due to the formation of a tetrahedral 'transition state analog' hemiacetal between the enzyme active-site serine hydroxyl (or cysteine thiol) group and the aldehyde.

The binding of non-specific substrate aldehyde analogs to α-chymotrypsin has recently been reported [4,5]. It was found that the binding of the non-specific aldehyde analog, hydrocinnamaldehyde, was only slightly better than that for the corresponding non-specific substrate, hydrocinnamide [5]. It was argued that becuase of the lack of specificity the hemiacetal formed between active-site serine-195 and hydrocinnamaldehyde would be a poor model for the transition state of α-chymotrypsin-catalyzed reactions whereas it was predicted that specific enzyme-hemiacetals would be good models for this transition state [5]. We have studied the binding of specific substrate aldehyde analogs to α-chymotrypsin in an attempt to test this prediction.

Aliphatic aldehydes exist as hydrates to a substantial extent in aqueous solution [6]. Recently it has been shown that the hydrated aldehydes and not the aldehydes are involved in the binding to the aliphatic amidase isolated from *Pseudomonas aeruginosa* [7]. This finding raises the question as to whether or not the binding of specific substrate aldehyde analogs to hydrolytic enzymes is actually due to hemiacetal formation. The binding of these aldehydes may be due to any of the following possibilities: (1) covalent hemiacetal formation (to either active-site serine or histidene residues); (2) non-covalent aldehyde hydrate binding; (3) non-covalent aldehyde binding. We have spectrophotometrically studied the binding of the non-specific substrated aldehyde analog, *p*-dimethylaminocinnamaldehyde, to α-chymotrypsin in an effort to distinguish which of these modes of binding is involved in the reversible inhibition.

2. Results and discussion

2.1. *The binding of specific and non-specific substrate aldehyde analogs to α-chymotrypsin*

The binding and rate of hydrolysis of α-chymotrypsin substrates is dependent upon the interaction of the α-acylamino group in addition to the primary hydrophobic interaction of the aromatic side chain with the enzyme [10]. This 'secondary specificity' conferred upon the substrate by the α-acylamino group is best illustrated by the increase in specificity constant (k_{cat}/K_M) with the addition of alanyl [11] or leucyl [12] residues. For example, the k_{cat}/K_M values (M^{-1} sec^{-1}) for *N*-acetyl-(L-alanyl)$_n$-L-phenylalanine methyl ester are: 55 000 for n = 0; 330 000 for n = 1; and 2 000 000 for n = 2 [11]. Similarly for *N*-acetyl-(L-leucyl)$_n$-L-tyrosine methyl ester the k_{cat}/K_M values are 185 700 for n = 0 and 2 900 000 for n = 1 [12] while the value for methyl hydrocinnamate is 4.6 [10].

The K_i values for the specific and non-specific

Table 1
Binding constants obtained for the binding of aldehyde substrate analogs to α-chymotrypsin[a]

Aldehyde analog	$K_i \times 10^{-5}$ M
Hydrocinnamaldehyde[b]	30.8
N-Acetyl-L-leucyl-L-phenylalainal	1.77
N-Acetyl-L-leucyl-L-leucyl-L-phenylalinal[c]	0.38
p-Dimethylamino-cinnamaldehyde	51.7

[a] The K_i values were obtained by the inhibition of the hydrolysis of *N*-benzoyl-L-tyrosine-*p*-nitroanilide at pH 7.7 in 8.82% dimethyl sulfoxide ($I = 0.2$) by steady state methods [9].
[b] The previously determined K_i values for this compound at pH 7.8 are 38×10^{-5} M [4] and 79×10^{-5} M [5].
[c] The binding constant for this compound have previously been found to be 5.2×10^{-5} M [8].

aldehyde substrate analogs are shown in table 1. It was found that the binding was enhanced by the addition of leucyl residues. This is the manner in which the binding would be expected to vary if the binding of the aldehyde substrate analogs parallelled the specificity of the corresponding substrates.

The binding of the non-specific substrate aldehyde analog, hydrocinnamaldehyde, has previously been shown to be pH dependent [5]. The K_i values were found to decrease from 5.8 to 0.62×10^{-3} M as the pH was increased from pH 4.5 to 8.3. We have observed a similar pH dependence for the binding of the specific substrate aldehyde analog, *N*-acetyl-L-leucyl-L-phenylalinal [8] α-chymotrypsin (table 2).

The pH dependence of the binding of hydrocinnamaldehyde to α-chymotrypsin was postulated to be due to formation of covalent enzyme–hemiacetal between the active-site serine hydroxyl and the aldehyde [5]. This interpretation of the effect of pH upon binding was based upon earlier observations that the non-covalent binding of specific amides such as *N*-acetyl-L-tryptophanamide to α-chymotrypsin is not pH dependent in a similar pH range. However, there are alternate explanations for the decreased binding of aldehyde substrate analogs at low pH which do not necessarily involve formation of an enzyme–hemiacetal intermediate. For example, the decreased binding at low pH could be due to disruption of hydrogen bonding between the aldehyde (or aldehyde hydrate) and protein amide hydrogens or to disruption of hydrogen bonding between the aldehyde or hydrate and histidine-57.

Table 2
The effect of pH upon the binding of *N*-acetyl-L-leucyl-L-phenylalinal to α-chymotrypsin[a]

$K_i \times 10^{-5}$ M	pH	Buffer
1.9	7.6	Phosphate
8.8	6.0	Phosphate
17.5	4.4	Acetate

[a] The K_i values were obtained from the inhibition of *N*-acetyl-L-tryptophan ethyl ester hydrolysis in 4.68% acetonitrile ($I = 0.2$).

Data from X-ray crystallographic studies of the complexes of α-chymotrypsin with *N*-formyl-L-tryptophan and *N*-formyl-L-phenylalanine indicates that one of the carboxylate oxygens of these inhibitors is hydrogen bonded to histidine-57 [13]. It is possible that aldehyde substrate analogs (either as the free aldehyde or hydrate) also hydrogen bond to histidine-57 and the lowered affinity of the enzyme for the aldehydes at low pH could be a result of the loss of this hydrogen bonding. A similar decreased affinity at low pH has been observed for the binding of substituted boronic acid inhibitors to α- and δ-chymotrypsin [14–16]. The data obtained from nmr studies of the binding of these inhibitors suggests that the decreased binding at low pH is due to loss of a hydrogen bond between the boronic acid oxygen and active-site histidine [16].

The specific substrate aldehyde analogs are the most efficient small molecular weight reversible inhibitors of α-chymotrypsin studied thus far ($K_i = 10^{-5} - 10^{-6}$ M, table 1). However they are not as effective as the papain and elastase specific substrate aldehyde analogs which had binding constants of $10^{-7} \sim 10^{-8}$ M. Indeed, the K_i value of *N*-acetyl-L-phenylalinal (table 1) is of the same order of magnitude as the non-covalent binding constant for the specific ester *N*-acetyl-L-leucyl-L-tyrosine methyl ester ($K_s = 4 \times 10^{-5}$ M) [12].

If these aldehyde specific substrate analogs actually formed hemiacetals with active-site serine-195 which were models for the catalytic transition state (i.e.

'transition state analogs') then the affinity of these aldehydes could be predicted to be several orders of magnitude greater than that observed. Two possible explanations for the discrepancy between the observed affinity of the aldehyde substrate analogs and that predicted by the 'transition state analog' theory are: (1) covalent enzyme hemiacetals are not formed and the binding is due to binding of the free aldehyde or the aldehyde hydrate; and (2) the hemiacetal formed does not approximate the catalytic transition state and therefore is not a good 'transition state analog'.

2.2. *Attempted spectrophotometric observation of intermediates in the binding of p-dimethylaminocinnamaldehyde to α-chymotrypsin*

In an attempt to determine which mode of binding (covalent hemiacetal formation, non-covalent aldehyde binding, or non-covalent binding of the aldehyde hydrate) is involved in teh reversible inhibition of α-chymotrypsin by aldehyde substrate analogs we have spectrophotometrically examined the binding of the chromophoric, unsaturated aldehyde, *p*-dimethylaminocinnamaldehyde. This approach was chosen because the unsaturated aldehydes have absorption maxima at longer wavelengths (~ 50 nm, $\Delta\epsilon \sim 20\,000$) than the analogous alcohols, dimethyl acetals and deoxy derivatives. Also, unsaturated aldehydes are less than 10% hydrated in aqueous solution and the exact extent of hydration may be determined spectrophotometrically [6]. *p*-Dimethylaminocinnamaldehyde was used because its absorption maxima (400 nm, $\epsilon = 27\,850$) should permit detection of the enzyme–hemiacetal intermediate in the presence of high concentrations of enzyme (10^{-3} M) and because of its favorable binding constant (5.17×10^{-4} M, table 1). If the enzyme formed a hemiacetal with this aldehyde the absorbance of the parent aldehyde would be expected to decrease ($\Delta\epsilon_{400} \sim 20\,000$) with the concomitant appearance of the hemiacetal absorbance ($\Delta\epsilon_{350} \sim 15\,000$) at shorter wavelength.

The reversible binding of aldehyde substrate analogs is described by equation 1 where EI is the Michaelis–Menten complex, EI′ is the enzyme–hemiacetal, and K_s is the non-covalent binding constant. Since for *p*-dimethylaminocinnamaldehyde the amount of covalent hemiacetal can be

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EI'; K_s = \frac{k_1}{k_{-1}} \qquad (1)$$

determined by the decrease in absorbance at 400 nm ($EI' = \Delta A_{400}/\Delta\epsilon_{400}$) then the non-covalent interaction may be determined from 2.

$$K_i = \frac{[E]\quad[I]}{[EI] + [EI']}; \quad [EI] = \frac{[E]\quad[I]}{K_i} \text{ —— } \frac{\Delta A_{400}}{\Delta\epsilon_{400}} \qquad (2)$$

If the predominant mode of binding is due to the enzyme–hemiacetal formation ($[EI'] >> [EI]$) then the expected absorbance change can be predicted from equation 3.

$$\Delta A_{400} \cong [EI']\ \Delta\epsilon_{400} \cong \frac{[E]\ [I]\Delta\epsilon_{400}}{K_i} \qquad (3)$$

The attempted spectrophotometric observation of intermediates was accomplished by comparing the spectra of *p*-dimethylaminocinnamaldehyde (3.0×10^{-5} – 1.2×10^{-4} M) in the presence and absence of 1×10^{-3} M α-chymotrypsin at pH 7.7 in 8.82% (v/v) dimethyl sulfoxide. Experimentally, no decrease in the absorbance at 400 nm or increase in the absorbance at 350 nm was observed. This indicates that the predominant mode of binding in this case is non-covalent and K_i approximates K_s.

Although direct evidence for the formation of a covalent hemiacetal was not experimentally obtained the involvement of an enzyme hemiacetal 'transition state analog' in the binding of aldehyde specific and non-specific substrate analogs to α-chymotrypsin cannot be ruled out. However it is possible that the affinity of the substrate aldehyde analogs can be totally accounted for by the following: (1) non-covalent interaction of the free aldehyde (or hydrate) moiety and the enzyme active-site; (2) hydrophobic interaction of the aromatic ring with the hydrophobic subsite; and (3) hydrophobic and hydrogen bonding interactions of the α-acylamino group with the amide binding subsite.

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