THE BINDING OF A NON-SPECIFIC 'TRANSITION STATE ANALOGUE' TO α-CHYMOTRYPSIN

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1. Introduction

The exceedingly good binding constants obtained for substrate analogues with the structural features of an enzyme-substrate reaction transition state have given important information on the mechanism of the enzyme's catalysis [1,2]. For the hydrolase enzymes papain and elastase, L-α-acylamidoaldehydes, which may be transition state analogues of specific substrates of these enzymes, were found to bind 10^3-10^4 times better than their analogous substrates [3,4]. We wish to report on the affinity of hydrocinnamaldehyde between pH 4.5 and pH 8.3 to the active site of the hydrolase enzyme α-chymotrypsin. This aldehyde has a similar structure to hydrocinnamate esters and amides, which are substrates of the enzyme [5,6]. Recently, the binding of this aldehyde to chymotrypsin has been studied at a single pH, and postulated to be a transition state analogue for this enzyme [7]. In contrast, we will argue that the hemiacetal form of this aldehyde is a poor model for the transition state of chymotrypsin catalyzed reactions. We will also offer an explanation for the good binding of aldehyde analogues of specific substrates to serine and cysteine proteases.

2. Results and discussion

The binding of hydrocinnamaldehyde to α-chymotrypsin may be a two step process (eq.1), analogous to the reaction of substrates with α-chymotrypsin [8]. The aldehyde, I, first binds reversibly to form

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

the Michaelis complex (EI), and in a second step forms a hemiacetal with the active site serine-195 (EI') having the tetrahedral configuration found in the enzyme-substrate transition state of chymotrypsin-catalyzed reactions [3,4,8–10]. This mechanism involving hemiacetal formation between aldehyde and the enzyme has been supported previously for the binding of aldehyde inhibitors to elastase [3], papain [4] and chymotrypsin [7]. Based on the scheme of equation 1, equation 2 may be derived. In equations 1 and 2 we make no assumptions as to whether the

$$K_I = \frac{(E)(I)}{(EI) + (EI')} = \frac{K_s}{1 + k_2/k_{-2}} \qquad (2)$$

hydrated or unhydrated form of the aldehyde binds to E. Thompson [3] has shown that for the binding of the hydrated aldehyde $K_{I(obs)} = K_I(1 + K_h)$, where K_h is the formation constant for the aldehyde hydrate in water. If we assume to a first approximation that K_h is independent of remote structural features of the aldehyde [3,11], then we may set $K_h = 1.4$, the constant found for the hydration of acetaldehyde in water [11]. Accordingly, if hydrated aldehyde binds, the true equilibrium binding constant, K_I, will be only 2.4 times smaller than $K_{I(obs)}$. Furthermore, if we assume that step k_2 is general base catalyzed and step k_{-2} general acid catalyzed

by the imidazole group of histidine-57 (pK_a = 7) (eq.3) analogous to the proposed mechanism of substrate catalysis [8,12,13], then the pH dependency of K_I will be predicted by equation 4. In contrast, if

$$k_2 = \frac{k_{2(\lim)}}{1 + (H^+)/K_a}; \quad k_{-2} = \frac{k_{-2(\lim)}}{1 + K_a/(H^+)} \qquad (3)$$

$$K_I = \frac{K_s}{1 + K'/(H^+)}; \quad \text{where } K' = \frac{k_{2(\lim)}}{k_{-2(\lim)}} \times K_a \qquad (4)$$

the aldehyde only bound non-covalently to the enzyme, one would predict the binding to be pH independent between pH 4.5 and pH 8.0, similar to that bound for the non-covalent binding of neutral compounds to the active site of α-chymotrypsin [14].

Fig.1 shows the theoretical curve for K_I vs. pH based on equation 4 and values of $K_a = 10^{-7}$, $K_s = 5.5 \times 10^{-3}$ M, and $k_{2(lim)}/k_{-2(lim)} = 5$. The values for K_s and $k_{2(lim)}/k_{-2(lim)}$ are those which gave the best fit to the experimental data. The points are the experimental values of K_I obtained in this work (table 1). A small deviation from the theoretical line is observed in the region of high pH (pH > 7) where the experimental values of K_I are slightly poorer than predicted. However, it has been previously reported that the binding of negatively charged molecules to

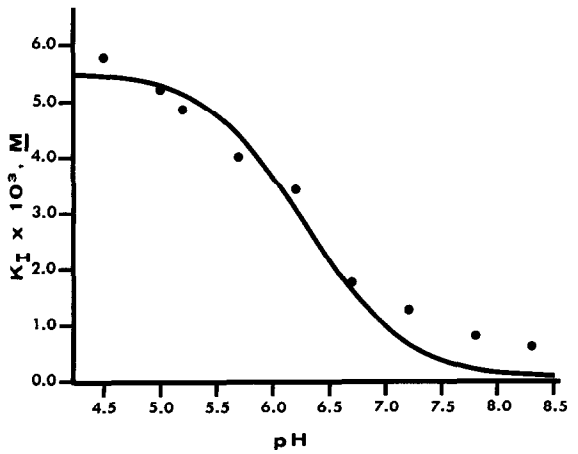

Fig.1. pH Dependency of K_I. Points are experimentally obtained values (see table 1). Line is calculated from equation 4 with $K_a = 10^{-7}$, $K_s = 5.5 \times 10^{-3}$ M, and $k_{2(lim)}/k_{-2(lim)} = 5$.

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

$K_I \times 10^3$, M	pH	Buffer
5.8 ± 0.3	4.5	acetate
5.2 ± 1.4	5.0	acetate
4.8 ± 0.7	5.2	acetate
4.0 ± 0.3	5.7	acetate
3.4 ± 0.1	6.2	phosphate
1.8 ± 0.2	6.7	phosphate
1.3 ± 0.2	7.2	phosphate
0.79 ± 0.15[b]	7.8	phosphate
0.62 ± 0.21[b]	8.3	pyrophosphate

[a] The K_I values were obtained from the inhibition of *N*-acetyl-L-tyrosine ethyl ester hydrolysis by standard steady state techniques [15,16]. Solution conditions: at 25°C, in 11.5% acetonitrile, 0.09 M NaCl, 0.044 M in buffer component.

[b] Corrected for the increase in K_s due to a group in the enzyme of pK_a 8.7 [8].

the active site in α-chymotrypsin is slightly poorer above pH 7 than below, due to the repulsion by a negative charge of the active site above pH 7 where the imidazole group of histidine-57 is uncharged [14,17]. Accordingly, a deviation above pH 7 from the theoretical line in fig.1 may occur if a partial negative or full negative charge is present in the complexes EI and/or EI'. The presence of such a charge in the tetrahedral configuration of the hemiacetal or bound hydrated form of the aldehyde is not unexpected, in view of proposed mechanisms for chymotrypsin which postulate a negative or partial negative charge in the tetrahedral transition state in substrate hydrolysis [10,13].

Bender et al. [12] have argued that for *N*-acetyl-L-tryptophane amide the ratio of first order rate constants for the enzyme-catalyzed hydrolysis, k_e, to the non-enzymic hydrolysis proceeding through the same mechanism, k_n, is 10^8. As hydrocinnamide is a non-specific substrate of the enzyme, the ratio k_e/k_n may be approx. 10^5. Accordingly, if hydrocinnamaldehyde were a good transition state analogue of this substrate, the transition state theory would predict [1,2] that K_I for hydrocinnamaldehyde would be smaller then the K_s for hydrocinnamide by a factor of 10^5. Surprisingly, the K_I found for the

binding of hydrocinnamaldehyde at pH 7.8 is only 7 times better than the binding constant found for the substrate hydrocinnamide [5]. In addition, the stability of EI′ relative to EI ($k_{2(lim)}/k_{-2(lim)} = 5$) is only 4 times that found for the aldehyde hydrate in water ($K_h \approx 1.4$ (11))*.

Accordingly, the covalent hemiacetal intermediate (EI′) for hydrocinnamaldehyde appears to have a stability similar in magnitude to the solution stability of a hydrated aldehyde, and the binding of the hydrated tetrahedral form of hydrocinnamaldehyde (K_s) is not much better than that for hydrocinnamide. These results indicate that α-chymotrypsin does not show any special binding strength to the sp^3 tetrahedral configuration as depicted by the hemiacetal structure of this aldehyde inhibitor. We believe the differences between the relatively strong binding found previously with aldehyde analogues of papain and elastase substrates [3,4], and the relatively poor binding found for hydrocinnamaldehyde to α-chymotrypsin in this work, reflect the differences in substrate specificity on the ratio $k_{2(lim)}/k_{-2(lim)}$. The analogy may be made to ester substrate hydrolysis by α-chymotrypsin, in which substrates form an sp^2 acylserine intermediate during their catalysis by the enzyme [8,12]. The ratio $k_{acylation}/k_{deacylation}$ may be 10^3 times greater for specific substrates of α-chymotrypsin than for less specific substrates, due to the greater effect of specificity on $k_{acylation}$ than $k_{deacylation}$ [12]. Methyl hydrocinnamate lacks the α-acylamido group of specific substrates and, accordingly, can not orient itself precisely in the Michaelis complex with respect to the serine-195 [18,19], leading to its slower rates of $k_{acylation}$ than for specific substrates [12]. It is inferred that the ratio $k_{2(lim)}/k_{-2(lim)}$ may similarly vary with specificity by 10^3; and this factor is reflected in the value of K_I according to eq.4. A factor of 10^3 will explain the differences in K_I found for aldehyde analogues of specific substrates to elastase and papain and of hydrocinnamaldehyde to α-chymotrypsin.

* Rawn and Lienhard [7] have calculated a factor of k_c/k_n of 10^3 for methyl hydrocinnamate. However, the non-enzymic model used was the hydroxide catalyzed hydrolysis of this methyl ester. We believe the non-enzymic model used by Bender [12] is more analogous to the actual enzymic mechanism. In addition, we believe these authors incorrectly compare K_I for hydrocinnamaldehyde to K_h, as they have not corrected for the contribution of the binding of the phenylethyl side chain to K_I. The correction is inherent in the comparison of $k_{2(lim)}/k_{-2(lim)}$ to K_h, as the aldehyde is already bound prior to step k_2.

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