NMR Evidence Against Covalent Attachment of an Aldehyde 'Transition-State' Analogue to α-Chymotrypsin

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Received August 4,1976

Summary. Proton NMR studies on the binding of trans-cinnamaldehyde to α-chymotrypsin indicates that the free aldehyde form of the "transition-state" analogue is complexed to the enzyme. Specifically, the aldehyde porton chemical shift does not change in the presence of enzyme, ruling out binding as the hydrate or hemiacetal. However, line broadening effects are observed, confirming fast to intermediate chemical exchange.

INTRODUCTION

Aldehyde substrate analogues have been shown to bind very effectively to proteolytic enzymes such as elastase [1], papain [2], and α-chymotrypsin [3-5]. It has been argued that aldehydes are tightly bound because they form stable hemiacetals or thiolacetals with an active site cysteine sulfhydry or serine hydroxyl group. These stable tetracovalent complexes can be considered analogues of the transition states in these proteases [6,7].

Recently, it has been shown that the observation of tight binding is not sufficient in itself to allow the classification of an aldehyde inhibitor as a true covalent transition state analogue [3]. Thus, it has been suggested that several specific and non-specific aldehyde substrate analogues do not form covalent hemiacetals with α-chymotrypsin [3]. We wish to present direct NMR evidence supporting the position that a non-specific aldehyde substrate analogue, trans-cinnamaldehyde, [3] binds as the free aldehyde to α-chymotrypsin (α-CT).

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*Fellow of the Alfred P. Sloan Foundation

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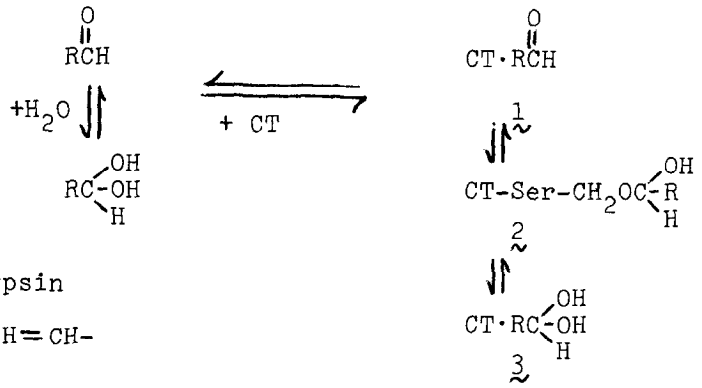
METHODS

Three-times recrystallized α-chymotrypsin (Sigma) was dialyzed at pD 3.00 and 4° vs. 0.01M EDTA (16 hrs.) in D_2O and doubly distilled D_2O (2x, 12hr each), followed by lyophilization.

The NMR samples contained 1 mM chymotrypsin, varing amounts of redistilled trans-cinnamaldehyde (Aldrich) and 1mM EDTA in 10% dimethyl sulfoxide-d_6/D_2O, .1M phosphate buffer. Proton NMR spectra were run on a Bruker HFX-90 spectrometer using block averaging in the Fourier Transform Mode, T = 25°.

RESULTS AND DISCUSSION

As shown in the following scheme, trans-cinnamaldehyde can possibly bind to α-chymotrypsin either as the free aldehyde, 1, hemiacetal, 2, or hydrate, 3.



CT= α-chymotrypsin

R= trans-$C_6H_5CH{=}CH$-

Binding as the hydrate must also be considered since it has recently been shown that actually the hydrated aldehydes are bound to an aliphatic amidase of Pseudomonas Aeruginosa [8].

The chemical shift of the aldehydic proton of the α-CT trans-cinnamaldehyde complex should provide direct evidence for the involvement of the hemiacetal or hydrate since the chemical shift of the directly bonded proton to the sp^3 hydridized carbons in 2 or 3 should be shifted 3-4 PPM upfield from the aldehyde proton [9]. Large shifts such as these are easily observed in enzyme-inhibitor NMR experiments [10].

As shown in Figures 1 and 2, within the experimental error (ca. ± .5 Hz) the chemical shift of the aldehyde proton does not shift with increasing enzyme to inhibitor (E_0/I_0) ratios. In fact, there may be a slight downfield shift with increasing E_0/I_0 ratios. Since the inhibition constant at pH 7.6

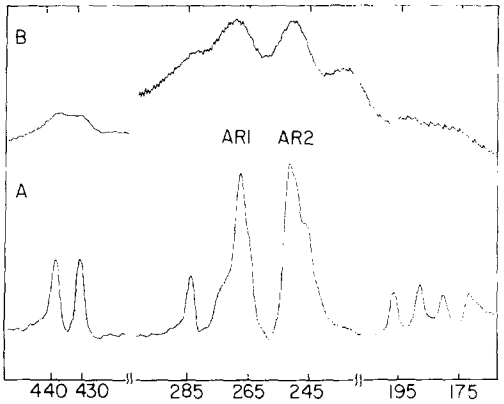


Figure 1. Aldehyde doublet (430-440 Hz from HDO), aromatic signals (245-285 Hz), and vinyl signals (175-195 Hz) of 2mM cinnamaldehyde (A) and 2mM cinnamaldehyde (A) and 2mM cinnamaldehyde, 1mM α-CT (B) in 10% DMSO-d_6/D_2O, pD = 5.5, 0.1 M phosphate.

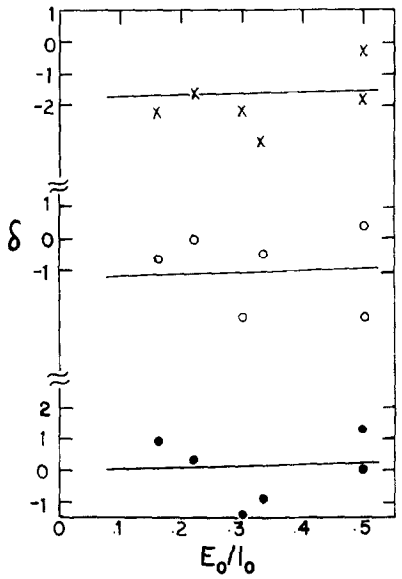


Figure 2. Chemical shift difference (in Hz, from free cinnamaldehyde) vs. enzyme to inhibitor ratio for aromatic signal AR2 (X), and aldehyde doublet (O, downfield signal; ●, upfield signal).

for trans-cinnamaldehyde is 2.6 mM [3], the α-CT will be nearly saturated with the inhibitor in the NMR experiments.* If the trans-cinnamaldehyde exchanges between the free solution environment and the active site of the enzyme:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot I \ , \qquad (1)$$

then the observed chemical shift of the aldehyde proton, δ_{obs}, will be a weighted average of the chemical shift of the E·I complex, δ_{EI}, and the free solution, δ_I. Assuming $(E \cdot I) \sim E_0$, it may be shown [10] that the observed chemical shift will be directly proportional to the differences in the chemical shifts between the two sites, $\Delta (= \delta_{E \cdot I} - \delta_I)$, and the E_0/I_0 ratio:

$$\delta_{obs} = \delta_I + \Delta \cdot E_0/I_0 . \qquad (2)$$

Since Δ should be 270-360 Hz (at 90 MHz) if the aldehyde were bound as either the hydrate or the hemiacetal, then upfield shifts of >100 Hz should be observed under the maximal conditions of the NMR study. The cinnamaldehyde must therefore bind only as the free aldehyde (at least >99% in the aldehyde form).

As already indicated, eq 2 is only valid under fast exchange conditions [11], where the rate of dissociation of the E·I complex (k_{-1}) satisfies $k_{-1} > 2\pi \cdot \Delta$. This condition is probably valid as suggested by comparing similar protein-small molecule rate constants [12]. However, a better verification of this condition is provided by analysis of the line-broadening effects. Although binding does not lead to any significant change in the chemical shift of the aldehydeic proton (or, as shown in Figure 2, even the aromatic or vinyl protons), the line widths of these signals does appreciably increase in the presence of enzyme (Figures 1 and 3). The line broadening in excess of the line widths of the free aldehyde, $\omega_{1/2}$, is approximately

* In the absence of DMSO. The value will be slightly different under the conditions of our experiment.

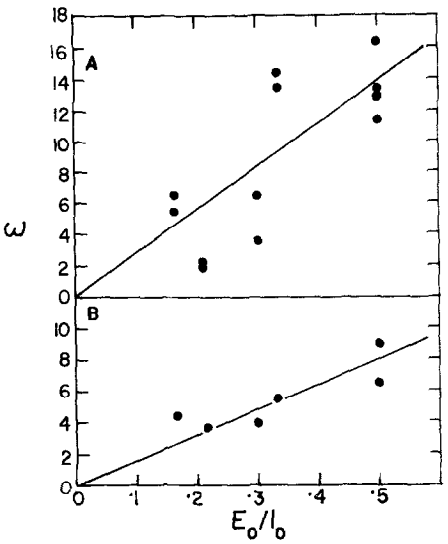


Figure 3. Excess line width vs. enzyme to inhibitor ratio for aromatic signals (upper plot) and aldehyde signals (lower plot).

given by

$$\omega_{1/2} = \Delta\omega_{1/2} \cdot E_0/I_0, \qquad (3)$$

where $\Delta\omega_{1/2}$ is the line width of the signal in the E·I complex in excess of the free solution signal line width. Again fast exchange and near saturating conditions are assumed* [12]. According to Eq. 3, a plot of the observed excess line broadening against E_0/I_0 should yield a straight line passing through the origin. The aldehyde and aromatic excess line widths for the E·I complex obtained from Figure 3 are 16 and 28 Hz respectively. Although the commercial enzyme titrates for only ca. 80% activity, no correction for

*These conditions will not strictly be valid for these experiments. However, the accuracy of the data does not warrant a more exact analysis [12-14]. Even though some small molecule inhibitors may bind to α-CT with exchange broadening contributing significantly to the line broadening [13,14], exchange averaging of signals is still always observed under these intermediate exchange conditions.

100% concentration of active sites [15] or dimerization [16] has been included in this analysis. The broadening of the aromatic signals (actually several overlapping lines) is similar to the effect of α-CT on trans-cinnamate [15], (both inhibitors having similar inhibition constants). As in the trans-cinnamate study [15], the line widths of the inhibitor are also broadened in the presence of the chemically modified enzyme, diisopropylphosphoryl chymotrypsin. However, the line broadening is independent of the E_0/I_0 ratios and ca. 80% smaller in the modified enzyme at $E_0/I_0 = .5$.

The observation of specific line broadening effects in the trans-cinnamaldehyde, α-CT solution thus confirms that exchange averaging is fast enough that chemical shift changes would be observed if the chemical shifts of the bound inhibitor were significantly different from those in the free solution. As earlier suggested [3,5], trans-cinnamaldehyde and smaller non-specific substrate analogues are likely not bound as tetrahedral, transition state analogues to α-chymotrypsin. Whether other more tightly bound, specific aldehyde substrate analogues also can no longer be considered adequate "transition-state" models, remains to be determined [18]. Certainly NMR studies should significantly contribute to a clarification of this problem.

ACKNOWLEDGEMENT. Support of this research by NIH (GM-17575) is gratefully acknowledged.

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