hydrolase is particularly pertinent. Several amino-hydrolases are known which catalyze the hydrolysis of cyclic amidine groups to amides in cytosine, purines, and their derivatives. Two enzyme reactions which involve amide aminolysis are the transfer of formyl groups from $N^{10}$-formyltetrahydrofolate to phosphoribosylaminoimidazolecarboxamide, on the pathway of purine biosynthesis, and from the same coenzyme to methionyl-tRNA, for the initiation protein synthesis in bacteria. There is apparently little information available on the mechanisms of these enzyme reactions at the present time. It is of interest that in recent work by Caplow the substituent effects on the hydrolysis of esters and anilides by chymotrypsin have been interpreted as evidence for the existence of tetrahedral intermediates during the enzymatic hydrolysis of these compounds.[36]

**Acknowledgments.** The author is grateful to Dr. W. P. Jencks for helpful discussions and for the use of a stopped-flow apparatus. The ultraviolet spectrum of N,N′-diphenyl-2-carboxyimidazolidine was kindly provided by Dr. R. G. Kallen. Competent technical assistance was given by Miss Libby Servello.

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# Nuclear Magnetic Resonance Studies of the Interaction of *trans*-Cinnamate with α-Chymotrypsin

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**Abstract:** A high-resolution proton magnetic resonance study of the Michaelis complex formed between cinnamate ion and the proteolytic enzyme, α-chymotrypsin, has been carried out. It has found that the aromatic proton resonances of the complexed acid anion are shifted upfield 0.6–0.8 ppm while the vinyl protons are changed very little. Line-broadening effects are consistent with the suggestion that this acid is bound tightly enough to the enzyme that it does not have any freedom of motion independent of the motion of the enzyme. Experiments with enzymes which have been chemically modified at the active site indicate that this site is also the locus of the cinnamic acid–enzyme interaction.

The mechanism of the α-chymotrypsin-catalyzed hydrolysis of esters and amides can be represented by eq 1.[2] Although likely an oversimplification, this representation is intended to portray, as the initial phase of the reaction, a reversible complexation of the substrate, S, with the enzyme, E, to give an enzyme–substrate complex, ES, within which the acyl group of the substrate is transferred to the protein and the alcohol or amino product ($P_1$) is released. The

$$E + S \rightleftharpoons ES \rightleftharpoons ES' \rightleftharpoons EP_2 \rightleftharpoons E + P_2 \quad (1)$$
$$\qquad\qquad\quad + P_1$$

resulting acylated enzyme, ES′, undergoes hydrolysis to form the product acid ($P_2$) and to regenerate the free enzyme. An additional equilibrium involving a complex between the enzyme and the acid product ($EP_2$) may intervene along the reaction paths leading from the acylated enzyme to products.

A reliable understanding of the physico-chemical interactions which are responsible for the specificity with which substrates and products are attracted to this enzyme is not yet available although a considerable amount of data has been accumulated. It now appears that magnetic resonance spectroscopy will play an important role in clarifying the nature of these interactions[3–6] since the parameters which characterize a magnetic resonance spectrum can reflect not only the nature of a spin's environment but also the time stability of this environment.

A number of elegant experiments by Bender and coworkers have shown that, when the acyl group is cinnamoyl, the corresponding acylated enzyme (ES′) is stable under a rather wide range of conditions.[7] Moreover, *trans*-cinnamic acid (I) is a good competitive inhibitor of the enzyme and thus participates in an enzyme–product equilibrium of the type shown in eq 1.[8,9] The cinnamoyl-α-chymotrypsin system is fairly well understood from the viewpoints of traditional kinetic and ultraviolet spectroscopic experiments and is, therefore, a logical choice for further investigation by magnetic resonance techniques. We describe here the results of a high-resolution nmr study of the interaction of the anion of cinnamic acid with bovine α-chymotrypsin.

(1) On leave from the College of Wooster, Wooster, Ohio, 1968–1969.
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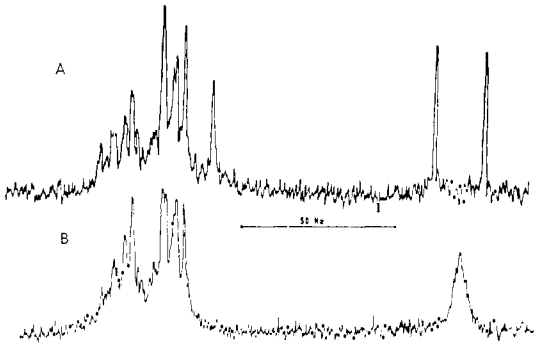
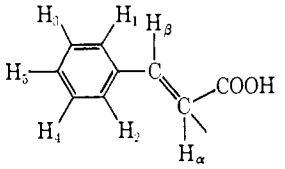


Figure 1. The pmr spectra of (A) *trans*-cinnamic acid and (B) β-deuterio-*trans*-cinnamic acid at 100 MHz.



## Results

The proton magnetic resonance spectrum of cinnamic acid at 100 MHz in a phosphate-buffered (pH 7.4) deuterium oxide solution is shown in Figure 1 (trace A). The vinyl resonances appear as an AB quartet; the high-field portion of this quartet is assigned to the proton nearest to the carboxyl group. The low-field doublet of this quartet corresponds to the β-vinyl proton and is partially intertwined with the signals of the aromatic ring protons. This assignment was confirmed by synthesis of *trans*-cinnamic acid specifically deuterated at the β-vinyl position. As can be seen in trace B of Figure 1, the spectrum of this deuterium-labeled compound is simplified to the extent that the upfield doublet collapses to a broad singlet and the downfield portion of the vinyl quartet disappears. The aromatic proton section of the pmr spectrum of β-D-*trans*-cinnamic acid was analyzed by the Swalen–Reilly method;[10] the resulting chemical shifts and coupling constants for these nuclei and the vinyl protons are recorded in Table I. Although high accuracy cannot be claimed for these data, they are sufficiently precise to identify the signals from each nucleus and to afford a computed theoretical spectrum of the aromatic proton that is in acceptable agreement with the experimental one (Figure 2).

The pmr spectrum of *trans*-cinnamic acid undergoes several interesting changes when the enzyme, α-chymotrypsin, is present in the solution; both line broadening and differential shifting of the peak positions are observed. The considerable line-broadening effect made it impossible to apply the iterative procedure in order to obtain the spectrum parameters in the presence of the protein.

Both the vinyl and aromatic parts of this acid are stereochemically rigid. We, therefore, made the reasonable assumption that the spin–spin coupling constants will not change when the carboxylic acid binds to the enzyme and supposed that the changes in the position of the major peaks of the spectrum reflect variations in chemical shifts induced by the enzyme.

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(11) S. L. Manatt and M. T. Bowers, *J. Amer. Chem. Soc.*, 91, 4381 (1969).

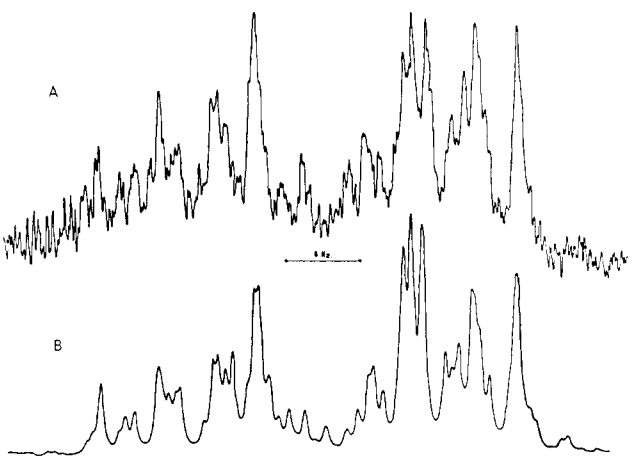

Figure 2. The aromatic region of the pmr spectrum of β-deuterio-*trans*-cinnamic acid. Ten scans were time averaged to give trace A. Trace B is the theoretical spectrum calculated with the parameters in Table I.

**Table I.** Proton Magnetic Resonance Parameters for *trans*-Cinnamic Acid[a]

| A. Chemical Shifts[b] | |
|---|---|
| $\nu_1 = \nu_2 = 4.886$ | $\nu_\alpha = 3.791$ |
| $\nu_3 = \nu_4 = 4.727$ | $\nu_\beta = 4.662$ |
| $\nu_5 = 4.704$ | |
| **B. Coupling Constant (Hz)[b]** | |
| $J_{12} = 1.94$ | $J_{34} = 1.44$ |
| $J_{13} = J_{24} = 7.92$ | $J_{35} = J_{45} = 6.96$ |
| $J_{14} = J_{23} = 0.3$ | $J_{\alpha,\beta} = 15.8$ |
| $J_{15} = J_{25} = 1.51$ | |

[a] A 0.08 *M* solution of *trans*-cinnamic acid or its β-deuterio derivative in deuterium oxide solution buffered to pH 7.4 (phosphate) was used. Internal dimethyl sulfoxide (5% by volume) was used to provide a reference and spectrometer lock signal. [b] Parts per million from internal dimethyl sulfoxide; estimated accuracy of the chemical shifts ±0.2 Hz and the coupling constants, ±0.1 Hz.

If the binding of cinnamic acid to α-chymotrypsin can be represented by the simple equilibrium below, then the change in a chemical-shift parameter relative

$$EA \underset{k_r}{\overset{k_f}{\rightleftharpoons}} E + A \qquad (2)$$

to its value in the absence of enzyme is given by eq 3,

$$\Delta = \frac{E_0 \delta_{EA}}{A_0 + K_A} \qquad (3)$$

where Δ is the observed change in the chemical shift, $E_0$ and $A_0$ are the total concentrations of enzyme and acid, respectively, $K_A$ ($= k_f/k_r$) is the dissociation constant characteristic of equilibrium 2, and $\delta_{EA}$ is the chemical shift of the nucleus under consideration in the enzyme–acid complex.[3,12] Equation 3 is valid only if the exchange rates are sufficiently rapid to average the

(12) (a) J. T. Gerig, *ibid.*, 90, 2681 (1968); (b) F. W. Dahlquist and M. A. Raftery, *Biochemistry*, 7, 3269 (1968).

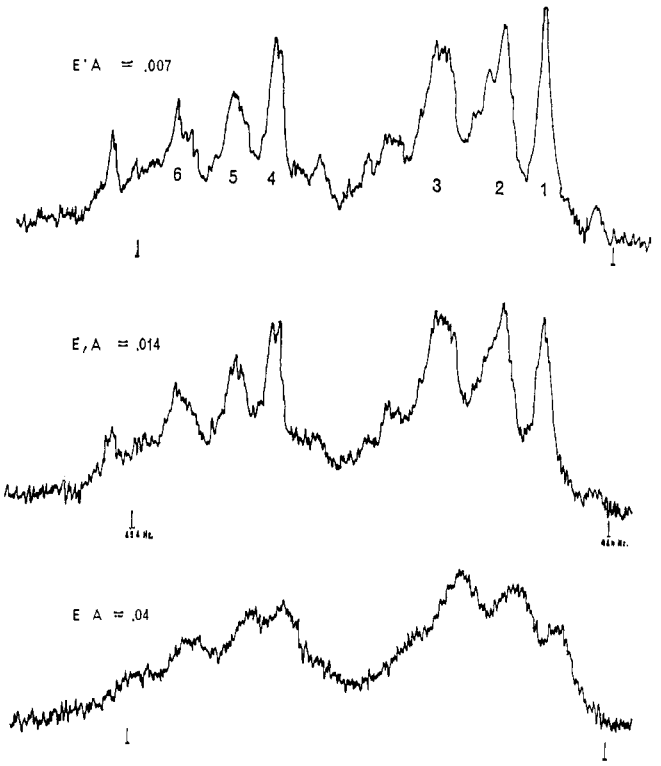


Figure 3. Several typical spectra illustrating the position and line-broadening effects of α-chymotrypsin on the aromatic proton region of β-deuteriocinnamic acid. The frequencies noted are relative to the DMSO lock signal.

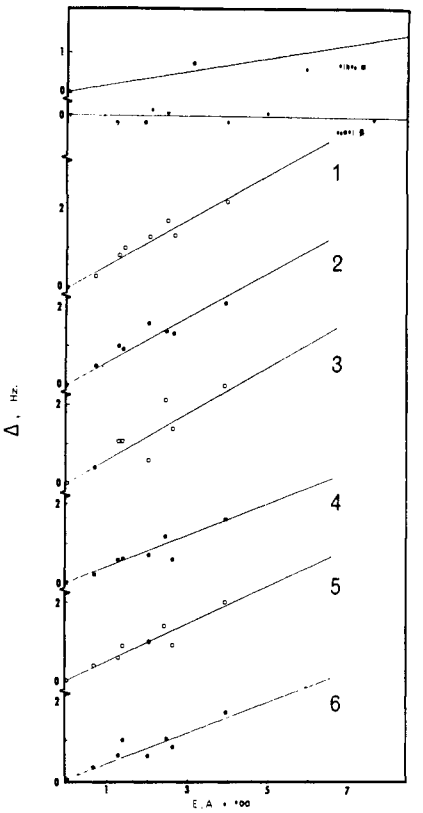


Figure 4. Plots of the enzyme-induced chemical shift changes, Δ, as a function of the enzyme:acid ratio. Additional data for the α-vinyl proton were obtained at higher E:A ratios than shown and lie on or near the line drawn in the figure.

chemical shifts of the free and bound forms of the acid. When $K_A$ is small relative to $A_0$, a plot of $\Delta$ *vs.* $E_0/A_0$ should be linear, pass through the origin, and have a slope equal to $\delta_{EA}$.

We have determined the chemical shifts of the various proton signals of cinnamate as a function of the enzyme–acid concentration ratio at pH 7.4; several typical spectra are shown in Figure 3. The vinyl chemical shifts were obtained by direct measurement while the positions of the six major bands indicated in Figure 3 in the aromatic region of the spectrum were taken to represent changes in the chemical shifts of these nuclei.

**Table II.** Chemical-Shift Parameters for the Cinnamic Acid–Chymotrypsin Complex[a]

| | $\delta_{EA}$ | Average, Hz | Corrected, Hz[b] |
|---|---|---|---|
| A. *ortho* protons | | | |
| Peak 6 | 41 | 45 | 60 |
| Peak 5 | 51 | | |
| Peak 4 | 42 | | |
| B. *meta, para* protons | | | |
| Peak 3 | 64 | 60 | 80 |
| Peak 2 | 57 | | |
| Peak 1 | 58 | | |
| C. β-Vinyl protons | ~0 | ~0 | ~0 |
| D. α-Vinyl protons | 14 | 14 | 19 |

[a] All shifts were to higher field relative to the position of the signal in the absence of enzyme. [b] Corrected to 100% concentration of enzymic active sites; only about 75% of the gravimetric concentration of the enzyme is catalytically active: *cf.*, M. L. Bender, *et al.*, *J. Amer. Chem. Soc.*, **88**, 5890 (1966).

The phenyl proton resonances were usually studied with the β-deuterated acid in order to avoid problems caused by the overlap of the low-field part of the vinyl quartet. Plots of $\Delta$ *vs.* $E_0/A_0$ for the two vinyl signals and the six components of the aromatic spectrum were prepared and, as shown in Figure 4, were found to be linear within the estimated accuracy of the experiment. The slopes of these plots are recorded in Table II. Because of the considerable scatter in the data, these slopes cannot be highly accurate. Nevertheless, the tendency for the aromatic proton chemical shifts to be much more sensitive to the concentration of protein than the chemical shifts of the vinyl protons is quite apparent.

Since its resonance pattern was simple, the doublet assigned to the α proton of the acid was used in an attempt to evaluate quantitatively the line-broadening effect. Under the same conditions for which eq 3 is valid, the line broadening in excess of the line widths of the free acid is given by

$$\delta_w = \frac{E_0}{A_0 + K_A} W_{EA} \qquad (4)$$

where $\delta_w$ represents the excess line width and $W_{EA}$ is the line width of the signal in the EA complex.[12] Theory predicts that the two lines of this doublet should have different widths[13] but no detectable difference was found in the $W_{EA}$ values for these two peaks. The slope of a plot of $\delta_W$ *vs.* $E_0/A_0$ was linear with slope 7.4 Hz (9.9 Hz, corrected to 100% enzyme activity).

(13) A. Abragam, "The Principles of Nuclear Magnetism," Oxford University Press, London, 1961, p 509.

The derivation of eq 3 and 4 rests on the assumption that the rate of interchange between the free and enzyme-bound environments of the cinnamate ion is rapid enough to average truly the nmr parameters characteristic of both states. For the chemical shifts, the requirement is met if [5,14]

$$k_f \gg \pi W_{EA},\delta_{EA} \quad (5)$$

We have examined representative enzyme–acid solutions over the temperature range 25–55° and find no significant variation in the line positions relative to those of the free acid. The rate constants for exchange, $k_f$ and $k_b$, should be temperature dependent and the fact that no large temperature effects on the enzyme–cinnamic acid spectra were observed would seem to indicate that these rate constants are of a magnitude such that the conditions expressed by eq 5 are, at least, approximately satisfied.

In order to help elucidate the locale on the chymotrypsin molecule which interacts with cinnamic acid, several chemically modified enzymes were examined. The serine-195 tosyl[15] and diisopropylphosphoryl[16] esters of the enzyme did not produce any chemical-shift effect. Likewise, the proteins obtained by alkylation of the methionine-192 residue with $\alpha$-bromacetanilide[17] or by alkylation of histidine-57 of the enzyme with L-1-tosylamido-2-phenylethyl chloromethyl ketone[18] were also ineffective in shifting the proton signals of cinnamic acid. The presence of any of these four modified enzymes did result in some line broadening in the spectrum, but, since this same effect could be obtained with an equivalent concentration of the presumably inert protein, ovalbumin,[19] it was ascribed to a bulk viscosity effect.

## Discussion

We have found that when *trans*-cinnamate ion is complexed with $\alpha$-chymotrypsin, the aromatic proton signals of the anion are shifted upfield 0.6–0.8 ppm while the vinyl protons are only slightly effected. A line-broadening effect also occurs. There is no evidence of these spectral changes when any of four enzymes chemically modified at the active site are used in place of the native enzyme, so that the enzymic active site must be included in any rationalization of these spectral effects.

The contribution of the dipolar mechanism to the nuclear relaxation rate, $1/T_2$, of a pair of AB nuclei is given by eq 6a and 6b,[13] where $r$ is the distance between the two nuclei, $\tau_c$ is a correlation time, and $\varphi$ is the angle whose tangent is the coupling constant, $J_{AB}$,

$$\frac{1}{T_2}\,(\text{strong lines}) = \frac{17\gamma^4h^2}{20r^6}\,\tau_c\left(1 + \frac{7}{17}\sin\varphi\right) \quad (6a)$$

$$\frac{1}{T_2}\,(\text{weak lines}) = \frac{17\gamma^4h^2}{20r^6}\,\tau_c\left(1 - \frac{7}{17}\sin\varphi\right) \quad (6b)$$

divided by the chemical-shift difference between the two nuclei. For the vinyl AB quartet of cinnamic acid, $\sin\varphi \approx 0.18$ at 100 MHz and it is computed that the strong central lines of this quartet should be about 12% broader than the weak outer lines when the acid is bound to $\alpha$-chymotrypsin. As indicated above, our measurements of line broadening were not precise enough to distinguish reliably any difference in the $W_{EA}$ parameters for the two $\alpha$-vinyl signals since it is likely that experimental errors lead to an uncertainty of at least this order of magnitude. Neglecting the part of eq 6 in the parentheses and taking 3.15 Å as the distance, $r$, between the vinyl protons in cinnamic acid, it can be computed from the line-width parameter recorded above that the correlation time for the vinyl portion of cinnamic acid in the enzyme–acid complex is $\sim 6 \times 10^{-8}$ sec. This quantity is close to that expected from hydrodynamic theory[20] and we conclude on this basis that *trans*-cinnamate, like D- and L-tryptophan,[12] is bound tightly enough to the enzyme in this complex that it has little motional freedom independent of the motion of the enzyme molecule as a whole. Other mechanisms, including dipolar coupling to the protons of the enzyme, may contribute to the relaxation of these nuclei and until further experimental information is available on this point, the conclusion must be regarded as tentative.[21]

The properties of the environment that hold the cinnamic acid molecule so firmly are indicated by the chemical-shift data reported above. The significant upfield chemical shifts experienced by the aromatic ring protons of the acid molecule upon complexation seem to be too large to ascribe to any influence other than a magnetic field associated with the induced "ring current" of an aromatic ring on the enzyme.[22]

Accepting the notion that a local interaction between cinnamic acid and an aromatic ring on the enzyme is responsible for the major portion of the observed chemical shifts in the EA complex and the validity of the classical electron-in-a-loop description of the ring current effect,[23] one can find several arrangements of two aromatic rings that semiquantitatively are consistent with the data presented above. Two such arrangements are sketched in Figure 5. No great reliance can be placed on the quantitative features of these sketches; the important points seem to be that the ring-current effect can quite reasonably account for the observed chemical-shift effects and that the distance between the aromatic ring on the enzyme responsible for these shifts and the phenyl ring of cinnamic acid is about 4 Å. Space-filling models[24] indicate that a benzene ring is about 3.1 Å "thick" and thus the interaction postulated here must involve near contact of the two aromatic rings. This situation would likely be the case if the two hydrocarbon components interact by a

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(17) H. Schramm and W. B. Lawson, *Z. Physiol. Chem.*, 332, 97 (1963).
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(21) It should be possible to confirm the magnitude of the correlation time by a study of the deuterium spectrum of the $\beta$-deuterated acid and/or the line shape of the $\alpha$-vinyl proton in the same compound; this possibility is being explored.
(22) (a) Reference 20, p 140; (b) J. D. Memory, "Quantum Theory of Magnetic Resonance Parameters," McGraw Hill Book Co., Inc., New York, N. Y., 1968, p 111.
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(24) Corey–Pauling–Koltun models were used.

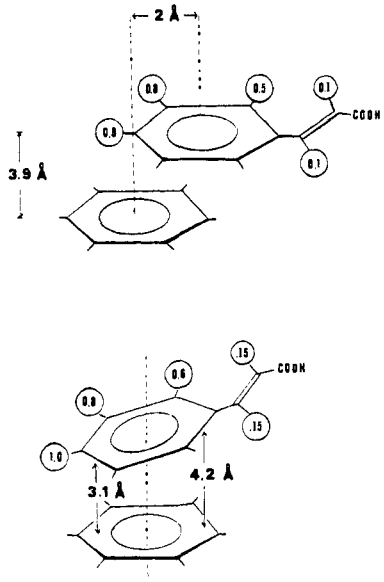


Figure 5. Two possible arrangements of a cinnamic acid–aromatic ring complex that are consistent with the chemical shifts reported in Table II.

water exclusion ("hydrophobic") mechanism. There is a considerable body of evidence that points to the existence of nonpolar binding locations at or near the active site of α-chymotrypsin[25–28] and it is reasonable to presume that one of these is used to aid in binding cinnamate to the enzyme.

It will be of interest to compare the observations and conclusions described here with those obtained from a similar study of the cinnamoyl acylated enzyme; work toward this end is now in progress.

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## Experimental Section

*trans*-**Cinnamic acid** was obtained from Matheson Coleman and Bell and used without further purification.

β-**Deuterio**-*trans*-**cinnamic acid** was synthesized by treating benzaldehyde-$d_1$[31] with a slight molar excess of the ylide derived from ethyl α-bromoacetate and triphenylphosphine.[32] The reaction mixture heated at reflux overnight. The benzene was removed by distillation whereupon water and 1.5 *M* $H_2SO_4$ were added to the cooled residue. The precipitate was stirred with petroleum ether (bp 30–60°); the petroleum ether was filtered and then removed by distillation. The residue was dissolved in aqueous sodium hydroxide solution and then precipitated with sulfuric acid. This process was repeated several times. The crude product melted at 130–132° and was recrystallized twice from methanol–water to give a white, crystalline material, mp 132–133.5°.

**Bovine α-chymotrypsin** (recrystallized three times) was obtained from Worthington Biochemical Corp.

A buffer solution (100 ml) was prepared by dissolving 5.0 ml of dimethyl sulfoxide, 2.72 g of $KH_2PO_4$, and 9.92 g of $K_2HPO_4 \cdot 3H_2O$ in deuterium oxide (Stohler Isotope Chemicals). The observed pH of the buffer (glass electrode) was 7.41. Stock solutions of the cinnamic acids (0.080–0.083 *M*) were prepared and used to dissolve known amounts of the crystalline enzyme.

The pmr spectra were obtained with a Varian Associates HA-100 equipped with a C-1024 computer-of-average transients. The dimethyl sulfoxide in the buffer solution was used as a lock signal. Ambient probe temperature was 33 ± 1°. The HOD signal was observed at 207 ± 1 Hz downfield from the lock; this signal was recorded before and after each spectrum was accumulated as a check for changes in resolution during the experiment. The output sweep ramp of the C-1024 was used to drive a Wavetek Model 111 voltage-controlled oscillator which was utilized as the observing frequency in frequency-sweep mode. The output of the oscillator was determined on several points in the sweep with a Varian frequency counter. It is estimated that the absolute accuracy of the determination of peak position is at least ±0.3 Hz, this being the reproducibility of absolute peak positions in a 0.082 *M* solution of cinnamic acid, observed a number of times over a period of 1 year.

**Acknowledgments.** We are indebted to the National Institutes of Health (Grant No. CA-11220 and FR-07099), the National Science Foundation (Grant No. GP-8166) and the Petroleum Research Fund (travel grant to J. D. R.), administered by the American Chemical Society, for support of this research.

(31) K. B. Wiberg, *J. Amer. Chem. Soc.*, 76, 5371 (1954).
(32) The authors thank Dr. P. O. Crews for supplying the sample of this ylide.