to give ii and either a sequential or concerted ring opening concomitant with the loss of a second molecule of methanol. In contrast, simple alkoxyfurans are known to hydrolyze by a process involving 1,4 addition of water: J. E. Garst, *J. Org. Chem.*, **39**, 2920 (1974).

(7) Although the conversion of **8b** to **11b** appears in a formal sense to be in violation of "Baldwin's rules", it has recently been pointed out that these rules do not necessarily obtain with oxygen nucleophiles under acid catalysis: J. E. Baldwin, R. C. Thomas, L. I. Kruse, and L. Silberman, *J. Org. Chem.*, **42**, 3846 (1977).

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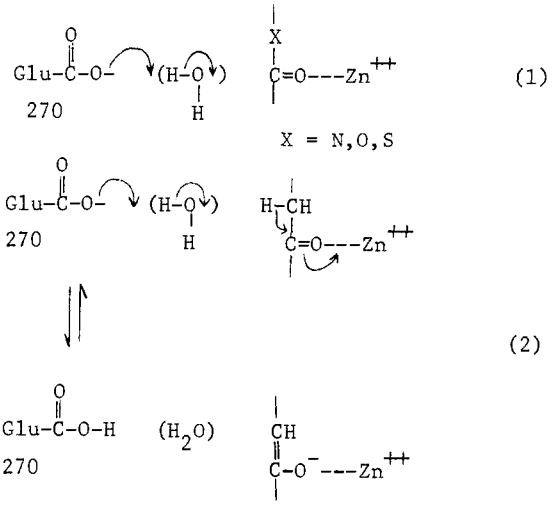
Carboxypeptidase A Catalyzed Enolization of a Ketonic Substrate. A New Stereochemical Probe for an Enzyme-Bound Nucleophile

Sir:

We report here our discovery that carboxypeptidase A (CPA) catalyzes stereospecifically the exchange of hydrogens present in an activated methylene group of a ketonic substrate with those of the aqueous solvent. Hydrolytic reactions at the active site of CPA have been postulated[1,2] to involve either nucleophilic attack by the γ-carboxylate group of Glu-270 or attack by water aided by this residue acting as a general base. The active site Zn(II) ion, which is essential to enzymic activity, is believed to assist the hydrolytic process by polarizing the substrate's carbonyl group. As illustrated, eq 1 and 2 in Scheme I, if a methylene analogue of a peptide or ester substrate were bound in the active site in a manner similar to that of the hydrolytically labile compound, then hydrogen abstraction from the methylene group of this ketone might be catalyzed by the aforementioned functional residues. According to this proposal, either the γ-carboxylate group of Glu-270 or a water molecule assisted by this residue might extract a proton from the methylene group of the ketonic substrate to produce an enolate anion which could be stabilized by the Zn(II) ion with its positive charge.

In order to test the hypothesis of Scheme I, we have examined the possibility that hydrogen-deuterium exchange at the methylene group of 3-*p*-methoxybenzoyl-2-benzylpropionic acid-3,3-d_2 (**1**-d_2) might be catalyzed by CPA_γ. This compound is an analogue of two categories of CPA substrates, *N*-acyl-L-phenylalanines or *O*-acyl-L-β-phenyllactates. Decarboxylation at 180 °C of the malonic acid derivative prepared by condensation of α-bromo-*p*-methoxyacetophenone

Scheme I



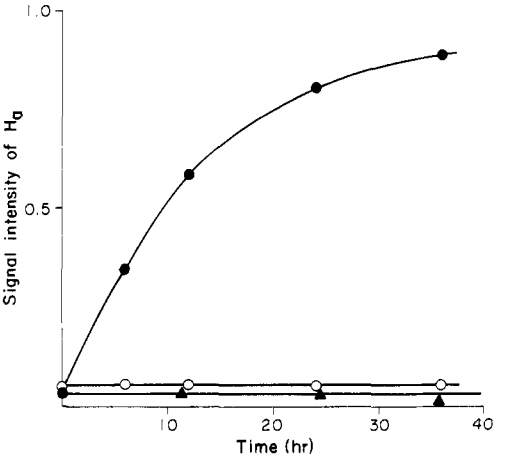


Figure 1. Time dependence of the signal intensity of the H_a proton: (●) (R)-(−)-**1**-d_2 (2.0×10^{-3} M), CPA_γ (9.5×10^{-5} M); (○) (*S*)-(+)-**1**-d_2 (2.0×10^{-3} M), CPA_γ (9.5×10^{-5} M); (▲) (*R*)-(−)-**1**-d_2 (2.0×10^{-3} M), CPA_γ (9.0×10^{-5} M), *dl*-benzylsuccinic acid (3.2×10^{-3} M).

(Aldrich) and diethyl benzylmalonate led to the synthesis of **1**. Anal. Calcd for $C_{18}H_{18}O_4$: C, 72.46; H, 6.08. Found: C, 72.50; H, 6.30. Deuterium was introduced into the methylene group at the 3 position of **1** by hydrogen-deuterium exchange in alkaline deuterium oxide solution (0.5 M NaOD, 12 h, 23 °C).

Resolution of **1**-d_2 was accomplished by repeated recrystallization from ethyl ether of the salt formed with enantiomerically pure methylbenzylamine (Aldrich). The enantiomers, (+)-**1**-d_2, mp 91.0–92.5 °C, α^{23}_D 19.9° (*c* 11.35, ethyl acetate) and (−)-**1**-d_2, mp 90.5–91.5 °C, α^{23}_D −19.3° (*c* 10.0, ethyl acetate) were obtained using (+)- and (−)-methylbenzylamine, respectively.

Hydrogen-deuterium exchange was initiated by adding **1**-d_2 to CPA_γ solutions.[3] By observation of the signal intensity of the ^1H NMR spectrum, incorporation of protons into the methylene group could be followed. At 270 MHz, the diastereotopic protons on the methylene group of **1** appear separately at 2.98 ppm (H_a) and 3.33 ppm (H_b).[4] Under the neutral pH conditions usually optimal for the hydrolytic action of CPA_γ (0.05 M Tris-HCl, 0.5 M NaCl, pH 7.5),[5] exchange at the H_a position could readily be observed in the case of (−)-**1**.[6,7]

Figure 1 shows the time course of the increase of the H_a signal in (−)-**1** under the above-mentioned conditions. The introduction of hydrogen from the solvent follows apparent first-order kinetics. At a CPA_γ concentration of 9.5×10^{-5} M, the observed k_{obsd} was found to be 1.75×10^{-5} s^{-1}. In contrast, under these conditions (+)-**1** did not exhibit any appreciable exchange.[6] This difference is consistent with the hypothesis that the exchange reaction is catalyzed in the asymmetric environment of the active site. As a test of the nature of the exchange process, 3.2×10^{-3} M *dl*-benzylsuccinic acid was added and it was found that the exchange at the H_a position of (−)-**1** was greatly retarded (Figure 1). Since *dl*-benzylsuccinic acid is a potent competitive inhibitor of the hydrolytic action of CPA ($K_i = 1.1 \times 10^{-6}$ M),[8] the inhibition seen for the exchange reaction at the H_a position provides strong evidence that the latter process occurs at the active site.

Binding of both (+)-**1** and (−)-**1** at the active site was examined in a study of inhibitory effects of these compounds on the hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate. At pH 7.5 (0.05 M Tris-HCl, 0.5 M NaCl, 25 °C), the inhibition constants (K_i) estimated were 4.9×10^{-5} M and 1.1×10^{-4} M, respectively.

The difference between the two enantiomers of **1** in the rate

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of exchange at the H_a position must be due to an important difference in the geometry of the enzyme–substrate complexes. We believe that the enantiomer having the configuration similar to that of a native peptide substrate is incorporated properly into the active site so that its 3-methylene group may be within reach of the catalytic groups of the enzyme (see Scheme I). On this basis, (−)-**1** has been assigned the *R* configuration and (+)-**1** the *S* configuration.

In contrast to what was observed for the H_a position, no significant exchange at the H_b site was observed for either of the enantiomers of **1.** This observation suggests that the exchange reaction at the H_a position in (*R*)-(−)-**1** proceeds highly stereospecifically with retention of configuration at the methylene carbon. It is improbable that complete inversion would be taking place because in this case both the H_a and H_b positions would have undergone exchange by the end of the reaction. Therefore, we envision that H_a, which faces basic group(s) in the active site, is predominantly abstracted and that the enolate intermediate formed is reprotonated on the same side with respect to its olefinic plane.

Turning to the kinetics of the exchange process, the reaction which we have observed can be treated as illustrated in eq 3. If we assume that the K_m value for **1**-*d* is the same as that for **1**-d_2, where **1**-*d* corresponds to **1** containing one deuterium at the methylene position, it can be easily shown that the rate expression for the exchange reaction is that given in eq 4. As predicted by eq 4, we have found that the exchange reaction follows an apparent first-order rate law and that the observed first-order rate constant, k_{obsd}, shows the expected inverse dependence on the initial substrate concentration up to substrate concentrations as high as 4.5×10^{-3} M. From the slope of a plot[9] of k_{obsd} vs. $1/[\mathbf{1}\text{-}d_2]_{initial}$, the k_{cat} value for the exchange of deuterium for hydrogen at the H_a position of (−)-**1**-d_2 is calculated to be 3.7×10^{-4} s^{-1} at pH 7.5. We have not been able to as yet obtain accurate measurements of the K_m value in exchange experiments. However, it may be reasonable to estimate K_m from the value of K_i measured for the inhibition by (−)-**1** of the CPA_γ catalyzed hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate (see above). Using the assumption that $K_m \approx K_i$, k_{cat}/K_m for exchange at the H_a position of **1**-d_2 is estimated to be 3.4 M^{-1} s^{-1} at pH 7.5, 25.0 °C. A direct comparison of the enzyme's catalytic effect on the proton-abstraction reaction with the behavior of a suitable model system is not feasible at the present time. However, if one considers the catalytic effect of acetate on hydrogen–deuterium exchange in acetone, for example, the second-order rate constant k_{OAc^-} is 2.5×10^{-7} M^{-1} s^{-1} at 25.0 °C.[10,11] Since the pK_a for the ionization of the γ-carboxylic acid group of Glu-270 is thought to be in the vicinity of 6.5 and since $\beta_B = 0.88$ for carboxylate ion catalyzed exchange in the case of acetone,[12] one can estimate that a model for the carboxylate component of the CPA active site would give catalysis of exchange with a rate constant of $\sim 10^{-4}$–10^{-5} M^{-1} s^{-1}. On this basis we estimate that CPA_γ catalyzes the hydrogen–deuterium exchange reaction of **1** at least 10^4–10^5 times more effectively than an appropriate carboxylate ion model system would.

$$\mathrm{E} + \mathbf{1}\text{-}d_2 \underset{K_m}{\rightleftharpoons} \mathrm{E}\cdot\mathbf{1}\text{-}d_2 \xrightarrow{k_{cat}} \mathrm{E} + \mathbf{1}\text{-}d \qquad (3)$$

$$v = \frac{k_{cat}[\mathrm{E}_0][\mathbf{1}\text{-}d_2]}{[\mathbf{1}\text{-}d_2]_{initial} + K_m} \qquad (4)$$

In this communication we have demonstrated that the active site of a hydrolytic enzyme is capable of catalyzing stereospecifically an enolization reaction. Compared with enzymic hydrolytic reactions which can involve the formation of several intermediates (e.g., tetrahedral adducts and an anhydride in the case of CPA catalysis), the enolization of a ketonic substrate is a relatively simple process. The study of such enolization reactions at the active sites of enzymes involved in acyl and phosphoryl transfer may allow us to examine the catalytic properties of enzyme-bound nucleophilic groups without having to consider the complications of the formation and breakdown of a multiplicity of intermediates along the reaction pathway. Studies of this type are underway in our laboratory.

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References and Notes

(1) J. A. Hartsuck and W. N. Lipscomb, *Enzymes*, 3rd ed., **3**, 1 (1971).
(2) E. T. Kaiser and B. L. Kaiser, *Acc. Chem. Res.*, **5**, 219 (1972).
(3) Carboxypeptidase A_γ, from bovine pancreas, was purchased as a crystalline suspension in toluene–water from Worthington Biochemical Corporation (Lot No. 1681 COA 37K678). Stock solutions of the enzyme were prepared as described by E. T. Kaiser and F. W. Carson, *J. Am. Chem. Soc.*, **86**, 2922 (1964).
(4) Assignment of the signals was made by a comparison of the NMR spectra of **1** and **1**-d_2. The other signals observed in the spectrum of **1** are (in parts per million from M_4Si in $CDCl_3$) 2.86 (1 H, dd, J = 9.0, 13.5 Hz), 3.19 (1 H, dd, J = 5.9, 13.5 Hz), 3.35 (1 H, m, overlap with H_b) 3.85 (3 H, s), 6.90 (2 H, d, J = 8.8 Hz), 7.20–7.27 (5 H, m), 7.87 (2 H, d, J = 11 Hz). In the spectrum of **1**-d_2, the multiplet at 3.35 ppm appeared as a double doublet (J = 6.3, 9.0 Hz), and the rest of the signals observed except those for H_a and H_b were identical with those seen in the NMR spectrum of **1**.
(5) J. Suh and E. T. Kaiser, *J. Am. Chem. Soc.*, **98**, 1940 (1976).
(6) After incubation at 25 °C for 36 h no appreciable decrease of the enzymic activity of CPA_γ was observed in the hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactic acid.
(7) The reaction was quenched by adding concentrated hydrochloric acid to a sample aliquot removed from the enzymic solution. Precipitated **1** was extracted using ethyl acetate. After the solution was dried over sodium sulfate, the solvent was removed by evaporation, and the NMR spectrum was recorded in deuteriochloroform solution.
(8) L. D. Byers and R. Wolfenden, *Biochemistry*, **12**, 2070 (1973).
(9) The good linearity observed in this plot also excludes the possibility of substrate inhibition which was observed in the CPA catalyzed hydrolysis of benzoyl esters at substrate concentrations above 10^{-3} M, according to J. W. Bunting, J. P. Murphy, C. D. Meyers, and G. G. Cross, *Can. J. Chem.*, **52**, 2658 (1974).
(10) R. P. Bell, "The Proton in Chemistry", 2nd ed., Cornell University Press, Ithaca, N.Y., 1973, p 150.
(11) R. P. Bell and P. Jones, *J. Chem. Soc.*, 88 (1953).
(12) R. P. Bell and O. M. Lidwell, *Proc. R. Soc. London, Ser. A*, **176**, 88 (1940).

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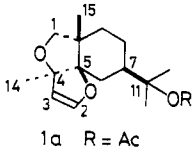
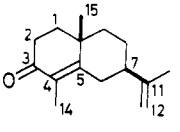
Synthesis of Phytuberin[1]

Sir:

Phytuberin[2] (**1a**) is a novel sesquiterpene qualified as a representative phytoalexin[3] together with rishitin[4] in the genus *Solanum* and characterized structurally by the presence of two fused hydrofuran rings. The proposed biogenesis[2b,5] suggested that the ring system in **1a** would be formed via oxidative cleavage of a C-1–C-2 bond of a hypothetical intermediate with an eudesmane skeleton. We describe herein a stereoselective synthesis of **1a,** which in its critical stages mimics the



1a R = Ac
1b R = H



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