Carboxypeptidase A Catalyzed α,β-Elimination Reactions: Rapid Catalysis of α,β-Elimination of Hydrogen Chloride from β-Chloro Ketone Substrates

Nashaat T. Nashed[†,§] and E. T. Kaiser*[‡]

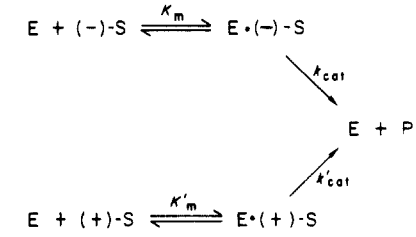
Contribution from the Department of Chemistry, University of Chicago, Chicago, Illinois 60637, and Laboratory of Biorganic Chemistry and Biochemistry, Rockefeller University, New York, New York 10021. *Received September 23, 1985*

Abstract: The proteolytic enzyme carboxypeptidase A (CPA) catalyzes α,β-elimination of hydrogen chloride from 2-benzyl-2-chloro-3-(4-methoxybenzoyl)propionic acid (I), 2-benzyl-2-chloro-3-benzoylpropionic acid (II), and 2-chloro-2-methyl-3-benzoylpropionic acid (III). The kinetic parameters for the CPA-catalyzed α,β-elimination of hydrogen chloride from compounds I–III reflected the known preference of CPA for substrates with a carboxy terminal aromatic side chain. Evidence that this reaction occurs at the hydrolytic active site is provided by the observation that *dl*-benzylsuccinic acid, a known potent competitive inhibitor for the hydrolytic activity, inhibits the CPA catalysis of the α,β-elimination reaction. In addition, the pH profile of k_{cat}/K_m for CPA-catalyzed α,β-elimination of HCl from I is a bell-shaped curve ($pK_{a_1} = 6.4 \pm 0.2$ and $pK_{a_2} = 9.5 \pm 0.2$), corresponding closely to the curves describing the pH–k_{cat}/K_m profiles for the CPA-catalyzed hydrolyses of specific ester and peptide substrates. The CPA-catalyzed α,β-elimination reaction is stereo- and regiospecific and yields (*Z*)-2-benzyl-3-(4-methoxybenzoyl)propenoic acid (VII) and (*Z*)-2-benzyl-3-benzoylpropenoic acid (VIII) from compounds I and II.

Carboxypeptidase A (peptidyl-L-amino acid hydrolase, EC 3.4 17.1) is a zinc-containing exopeptidase that catalyzes the hydrolysis of peptides and proteins at their carboxy termini. In addition, this enzyme has been shown to cleave efficiently a wide variety of ester substrates. The catalytic action of carboxypeptidase A (CPA) has been studied extensively, and much information has been obtained through kinetic studies[1,2] and from the elucidation of the enzyme's crystal structure.[3-5] It has been postulated that peptide substrates bind to CPA in a manner such that the carbonyl oxygen of the scissile amide bond is coordinated to the catalytically essential Zn ion, the terminal carboxylate group is in close proximity to the positively charged Arg-145 side chain, and the aromatic side chain of the carboxy terminal amino acid occupies a hydrophobic pocket large enough to contain a tryptophan side chain.[3-5] The catalytic role of the caroxylate side chain of Glu-270, which is located close to the scissile carbonyl carbon, has been under investigation in our laboratory. The special chemical reactivity and selectivity of this carboxylate residue was examined by the ability of CPA to catalyze stereo- and regioselective hydrogen–deuterium exchange at the methylene group α to the ketone of (−)-2-benzyl-3-(4-methoxybenzoyl)propionic acid.[6,7] This suggested that CPA can catalyze the formation and stabilization of an enolate anion. Subsequently, we discovered that CPA also catalyzes the α,β-elimination reaction of 4-nitrothiophenol from (+)-3-benzoyl-2-[(4-nitrophenyl)thio]propionic acid.[8] Interestingly, the ability to catalyze α,β-elimination reactions may be a general feature of zinc-containing proteases since we have shown recently that angiotensin-converting enzyme can similarly catalyze α,β-elimination of 4-nitrothiophenol from a ketone substrate.[9]

Examination of a possible structure of (+)-3-benzoyl-2-[(4-nitrophenyl)thio]propionic acid when bound in the CPA active site (Figure 1A) reveals that the incipient enolate anion p orbital would lie in a plane perpendicular to that of the leaving group.[10] This suggests that the elimination of 4-nitrothiophenol from (+)-3-benzoyl-2-[(4-nitrophenyl)thio]propionic acid catalyzed by CPA may be retarded by the geometrical arrangement of the enolate anion. It seemed likely that an analogue of (+)-3-benzoyl-2-[(4-nitrophenyl)thio]propionic acid that would have a leaving group in the position of the methine hydrogen (Figure 1A) would be a substantially better substrate for the CPA-catalyzed α,β-elimination reaction than the former. In such a case the incipient p orbital would be coplanar to the leaving group. In this study we report that CPA catalyzes the stereo- and regiospecific α,β-elimination of hydrogen chloride from three ketone substrates (I–III). The enzyme catalyzes this process quite efficiently; in fact, the efficiencies of these eliminations are comparable to those of the CPA-catalyzed hydrolysis of esters having similar structures.[11]

Scheme I



Results

The proteolytic enzyme CPA catalyzes the α,β-elimination of HCl from the β-chloro ketones I–III according to eq 1. Under substrate in excess conditions, saturation kinetics were observed for the CPA-catalyzed elimination of HCl from (±)-I and (±)-II.

[†] University of Chicago.
[‡] Rockefeller University.
[§] Present address: National Institutes of Health, NIADDK, Bethesda,MD 20892.

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(10) While this paper was in preparation, we were informed by Professor W. N. Lipscomb that the X-ray structure of the complex between CPA and (−)-2-benzyl-3-(4-methoxybenzoyl)propionic acid has been determined at 1.54-Å resolution: Christianson, D. W.; Kuo, L. C.; Lipscomb, W. N. *J. Am. Chem. Soc.* **1985**, *107*, 8281. In this structure the ketone carbonyl hydrogen bonds to the guanidinium moiety of Arg-127 rather than to the zinc ion. Thus, a possibility that must be considered is that in Figure 1 instead of showing interaction of the substrate carbonyls with the zinc ion we could show their interactions with Arg-127. In any event, the stereochemical outcome of the α,β-elimination reactions would remain the same.
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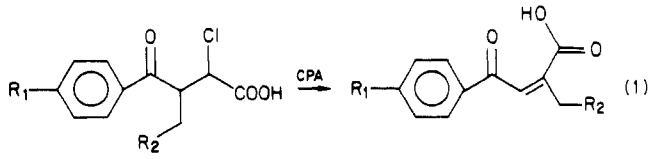


(1)

compd	R_1	R_2	compd	R_1	R_2
I	OCH_3	C_6H_5	IV	OCH_3	C_6H_5
II	H	C_6H_5	V	H	C_6H_5
III	H	H	VI	H	H

In all cases where racemic β-chloro ketones were used as substrates initially, 50% of the compound reacted with the enzyme, followed by a substantially slower reaction. Values of k_{cat}, K_m, and k_{cat}/K_m obtained for the CPA-catalyzed elimination of HCl from the β-chloro ketones I–III are listed in Table I. For ease of comparison, also included in Table I are the kinetic parameters for the CPA-catalyzed hydrolysis of *O*-(*p*-nitrobenzoyl)-L-β-phenyllactic acid, the CPA-catalyzed deuterium–hydrogen exchange of the ketonic substrate (−)-2-benzyl-3-(*p*-methoxybenzoyl)propionic acid-*3,3-d*$_2$ in H_2O, and the CPA-catalyzed elimination of *p*-nitrothiophenol from (+)-3-benzoyl-2-[(4-nitrophenyl)thio]propionic acid.

The simple general kinetic scheme (Scheme I) for the reaction of an enzyme with a racemic substrate is sufficient to fit the experimental results. For a reaction as seen in Scheme I, it can be shown that

$$\frac{d[(\pm)\text{-S}]}{dt} = \frac{k_{cat}[E][(-)\text{-S}]}{K_m(1 + [(+)\text{-S}]/K'_m) + [(-)\text{-S}]} + \frac{k'_{cat}[E][(+)\text{-S}]}{K'_m(1 + [(-)\text{-S}]/K_m) + [(+)\text{-S}]} \quad (2)$$

From the kinetic parameters of the less reactive enantiomers (+)-I and (+)-II listed in Table I, it is clear that $k_{cat} \gg k'_{cat}$ and $K_m \ll K'_m$. Therefore, eq 2 may be simplified to give eq 3. Thus,

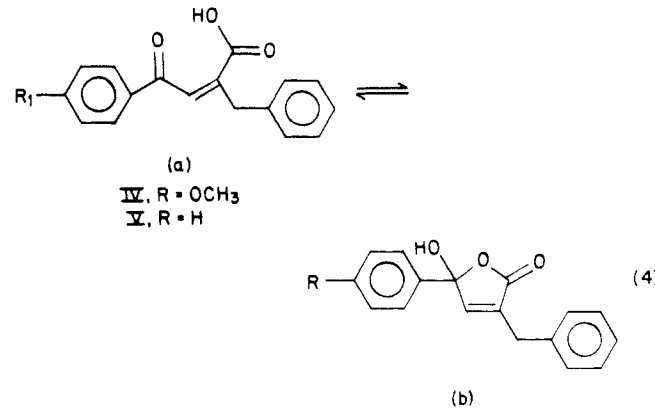
$$\frac{d[(-)\text{-S}]}{dt} = \frac{k_{cat}[E][(-)\text{-S}]}{K_m + [(-)\text{-S}]} \quad (3)$$

the kinetic parameters obtained for the more reactive enantiomers (−)-I and (−)-II were not complicated by the presence of the less reactive enantiomers.

In all cases, the CPA-catalyzed α,B-elimination of HCl from the β-chloro ketones were totally inhibited upon the addition of 2 mM *dl*-benzylsuccinic acid. Since *dl*-benzylsuccinic acid is a potent inhibitor of the hydrolytic action of CPA (K_i = 1.1 μM),[12] the inhibition observed for the elimination reaction provides strong evidence that the latter process occurred at the active site. This conclusion was strengthened by the fact that apo-CPA does not catalyze the elimination of HCl from (±)-I and (±)-II.

From the pH profile for the kinetic parameter k_{cat}/K_m for the CPA-catalyzed α,β-elimination of HCl from (±)-I the values calculated for the controlling pK_a's for the bell-shaped curve are 6.4 ± 0.2 and 9.5 ± 0.2. These values closely correlate with the pK_a values for the bell-shaped curves arising from the pH profiles of the kinetic parameter k_{cat}/K_m for the CPA-catalyzed hydrolysis of specific ester, peptide, and ketone substrates.[1,2,13-16]

Product Analysis. The only products for the CPA-catalyzed elimination of HCl from (−)-I and (+)-I and from (−)-II and (+)-II are IV and V, respectively. The characterization of IV and V was complicated by the dynamic equilibrium shown in eq 4. Evidence that this dynamic equilibrium was important came first from the ^{1}H NMR spectrum of IV. The ^{1}H NMR spectrum of IV in $CDCl_3$ had broad absorption peaks for the benzylic, vinyl, and some aromatic protons. The ^{1}H NMR ($CDCl_3$) chemical shifts of key protons in the *Z* isomers IV and V and in the corresponding *E* isomers VII and VIII along with their carbonyl IR stretching vibrations in chloroform are listed in Table II. Not surprisingly, the position of the equilibrium of eq 4, along with the kinetics by which the equilibrium is established, could be altered by changing the solvent and/or adding base. In Me_2SO-d_6–D_2O (3:1, v/v), compound IV is mostly in the lactone form: δ 3.67 (br s, 2 H), 3.78 (br s, 3 H), 7.0 (m, 3 H), 7.3 (m, 5 H), 7.9 (br, 2 H). When a molar excess of NaOD is added, the broad absorptions became sharper: δ 3.71 (s, 2 H), 3.81 (s, 3 H), 7.01 (d, *J* = 8 Hz, 2 H), 7.25 (m, 5 H), 7.55 (s, 1 H), 7.93 (d, *J* = 8 Hz, 2 H).



(a)

IV, R = OCH_3
V, R = H

(4)

(b)

The γ-lactone structures for IV and V were further supported by the observation of carbonyl stretching vibrations in the IR spectrum at 1772 and 1765 cm^{-1}, respectively, that can be assigned unequivocally to the α,β-unsaturated γ-lactone function.[17-19] On the other hand, the IR spectra of the *E* isomers VII and VIII show two different carbonyl stretching vibrations (see Table II) that can be assigned to the α,β-unsaturated ketone moiety and to the α,β-unsaturated carboxylic acid.[18,19]

The position of the double bond in structures IV, V, VII, and VIII was established by deuteration of the double bond by catalytic reduction with deuterium gas and/or diimide reduction in a deuterated medium. Reduction of compounds IV and VI produced compound IX and reduction of compounds V and VII produced compound X. All of the peaks for the methylene and methine protons of IX are resolved in a 500-MHz ^{1}H NMR spectra, and all were assigned previously.[7,16] The doublet of doublets at 2.86 and 3.19 ppm was assigned to the benzylic hydrogens, the doublet of doublets at 2.98 and 3.33 ppm was assigned to the *pro-R* and *pro-S* hydrogens on carbon-3, respectively, and the multiplet at 3.35 was assigned to the methine proton on carbon-2. The ^{1}H NMR spectra of IX produced from the catalytic reduction with deuterium of IV and VII were identical and showed two simple doublets at 2.98 and 3.19 ppm for the benzylic protons, each corresponding to one proton, two other doublets at 2.98 and 3.33 ppm corresponding to one-half proton each, and no absorption at 3.35 ppm. The diimide reduction of VII and VIII in deuterated medium provided only one pair of diastereomers corresponding to IX and X, respectively. Comparison of the NMR spectra of these products indicated that the configurations of the diastereomeric pair are 2*S*,3*R* and 2*R*,3*S* in both cases. The reduction of IV and V should have produced the other diastereomeric pair (2*R*,3*R* and 2*S*,3*S*), but unfortunately IV and V partially isomerized under the diimide-reduction conditions.

Discussion

The results described above show that CPA catalyzed the α,β-elimination of HCl from (±)-I, (±)-II, and (±)-III at its hydrolytic active site. The low reactivity of (±)-III toward the CPA-catalyzed α,β-elimination of HCl as compared with (−)-I and (−)-II is consistent with the known preference of CPA for substrates with an aromatic side chain in the group α to the

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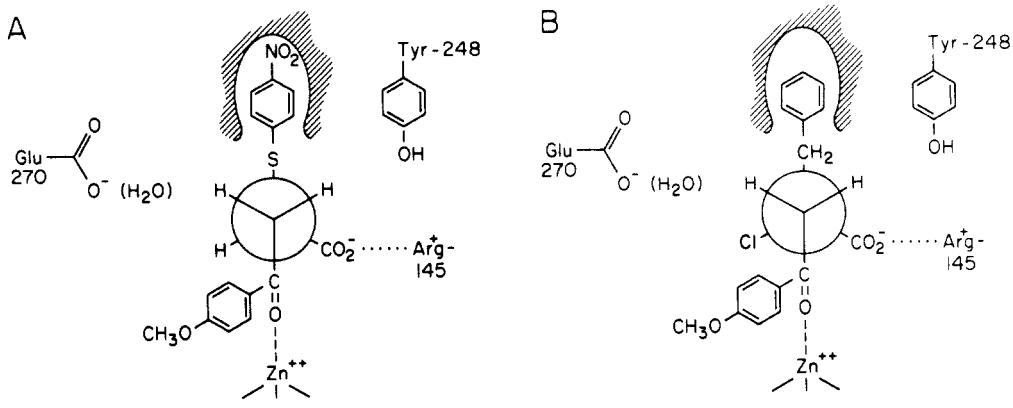
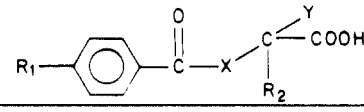


Figure 1. Proposed modes for productive binding to the active site of CPA: (A) (+)-3-benzyl-2-[(4-nitrophenyl)thio]propionic acid; (B) (−)-2-benzyl-2-chloro-3-(4-methoxybenzoyl)propionic acid.

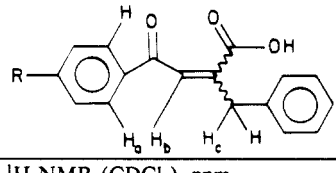
Table I



no.	compd R_1	compd R_2	compd X	compd Y	isomers	$10^3 k_{cat}$, s^{-1}	K_m, μM	k_{cat}/K_m, M^{-1} s^{-1}	$k_{[OH^-]}$, M^{-1} s^{-1}
BNT	H	p-S-C_6H_4-NO_2	CH_2	H	+	1.44 ± 0.09	420 ± 10	3.52 ± 0.25[a]	4.7 ± 0.1
(−)-IX-d_2	OCH_3	$CH_2C_6H_5$	CD_2	H	−	0.37	110	3.4[b]	
(±)-IX	OCH_3	$CH_2C_6H_5$	CH_2	H	±				0.0030[c] ± 0.0001
	NO_2	$CH_2C_6H_5$	O	H		3500	310	11 400[d]	
(±)-III	H	CH_3	CH_2	Cl	±		>1000	0.31 ± 0.01[e]	
(+)-II	H	$CH_2C_6H_5$	CH_2	Cl	+	6.92 ± 0.69	2540 ± 380	2.73 ± 0.27[f]	
(−)-II	H	$CH_2C_6H_5$	CH_2	Cl	−	222 ± 20	20.5 ± 2	10400 ± 1000[g]	0.36 ± 0.01
(+)-I	OCH_3	$CH_2C_6H_5$	CH_2	Cl	+		>1000	1.10 ± 0.01[f]	
(±)-I	OCH_3	$CH_2C_6H_5$	CH_2	Cl	±	88.9 ± 3.6	27.0 ± 2.3	3300 ± 300[g]	
(±)-I	OCH_3	$CH_2C_6H_5$	CH_2	Cl	±			3650 ± 50[h]	0.182 ± 0.004
(±)-I-d_2	OCH_3	$CH_2C_6H_5$	CD_2	Cl	±			465 ± 10[h]	0.032 ± 0.001

[a] pH 7.50, 0.5 M NaCl, 0.05 M Tris-HCl, 6.3% acetonitrile at 25 °C.[8] [b] pH 7.50, 0.5 M NaCl, 0.05 M Tris-HCl.[6] [c] $k_{[OD^-]}$ at ambient temperature. [d] pH 7.50, 0.5 M NaCl, at 25 °C.[11] [e] pH 7.50, 0.5 M NaCl, 0.05 M Tris-HCl, 0.3% DMF at 25 °C. [f] pH 8.20, 0.5 M NaCl, 0.05 M Tris-HCl, 6% dioxane at 25 °C. [g] pH 8.20, 0.5 M NaCl, 0.05 M Tris-HCl, 3% dioxane at 25 °C. [h] From measurements under conditions where [S] ≪ K_m and pseudo-first-order kinetics was observed, 0.05 M Tris-HCl buffer, 3% dioxane at 25 °C.

Table II



compd	^{1}H NMR ($CDCl_3$), ppm a	b	c	IR ($CHCl_3$), cm^{-1} (carbonyl abs)
IV	7.64 br	6.71	3.72 br	1772 br, 1725 br
V	7.46	6.70	3.57	1765
VII	7.96	7.88	3.96	1692, 1655
VIII	7.97	7.92	4.00	1700, 1665

carboxyl end of a peptide or an ester substrate. This and the high enantioselectivity of CPA in catalyzing the enolization of compounds IX and X, as well as the α,β-elimination reactions of I–III and (+)-3-benzoyl-2-[(4-nitrophenyl)thio)]propionic acid (BNT), suggest that (−)-I and (−)-II bind to the active site of CPA in a manner similar to that of peptide, ester, and previously studied ketone substrates. Therefore, it is reasonable to assume that (−)-I and (−)-II have the S configuration. In this event when bound by the enzyme active site,[10] the benzyl group would be anchored in the hydrophobic pocket, the carboxylate anion of the substrate with its negative charge interacting with the positively charged side chain of Arg-145, and if the carbonyl oxygen of the substrate were coordinated to the active-site zinc ion, a hydrogen of the α-methylene group would be within striking distance of the γ-carboxylate group of Glu-270 (Figure 1B). According to this proposal, either the γ-carboxylate of Glu-270 or that residue acting through a water molecule would be the species removing the hydrogen from the α-methylene position of the substrate. Fitting the R configuration of I or II to the active site of CPA would require the absence of at least one of these important interactions for catalytic activity. This would be manifested in the kinetic parameters in the form of a larger value for K_m and a smaller value for k_{cat} as was observed for the kinetic parameters for the the CPA-catalyzed α,β-elimination of HCl from (+)-I and (+)-II.

The k_{cat}/K_m values for the catalytic action of CPA on (−)-I and (−)-II are comparable to the k_{cat}/K_m value measured for the CPA-catalyzed hydrolysis of O-(4-nitrobenzoyl)-3-phenyllactic acid,[11] which is the ester substrate analogous to the substrates undergoing elimination. This is a rate enhancement 10^4–10^5 over the second-order rate constant for the hydroxide ion catalyzed elimination of HCl from (±)-I and (±)-II and an increase of at least 10^6–10^7 over the rate constant for the acetate ion catalyzed reaction ($k_{AcO^-} < 0.001$ M^{-1} s^{-1}). The reaction of CPA with (−)-I and (−)-II is much more rapid than the previously reported CPA catalysis of the α,β-elimination of p-nitrothiophenol[8] from (+)-3-benzoyl-2-[(4-nitrophenyl)thio]propionic acid, (+)-BNT, in which the kinetic parameter k_{cat}/K_m is comparable in value to the second-order rate constant for the hydroxide ion catalyzed reaction (see Table I). This large difference in the rates of the CPA-catalyzed reactions does not appear to be due to the relative intrinsic chemical reactivities of BNT and II because BNT is 13 times more reactive than II toward the hydroxide ion catalyzed elimination reaction. However, this difference may be accounted for by the following stereoelectronic argument. Possible schematic representations for the binding of (+)-BNT and (−)-II to the active-site region of CPA, assuming that the absolute configurations of (+)-BNT and (−)-II correspond to the R and S con-

figurations, respectively, and that the binding of (+)-BNT and (−)-II to CPA in the reactive complexes corresponds to the reactive arrangement deduced for ester and peptide substrates on the basis of the X-ray crystallographic structure of the Gly–Tyr complex with CPA, are each shown in Figure 1. The removal of a proton by Glu-270 or a water molecule assisted by Glu-270 from the activated methylene group of (+)-BNT leads to an enolate anion in which the dihedral angle between the newly formed p orbital and the leaving group is 90°. This should impede the reaction substantially and would be reflected in the kinetic parameters for the reaction. On the other hand, the enolate anion that is formed from (−)-II has its p ortibal coplanar to the leaving group and thus should lead to a more facile elimination reaction.

This postulated conformation of the elimination substrates at the active site of CPA (Figure 1) not only accounts for the large difference in reactivities of the CPA-ctalyzed α,β-elimination reactions from (+)-BNT and (−)-II but also makes the correct prediction about the stereochemistry of the products. The α,β syn elimination of *p*-nitrothiophenol from (+)-BNT (Figure 1A) should lead to (*E*)-benzoylpropenoic acid, and this result has been confirmed.[8] Also, the α,β syn elimination of HCl from (−)-II should and does lead to compound V. This is the only product obtained from the CPA-catalyzed α,β-elimination of HCl from (±)-II. The hydroxide ion catalyzed α,β-elimination of HCl from II yielded both V and VIII.

Mechanism. Since we have data only for the elimination of HCl from I and II, we cannot assign Hammett ρ values for the reactions of these compounds with confidence. Nevertheless, we have estimated that the Hammett ρ values are 1.1 and 1.8 for the hydroxide ion and CPA-catalyzed α,β-elimination reactions, respectively. These estimates suggest that the mechanism for the α,β-elimination reaction is of the carbanion type. As discussed by Bordwell,[20] the carbanionic mechanisms for elimination reactions are as follows: first-order anion $(E1)_{anion}$; preequilibrium anion or reversible anion $(E1cB)_R$; preequilibrium ion pair or tightly solvated anion $(E1cB)_{ip}$; $(E1cB)_I$. The $(E1)_{anion}$ mechanism was removed from contention for two reasons: First, second-order kinetics is observed for the hydroxide ion catalyzed α,β-elimination of HCl from I and II, not first-order kinetics as might be anticipated for an $(E1)_{anion}$ mechanism. Second, no deuterium is incorporated into unreacted I when I is allowed to react in deuterated medium for a time corresponding to 1 half-life of the elimination reaction. This fact also makes an $(E1cB)_R$ mechanism unlikely since the mechanism would require an equilibrium between a carbanion and the starting material prior to the rate-limiting step, and hence hydrogen–deuterium exchange should be found in the unreacted substrate. The substantial primary kinetic isotope effects shown in Table I (k_H/k_D = 5.7 and 7.8 for the hydroxide ion and the CPA-catalyzed α,β-elimination of HCl from I, respectively) are most consistent with an $(E1cB)_I$ mechanism for the hydroxide and CPA-catalyzed elimination reactions. A smaller primary kinetic isotope effect (k_H/k_D = 1–2) might have been expected for an $(E1cB)_{ip}$ mechanism.

Experimental Section

Materials and Methods. ^{1}H NMR spectra were recorded at 500 MHz on the University of Chicago spectrometer equipped with a Nicolet data processor system. Tetramethylsilane was used as an internal standard. IR spectra were recorded on a Perkin-Elmer 283 infrared spectrometer. Ultraviolet spectra were recorded on either a Cary-219 or a Beckman ACTA MVI spectrometer, each equipped with a thermostated cell compartment. Mass spectra were recorded on a Finnigan 1015 mass spectrometer. Optical rotations were measured with a Perkin-Elmer 141 polarimeter employing a 10-cm path-length cell. All melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN.

The following chemicals were purchased from Aldrich and used without further purification: acetic acid-*d*, anisole, azodicarbonamide, α-bromoacetophenone, α-bromo-*p*-methoxyacetophenone, chloroform-*d*, ethyl pyruvate, methanol-*d*, sodium deuteroxide 40% weight solution in D_2O, palladium on activated carbon, the sodium salt of phenylpyruvic acid monohydrate, tetramethylsilane, trifluoroacetic acid, triphenylphosphine. Hydrogen gas and hydrogen chloride were purchased from Matheson. Deuterium gas and deuterium chloride were purchased from Merck Isotopes. Isobutylene was purchased from the Union Carbide Co. A prepacked silica column and EM silica were purchased from Merck. Silica gel TLC plates were purchased from Eastman. All buffers were purchased from Sigma Chemical Co. Azodicarboxylate was synthesized by the potassium hydroxide catalyzed hydrolysis of azodicarbonamide as described by Berson et al.[21]

Carboxypeptidase A, type II, from bovine pancreas was purchased from Sigma Chemical Co. The enzyme stock solution was prepared as described by Kaiser and Carson.[22] The zinc ion was removed from CPA to form apo-CPA by the method of Davies et al.[23]

Kinetics. The elimination reactions were monitored spectrophotometrically by following the increase of absorption at either 315 nm ($\Delta\epsilon$ = 9000 cm^{-1} M^{-1}) for I or 300 nm ($\Delta\epsilon$ = 3960 cm^{-1} M^{-1}) for II and III. In a typical kinetic measurement, 3 mL of 0.05 M buffer–0.5 M NaCl was incubated at 25.0 °C for 15 min. Then, the substrate was added in 100 μL of dioxane. The reaction was initiated by adding 100 μL of CPA stock solution. The nonenzymatic rate of reaction was less than 1% of the enzymatic rate under all conditions employed at pH values up to pH 9. Above pH 9, the data were corrected for the nonenzymatic reaction. Zinc chloride (10 μM) was added to all solutions below pH 7.5 employed for kinetic measurements. The kinetic parameters reported here were calculated by two methods: (a) Initial rates, which were obtained from the initial 5% of the reactions, were fitted to hyperbolic kinetics by using an iterative curve-fitting computer program obtained from Dr. John Westley, Department of Biochemistry, University of Chicago. This program followed the methodology of G. N. Wilkinson.[24] (b) The total time course of the reaction was fitted to a hyperbolic function by using a nonlinear least-squares computer program obtained from B. A. Blumenstein, Emory University. Values derived from one method were consistent with the values derived from the other method in all cases. When $[S] \ll K_m$, good first-order kinetics was obtained. No buffer inhibition was observed with any buffer used in studying the pH–rate profile.

The second-order rate constants for the hydroxide ion catalyzed elimination of HCl from (±)-I and (±)-I-d_2 were obtained by measuring the pseudo-first-order rate constant at different hydroxide ion concentrations. Over a range of hydroxide ion concentrations from 33 to 100 mM pseudo-first-order kinetics was observed for at least 3 half-lives. A plot of k_{obsd} vs. $[OH^-]$ is linear, and the line obtained goes through the origin. The slope of that line is the reported second-order rate constant.

The second-order rate constant for the deuteroxide ion catalyzed hydrogen–deuterium exchange in IX was measured by monitoring the disappearance of ^{1}H NMR signals for the methylene protons (in deuterium oxide). The sodium deuteroxide solutions were prepared from a 40% deuteroxide solution and deuterium oxide. The concentration of a sodium deuteroxide varied from 8.2 to 73.5 mM. The disappearance of the ^{1}H signals followed apparent first-order kinetics for at least 3 half-lives. A plot of k_{obsd} vs. $[OD^-]$ is linear, and the slope of the line gives the reported second-order rate constant.

Syntheses. (+)- and (−)-2-benzyl-3-(4-methoxybenzoyl)propionic acids, IX, were prepared and resolved as described previously.[6] The ylides, 1-phenyl-2-(triphenylphosphoranylidene)ethanone and 1-(4-methoxyphenyl)-2-(triphenylphosphoryanylidene)ethanone, were prepared as described by Ramirez and Dershowitz.[25]

***tert*-Butyl Phenylpyruvate.** The sodium salt of phenylpyruvic acid (6 g, 30 mmol) was treated with 160 mL of 6 M HCl). The free acid was extracted from the aqueous mixture with diethyl ether. The ether solution was washed with 0.1 M HCl followed by saturated sodium chloride and then dried over magnesium sulfate. The ether was removed under vacuum, and the residue was dissolved in dichloromethane. The dichloromethane solution was added to a mixture of 50 mL of isobutylene and 0.5 mL of sulfuric acid in a pressure bottle cooled in a dry ice–acetone bath. After the bottle was capped, the reaction mixture was allowed to rise to room temperature. The reaction mixture was then stirred for 3 h and poured into 250 mL of diethyl ether cooled in an ice bath. The ether solution was washed with water, 5% sodium bicarbonate, and saturated sodium chloride and then dried over magnesium sulfate. Solvent was removed under reduced pressure at room temperature. The residue was used without any further purification. ^{1}H NMR data were

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consistent with that of the product containing an equilibrium mixture of keto and enol forms in about a 1:1 ratio. ^{1}H NMR ($CDCl_3$): δ 1.50 (s, 4.5 H), 1.60 (s, 4.5 H), 4.23 (s, 1.4 H), 6.5 (br s, 0.5 H), 6.75 (s, 0.5 H), 7.40–8.20 (m, 5 H).

***tert*-Butyl (*E*)-2-Benzyl-3-benzoylpropenoate.** A solution of *tert*-butyl phenylpyruvate (9.7 g, 44 mmol) and 1-phenyl-2-(triphenylphosphoranylidene)ethanone (15.2 g, 40 mmol) in 100 mL of chloroform was incubated at room temperature for 5 days. The chloroform was removed under vacuum at ambient temperature. On treatment of the residue with hexane, triphenylphosphine oxide precipitated and was removed by filtration. The hexane was removed under vacuum at ambient temperature to yield 9.5 g (74%) of yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.40 (s, 9 H), 4.00 (s, 2 H), 7.15–7.60 (m, 8 H), 7.74 (s, 1 H), 7.91 (d, J = 7 Hz, 2 H). IR (neat): 1712, 1668, 1592 cm^{-1}. Mass spectrum: M^+/e 322 (molecular ion), 266, 248, 221, 105, 91, 77, 57.

***tert*-Butyl (*E*)-2-Benzyl-3-(4-methoxybenzoyl)propenoate.** This compound was prepared as described above in a 72% yield by using *tert*-butyl phenylpyruvate and 1-(*p*-methoxyphenyl)-2-(triphenylphosphoranylidene)ethanone. ^{1}H NMR ($CDCl_3$): δ 1.40 (s, 9 H), 3.82 (s, 3 H), 3.97 (s, 2 H), 6.93 (d, J = 8.9 Hz, 2 H), 7.00–7.25 (m, 5 H), 7.72 (s, 1 H), 7.97 (d, J = 8.9 Hz, 2 H). IR (neat): 1709, 1650, 1596 cm^{-1}. Mass spectrum: M^+/e 352 (molecular ion), 296, 278, 251, 135, 91, 77, 57.

(*E*)-2-Benzyl-3-benzoylpropenoic Acid (VIII). A solution of *tert*-butyl (*E*)-2-benzyl-3-benzoylpropenoate (5 g, 15.5 mol) in 20 mL of trifluoroacetic acid containing 1 mL of anisole was stirred at room temperature for 30 min. The trifluoroacetic acid was removed under vacuum at ambient temperature, and the residue was dissolved in ether. When petroleum ether was added, 2.5 g (61%) of a yellow crystalline material was obtained; mp 127–130 °C. ^{1}H NMR ($CDCl_3$): δ 4.00 (s, 2 H), 7.14–7.62 (m, 8 H), 7.92 (s, 1 H), 7.97 (d, J = 8.5 Hz, 2 H). IR ($CHCl_3$): 1700, 1655, 1598 cm^{-1}. Mass spectrum: M^+/e 266 (molecular ion), 248, 221, 105, 91, 77. Anal. Calcd for $C_{17}H_{14}O_3$: C, 76.68; H, 5.30. Found: C, 76.97; H, 5.46.

(*E*)-2-Benzyl-3-(4-methoxybenzoyl)propenoic Acid (VII). A solution of (5 g, 14.2 mmol) of *tert*-butyl (*E*)-2-benzyl-3-(*p*-methoxybenzoyl)-propenoate in trifluoroacetic acid containing 5% anisole was stirred at room temperature for 30 min. The trifluoroacetic acid was removed under vacuum, and the residue was dissolved in ether. The ether solution was washed with water and saturated NaCl, dried over $MgSO_4$, and concentrated under vacuum. Upon the addition of petroleum ether, 2.8 g (67%) of the acid crystallized; mp 121–123 °C. ^{1}H NMR ($CDCl_3$): δ 3.88 (s, 3 H), 3.96 (s, 2 H), 6.96 (d, J = 8.7 Hz, 2 H), 7.16–7.30 (m, 5 H), 7.88 (s, 1 H), 7.96 (d, J = 8.7 Hz, 2 H). IR (KBr): 3000 br, 1692 s, 1655, 1591 cm^{-1}. Mass spectrum: M^+/e 296 (molecular ion), 279, 251, 135, 91, 77.

2-Benzyl-2-chloro-3-benzoylpropionic Acid (II). A solution of 5 g (15.5 mol) of *tert*-butyl (*E*)-2-benzyl-3-benzoylpropenoate in 50 mL of benzene was saturated with dry HCl at ice bath temperature. The reaction mixture was cooled in an ice bath for 3 h. Then, the reaction mixture was diluted with 100 mL of ether, and the resultant mixture was washed three times with water and saturated NaCl and dried over $MgSO_4$. After removal of solvent under vacuum, the residue was treated with 50 mL of 5% anisole in trifluoroacetic acid for 30 min. After evaporation of trifluoroacetic acid under vacuum at ambient temperature, the residue was dissolved in diethyl ether, and the solution was washed with water and saturated NaCl, dried over $MgSO_4$, and evaporated under vacuum at ambient temperature. Upon addition of petroleum ether, 2.5 g (53%) of white crystals was obtained; mp 103 °C dec. ^{1}H NMR ($CDCl_3$): δ 3.56 (d, J = 18 Hz, 1 H), 3.57 (d, J = 15 Hz, 1 H), 3.68 (d, J = 15 Hz, 1 H), 3.78 (d, J = 18 Hz, 1 H), 7.22–7.60 (m, 8 H), 7.88 (d, J = 7.6 Hz, 2 H). IR (KBr): 2900 br, 1705 s, 1678 cm^{-1}. Anal. Calcd for $C_{17}H_{15}ClO_3$: C, 67.44; H, 4.99; Cl, 11.71. Found: C, 67.41; H, 4.99; Cl, 11.73. Mass spectrum: M^+/e 267 (M – Cl), 221, 144, 131, 115, 105, 91.

2-Benzyl-2-chloro-3-(4-methoxybenzoyl)propionic Acid (I). A solution of 8 g (22.7 mmol) of *tert*-butyl (*E*)-2-benzyl-3-(*p*-methoxybenzoyl)-propenoate in benzene was saturated with dry HCl at ice bath temperature. The reaction mixture was stirred for 3 h. Yellow needles (0.5 g) precipitated and were collected by filtration. The ^{1}H NMR, IR, and mass spectral data of the yellow crystalline material were consistent with that of the structure of 5-(4-methoxyphenyl)-3-(phenylmethylene)-2-(3*H*)-furanone. ^{1}H NMR ($CDCl_3$): δ 3.85 (s, 3 H), 6.81 (s, 1 H), 6.97 (d, J = 8.7 Hz, 2 H), 7.38 (s, 1 H), 7.40–7.48 (m, 3 H), 7.63 (d, J = 7.6 Hz, 2 H), 7.71 (d, J = 8.7 Hz, 2 H). Mass spectrum: M^+/e 278, 135, 107, 91, 77. Mass for $C_{18}H_{14}O_3$: calcd 278.0943; found 278.0942. As expected, the lactone linkage was cleaved on treatment with ethanolic KOH to yield VII.

The filtrate was diluted with 100 mL of diethyl ether, washed with water and saturated NaCl, dried over $MgSO_4$, and decolorized over activated carbon. Next, the solvent was removed under vacuum at ambient temperature, and the residue was treated with 50 mL of 5% anisole in trifluoroacetic acid for 30 min. Trifluoroacetic acid was removed under reduced pressure at ambient temperature. Upon the treatment of the residue with 20 mL of diethyl ether, 2.0 g of 5-(4-methoxyphenyl)-3-(phenylmethylene)-2(3*H*)-furanone crystals was obtained, and these were collected by filtration. The filtrate was decolorized again with activated carbon. Upon the addition of petroleum ether and cooling, 2 g (26%) of 2-benzyl-2-chloro-3-(*p*-methoxybenzoyl)propionic acid crystallized and was collected by filtration; mp 60 °C dec. ^{1}H NMR ($CDCl_3$): δ 3.49 (d, J = 17.7 Hz, 1 H), 3.53 (d, J = 14.5 Hz, 1 H), 3.63 (d, J = 14.5 Hz, 1 H), 3.72 (d, J = 17.7 Hz, 1 H), 3.86 (s, 3 H), 6.91 (d, J = 8.7 Hz, 2 H), 7.1–7.30 (m, 5 H), 7.84 (d, J = 8.7 Hz, 2 H). IR (KBr): 3000 br, 1733 s, 1665 s, 1600 cm^{-1}. Mass spectrum: M^+/e 297 (M – Cl), 149, 135, 107, 91, 77. Anal. Calcd for $C_{18}H_{17}ClO_4 \cdot H_2O$: C, 61.63; H, 5.46; Cl, 10.11. Found: C, 61.93; H, 5.44; Cl, 10.36.

2-Benzyl-2-chloro-3-benzoylpropionic Acid-3,3-d_2 (II-d_2). This compound was synthesized as described above in 20% yield by using DCl instead of HCl; mp 62 °C dec. ^{1}H NMR ($CDCl_3$): δ 3.52 (d, J = 14.5 Hz, 1 H), 3.64 (d, J = 14.5 Hz, 1 H), 3.70 (s, 0.1 H), 3.77 (s, 0.1 H), 3.85 (s, 3 H), 6.89 (d, J = 8.8 Hz, 2 H), 7.20–7.25 (m, 5 H), 7.83 (d, J = 8.8 Hz, 2 H). Mass spectrum: M^+/e 299, 298, 297 (M – Cl) [1:0.35:0.11], 152, 135, 107, 91, 77. Both ^{1}H NMR and mass spectroscopy results have indicated that the isotopic purity at the activated methylene group is only 80% in deuterium. This conclusion is confirmed by the kinetics of both the hydroxide ion and CPA-catalyzed α,β-eliminations of HCl, which are characterized by biphasic kinetics (the α,β-elimination of HCl from the undeuterated compound follows apparent first-order kinetics under the same conditions). The initial burst reaction corresponds to 20% of the substrate, followed by a slower reaction corresponding to the remaining 80%.

Ethyl (*E*)-2-Methyl-3-benzoylpropenoate. A solution containing 29.4 g (77.4 mol) of 1-phenyl-2-(triphenylphosphoranylidene)ethanone and 8.9 g (76.7 mmol) of ethyl pyruvate in 200 mL of chloroform was stirred at room temperature for 24 h. The chloroform was removed under vacuum and the residue dissolved in hexane. Triphenylphosphine oxide crystallized and was removed by filtration. The filtrate was passed over a short silica column to remove the remaining triphenylphosphine oxide. The solvent was removed under vacuum to yield 13.8 g (82%) of light yellow oil. ^{1}H NMR ($CDCl_3$): δ 0.97 (t, J = 6.6 Hz, 3 H), 2.25 (s, 3 H), 3.98 (q, J = 6.6 Hz, 2 H), 6.99–7.20 (m, 3 H), 7.75 (s, 1 H), 7.84 (d, J = 8.2 Hz, 2 H). IR (neat): 1710, 1675 cm^{-1}.

(*E*)-2-Methyl-3-benzoylpropenoic Acid. A solution of 7.1 g (32.6 mmol) of ethyl (*E*)-3-benzoyl-2-methylpropenoate and 10 g of Na_2CO_3 in 200 mL of 40% aqueous ethanol was heated under reflux for 3 h. The reaction mixture was concentrated under reduced pressure and washed once with ether. Upon acidification of the aqueous layer with 6 M HCl and cooling, 4.8 g (77.5%) of yellow needles was obtained. This material was recrystallized from ethyl acetate and petroleum ether; mp 100–102 °C. ^{1}H NMR ($CDCl_3$): δ 2.19 (s, 3 H), 7.48–7.60 (m, 3 H), 7.83 (s, H), 7.97 (d, J = 7.7 Hz, 2 H). IR (KBr): 2950 br, 1688 s, 1662 s, 1590 m cm^{-1}. Mass spectrum: M^+/e 190 (molecular ion), 172, 145, 105, 77.

2-Chloro-2-methyl-3-benzoylpropionic Acid (III). A solution of 1.1 g (5.8 mmol) of (*E*)-3-benzoyl-2-methylpropenoic acid in 10 mL of acetic acid was saturated with dry HCl and stirred at room temperature for 2 h. Upon the addition of 100 mL of water and cooling, the product crystallized. Recrystallization from a diethyl ether–petroleum ether mixture produced 0.5 g (38%) of colorless crystals, mp 100 °C dec. ^{1}H NMR ($CDCl_3$): δ 1.91 (s, 3 H), 3.74 (d, J = 17.9 Hz, 1 H), 3.97 (d, J = 17.9 Hz, 1 H), 7.40–7.62 (m, 3 H), 7.94 (d, J = 7.5 Hz, 2 H). IR (KBr): 2950 br, 1703 s, 1685 s cm^{-1}. Mass spectrum: M^+/e 191 (M – Cl), 173, 147, 145, 105, 77. Anal. Calcd for $C_{11}H_{11}ClO_3$: C, 58.29; H, 4.89; Cl, 15.64. Found: C, 58.96; H, 4.99; Cl, 15.50.

Product Analysis

CPA-Catalyzed Elimination Using 2-Benzyl-2-chloro-3-(4-methoxybenzoyl)propionic Acid (I) as the Substrate. A solution of 0.5 g (1.5 mol) of (±)-I in 150 mL of freshly distilled dioxane was added to 2 L of 3 μM CPA in Tris-HCl buffer, pH 8.4, and 0.5 M NaCl at ambient temperature. The CPA-catalyzed elimination of HCl from (±)-I was followed spectrophotometrically at 315 nm. Upon the completion of the reaction, the reaction mixture was acidified to pH 2 with 2 M HCl and extracted with diethyl ether. The ether solution was washed with 2 M HCl and then with saturated NaCl and dried over $MgSO_4$. After evaporation of solvent under reduced pressure at ambient temperature, the residue was chromatographed on a silica column and eluted with a solution containing 71.5% hexane, 25% ethyl acetate, and 3.5% acetic acid to yield 175 mg (70%) of (+)-I, $[\alpha]^{25}_D$ = 129

(c 1.7, dioxane), and 160 mg (75%) of (Z)-2-benzyl-3-(4-methoxybenzoyl)propenoic acid (IV) as the only detectable products. Compound (+)-I has NMR, IR, mass spectrum, and chromatographic behavior identical with that of (±)-I. Compound IV has different spectral and chromatographic behavior than (E)-2-benzyl-3-(4-methoxybenzoyl)propenoic acid (VII). ^{1}H NMR ($CDCl_3$): δ 3.72 (br s, 2 H), 3.81 (s, 3 H), 6.71 (s, 1 H), 6.87 (d, J = 8.9 Hz, 2 H), 7.22–7.32 (m, 5 H), 7.64 (br, 2 H). IR ($CHCl_3$): 3420 m, 1772 s, 1725 m, 1600 s cm^{-1}. Mass spectrum: M^+/e 296 (molecular ion), 278, 251, 161, 135, 107, 91, 77.

CPA-Catalyzed Elimination Using 2-Benzyl-2-chloro-3-benzoylpropionic Acid (II) as the Sustrate. By use of a procedure similar to that for (±)-I for the CPA-catalyzed elimination of HCl from (±)-II, compound (+)-II $[\alpha]^{25}_D$ = 62.0 (c 1.4, chloroform) and (Z)-2-benzyl-3-benzoylpropenoic acid (V) were isolated in over 70% yield by using 88% hexane, 10% ethyl acetate, and 2% acetic acid to elute the products from the silica column. No other product was detected. Compound (+)-II has NMR, IR, mass spectrum, and chromatographic behavior identical with that of (±)-II. Compound V has different NMR, IR, and chromatographic behavior than (E)-2-benzyl-3-benzoylpropenoic acid (VIII). ^{1}H NMR ($CDCl_3$): δ 3.57 (s, 2 H), 6.70 (s, 1 H), 7.13–7.35 (m, 8 H), 7.46 (d, J = 6.0 Hz, 2 H). Mass spectrum: M^+/e 266 (molecular ion), 248, 221, 105, 91, 77. IR ($CHCl_3$): 3565 m, 3280 m, br, 1765 s, 1600 m cm^{-1}.

Deuteration of Alkenes. (a) Deuteration Employing Diimide. Deuteration using reduction with diimide was carried out as described by Hamersma and Snyder.[26] In a typical deuteration experiment, a solution of 60 mg (0.2 mmol) of VII and 838 mg (4.3 mmol) of dipotassium azodicarboxylate was prepared in 4 mL of methyl alcohol-*d* under nitrogen. To the reaction mixture was added 145 μL (7.96 mmol) of deuterium oxide (or acetic acid-*d* for the reduction of V) in portions over 2–3 h and the resultant mixture allowed to stand for another 1 h. Then, the reaction was quenched with 10 mL of water and extracted with ethyl acetate. The ethyl acetate solution was washed with 2 M HCl followed by saturated NaCl and dried over $MgSO_4$. After solvent was evaporated under reduced pressure at ambient temperature, the residue was chromatographed on a silica column with a mixture of 76% hexane, 20% ethyl acetate, and 4% acetic acid to elute first the starting material, followed by the product.

(b) Catalytic Reduction. Compounds IV, V, VII, and VIII were reduced by deuterium gas in benzene by using palladium on carbon as the catalyst. In a typical reduction, 123 mg (46 mmol) of the alkene in 100 mL of benzene was treated with deuterium gas in the presence of 50 mg of 5% palladium on carbon for 90 min. Then, the catalyst was removed by filtration, and the solvent was evaporated under vacuum at ambient temperature. The residue was crystallized from chloroform and petroleum ether to yield 98 mg (71% yield) of product. The product of the reduction was chromatographically indistinguishable from 2-benzyl-3-(4-methoxybenzoyl)propionic acid (IX) when IV and VII were reduced and from 2-benzyl-3-benzoylpropionic acid (X) when V and VIII were reduced.

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Molecular Structure and Dynamics of Crystalline *p*-Fluoro-D,L-phenylalanine. A Combined X-ray/NMR Investigation

Yukio Hiyama,[†] James V. Silverton,[‡] Dennis A. Torchia,*[†] J. T. Gerig,[§] and S. J. Hammond[§]

Contribution from the Mineralized Tissue Research Branch, National Institute of Dental Research, Laboratory of Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205, and Department of Chemistry, University of California, Santa Barbara, California 93106. *Received June 28, 1985*

Abstract: We report the molecular structure and phenyl-ring dynamics of crystalline *p*-fluoro-D,L-[2,3,5,6-$^2H_4$]phenylalanine. Crystals of this molecule have space group $P\bar{1}$ and unit cell dimensions *a* = 5.1927 (6) Å, *b* = 5.4246 (4) Å, *c* = 16.1318 (15) Å, α = 96.752 (8)°, β = 94.060 (7)°, and γ = 110.153 (7)°. In the crystals the molecules pack so that layers of strongly hydrogen-bonded atoms alternate with layers composed of the nonpolar phenyl rings. Phenyl-ring dynamics in the crystalline state was studied by ^{2}H and ^{19}F nuclear magnetic resonance (NMR) spectroscopy. Analysis of NMR line shapes and spin–lattice relaxation times (T_1) showed that the phenyl ring exhibited two types of motion: (a) a rapid, small-amplitude rolling motion about the Cβ–C1 bond axis and (b) a slower 180° flip about the Cβ–C1 axis. The rms amplitude of the rolling motion increased from less than 5° at 22 °C to 17° at 143 °C. At 143 °C the correlation time for the motion was less than 10^{-11} s. The correlation time for the 180° ring flip decreased from ca. 10^{-4} s at 22 °C to 4.8 × 10^{-8} s at 160 °C. The temperature dependence of the correlation time in the 100–160 °C temperature range was fit by using a simple activation equation that yielded an apparent activation energy of 47 kJ/mol and a preexponential factor of 4 × 10^{-14} s for the phenyl-ring flip process. ^{2}H and ^{19}F spectra of twinned crystals were used to determine the approximate orientation of the ^{19}F chemical shift tensor. The most shielded tensor component was found to be normal to the phenyl ring while the intermediate shielded component was parallel to the C4–F bond axis.

Over the past decade various investigators have used nuclear magnetic resonance (NMR)[1] spectroscopy to study the motions of aromatic rings in amino acids, peptides, proteins, and synthetic polymers.[2-8] These studies have shown that, in addition to rapid, small-amplitude fluctuations, aromatic rings undergo 180° flips. The rates of ring flipping and the apparent activation energies

[†] National Institute of Dental Research.
[‡] National Heart, Lung, and Blood Institute.
[§] University of California.

(1) Abbreviations used: NMR, nuclear magnetic resonance; PFF, *p*-fluoro-D,L-phenylalanine; d_4PFF, *p*-fluoro-D,L-[2,3,5,6-$^2H_4$]phenylalanine; d_7PFF, *p*-fluoro-D,L-[2,3,5,6,*N*,*N*,*N*-$^2H_7$]phenylalanine; d_3PFF, *p*-fluoro-D,L-[*N*,*N*,*N*-$^2H_3$]phenylalanine; EFG, electric field gradient; T_1, spin–lattice relaxation time; T_2, transverse relaxation time; UV, ultraviolet spectroscopy.

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