Evidence against a Crucial Role for the Phenolic Hydroxyl of Tyr-248 in Peptide and Ester Hydrolyses Catalyzed by Carboxypeptidase A: Comparative Studies of the pH Dependencies of the Native and Phe-248-Mutant Forms

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Abstract: The kinetics of ester and amide hydrolysis by a genetically engineered carboxypeptidase A containing a phenylalanyl residue in place of Tyr-248, a putative catalytic group, have been studied over the pH range 5–10.5 at 25.0 °C. The pH dependencies of the steady-state parameters, k_{cat} and k_{cat}/K_m, for the cleavage of carbobenzoxyglycylglycyl-L-phenylalanine are similar to the corresponding dependencies obtained with the native enzyme, although the limiting values of the rate constants are respectively 2.5- and 12-fold lower in the case of the mutant enzyme. At pH 7.5, the Tyr-248-to-Phe conversion also displaces the substrate inhibition profile to at least 10-fold higher concentrations of tripeptide. In contrast, neither the steady-state rate constants nor the characteristic substrate inhibition profile measured for hydrolysis of the ester, *O*-(benzoylglycyl)-L-phenyllactate, at pH 7.5 are affected by this amino acid substitution. Another ester substrate, *O*-(*trans*-*p*-chlorocinnamoyl)-L-phenyllactate, does not display substrate inhibition with either the mutant or the wild-type enzymes. The shapes of the k_{cat}/K_m vs. pH profile for hydrolysis of this compound by the two catalysts are comparable, although the limiting value of k_{cat}/K_m is 35-fold smaller for the mutant. Thus, these studies eliminate the possibility that ionization of Tyr-248 is responsible for the decrease in activity seen above pH 8.5 in the k_{cat}/K_m profiles for ester *or* amide hydrolysis by carboxypeptidase A. The Tyr-248-to-Phe replacement does alter the k_{cat} vs. pH profile for hydrolysis of the cinnamate ester above pH 8.5 by suppressing the characteristic steeply rising basic limb. However, studies on other modified carboxypeptidases mitigate against assignment of the ionizing group of the EHS form of the wild-type enzyme–substrate complex to the phenolic hydroxyl of Tyr-248. Our finding that Tyr-248 can be replaced by Phe without substantial loss of either peptidase or esterase activity thus demonstrates that this residue is not required for catalysis of substrate cleavage by the enzyme. Nevertheless, Tyr-248 does seem to be important for ligand binding, and this role is briefly discussed.

Carboxypeptidase A (CPA)[1] is a zinc-dependent enzyme that cleaves amide and ester bonds of *N*-acyl-α-amino acids and *O*-acyl-α-hydroxy acids adjacent to the terminal free carboxyl groups. A tyrosyl residue at the active site of the protein, Tyr-248, has been widely believed to be important for the hydrolysis of peptide substrates, although its precise role in the hydrolytic mechanism has remained a matter of some controversy.[2] Chemical modification of this amino acid generally has a deleterious effect on the peptidase activity of the protein but not on its esterase action. Selective blocking of Tyr-248 with Co(III),[3] for example, has been reported to abolish peptidase activity without affecting the ability of the enzyme to bind peptides; ester substrates are cleaved by this modified CPA. Similarly, tyrosine nitration does not alter the pH–rate profiles for ester hydrolysis[4] but causes the basic limb of the k_{cat}/K_m profile for hydrolysis of a tripeptide to shift almost 3 pK units toward acid.[5] The X-ray diffraction analysis of several CPA–ligand complexes indicates further that substrate binding causes the phenolic side chain of Tyr-248 to move from the surface of the enzyme to within hydrogen-bonding distance of the scissile peptide bond.[6]

Consistent with these observations, Tyr-248 has been suggested[7] to function as a general-acid catalyst during cleavage of peptide substrates. The phenolic hydroxyl group could facilitate breakdown of the tetrahedral intermediate, generated by either a nucleophilic or a general-base pathway for peptide cleavage, by providing a proton to the amine leaving group. Because an amine anion requires greater stabilization than an alkoxide, the peptidase activity of the enzyme is expected to be more dependent on general-acid catalysis by Tyr-248 than its esterase action and hence sensitive to chemical modification of this amino acid. In another proposal,[8] torsional destabilization of the amide bond in peptide substrates bound at the active site of CPA has been inferred from the crystal structures of CPA–ligand complexes. The ensuing rehybridization of the amide π-bond orbitals has been argued to require a "reverse protonation" mechanism[5] in which Glu-270 acts as the proton donor to the leaving group, while Tyr-248, in its ionized form, serves as a general base to deliver water to the scissile amide bond. However, the twisting of the amide bond of the substrate might be induced by the change in enzyme conformation and need not depend explicitly on the ionization of the phenolic hydroxyl moiety.[9] The deleterious effects of chemical modification on peptidase activity, according to this suggestion, would result from unfavorable steric or electrostatic interactions introduced by the derivatization procedure.

We have investigated the mechanistic function of Tyr-248 in CPA-catalyzed hydrolyses using the technique of site-directed mutagenesis.[10] The rat pancreatic CPA cDNA[11] was modified

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(1) The Abbreviations used are: CPA, carboxypeptidase A; CPA-WT, rat wild-type carboxypeptidase A expressed in yeast; CPA-Phe-248, rat variant (Phe-248)carboxypeptidase A expressed in yeast; Cbz-Gly-Gly-Phe, carbobenzoxyglycylglycyl-L-phenylalanine; Bz-Gly-OPhe, *O*-(benzoylglycyl)-L-phenyllactic acid; ClCPL, *O*-(*trans*-*p*-chlorocinnamoyl)-L-phenyllactic acid.

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Table I. Kinetic Parameters for Substrate Hydrolyses by Unmodified and Acetylated CPA-WT and CPA-Phe-248[a]

substrate	kinetic parameter	CPA-WT	acetyl-CPA-WT	CPA-Phe-248	acetyl-CPA-Phe-248
Cbz-Gly-Gly-Phe	k_{cat}, s^{-1}	50.3 ± 2.3	14.2 ± 0.4	17.9 ± 1.1	14.4 ± 0.5
	K_m, μM	31.6 ± 3.9	96.1 ± 8.8	162 ± 34	182 ± 18
	$10^{-5}k_{cat}/K_m$, M^{-1} s^{-1}	15.9	1.48	1.11	0.790
Bz-Gly-OPhe	k_{cat}, s^{-1}	1040 ± 29	1150 ± 31	1090 ± 34	1030 ± 62
	K_m, μM	98.9 ± 8	368 ± 31	105 ± 8	124 ± 20
	$10^{-5}k_{cat}/K_m$, M^{-1} s^{-1}	105	31.2	104	82.8
ClCPL	k_{cat}, s^{-1}	13.8 ± 1.4		3.26 ± 0.5	
	K_m, μM	26.4 ± 0.71		225 ± 17	
	$10^{-5}k_{cat}/K_m$, M^{-1} s^{-1}	5.23		0.145	

[a] Assayed in aqueous buffer (50 mM Tris-HCl, 0.5 M NaCl, pH 7.5) at 25 °C as described in the text.

so that the codon for Tyr-248 would direct the incorporation of a phenylalanine. The mutagenized and wild-type templates were expressed in yeast via the α-factor system[12,13] to yield CPA-Phe-248 and CPA-WT, respectively. This methodology has considerable advantages over more traditional chemical modification protocols. It affords a means of accomplishing a rather conservative change, namely, specific replacement of the phenolic hydroxyl group of Tyr-248 with a hydrogen, that is not possible with current chemical methodology. Comparison of the detailed kinetic behavior exhibited by CPA-WT and CPA-Phe-248 has allowed us to discriminate among the postulated roles of Tyr-248. We have found that both purified proteins possess esterase and peptidase activity at pH 7.5, demonstrating that under the given conditions Tyr-248 is not essential for catalytic activity.[10] However, it seemed possible that incorporation of a phenylalanyl residue at the active site could alter the pH profile in such a way as to mask the deleterious effects of the mutation. We have now examined the properties of the mutant over a broad pH range and find that Tyr-248 ionization is not reflected in the kinetics of peptide or ester scission as catalyzed by CPA.

Experimental Section

Materials. The buffers, salts, and standard titrants used in these studies were all of the highest grade available. Carbobenzoxyglycyl-glycyl-L-phenylalanine (Cbz-Gly-Gly-Phe) and the sodium salt of *O*-(benzoylglycyl)-D,L-phenyllactic acid (Bz-Gly-OPhe) were purchased from Sigma Chemical Co. and used without further purification. *O*-(*trans*-*p*-Chlorocinnamoyl)-L-phenyllactic acid (ClCPL) was prepared according to the published procedure,[4] purified by preparative HPLC (C-18 column; CH_3CN:H_2O 58:42), and subsequently recrystallized from $CHCl_3$ [mp 126–126.5 °C (lit.[4] mp 125–126.5 °C)]. It was converted to the sodium salt prior to use.

Enzymes. Wild-type rat pancreatic carboxypeptidase A (CPA-WT) and the Tyr-248-to-Phe mutant enzyme (CPA-Phe-248) were synthesized in yeast and purified to homogeneity as previously described.[10] Stock solutions of relatively concentrated enzyme (ca. 10^{-5} M) were stored in aqueous buffer (50 mM Tris-HCl, 0.5 M NaCl, pH 7.5) at 4 °C for several months without appreciable loss of activity. Dilute enzyme solutions were prepared as needed. Enzyme concentrations were determined spectrophotometrically for both CPA-WT and CPA-Phe-248 by using the value of 6.42×10^4 M^{-1} cm^{-1} as the extinction coefficient at 278 nm.[14] The concentrations measured in this way were within 10% of those determined by amino acid analysis.

Acetylation of both CPA-WT and CPA-Phe-248 was effected by incubating the enzyme (ca. 1 mg/mL) with 5 mM *N*-acetylimidazole for 1 h at room temperature in aqueous buffer (20 mM HEPES, 1 M NaCl, pH 7.5). Excess reagent was removed by dialysis against two changes of buffer at 4 °C. Protein concentration was estimated by the absorbance at 280 nm (ϵ 5.92×10^4 M^{-1} cm^{-1}).[15]

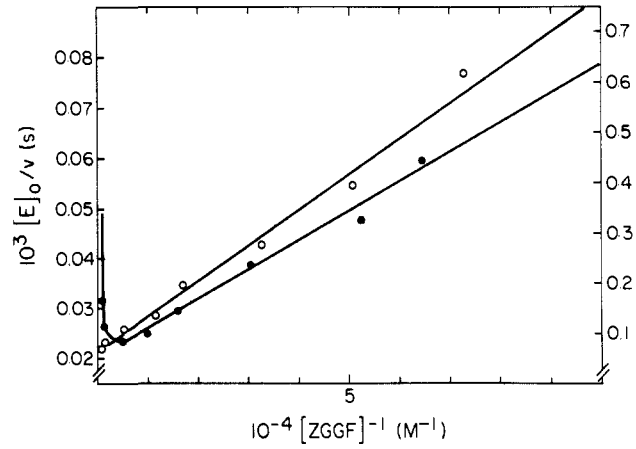

Figure 1. Effect of Cbz-Gly-Gly-Phe (ZGGF) concentration on the rate of its hydrolysis by CPA-WT (●, left scale) and acetylated CPA-WT (○, right scale) at 25.0 °C, pH 7.5, Tris-HCl buffer (50 mM), containing 0.5 M NaCl.

Kinetic Measurements. The rate of hydrolysis of each substrate was continuously measured spectrophotometrically at 25.0 ± 0.1 °C. The wavelengths used to monitor the various reactions were as follows: Bz-Gly-OPhe, 250–285 nm; Bz-Gly-Phe, 254 and 281–289 nm; Cbz-Gly-Gly-Phe, 223–228 nm; ClCPL, 300–330 nm. The absorbance change at a given wavelength was followed for at least 1 or 2 half-lives of the reaction. In random cases, infinity absorbance readings were taken and found to agree within 5% with the values expected for complete hydrolysis of the respective substrate. Initial rates, determined graphically from the first 5–10% of reaction, were obtained at substrate concentrations bracketing the apparent K_m for each pH. The values of the kinetic parameters, k_{cat} and K_m, were then obtained by direct fit of 5–6 points to eq 1 using an iterative nonlinear least-squares computer program.[16]

$$V_0/[E_T] = k_{cat}[S]/(K_m + [S]) \quad (1)$$

In order to detect systematic deviations from standard Michaelis–Menten kinetics, the data were also converted to double reciprocal plots. Product inhibition was neglected in our study, since only initial rates were used in the calculation of the kinetic constants.

MES, Tris, and ammediol buffers (0.05 M) containing sodium chloride (0.5 M) were used in the determination of the pH–rate profiles. Buffers below pH 6.0 also contained 10^{-4} M $ZnCl_2$.[17] An iterative curve-fitting program was used to fit the pH vs. rate data to the equations described in the text.[16]

Results

Comparison of Amide Hydrolysis by CPA-WT and CPA-Phe-248. A preliminary report describing the steady-state rate constants for hydrolysis of typical amide and ester substrates by CPA-WT and CPA-Phe-248 at pH 7.5 has already appeared.[10] The kinetic parameters for some of the substrates were redetermined here with fresh preparations of the two enzymes (Table I), and the results obtained are in excellent agreement with our

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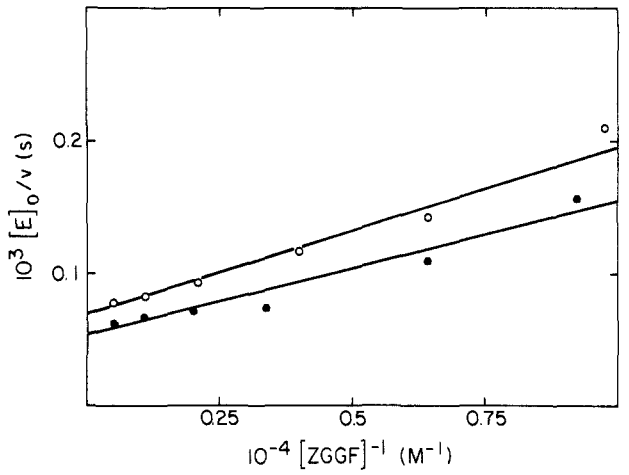

Figure 2. Effect of Cbz-Gly-Gly-Phe (ZGGF) concentration on the rate of its hydrolysis by CPA-Phe-248 (●) and acetylated CPA-Phe-248 (○) at 25.0 °C, pH 7.5, Tris-HCl buffer (50 mM), containing 0.5 M NaCl.

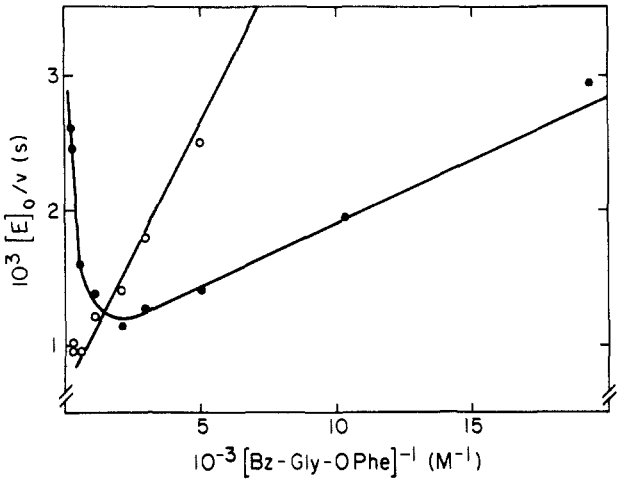

Figure 3. Effect of Bz-Gly-OPhe concentration on the rate of its hydrolysis by CPA-WT (●) and acetylated CPA-WT (○) at 25.0 °C, pH 7.5, Tris-HCl buffer (50 mM), containing 0.5 M NaCl. One low substrate point for CPA-WT and three low substrate points for acetyl-CPA-WT lie on the linear portion of the respective curves but fall outside the scale of the plot.

previously published values. Comparison of the ability of the two enzymes to hydrolyze peptide and ester substrates reveals that the major features of their reactivities are similar, indicating that Tyr-248 cannot be essential for catalytic activity. However, in some cases there are appreciable differences in detail between the two enzymes, which are manifested in the substrate–velocity profiles as well as the values of the steady-state kinetic parameters.

Kinetic anomalies are well precedented in CPA-catalyzed reactions,[15,18] and we find that the hydrolysis of Cbz-Gly-Gly-Phe by rat CPA-WT is complicated by substrate inhibition at peptide concentrations above 0.2 mM (Figure 1). However, the K_m value for this reaction appears to be only about 30 μM, so the kinetic constants can be extrapolated without difficulty with substrate concentrations below 0.2 mM (Table I). Tyrosine acetylation in bovine CPA has been shown to decrease peptidase activity significantly under standard assay conditions,[14] in addition to displacing the substrate–inhibition profile to much higher substrate concentrations.[19] Treatment of rat CPA-WT with *N*-acetylimidazole similarly reduces its activity toward Cbz-Gly-Gly-Phe. The value of k_{cat}/K_m for the acetylated rat enzyme is approximately 10% of that found for the unmodified protein due to a 3.5-fold decrease in k_{cat} and a 3-fold increase in K_m (Table I). Furthermore, substrate inhibition cannot be detected with peptide concentrations up to 2 mM (Figure 1).

Replacement of Tyr-248 by Phe causes the k_{cat} value measured at pH 7.5 for hydrolysis of Cbz-Gly-Gly-Phe to decrease only by a factor of 2.8 compared to the value obtained with CPA-WT (Table I). Since this parameter presumably reflects the efficiency of the catalytic step, a rate-determining proton transfer involving the Tyr-248 phenolic hydroxyl is ruled out at this pH for peptide cleavage. The K_m value also increases almost 5-fold, so the activity of the mutant protein, as reflected by the k_{cat}/K_m parameter, is more than 10-fold lower than that of the wild-type enzyme. Substrate inhibition is not observed in the CPA-Phe-248-catalyzed cleavage of Cbz-Gly-Gly-Phe using peptide concentrations of at least 2 mM (Figure 2). Removal of the phenolic hydroxyl moiety therefore appears to have much the same effect on the kinetics of peptide hydrolysis as acetylation of the native enzyme. Because the acetylation protocol is nonspecific,[14] however, it had never been possible to correlate the kinetic consequences of protein modification with the derivatization of a single tyrosyl residue. Now it is clear that the alteration in the kinetic behavior of the protein toward peptide substrates is primarily due to acetylation of Tyr-248. Treatment of CPA-Phe-248 with *N*-acetylimidazole causes k_{cat} and k_{cat}/K_m to decrease modestly, probably as a consequence of acetylation of the remaining active-site tyrosine, Tyr-198 (Table I).

Comparison of Ester Hydrolysis by CPA-WT and CPA-Phe-248. It has been suggested[20] that CPA catalyzes the cleavage of amides and esters by different mechanisms. The fact that chemical modification of Tyr-248 affects the esterase and peptidase actions of the enzyme differently has been used to support this hypothesis.[2b,c] The detailed kinetic consequences of replacing Tyr-248 with Phe on the hydrolysis of ester substrates were therefore of considerable interest.

As we reported previously,[10] the ester Bz-Gly-OPhe is efficiently hydrolyzed by CPA-WT at pH 7.5 (Table I), although the enzyme displays marked substrate inhibition at substrate concentrations above 0.5 mM (Figure 3). Racemic Bz-Gly-OPhe was employed in this study, since the D-isomer of this substrate is known to have no effect on the kinetics of the reaction.[15] The value of K_m measured in the hydrolysis of this ester was calculated, assuming that only the L-isomer binds to the enzyme and was estimated to be ca. 100 μM, about 5 times smaller than the substrate concentrations that cause inhibition. A comparable substrate–velocity profile is obtained when Bz-Gly-OPhe is hydrolyzed by CPA-Phe-248 (Figure 4), and the extrapolated kinetic constants for the mutant and native enzymes are well within experimental error (Table I). Thus, in contrast to the situation observed for the hydrolysis of Cbz-Gly-Gly-Phe, removal of the phenolic hydroxyl group does not alter the pattern of substrate inhibition by this ester or affect the value of K_m.

Treatment of bovine CPA with *N*-acetylimidazole has been shown to displace the inhibition due to excess Bz-Gly-OPhe to much higher substrate concentrations.[15,18] Acetyl-CPA-WT behaves analogously, with no kinetic evidence for substrate inhibition at pH 7.5 with ester concentrations below 1 mM (Figure 3). Comparison of the kinetic constants with those for CPA-WT (Table I) shows that the value of k_{cat} is little changed as a result of chemical modification. The primary effect of acetylation on enzymatic activity is an approximately 4-fold increase in the apparent K_m. In the case of CPA-Phe-248, however, treatment with *N*-acetylimidazole does not suppress substrate inhibition at all (Figure 4), and the extrapolated kinetic constants are comparable to those determined with the unmodified enzyme (Table I). We conclude that derivatization of Tyr-248 is solely responsible for the observed alteration of the kinetic profile for CPA-WT (Figure 3).

We have also examined the hydrolysis of the specific ester substrate ClCPL that has been reported[4] to be relatively free of kinetic anomalies. We did not detect substrate activation or substrate inhibition in the reaction of ClCPL with either CPA-WT

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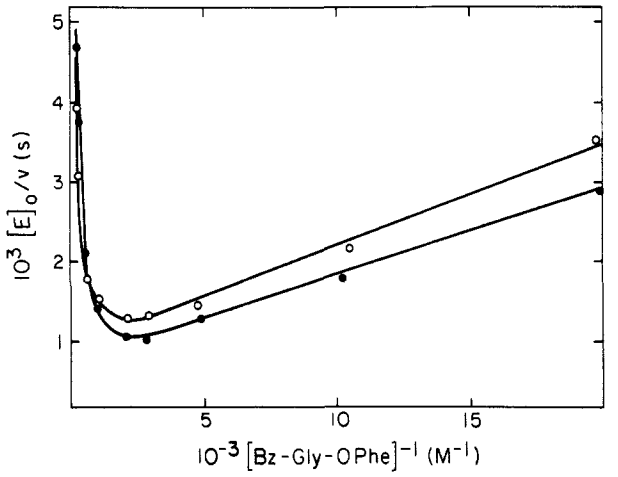

Figure 4. Effect of Bz-Gly-OPhe concentration on the rate of its hydrolysis by CPA-Phe-248 (●) and acetylated CPA-Phe-248 (○) at 25.0 °C, pH 7.5, Tris-HCl buffer (50 mM), containing 0.5 M NaCl. For both profiles, an additional low substrate point was determined that lies on the linear portion of the curves but falls outside the scale of the plot.

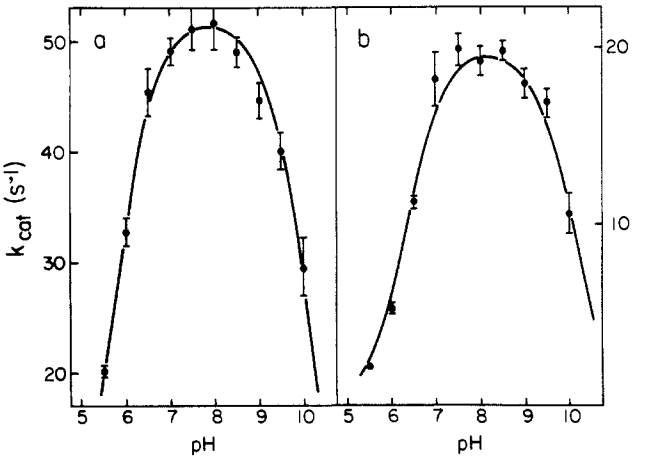

Figure 5. k_{cat} vs. pH for the hydrolysis of Cbz-Gly-Gly-Phe catalyzed by (a) CPA-WT and (b) CPA-Phe-248 at 25.0 °C. The experimentally determined values (●) were fitted in both cases to eq 2 by an iterative curve-fitting program. Standard deviation estimates are indicated at each point by the error bars.

or CPA-Phe-248 up to substrate concentrations of about 2 mM. The mutant enzyme is, however, significantly less active toward this ester than CPA-WT. As seen in Table I, the Tyr-248-to-Phe replacement causes an approximately 4-fold decrease in k_{cat} and a 10-fold increase in K_m at pH 7.5.

pH Dependence of Tripeptide Hydrolysis. Although CPA-Phe-248 displays considerable activity toward peptide substrates at pH 7.5, it is important to examine the ability of this mutant to cleave substrates over a wider range of pH. Circumstantial evidence has been used to implicate Tyr-248 as the ionizing group responsible for the drop in peptidase activity (as measured by k_{cat}/K_m) above pH 8.5.[2,5] If this assignment is correct, we would predict that this inflection in the k_{cat}/K_m profile would be absent in the reactions catalyzed by the mutant enzyme.

In Figure 5, plots of the pH dependency of k_{cat} for the cleavage of Cbz-Gly-Gly-Phe by both CPA-WT and CPA-Phe-248 are displayed, and the variation of k_{cat}/K_m with pH for the two enzymes is shown in Figure 6. The experimental data were fitted to eq 2 and 3, and the resulting curves are illustrated in the figures.

$$k_{cat} = \frac{(k_{cat})_{lim}}{1 + [H^+]/K_{EH_2S} + K_{EHS}/[H^+]} \quad (2)$$

$$k_{cat}/K_m = \frac{(k_{cat}/K_m)_{lim}}{1 + [H^+]/K_{EH_2} + K_{EH}/[H^+]} \quad (3)$$

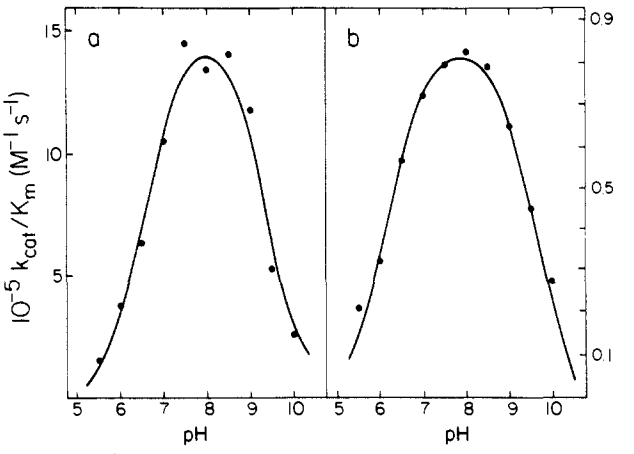

Figure 6. k_{cat}/K_m vs. pH for the hydrolysis of Cbz-Gly-Gly-Phe catalyzed by (a) CPA-WT and (b) CPA-Phe-248 at 25.0 °C. The experimentally determined values (●) were fitted to eq 3 by an iterative curve-fitting program.

Scheme I

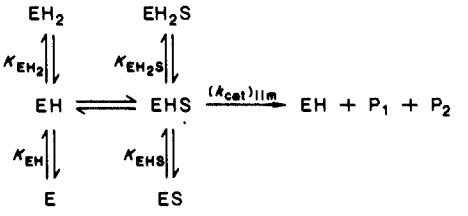

Table II. Parameters for pH Dependence of Hydrolysis of Cbz-Gly-Gly-Phe and ClCPL by CPA-WT and CPA-Phe-248[a,b]

	Cbz-Gly-Gly-Phe		ClCPL	
parameter[b]	CPA-WT	CPA-Phe-248	CPA-WT	CPA-Phe-248
pK_{EH_2S}	5.73 ± 0.05	6.35 ± 0.05	6.02 ± 0.17	6.75 ± 0.06
pK_{EHS}	10.1 ± 0.05	10.1 ± 0.06	>10	
$(k_{cat})_{lim}$	51.9	20.0	14.0	3.88
pK_{EH_2}	6.56 ± 0.10	6.16 ± 0.06	6.19 ± 0.07	5.74 ± 0.12
pK_{EH}	9.32 ± 0.11	9.53 ± 0.05	9.46 ± 0.07	9.64 ± 0.11
$10^{-5}(k_{cat}/K_m)_{lim}$	15.0	0.843	5.31	0.146

[a] Measurements at 25.0 °C in aqueous buffer, ionic strength 0.5. The parameters were determined from eq 2 and 3 as described in the text. The errors are at the 99% confidence level. [b] Units for $(k_{cat})_{lim}$ and $(k_{cat}/K_m)_{lim}$ are s^{-1} and M^{-1} s^{-1}, respectively.

The various ionization constants, defined in the minimum scheme (Scheme I), are summarized in Table II.

Although the value of k_{cat} for Cbz-Gly-Gly-Phe hydrolysis by bovine CPA is reported[21] to be constant over the pH range 7.5–10, this is not true for the enzymes of rat origin. As seen in Figure 5, the plot of k_{cat} vs. pH is bell-shaped for both CPA-WT and CPA-Phe-248. The limiting value of this rate constant in the case of the mutant enzyme is only 2.5-fold smaller than that for the reaction catalyzed by CPA-WT. Furthermore, the apparent pK_a's that control k_{cat} are also similar for the two enzymes. The small upward shift observed in the value of pK_{EH_2S} upon replacement of Tyr-248 with Phe may reflect the greater hydrophobicity of the active site of the mutant enzyme.

The K_m value exhibited by the bovine enzyme for the cleavage of Cbz-Gly-Gly-Phe is reportedly constant from pH 5 to pH 8, while k_{cat} depends strongly on pH.[21] This observation provides strong evidence[22] that, at least for bovine CPA, the Michaelis constant is proportional to the dissociation constant, K_s, of the enzyme–substrate complex below pH 8. In contrast, we find that the value of K_m of tripeptide hydrolysis by both CPA-WT and CPA-Phe-248 varies over the entire pH range studied. Thus, no

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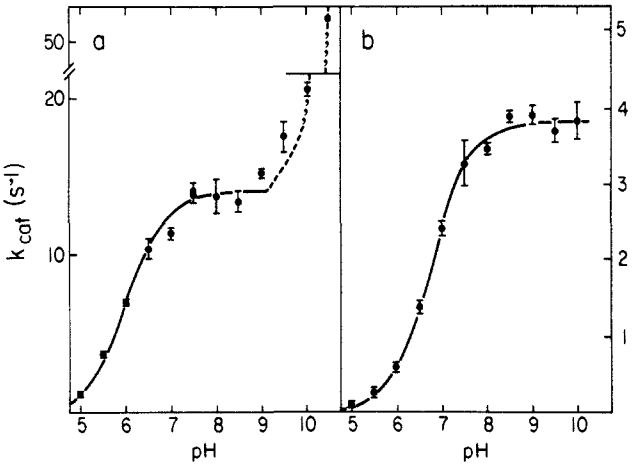

Figure 7. k_{cat} vs. pH for the hydrolysis of ClCPL catalyzed by (a) CPA-WT and (b) CPA-Phe-248 at 25.0 °C. The solid line represents a theoretical sigmoidal curve that was fitted to the indicated experimental points (●) according to eq 4. The broken line was fitted by eye to the high pH data in the CPA-WT-catalyzed reaction. Standard deviation estimates are indicated at each point by the error bars. The standard deviation on the pH 10.5 point in (a) is ±12.8 s^{-1}.

simple relationship between it and the dissociation constant is apparent in the reactions catalyzed by either of the rat enzymes.

The plot of k_{cat}/K_m vs. pH for tripeptide cleavage by rat CPA-WT resembles that previously seen[21] for this substrate with bovine CPA, and the data, fitted to eq 3, yield apparent pK_a's of about 6.6 and 9.3 for two ionizing groups on the protein (Table II). The pH profile obtained with CPA-Phe-248 is qualitatively similar, although the pK_a values shift slightly and $(k_{cat}/K_m)_{lim}$ is decreased more than 15-fold compared to the wild-type enzyme. Despite the overall decrease in the limiting value of the rate constant, the fact that the pH profiles for the mutant and wild-type catalysts display comparable inflections demonstrates conclusively that ionization of Tyr-248 is not responsible for the decrease in peptidase activity above pH 8.5 (or below 7.0).

pH Dependence of Ester Hydrolysis. The hydrolysis of ClCPL was examined over the pH range 5.5–10.5. The pH dependencies of the steady-state parameters, k_{cat} and k_{cat}/K_m, obtained in the cleavage of this substrate by the rat enzymes are presented in Figures 7 and 8, respectively.

As observed for bovine CPA,[4] the value of k_{cat} for the hydrolysis of ClCPL by CPA-WT shows a sigmoidal increase as the medium becomes more basic. The curve begins to level off at pH 8.5 before rising steeply above pH 9 (Figure 7a). The acidic limb of this curve was fitted to eq 4 and seems to depend on an ionizing group

$$k_{cat} = \frac{(k_{cat})_{lim}}{1 + [H^+]/K_{EH_2S}} \quad (4)$$

with pK_{EH_2S} of about 6.0. The value of pK_{EHS} could not be reliably estimated because of the difficulty of collecting data above pH 10.5. The k_{cat} vs. pH profile of the CPA-Phe-248 catalyzed reaction (Figure 7b) is also sigmoidal, with an inflection corresponding to an apparent pK_a of about 6.8, which is somewhat higher than that found for the wild-type enzyme. Thus, for both peptide and ester substrates, the Tyr-248-to-Phe substitution causes the value of pK_{EH_2S} to increase by 0.6–0.8 pK unit. The most profound consequence of mutation is the apparent suppression of the basic limb of the k_{cat} profile for hydrolysis of ClCPL (Figure 7b). However, we were unable to extend our measurements to pH 10.5, because of the limited solubility of the ester and the very high K_m for this substrate under these conditions. It is possible that the pK_{EHS} ionization is raised by several units as a consequence of Tyr-248 replacement, in which case the basic limb would be shifted to an experimentally inaccessible pH range.

The k_{cat}/K_m vs. pH profiles for ClCPL hydrolysis by CPA-WT and CPA-Phe-248 are bell-shaped with two well-separated ionizations (Figure 8). Although the limiting value of this rate constant differs by a factor of about 40 for the two enzymes, the

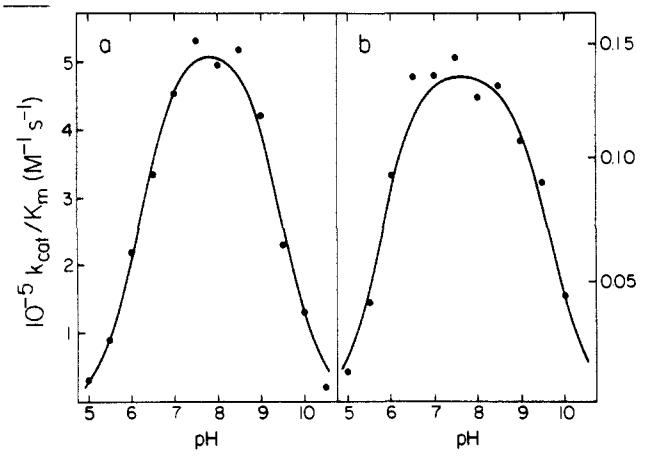

Figure 8. k_{cat}/K_m vs. pH for the hydrolysis of ClCPL catalyzed by (a) CPA-WT and (b) CPA-Phe-248 at 25.0 °C. The experimentally determined values (●) were fitted to eq 3 by an iterative curve-fitting program.

pK_a values for the acid and the base limbs are comparable. Moreover, the ionizations are similar to those observed in hydrolyses of cinnamate esters catalyzed by bovine CPA.[4,23] The state of ionization of Tyr-248 thus appears not to affect k_{cat}/K_m in the hydrolysis of this ester.

Discussion

Zinc(II) and the side chains of Glu-270 and Tyr-248 at the active site of CPA have been implicated as functional groups in catalysis of substrate hydrolysis.[2] Despite extensive study, however, a general consensus has not yet been reached concerning the specific roles of these groups. Thus, the controversy concerning whether the hydrolytic pathway involves nucleophilic or general-base catalysis on the part of Glu-270 has not been settled. A zinc hydroxide mechanism has not been ruled out either, although the stereochemistry of CPA catalysis in the enolization of 2-benzyl-3-(*p*-methoxybenzoyl)propionic acid argues against such a pathway in that particular case.[24] In this report we have examined the putative role of Tyr-248.

Previous conclusions regarding the mechanism of action of CPA derive from extensive work with enzyme isolated from bovine pancreas. The cloned enzymes we have studied, however, were constructed by using the gene-encoding rat pancreatic CPA. Nevertheless, the amino acid sequences of rat and bovine CPA-WT are 78% homologous, and the residues previously implicated in catalysis and ligand binding are conserved between the two species. The kinetic properties of both wild-type enzymes, including substrate–inhibition profiles and pH–rate dependencies, also appear to be similar. The values of k_{cat} and K_m for the hydrolysis of Bz–Gly-OPhe, for example, are roughly the same for rat[10] and bovine[15] CPA. The k_{cat}/K_m values are also of similar magnitude (within a factor of 4) for both enzymes in the hydrolysis of Bz-Gly-Phe,[18] Cbz-Gly-Gly-Phe[19] and ClCPL,[4] although both k_{cat} and K_m for these substrates are 5–20-fold lower for rat CPA-WT as compared to the bovine catalyst. In addition, nitration[10] and acetylation of Tyr-248 of rat CPA-WT have the same effect on esterase and peptidase activities as previously reported for the bovine enzyme.[15,33] Thus, the role of Tyr-248 is likely to be the same for the rat and bovine catalysts.

Our results show that the phenolic hydroxyl group of Tyr-248 is not essential for the cleavage of ester *or* peptide substrates over the pH range 5–10. The catalytic rate constant, k_{cat}, does not decrease significantly upon replacement of Tyr-248 with a phenylalanyl residue. The k_{cat} values obtained at pH 7.5 for the hydrolysis of the ester Bz-Gly-OPhe and its exact dipeptide analogue, Bz-Gly-Phe,[10] by CPA-Phe-248 are the same, or even slightly greater, than those determined for the wild-type enzyme.

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With a tripeptide substrate, Cbz-Gly-Gly-Phe, the Tyr-248-to-Phe substitution causes the limiting value of k_{cat} to decrease only 2.5-fold. The pH profiles for k_{cat} in the hydrolysis of the latter substrate by the mutant and wild-type enzymes are both bell-shaped with similar inflections. The slightly higher value of pK_{EH_2S} seen in the CPA-Phe-248 catalyzed reaction may reflect the more hydrophobic active site of this protein. In any case, it is clear that the pK_a's that characterize the k_{cat} profile for peptide hydrolysis catalyzed by CPA-WT cannot be due to ionization of Tyr-248.

The considerable activity displayed by the phenylalanine-containing mutant toward peptide substrates contrasts with the inactivity of some chemically modified CPA derivatives.[3,14] Although diazotization of Tyr-248 with arsanilic acid does not significantly diminish the peptidase activity of bovine CPA,[25] further coordination of the phenolic oxygen of the arsanilazo-Tyr-248 residue to Co(III) yields a modified enzyme that cannot cleave peptides.[3] This Co(III) derivative appears to bind peptides as tightly as the unmodified protein and retains the ability to bind and hydrolyze esters. The authors claim that only Tyr-248 is affected by their chemical modification protocol, and reduction of the complex to Co(II) completely restores peptidase action. Our results demonstrate that the loss of activity observed for this particular Tyr-248 derivative is not simply a consequence of blocking the phenolic hydroxyl group, since CPA-Phe-248, which lacks this moiety, is still quite active toward peptide substrates. Rather, the change in kinetic behavior subsequent to chemical modification of Tyr-248 must reflect the novel steric and electronic properties of the arsanilazo modification.

The active-site mutation that we have studied has a more profound effect on the k_{cat} parameter for cleavage of the specific ester substrate, ClCPL. The rate constant shows a sigmoidal dependence on pH in the region 5.0–8.5 with CPA-WT and CPA-Phe-248, and as seen with the pH–rate data for tripeptide hydrolysis, the apparent pK_a for the EH_2S form of the mutant is somewhat higher than that of the wild-type enzyme. The limiting value of the catalytic rate constant decreases roughly 4-fold upon substitution of Tyr-248 with Phe. However, the most significant consequence of this mutation is the suppression of the basic limb of the k_{cat} vs. pH profile. Although the value of the rate constant increases steeply above pH 8.5 for CPA-WT, the steep rise is absent in the rate profile for the reaction catalyzed by CPA-Phe-248.

The difference in the pH dependencies of k_{cat} for the hydrolysis of ClCPL by the mutant and wild-type enzymes could be considered to support the assignment of Tyr-248 as the ionizing group with pK_{EHS} greater than 10 in the CPA-WT complex with substrate. However, we feel that this explanation of the experimental data is unlikely. The intrinsic pK_a of Tyr-248 can be lowered to 6.3 or 7.7 by specific nitration[26] and diazotization,[25,27] respectively, but the basic limb of the k_{cat} vs. pH profiles for the hydrolysis of ClCPL by nitro-Tyr-248-CPA and arsanilazo-Tyr-248-CPA has been shown to be unaffected by such chemical modification.[4] In addition, we have used site-directed mutagenesis to prepare another CPA mutant in which Tyr-198 is replaced with a phenylalanine.[28] This tyrosyl residue is located near the active site and has been implicated as a potential catalytic group by chemical modification studies.[29] We have found that the k_{cat} parameter for CPA-Phe-198 catalyzed cleavage of ClCPL is independent of pH in the range 7.5–10.5, as well, yielding a limiting value of the rate constant of 2.5 ± 0.2 s^{-1} at 25 °C. Since Tyr-198 and Tyr-248 cannot both be responsible for the pK_{EHS} ionization, another explanation must be sought. It is possible that replacement of Tyr-248 or Tyr-198 with a phenylalanyl residue displaces the pK_a of the ionizing group that actually controls the basic limb in the enzyme–substrate complex to an experimentally inaccessible pH region. Another possibility is that the apparent pK_a observed in the reaction catalyzed by the wild-type enzyme does not reflect the intrinsic pK_a of an ionizing group at all but is rather a composite term that depends on various rate constants in a complex way.[30] The apparent loss of the basic limb seen with CPA-Phe-248 and CPA-Phe-198 might therefore reflect the fact that mutation changes the rate-determining step in the ClCPL hydrolysis pathway.

Our finding that the catalytic rate constant, k_{cat}, for peptide hydrolysis is little affected by the Tyr-248-to-Phe mutation demonstrates that Tyr-248 cannot be involved in an essential proton-transfer step during hydrolysis. The mechanistic suggestions[7] that invoke Tyr-248 as a general-acid catalyst which facilitates breakdown of the tetrahedral intermediate are therefore inadequate. Similarly, a "reverse-protonation" mechanism[5,8] in which Tyr-248, in its ionized form, is suggested to act as a general base cannot be operative. If this residue is involved in a proton-transfer step at all, then this step cannot be rate controlling for catalysis by CPA, and the phenolic hydroxyl of Tyr-248 can be replaced by water or possibly by another active-site residue without comprising the value of the catalytic rate constant, k_{cat}.

The question is what then is the role of Tyr-248. It may be that a significant function of this residue is to provide a hydrophobic "lid" for the binding of substrates and that conversion of this residue to Phe does not have much effect on this phenomenon. It is also possible that Tyr-248 is involved in the twisting of the scissile-substrate bond prior to attack of water or an enzyme nucleophile, as has been postulated previously.[9] This residue does seem to be important for the binding of at least some ligands. We have shown[10] that a specific carboxypeptidase inhibitor from potatoes (PCI) binds 70 times less tightly to CPA-Phe-248 than to the wild-type enzyme. The crystal structure[31] of the complex between PCI and bovine CPA reveals two intermolecular hydrogen-bonding interactions between Tyr-248 and the bound inhibitor, and the lessened affinity of the mutant enzyme for PCI is consistent with the estimated[32] loss in stabilizing energy of approximately two hydrogen bonds (2.5 kcal mol^{-1}).

Although the Michaelis constant is likely to be a complex kinetic parameter, the increased K_m values obtained for hydrolysis of Bz-Gly-Phe,[10] Cbz-Gly-Gly-Phe, and ClCPL by CPA-Phe-248 at pH 7.5 are also consistent with the notion that the Tyr-248 hydroxyl group somehow participates in substrate binding. In contrast, binding of Bz-Gly-OPhe to the enzyme does not require hydrogen-bonding interactions with Tyr-248. The Michaelis constant measured for hydrolysis of this ester substrate is not changed substantially by removal of the Tyr-248 phenolic hydroxyl group. Bz-Gly-OPhe must bind close to Tyr-248, however, since acetylation of this amino acid causes the K_m parameter to increase almost 4-fold (Figure 3). This effect is probably due to the incorporation of unfavorable steric interactions in the vicinity of the ester binding site rather than due to the interruption of a stabilizing hydrogen bond, because it is not seen for CPA-Phe-248.

Substrate inhibition, observed at high concentrations of Cbz-Gly-Gly-Phe with CPA-WT, also appears to involve a hydrogen-bonding interaction with Tyr-248. Replacement of the phenolic hydroxyl group of this residue with a hydrogen atom causes the inhibition profile to be abolished or at least displaced to peptide concentrations greater than 2 mM. Thus, the K_i for Cbz-Gly-Gly-Phe is increased more than 10-fold compared to K_i the wild-type enzyme. Again, the nature of the interaction of Bz-Gly-OPhe with Tyr-248 is appreciably different. Substrate inhibition caused by this ester cannot involve a hydrogen bond to Tyr-248, since removal of the phenolic hydroxyl does not alter the inhibition profile at all. The displacement of the substrate–inhibition profile following treatment of CPA-WT with *N*-acetylimidazole presumably reflects a steric bias.

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Reaction of Cbz-Gly-Gly-Phe and ClCPL with both CPA-WT and CPA-Phe-248, as reflected in the k_{cat}/K_m vs. pH profile, is controlled by a base with $pK_{EH_2} = 6.2 \pm 0.3$ and by an acid with $pK_{EH} = 9.4 \pm 0.2$. These ionizations are similar to those observed for the hydrolysis of tripeptides[21] and cinnamate esters[4,23] as catalyzed by bovine CPA. Nitration of the bovine enzyme,[5] however, has been shown to alter significantly the shape of the pH dependence of the k_{cat}/K_m parameter for Cbz-Gly-Gly-Phe hydrolysis without changing the limiting value of this rate constant. This chemical modification is reasonably specific for Tyr-248[33] and causes the pK_{EH} for the basic limb in the pH profile to shift from a value of 9.0 for the native enzyme to 6.60 ± 0.17. The latter value correlates well with the spectroscopic pK_a assigned to the nitro-Tyr-248 residue,[5,26] and these findings have been frequently cited as evidence in support of the assignment of Tyr-248 as the ionizing group responsible for the inflection at pH 9 in the k_{cat}/K_m profile for peptide hydrolysis by the native enzyme.[2,5] In contrast, the decrease in esterase activity above pH 8.5 has been shown[4] not to be due to ionization of Tyr-248, since the basic limb of the pH-rate profile for ClCPL hydrolysis is not shifted subsequent to nitration of Tyr-248. Our results now demonstrate unequivocally that ionization of this residue cannot be responsible for the decrease in peptidase activity seen for CPA-WT above pH 8.5 either. Chemical modification of Tyr-248 apparently perturbs the system significantly, possibly by favoring the binding of the phenolate form of the modified Tyr-248 as a ligand to the active-site zinc ion.[34,35]

Elimination of the possibility that the ionizing group with pK_a at about 9 is Tyr-248 leaves open the question of the identity of the functional group responsible for this ionization. The most obvious candidates based on structural information are the zinc-water complex and an arginine residue involved in binding. We believe the assignment of this pK to the metal-bound water molecule is more probable. Chelation of arsanilazo-Tyr-248 to the active-site zinc ion of bovine CPA has been shown to be controlled by ionizations at pH 7.7 and 9.5.[25,27] The former correlates well with the spectroscopically determined value of the pK_a of the modified Tyr-248, while the latter has been argued[4] to be due to ionization of the zinc-bound water. It seems unlikely that the pK_a of an Arg residue would be lowered to the extent necessary to account for the observed ionization.

In conclusion, site-directed mutagenesis of Tyr-248 in CPA, in conjunction with measurements comparing the rates and pH dependencies for the catalytic action of the native and mutant enzymes, has demonstrated that the ionization state of the phenolic hydroxyl of the Tyr residue cannot be crucial for catalysis by CPA. This finding rules out the mechanistic proposals that have invoked Tyr-248 as an essential general acid or as a general base in the hydrolysis of amides and esters. Through related studies of other residues in the binding and catalytic sites of CPA, we hope to probe further the precise roles of functional groups in this hydrolytic enzyme.

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Registry No. CPA, 11075-17-5; ClCPL, 61556-61-4; L-Tyr, 60-18-4; L-Phe, 63-91-2; Cbz-Gly-Gly-Phe, 13171-93-2; Bz-Gly-OPhe, 3675-74-9.

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A Thorough Study of the Stereochemical Consequences of the Hydration/Dehydration Reaction Catalyzed by β-Hydroxydecanoyl Thioester Dehydrase

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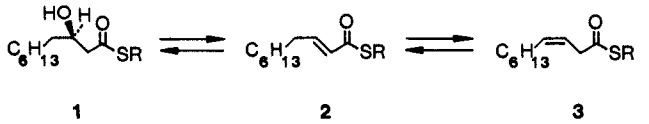
Abstract: β-Hydroxydecanoyl thioester dehydrase is the pivotal enzyme in the biosynthesis of unsaturated fatty acids under anaerobic conditions, catalyzing the equilibration of thioesters of (*R*)-3-hydroxydecanoic acid, (*E*)-2-decenoic acid, and (*Z*)-3-decenoic acid. Substrates chirally labeled with deuterium have been synthesized and incubated with dehydrase. Analysis of labeled products by ^2H NMR spectroscopy has shown that the *pro*-2*S* hydrogen is removed in the course of the dehydration reaction, which is therefore a syn elimination. In the complementary experiment, the *N*-acetylcysteamine thioesters of (*E*)-2-[2-^2H]decenoic acid and unlabeled (*E*)-2-decenoic acid were hydrated by dehydrase in ^1H_2O- and ^2H_2O-based buffers, respectively. Analysis of the resulting products by ^1H NMR spectroscopy demonstrated that the hydration is a synfacial process, with addition of the elements of water to the *si* face of the C-2/C-3 double bond.

β-Hydroxydecanoyl thioester dehydrase,[1] the crucial enzyme in the biosynthesis of unsaturated fatty acids under anaerobic conditions, catalyzes the equilibration of thioesters of (*R*)-3-hydroxydecanoic acid (**1**), (*E*)-2-decenoic acid (**2**), and (*Z*)-3-decenoic acid (**3**). In vivo, acyl carrier protein (ACP) thioesters are utilized, although other thiol moieties, including *N*-acetylcysteamine ($HSCH_2CH_2NHAc$; NAC), function acceptably in vitro.[2]



1 **2** **3**

Herein, we report the results of experiments that have defined the dehydrase-catalyzed hydration-dehydration as a syn addition-elimination, a finding that is mechanistically consistent with the stereochemical course of the allylic rearrangement, **2** to **3**, as reported previously.[3]

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