Use of Directed Mutagenesis to Probe the Role of Tyrosine 198 in the Catalytic Mechanism of Carboxypeptidase A*

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Derivatization of Tyr198 in carboxypeptidase A (CPA) results in lowered catalytic activity toward peptide substrates (Cueni, L., and Riordan, J. F. (1978) Biochemistry 17, 1834–1842). We have synthesized via directed mutagenesis a rat CPA variant [Phe198]CPA containing a Tyr198-to-Phe substitution in order to test whether the phenolic hydroxyl plays a critical role in catalysis. A double mutant [Phe198,Phe248]CPA in which both Tyr198 and Tyr248 have been replaced by phenylalanine has also been engineered. Enzymatic characterization of [Phe198]CPA indicates that the Tyr198 hydroxyl is not obligatory for the hydrolysis of peptide and ester substrates. Furthermore, parallel studies with [Phe198,Phe248]CPA show that simultaneous removal of both the Tyr198 and Tyr248 hydroxyls does not abolish catalytic activity. Analysis of the acetylated derivatives of [Phe198]CPA, [Phe248]CPA, and [Phe198,Phe248]CPA establishes that Tyr198 and Tyr248 are the active site tyrosines which are modified by N-acetylimidazole. In addition, the perturbations of enzymatic activity which accompany acetylation of native CPA can be largely assigned to derivatization of Tyr248. The changes in the kinetic constants of substrate hydrolysis due to the Tyr198-to-Phe substitution are manifested as small decreases in the kₐ values, but the kₐ values are essentially unaffected. This exclusive effect on the kₐ values suggests that the Tyr198 hydroxyl participates in catalysis by stabilizing the rate-determining transition-state complex.

A wide variety of chemical, kinetic, and structural studies have investigated the catalytic mechanism of CPA (Quiocho and Lipscomb, 1971). Model building (Quiocho and Lipscomb, 1971) and the structure of bovine CPA complexed with the potato carboxypeptidase inhibitor (Rees and Lipscomb, 1982) show that Tyr198 occupies a position in the secondary recognition site involved in the binding of extended substrates. Chemical modification studies suggest that this residue nevertheless plays a significant role in catalysis. Nitration of Tyr198 has no effect on the esterase activity but decreases the peptidase activity by 10-fold in standard assays (Cueni and Riordan, 1978). In addition, Tyr198 apparently exhibits marked reactivity toward the acetylating reagents, N-acetylimidazole (Simpson et al., 1985) and acetic anhydride (Riordan and Vallee, 1963). The consequences on activity specifically due to acetylation of Tyr248 could not be established due to the accompanying derivatization of Tyr248 in these experiments.

We have previously shown that Tyr248 is not required for the hydrolysis of peptide or ester substrates (Gardell et al., 1985; Hilvert et al., 1986). We wished to test whether Tyr198 might fulfill that function, perhaps by a structural rearrangement which is not evident from the crystal structure or via the intercession of water molecules. The selective inhibition of peptidase activity due to nitration of Tyr198 (Cueni and Riordan, 1978) could be consistent with this residue acting as the proton donor in light of the relative stabilities of the leaving groups generated during the turnover of ester and peptide substrates.

In this paper, we characterize the enzymatic activities of the [Phe198]CPA and [Phe198,Phe248]CPA mutant enzymes in order to evaluate the importance of the Tyr198 hydroxyl in the catalytic mechanism of CPA. Furthermore, these CPA variants and [Phe248]CPA enable the elucidation of the consequences on activity which accompany specific acetylation of events which occur during catalysis are not fully understood.

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The abbreviations used are: CPA, carboxypeptidase A; WT, wild type; CBz-Gly-Gly-Phe, carbobenzoxyclyglycylglycyl-L-phenylalanine; 6-11-Gly-Phe, O-benzoxyclyglycyl-L-phenylalanine; CBz-Gly-Phe-O-benzoxyclyglycylglycyl-L-phenylalanine; ClCPL, O-trans-p-chlorocinnamoyl-L-phenylalanine; ClCPL, O-trans-p-chlorocinnamoyl-L-phenylalanine; MES, (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; AcCPA, acetylated derivative of carboxypeptidase A; TETOPL, O-3-(2,2,5,5-tetramethylpyrroolyl-1-oxyl)-pro-pen-2-oyl-1-2-phenylalactate.
either Tyr$^{106}$ or Tyr$^{248}$ and thereby provide an additional avenue for illuminating the events which occur during catalysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cbz-Gly-Gly-Phe and the sodium salt of Bz-Gly-OPhe were purchased from Sigma and used without further purification. CICPL was prepared according to the published procedure (Suh and Kaiser, 1976). 1-Acetylimidazole, hydroxylamine HCl, and deuterium oxide (99.8 atom % deuterium) were obtained from Aldrich.

Restriction enzymes, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were obtained from Boehringer Mannheim. The synthetic oligonucleotides were made with a Beckman System I DNA synthesizer.

**Methods**—Construction of the [Phe$^{106}$]CPA and [Phe$^{248}$]Phe$^{248}$ CPA expression vectors was similar to that detailed for pYocCPA-WT and pYocCPA-Phe$^{248}$ (Gardell et al., 1985). Yeast strain BJ1994 (MATa, leu2, trp1, his3-112,200;p4-4) was transformed (Ito et al., 1985) with either pYocCPA-Phe$^{198}$ or pYocCPA-Phe$^{106}$Phe$^{248}$ and leucine prototrophs were selected. Transformants were grown in synthetic complete medium lacking leucine (Sherman et al., 1976). 1-Acetylimidazole, hydroxylamine HCl, and deuterium oxide were purchased from Sigma and used without further purification.

Fractions containing activity were pooled and dialyzed exhaustively against a mixture of 0.5 M Na$_2$CO$_3$, 0.5 M NaCl and 20 mM Na$_2$SO$_4$. Protein was eluted with 0.1 M Na$_2$CO$_3$, 0.5 M NaCl (pH 8.0), and the solution was applied to an affinity column (6 cm x 4.9 cm bed volume) equilibrated in 20 mM Tris-HCl, 0.5 M NaCl (pH 8.0). Protein was eluted with a linear NaCl gradient (20-250 mM). Fractions containing activity were pooled and dialyzed exhaustively against 20 mM MESS, 0.1 M NaCl (pH 6.0) at 4°C. Preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis; [Phe$^{106}$]CPA and [Phe$^{106}$,Phe$^{248}$]CPA comigrated during sodium dodecyl sulfate-polyacrylamide gel electrophoresis; [Phe$^{106}$,Phe$^{248}$]CPA involved affinity chromatography using the carboxylic group of the CPA-WT. CPA variants were eluted with 0.1 M Na$_2$CO$_3$, 0.5 M NaCl (pH 8.0), and the solution was applied to an affinity column containing immobilized glycyl-L-tyrosyl-azo-benzylsuccinate (Cuoni et al., 1980) (Pierce). Protein was eluted with 20 mM Tris-HCl, 0.2 M NaCl (pH 8.0) and monitored for Bz-Gly-OPhe hydrolyzing activity.

**Directed Mutagenesis and Heterologous Expression in Yeast**—Conversion of the Tyr$^{106}$ codon (TAT) to phenylalanine (TTT) in the cDNAs for CPA-WT and [Phe$^{248}$]Phe$^{248}$ CPA was accomplished by oligonucleotide-directed mutagenesis (Zoller and Smith, 1984; Crak, 1985). To each template was annealed an oligonucleotide, 5'AGAGCAGAGCTGGAAGCTG-TGGATG3', which introduces an A→T transition in the Tyr$^{106}$ codon, and in addition a "silent" T→G transition in order to facilitate sequencing of the mutant template. The cDNAs coding for [Phe$^{106}$]CPA and [Phe$^{106}$,Phe$^{248}$]CPA were sequenced in their entirety by the dyeoxy termination method (Sanger et al., 1977) to confirm that only the desired nucleotide changes had been introduced. Expression of CPA variants was carried out in yeast via the a factor system (Brake et al., 1984) as described previously for CPA-WT and [Phe$^{248}$]CPA (Gardell et al., 1985). This system directs the synthesis and secretion of the proenzyme forms of the various carboxypeptidases. [Phe$^{106}$]CPA and [Phe$^{106}$,Phe$^{248}$]CPA were purified to homogeneity following in vitro zymogen activation by trypsin.

**Directed Mutagenesis of Carboxypeptidase A**—The pH dependence of $k_{cat}/K_m$ for the hydrolysis of CICPL was also fitted to Equation 2. The acidiic limb of the $k_{cat}$ versus pH profile for the hydrolysis of CICPL was fitted to Equation 3.

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*The buffer for the solvent kinetic isotope effect study was prepared by lyophilizing a known volume of 50 mM Tris-HCl, 0.5 M NaCl (pH 7.5), adding D$_2$O to the original volume, and titrating to pH 8.0 with DCl (pD = meter reading + 0.6) (Glaser and Long, 1960); pH 8.0 is equivalent to pH 7.5 in terms of the ionization of oxygen and nitrogen bases (Schoven, 1977). D$_2$O solutions were stored under argon.*
The rapid increase in the acetylation profile identical to that exhibited by [Phe'98,Phe248]CPA-WT (Hilvert et al., 1986) in the presence of N-acetylimidazole in the presence of phenylpropionate, subsequent incubation with hydroxylamine results in a deacetylation of O-acetyltyrosine at 278 nm (Simpson et al., 1963). Previous studies on bovine CPA demonstrated that four tyrosines are modified by N-acetylimidazole (Simpson et al., 1963); protection experiments with β-phenylpropionate indicate that two of these are situated in the active site. The rate of deacetylation of the modified active-site tyrosines is faster than the other two; hence a biphasic deacetylation profile is observed. The rapid increase in A278 during treatment of AcCPA-WT with hydroxylamine (Fig. 3) corresponds to the deacetylation of two modified tyrosines. The burst phase during deacetylation of either [Phe'98]AcCPA or [Phe248]AcCPA resembles that previously demonstrated for [Phe248]CPA (Hilvert et al., 1986). The pK_m values of [Phelg8]CPA and [Phe'98,Phe248]CPA were assayed in aqueous buffer (50 mM Tris-HCl, 0.5 mM NaCl, pH 7.5) at 5°C as described elsewhere (Hilvert et al., 1986).

### Table I

| Substrate                  | Kinetic parameter | [Phe'98]CPA | [Phe']Acetyl-CPA | [Phe248]CPA | [Phe'98,Phe248]Acetyl-CPA |
|----------------------------|-------------------|-------------|------------------|-------------|-------------------------|
| Cbz-Gly-Gly-Phe            | \(k_{cat}(s^{-1})\) | 34.1 ± 1.0  | 10.4 ± 0.2       | 12.6 ± 0.73 | 13.0 ± 0.6              |
|                            | \(K_m(μM)\)      | 34.5 ± 2.7  | 136 ± 9          | 218 ± 39    | 200 ± 26                |
|                            | \(10^{-6} k_m/K_m(M^{-1} s^{-1})\) | 99.8        | 0.764            | 0.569       | 0.649                   |
| Bz-Gly-OPhe                | \(k_{cat}(s^{-1})\) | 1052 ± 73   | 1376 ± 70        | 1253 ± 83   | 1089 ± 50               |
|                            | \(K_m(μM)\)      | 118 ± 20    | 459 ± 61         | 144 ± 21    | 122 ± 15                |
|                            | \(10^{-6} k_m/K_m(M^{-1} s^{-1})\) | 89.1        | 30.0             | 87.1        | 88.2                    |
| CICPL                      | \(k_{cat}(s^{-1})\) | 2.68 ± 0.11 | 0.682 ± 0.018    |             |                         |
|                            | \(K_m(μM)\)      | 17.2 ± 2.3  | 203 ± 13         |             |                         |
|                            | \(10^{-6} k_m/K_m(M^{-1} s^{-1})\) | 1.56        | 0.0336           |             |                         |

The number of tyrosines which are derivatized following treatment of CPA with N-acetylimidazole can be determined by treating the modified enzyme with hydroxylamine and monitoring the deacetylation of O-acetyltyrosine at 278 nm (Simpson et al., 1963). Previous studies on bovine CPA demonstrated that four tyrosines are modified by N-acetylimidazole (Simpson et al., 1963); protection experiments with β-phenylpropionate indicate that two of these are situated in the active site. The rate of deacetylation of the modified active-site tyrosines is faster than the other two; hence a biphasic deacetylation profile is observed. The rapid increase in A278 during treatment of AcCPA-WT with hydroxylamine (Fig. 3) corresponds to the deacetylation of two modified tyrosines. The burst phase during deacetylation of either [Phe'98]AcCPA or [Phe248]AcCPA resembles the presence of a single O-acetyltyrosine. Treatment of [Phe'98,Phe248]AcCPA with hydroxylamine does not cause a rapid increase in A278. If CPA-WT is treated with N-acetylimidazole in the presence of β-phenylpropionate, subsequent incubation with hydroxylamine results in a deacetylation profile identical to that exhibited by [Phe'98,Phe248]AcCPA (data not shown). The increases in A278 displayed by AcCPA-WT, [Phe'98]AcCPA, and [Phe248]AcCPA following treatment with hydroxylamine are accompanied by a return to activity characteristic of the unmodified enzymes.

The changes in the kinetic constants of [Phe'98]CPA due to treatment with N-acetylimidazole (Table I) are similar to those observed following acetylation of CPA-WT (Hilvert et al., 1986). For example, [Phe'98]AcCPA exhibits a 3.4-fold decrease in the \(k_{cat}\) value and a 4-fold increase in the \(K_m\) value for Cbz-Gly-Gly-Phe when compared to the corresponding values of [Phe'98]CPA. In contrast, acetylation of [Phe248]CPA resulted in only a small decrease in \(k_{cat}\) with no corresponding effect on \(K_m\) (Hilvert et al., 1986). The ability of the double mutant [Phe'98,Phe248]CPA to hydrolyze Cbz-Gly-Gly-Phe was unaffected by treatment with N-acetylimidazole (Table I). The \(k_{cat}\) values exhibited by CPA-WT or its variants for the hydrolysis of Bz-Gly-OPhe were essentially unchanged following acetylation. Nevertheless, both AcCPA-WT (Hilvert et al., 1986) and [Phe'98]AcCPA displayed an approximate 3.8-fold increase in \(K_m\) toward Bz-Gly-OPhe; the corresponding values of [Phe'98]AcCPA and [Phe188,Phe248]AcCPA were unaffected.

The hydrolysis by CPA of N-substituted dipeptides (Auld and Vallee, 1970) and their ester analogs (McClure et al., 1964) is typically associated with kinetic anomalies. For example, bovine CPA (McClure et al., 1964) and rat CPA (Hilvert et al., 1986) are inhibited by concentrations of Bz-Gly-OPhe above 0.5 mM. [Phe'98]CPA and [Phe248]CPA (Fig. 4) as well as [Phe248]CPA (Hilvert et al., 1986) are similarly affected by elevated levels of this substrate. It was previously demonstrated that nitration (Riordan et al., 1967) or acetylation (Whitaker et al., 1966) of bovine CPA shifts the onset of substrate inhibition to higher Bz-Gly-OPhe concentrations. Likewise, acetylation of CPA-WT (Hilvert et al., 1986) or [Phe'98]CPA (Fig. 4A) by N-acetylimidazole abolishes substrate inhibition at concentrations of Bz-Gly-OPhe up to 2 mM. In contrast, treatment of either [Phe248]CPA (Hilvert et al., 1986) or [Phe188,Phe248]CPA (Fig. 4B) with N-acetylimidazole has no effect on substrate inhibition by Bz-Gly-OPhe. Another kinetic anomaly which we investigated was substrate inhibition by elevated levels of Cbz-Gly-Gly-Phe (Auld and Vallee, 1970). The absence of the Tyr198 phenolic hydroxyl does not change the onset of this kinetic anomaly (Fig. 5A) from that observed with CPA-WT (Hilvert et al., 1986). Nevertheless, [Phe'98,Phe248]CPA (Fig. 5B) is similar to [Phe248]CPA (Hilvert et al., 1986) in that substrate inhibition is not exhibited even at 2 mM Cbz-Gly-Gly-Phe. As previously shown for CPA-WT (Hilvert et al., 1986), treatment of [Phe188]CPA with N-acetylimidazole suppresses the onset of substrate inhibition (Fig. 5A). Similar treatment of [Phe'98,Phe248]CPA does not affect the hydrolysis of Cbz-Gly-Gly-Phe (Fig. 5B).

**DISCUSSION**

We have used directed mutagenesis of the rat CPA cDNA and heterologous expression in yeast to synthesize a CPA variant containing a Tyr256-to-Phe replacement and a double mutant in which both Tyr198 and Tyr248 have been substituted by phenylalanine. [Phe'98]CPA and [Phe188,Phe248]CPA were purified to homogeneity and characterized enzymatically in order to investigate the possibility suggested by chemical modification studies that Tyr198 may play an important role in catalysis (Cueni and Riordan, 1978).

The catalytic activity of CPA toward peptide and ester substrates is not abolished by the Tyr198-to-Phe replacement; thus Tyr198 does not play a crucial catalytic role such as mediating general acid catalysis. This same conclusion was reported previously for Tyr248 (Gardell et al., 1985; Hilvert et al., 1986). The ability of [Phe'98,Phe248]CPA to catalyze substrate hydrolysis eliminates the possibility that proton dona-
tion to the leaving group can be mediated independently by
either Tyr198 or Tyr248.

The shape of the $k_{cat}/K_m$ versus pH profile for the hydrolysis of the ester substrate, CICPL, is essentially unchanged in spite of the Tyr198-to-Phe substitution. Hence, the ionization of Tyr198 does not govern the basic limb of this pH-rate profile as suggested by Makinen and co-workers (Makinen et al., 1985). The pH dependencies of both $K_m$ and $k_{cat}/K_m$ for the hydrolysis of Cbz-Gly-Gly-Phe are also unaffected by the Tyr198-to-Phe substitution. In contrast to CPA-WT, the $k_{cat}$ versus pH profile for the hydrolysis of CICPL by CPA-Phe198 does not exhibit a steeply ascending arm at pH values greater than 9. It is unlikely that ionization of Tyr198 is responsible for the increase in $k_{cat}$ in this pH range since the Tyr248-to-Phe substitution has a similar effect (Hilvert et al., 1986).

The free energy changes associated with the binding of the rate-determining transition state in the hydrolysis of Cbz-Gly-Gly-Phe suggest that there is no coupling between the Tyr198 and Tyr248 mutations (Fig. 6). Specifically, the Tyr248 hydroxyl of CPA-WT or [Phe198]CPA each contribute 1.6 kcal/mol to binding of the rate-determining transition state. Similarly, the Tyr248 hydroxyl contributes 0.4 kcal/mol to transition-state binding regardless of whether tyrosine or phenylalanine is in position 248. In addition, the decrease in the binding free enzyme exhibited by the double mutant, 2.0 kcal/mol, is the algebraic sum of the changes of the two single mutants. This comparison suggests that the replacement of either Tyr198 or Tyr248 with phenylalanine does not result in an extensive perturbation of enzyme structure.

The present study establishes that Tyr198 and Tyr248 exhibit a marked reactivity toward acetylation and that the effects on substrate hydrolysis due to acetylation of CPA-WT are largely a result of Tyr248 modification. The ability of the [Tyr248 Phe198]Ac derivative to hydrolyze peptide and ester substrates supports our previous conclusion that the Tyr248 hydroxyl is not required for catalysis (Gardell et al., 1985; Hilvert et al., 1986). Acetylation of Tyr248 or removal of the Tyr248 hydroxyl (Gardell et al., 1985; Hilvert et al., 1986) results in similar changes in the $k_{cat}$ and $K_m$ values for Cbz-Gly-Gly-Phe. The comparable effect on peptidase activity due to these different Tyr248 alterations suggests that the hydroxyl of Tyr248 may be involved in hydrogen-bonding interactions that contribute to the hydrolysis of peptide substrates. In contrast, the Tyr248-to-Phe replacement and acetylation of Tyr248 have dissimilar effects on the hydrolysis of the ester substrate, Bz-Gly-OPhe. The $K_m$ value for Bz-Gly-OPhe is unaffected by removal of the Tyr248 hydroxyl but increases approximately 4-fold by acetylation.

The effect of acetylation of CPA-WT on the suppression of substrate inhibition by Bz-Gly-OPhe and Cbz-Gly-OPhe is also attributed to the derivatization of Tyr248 by parallel studies with [Phe198]CPA and [Phe248]CPA. This conclusion is consistent with previous studies on bovine CPA which showed that Tyr248-specific chemical modifications displace the onset of substrate inhibition by Bz-Gly-OPhe to higher substrate concentrations (Riordan et al., 1967; Urdea and Legg, 1979). Removal of either the Tyr198 or the Tyr248 hydroxyl does not affect the kinetic anomaly observed at elevated concentrations of Bz-Gly-OPhe. In contrast, removal of the Tyr248 hydroxyl mimics the effect of acetylation in suppressing the substrate inhibition observed at elevated concentrations of Cbz-Gly-Gly-Phe. The alternative substrate binding mode of Cbz-Gly-Gly-Phe responsible for this kinetic
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FIG. 2. pH dependence for the hydrolysis of CICPL catalyzed by [Phe\textsuperscript{188}]CPA. A, \(k_{\text{cat}}\) versus pH profile. The experimentally determined values (●) were fitted to Equation 3 (see "Experimental Procedures"). Standard deviation estimates are indicated at each point by the error bars. B, \(k_{\text{cat}}/K_m\) versus pH profile. The experimentally determined values (●) were fitted to Equation 3.

Anomaly thus appears to involve hydrogen-bonding interactions with the phenolic hydroxyl of Tyr\textsuperscript{188}.

Although the Tyr\textsuperscript{188} hydroxyl is not crucial for catalysis, its removal results in significant decreases in the \(k_{\text{cat}}\) values of Cbz-Gly-Gly-Phe and CICPL (Table 1). Interestingly, there are no accompanying changes in their \(K_m\) values. This selective effect on the \(k_{\text{cat}}\) values is consistent with the conclusions from a previous investigation of the influence of substrate structure on the Michaelis-Menten parameters exhibited by bovine CPA (Abramowitz \textit{et al.}, 1967). It was shown that the \(K_m\) value of a peptide substrate is largely determined by the structure of its COOH-terminal moiety. In contrast, the \(k_{\text{cat}}\) value is largely determined by the steric bulk of that part of the substrate which interacts with the \(S_2\) subsite. Model building of extended substrates onto the CPA structure indicates that the side chain of Tyr\textsuperscript{188} constitutes part of the \(S_2\) subsite (Quiocho and Lipscomb, 1971). Hence the removal of the Tyr\textsuperscript{188} phenolic hydroxyl may alter the complementarity between enzyme and substrate in this critical region.

The exclusive effect of the Tyr\textsuperscript{188}-to-Phe substitution on

FIG. 3. Titration of O-acetyltirosines following deacetylation by hydroxylamine. The deacetylation profiles corresponding to acetylated CPA-WT (AcCPA-WT) or its genetically engineered variants are as labeled.

FIG. 4. Effect of Bz-Gly-OPhe concentration on the rate of its hydrolysis by [Phe\textsuperscript{188}]CPA, [Phe\textsuperscript{188},Phe\textsuperscript{248}]CPA, and their acetylated derivatives. A, hydrolysis of Bz-Gly-OPhe by [Phe\textsuperscript{188}]CPA (●) and [Phe\textsuperscript{188},Phe\textsuperscript{248}]CPA (●). B, hydrolysis of Bz-Gly-OPhe by [Phe\textsuperscript{188},Phe\textsuperscript{248}]CPA (●) and [Phe\textsuperscript{188},Phe\textsuperscript{248}]AcCPA (○). Assays were carried out at 25 °C in 50 mM Tris-HCl, 0.5 M NaCl (pH 7.5).
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by preferentially binding to the rate-determining transition state structures of these substrates, thereby decreasing the free energy difference between the enzyme-substrate complex and the transition-state complex. The rate-determining transition state for the hydrolysis of CICPPL appears to be the same for both CPA-WT and [Phe188]CPA since they exhibit similar kinetic solvent isotope effects. Previous workers had proposed that the rate-determining step for the turnover of CICPPL was the hydrolytic breakdown of a mixed-anhydride intermediate (Makinen et al., 1976). The free energy contributed by the Tyr189 hydroxylation to transition-state binding, 0.8 kcal/mol, may be manifested as stabilization of the transition state which results during the hydrolysis of the putative acyl-enzyme intermediate. Interestingly, Tyr189 was recently implicated on the basis of magnetic resonance and molecular modeling techniques to be a determinant of the strain which is imposed upon a spin-label substrate, TEPOPL, following its interaction with the extended binding cleft of CPA (Kuo et al., 1983).

The present study shows that the Tyr189 phenolic hydroxyl is not obligatory for the hydrolysis of peptide or ester substrates. Simultaneous removal of the Tyr189 and Tyr448 phenolic hydroxyls also does not abolish catalytic activity. Nevertheless, in spite of the apparent distance of Tyr189 from the site of bond scission, it appears to play a role in catalysis as measured by the specific effect on $k_{cat}$ due to the Tyr189-to-Phe substitution. The Tyr189 hydroxyl may facilitate substrate hydrolysis by participating in the stabilization of the rate-determining transition state. In addition, this study illustrates the utility of directed mutagenesis to clarify the effects of chemical modification and hence to increase the effectiveness of chemical modification as a probe of protein structure and function.

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