The Role of Tyr248 Probed by Mutant Bovine Carboxypeptidase A: Insight into the Catalytic Mechanism of Carboxypeptidase A†

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Received April 20, 2001; Revised Manuscript Received June 18, 2001

ABSTRACT: We have investigated the function of Tyr248 using bovine wild-type CPA and its Y248F and Y248A mutants to find that the K_M values were increased by 4.5−11-fold and the k_{cat} values were reduced by 4.5−10.7-fold by the replacement of Tyr248 with Phe for the hydrolysis of hippuryl-L-Phe (HPA) and *N*-[3-(2-furyl)acryloyl]-Phe-Phe (FAPP), respectively. In the case of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate (ClCPL), an ester substrate, the K_M value was increased by 2.5-fold, and the k_{cat} was reduced by 20-fold. The replacement of Tyr248 with Ala decreased the k_{cat} values by about 18- and 237-fold for HPA and ClCPL, respectively, demonstrating that the aromatic ring of Tyr248 plays a critical role in the enzymic reaction. The increases of the K_M values were only 6- and 5-fold for HPA and ClCPL, respectively. Thus, the present study indicates clearly that Tyr248 plays an important role not only in the binding of substrate but also in the enzymic hydrolysis. The kinetic results may be rationalized by the proposition that the phenolic hydroxyl of Tyr248 forms a hydrogen bond with the zinc-bound water molecule, causing further activation of the water molecule by reducing its pK_a value. The pH dependency study of k_{cat} values and the solvent isotope effects also support the proposition. A unified catalytic mechanism is proposed that can account for the different kinetic behavior observed in the CPA-catalyzed hydrolysis of peptide and ester substrates.

Carboxypeptidase A (CPA,[1] EC 3.4.17.1) is a zinc-containing metalloprotease that removes the C-terminal amino acid residue having an aromatic or branched aliphatic side chain from peptide substrate (*1, 2*). As one of the most intensively studied enzymes, CPA has contributed enormously to the elucidation of the catalytic mechanism of other zinc-containing proteolytic enzymes and served as a prototypical enzyme for a large number of pathologically important metalloenzymes. CPA has also been used as a model in the development of inhibitor design strategies that can be useful in designing inhibitors effective for zinc proteases of medicinal importance (*3*−*7*).

The important binding sites and key catalytic groups of CPA may be summarized as the following: The hydrophobic pocket at the S_1' subsite and the guanidinium moiety of Arg145 are responsible for the substrate specificity. The former accommodates the aromatic side chain of the P_1' residue, and the latter forms bifurcated hydrogen bonds with the terminal carboxylate of substrate. The zinc ion coordinated tightly to the backbone amino acid residues of His69, Glu72, and His196 is essential for the enzymic hydrolysis reaction. A water molecule is bound loosely to the zinc ion as the fourth ligand. The zinc-bound water molecule and the carboxylate of Glu270 are known to be intimately involved in the cleavage of the scissile bond of the enzyme-bound substrate. However, a detailed catalytic mechanism defining the role of each catalytic residue remains incomplete.

The role of Tyr248 at the active site has been the subject of much debate over the years. X-ray crystal structures of CPA complexed with Gly-Tyr, a slowly hydrolyzed substrate, revealed that Tyr248 undergoes a substrate-induced conformational change causing the phenolic hydroxyl of the Tyr residue to move from the surface of the enzyme to within hydrogen bonding distance of the scissile peptide bond (*8, 9*). It was thus proposed that Tyr248 plays a role as a proton donor (general acid) to the departing amino group of the C-terminal residue in the enzymatic hydrolysis of peptide substrate (*8, 9*). However, the subsequent high-resolution X-ray crystallographic study of the complex failed to confirm the proposition, but demonstrated that the Tyr phenolic hydroxyl instead forms a hydrogen bond with the terminal carboxylate of the substrate (*10*). The kinetic study performed with the mutant rat CPA in which Tyr248 is replaced with Phe showed almost normal k_{cat} values in the hydrolysis of peptide as well as ester substrates, suggesting that Tyr248 is not required for the catalytic process (*11, 12*). Accordingly, it is now generally believed that Tyr248 is involved only in

† This work was supported by the Korea Research Foundation through CBM at Pohang University of Science and Technology during the period of 1996−1999.

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[1] Abbreviations: CPA, carboxypeptidase A; ClCPL, *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate; HPA, hippuryl-L-Phe; FAPP, *N*-[3-(2-furyl)acryloyl]-Phe-Phe; BEBA, 2-benzyl-3,4-epoxybutanoic acid; CD, circular dichroism; HPLC, high-pressure liquid chromatography; pSK(−), *p*Bluescript SK(−); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

10.1021/bi010807j CCC: $20.00
Published on Web 08/02/2001

the ligand binding, its side chain capping the ligand-occupied hydrophobic pocket at the S_1' subsite (*1*).

It should be, however, noted that the kinetic studies aimed at addressing the role of Tyr248 were performed with rat CPA and its Y248F mutant, but most of the other studies including the X-ray crystal structures were carried out with CPA obtained from the bovine pancreas. Rat CPA has 78% amino acid homology with bovine CPA (*13*). Although the catalytic residues are conserved, detailed modes of binding and transition state stabilization of the rat CPA might be somewhat different from those of bovine CPA. It was thus thought to be of importance to probe the role of Tyr248 using bovine CPA and its mutant having Phe in place of the Tyr. The CPA having Ala at the 248 position is also thought to be of much interest in understanding the catalytic mechanism since this mutant may provide some insight into the role of the aromatic ring of Tyr248 in the catalytic pathway (*14*).

We have reexamined in the present study the role of Tyr248 using bovine wild-type CPA and its Y248F and Y248A mutants. Contrary to the results obtained with rat CPA, we have found that *Tyr248 is essential for the catalytic activity*. Furthermore, we have demonstrated for the first time that the aromatic ring of Tyr248 is crucial for the enzyme to perform the catalytic activity. On the basis of our results and those of others reported in the literature, a unified catalytic mechanism is proposed that can account for the observed kinetic difference between peptide and ester substrates in the CPA-catalyzed hydrolysis.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Replacement of Tyr248 with such amino acids as Phe or Ala was carried out as described previously (*15*). The entire gene encoding bovine proCPA isolated previously (*16*) was subcloned into pBluescript SK-(−) plasmid. Single-stranded uracil-containing template DNA was isolated from *Escherichia coli* RZ1032 (*Hfr KL 16 PU/45 [lys A(61−62) dut 1 ung 1 thil, rel A1, zbd-279::Tn10, supE44*] after infection with helper phage M13K07 (Pharmacia). The following synthetic oligonucleotides were used to introduce the mutation (the underlined nucleotides represent the ones changed by point mutation):

Y248F: 5′-ATC-ACA-ACA-ATT-<u>TT</u>C-CAA-GCT-TCT-GAA-3′

Y248A: 5′-ATC-ACA-ACA-ATT-<u>GC</u>C-CAA-GCT-TCT-GAA-3′

The intended mutations were confirmed by determining the nucleotide sequence of the entire gene. The mutated gene in pSK(−) was digested with *Bam*HI and *Eco*RI to isolate the inserted DNA fragment and then the insert subcloned into the same sites of pRSET-A.

Expression and Purification of CPAs. The proCPA gene with the N-terminal 6X-His tag region in pRSET-A was expressed in *E. coli* BL21(DE3) (Novagen). Mutant and wild-type proCPAs were purified by Ni-column chromatography. The mature CPAs were obtained by digestion with trypsin as described in the literature (*17*) and purified by HPLC using a Mono Q column (Pharmacia). The purified CPA was homogeneous as judged by 15% SDS−PAGE.

Determination of Kinetic Parameters. Hippuryl-L-phenylalanine (HPA), *N*-[3-(2-furyl)acryloyl]-Phe-Phe (FAPP), and hippuryl-D,L-β-phenyllactate (HPL) were purchased from Sigma and used without purification. *O*-(*trans-p*-Chlorocinnamoyl)-L-β-phenyllactate (ClCPL) was prepared according to the published method (*18*). 2-Benzyl-3,4-epoxybutanoic acid (BEBA) was synthesized as described previously (*19*). Stock solutions of relatively high concentration of CPA (ca. 5×10^{-5} M) in aqueous buffer containing 50 mM Tris, pH 7.5, and 500 mM NaCl were stored at 4 °C and diluted before use. Because of the limited solubility of the substrates and BEBA in water, dimethyl sulfoxide (DMSO) was used as a cosolvent. Final concentration of DMSO in the assay buffer was less than 2%. Final enzyme concentrations were determined using the Coomassie protein assay reagent (Pierce) according to the procedure of the supplier. The rate of hydrolysis of each substrate was continuously monitored spectrophotometrically at 25.0 ± 0.1 °C by measuring the UV absorbance. The wavelength used to monitor the reaction of HPA was 254 nm and that of ClCPL 320 nm. The kinetic parameters (K_M and k_{cat}) were obtained by fitting the initial rate data to eq 1 using a nonlinear least-squares program:

$$V_o/[E]_T = k_{cat}[S]/(K_M + [S]) \quad (1)$$

in which V_o and $[E]_T$ represent the initial velocity of enzymatic reaction and total enzyme concentration, respectively. The kinetic parameters K_I and k_{inact} for the inactivation of CPA and its Y248 mutants by (2*R*,3*S*)-BEBA were determined by the substrate competitive assay method as described previously (*19*). The change in absorbance at 254 nm was recorded with a time interval of 0−10 min at 25.0 ± 0.1 °C. The values of K_I and k_{inact} were determined from the slope and *y*-intercept, respectively, of the double reciprocal plot according to eq 2:

$$1/k_{obs} = (K_I/k_{inact})\{1 + ([S]_0/K_M)\}/[I]_0 + 1/k_{inact} \quad (2)$$

in which $[S]_0$ and $[I]_0$ are the initial concentrations of substrate and inhibitor, respectively. The values of k_{obs}, the apparent inactivation rate constant, were obtained from the progress curve data of the enzymic reaction in the presence of the inhibitor using nonlinear least-squares fitting to eq 3:

$$\Delta A = (V_0/k_{obs})\{1 - \exp(-k_{obs}t)\} \quad (3)$$

where ΔA is the change of absorbance during reaction time.

pH Dependence of Kinetic Parameters. The compositions of the buffer solutions used for the determination of the pH−rate profiles were as follows: 0.5 M NaCl and 0.05 M MES for the pH range of 5.55−7.00; 0.5 M NaCl and 0.05 M Tris for the pH range of 7.53−9.00; and 0.5 M NaCl and 0.05 M 2-amino-2-methyl-1,3-propanediol for the pH range of 9.10−9.50. Since the enzyme loses activity continuously at pH below 6 in the absence of excess metal ion (*20*), zinc chloride was added to the buffer while maintaining the zinc concentration at 10^{-4} M. The acidic limb of the pH profiles observed for k_{cat} was fitted to eq 4 (*21*):

$$k_{cat} = (k_{cat})_{lim}/(1 + [H^+]/K_{EH2S}) \quad (4)$$

Deuterium Isotope Effect. Tris buffer solution was lyophilized and then dissolved in the D_2O solution containing

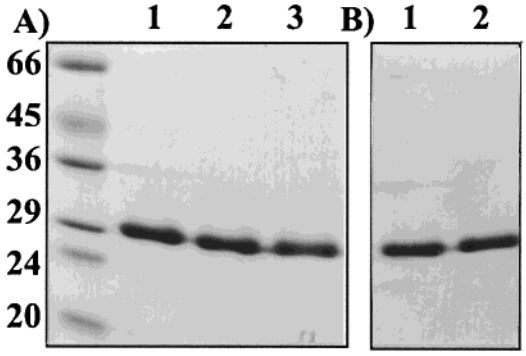

FIGURE 1: SDS−PAGE analysis of bovine wild-type CPA and its mutants. The migration of purified CPA on SDS−PAGE is rather aberrant. The protein bands of CPA and its mutants do not reflect the actual size (34 kDa) on SDS−PAGE. The migation of purified CPA corresponds to that of commercial CPA (Sigma). (A) Lane 1, wild-type; lane 2, Y248F; lane 3, Y248A. (B) Lane 1, purified CPA; lane 2, commercial CPA.

0.5 M NaCl. ClCPL was dissolved in $(CD_3)_2SO$. Stock solutions of the protein were equilibrated prior to use in the freshly prepared D_2O buffer. The pD of the buffer was determined using the equation: $pD = pH_{obs} + 0.4$ (*22*).

RESULTS AND DISCUSSION

The entire gene encoding bovine proCPA (*16*) was subjected to the mutation at the specific amino acid site. Both wild-type and the mutated bovine CPA genes were expressed in *Escherichia coli* (*E. coli*) BL21(DE3) to obtain the wild-type CPA and its mutants, CPA-Y248F and CPA-Y248A. The expression of His_6-proCPA gene, a proCPA gene with the histidine tag, was induced at 27 °C in *E. coli* by addition of isopropyl-*β*-*O*-thiogalactopyranoside to a final concentration of 1 mM. The major protein in the total cell lysate and cytosolic fraction was found to be His_6-proCPA having a molecular mass of 49 kDa. Most of the protein impurities were removed by Ni-column chromatography, and the His_6-proCPA protein was purified to homogeneity using Superose 12 gel chromatography.

Activation of the proCPA was a delicate process not like other zymogens possibly due to its long N-terminal pro-region. The cleavage of the proCPA by trypsin to generate CPA was fast (in about 5 min), but prolonged digestion longer than 5 h was necessary to obtain the fully active CPA. Chapus et al. reported a similar observation and ascribed the delayed activation to the inhibitory effect of the peptide generated during the activation of CPA, which has a tight globular structure resistant to tryptic digestion (*17*). After the tryptic digestion, the mixture was applied onto a Mono-Q chromatographic column to separate the mature CPA. About 500 *μ*g of CPA could be obtained from 1 L of *E. coli* culture. The CPA thus obtained was homogeneous as confirmed by SDS−PAGE analysis (Figure 1) and by isoelectric focusing electrophoresis (data not shown). In contrast to the commercially available CPA that exhibits more than 10 protein bands on the isoelectric focusing gel, the purified CPA showed 1 major band accompanied by a minor one. Essentially identical CD spectra (data not shown) were obtained for both the wild-type CPA and its mutants, indicating that the mutation of Tyr248 does not cause any significant structural change of the enzyme.

Table 1: Kinetic Parameters for Substrate Hydrolysis Catalyzed by Bovine CPA and Its Tyr248 Mutants

CPA	substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\times 10^{-5}$ M^{-1} s^{-1})
wild-type	HPA	0.88 ± 0.05	60 ± 0.8	0.68
	FAPP	0.05 ± 0.001	340 ± 7.0	68
	ClCPL	0.11 ± 0.002	120 ± 1.2	11
	HPL	0.1 ± 0.01	1300 ± 110	130
Y248F	HPA	4.0 ± 0.42	5.6 ± 0.75	0.014
	FAPP	0.55 ± 0.053	76 ± 7.7	1.4
	ClCPL	0.27 ± 0.04	5.8 ± 0.43	0.21
	HPL	0.39 ± 0.031	780 ± 3.1	20
Y248A	HPA	5.1 ± 0.055	3.3 ± 0.04	0.006
	FAPP	1.0 ± 0.1	27 ± 3.0	0.26
	ClCPL	0.56 ± 0.12	0.49 ± 0.04	0.008
	HPL	ND	ND	ND

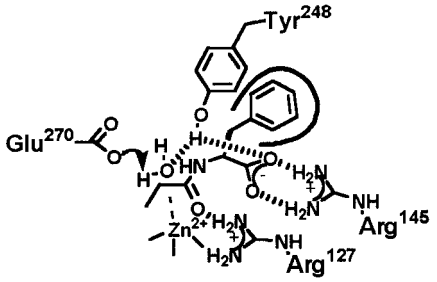

FIGURE 2: Schematic representation of putative CPA·peptide substrate complex. In the figure, the zinc-bound water molecule is not meant to rest above the molecule of substrate. However, the water molecule may be pushed to some extent by the substrate upon its binding toward the phenolic hydroxyl of Tyr248 forming a hydrogen bond (see discussion in the text). It may be likely that the relocated water molecule is in close proximity to the carbonyl group of the scissile peptide bond of the CPA-bound substrate, so that it attacks the carbonyl carbon to generate a tetrahedral transition state.

The kinetic parameters obtained for the hydrolysis of amide and ester substrates in the presence of the wild bovine CPA and its mutants are listed in Table 1. As shown in Table 1, both K_M and k_{cat} values are changed substantially by the substitution of Tyr248 with Phe (*11*, *12*). While the K_M values are increased by about 4.5−11-fold, the k_{cat} values are reduced by 4.5−10.7-fold for the hydrolysis of HPA and FAPP catalyzed by bovine CPA-Y248F. The reduction of the k_{cat} value by 10.7-fold in the case of HPA hydrolysis by the mutant suggests strongly that the phenolic hydroxyl group plays an important role in the proteolytic process. These observations may be envisioned on the basis of the proposition that the phenolic hydroxyl of the down-positioned Tyr248 forms a hydrogen bond not only with the carboxylate of the substrate but also with the zinc-bound water molecule as depicted in Figure 2. Consequently, the pK_a value of the water molecule would be lowered further (*23*, *24*), and thus the Glu270 carboxylate that is engaged in the hydrogen bonding with the zinc-bound water molecule in the native CPA can now abstract a proton from the molecule to generate a hydroxide ion that attacks the carbonyl carbon of the CPA-bound substrate. The tetrahedral transition state generated by the hydroxyl attack is known to be stabilized by the guanidinium moiety of Arg127 (*1*, *25*). The increase of the K_M value and the decrease of the k_{cat} value observed for the hydrolysis of the peptide substrate in the presence of CPA-Y248F may, accordingly, be ascribed to the disappearance

of the hydrogen bonds between the zinc-bound water and the phenolic hydroxyl in the mutant CPA.

The X-ray crystal structure of the native CPA showed that the zinc-bound water molecule is distributed between two positions at pH 7.5: a water molecule (Wat571) at site 1 is separated by 2.0 Å from the zinc ion and hydrogen-bonded to O_ϵ 1 of Glu270, and that (Wat567) at site 2 is distanced by 3.2 Å from the zinc and hydrogen-bonded to Glu270 and the carbonyl group of Ser197 (*26*). This observation suggests that the water molecule is possibly not so tightly held by the zinc ion and could be pushed toward the substrate to the site proximal to the side chain of the down-positioned Tyr248, resulting in formation of additional hydrogen bonding with the phenolic hydroxyl. It has been reported that in other zinc proteases the zinc-bound water molecule changes its position away from the zinc upon binding a ligand to the enzyme (*27*). Furthermore, the zinc-bound water molecule in zinc proteases such as thermolysin and carbonic anhydrase is always found to be hydrogen bonded to a nearby residue, whereby the nucleophilicity of the water molecule is enhanced (*28*).

No single catalytic pathway can account for all the kinetic, spectroscopic, and X-ray crystallographic data accumulated for the enzymic reaction of CPA (*29, 30*). It is generally believed that while the peptide substrate is hydrolyzed by the general base mechanism, the ester substrate is hydrolyzed by an alternative mechanism in which the Glu270 carboxylate attacks directly the scissile ester carbonyl carbon with generation of an anhydride type acyl-CPA intermediate. The latter is subsequently hydrolyzed to yield product with regeneration of the enzyme (*31*−*34*). The anhydride pathway may be understood in terms of the different binding mode of ester substrate from that of peptide (*35*): While the carbonyl group of the scissile amide bond of peptide substrate forms a hydrogen bond with the guanidinium group of Arg127 (*25*), the carbonyl of the ester substrate has been claimed to ligate to the zinc ion at the active site (*35*, *36*). Thus, the zinc-bound water molecule in the CPA·ester complex is thought to be displaced by the ester carbonyl upon its binding to CPA from the zinc. As a consequence, the carboxylate of Glu270 may no longer serve as a base to abstract a proton from the water molecule but to function instead as a nucleophile, attacking the carbonyl to generate the acyl-enzyme intermediate. In this catalytic pathway, the hydrolysis of the acyl-CPA intermediate has been suggested to be the rate-determining step (*37*).

As can be seen in Table 1, upon the replacement of Tyr248 with Phe, the K_M value for ClCPL was increased by 2.5-fold but the k_{cat} value decreased as much as 20-fold compared with those obtained with the wild type. The 20-fold decrease of the k_{cat} value is remarkable, and suggests strongly that the phenolic hydroxyl of Tyr248 plays an important role also in the CPA-catalyzed hydrolysis of the ester substrate. In this regard, it may be worth noting that Gardell et al. (*11*) reported that the K_m value increases by 9.6-fold and the k_{cat} value decreases by 4.2-fold for the hydrolysis of ClCPL by the rat mutant CPA in which Tyr248 is replaced with Phe, thus observing the same trend as we did, albeit weak, in the hydrolysis of the substrate. In the hydrolysis of HPL, we observed that the k_{cat} decreases only by 1.65-fold. A similar result has been reported for the hydrolysis with rat CPA and its mutant. HPL is known to be an anomalous substrate

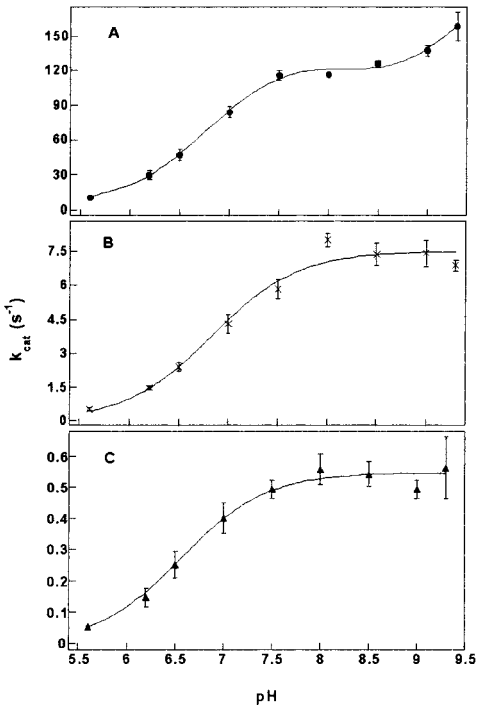

FIGURE 3: k_{cat} vs pH profiles for the hydrolysis of ClCPL catalyzed by CPA-WT (A), CPA-Y248F (B), and CPA-Y248A (C) at 25 °C. The experimental data were fitted to eq 3 by an iterative curve-fitting program. The error bars indicate ±1 standard deviation at each point.

showing a marked substrate inhibition for CPA (*12*). The pH dependency of the steady-state kinetic parameter, k_{cat}, was examined for the CPA-catalyzed ester hydrolysis. The k_{cat} value for the hydrolysis of ClCPL by CPA-WT shows a sigmoidal increase as the pH of the medium increases up to about 7, and then levels off before it rises steeply at about pH 9 (Figure 3). Such a steep rise of the k_{cat} value in the basic medium was not observed in the hydrolysis catalyzed by the mutant CPAs as can be seen in Figure 3B,C. The pH dependence of k_{cat} in the alkaline region reflects the pK_{ESH} of the enzyme−substrate complex (*38*). The rise of the k_{cat} value observed only in the CPA-WT-catalyzed hydrolysis indicates that the phenolic hydroxyl plays an important role in the hydrolysis, consistent with the conclusion derived from the kinetic study. The observation may be rationalized again with the proposition that the phenolic hydroxyl activates the water molecule that is relocated by the ester substrate through hydrogen bonding. The pK_a value of Tyr248 was reported to be 10.06 (*36*). Therefore, under the alkaline conditions, the hydrogen bonding remains and the activated water molecule may be deprotonated under the alkaline conditions to generate a hydroxide ion that attacks the acyl-CPA intermediate. However, in cases of mutant CPAs where the phenolic hydroxyl is absent, the water molecule fails to be activated by the hydrogen bonding and thus may not be deprotonated to generate the strong nucleophile that hydrolyzes the intermediate. Consequently, no increase in the k_{cat} value results.

2-Benzyl-3,4-epoxybutanoic acid (BEBA) is a pseudo-mechanism-based inactivator for CPA which covalently modifies the catalytic carboxylate of Glu270 (*19, 40, 41*). It has been proven by the X-ray crystallographic study that the nucleophilic attack by the Glu270 carboxylate takes place at the 3-position of the oxirane ring of BEBA bound to CPA,

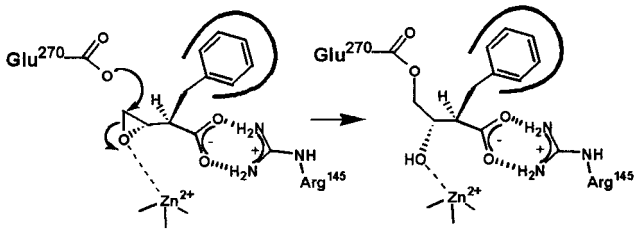

FIGURE 4: Schematic representation of the inactivation pathway of CPA-WT by 2-benzyl-3,4-epoxybutanoic acid. The nucleophilic attack of the carboxylate of Glu270 on the oxirane ring of the inactivation results in the covalent modification of the carboxylate in the form of an ester with concomitant ring opening.

Table 2: Kinetic Parameters for the Inactivation of Bovine CPA and Tyr248 Mutants by (2*R*,3*S*)-2-Benzyl-3,4-epoxybutanoic Acid[a]

CPA	k_{inact} (min^{-1})	K_I (mM)	k_{inact}/K_I (M^{-1} s^{-1})
wild-type	1.20 ± 0.09	0.35 ± 0.01	58
Y248F	0.80 ± 0.02	0.30 ± 0.05	44
Y248A	0.53 ± 0.07	1.1 ± 0.08	8.4

[a] Assays were performed in a solution of 50 mM Tris-HCl (pH 7.5) and NaCl (0.5 M) at 25 °C.

resulting in the formation of an ester bond with concurrent opening of the epoxide ring (*40, 41*) (Figure 4). The wild-type CPA and both Tyr248 mutants were inactivated by BEBA. The kinetic parameters obtained from the respective Kitz−Wilson plot are listed in Table 2. The K_I and k_{inact} values were not changed significantly upon the substitution of Tyr248 with Phe. However, the replacement of the Tyr with Ala reduced the k_{inact} by 2.3-fold and increased the K_I value by 3-fold. These observations imply that Tyr248 is not involved in the acyl-CPA intermediate formation in the CPA-catalyzed ester hydrolysis, but suggest that Tyr248 plays an important role in the later stage of the enzymic hydrolysis pathway, that is, the hydrolysis of the acyl-CPA intermediate. This is in line with the conclusion derived from the pH-dependent k_{cat} study.

The solvent isotope effect of 2.09 was obtained for the CPA-WT-catalyzed hydrolysis of ClCPL, which is in agreement with that obtained by Kaiser and Kaiser, who reported the solvent effect of 1.9 for the CPA-catalyzed hydrolysis of *O*-(*trans*-cinnamoyl)-L-β-phenyllactic acid (*42*). The isotope effects for the ClCPL hydrolysis by CPA-Y248F and CPA-Y248A were found to be 1.30 and 1.03, respectively. The decrease of the isotope effects observed with the mutants also suggests that the phenolic hydroxyl is involved in the hydrolysis of the ester substrate, most likely in the stage of the hydrolysis of the acyl-CPA intermediate, i.e., the rate-determining step in the enzymic hydrolysis of the ester. We are tempted to propose a mechanism that can account for the foregoing results obtained in the CPA-catalyzed hydrolysis of ester substrate: As discussed earlier, the scissile ester bond is subjected to nucleophilic attack by the Glu270 carboxylate, generating a tetrahedral transition state that collapses to form the acyl-CPA intermediate. The alkoxide thus generated would pick up a proton most likely from the activated water. The hydroxide that was generated in the process, in turn, abstracts the phenolic acidic proton of Tyr248 to yield a phenoxide anion (Figure 5). The loss of the hydroxyl proton of Tyr248 would result in the disruption of the hydrogen bonding between the hydroxyl of Tyr248 and the carboxylate of product. The repulsive electrostatic interaction imparted between the phenoxide and the carboxylate of the product upon the hydrogen bonding disruption would facilitate the departing of the product from the enzyme. In the subsequent step, the phenoxide ion would abstract a proton from the water molecule to produce a hydroxide that can attack the carbonyl carbon of the acyl-CPA intermediate, thus to complete the enzymic hydrolysis reaction (Figure 5). It thus seems that the deprotonation of the water molecule by the phenoxide of Tyr248 is responsible

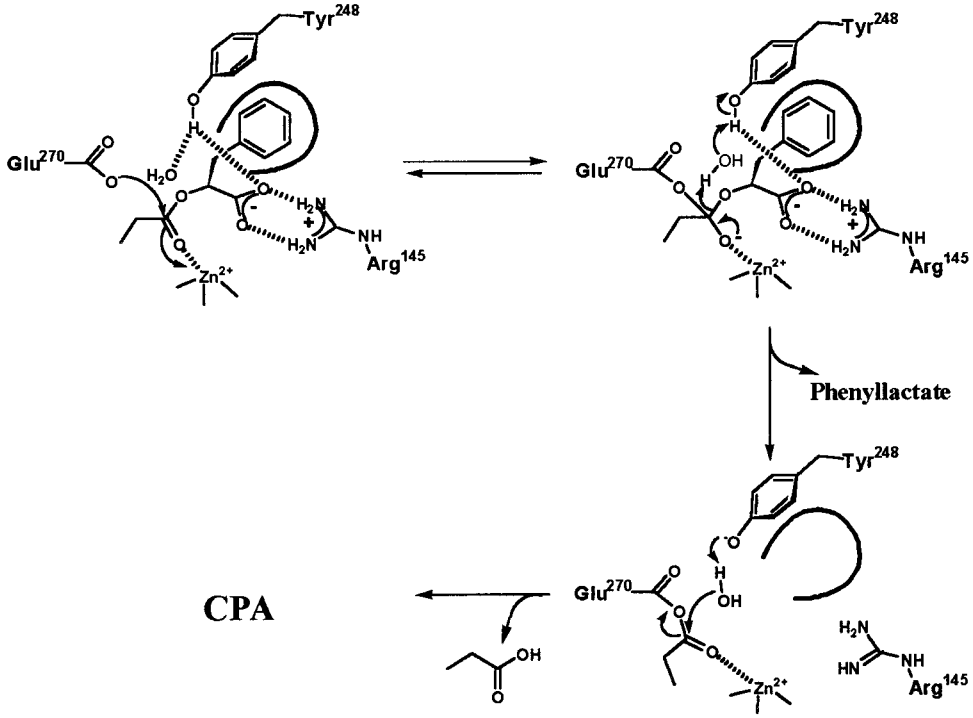

FIGURE 5: Mechanism for the CPA-catalyzed hydrolysis of the ester substrate.

for the isotope effect observed in the CPA-WT-catalyzed hydrolysis of ClCPL. In the ester hydrolysis catalyzed by the mutants, the water molecule rather than the hydroxide ion is expected to attack the acyl-CPA intermediate, whereby no isotope effect is expected. The proposition explains not only the isotope effect observed in the CPA-WT-catalyzed hydrolysis of ClCPL but also the reduced k_{cat} value observed in the hydrolysis catalyzed by CPA-Y248F. Christianson and Lipscomb have previously suggested the possibility of the zinc-bound water molecule to serve as the proton donor for the leaving alkoxide in the CPA-catalyzed hydrolysis of ester substrate (*43*).

The replacement of Phe248 with Ala brought about a further reduction of the k_{cat} value for the ester hydrolysis by 12-fold, but the K_M value was increased by only 2-fold (Table 2). The profound reduction of the k_{cat} value by the Phe → Ala replacement indicates that the aromatic ring of Tyr248 plays an important role in the ester hydrolysis. The reason for the decrease of the k_{cat} value is not apparent to us presently, but may possibly have arisen as a result of the reduced hydrophobic environment surrounding the zinc-bound water molecule. This notion is consistent with the report that hydrophobicity surrounding the metal bound water molecule enhances the acidity of the water molecule (*44*−*46*).

In summary, the present study carried out with bovine CPA and its mutants, CPA-Y248F and CPA-Y248A, indicates that Tyr248 plays a critical role not only in the binding of substrate to the enzyme but also in the catalytic process in the enzymic hydrolysis reaction. The results of kinetic and solvent isotope effect studies may be accounted for by the proposition that the phenolic hydroxyl of Tyr248 hydrogen-bonds to the zinc-bound water molecule upon substrate binding of the enzyme. On the basis of the proposition, a unified catalytic mechanism for the enzymic hydrolysis of peptide and ester substrates is proposed.

ACKNOWLEDGMENT

We thank Dr. Sang J. Chung for providing 2-benzyl-3,4-epoxybutanoic acid.

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BI010807J