Arginine 127 Stabilizes the Transition State in Carboxypeptidase*

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Crystallographic studies suggest that Arg-127 is a key amino acid in the hydrolysis of peptides and esters by carboxypeptidase A. The guanidinium group of Arg-127 is hypothesized to stabilize the oxyanion of the tetrahedral intermediate formed by the attack of water on the scissile carbonyl bond. We have replaced this amino acid in rat carboxypeptidase A1 with lysine (R127K), methionine (R127M), and alanine (R127A), in order to define the role of Arg-127 in carboxypeptidase catalyzed hydrolysis. The wild-type and mutant enzymes were expressed in yeast and purified. Kinetic studies show that Arg-127 substitution decreases $k_{cat}$ for both ester and amide substrates, whereas $K_m$ is relatively unchanged; for R127M and R127A this corresponds to a 6 kcal/mol decrease in transition state stabilization of the rate-limiting step. The binding affinity for the phosphonate transition state analog, Cbz-Phe-Ala(P)-OAla, was decreased by 5.4 kcal/mol, whereas binding affinity for the ground state inhibitor, DL-benzylsuccinic acid, was decreased by only 1.7 kcal/mol for R127M. Electrostatic calculations employing a finite difference solution to the Poisson-Boltzmann equation predict that the positive charge of Arg-127 should stabilize the transition state by 6–8 kcal/mol. Therefore, the experimental and theoretical data suggest that the primary role of Arg-127 is stabilization of the transition state through electrostatic interaction with the oxyanion.

Pancreatic CPA is a $Zn^{2+}$-dependent exopeptidase which cleaves carboxy-terminal aromatic or aliphatic amino acids from peptides. CPA has been extensively studied by a wide range of techniques that include crystallography, spectroscopy, kinetics, and site-directed mutagenesis (1–3, 8, 11, 19).

The key functional groups positioned near the scissile bond are $Zn^{2+}$, Glu-270, and Arg-127 (Fig. 1). $Zn^{2+}$ is coordinated to the enzyme by residues Glu-72, His-69, and His-196 (Fig. 1). A hydrophobic cleft forms the binding pocket of the terminal hydrophobic side chain and provides substrate specificity. The terminal carboxylate forms a bifurcated hydrogen bond to Arg-145 and a single hydrogen bond to Tyr-248 (1). Additional contacts with Arg-71, Tyr-198, and Phe-279 form an extended binding site for larger substrates (1).

The proposed mechanism of CPA hydrolysis is a $Zn^{2+}$-promoted attack of water on the scissile carbonyl bond with Glu-270 assisting as a general base (Fig. 2) (1). The enzyme must additionally stabilize the oxyanion in the tetrahedral intermediate. This role has classically been assigned to $Zn^{2+}$. However, Christianson and Lipscomb (1) argue that interaction of $Zn^{2+}$ with the scissile carbonyl oxygen would decrease the nucleophilicity of the $Zn^{2+}$ bound water; therefore, $Zn^{2+}$ would not be a good candidate for the electrophilic catalyst. Instead, they suggest that the positive charge of Arg-127 may stabilize the oxyanion (4, 5). Crystallographic studies show that Arg-127 forms a hydrogen bond to the tetrahedral oxygen of the transition state analog Bop (the ketone analog of Bz-Gly-Phe) and Zgp (the phosphonamide analog of Cbz-Gly-Phe) (Fig. 1). Chemical modification of an unidentified arginine residue abolished peptide hydrolysis without affecting ester hydrolysis (6). Peptides can bind to the apoenzyme, whereas esters cannot (7), and it has been hypothesized that a binding mode involving Arg-127 exists for peptides but not esters. These results suggest that Arg-127 will have a more important function in the hydrolysis of peptides than for esters (1).

To elucidate the role of Arg-127 in peptide and ester hydrolysis, we have replaced Arg-127 with Lys (R127K), Met (R127M), and Ala (R127A) by site-directed mutagenesis of the rat enzyme. The wild-type and mutant enzymes were expressed in yeast, purified, and evaluated by kinetic methods. These studies reveal an important catalytic role for Arg-127.

**EXPERIMENTAL PROCEDURES**

**Materials**

Fast blue salt, CPA substrates, and CPA inhibitors were purchased from Sigma with the exceptions of CLCLP, which had been prepared previously (8), carboxanphosphorylalanine, which was purchased from Pfaltz and Bauer, Inc., Waterbury, CT, and Cbz-Phe-Ala(P)-OAla, which was a generous gift from Dr. Paul Bartlett, University of California, Berkeley.

**Mutagenesis**

The gene encoding rat proCPA (9) was cloned into Bluescript (Stratagene). Single-stranded DNA was isolated from Escherichia coli strain RZ1032 (Hfr K1, 16--111/45 lys A61-62) by the method of Kunkel (10). The mutagenic primers were as follows: (The underlined
Fig. 1. The superimposed structures of CPA bound to Bop and CPA bound to Zgp. Only residues within 5 Å of Zn$^{2+}$ are shown. The Zgp-CPA structure is in red, and the Bop-CPA structure is in blue.

Fig. 2. Proposed mechanism of CPA-catalyzed hydrolysis. The reaction proceeds by the attack of Zn$^{2+}$-bound water on the scissile carbonyl. A tetrahedral intermediate is formed in the transition state. This collapses to form product.

bases encode the new amino acid at amino acid position 127, and additional changes that did not affect the amino acid sequence were made to introduce diagnostic restriction sites.)

To avoid sequencing the entire CPA gene for each mutant, 300-base pair BstEII-StuI fragment containing the Arg-127 mutation was subcloned into the proper context of the wild-type gene. The BstEII-StuI cassette was sequenced in its entirety to verify that only the desired mutations had been produced.

Construction of the Wild-type CPA Shuttle Vector Which Contains Yeast Transcription, Translation, and Secretion Signal—A yeast expression vector containing the ADH/GAPDH regulatory and promoter region and rat proCPA fused in frame to the α-factor leader sequence (the α-factor leader sequence directs secretion of the protein into the media) was constructed as follows: step 1, rat proCPA (11) was fused in frame to the α-factor leader by three piece ligation of the 1.2-kb HindIII/StuI fragment, containing the gene for proCPA, with the large KpnI-Sall fragment of pRR34 (alkaline phosphatase promoter and regulatory regions and α-factor leader) (12) and a HindIII-KpnI oligonucleotide adapter. The nucleotide adapter was formed by the annealing of the nonphosphorylated oligonucleotide 5‘CTTGGAGATTTAGAGA3‘ (coding strand) and 5‘AGCTTTGCA- TTATGTTGAGCTAC3‘ (noncoding strand). The adapter contains the amino acid sequence required for processing of the α-factor leader by the cell, the underlined triplet coding for arginine is the site of α-factor cleavage (13); step 2, the 1.2-kb HindIII-Sall fragment containing rat proCPA was also cloned 3‘ to the ADH regulatory region and GAPDH promoter sequences, by three piece ligation with the large NcoI-StuI fragment from pBS100 (14) and a NcoI-HindIII oligonucleotide adapter. The adapter was formed by the annealing of the nonphosphorylated oligonucleotides 5‘CATGGGACTCGA3‘ and 5‘AGCTTGTGAGCTAC3‘; step 3, the α-factor leader sequence was inserted into the clone containing the ADH/GAPDH promoter sequences. This was achieved by replacing the 0.8-kb NcoI fragment from the plasmid constructed in step 2 with the 1.1-kb NcoI fragment (the α-factor leader and the 5‘ end of rat CPA) from the plasmid constructed in step 1. The 3.6-kb BamHI fragment containing ADH/GAPDH promoter, α-factor leader, and proCPA was cloned into the BamHI site of a modified Bluescript vector (the restriction sites between and including EcoRV to KpnI had been destroyed) to produce pMP36.

Construction of Yeast Expression Vectors for Wild-type CPA—The BamHI fragments, containing the yeast promoter regions and proCPA from pMP36, were cloned into the BamHI site of the 1.2-kb yeast 2-μm plasmid PC1/1 (13) to produce pMP32 (Fig. 3). PC1/1 contains the yeast and bacterial replication origins, the ampicillin resistance gene, and the leu2d gene.

Construction of the Yeast Expression Vector for the Arg-127 Mutants of CPA—The three Arg-127 mutants were cloned into the KpnI-Sall sites of pMP36 by three piece ligation with the KpnI-HindIII adapter described above. The resulting BamHI fragments were then cloned into PC1/1 to yield the final yeast expression vector for the three mutants (R127K, pMP47; R127M, pMP48; R127A, pMP49).

Expression of Rat ProCPA in Yeast

Yeast expression plasmids were transformed into yeast strain DLM101 (Mat a, leu 2-3,112 his2 3-11,15 can1, urs 3D, pep4D [cir5]; DM23) by the method of spheroplasting (15). The resulting transformants were selected for by growth on plates of synthetic complete medium lacking leucine (16). The growth conditions used to induce the production of proCPA from the ADH/GAPDH promoter were as described (16).

Purification of CPA

The yeast media containing the secreted protein was collected by centrifugation and brought to 0.75 mM ammonium sulfate, 20 mM sodium phosphate, pH 7.0, 0.1 mM ZnCl$_2$ (buffer A) and then loaded directly onto a 50-ml phenyl-Sepharose (Sigma) column equilibrated in buffer A. The protein was eluted with a 120-ml linear gradient of 0-10% ethanol, 0.75-0 M ammonium sulfate. ProCPA activity eluted at the end of the gradient. The fractions containing proCPA were pooled and dialyzed against 20 mM MES, pH 5.5, 50 mM NaCl, 1 mM ZnCl$_2$ (buffer C).

ProCPA was cleaved to CPA by incubation with trypsin (2 μg/ml) at 37°C for 30 min. Phenylmethylsulfonylfluoride (0.5 mM) was added to inactivate the trypsin and the preparation was re-dialyzed against buffer C.

The activated CPA was loaded onto a p-amino benzenesulfonylic acid affinity column. The column was washed with 20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 0.1 mM ZnCl$_2$ (buffer D) and then with 20 mM Tris-HCl, pH 7.5, 1 M NaCl, 0.1 mM ZnCl$_2$ to elute the carboxypeptidase. The peak fractions were pooled and dialyzed against buffer D. The column was made by incubating 250 mg of p-amino benzenesulfonylic acid (Sigma) with 25 ml of Affigrop 10 support (Bio-Rad) in 50 mM
HEPES, pH 7.5, at 4 °C overnight, and the resin was treated with hydroxylamine and equilibrated in buffer C.

The CPA was applied to a Mono Q column (Pharmacia LKB Biotechnology Inc.) equilibrated in buffer D and eluted with a 30-ml linear gradient of 0-250 mM NaCl. The purified protein was stored at 4 °C in the presence of 10⁻⁴ M ZnCl₂.

**Enzyme Assays**

CPA activity was measured spectrophotometrically as described (17) with the exception of the activity of mutant enzymes on the substrate Bz-Gly-Phe, which was measured by HPLC analysis (18). Assays were performed in 50 mM Tris-HCl, pH 7.5, 500 mM NaCl at 25 °C.

**Electrophoresis**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described (19). Isoelectric focusing was performed using preprepared gels, pI range 3.5-9.5 (Pharmacia), as recommended by Pharmacia. Activity stains were a modification of a previously reported method (20). The isoelectric focusing gel was soaked for 15-min intervals at 37 °C in three changes of 1 mg/ml carbamoylphosphohexosamine and 1 mg/ml fast blue salt dissolved in 50 mM Tris-HCl, pH 7.5, 500 mM NaCl. CPA hydrolyzes carboxamidophosphohexosamine and produces naphthol, which reacts with the diazonium salt to form a purple precipitate.

**Molecular Modeling and Electrostatic Calculations**

Structures were displayed with the INSIGHT program (Biosym Technologies). The coordinates for the complexes of cow CPA with Bop and Zgp were provided by Dr. David Christianson (University of Pennsylvania). Electrostatic calculations were performed with the DelPhi program which uses the finite difference method to solve the Poisson-Boltzmann equation. This method has been described in a number of publications and is valuable for identifying the electrostatic potential over the enzyme's surface on a per residue basis (21-23). We did not include the crystallographically determined solvent positions in the calculation. The calculation parameters were assigned the standard values, except that the dielectric constant for CPA was taken to be 4.0 and 80.0 was used for the solvent. Charges were assigned as follows: arginine, 0.5 centered at each of the two terminal guanidinium nitrogens; lysine, 1.0 centered at the terminal primary nitrogen; glutamic and aspartic acid, -0.5 centered on each carboxylate oxygen; Zn²⁺, +2 and; for the inhibitors Bop and Zgp, -0.5 centered on each carboxylate of the C-terminal amino acid. To represent the oxyanion which is formed during hydrolysis, O(2) was assigned a -1 charge (defined in Fig. 2). A positive charge will also be formed in the transition state as a result of the attack of water on the scissile carbonyl bond. The position of this charge is not known so we used several probable locations and evaluated the electrostatic potentials for each model; a +1 charge was assigned to either O(1) of the inhibitor or to a hydrogen modeled half-way between the nearest oxygen of Glu-270 and O(1) of the inhibitor (defined in Fig. 2). The calculation to obtain the contribution of Arg-127 at any particular site was done as described (21). All modeling and electrostatic calculations were based on the structure of cow CPA because a structure for rat CPA is unavailable. However, based on the observation that rat and cow CPA have strong sequence similarity (79 % of the amino acid residues are similar (24)) and all catalytically important residues are invariant, we make the assumption that the analysis will be valid for rat CPA as well. This assumption is valid in the comparison of the active site electrostatic potentials for cow and rat trypsin (21).

**RESULTS**

**Mutagenesis, Expression, and Purification of CPA—**Arg-127 was replaced by lysine (R127K), methionine (R127M), and alanine (R127A) as described under "Experimental Procedures." The wild-type and mutant CPA genes were cloned into the ADH/GAPDH yeast expression vector (Fig. 3) and purified as described under "Experimental Procedures." The yield of proCPA is approximately 10 mg/liter of yeast culture. This represents a 25-fold increase in CPA expression over our previous system and will provide sufficient material for structural characterization of CPA and its mutants (11). Unlike the wild-type enzyme, the purified Arg-127 mutants were unstable when stored for prolonged periods (days to weeks) in the absence of ZnCl₂; the mutant enzymes were subsequently stored in the presence of 10⁻⁴ M ZnCl₂. CPA purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (data not shown) and by isoelectric focusing. Fig. 4 shows an isoelectric focusing gel of wild-type CPA, R127K, R127M, and R127A. The gel was stained with Coomassie Blue (Fig. 4A) and for CPA activity (Fig. 4B). Wild-type CPA and R127K migrated with the same mobility in the gel (pl 6.1), whereas R127M and R127A both shifted toward the anode (pl 5.6). The band observed by the activity staining co-migrated with the major Coomassie Blue staining protein band in each case and was observed only in lanes containing CPA. Several minor bands were observed in all lanes. These bands may represent deamidation (they have more acidic pl values) or alternate cleavage sites for propetide removal and it is not surprising that they also show activity.

**The Effect of Arg-127 Mutagenesis on Kcat and Km**—The
effect of Arg-127 mutagenesis on CPA catalyzed hydrolysis of peptides and ester varied with the substrate as shown in Table I. The $k_{cat}$ values for R127K hydrolysis of Bz-Gly-Phe, Bz-Gly-OPhe and Cbz-Gly-Gly-Phe are decreased on average 150-fold, whereas the $K_m$ values are increased on average 10-fold. The $k_{cat}$ values for the hydrolysis of these substrates by R127M or R127A are 1500- and 3000-fold lower than for wild-type CPA, respectively, whereas the $K_m$ values are increased about 10-fold.

In contrast to the three substrates described above, hydrolysis of Cbz-Gly-Phe and CLCPL by R127K was only mildly affected. The $k_{cat}$ values were reduced by 25- and 50-fold, respectively (Table I).

For wild-type CPA, the ratio of the $k_{cat}/K_m$ values (specificity constant) for Cbz-Gly-Gly-Phe and Cbz-Gly-Phe is 6.0, indicating a preference for Cbz-Gly-Phe. In contrast, the mutant enzymes have a reversed preference with the ratio of the specificity constants averaging 0.1. These trends are also observed for Cbz-Gly-Gly-Tyr and Cbz-Gly-Tyr (data not shown). Even more surprisingly, the ratio of the specificity constants for the structurally similar substrates, Cbz-Gly-Phe and Bz-Gly-Phe, has gone from 1.4 for the wild-type enzyme to an average of 100 for the mutants.

**Electrostatic Calculations**—The value of the electrostatic potential generated by all charged amino acids in Bop-CPA was calculated by the DelPhi program (21-23). Charges were assigned as described under “Experimental Procedures.” Fig. 5A displays the +3.6 kcal/mol contours of the electrostatic potential for the Bop-CPA structure and Fig. 5B displays the +3.6 kcal/mol contours for the Bop CPA structure in which Arg-127 was replaced by Met. The active site has a prominent region of positive potential generated by arginines 145, 127, and Arg-130. Interestingly, all 6 of these residues are conserved among the pancreatic carboxypeptidases of known sequence (24). The replacement of Arg-127 with Met disrupts the positive potential field within the active site (Fig. 5 A and B). To calculate the electrostatic potential of Arg-127 alone, each guanidinium nitrogen was assigned a charge of 0.5, and all other charges were set at 0 for the DelPhi calculation. The electrostatic effect of Arg-127 could then be sampled at any position within the structure. The hydrogen atom, derived from the dissociation of water in the catalytic attack, was built into the Bop and Zgp structures halfway between the oxygen of Glu-270 and the O(1) of the inhibitor. The results are summarized in Table II. The large difference in potential at the Zn$^{2+}$ atom found between the Bop-CPA complex and the Zgp-CPA complex is due to a difference in the position of the inhibitor and Arg-127.

**Solvent Isotope Effects**—Deuterium solvent isotope effects were measured for the wild-type and mutant enzymes. The results observed for the wild-type CPA hydrolysis of CLCPL ($k_H/k_D = 2.3 \pm 0.1$) are consistent with the previously reported values (8, 29). The results for the wild-type CPA hydrolysis of Cbz-Gly-Phe ($k_H/k_D = 1.2 \pm 0.1$) and Cbz-Gly-Gly-Phe ($k_H/k_D = 1.2 \pm 0.1$) by wild-type CPA are likewise in agreement with the reported values for peptide substrates (25). In addition, we measured an isotope effect for the hydrolysis of Bz-Gly-OPhe, by wild-type CPA, that was equal in magnitude to that reported for CLCPL ($k_H/k_D = 2.5 \pm 0.1$). Deuterium solvent isotope effects similar to those observed for wild-type CPA were observed for the hydrolysis of both ester and peptide substrates by R127K and R127M. These data imply that no change in the rate-limiting step has occurred for the hydrolysis of the ester substrates. No information could be obtained from this study regarding the rate-limiting step for the peptide substrates, because a significant isotope effect was not observed.

**The Effect of Arg-127 Mutation on the Binding of Inhibitors**—The effects on the binding of three competitive inhibitors of CPA hydrolysis were tested for the Arg-127 mutant enzymes; BzSA and PCl are ground state inhibitors and Cbz-Phe-Ala(P)-OAla is a transition state analog.

BzSA has a $K_J$ of 0.2 $\mu$m for cow CPA and is believed to

| TABLE I | Kinetic parameters of wild-type and Arg-127 mutant CPAs |
|---------|------------------------------------------------------|
| **Enzyme** | **Substrate** | **Bz-Gly-Gly-Phe** | **Bz-Gly-OPhe** | **Bz-Gly-Phe** | **Cbz-Gly-Phe** | **CLCPL** |
| | | $k_{cat}$ | $K_m$ | $(k_{cat}/K_m) \times 10^5$ | $k_{cat}$ | $K_m$ | $(k_{cat}/K_m) \times 10^5$ | $k_{cat}$ | $K_m$ | $(k_{cat}/K_m) \times 10^5$ | $k_{cat}$ | $K_m$ | $(k_{cat}/K_m) \times 10^5$ |
| Wild-type | 53 ± 3 | 1220 ± 70 | 12 ± 2 | 15 ± 1 | 9.3 ± 0.5 |
| R127K | 36 ± 4 | 82 ± 6 | 36 ± 3 | 61 ± 6 | 28 ± 3 |
| R127M | 1.10 ± 0.03 | 4.8 ± 0.3 | 0.09 ± 0.01 | 5.70 ± 0.03 | 2.3 ± 0.1 |
| R127A | 380 ± 30 | 440 ± 40 | 450 ± 80 | 230 ± 20 | 83 ± 6 |
| | 3.1 ± 0.2 | 11 ± 1 | 0.20 ± 0.01 | 25.0 ± 0.2 | 28 ± 2 |
| | 3.6 | 4.3 | 4.3 | 1.4 | 1.5 |
| Wild-type | 0.049 ± 0.01 | 0.33 ± 0.06 | 0.010 ± 0.002 | 0.64 ± 0.01 | 0.19 ± 0.001 |
| R127K | 0.12 ± 0.03 | 1.0 ± 0.3 | 0.02 ± 0.01 | 1.7 ± 0.2 | 1.1 ± 0.2 |
| R127M | 5.5 | 5.7 | 5.6 | 2.9 | 3.4 |
| R127A | 0.03 ± 0.01 | 0.19 ± 0.02 | ND | 0.30 ± 0.05 | ND |
| | 400 ± 100 | 500 ± 100 | 500 ± 200 | 380 ± 40 | 160 ± 90 |
| | 0.13 ± 0.03 | 1.0 ± 0.3 | 0.02 ± 0.01 | 1.7 ± 0.2 | 1.1 ± 0.2 |
| | 5.5 | 5.7 | 5.6 | 2.9 | 3.4 |
| | 0.072 ± 0.04 | 0.6 ± 0.1 | 0.8 ± 0.2 | 0.8 ± 0.2 |
| | 5.9 | 6.0 | 5.4 | 3.4 | 3.4 |
interact with both Zn$^{2+}$ and the hydrophobic specificity pocket (26). BzSA is a competitive inhibitor for hydrolysis of Cbz-Gly-Phe, Bz-Gly-OPhe, and Cbz-Gly-Gly-Phe ($K_i = 0.06 \mu M$) by rat wild-type CPA and for the hydrolysis of Cbz-Gly-Gly-Phe and Bz-Gly-OPhe by R127K. In contrast, BzSA is a noncompetitive inhibitor of R127K hydrolysis of Cbz-Gly-Phe. The average $K_i$ for R127K is increased 4-fold over the wild-type value and for R127M it is increased 20-fold.

PCI is a 39-amino acid peptide inhibitor, with a $K_i$ of 3 nM for rat CPA (11, 27). A large decrease in the binding affinity of PCI was observed for the Arg-127 mutant CPAs; the $K_i$ for inhibition of Cbz-Gly-Phe hydrolysis by R127K is 2 \mu M and by R127M is \(3 \mu M\). Similar results were obtained for inhibition of Bz-Gly-OPhe and Cbz-Gly-Gly-Phe hydrolysis.

Cbz-Phe-Ala(P)-OAla, the phosphonate analog to the substrate Cbz-Phe-Ala-OAla, has been shown previously to be a transition state analog of CPA catalyzed hydrolysis (28). The phosphonate inhibitors are the tightest binding class of inhibitors to CPA known; Cbz-Phe-Ala(P)-OAla is a relatively weak binding member of this class with a $K_i$ of 56 nM for cow CPA (28). The $K_i$ for the inhibition of Cbz-Gly-Phe hydrolysis by wild-type rat CPA is 30 nM. In contrast, the binding affinity of the R127K and R127M for Cbz-Phe-Ala(P)-OAla is greatly reduced; the $K_i$ values for the inhibition of Cbz-Gly-Phe hydrolysis by R127K and R127M are 75 and 300 pM, respectively (Fig. 6).

Criteria for Mutant Purity—A potential problem when evaluating mutants with such low levels of activity is contamination from wild-type enzyme or second site revertants caused by transcriptional or other procedural errors (29). The evidence indicates that the hydrolytic activity of R127K, R127M, and R127A must result from catalysis by these enzymes and not by a more active contaminant. The major Coomassie Blue staining protein band on isoelectric focusing gels also stains with the CPA activity reagents for both wild-type CPA and the Arg-127 mutants. Furthermore, the $K_i$ for inhibition of the Arg-127 mutant enzymes by PCI and Cbz-Phe-Ala(P)-OAla are over 1000 times higher than that of the wild-type enzyme; therefore, the mutant enzymes are still active at concentrations of inhibitors that completely inactivate wild-type CPA. Additionally, the $K_i$ values of the mutant enzymes are higher than the wild-type $K_i$ values for the same substrates and a change in substrate specificity has occurred.

### Table II

| Sampled residue                      | $B_{op}$ | $Z_{op}$ |
|--------------------------------------|----------|----------|
| Oxyanion (O(2))                       | -12.3    | -12.1    |
| Oxygen from Zn$^{2+}$-bound H$_2$O (O(1)) | +5.9     | +5.6     |
| Proton from Zn$^{2+}$-bound H$_2$O     | +4.0     | +3.7     |
| Zn$^{2+}$                             | +17.6    | +13.6    |

**FIG. 6. Binding of the phosphonate inhibitor Cbz-Phe-Ala(P)-OAla.** The percent activity remaining is plotted versus inhibitor concentration. Activity was measured by following hydrolysis of 250 \(\mu M\) Cbz-Gly-Phe. The $K_i$ for wild-type was measured to be 30 nM, for R127K, 75 \(\mu M\); and for R127M, 300 \(\mu M\).
DISCUSSION

Although much is known about CPA, the mechanism of hydrolysis is still not well understood. Hydrolysis is believed to proceed by attack of a Zn$^{2+}$ bound water on the peptide bond to form a tetrahedral intermediate, but the exact nature and function of the amino acids involved is still unclear. The enzyme must stabilize the developing oxyanion to facilitate formation of the tetrahedral intermediate. Arg-127 was replaced with Lys, Met, and Ala by site-directed mutagenesis. These three amino acids were chosen to evaluate the contribution to catalysis of the size, shape, and charge of Arg-127.

The data collected for the three Arg-127 mutant enzymes, R127K, R127M, and R127A, indicate that Arg-127 functions primarily by stabilizing the rate-limiting step. The loss in binding energy ($\Delta G$) of the rate-limiting transition state is substantial, ranging from 4.1 kcal/mol for R127K to 6.0 kcal/mol for R127M and R127A hydrolysis of Cbz-Gly-Gly-Phe, Bz-Gly-Phe, and Bz-Gly-OPhe. Peptide and ester substrates are equally dependent on the contributions of Arg-127 to catalysis, as illustrated by the similar reduction in $k_{cat}$ for the hydrolysis of the matched peptide-ester pair Bz-Gly-Phe and Bz-Gly-OPhe. Therefore, the initial prediction that Arg-127 would only be important for peptide hydrolysis is not supported by our experimental data. However, mutation of Arg-127 does not affect all substrates equally. The $k_{cat}$ values are decreased substantially for hydrolysis of Cbz-Gly-Gly-Phe, Bz-Gly-Phe, and Bz-Gly-OPhe by the mutant CPAs, whereas only a mild reduction in the $k_{cat}$ values for the hydrolysis of Cbz-Gly-Phe and CLCPL occurred. Although $k_{cat}$ is lowered dramatically, $K_a$ is increased on average only 8-fold. If $K_a$ equals the true dissociation constant ($K_a$) for enzyme and substrate this would correspond to a 1.2 kcal/mol loss in binding energy of the ground state structure. This assumption is valid for cow CPA, although it has not been demonstrated for rat CPA (17).

The observation that the major effect of Arg-127 mutation is on $k_{cat}$ implies that the primary role of Arg-127 is transition state stabilization. This conclusion is further supported by the decreased binding of the transition state analog Cbz-Phe-Ala(P)-OAla to the mutant enzymes. Structural data revealed that Arg-127 interacts tightly with the phosphonate anion of a similar phosphonamidate inhibitor, Zgp. This interaction may account for the unusual strength of the binding interaction of phosphates to CPA (5). The binding affinity of these inhibitors to the Arg-127 mutants would be expected to decrease in parallel with $k_{cat}/K_a$; the loss in binding energy for Cbz-Phe-Ala(P)-OAla ranges from 4.6 kcal/mol for R127K to 5.4 kcal/mol for R127M and is in excellent agreement with the observed decreases in $k_{cat}/K_a$. In contrast, the loss in binding energy for the ground state inhibitor, BzSA, ranges from 0.8 kcal/mol for R127K to 1.7 kcal/mol for R127M, similar to the changes observed for substrate binding.

Electrostatic calculations also suggest that the main role of Arg-127 should be transition state stabilization. The theoretical contribution of Arg-127 to transition state stabilization was calculated by totaling the electrostatic potentials generated by Arg-127 on the oxyanion (O(2)) and on the attacking water molecule (the positive charge was positioned either on O(1) or the modeled proton, Fig. 2 and Table II). This predicts that Arg-127 stabilizes the transition state by 6–8 kcal/mol and is in reasonable agreement with the experimental result. This large electrostatic interaction energy may result because Arg-127 is buried within the protein. Modeling reveals that in the structure of free CPA, Arg-127 is 80% solvent inaccessible, whereas in the Bop and Zgp complexes it is 90% solvent inaccessible. Typical arginine residues are instead 90% solvent accessible (30).

Although the electrostatic calculations successfully predict the contribution of Arg-127 to transition state stabilization, they overestimate the contribution of Arg-127 to the binding of transition state analogs. Arg-127 is predicted to contribute 12 kcal/mol toward the binding energy of the Zgp through interaction with the oxyanion (O(2), Table II), whereas the observed decrease in binding energy for Cbz-Phe-Ala(P)-OAla is only 5.4 kcal/mol. However, these calculations have not accounted for the desolvation energy of Arg-127 upon inhibitor binding. This factor will reduce the contribution of the positive charge on Arg-127 to the binding energy of the phosphonate and phosphamidate inhibitors. Since desolvation occurs in the ground state, it is irrelevant to transition state stabilization.

Having established that the positive charge of Arg-127 is important to CPA-catalyzed hydrolysis, can we learn anything further about the essential nature of arginine in this position? In addition to the positive charge, arginine has other important properties not shared by Lys, Met, or Ala. 1) It can form spatially separate hydrogen bonds, and 2) it disperses the charge over the entire guanidinium group. If Lys occupied the same position as arginine, the positive charge would be centered at the position of CZ (the carbon within the guanidinium group) of arginine and electrostatic calculations predict that substitution of Arg with Lys would only destabilize the rate determining transition state by 1 kcal/mol; a decrease of up to 4 kcal/mol is actually observed. Why then is the positive charge on Lys unable to mimic that of Arg? When substrate is bound, Arg-127 forms two hydrogen bonds, one to the scissile carbonyl and the second to Asp-142. Modeling of Lys into the space vacated by Arg in the Bop-CPA structure, predicts that Lys can form a hydrogen bond to Asp-142, but not to the scissile carbonyl. This second hydrogen bond may be important for proper orientation of the substrate relative to other important catalytic residues. It is, therefore, likely that hydrogen bonds formed by Arg-127 in the active site and its size and hydrophobicity contribute to the optimal positioning of the charge relative to the scissile carbonyl bond and to stabilizing the active site geometry. In the absence of these additional factors, proper transition state stabilization cannot be achieved. The actual position of Lys in the active site cannot be predicted, and structural characterization of the mutant enzymes will be required to completely understand these observations.

The binding affinity of PCI toward the Arg-127 mutant enzymes has been significantly altered when compared with wild-type CPA; the loss in PCI binding energy for the mutant enzymes is 4.1 kcal/mol. The carboxyl-terminal glycine is rapidly cleaved from the inhibitor by cow CPA; thus it is likely that the measured $K_a$ is to this species of the inhibitor (27). Because PCI is a product, the carboxyl-terminal carboxylate will be positioned near Arg-127 instead of Arg-145, as observed for substrate and substrate analogs. However, PCI is distinguished from product in that, due to extensive contacts with the enzyme, it binds very tightly. This property of PCI makes it an unusual ground state inhibitor and may explain why it does not behave similarly to other ground state structures following Arg-127 mutagenesis. Arg-127 is not within hydrogen bonding distance of the terminal carboxylate, although a strong electrostatic interaction is predicted (31). The loss of this electrostatic interaction may have a role in the decreased binding affinity of PCI toward the mutant enzymes. Additionally, the positions of water molecules are not included in the structure and therefore, Arg-127 may...
interact with PCI via a bridge with water.

The large discrepancy in binding energy for the transition state of Bz-Gly-Phe (Bz = PhCO) and Cbz-Gly-Phe (Cbz = PhCH₂OCO) by the R127 mutant enzymes merits further discussion. These substrates have very similar structures and are hydrolyzed equally well by wild-type CPA. Electrostatic calculations on the structures of Bop and Zgp bound to CPA predict that the transition state of both substrates should be equally stabilized by Arg-127, yet this is not observed (Table II). The structure of Zgp (transition state analog of Cbz-Gly-Phe) bound to wild-type CPA shows that it binds abnormally when compared with Bop (transition state analog of Bz-Gly-Phe) and several other inhibitors (Fig. 1, Ref 5). A likely explanation for the insensitivity of Cbz-Gly-Phe hydrolysis to Arg-127 replacement is the existence of yet another alternate binding mode for this substrate on the mutant enzymes. This is supported by the BzNA inhibition data, which shows that noncompetitive inhibition is observed for hydrolysis of Cbz-Gly-Phe by R127K but not for any other tested substrate. Within this binding mode the oxyanion may be partially stabilized by another active site residue. A change in the rate-limiting step for Cbz-Gly-Phe hydrolysis by the mutant enzymes could also account for these differences.

The structural data on the inhibitor analogs Bop and Zgp suggests an additional possibility. The tetrahedral oxygen (O(2)) of Zgp, the transition state analog of Cbz-Gly-Phe, is closer to Arg-127 (Figs. 1 and 2, distance 2.6 Å) than to Zn²⁺ (Figs. 1 and Fig. 2, distance 3.5 Å), while the tetrahedral oxygen (O(2)) of Bop, the transition state analog of Bz-Gly-Phe, is closer to Zn²⁺ (Figs. 1 and 2, distance 2.5 Å) than Arg-127 (Figs. 1 and 2, distance 3.2 Å). By inference Cbz-Gly-Phe would have the strongest interaction with Arg-127 and Bz-Gly-Phe the weakest, yet hydrolysis of Cbz-Gly-Phe is the least affected by the loss of Arg-127. Thus, one role of Arg-127 may be to prevent the exclusive interaction of the scissile carbonyl oxygen with Zn²⁺. Such an interaction could decrease the acidity of the Zn²⁺ bound water and hinder nucleophilic attack of water on the peptide bond. This function may be more essential for those substrates that prefer the putative Zn²⁺-O(2) binding mode over the Arg-127-O(2) binding mode. Supporting this possibility, studies on model compounds in dicoce that the pKₐ of Zn²⁺ bound water can vary 2–3 pKₐ units depending on the type of Zn²⁺ ligands and the coordination environment. Furthermore, rate enhancement of peptide bond cleavage was dependent on the formation of Zn²⁺-bound hydroxide as the pH rate profiles contained an inflection point which corresponded to titration of Zn²⁺-bound water (32, 33). However, it is also possible that the different binding modes of the two inhibitors do not reflect the actual substrate binding modes; alternatively they may reflect differences between a phosphonamidate (Zgp) and a ketone (Bop).

In summary, the evidence indicates that Arg-127 stabilizes the rate determining transition state by 6 kcal/mol, whereas it plays a more minor role in binding of the ground state structure. Arg-127 apparently functions as an electrophilic catalyst through interaction of the positively charged guanidinium group with the oxyanion. The importance of Arg-127 in CPA catalyzed hydrolysis is further underscored by comparison to the effect observed for mutation of Glu-270; reductions in kₐ for the Glu-270 mutants were of similar magnitude to that observed for the Arg-127 mutants. Structural data on rat CPA and the Arg-127 mutant enzymes will be required to provide further insight into the observations made in these studies.

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