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MODIFICATION OF THE CARBOXYPEPTIDASE A ACTIVE SITE RESIDUE Glu-270 PREVENTS INTERACTION WITH A PROTEIN PROTEASE INHIBITOR FROM *ASCARIS* *

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Summary

The involvement of two active site residues of carboxypeptidase A in binding a protein inhibitor from *Ascaris* was studied. Glu-270 was modified with *N*-ethyl-5-phenylisoxazolium-3′-sulfonate and Tyr-248 was modified with tetranitromethane or diazotized arsanilic acid. Modification of Glu-270 abolished protein inhibitor binding and Glu-270 was protected from modification when the enzyme was bound to the protein inhibitor. In contrast, modification of Tyr-248 did not abolish protein inhibitor binding, nor did such binding protect Tyr-248 from modification. The absorption isosbestic point of arsanilazocarboxypeptidase A (Tyr-248 chemically modified) underwent a blue shift from 428 to 416 nm when the modified enzyme was bound to the protein inhibitor between pH 7.7 and 9.0. The 416 nm isosbestic point is characteristic of the loss of interaction between modified Tyr-248 and the active site zinc ion. These results with a protein inhibitor can be compared to substrate catalysis in which Tyr-248 moves away from the active site zinc ion of carboxypeptidase A when substrate binds. The close association of Glu-270 with *Ascaris* inhibitor interaction is consistent with other results which show that of the active site residues, only the modification of Glu-270 completely abolishes catalysis.

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Introduction

Studies with the protein inhibitors of serine endopeptidases have shown that the region of the inhibitor which comes in contact with the active site of the protease has the properties of a substrate. Peptide bond hydrolysis can occur under the appropriate conditions at that region on the inhibitor [2]. This implies that the functional groups required for catalysis by the endopeptidase are involved in interaction with the inhibitor. A similar mechanism for exopeptidase inhibitors was suggested by the covalent addition of leucyl residues to the α-carboxylate of the carboxypeptidase inhibitor from potatoes, which destroyed inhibitor activity toward carboxypeptidase A. Selective removal of these leucyl residues regenerated inhibitory activity [3]. This implies that the α-carboxylate of the terminal residue is required for inhibitor interaction with carboxypeptidase A just as it is required for substrate binding to the enzyme [4]. Hydrolysis of the carboxyl-terminal residue of the inhibitor catalyzed by the carboxypeptidase would prove this hypothesis. Since this has not yet been accomplished the proof offered is indirect.

Recent studies with a protein isolated from *Ascaris lumbricoides* var. *suum* which inhibits porcine, human and bovine carboxypeptidases [5] reveal that this inhibitor, when bound to enzyme, covers the active site cavity. The observations that support this conclusion are: (a) the zinc ion cannot be removed from the complex between carboxypeptidase A and *Ascaris* protein with 1,10-phenanthroline [6]; (b) the zinc ion cannot reassociate with the reactive site when apocarboxypeptidase A and the *Ascaris* protein form a complex; and (c) the *Ascaris* protein competitively inhibits porcine carboxypeptidase B, an enzyme whose structure and active site region are similar to bovine carboxypeptidase A.

In this work we suggest that the *Ascaris* inhibitor does not merely cover the active site but rather directly interacts with active site residue(s) of the enzyme. If the enzyme active site is merely blocked by the inhibitor one would expect that chemical modification of the catalytic residues of the enzyme would not affect the binding affinity of inhibitor toward enzyme. On the other hand, if the inhibitor when bound to the enzyme comes directly in contact with the active site residues, one would expect the chemical modification of the active site residues to significantly affect the inhibitor binding affinity. Two residues, Glu-270, a suspected nucleophile, and Tyr-248, a suspected proton donor, have been implicated in catalysis by X-ray diffraction studies [7—9]. Interactions between these residues and substrates have been described [10], and separate chemical modifications of these residues have been shown to be specific [11—14]. We report the effects of chemical modifications of these two enzyme residues on binding the inhibitor to the enzyme derivatives. Based on this we suggest the extent to which these residues of carboxypeptidase A which are involved in catalysis are involved in interaction with the *Ascaris* inhibitor.

Experimental procedures

Materials

Ascaris carboxypeptidase inhibitors were prepared as described [5]. Bovine

carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.17.1) (45.5 units per mg) was purchased from Worthington Biochemical Company. Carbobenzoxyglycyl-L-phenylalanine and hippuryl-β-L-phenyllactic acid were obtained from Cyclo Chemical Company, *p*-arsanilic acid from Eastman Organic Chemicals, tetranitromethane and *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K) from Aldrich. Bio-Gels P-4 and P-100 (100—200 mesh) and ninhydrin were from Bio-Rad Laboratories.

Methods

Enzymatic activity of carboxypeptidase A or the derivatives was determined with both peptide and ester substrates. In the exopeptidase assay [5] the rate of L-phenylalanine release from *N*-carbobenzoxyglycyl-L-phenylalanine was measured by the ninhydrin reaction. Esterase activity was determined by a pH-stat assay in which the substrate was hippuryl-β-L-phenyllactic acid (10 mM) dissolved in 5 mM Veronal buffer (pH 7.5) containing 45 mM NaCl [15].

Concentrations of enzyme and its derivatives were based on a molecular weight of 34400 [16] and a molar extinction at 278 nm of $6.42 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [17] except for *p*-azobenzenearsonate-treated enzyme where $7.32 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [18] was used. Inhibitor concentrations were based on an $A^{1\%}_{280nm}$ of 14.6.

Modification of enzyme with Woodward's reagent K has been shown to modify Glu-270 and to inactivate the enzyme [19]. The derivative was prepared according to the published procedure [12] and will be referred to as WRK-enzyme.

Modification of enzyme with tetranitromethane has been shown to specifically nitrate Tyr-248 [20]. This derivative, referred to as nitroenzyme, was prepared by the published procedure [13]. The molar extinction at 428 nm and pH 8 was identical to the value reported for the characterized derivative [21].

Modification of enzyme with *p*-azobenzenearsonate has been shown to be specific for Tyr-248 [22]. This derivative was prepared by the published procedure [18] and will be referred to as the azoenzyme. The molar extinction of the azoenzyme at 485 nm and pH 13 was 104% of the value published [18].

Results

Characterization of carboxypeptidase A derivatives

The activities of the carboxypeptidase A derivatives were similar to the literature values [14,23]. The peptidase or esterase activities relative to native enzyme were respectively: for nitroenzyme, 15 and 150%; for azoenzyme, 48 and 98%; and for WRK-enzyme, insignificant activity toward either substrate. Lineweaver-Burk analysis [24] was performed on data obtained from assays with concentrations of hippuryl-β-L-phenyllactic acid between 0.067 and 1 mM [25]. The K_m value for azoenzyme agreed with the literature value, but the K_m value for nitroenzyme (0.34 mM) was lower than the value reported [23].

Interaction between carboxypeptidase A derivatives and inhibitor studied by titration

Inhibitor was added to the enzyme or derivative and the residual enzymatic

activity toward peptidase or esterase substrate was determined. Enzymatic activity was plotted versus quantity of inhibitor as shown in reference [5] *. Such treatment showed that nitroenzyme bound to inhibitor as tightly as native enzyme. The concentration of free native enzyme or free nitroenzyme was decreased to 15 nM in a fashion directly dependent on the amount of inhibitor. Addition of more inhibitor then decreases the free enzyme concentration only slightly. Azoenzyme did not bind as tightly to inhibitor. The concentration of free azoenzyme could be decreased only to 170 nM.

Interaction between carboxypeptidase A derivatives and inhibitor studied by disc gel electrophoresis

If an enzyme derivative-inhibitor complex withstood disc gel electrophoresis at pH 8.2 [27], the interaction was considered to be strong and within the range of the interaction between native enzyme and inhibitor. Interaction was indicated by the disappearance of free inhibitor from gels corresponding to mixtures where less than saturating levels of inhibitor were added to enzyme or enzyme derivative [5]. The gel patterns showed that nitroenzyme bound inhibitor as tightly as native enzyme. A fraction of the nitroenzyme was cross-linked and smeared on the polyacrylamide gels. This decreased the amount of inhibitor required to saturate the enzyme. However, the observation that the rest of the nitroenzyme-inhibitor complex withstood electrophoresis was indicative of native type binding. The interaction between azoenzyme or WRK-enzyme and inhibitor was too weak for electrophoresis of the complex.

Interaction of WRK-enzyme with inhibitor

WRK-enzyme, in which Glu-270 is modified, is catalytically inactive [12]. The interaction with inhibitor was therefore investigated by comparing its elution from a column of Bio-Gel P-100 with the elution of samples of enzyme, inhibitor, or enzyme-inhibitor complex. When WRK-enzyme and inhibitor were mixed, incubated for 24 h, and applied to the column, two peaks were eluted. They corresponded to peak B, the WRK-enzyme and peak C, the inhibitor (top of Fig. 1). Since the reagent may modify residues other than Glu-270 which could interfere with binding of the inhibitor, additional experiments were necessary. A partially modified enzyme derivative was prepared, in which all available carboxyl groups except Glu-270 were modified. Glu-270 was protected by adding β-phenylpropionate (2 mM final concentration) to native enzyme 1 h prior to addition of reagent K. β-Phenylpropionate, an inhibitor, binds to the enzyme to protect Glu-270 from exposure to this reagent [19]. After the inhibitor and excess reagent were removed, this partially modified derivative retained all of the native esterase activity [19], and therefore, Glu-270 was not modified. This partially modified derivative could still interact

* In Ref. 5 a stoichiometry of two molecules of inhibitor per carboxypeptidase A molecule was suggested because the molecular weight of the *Ascaris* inhibitor was half of that required for unit stoichiometry. It must be emphasized that in the absence of more rigorous evidence, this suggestion was prompted only by the molecular weight determinations. Preliminary sequencing data are consistent with the earlier molecular weight determinations. Since an atypical stoichiometry would make an apparent K_i value meaningless, we have used the Green and Work titration method [26] for comparative, not absolute, measurements. The binding of inhibitor to these derivatives was compared to the binding of inhibitor to native enzyme (by comparing the point where the binding of inhibitor deviates from linearity).

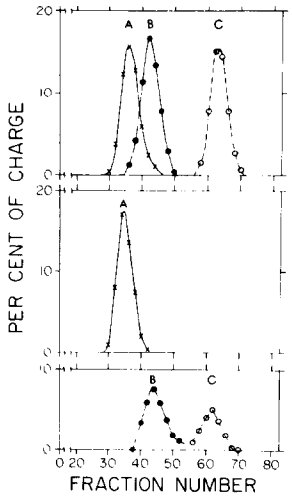


Fig. 1. Interaction of WRK-enzyme and inhibitor. Upper panel (control): shows the eluation of a complex of native enzyme with inhibitor (Peak A, X———X), native enzyme (Peak B, •———•), and inhibitor (Peak C, ○- - - - -○), obtained in separate experiments. 1-ml solutions were chromatographed on a 2.5 × 40 cm column of Bio-Gel P-100, equilibrated and eluted with 50 mM potassium phosphate buffer, pH 6.4, containing 1 M NaCl. Each fraction, 2.0 ml. The ordinate is per cent of the total protein determined at A_{230nm}. Middle panel: enzyme (0.64 mg) modified by Woodward's reagent K in the presence of β-phenylpropionate was exhaustively dialyzed and then reacted with inhibitor (0.28 mg), and chromatographed. Lower panel; enzyme (0.64 mg) modified by Woodward's reagent K in the absence of β-phenylpropionate was reacted with inhibitor (0.28 mg). A precipitate (about 40% of charge) appeared 24 h after mixing WRK-enzyme and inhibitor and was removed prior to chromatography. Inhibitor was assigned by assay. Native enzyme and WRK-enzyme coeluted.

with inhibitor, as shown by mixing the derivative and *Ascaris* inhibitor and chromatographing the solution after 24 h incubation. The resulting elution profile (center panel, Fig. 1) showed that a complex formed. Therefore, this partially modified derivative could be used to determine if specific modification of Glu-270 prevented interaction of enzyme with inhibitor. The partially modified enzyme was again treated with reagent K to modify Glu-270. The resulting completely modified derivative had no esterase activity. When this derivative was mixed with inhibitor, incubated 24 h, and chromatographed, no interaction occurred (lower panel, Fig. 1), confirming that singular modification of Glu-270 prevents enzyme-inhibitor interaction.

Protection of Glu-270 by inhibitor

To test this possibility, reagent K was added to native enzyme, inhibitor, or the native enzyme-inhibitor complex, and the absorbance A^{1cm}_{340nm} was determined after 10 min. A resulting increase in absorbance indicated modification [12]. The absorbance of the complex was less by 0.21 than the sum of the absorbances of reagent K treated enzyme and inhibitor solutions. Although this indicated that bound inhibitor protected otherwise susceptible residues (on either protein or inhibitor), it could not be assumed that Glu-270 was one of these. To determine whether inhibitor could specifically protect Glu-270 from reacting with reagent K, the partially modified enzyme derivative described in

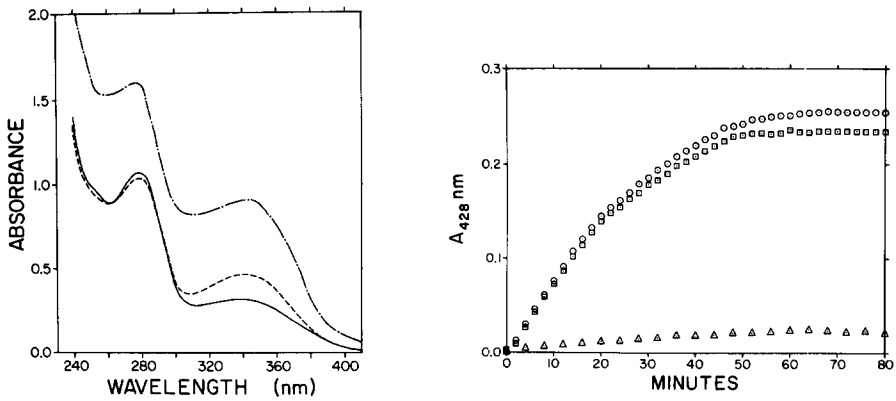


Fig. 2. Protection by inhibitor against modification of Glu-270 by Woodward's reagent K. 0.28 mg inhibitor was allowed to react with reagent K to modify all available groups. Next, 0.64 mg enzyme preincubated with 1 mM β-phenylpropionate (to protect Glu-270) was allowed to react with reagent K. The proteins were dialyzed to remove excess reagent K or β-phenylpropionate. The solutions were then mixed and their spectra recorded (———). (Interaction between modified inhibitor and partially modified enzyme occurred as demonstrated by an elution profile similar to the middle panel (Peak A) in Fig. 1. Reagent K was then added to this partially modified enzyme (Glu-270 protected) and modified inhibitor complex; the mixture was dialyzed and that spectra recorded (- - - - -). A sample of the partially modified enzyme (Glu-270 protected) was then modified with Woodward's reagent K in the absence of inhibitor, dialyzed and its spectra recorded (· — · — ·).

Fig. 3. Nitration of Tyr-248 of carboxypeptidase A in the presence and absence of inhibitor. Tetranitromethane solution (25 μl of an 800-fold dilution) was added to solutions of native enzyme (1.36 mg), or inhibitor (0.60 mg), or a mixture of native enzyme and inhibitor at those concentrations in 1.0 ml of 50 mM Tris buffer, pH 8.0, containing 1.0 M NaCl. The progress of the reaction at 25°C with enzyme (○ ○ ○ ○); inhibitor (△ △ △ △); and enzyme-inhibitor complex (□ □ □ □) was monitored at 428 nm.

the preceding section was used. To be sure that the complex formed between this derivative and inhibitor does not dissociate when treated with reagent K allowing Glu-270 to be modified, inhibitor (0.56 mg/ml) was separately incubated with reagent K and dialyzed [12]. This inhibitor derivative and partially modified enzyme derivative were then mixed, incubated 24 h, and chromatographed. The elution profile was similar to the middle panel of Fig. 1. This showed that these two proteins interacted. After the spectrum of this complex was obtained (Fig. 2), it was treated with reagent K and the reaction mixture was dialyzed. The spectrum of this product is compared to the spectrum of completely modified enzyme in Fig. 2. Although some additional modification of the complex did occur (perhaps due to forced interaction at secondary sites), the magnitude of the absorbance change was much less than that seen in the absence of inhibitor. This suggests that the interaction of inhibitor with enzyme occurs at the active site and makes Glu-270 inaccessible to modification.

Reaction of enzyme-inhibitor complex with tetranitromethane

The reaction of native enzyme, inhibitor and enzyme-inhibitor complex with tetranitromethane is shown in Fig. 3. The free inhibitor as expected from the absence of tyrosine [5] showed only a slight increase in absorbance. Based on the molar extinction of 3-nitrotyrosine [21] at 428 nm and pH 8 the nitro-

enzyme contained 1.0 modified tyrosyl residue. Quantitation of 3-nitrotyrosine by amino acid analysis confirmed that 0.87 residue was modified. The complex, surprisingly, was modified to the same extent as native enzyme. This indicated that in the complex Tyr-248 is still fully accessible to modification. The possibility that modification of inhibitor caused dissociation of the complex and allowed modification of Tyr-248 was considered. However, inhibitor treated with tetranitromethane still inhibited native enzyme.

Spectral perturbation of nitroenzyme by Ascaris inhibitor

The summed absorbance spectra between 520 and 360 nm of tandem solutions in a two compartment reference cell (nitroenzyme in one compartment and inhibitor in the other) was compared to the spectrum of a solution of nitroenzyme-inhibitor complex in the sample cell. This difference spectrum is shown in Fig. 4. Addition of inhibitor decreased the absorbance of the 428 nm band of the nitroenzyme by 14%. This effect is also seen in Fig. 3 where nitration of the enzyme-inhibitor complex showed a smaller absorbance increase than the enzyme. This indicated that the interaction of nitroenzyme with inhibitor changed the chemical environment of the tyrosyl chromophore, as observed when β-phenylpropionate and other small inhibitors and substrates were added [13,14].

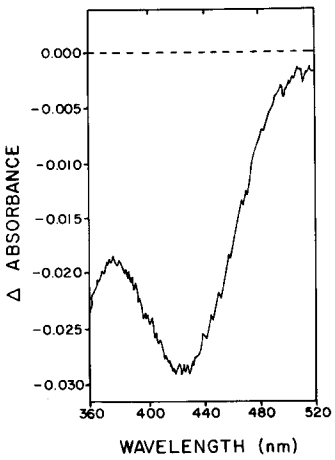
Reaction of enzyme-inhibitor complex with p-azobenzenearsonate

When diazobenzenearsonate reagent was added to solutions containing either native enzyme or native enzyme-inhibitor complex, the chromophore developed. After one hour the reaction mixtures were dialyzed [14] and the molar extinctions at 485 nm and pH 11 were determined. The complex had a value of 11 000 $M^{-1} \cdot cm^{-1}$, nearly identical to the experimental value for the modified free enzyme, 10 900 $M^{-1} \cdot cm^{-1}$, and to the literature value of 10 500 $M^{-1} \cdot cm^{-1}$ [18]. These data support the contention already suggested by the nitration experiments that the interaction between native enzyme and inhibitor leaves Tyr-248 accessible to the media.

Spectral perturbation of azoenzyme by Ascaris inhibitor

The azo-Tyr-248 chromophore of carboxypeptidase A is a better characterized active site probe [14,18,21,22,28,29] than nitrotyrosyl-248. In solution, azo-Tyr-248 participates in an intramolecular coordination complex with the active site zinc. This is responsible for the 510 nm absorption band [14]. When zinc is removed or this complex is disrupted, the 510 nm band is abolished and a band at 485 nm appears in the appropriate pH range (characteristic of the degree of ionization of azo-Tyr-248). The spectra of solutions of azoenzyme and of azoenzyme-inhibitor complex obtained between pH 7.7 and 9.0, showed that the 510 nm and 485 nm absorbances of the azoenzyme-inhibitor complex were always lower than those of azoenzyme (Fig. 5). The 510 nm absorption band disappears from these spectra of the azoenzyme-inhibitor complex while a band at 485 nm becomes more prominent. In apoazocarboxypeptidase A, devoid of the zinc-azo-Tyr-248 interaction, the 510 nm band is also absent and a 485 nm band becomes prominent at pH 8.5 [18].

The solid lines in Fig. 5 trace out an isosbestic point of 428 nm for free azo-



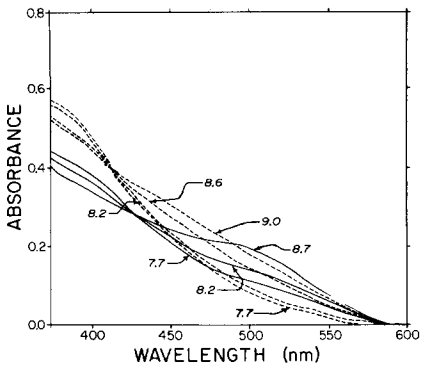


Fig. 4. Spectral perturbation at pH 8.0 of nitroenzyme by inhibitor. One compartment of the tandem sample cell contained a mixture (1.0 ml) of nitroenzyme (1.85 mg, A_{428nm} = 0.216 at pH 8.0) and 0.81 mg inhibitor; the other compartment contained buffer, 50 mM Tris/1.0 M NaCl, pH 8.0. The tandem reference cell contained the same concentrations of the two proteins, in separate compartments. The difference spectra are redrawn from data obtained on a Cary model 1501 spectrophotometer.

Fig. 5. Absorbance isosbestic points of azoenzyme and azoenzyme-inhibitor complex. 1.0 ml solutions of 1.14 mg azoenzyme and of 1.14 mg azoenzyme and 0.50 mg inhibitor were prepared in 5 mM Tris buffer containing 1 M NaCl at different pH values. The spectra for azoenzyme (———) and for azoenzyme-inhibitor complex (- - - - - -) are identified by pH. The isosbestic point for azoenzyme is 428 nm; for the complex 416 nm.

enzyme between pH 7.7 and 8.7. This value is identical to the literature value [18]. Over the pH range 7.7—9.0, the spectra for the azoenzyme-inhibitor complex shows an isosbestic point shifted to 416 nm, which again is identical to the value for apoazocarboxypeptidase A [18]. These data suggest that in the azoenzyme-inhibitor complex the environment of azo-Tyr-248 has changed as ionization of the chromophore has decreased and that azo-Tyr-248 no longer interacts with the active site zinc ion.

Discussion

Two of the residues in carboxypeptidase A, Glu-270 and Tyr-248, are associated with substrate binding and catalysis. When Glu-270 is modified, substrate activity is completely abolished. Modification of Tyr-248 does not destroy catalytic activity but alters binding and kinetic parameters of substrates. Derivatives of carboxypeptidase A in which these residues were modified were used to study binding to *Ascaris* inhibitor.

Treatment of carboxypeptidase A with either Woodward's reagent K or 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide methyl-*p*-toluenesulfonate modifies carboxyl groups including Glu-270 [11]. These derivatives are inactive against substrates [11] and failed to bind *Ascaris* inhibitor. It may be argued that modification with Woodward's reagent K physically prevented interaction with *Ascaris* inhibitor by sterically blocking the binding site of the enzyme.

However, the modifying group is not large enough to extend from the binding cavity to beyond the surface of the enzyme to prevent interaction with inhibitor. From this it may be inferred that the inhibitor must penetrate into the binding cavity of the native enzyme, to where a portion of its surface is physically near Glu-270. In contrast, it has been suggested that the inhibitor from potatoes does not penetrate into the binding cavity of carboxypeptidase A but does bind in the active site region [30].

Studies to determine what role Tyr-248 in the active site of carboxypeptidase A plays in binding the enzyme to the inhibitor were performed with two derivatives of Tyr-248. Nitroenzyme bound to inhibitor as tightly as did native enzyme but azoenzyme did not. This reduced affinity of azoenzyme for inhibitor is caused either by the bulkiness of the azophenol group or by the ionization of Tyr-248 which allows the azophenolate to act as a strong competitive inhibitor preventing binding of some substrates [28] and, in this experiment, *Ascaris* inhibitor.

In azoenzyme, azo-Tyr-248 is near enough to the active site zinc to form an intramolecular coordination complex with a prominent absorption band at 510 nm [18]. This prominent band is abolished by removal of zinc, denaturation or crystallization of the enzyme, or addition of substrate or inhibitor [14,18]. When zinc is removed from azoenzyme not only is the prominent absorption band at 510 nm diminished but a new band is formed at 485 nm and the absorption isosbestic point shifts from 428 to 416 nm. The addition of *Ascaris* inhibitor to azoenzyme decreased the 510 nm absorbance band just as β-phenylpropionate and glycyl-L-tyrosine do [14,18]. In the complex with *Ascaris* inhibitor a new band is formed at 485 nm. The isosbestic point of the complex between *Ascaris* inhibitor and azoenzyme is at 416 nm and not at 428 nm as in azoenzyme or when azoenzyme is bound to β-phenylpropionate or glycyl-L-tyrosine. The complex between carboxypeptidase A and *Ascaris* inhibitor reacts as easily and to exactly the same extent with tetranitromethane and *p*-azobenzenearsonate as does free enzyme. β-Phenylpropionate and glycyl-*L*-tyrosine protect the enzyme from these two modifications. The spectral data show that the chemical environment of Tyr-248 (in either nitroenzyme or azoenzyme) is changed by complex formation with *Ascaris* inhibitor. The protection experiments suggest that in the enzyme-inhibitor complex Tyr-248 is displaced into the media by the inhibitor and the isosbestic point shift argues that it cannot interact with the zinc ion.

Although it may seem strange that a suggested displacement of a tyrosyl residue into the aqueous media would decrease its ionization-dependent absorbance, it should be noted that Tyr-248 is thought to have an abnormally low pK value. From the 485 nm absorbance of the azoenzyme-inhibitor complex, an apparent pK of 8.9 was determined for the interaction. This value is higher than the value of 7.7 for the formation of the coordination complex of Tyr-248 with zinc and lower than the value of 9.5 for dissociation of the complex [29,31]. Since zinc is not interacting with Tyr-248 in the complex of inhibitor and azoenzyme, the apparent pK of 8.9 is based on a simple, single proton dissociation. In free modified enzyme, the pK is due to the interconversion of multiple forms including azophenol, azophenolate and the coordination complex with zinc. Therefore, the apparent pK of 8.9 for the

complex should not be compared to the apparent pK for these multiple forms. Neither should a comparison be made to the apparent pK of 9.4 of apoazocarboxypeptidase A [18], since azo-Tyr-248 in this species can still interact with Arg-145 or other residues in the active site. The apparent pK reported here should be compared only to the value of 8.8 for monoazotyrosine [32] or the value of 9.0 for mono-tetrazolyl-*N*-carbobenzoxytyrosine [31] or arsanilazo-*N*-acetyltyrosineamide [18] as model compounds in aqueous solution. This comparison suggests that azo-Tyr-248 in a complex of azoenzyme with *Ascaris* inhibitor is fully exposed to the media. All the data are consistent with this proposal.

It should not be surprising that although the *Ascaris* inhibitor perturbs Tyr-248, this residue is unimportant for binding inhibitor. As mentioned, Tyr-248 can be modified without loss of activity toward substrate. Although the interaction of substrate or *Ascaris* inhibitor with enzyme may involve some interaction with Tyr-248, this residue is dispensible in these interactions. The very tight association and slow dissociation of the *Ascaris* inhibitor may make the involvement of certain residues (e.g., Tyr-248) less important for interaction than these same residues may be for weaker-binding, faster-dissociating substrates.

In summary, our data have demonstrated that *Ascaris* inhibitor binds at the active site of carboxypeptidase A near Glu-270. Further, this binding of inhibitor may cause Tyr-248, a suspected proton donor, to move away from interaction at the active site into the aqueous environment.

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