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RELATIONSHIP OF THE THREE DIMENSIONAL STRUCTURE OF CARBOXYPEPTIDASE A TO CATALYSIS

POSSIBLE INTERMEDIATES AND pH EFFECTS

WILLIAM N. LIPSCOMB

Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138, U.S.A.

Abstract—A brief discussion is presented of activity, conformational changes, possible mechanisms, intermediates, and pH effects in the peptidase activity of the hydrolytic enzyme, carboxypeptidase A.

Activities in solution and in crystals

Enzymes, and many other proteins, bind certain smaller molecules so well and with such specificity that at least in the binding region they have very well defined 3-dimensional structures. Recent X-ray diffraction studies have elucidated many of these protein structures to atomic resolution. These studies have also shown that protein crystals, unlike most crystals of small molecules, have an average water content[1] of about 43% by volume (range, 27 to 65%), leaving most of the surface of the protein, perhaps 70%, in contact with solvent. Usually, although not always, the binding site for ligand molecules is not blocked by intermolecular contacts. In many instances, the enzymes which were studied show comparable enzymatic activity in the crystals and in solution. It therefore seems reasonable to employ 3-dimensional X-ray diffraction studies for interpreting the biochemical behavior in solution and in physiological situations. Even when intermolecular contacts restrict access of long substrates in the crystal structure, the correlation of 3-dimensional structure with biochemical function has been strikingly successful.[2]

Even so, no general statement can be made about comparisons of behavior of enzymes and other proteins in crystals and in solutions. Most examples, so far, consist of single polypeptide chains.[3,4] Activities of proteins in which allosteric changes can be induced usually produce such large structural changes that separate crystal structures are required, before and after such changes, in order to elucidate the structural basis of the mechanisms of catalysis and control.

However, the enymatic activity of carboxypeptidase A_α toward a small substrate, such as carbobenzoxyglycylphenylalanine, is less than that in solution by only a factor of three[5] in the crystals on which the X-ray diffraction studies[6,7] were carried out. It is therefore expected that the binding and catalytic groups as deduced from these X-ray diffraction studies to 2 Å of carboxypeptidase A, of its complex with glycyl-L-tyrosine, and of a number of associated studies at lower resolution, are highly relevant to both crystalline and solution states and most likely to physiological conditions.

Catalytic groups and conformational changes

Carboxypeptidase A hydrolyzes the C-terminal amino acid from a polypeptide substrate. This hydrolysis is most rapid when R_1' (Fig 1) is a neutral aromatic, or large aliphatic, side chain. Effects of various substituents, R_1', R_1, R_2, ... etc., indicate[8] that five (R_1' to R_4) side chains of the substrate influence binding and catalysis, the first three more than the last two.

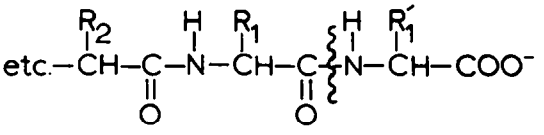

Fig 1. Substrate for carboxypeptidase A, showing the point of cleavage as a wavy line.

The polypeptide chain (Fig 2) of the enzyme has 17% of β-sheet structure in which approximately half of the adjacent strands are parallel and half antiparallel, and about 38% of the helix, nearly all distorted. Aside from the water-like structure present in the active site cavity before substrate enters, there are ten individual water molecules inside the enzyme itself, apparently involved in the protein structure.[9]

This large active site cavity is shown in Fig 3a, where all side chains of this quarter of the protein structure are included. Structural rearrangements which occur upon binding of Gly–Tyr (Fig 3b), and other substrates, appear to enfold the C-terminal part of the substrate, and are most probably induced by the presence of the substrate itself. Carboxypeptidase A is thus the first striking example of the "induced fit" hypothesis recently most clearly formulated by Koshland.[10] A major consequence of the binding of substrate, and the associated motions of side chains of the protein (Fig

Fig 2. Polypeptide model of the amino acid backbone of carboxypeptidase A_α (307 residues). The N-terminus is some 25 Å from Zn, and the only two cysteinyl side chains are linked as a disulphide bond which is about 20 Å from Zn. Ligands to Zn, shown near the center of the diagram, are His 69, Glu 72 and His 196. This complex is referred to as $[ZnL_3]^+$ in this paper.

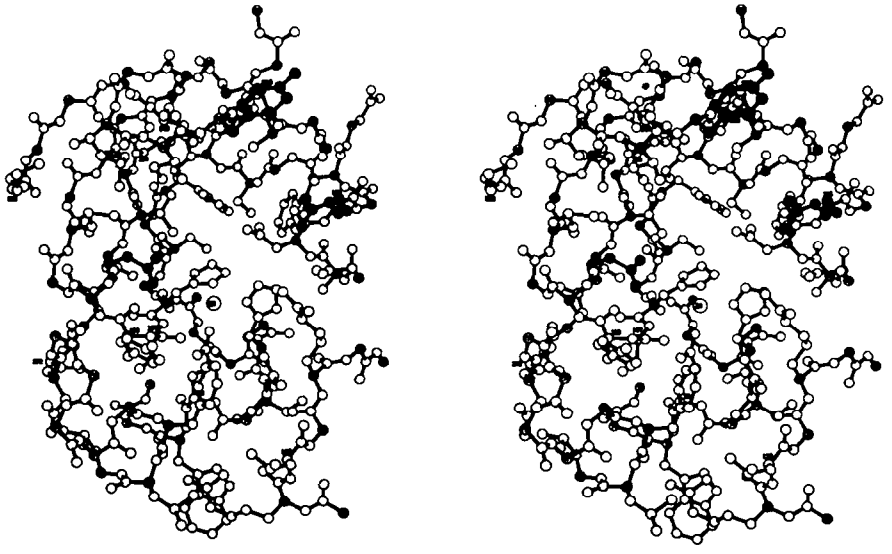

Fig 3a. Stereoview of about one-quarter of the carboxypeptidase A molecule. The Zn, labelled near the center, is bound to His 69, Glu 72, His 196 and at low pH to a water molecule (not shown). Solid circles indicate Arg 145 (right), Tyr 248 (above) and Glu 270 (left). The enzyme structure continues toward the left, the bottom of the diagram, and away from the reader. At the right, the top of the diagram, and toward the reader the isolated enzyme molecule is in contact with solution.

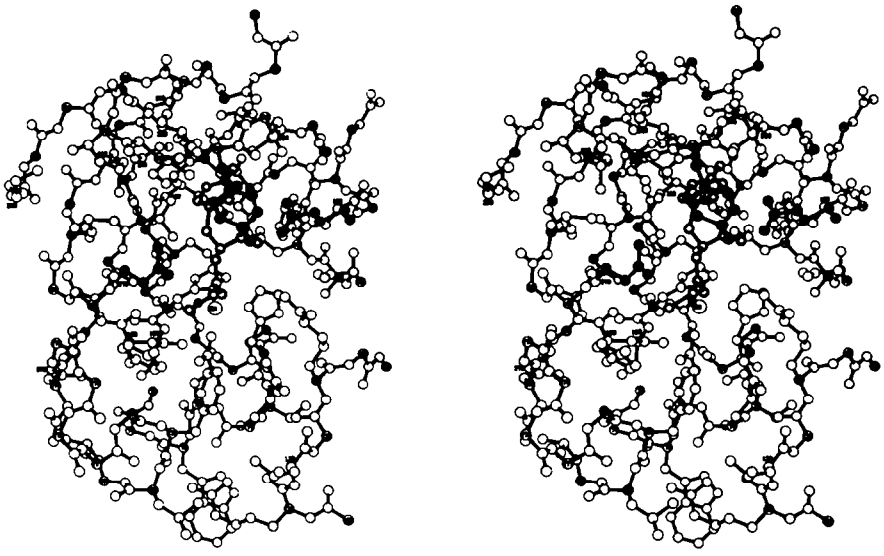

Fig 3b. Binding of glycyl-L-tyrosine to carboxypeptidase A, showing conformational changes, especially of Tyr 248, Arg 145 and Glu 270. Besides hydrophobic interactions in the pocket and elsewhere, specific binding occurs so that (a) the carbonyl group of the substrate binds to Zn, displacing a water molecule, (b) two hydrogen bridges occur between Arg 145 and the substrate's C-terminal carboxylate ion, (c) a hydrogen probably exists between Tyr 248 and the substrate's NH group, and (d) in an interaction possibly special to free NH_2 (or NH_3^+) groups of a dipeptide substrate, an NH_2. . .OH_2. . .Glu 270 hydrogen bond system is present. Largest movements are 2 Å for end atoms of Arg 145 and Glu 270, and 12 Å for the OH of Tyr 248. An earlier stage of binding might possibly involve a hydrogen bond, N—H. . .O, between the NH of the scissile peptide bond and the OH of Tyr 248.

3) is the exclusion of most of the water molecules from the active site cavity, which is thereby transformed to a hydrophobic-like region. More detailed rate studies may confirm that these structural changes, particularly of Tyr 248, are initiated or triggered by the formation of the salt link between the guanidinium group of Arg 145 and the carboxylate group of the substrate.[6,11] Other conformational changes also occur, most notably the movement by 1 to 2 Å of the polypeptide chain in the region of 247–249, and nearby. In fact, this region of the carboxypeptidase A molecule seems flexible, while that part associated with the β-sheet structure seems comparatively rigid, in our structural models.[11]

The X-ray diffraction studies show that the only side chains of the enzyme which can approach within 3 Å of the peptide bond to be cleaved are[6,7] Glu 270 and Tyr 248. Mechanisms discussed below therefore involve only these two groups, and water. One specific group not far away is Zn, which is believed to produce a kind of electronic strain (covalency, polarization) rendering the CO group of the scissile bond more polar, and therefore the C atom of this group more susceptible to nuclophilic attack. This binding, along with H— bonds involving Tyr 248, and the salt link of the carboxylate terminus to Arg 145, together with the more general hydrophobic and steric interactions, appear to twist the scissile peptide bond, and to make its CO carbon and its imino nitrogen non-coplanar. The reader is cautioned that details of these conclusions come primarily from an examination of the model, not from a precise location of atoms in the X-ray diffraction study, which yields positions only to about 0·4 Å.

Aside from the atomic proximities outlined above, some of the well known chemical properties of carboxypeptidase A play a role in the interpretations below. The pH rate profile for peptide hydrolysis rises with an inflection at about an apparent pK_a of 6·7, peaks at about pH 7·5, and falls with an inflection having an apparent pK_a of about 8·7. In this range, Glu 270 is expected to be anionic, and Tyr 248 is expected to retain its proton in the binding stage. Hence, the nucleophile is taken as either Glu 270 or oxygen of H_2O, and the proton donor taken as Tyr 248 (or H_2O) for peptide substrates. Another feature included below is a binding function, deduced from model building, for the NH of the penultimate peptide bond or the NH_2 terminus of a dipeptide, in view of the large reduction in rate when this NH is substituted.[12,13]

Comments on possible tetrahedral intermediates

Repeated attempts to observe an intermediate in the hydrolysis of substrate by carboxypeptidase A have failed so far.[14] Some esters[15,16] of L-β-

phenyllacate show deuterium isotope effects of about 2, but peptide substrates usually have effects close to unity.[14]. Thus, peptide hydrolysis has a rate determining step less dependent upon proton transfer than does hydrolysis of these esters of L-β-phenyllactate. However, peptides and some esters[17] are not hydrolyzed when the enzyme (Tyr 248) is acetylated, although most esters are.[18] Hydrolysis of various esters by carboxypeptidase A shows other anomalies such as the pK_a of 7·9 for the high pK_a of k_{cat} for acetylmandelate.[19] Hence we comment below only on probable modes of peptide hydrolyses. Transfer reactions involving substrates and fragments of substrates have also failed so far. However, carboxypeptidase B has been used to replace Arg by Lys in the trypsin inhibitor from soybean.[20]

Any proposal for intermediates must therefore provide for their facile reaction to products. This, facilitation is attributed to Zn in the proposed complexes (Fig 4). Also, because the $(ZnL_3)^+$ complex is covered by the substrate, intermediates have been chosen here in order to render the complex neutral in 4(a–c), but not in the more open structure of 4(d).

The anhydride intermediate (Fig 4a) for the acylenzyme is that proposed when the first evidence was obtained for probable involvement of a carboxylate group.[6] Attack of H_2O, promoted by

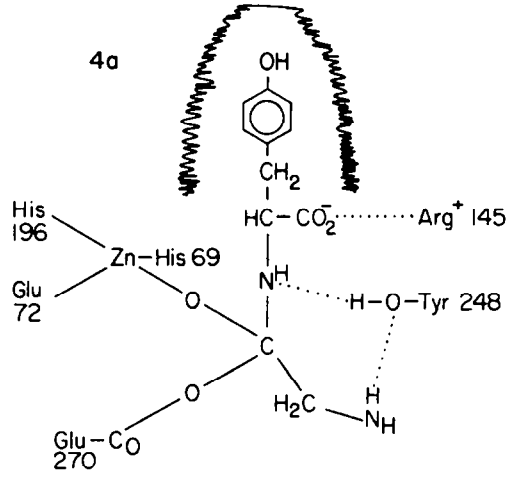

Fig 4a. Proposed neutral non-observed tetrahedral complex. Originally the negative charge was on Glu 270, which becomes even more buried as the substrate approaches. This charge has now been transferred to the $[ZnL_3]^+$ complex, where one positive charge of Zn has previously been neutralized by Glu 72. Thus, when all covalent bonds are formed as shown all atoms are neutral, except for atoms in the doubly hydrogen bonded salt link between Arg 145 and the substrate's terminal carboxylate group. Bonds to Zn^{+2} are partly ionic, more so toward O than toward N. In an alternative tetrahedral complex Glu 270 promotes the attack of O of H_2O on the carbonyl C atom of the scissile peptide bond.

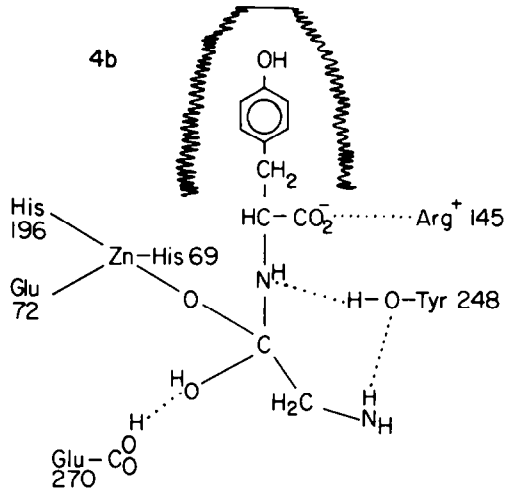

Fig 4b. Alternative neutral tetrahedral intermediate in which Glu 270 anion promotes the attack of O of H_2O at the carbon atom of the substrate's scissile bond.

Glu 270, shown in Fig 4(b), is an alternative that cannot be excluded on the basis of present evidence. Hydrolysis of the acylenzyme of Fig 4(a) either by attack of O of H_2O (with proton loss) at $O^{(1)}$ or $O^{(2)}$ is suggested in Fig 4(c), while incipient attack of O from H_2O bound to Zn on the closer CO group of the acylenzyme is illustrated in Fig 4(d). Steps 4(a) and 4(b) are alternatives, Step 4(d) could follow 4(a), and Step 4(c) could follow 4(a) or 4(d).

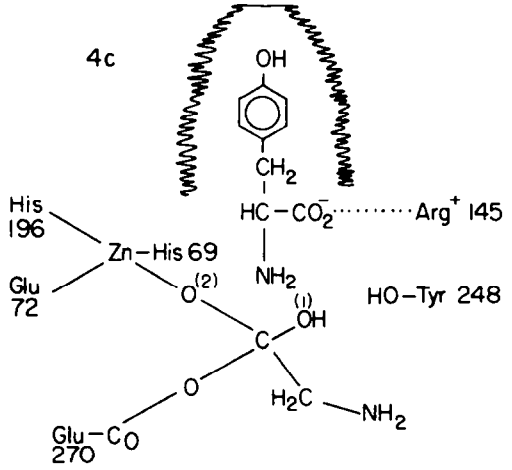

Fig 4c. Proposed neutral tetrahedral complex in the deacylation of the non-observed intermediate. Following cleavage of the scissile peptide bond, attack of O of H_2O could occur at the carbon of the incipient carboxylate group either directly at position (1) or promoted by first bonding to Zn at position (2). The alternative in which attack occurs at the carbon of the carboxylate group of Glu 270 is also possible, and would incorporate labeled oxygen into the enzyme in a tracer experiment not yet performed.

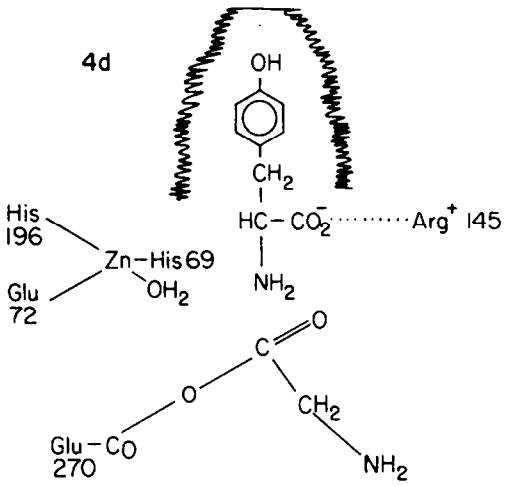

Fig 4d. Non-neutral complex after dissociation of the acylenzyme (anhydride) from Zn, followed by attachment of H_2O to Zn and then proton loss to achieve near local neutrality. If the $H_2O[ZnL_3]^+$ complex has lost a proton, as supposed for carbonic anhydrase, the carbonyl carbon of the glycine part of the substrate could be attacked by a lone pair of the ZnOH or by a H_2O molecule intervening between the ZnOH and this carbonyl group. However, considerations (in the text) of pK_a values suggest that the more likely Zn complex is $H_2O[ZnL_3]^+$, which could deacylate the anhydride directly or through an intervening water molecule.

The alternative of hydrolysis of the anhydride at the CO of Glu 270 is conceivable, but this CO cannot be linked to the Zn directly, and hence would have to be attacked by the O of a $H_2O(ZnL_3)^+$ complex as an alternative to Fig 4(d).

Effects of pH

The great difficulties associated with assignment of pH effects to specific ionizations in an enzyme system arise because of influence of environment, conformational changes, and different binding modes of ligand (i.e. substrate, product, inhibitor analogue, or effector). Although I attempt here to discuss pH effects in a limited way,[11] most of these speculative comments are derived from model-building in the active center of carboxypeptidase A. Intended only as a guide for future experiments, these comments do not have the experimental status of difference electron densities. They may illustrate the usefulness of the 3-dimensional structure of the enzyme and its complex with glycyl-L-tyrosine in one of the more difficult areas of interpretation. Ideally, one would like a 3-dimensional structure at each pH, in the presence and absence of the ligand. Perhaps this discussion will stimulate such studies, but at present the structures are available only under the single set of conditions, pH 7·5, 0·02 M Tris Cl, 0·2 M LiCl.

Elements of rigidity and flexibility of different parts of the enzyme are derived from the 3-dimensional structures at 2·0 Å resolution of carboxypeptidase A and its complex with glycyl-L-tyrosine, and from a number of other studies at lower resolution of other ligands bound to the enzyme. The 2 Å movements of the side chains of Glu 270 and of Arg 145, and the 12 Å movement of the OH of Tyr 248 have been described above in connection with binding and catalytic steps.

In addition, the 3-dimensional electron density map provides support for the further movement of the OH of Tyr 248 by a total of 17 Å, so that in the absence of substrate, there is some 15–25% of this form present under the conditions of the crystallographic study.[11] In this form an O^-—Zn covalent bond is formed in the internal complex (Tyr 248) $O^-[ZnL_3]^+$ in the pH range from $pK_1 = 7·8$ to $pK_2 = 9·5$ in the arsanilazo Tyr 248 derivative of carboxypeptidase A.[5,21,22] The formation of this internal complex is characterized by a pK_1 value of 7·5 in carboxypeptidase B.[23] It seems probable that the formation of this O^-—Zn bond displaces H_2O from $(ZnL_3)^+$, and that this O^-—Zn bond is cleaved by OH^-. If so, the ionization of $H_2O[ZnL_3]^+$ to $HO^-(ZnL_3)^+$ most probably has a pK_a in the range of 7·5 to 9·5, suitably higher than the probable pK_a of 7·0 assigned tentatively to the ionization of the $H_2O[ZnL_3]^{++}$ portion of carbonic anhydrase,[24] where all three ligands L are His. Because little change occurs until about pH 9, one might guess the pK_a of the $H_2O[ZnL_3]^+$ complex in carboxypeptidase A to be on the high side of the range from 7·5 to 9·5. Probably then, this ionization, and that of Tyr 248 having a pK_a of 9·5 in the apo arsanilazo Tyr 248 enzyme,[22] are to be associated with the inflection in the descending limb of the pH-rate profile at about 8·7 for most peptide substrates. A pK_a of 9·3 has been attributed[25] to ionization of the H_2OMn complex in the hydrolysis of O-(transcinnamoyl)-L-β-phenyllactate in Mn-carboxypeptidase A, in which Tyr 248 plays certainly no more than an equal role in the mechanism. This value is in agreement with a less quantitative conclusion from NMR dispersion studies[26] on Mn-carboxypeptidase A. Furthermore the pK_2 of 9·2 found[25] for hydrolysis of this substrate by carboxypeptidase A itself is probably an average of the pK_a of about 9·5 for Tyr 248 and the pK_a for $H_2O[ZnL_3]^+$ which would then be roughly 8·9.

On the acidic side, the lower limb of the pH rate profile having a pK_a of about 6·7 for most peptide substrates is most likely to be associated with Glu 270. Although perhaps about two pK_a units higher than "normal," Glu 270 is rather well buried by glycyl-L-tyrosine, and probably by most substrates. For comparison, Glu 35 has a pK_a of 6·1 in lysozyme and 6·6 in the lysozyme-substrate complex.[27] Inactivations of Glu 270 of carboxypeptidase A by the reagents N-ethyl-5-phenylisoxazolium-3'-sulfonate[28,29] and N-bromoacetyl-N-methyl-L-

phenylalanine[30] show a pH dependence having a pK of about 7. Inasmuch as Glu 72, His 69 and His 196 are ligands to Zn, and therefore expected not to dissociate above about pH 5, Glu 270 is probably the best candidate for this pK_a and therefore for the pK_a of about 6·7 associated with enzymatic activity. Special situations such as hydrogen bonding of Glu 270 to a water molecule hydrogen bonded in turn to the N-terminus of glycyl-L-tyrosine, or of hydrogen bonding of Glu 270 in the free enzyme to a water molecule attached to $[ZnL_3]^+$ may produce somewhat different pK_a values. In particular, a pK_a lower than 6·7 would be expected for the ionization of (Glu 270)$CO_2H \cdots H_2O[ZnL_3]^+$ to (Glu 270)$CO_2^- \cdots H_2O[ZnL_3]^+$. Indeed, (Glu 270)$CO_2H$ may or may not be hydrogen bonded to $H_2O[ZnL_3]^+$, but the X-ray study suggests that this hydrogen bond probably does exist above pH 6·5, i.e. after proton loss from Glu 270. Certainly this H—bond is broken by addition of glycyl-L-tyrosine, or by formation of the (Try 248)O^-—$[ZnL_3]^+$ bond. It is not possible, however, for Glu 270 to form a covalent bond to Zn in view of the rigidity of the pleated sheet structure, and of the limited 0·5 Å motion of the $[ZnL_3]^+$ complex noted when substrates bind.

Modification of Glu 270 by a fairly small group would probably prevent formation of its H— bond to $H_2O[ZnL_3]^+$, and would also interfere sterically with formation of the (Tyr 248)$O^-[ZnL_3]^+$ bond. If the modification is not too bulky, the $H_2O[ZnL_3]^+$ complex would then remain, and its ionization to $HO^-[ZnL_3]^+$ could be studied upon Cl^- displacement by NMR methods currently in use.[31] Also, modification of Glu 270 to Gln 270 would be of very great interest for both steric and mechanistic reasons, if it could be done.

The presence of a substrate longer than a dipeptide having a free amino group provides no additional specific interactions in the region described below, except possibly an additional H— bond from the NH of the penultimate peptide bond to a lone pair on the OH of Tyr 248. However, the amino terminus of Gly–Tyr is believed to be H— bonded to a water molecule which is in turn H— bonded to Glu 270. The level of protonation is assigned below on the probable basis that the anionic form[32] of Gly–Tyr is bound to carboxypeptidase A.

Expanding somewhat a preliminary discussion[11] of levels of protonation, designated as a, b, c . . . , I suggest the following models.

1. Absence of substrate or product
(a1). (Tyr 248)OH, (Glu 270)$CO_2H \cdot ? \cdot H_2O[ZnL_2]^+$, HL^+(His)
(b1). (Tyr 248)OH, (Glu 270)$CO_2H \cdot ? \cdot H_2O[ZnL_3]^+$
{(b2). (Tyr 248)OH, (Glu 270)$CO_2^- \cdots H_2O[ZnL_2]^+$, HL^+(His)}
(c1). (Tyr 248)OH, (Glu 270)$CO_2^- \cdots H_2O[ZnL_3]^+$
{(c2). (Tyr 248)OH, (Glu 270)$CO_2H \cdots {}^-HO[ZnL_3]^+$}
(c3). (Tyr 248)OH, (Glu 270)CO_2^-(modified), $H_2O[ZnL_3]^+$
(d1). (Tyr 248)OH, (Glu 270)$CO_2^- \cdots HO^-[ZnL_3]^+$
(d2). (Glu 270)CO_2^-, (Tyr 248)$O^-[ZnL_3)^+$
(d3). (Tyr 248)OH, (Glu 270)CO_2^-(modified), $HO^-[ZnL_3]^+$
(e1). (Tyr 248)O^-, (Glu 270)$CO_2^- \cdots HO^-[ZnL_3]^+$

2. Substrate present: (Substrate)$CO_2^- \cdots$ Arg 145.
(b3). (Tyr 248)OH, (Glu 270)CO_2H, (Substrate)$CO[ZnL_3]^+$
(c4). (Tyr 248)OH, (Glu 270)CO_2^-, (Substrate)$CO[ZnL_3]^+$
(d4). (Tyr 248)O^-, (Glu 270)CO_2^-, (Substrate)$CO[ZnL_3]^+$
(b'4). (Tyr 248)OH, (Glu 270)$CO_2^- \cdots H_2O \cdots {}^+H_3N$(Gly–Tyr)$CO[ZnL_3]^+$
(c5). (Tyr 248)OH, (Glu 270)$CO_2^- \cdots H_2O \cdots H_2N$(Gly–Tyr)$CO[ZnL_3^+]$

3. Product present: (Product)$CO_2^- \cdots$ Arg 145. See Fig. 5
(b'5). (Tyr 248)OH, (Glu 270)CO_2^-, (L-Phe)$NH_2^+ \cdots H_2O[ZnL_3]^+$
(c6). (Tyr 248)OH, (Glu 270)CO_2^-, (L-Phe)$H_2N[ZnL_3]^+$
{(c7). (Tyr 248)OH, (Glu 270)CO_2^-, (L-Phe)$NH_3^+ \cdots HO^-[ZnL_3]^+$}

The question mark refers to whether or not the H— bond, indicated by a dotted line, exists between (Glu 270)OH and the H_2O bound to Zn. Substrate or product brings additional ionizable groups to the region. Assuming normal binding for peptides the substrate or product CO_2^- is doubly H— bonded to the guanidinium group of Arg 145. Other levels of protonation, involving (Glu 270)CO_2H or free (Tyr 248)O^- are not explicitly given for the substrate Gly–Tyr or the product L–Phe, but are to be understood. Less likely states are enclosed in braces.

Probable values for those pK_a's not implied by pH profiles, or not directly measured, are even harder to guess than are the levels of protonation themselves. Some comments on specific ionizations are as follows:

a1←b1 $pK_a < 5$; probably associated with Zn^{+2} loss.

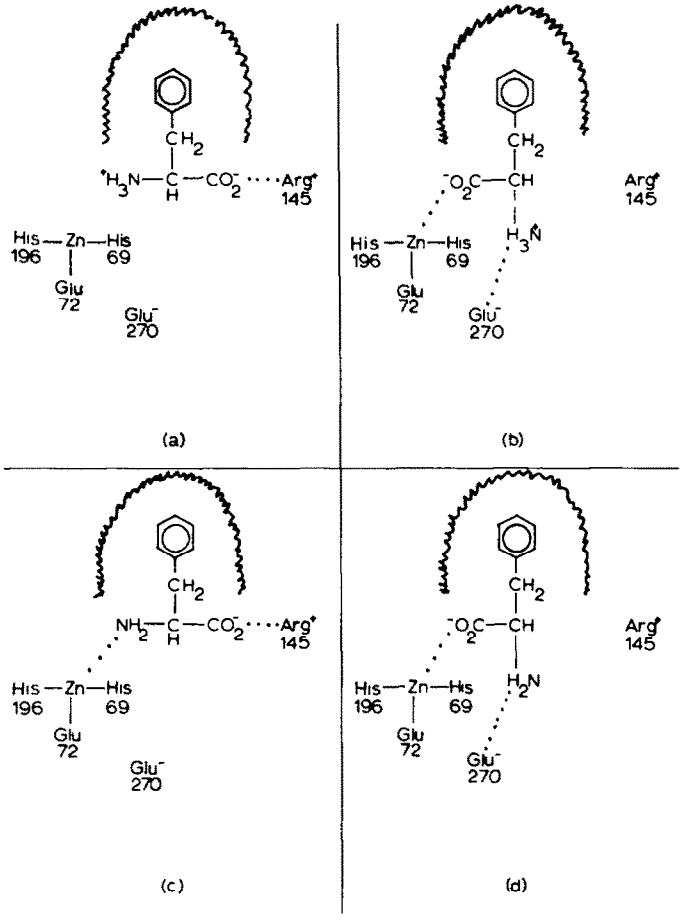

Fig 5. Probable binding of (a) L-Phe at pH 7·5, (b) D-Phe at pH 7·5, (c) L-Phe at pH 9·0 and (d) D-Phe at pH 9·0. Inhibition (a) is one-eighth that of (b), and is dependent on phosphate buffer concentration. At pH 9·0, L-Phe is about as effective an inhibitor as is D-Phe. Binding modes (a) and (b) are supported by the observation[25] of a conformational change of Tyr 248 at 6 Å resolution (and pH 7·5); however, this resolution is insufficient to prove the detailed mode of binding suggested here. The binding modes (c) and (d) are from model building.

b1→c1 $pK_a \sim 5{\cdot}0$ to 6·5; should be lower than 6·7 because the (Glu 270)CO_2^- state is favored by the positive charge.

b3→c4 $pK_a \sim 6{\cdot}7$, the lower limb of peptide pH profile.

b'4→c5 $pK_a \sim 7$, lower than the "free" amino value of 8·2 for dipeptides because of other positive charges in the region.

b'5→c6 $pK_a \sim 7$–8; the pK_2 of 9·2 for Phe is probably shifted downward if Zn binds covalently to the NH_2 group.

c1→d2 $pK_a = 7{\cdot}8$, measured on arsanilazo Tyr 248 enzyme.

c3→d3 $pK_a \sim 8$–9·5, the expected range for H_2OZn ionization in the absence of interactions with Glu or Tyr 248; probably near 8·9.

d1→e1 $pK_a = 9{\cdot}5$, measured on arsanilazo Tyr 248 enzyme, and on apo arsanilazo Tyr 248 enzyme.

In summary, these proposals represent an attempt to combine the 3-dimensional crystallographic results with known chemical results in a way which may lead to further understanding of the enzymatic behavior of carboxypeptidase A.

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