Proc. Natl. Acad. Sci. USA
Vol. 77, No. 7, pp. 3875–3878, July 1980
Biochemistry

Carboxypeptidase A mechanisms

(enzyme activity/x-ray crystallography of an enzyme/protein structure/solid-state activity of enzymes)

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ABSTRACT **The mode of binding of a ketonic substrate, which is an analogue of esters in which the O of the scissile bond is replaced by CH_2, to carboxypeptidase A is similar to that of Gly-Tyr. The site is S_1', with the side chain in the pocket of the enzyme, the carboxylate salt-linked to Arg-145, and the carbonyl group bound to Zn. Thus, esters are probably cleaved at the peptide cleavage site, although not necessarily with the same rate-controlling step or by the same detailed mechanism. The large differences found between the behavior of the enzyme in solution and in one crystalline phase do not apply to a different crystalline phase.**

For those enzymes for which x-ray diffraction studies have been made on the three-dimensional structures of complexes with ligands, a new level of mechanistic questions arises, related to the detailed succession of binding and catalytic steps. The structures of the long-lived complexes of Gly-Tyr, Phe-Gly-Phe-Gly, and other ligands with carboxypeptidase A, for example, along with a wealth of other chemical evidence, have led to detailed proposals (1–4) for the mechanisms of cleavage of peptides and esters. The principle of charge neutrality has been used to treat the general acid-base catalyzed or, alternatively, nucleophilic, first step, and to discuss possible deacylation steps (5).

In this paper, the relationship between structural studies and more recent biochemical studies is discussed, with emphasis on the ambiguities in the mechanisms of action of this enzyme.

Mechanistic deductions using x-ray results

Hydrolytic cleavage of the COOH-terminal peptide bond of the substrate involves nucleophilic attack at the carbonyl carbon and electrophilic attack at the amide NH of this bond. Candidates for nucleophilic attack are Glu-270 to form an anhydride, Glu-270-promoted attack by H_2O, or (with considerable substrate distortion) a Zn-bound H_2O or OH^- which is not present in the initial complexes of the x-ray diffraction studies. Candidates for proton donation are Tyr-248 or, less likely, H_2O. The Zn binds to the carbonyl oxygen of these substrates and initially serves to polarize this C=O bond. If an anhydride is formed, deacylation could occur by attack of Zn-bound water or hydroxyl ion (3, 5).

The productive binding site S_1' places the COOH-terminal side chain in a pocket of the enzyme and binds the substrate's COOH-terminal carboxylate group by two hydrogen bonds to Arg-145 (Fig. 1). Extension of this site shows Arg-127 (S_1) 4 Å away from Arg-145 and Arg-71 (S_2) an additional 4 Å away near Phe-279 and Tyr-198 (S_2 to S_3). This last region may be a recognition site initiating a sliding mechanism for movement of substrate into the pocket (6, 7) and is probably the site of kinetic anomalies for *N*-acyl-dipeptides (1). The extended binding site is five amino acids in length (8, 9), and it is known that longer substrates do not show these kinetic anomalies (8, 9).

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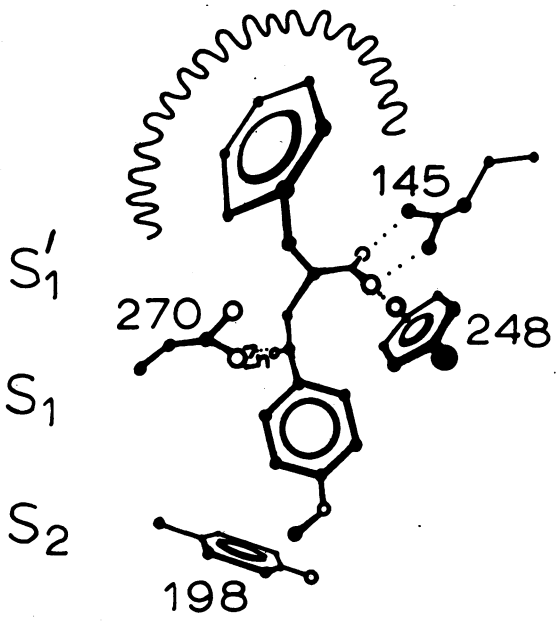

FIG. 1. Complex of (−)-2-benzyl-3-*p*-methoxybenzoylpropionic acid with the active site region of carboxypeptidase A. The amide NH or ester O of a cleavable substrate is here replaced by CH_2 in which the R proton (that nearer to Glu-270) is exchanged stereospecifically.

Products of rapidly cleaved esters and peptides—e.g., β-phenyllactate and L-phenylalanine—bind in S_1', the pocket region of the enzyme (4). Moreover, specificities for ester and peptide substrates are very similar. On the other hand, there are striking differences between binding and kinetic parameters for ester and peptide substrates (10, 11). However, these differences do not necessarily require the proposed (12) different sites for catalysis of esters and peptides, inasmuch as an explanation based upon different rate-controlling steps for esters and peptides seem more reasonable (13) in view of the x-ray diffraction studies.

A ketonic substrate

Structures of active complexes of substrates are obtainable by lowering the temperature, which may alter the rate-determining steps, or by choosing a poor substrate, such as Gly-Tyr. The structure of this complex at 4°C shows that the NH_2-terminus of Gly-Tyr is hydrogen-bonded through a H_2O molecule to the catalytic group Glu-270. This mode of bonding is not available to longer substrates and may explain the low rate of cleavage of nonacylated dipeptides. Esters are cleaved much more rapidly than are analogous peptides by carboxypeptidase A. Hence, the appropriate models are those in which the ester oxygen is replaced by CH_2, which prevents cleavage and retains some of the flexibility of ester analogues.

The ketone 3-*p*-methoxybenzoyl-2-benzylpropionic acid, which is analogous to an *N*-acyl-L-phenyllactate substrate, has been shown to undergo stereospecific exchange of the H_a proton

in the $R(-)$ position of the 3-CH_2 group (14, 15). The suggestion (14, 15) that this ester analogue is bound in site S_1' has been verified by x-ray diffraction (16). In common with Gly-Tyr, the COOH-terminal aromatic group is in the pocket, the carboxylate group is bound to Arg-145, and the ketonic carbonyl group is bound to Zn. Enolization is thus promoted, facilitating proton exchange to Glu-270 or to a water molecule adjacent to Glu-270. This study strengthens the view that esters are also productively bound at S_1'.

An anhydride intermediate in ester hydrolysis

For the specific substrate, *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate, an anhydride at Glu-270 has been isolated as an intermediate in hydrolysis by carboxypeptidase A (17, 18). The reaction is unaffected by acetylation of Tyr-248, which is required for hydrolysis of peptides but not for some esters. Deacylation at −40°C depends on pK_as of 7.65 for the Zn enzyme and 6.33 for the Co enzyme, and thus probably on the L_3ZnOH unit, which has lost a proton in this hydrophobic environment (L is a protein ligand to Zn). It is not established whether L_3ZnOH deacylates directly or through a water molecule, or whether one carbonyl group of the anhydride remains bound to Zn which would then be 5-coordinated in this step of catalysis (5). The activity of the VO^{+2} enzyme (19) may be related to this possibility or may proceed by the nonanhydride pathway. Further studies are needed to determine if other esters form anhydride intermediates when hydrolyzed by carboxypeptidase A.

Comparison with thermolysin

This enzyme is an endopeptidase, not an exopeptidase, although its active site has converged (20) to one somewhat like that of carboxypeptidase A. The Zn ligands are two His and one Glu, the potential nucleophile is Glu-166, the proton donor is probably His-231, and substrate analogues bind with their carbonyl group and displace a H_2O from 4-coordinated Zn (21). Although an anhydride pathway is not eliminated, the long distance of Glu-166 from the substrate's carbonyl carbon and the intervening water molecule make the general base (nonanhydride) pathway also possible. Proton donation by His-231 is supported by chemical modification studies (22), which block peptidase activity. The esterase activity of this modified thermolysin has not been examined. Although the most rapidly cleaved esters are more rapidly hydrolyzed than their peptide analogues by carboxypeptidase A, the reverse is true for thermolysin (23). Perhaps this difference is due to a less favorable anhydride pathway for ester hydrolysis by thermolysin or to the more efficient donation of a proton from His in thermolysin compared to Tyr in carboxypeptidase A.

Solid State Anomalies? The relevance of x-ray diffraction studies has been questioned (24–33) on the basis of low reactivity (1/300) of one crystalline phase (a = 50.9 Å, b = 57.9 Å, c = 45.0 Å, and β = 94°40′) (34, 35). However, the x-ray diffraction studies have been made on a different crystalline phase (a = 51.41 Å, b = 59.89 Å, c = 47.19 Å, and β = 97°35′) which has an activity of about one-third of that in solution (36).

Great care is needed in describing the appropriate amount of flexibility or of conformational states in the active site. The free energies of crystallization are particularly small because the crystals contain about 45% water and must be maintained in contact with solution to retain stability. When substrates bind those conformational changes that are observed—Tyr 248 and some of its polypeptide backbone, Glu-270 and Arg-145—destroy the crystals unless care is taken and low occupancy (one-third) is maintained. Other large distortions of the active site upon crystallization are not expected, especially in view of the almost identical conformation in the remainder of the active site of carboxypeptidase B, which has very different intermolecular contacts in its crystalline phase (37) from those in carboxypeptidase A. In addition, the molecular structure of carboxypeptidase A in its complex with the inhibitor from potato is similar to that of the unliganded enzyme (38). Thus, the forces of crystallization can be expected to influence rotations about single bonds in side chains and the few flexible polypeptide backbone regions when these parts are involved in intermolecular interactions. Too many accessible configurations or too much flexibility may require substrate binding to overcome too much entropy of organization of the active site, as it does, for example, in trypsinogen (39).

The two major reasons, then, for reduction of activity upon crystallization are the restriction of motion of essential binding or catalytic side chains, or associated polypeptide backbone, and the blocking of access of the substrate to the active site by intermolecular contacts. In the first the forces are comparable with single-bond rotations, and in the second the final step of diffusion is prevented although the large channels of water in the crystal may still be present. Moreover, one cannot expect crystallization of an enzyme to select a small percentage of a conformation which is not readily accessible in solution, in view of the large aqueous content and small free energy differences involved in crystallization. Finally, to the extent that the Gly-Tyr complex with carboxypeptidase could have been built as a model from the knowledge of the structure of the enzyme only, and the associated kinetics results, the present starting point could have been reached whether or not one of the crystalline phases showed substantial enzymatic activity.

Different catalytic sites for esters and peptides?

The x-ray studies suggest that S_1', the pocket of the enzyme, is the catalytic site for both esters and peptides, although they may have very different kinetic parameters. The K_ms for BzGly-Gly-L-Phe are similar for the Co, Zn, Mn, and Cd enzymes; the relative Vs are 5, 1, 0.2, and 0.034, respectively. For the corresponding L-β-phenyllactate ester the relative Vs are similar but the K_ms vary from 1/2 to 10 times those of the amide (10). Also, noncompetitive inhibitors of peptide hydrolysis are competitive inhibitors of ester hydrolysis (10). Furthermore, the apoenzyme binds peptides as tightly as does the Zn enzyme, but it binds esters much more weakly. These results have been interpreted as indicating nonidentical but overlapping binding of dipeptide and analogous ester sites, with different mechanisms for these two classes of substrates (10–12).

However, the kinetic results for esters and peptides can be fitted to a common sequential mechanism,

$$E_1 \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} E_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_3 \xrightarrow{k_5} E_1,$$

in which k_3 (strain on the peptide bond) is rate limiting in the precatalytic step for peptides and k_5 (independent of the metal) is rate limiting for esters (13). Ester substrates are easily distorted and hence proceed through initial binding transfer to S_1' and to the transition state; therefore, they should compete easily for inhibitors that bind in S_1'. Peptides, which are less readily strained, also bind initially and move along the subsites but are reluctant to enter S_1' where the scissile bond must be strained; hence, they may show noncompetitive inhibition with inhibitors which are known (4) to bind in S_1'. Removal of the metal would thus influence peptide binding less than ester binding, especially if the metal is partly involved in the strain processes. Although there may be more than five subsites, especially in ES_2 complexes (40, 41), the five subsites S_4, S_3, S_2, S_1, and S_1' may accommodate the need for separate but overlapping binding sites

for certain peptides and esters. However, the need for separate catalytic sites for these two classes of substrates is not compelling, although their detailed mechanistic steps at S_1' may differ.

Catalytic steps, pK_as, and strain

Values of pK_as for nonspecific esters, specific esters, and peptides are (42–44), respectively; pK_{EH_2}, 5–7 (substrate-dependent), ≈6, ≈6; pK_{EH}, ≈9, ≈9, ≈9; pK_{EH_2S}, 6–7, absent (≈6 for cinnamoyl esters), 6.3; and pK_{EHS}, ≈9, absent, absent. The low values, ≈6, for pK_{EH_2} and pK_{EH_2S} are metal dependent, and so is the low value for the deacylation of the anhydride intermediate for the ester *O*-(*trans-p*-chlorocinnamoyl)-L-β-phenyl lactate (18). Except for this anhydride, this low pK_a may also be associated with Glu-270 (45). Besides metal dependence, the pK_as may depend on hydrophobic coverage which should increase the pK_a of Glu-270 but decrease that of the $ZnOH_2$ group. The high values may be due to Tyr-248, Tyr-198, or L_3ZnOH_2 in a hydrophilic environment, not yet covered by substrate or covalent intermediate (5, 42). However, nitration of Tyr-248, which lowers its pK_a, has little effect on the kinetics of hydrolysis of the ester *O*-(*trans-p*-chlorocinnamoyl)-L-β-phenyl lactate (46). Caution in relating pK_as to specific groups applies to the values and especially to the thermodynamic aspects (47).

Tyr-248 has now been located in x-ray diffraction studies in five different sites. The original "up" position (a) and the "down" position (b) hydrogen bonded to the NH of Gly-Tyr have now been supplemented by (c) direct bonding to Zn (48) first found in the arsanilazo derivative (24), (d) the "down" unbonded position with low electron density in carboxypeptidase B (37), and (e) hydrogen bonded to the carboxylate group of the ketonic substrate (16). There is no evidence in the kinetics for the pK_a of 7.7 of the Zn–Tyr-248 interaction, which is easily broken by substrates or inhibitors. Thus, Tyr-248 becomes protonated before catalysis occurs. The rate of formation of this Zn–Tyr-248 complex when the pH is increased from 6.7 to 8.4 is 6.1 sec^{-1} at 3.0 mg of enzyme per ml and 7.2 sec^{-1} at 1.6 mg of enzyme per ml (36). Because rapidly cleaved substrates have k_{cat}s of 100–200 sec^{-1}, it is unlikely that this complex is involved after the initial turnover of these substrates. It is clear from the structure that a much more extensive movement of the polypeptide backbone in the vicinity of Tyr-248 is required to bind Tyr-248 to Zn, compared with binding it to the NH group of a peptide substrate.

The roles assigned to Tyr-248 in the cleavage of peptide substrates in the x-ray study include formation of a hydrophobic environment, hydrogen bonding to the NH of the scissile peptide bond, receptor of a hydrogen bond from the penultimate peptide bond, and proton donor to the NH of the scissile bond (H_2O is an alternative, but less likely, donor). The positions of these bonds, the pocket, the Zn and the salt link to Arg-145 develop, at the scissile peptide bond, a twist that is inferred from the positions of these binding sites. Nucleophilic attack by Glu-270 or attack of a lone pair from H_2O promoted by Glu-270 can then initiate the reaction.

An interesting alternative method of development of strain is that, after the carbonyl oxygen of the substrate displaces H_2O from the fourth coordination site of Zn, the environment has become so hydrophobic that H_2O then binds to Zn as OH^-, after losing a proton, thus leaving the L_3ZnOH^- complex as neutral. Whether or not the substrate oxygen remains on Zn to make it 5-coordinated would then be uncertain. Displacement of the substrate's carbonyl oxygen by OH^- would twist the peptide bond, and then the question (1) of a zinc-hydroxyl attack on the carbonyl carbon of the substrate would be revived. Alternatively, Glu-270 could still function in nucleophilic or general base attack. These mechanisms give a prominent role to the metal dependences of the low pK_as and limit functional metals to those that have a high pK_a in the enzyme and a low pK_a in catalysis in a hydrophobic environment. It does seem clear that this pK_a effect is present in the hydrolysis of the acyl enzyme intermediate of one specific ester (18). Some caution is urged about the properties of various metalloenzymes reconstituted from apo(Zn-free) enzyme, in view of the results of Zisapel (49) on carboxypeptidase B. In apocarboxypeptidase A, one might expect a slow *cis*-to-*trans* conversion of the *cis* peptide bond (2) between Ser-197 and Tyr-198, near the active site.

Although Gly-Tyr and the ketonic substrate showed no differences in their binding to L_3Zn, one might argue that these are Michaelis complexes. Nevertheless, if the alternative mechanism of the previous paragraph is the preferred one, this uncleavable flexible ketonic substrate, which only exchanges a proton, might have shown the distortion described above as a displaced carbonyl group. Perhaps a tentative preference can be expressed for direct strain, without displacement of the carbonyl group by OH^-, as suggested by the x-ray study.

Although an acyl-enzyme intermediate has been demonstrated for one specific ester substrate, generalizations are not yet possible. Esters are more flexible than peptides, are slightly weaker ligands to Zn, form an anhydride much more easily, are more easily hydrolyzed in the absence of an enzyme (O^- can leave, but NH^- cannot, at the scissile bond), are more easily protonated at O than is the NH of the planar peptide bond, and require one less proton. In the hydrolysis of Gly-Gly-Leu in ^{18}O-enriched water, no oxygen is incorporated into Glu-270, and thus either the general base mechanism applies or the anhydride is cleaved asymmetrically (50). (An ester substrate was not tested.) Hydrolysis by the general base pathway for peptides and by the anhydride (and $Zn\text{-}H_2O$) pathway for some esters is suggested from the observation that ^{18}O is exchanged from a substrate at about the rate of resynthesis of the peptide bond and thus occurs only when product of hydrolysis is present (51, 52). However, these authors recognize that, if the water molecule formed upon synthesis also is used in the hydrolysis direction, no exchange would be observed and the mechanism is then ambiguous (52). Moreover, in the hydrolysis direction water cannot be replaced by methanol, hydroxylamine, ammonia, or borohydride ion.

In summary, the mechanisms of hydrolysis of ester and peptide substrates by carboxypeptidase A remain ambiguous. An anhydride intermediate has been shown for one ester substrate, and an analogue for esters binds in the same manner as do peptides in the catalytic site S_1'. However, the function of Glu-270 as a general base catalyst for attack of H_2O on the substrate's carbonyl carbon also remains as a reasonable possibility for peptides and some esters. Deacylation by $ZnOH^-$, or promoted by this group, is probable for hydrolysis of the anhydride, and may involve 5-coordinated Zn (53). This $ZnOH^-$ mechanism is not out of the question earlier in the reaction, either involving 5-coordinated Zn or displacement of the carbonyl group of the substrate from the Zn, although the binding of the ester analogue gives no direct support to this last alternative in the initial stage of the reaction.

I wish to acknowledge support by the National Institutes of Health (GM 06920) and to thank the Humboldt Foundation for an award in aid of this research.

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