The Structure of Carboxypeptidase A

V. STUDIES OF ENZYME-SUBSTRATE AND ENZYME-INHIBITOR COMPLEXES AT 6 Å RESOLUTION*

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SUMMARY

Model peptide substrates, e.g. glycyl-L-tyrosine, and inhibitors, e.g. β-(p-iodophenyl)propionate, are shown at 6 Å resolution to bind to crystalline carboxypeptidase A in regions near the zinc atom. Although the question of bond formation of these added molecules to zinc must await studies at higher resolution, the present studies are not inconsistent with a direct role for the metal in enzymic reactions. Binding of glycyl-L-tyrosine to either native or acetylated crystals produces an increase in electron density in two adjacent regions (A and B) near the zinc. The main region (A) extends into the enzyme's pocket from above the zinc and is attributed to Gly-Tyr bound with its tyrosyl side chain projected into the pocket. Gly-Tyr bound to either apo- or copper-carboxypeptidase increases electron density only in Region A. Furthermore, there appears to be a structural change in the enzyme when substrates are bound to the native or modified enzyme.

As part of a continuing study of the structure of bovine pancreatic carboxypeptidase A (1–3), we are investigating the interaction of carboxypeptidase crystals with substrates and inhibitors. The structure of a glycyl-L-tyrosine complex has already been reported in a preliminary communication (2). The present paper is a full account of three-dimensional x-ray diffraction studies at 6 Å resolution of a number of complexes which are formed when crystals of CPA, modified CPA, or native CPA, bind model peptide substrates and inhibitors. In particular, we examine the complexes of glycyl-tyrosine with native, apo-, copper, and acetylcarnocarboxypeptidases, and the complex of hippuryl-L-phenylalanine with acetyl-CPA. In addition, we present studies of CPA-inhibitor complexes in which the inhibitor is L-lysyl-L-tyrosineamide, β-(p-iodophenyl)propionate, or L-phenylalanine.

The structures of crystals containing bound inhibitors and substrates have been determined by the difference Fourier method. This procedure was first applied to the location of light atom molecules bound to proteins by Stryer, Kendrew, and Watson (4) and has subsequently been used by Johnson and Phillips (5) to study the interaction of substrate analogues with lysozyme. In difference Fourier maps the enzyme structure has been subtracted from the structure of the complex, giving an image of the added small molecule. Alterations of a small fraction of the protein structure can also be elucidated by this method (6).

In order to determine structures of enzyme-substrate complexes, a significant amount of substrate must still be bound to the molecules in the crystal at the end of the measuring time, which is approximately 2 days in our experiments. To attain a turnover time of this order, both exceedingly poor substrates and modified proteins have been used. For our purpose it is fortunate that crystallization itself decreases the enzymic activity appreciably; for example, Quiocio and Richards (7) have shown that the hydrolysis of carbobenzoxyglycyl-L-phenylalanine by crystals proceeds at 1/3 the rate found in solution. As a result of the reduction in activity accompanying crystallization, the complex of Gly-Tyr with cross-linked native CPA has proven sufficiently stable for x-ray studies. Of the available modified forms of CPA (10–11) we have so far utilized the apo-, copper, and acetyl enzymes. Apo- and copper-carboxypeptidase are enzymically inactive (10, 11) but are still able to bind peptides in solution (12) and in the crystal (2). We find that acetylated CPA crystals, which have been shown to have reduced peptide activity (13), are capable of binding peptides. The structures of such inactive substrate complexes may, of course, provide further information about the mechanism of catalysis.

Our previous studies (1) have shown that the metal atom required for enzymic activity lies in a depression in the molecular surface and is adjacent to a pocket extending into the interior. Now, we have found that both substrates and competitive inhibitors bind near the zinc and that, in one mode of binding, the pocket is occupied. The binding of substrates appears to be accompanied by conformational changes in the protein, occurring in regions adjacent to the substrate.

EXPERIMENTAL PROCEDURE

Bovine carboxypeptidase A crystals were prepared as described previously (1). CPA crystallizes in space group P2₁,
Unit cell parameters are \( a = 51.4, b = 59.9, c = 47.2 \), \( \alpha = 97.35^\circ \). The \( \beta \)-p-iodophenylpropionate was prepared by iodination of \( \beta \)-phenylpropionate (14) and after recrystallization was found to have a melting point of 139–140\(^\circ\). Other reagents were obtained from the following companies: Gly-Tyr, Sigma; Lys-Tyr-amide, Cyclo Chemical Corporation; L-phenylalanine, Nutritional Biochemicals; N-acetyl-l-tyrosine, K and K Laboratories; hippuryl-L-phenylalanine, Mann.

All experiments, including modification reactions, assays, and the collection of x-ray data, have been carried out at cold room temperatures (approximately 4\(^\circ\)).

Crystals of apo-CPA were prepared by immersing native CPA crystals in a solution containing 5 \( \times \) \( 10^{-5} \) M 8-hydroxyquinolinesulfonate and 0.04 M tris(hydroxymethyl)aminomethane, pH 7.5, for 2 to 3 weeks (1). Crystals of copper-CPA were then obtained by placing the apo-CPA crystals in a solution of 10\(^{-5} \) M CuCl\(_2\)-0.02 M Tris, pH 7.5. After addition of CuCl\(_2\) to the apo-enzyme crystals, the intensities in the h01 zone were not visually different from those of zinc-CPA.

Assays for enzymic activity of CPA crystals were performed at 4\(^\circ\) following either of the two methods developed in Richards' laboratory (7, 15). In the assay with a column of packed crystals (7) the substrate solution consisted of 0.02 M carboxenzoxy-glycyl-L-tyrosine, 0.2 M LiCl, and 0.02 M Tris, pH 7.5. When glycyl-L-tyrosine was used as substrate, several cross-linked CPA crystals were suspended in 0.7 ml of 0.05 M Gly-Tyr, 0.2 M LiCl, and 0.02 M Tris, pH 7.5, and rotated in a tube for 20 days, at which time hydrolysis was 10\% complete. After the crystals had been allowed to settle, crystal-free substrate solution was removed, and the products, glycine and tyrosine, were analyzed with the use of an automatic amino acid analyzer. The rest of the separated substrate solution was incubated for 10 days and then analyzed as before. No increase in product concentration could be detected, indicating the absence of dissolved CPA.

Intermolecular cross-linking of CPA crystals was essential for most of our experiments, either to keep the crystals suspensions free of dissolved enzyme, which would consume substrates, or to prevent solution of crystals at moderate ionic strengths. Native crystals used in these experiments were cross-linked according to the procedure of Quiocho and Richards (16). Crystals were suspended in a solution of 0.1% glutaraldehyde, 0.1 M LiCl, and 0.02 M sodium Veronal, pH 7.5, and gently rotated. The glutaraldehyde solution was changed at least twice during a period of 6 hours. Excess aldehyde was removed after the reaction by repeated washing with 0.1 M LiCl and 0.02 M sodium Veronal, pH 7.5. Comparison of photographs of cross-linked crystals and of native CPA crystals showed only small intensity changes.

Acetylated CPA crystals were prepared by placing cross-linked native crystals into a tube containing 0.01 M N-acetyl-l-tyrosine, 0.02 M sodium Veronal, and 0.02 M sodium glycin at pH 7.5, and gently rotating the tube; after several hours the solution was replaced by a freshly prepared solution of the same composition. The reaction was allowed to continue for about 12 hours, after which the crystals were washed with buffer. Acetylated crystals yielded a diffraction pattern very similar to that of native cross-linked crystals. Pepsidase assays, using a column of crystals as described by Quiocho and Richards (7, 13), showed that after acetylation the crystals retained 10 to 15\% of their initial activity.

All of the CPA substrate and inhibitor complexes were prepared by placing crystals into fresh substrate or inhibitor solutions for about 12 hours, after which the crystals were mounted and the data were collected. The presence of salt (0.1 to 0.2 M LiCl) is required in these experiments. When crystals are placed in buffered substrate or inhibitor solutions (ionic strength below 0.05) without added salt, disordered x-ray patterns are obtained. The preparations of the various inhibitor and substrate complexes are summarized in Table I. Substitution of mercury for zinc produces no changes in cell dimensions, and cross-linked crystals soaked in Gly-Tyr show lattice changes no greater than 0.3 to 0.4\%. The maximum cell change observed with cross-linked, acetylated crystals in the presence of hippuryl-phenylalanine, was 0.6\% in c axis.

All x-ray diffraction data were collected on Supper-Pace diffractometers, with the use of copper target x-ray tubes with nickel filters. For each of the complexes reported here the 6 A three-dimensional data were obtained from a single crystal within about 2 days. Corrections for Lorentz and polarization factors and for absorption were made, after which three-dimensional electron density maps were computed at 6 A resolution, using our best set of native protein phases, \( \alpha \), to compute the Fourier coefficients

\[
A = n|{F}_p| - |{F}_o| \cos \alpha \\
B = n|{F}_p| - |{F}_o| \sin \alpha
\]

where \( |{F}_p| \) is the measured amplitude of a given reflection from a derivative crystal, \( |{F}_o| \) is the corresponding amplitude for native CPA, and \( n \) is the figure of merit for the reflection (17). The phases from the protein structure determination are thus assumed to be valid for the amplitudes of x-ray scattering from the complex. In the absence of rigorous criteria for isomorphism, one depends ultimately on chemical sense in the interpretation, internal consistencies among related studies, smallness of cell parameter changes, and low background of the difference electron density maps in order to assess the validity of the results.

**RESULTS**

Relevant portions of difference maps are summarized in Figs. 1 to 4; peak positions and occupancies are given in Table 1.

**Glycyl-L-tyrosine on Native CPA**—The binding of glycyl-L-tyrosine to crystalline native CPA yields increased electron density in two regions, A and B, near the zinc. These regions are shown as dotted contours on a superposition of several sections of the 6 A map (solid contours) in Fig. 1. The placement of Region A (striped) and Region B (dotted) in a balsa model of the 6 A contours is shown in Fig. 5, and the effect of change of concentration of Gly-Tyr from 0.05 M to 0.01 M is compared in Fig. 2 (Parts 1 and 2). The major region, A, extends into the pocket of the enzyme from above the zinc. Region B is approximately normal to the plane of Fig. 1, extending upward toward the reader, and hence is roughly perpendicular to the long direction of Region A. Region B has a lower density than Region A (Table I). The occurrence of negative peaks near B makes the interpretation that B is a second substrate molecule somewhat uncertain. The peaks in A and B do not represent bound products of the enzymic reaction, because of difference electron density map using data taken from a cross-linked CPA crystal soaked in 0.05 M glycine

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* X-Ray data have been deposited as Document 0642 with the American Documentation Institute, Auxiliary Publication Project, Photoduplication Service, Library of Congress, Washington, D. C. 20540. A copy may be secured by citing the document number and remitting $16.25 for photoprints or $5.00 for 35-mm microfilm. Advance payment is required. Make checks or money orders payable to: Chief, Photoduplication Service, Library of Congress.
Table I

Summary of difference electron density maps

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrate or inhibitor</th>
<th>Modification</th>
<th>Site</th>
<th>Coordinates of peak</th>
<th>Peak density&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Electron count&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td>y</td>
<td>z</td>
</tr>
<tr>
<td>1</td>
<td>Gly-Tyr, 0.05 m</td>
<td>Cross-link</td>
<td>A</td>
<td>0.95</td>
<td>0.48</td>
<td>0.78</td>
</tr>
<tr>
<td>2</td>
<td>Gly-Tyr, 0.01 m</td>
<td>Cross-link</td>
<td>A</td>
<td>0.95</td>
<td>0.48</td>
<td>0.80</td>
</tr>
<tr>
<td>3</td>
<td>Gly-Tyr, 0.05 m</td>
<td>Cross-link</td>
<td>A</td>
<td>0.95</td>
<td>0.47</td>
<td>0.82</td>
</tr>
<tr>
<td>4</td>
<td>Gly-Tyr, 0.01 m</td>
<td>Copper CPA</td>
<td>A</td>
<td>0.96</td>
<td>0.47</td>
<td>0.86</td>
</tr>
<tr>
<td>5</td>
<td>Gly-Tyr, 0.01 m</td>
<td>Apo-CPA</td>
<td>A</td>
<td>0.92</td>
<td>0.47</td>
<td>0.84</td>
</tr>
<tr>
<td>6</td>
<td>Lys-Tyr-NH&lt;sub&gt;2&lt;/sub&gt; 0.15 m</td>
<td>Cross-link</td>
<td>A, B</td>
<td>0.083</td>
<td>0.52</td>
<td>0.13</td>
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<tr>
<td>7</td>
<td>Hippurylphenylalanine, 0.05 m</td>
<td>Cross-link</td>
<td>A</td>
<td>0.39</td>
<td>0.47</td>
<td>0.82</td>
</tr>
<tr>
<td>8</td>
<td>IPP, 0.01 m</td>
<td>Cross-link</td>
<td>1</td>
<td>0.93</td>
<td>0.48</td>
<td>0.68</td>
</tr>
<tr>
<td>9</td>
<td>IPP, 0.05 m</td>
<td>Cross-link</td>
<td>1</td>
<td>0.93</td>
<td>0.48</td>
<td>0.68</td>
</tr>
<tr>
<td>10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>IPP, 0.05 m</td>
<td>Apo-CPA</td>
<td>1</td>
<td>0.90</td>
<td>0.48</td>
<td>0.75</td>
</tr>
<tr>
<td>11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>IPP, 0.10 m</td>
<td>Apo-CPA</td>
<td>1</td>
<td>0.90</td>
<td>0.47</td>
<td>0.77</td>
</tr>
<tr>
<td>12</td>
<td>Phenylalanine, 0.05 m</td>
<td>Native CPA</td>
<td>A</td>
<td>0.95</td>
<td>0.47</td>
<td>0.82</td>
</tr>
</tbody>
</table>

<sup>a</sup> Peak density in electrons per cubic angstrom (e/A<sup>3</sup>) and electron counts are recorded here as twice the observed values in order to correct the scaling error introduced by use of the native phases, and have been divided by the average figure of merit of 0.89. The occupancy of the mercury which replaces the zinc was set at 50 electrons after refinement at 2.0 Å resolution, in order to calibrate the absolute scale. For light atom substrates, the background density is approximately one-half of the density in Site A.

<sup>b</sup> No LiCl is present in Experiments 10 and 11, but 0.02 M Tris (pH 7.5) is present. In all other experiments 0.1 to 0.2 M LiCl and 0.02 M Tris (pH 7.5) were also present.

![Fig. 1. Contours from the glycyl-L-tyrosine difference electron density map on contours of enzyme (6 Å resolution). Dashed contour from sections y = 14/30 to 17/30 of the difference map calculated with data from a native crystal soaked in 0.05 M Gly-Tyr have been superimposed on the smooth contours of sections y = 13/30 to 17/30 of the 6 Å native protein map. Neighboring protein molecules have been omitted. The two regions of added density are labeled A and B.](http://www.jbc.org/)

and saturated (0.004 M) tyrosine showed no significant peaks in either Region A or Region B. Several other peaks, with densities approximately 60% of Site A, occur in regions removed from the active site and are known to represent cross-links.

**Glycyl-L-tyrosine on Acetyl-CPA**—The electron density difference map (Fig. 2, Part 3) shows that Gly-Tyr is bound to CPA which had been acetylated with N-acetylimidazole. The occupancy of Gly-Tyr in this experiment is essentially the same as that found with the native enzyme (Table I), while the activity of the crystals toward carbobenzoxyglycyl-L-phenylalanine has been reduced by at least 85 to 90%. The resolution (6 Å) is not sufficient to detect small shifts from the position occupied by Gly-Tyr in native CPA, nor can any new peaks attributable to acetylation be observed.

**Glycyl-L-tyrosine on Copper- and Apo-CPA**—The binding of Gly-Tyr to copper-CPA and apo-CPA, shown in Fig. 2 (Parts 4 and 5, respectively), produces increased electron density only in Region A at a concentration of 0.01 M Gly-Tyr. The peak density in Region A is shifted some 3 or 4 Å in both of these examples from the position found for Gly-Tyr bound to native CPA. Region B shows no increase in electron density when 0.01 M Gly-Tyr binds to copper-CPA or apo-CPA.

**L-Lysyl-L-tyrosinamide on Native CPA**—An attempt was made to examine the effect of converting the charged carboxylate group to a neutral amide, since amides are known to bind to CPA (12) but are not substrates. L-Lysyl-L-tyrosinamide was chosen...
rather than glycyltyrosinamide because of the greater solubility conferred by the lysyl residue. The electron density difference map (Fig. 2, Part 6) indicates about twice as much density in Region B as in Region A, in contrast with the densities in these two regions when Gly-Tyr binds.

\( \beta \)-\( (p\text{-iodophenyl}) \text{propionate on CPA and apo-CPA} \)--The competitive inhibitor \( \beta \)-phenylpropionate (19, 7) has been modified for x-ray study to IPP in order to locate the molecule more precisely from the heavy atom position. At 0.05 M, IPP binds to crystalline native CPA at four sites, identified in Table I by coordinates of iodine positions. Two of these sites, 1 and 2, are near Regions A and B, respectively, where Gly-Tyr binds to CPA. In apo-CPA site 2 does not bind IPP even at concentrations as high as 0.1 M IPP, while Site 1, near Region A, is shifted and reduced in height in the absence of zinc. Site 4 is reasonably near zinc (Table I), but Site 3 is far removed from the active site region.

\( L\)-Phenylalanine on Native CPA—\( L\)-Phenylalanine, a product of the hydrolysis of some CPA substrates, does bind to CPA crystals, but only in Region A (Table I, Fig. 4a).

Hippuryl-\( L\)-phenylalanine on Acetyl-CPA—We examined hippurylphenylalanine in an effort to study more reactive peptide substrates. X-ray photographs of the hippuryl-\( L\)-phenylalanine-CPA complex are different from those of the phenylalanine-CPA complex. In the hippuryl-\( L\)-phenylalanine difference map there is some increase in density in Region B, as well as in Region A, in contrast to the phenylalanine map (Table I, Fig. 4). However, the general similarity of the \( L\)-phenylalanine and hippuryl-\( L\)-phenylalanine difference maps does raise a question as to whether some formation and binding of product occurred during the later stages of the data collection.

Conformational Changes—In addition to Regions A and B described above we consistently observe another positive and an adjacent negative region in eight of the difference maps. In the most favorable example (Fig. 4b), hippuryl-\( L\)-phenylalanine is complexed with acetylated CPA; here the depth of the hole in the difference map is about 1.5 times the magnitude of the next highest unexplained feature of the map, and the centers of the peak and hole are separated by about 4 or 5 Å. For comparison, sections through the \( L\)-phenylalanine difference map showing the peak and hole are presented in Fig. 4a. While in most other cases this peak and hole are the next largest features (after the substrate peaks themselves), they are not significantly above background. However, this peak and hole appear consistently when Region A is occupied, and the appearance of these features can be explained if a portion of the CPA molecule moves when
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The movement of protein which occurs when substrates are bound is indicated by the arrow. The black spot below Region A is the zinc atom. The crystallographic b axis is along the directions of the vertical support rods, while the a axis is along the front edge of the base.

Our present results show that model peptide substrates and competitive inhibitors are bound to crystalline CPA in regions which are sufficiently near the zinc to permit these added molecules to interact with the metal atom when it is present. We must leave a direct verification as to whether or not a bond is formed between zinc and substrate for experiments, now in progress, at higher resolution. However, the present studies provide some support for the hypothesis that the metal atom is involved at some step in the complete enzymic reaction.

Studies of the binding of \( \beta \)-(p-iodophenyl)propionate to the native enzyme have allowed us to draw some conclusions concerning the orientation of inhibitors and substrates, since the heavy atom on IPP permits us to locate one end of the bound inhibitor accurately and distinguishes the added compound from alterations in the protein structure. Of the four IPP sites, we shall discuss only Sites 1 and 2, since they are most closely related, although not identical, to Regions A and B of the Gly-Tyr maps. Assuming that the centers of the strongest peaks on the difference maps correspond to the iodine atoms, we have calculated that the iodine atoms in Sites 1 and 2 are 10.5 to 11 Å from the zinc atom. This is the zinc to iodine distance expected for a fully extended IPP ion the carboxylate group of which is a zinc ligand. Our observations that the binding of IPP at Sites 1 and 2 is reduced by the removal of zinc, while very little structural change takes place in the enzyme itself when zinc is removed (1), are consistent with the proposal that the carboxylate group of
the inhibitor is attached to the zinc. These results are also consistent with a nuclear magnetic resonance study (18) which indicates that a water molecule is displaced from the manganese of manganese-CPA when β-phenylpropionate is added.

Furthermore, if the carboxylate groups of IPP in both Sites 1 and 2 are zinc ligand, then binding to one site should exclude binding at the other. We have looked for evidence of competition between these sites by predicting the relative occupancies of Sites 1 and 2 at 0.05 m IPP, using $K_r$ values derived from the occupancies observed at 0.01 m IPP. The results (Table II) tend to support competition between Sites 1 and 2, in agreement with the hypothesis based upon steric arguments.

The $K_r$ values for Sites 1 and 2, assuming overlapping sites, are $2.2 \times 10^{-5}$ m for Site 1 and $1.7 \times 10^{-5}$ m for Site 2. These constants are similar to the $K_r$ of β-phenylpropionate ($10^{-3}$ m) in CPA crystals (7) but very different from the $K_r$ of β-phenylpropionate ($6 \times 10^{-5}$ m) in solution (13). We cannot say, however, whether the $K_r$ values of both Sites 1 and 2 change upon going from solution to crystal.

It is more difficult to give equivalent details about light atom substrates at 6 A resolution. Comparison of IPP contours in Site 1 with those of Gly-Tyr in Region A is shown in Fig. 3a, and of IPP contours in Site 2 with those of lysyltyrosinamide in Region B in Fig. 3b. Since the iodine of the IPP in Site 1 is near Region A, the phenol side chain of bound Gly-Tyr probably extends into the pocket, but a detailed model is not inconsistent with a slight displacement of the IPP phenyl group from that of Gly-Tyr. Also, the results for the IPP are not to be extrapolated to indicate that the carboxyl group of Gly-Tyr in Region A is bound to the zinc. The steric relation of iodine of IPP in Site 1 to Region A is quite reasonable. On the other hand, Region B cannot be superimposed on an IPP molecule extending from the iodine of Site 2 to the zinc, so that some doubt remains as to whether Region B is a second substrate site for dipeptides, or whether it is some evidence of conformational changes in the protein. Of course, the absence of density in Region B when Gly-Tyr interacts with apo-CPA or with copper-CPA and the observation that the largest peak in the lysyltyrosinamide difference map occurs in Region B favor the assignments of Region B as a second substrate site, perhaps slightly shifted from Site 2 of IPP. Multiple binding sites for substrates might be expected, since substrate inhibition of CPA has been demonstrated both in solution (20) and in the crystal (7). However, the kinetic data of Izumiya and Uchio (21) show no evidence for substrate inhibition by Gly-Tyr. Studies at higher resolution will be required to demonstrate conclusively whether Region B is a second substrate site.

The question of whether these dipeptide complexes are "productive" is also difficult to answer unequivocally. In lysozyme crystals, a stable complex, apparently nonproductive, is formed with tri $N$ acetylglucosamine (6), which is actually a poor substrate in solution (21). Nevertheless, several observations suggest that the structures of the CPA complexes may be relevant to the enzyme mechanism. The apoenzyme is inactive both in solution and in the crystal (10, 13); the zinc is required for peptidase activity. If we suppose that the zinc is not involved directly with the substrate during binding or catalysis or both then it must induce activity by maintaining a tertiary protein structure which is necessary for activity. However, at least to 6 A resolution, there is no evidence of structural change when zinc is removed (1), except possibly for slight repositioning of metal ligands. Also a 2 A resolution difference map of mercury-CPA provides no indication of any protein structural changes when mercury is exchanged for zinc. Thus the "productive" binding site is probably near the zinc, which probably has a direct function in substrate binding or catalysis or both. This conclusion is also supported by the observation that the competitive inhibitor, L-phenylalanine, binds only at Site A. Higher resolution maps of both the protein and substrate complexes should help to ascertain whether the enzyme is capable of splitting a peptide bound at either A or B.

Substrate binding to acetylated CPA is discussed here on the assumption that results from experiments in solution also apply to the crystalline enzyme. It has been shown in solution that when Gly-Phe binds to copper-CPA the rate of exchange of copper for zinc is slowed, but that after acetylation of CPA this substrate no longer affects zinc exchange; these results have been used to imply a failure of the substrate to bind to acetylated CPA (8). On the contrary, we find that Gly-Tyr binds to crystalline acetylated CPA near zinc. It therefore seems reasonable to suggest that some small structural differences do exist between CPA and acetyl-CPA complexes with Gly-Tyr, although these differences are below our limits of observation in difference electron density maps at 6 A resolution. If we are indeed looking at a productive complex between native CPA and Gly-Tyr, then the decrease in peptidase activity after acetylation must result either from changes in the binding below our limits of resolution or from specific effects on catalytic groups.

The apparent conformational change that occurs when substrate is bound in Region A is perhaps the most interesting result of our present work. It occurs to varying extents, most clearly for hippuryl-L-phenylalanine (Fig. 4b). The characteristic positive ($P$) and negative ($M$) peaks (Fig. 4a) are clearly observed when phenylalanine is bound in Region A, and in the 0.05 m iodonophenylpropionate map these density changes of $P$ and $M$ are also present. However, no conformational changes detectable at 6 A resolution are produced by binding of lysyltyrosinamide. The separation of the positive ($P$) from the negative ($M$) region, approximately 4 to 5 A, does not necessarily mean that an entire segment of the peptide chain has moved that distance. For example, these features could result from a smaller motion of the main chain which displaces residues on opposite sides of the chain. Data on changes in ultraviolet absorption on binding of β-phenylpropionate (23) and on the absorbance of nitrotyrosyl residues after addition of inhibitor (24) may reflect the changes we have observed but cannot in themselves be uniquely interpreted as showing structural changes.

<table>
<thead>
<tr>
<th>Site</th>
<th>Observed</th>
<th>Competing sites</th>
<th>Independent sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.32</td>
<td>0.38</td>
<td>0.60</td>
</tr>
<tr>
<td>2</td>
<td>0.53</td>
<td>0.49</td>
<td>0.67</td>
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</tbody>
</table>

* Dissociation constants are calculated from the fractional occupancy of IPP at 0.01 m IPP, assuming competition between Sites 1 and 2 and assuming no competition. These constants are then used to predict the occupancy of IPP at 0.05 m IPP.
The details of conformational changes in proteins are now being elucidated by crystallographic studies. A large relative movement (approximately 8 Å) of the β chains occurs upon deoxygenation of hemoglobin (25). Also, small structural changes (less than 1 Å) in lysozyme, as a result of inhibitor binding (6), and in chymotrypsin, due to acylation of serine 195 (26), have been documented. On the other hand, the protein movement which we observe when CPA binds substrates or some inhibitors is larger (several angstroms) than that which occurs in the other single subunit enzymes which have been studied. We expect that study of these and other complexes at high resolution will enable us to comment on the specific nature and function of the structural change as well as its relevance to the “induced fit” theory advanced by Koshland (27, 28).

Preliminary Results at Higher Resolution—In collaboration with Hartsuck and Reeke, we have recently computed a 2.0 Å three-dimensional electron density map of native CPA. We have also computed a 2.8 Å three-dimensional difference map between the Gly-Tyr complex with CPA and the native CPA. In the map of native CPA, we have located a region whose density is consistent with the sequence Ile-Tyr-Gln-Ala (or structurally similar residues) shown by Roholt and Pressman (29) to contain the tyrosine essential for peptidase activity. The tyrosyl side chain of roughly 8 Å from its position in the native enzyme. In addition to side chain motion, the peptide substrate complexes. We are especially indebted to C. H. W. Hirs, who has provided us with pancreatic juice and procarboxypeptidase A.

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The Structure of Carboxypeptidase A: V. STUDIES OF ENZYME-SUBSTRATE AND ENZYME-INHIBITOR COMPLEXES AT 6 Å RESOLUTION
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