Binding of the Biproduct Analog L-Benzylsuccinic Acid to Thermolysin Determined by X-ray Crystallography*

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Benzylsuccinic acid, a competitive inhibitor of carboxypeptidase A, is shown to also inhibit thermolysin. The mode of binding of this "biproduct analog" to crystalline thermolysin has been determined to a resolution of 2.3 Å by X-ray crystallography. The inhibitor binds to the enzyme in a manner analogous to that of dipeptide inhibitors previously reported. One carboxyl oxygen displaces a water molecule from the zinc, forming a fourth zinc ligand; the second carboxyl interacts with Arg 203 and Asn 112, and the benzyl group occupies the hydrophobic specificity pocket. Benzylsuccinic acid differs from a dipeptide in having a methylene group in place of the peptide nitrogen, and, apparently as a consequence of this tetrahedral center, which somewhat resembles the presumptive transition state, the benzyl ring of the inhibitor is displaced in the specificity pocket relative to the position occupied by normal dipeptides.

The close similarity of the active sites of carboxypeptidase A and thermolysin makes it possible to infer the probable mode of binding of benzylsuccinic acid to the former enzyme. The predicted mode of binding is consistent with the known geometry of the carboxypeptidase A active site and also explains the observed behavior of a number of analogs of benzylsuccinic acid. The results obtained here are consistent with a general base mechanism of peptide hydrolysis by both thermolysin and carboxypeptidase A, in which a glutamic acid promotes the attack of a water molecule on the peptide carbonyl carbon, but do not rule out an anhydride mechanism.

A mechanism of action for thermolysin has been proposed (5), involving primarily three groups: the zinc ion, Glu 143, and His 231. It is proposed that the scissile peptide bond is aligned in the active site by the binding of the carbonyl oxygen to the zinc and that Glu 143 acts as a general base promoting a nucleophilic attack of a water molecule on the polarized carbonyl carbon. In concert with this, or immediately thereafter, His 231 donates a proton to the substrate nitrogen, yielding a transition state in which both carbon and nitrogen of the scissile bond are tetrahedral (6). It seems likely that carboxypeptidase A also has a similar mechanism. Breslow and Wernick (7, 8) have recently obtained evidence from oxygen-isotope exchange studies that carboxypeptidase A-catalyzed hydrolysis of peptides proceeds by a general base mechanism. Such a mechanism is consistent with structural studies of carboxypeptidase A (9, 10), although such studies indicated that hydrolysis might equally well proceed by a nucleophilic pathway, with Glu 270 forming an anhydride intermediate.

These observations suggested that L-benzylsuccinic acid (2R)-benzyl-3-carboxypropionic acid), an unusually strong competitive inhibitor of carboxypeptidase A (Kᵢ = 0.45 μM), might contain chemical and steric features which would make it also an inhibitor of thermolysin. This has, in fact, proved to be the case. L-Benzylsuccinic acid (Fig. 1) is structurally analogous to L-phenylalanine and was introduced by Byers and Wolfenden (11, 12) as a specific inhibitor of carboxypeptidase A. It contains a tetrahedral center at C2 which resembles the tetrahedral nitrogen of the presumptive transition state for peptide hydrolysis. Nevertheless, L-benzylsuccinic acid is not a true transition state analog and is better described, as by Byers and Wolfenden, as a "biprodut analog," resembling the collected products of peptide hydrolysis.

Thus, the x-ray structure determination of the complex between thermolysin and L-benzylsuccinic acid, described in this communication, reveals the mode of binding of a molecule which resembles both the transition state and the products of the enzymatic reaction.

** Experimental Procedures

Thermolysin, purchased from Calbiochem, was crystallized according to Colman et al. (13). Crystals stored in 0.01 M Tris/acetate, 0.01 M calcium acetate, 5% dimethylsulfoxide (v/v) were used for all the experiments described here.

Benzylsuccinic acid, a kind gift of Dr. R. Wolfenden, University of North Carolina, was dissolved in the same buffer medium at pH 7.2. Thermolysin crystals showed a tendency to crack if transferred directly to the inhibitor solution. For this reason the crystals were first soaked in two solutions at intermediate concentration for about 20 min each. In preliminary experiments, concentrations of the inhibitor

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1 As discussed in a note of correction (1973) Biochemistry, 12, 3864.) Byers and Wolfenden suggest that benzylsuccinic acid be described as a "biprodut" or "two-product" analog, emphasizing the notion that it resembles the two products of enzymatic hydrolysis, and not a "by-product" analog as appears in Ref. 12.
RESULTS

The ($F_{PL} - F_P$) electron density map showing the difference in density between inhibited and native crystals has its highest peaks close to the zinc (11σ), and in the thermolysin specificity pocket (8σ). Also, the difference density has a strong negative feature (−8σ) close to the zinc which has been observed with all inhibitors bound to crystalline thermolysin (5, 6) and is attributed to the displacement of water molecules and also a Tris molecule bound to the native enzyme. Peaks due to small movements of side chains, in particular Asn 112, Val 139, Glu 143, Leu 202, and Met 120 (−7σ to +7σ), can also be seen in the difference map. Away from the active site region, the highest peak is about 3σ. (The standard deviation, σ, was calculated as the root mean square value of the difference density within an arbitrarily large volume of the unit cell not including the active site region.)

The Fourier synthesis with coefficients ($2F_{PL} - F_P$) shows density corresponding to the whole of the L-benzylsuccinic acid molecule. This map, shown in Fig. 2, was used to estimate the approximate atomic coordinates of the inhibitor by placing markers on the map sections (5). The idealized inhibitor coordinates are given in Table II and the mode of binding illustrated in Figs. 2 and 3. The mode of binding of L-benzylsuccinic acid to thermolysin is very similar to that of other inhibitors, such as β-phenylpropionyl-L-phenylalanine and L-phenylalanine (5). L-Benzylsuccinic acid displaces the water molecule coordinated to the zinc ion and occupies the fourth coordination position with oxygen, O1, 2.1 Å from the zinc, yielding a slightly distorted tetrahedral coordination. The second oxygen, O2, is at hydrogen bonding distance (3.2 Å) from a water molecule, HO(A), which in turn is 2.6 Å from O1 of Glu 143. This water molecule is also in a good position to accept a hydrogen bond from the peptide nitrogen of Trp 115 (distance 2.9 Å). At the pH of our experiment, benzylsuccinic acid in solution is fully ionized (12), although its state of ionization when bound to thermolysin is not certain. The distance from O1 of Glu 143 to O2 of the C1 carboxyl of the inhibitor is 3.5 Å, so that there does not appear to be a direct interaction between these two carboxyl groups. On the other hand, the measured distance from O2 to the backbone carboxyl oxygen of Ala 113 is 2.7 Å, indicative of a hydrogen bond, suggesting that the C1 carboxyl may be protonated. The fact that the estimated errors in both the inhibitor and the protein coordinates are about 0.3 Å means that no great weight can be placed on such inferences.

The second carboxylate group of the inhibitor, C4, forms a salt link with Arg 203 and a hydrogen bond with N8 of Asn 112. These interactions are characteristic of thermolysin inhibitors with a free carboxyl group at the $R'_1$ position.

The phenyl ring binds in the hydrophobic pocket, between

![Fig. 1. Fisher projection of L-benzylsuccinic acid showing the atom numbering used.](http://www.jbc.org/)

The inhibition constant ($K_I$) for the racemic mixture of the inhibitor was evaluated using the commercial enzyme. Owing to the low solubility of the substrate furylacryloyl-Gly-Leu-NH$_2$, and to high $K_m$ values, the reaction was studied under pseudo-first order conditions (18, 19). Enzyme, substrate, and inhibitor were dissolved in 10⁻¹ M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer at pH 7.2 and in the thermolysin specificity pocket.

### Table 1

| Cell dimensions | L-BZSA$^*$ |
|-----------------|------------|
| $a$, b          | 94.2 Å     | 94.2 Å     |
| $c$             | 131.4 Å    | 131.6 Å    |

### Intensity statistics$^a$

| Films           | 24          | 18         |
|-----------------|-------------|------------|
| Average $R_{sym}$ | 0.054       | 0.061      |
| Average $R_{ave}$ | 0.053       | 0.037      |
| $R_{merge}$     | 0.020       | 0.022      |
| Average isomophous difference (%) | 8.1         |
| Reflections      | 14,210      | 11,809     |

$^a$L-BZSA, L-benzylsuccinic acid.

$^b$The respective $R$ values are as defined previously (13). $R_{sym}$ measures the agreement between symmetry-related reflections recorded on the same film (usually four reflections); $R_{ave}$ gives the agreement between symmetry-averaged reflections recorded on the stronger and weaker films in a film pack; and $R_{merge}$ gives the agreement between structure amplitudes measured on different films.

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Leu 202 and Val 139 side chains, as has been observed for several other inhibitors with large hydrophobic residues in position $R_I^*$ (5).

The $(F_{o}-F_{c})$ difference map indicates that there are some movements of the protein associated with the binding of L-benzylsuccinic acid. The most obvious changes are in the region of the hydrophobic pocket and appear to involve Val 139 and Met 120 on one side and Leu 202 on the opposite side of the binding pocket. Analysis of the maps suggests that the extremity of the benzyl group of L-benzylsuccinic acid is located about 0.7 Å further away from Leu 202 and closer to Val 139 than is the case with the dipeptide inhibitors studied previously. The change in geometry is shown (Fig. 4) by the difference in density between [L-benzylsuccinic acid:thermolysin] and [L-phenylpropionyl-L-phenylalanine:thermolysin]. Adjacent positive and negative features show that there is a concerted movement of density away from Leu 202 and toward Val 139 when L-benzylsuccinic acid is bound to the enzyme, presumably due to the tetrahedral center at C2. In contrast, this position is occupied by the planar peptide nitrogen, hydrogen bonded to the carbonyl oxygen of Ala 113, when dipeptide inhibitors are bound to the enzyme.

In addition to the above movements, a number of other side chains move slightly, although there is no indication of a movement of the zinc ion. Close to the carboxylate C4 of the inhibitor there is additional density (Fig. 2), also seen with other inhibitors, which is interpreted as bound solvent, and a water molecule, $H_2O(B)$, was introduced at this position.

In both the $(F_{o}-F_{c})$ and $(2F_{o}-F_{c})$ maps, there is weak density above the zinc ion, close to Phe 114 (Fig. 2). This is the location of the $R_I$ subsite and might indicate a secondary binding mode of L-benzylsuccinic acid, although it was not possible to place a model to coincide with the feature. Additional density in the same location was also seen when alanyl-L-phenylalanine was bound to the enzyme (5), and might be attributed to partial ordering of solvent induced by the binding of the inhibitor. From comparison of the electron density of the inhibitor with that of the protein, the occupancy of L-benzylsuccinic acid in crystalline thermolysin is estimated to be about 70%.

**Table II**

Coordinates for benzylsuccinic acid Coordinates are in angstroms relative to the orthogonal coordinate frame used previously (21).

| Benzylsuccinic acid* | X    | Y    | Z    |
|----------------------|------|------|------|
| C1 (C)               | 53.7 | 15.9 | -3.4 |
| O1 (O)               | 53.0 | 19.4 | -6.2 |
| O2                   | 51.0 | 18.9 | -5.5 |
| C2 (N)               | 52.2 | 18.6 | -5.7 |
| C3 (CA)              | 53.4 | 17.3 | -4.9 |
| C4 (C)               | 52.7 | 17.2 | -5.3 |
| O3 (O)               | 54.8 | 15.7 | -2.9 |
| O4                   | 59.8 | 15.0 | -3.6 |
| C5 (CB)              | 52.4 | 18.1 | -2.9 |
| C6 (CG)              | 53.2 | 18.6 | -1.8 |
| C7 (CD1)             | 52.9 | 18.3 | -0.5 |
| C8 (CE1)             | 53.6 | 18.8 | 0.6  |
| C9 (CZ)              | 54.7 | 19.6 | 0.4  |
| C10 (CR2)            | 55.1 | 19.9 | -0.9 |
| C11 (CD2)            | 54.4 | 19.4 | -2.0 |
| H$_2$O(A)            | 50.9 | 21.3 | -7.6 |
| H$_2$O(B)            | 52.7 | 12.8 | 6.2  |
| Asn 112              |      |      |      |
| N                    | 47.7 | 14.9 | -0.1 |
| C                    | 48.4 | 16.0 | -0.7 |
| O                    | 47.7 | 17.3 | -0.9 |
| O                    | 46.5 | 17.8 | -0.6 |
| CD                   | 45.1 | 16.0 | -2.2 |
| CG                   | 49.3 | 15.4 | -3.0 |
| ND                   | 50.3 | 16.2 | -2.9 |
| OD                   | 49.3 | 14.0 | -3.9 |
| Val 139              |      |      |      |
| CA                   | 54.5 | 23.3 | 1.0  |
| CB                   | 53.3 | 22.6 | 1.7  |
| CG1                  | 52.2 | 22.0 | 0.7  |
| CG2                  | 53.8 | 21.6 | 2.7  |

*The atom numbering is defined in Fig. 1, and the atom identification given in parenthesis is for a corresponding dipeptide.

**Fig. 2.** Stereo diagram showing the $(2F_{o}-F_{c})$ density superimposed on the bound inhibitor. For clarity, the density for the protein molecule has been omitted. In this and the other stereo drawings, the bonds for the inhibitor and for the backbone of the protein are drawn solid. $\bullet$, oxygen atoms; $\bigcirc$, carbon atoms; $\bullet$, nitrogen atoms.

**Table II**

**Discussion**

As pointed out by Byers and Wolfenden (11, 12), L-benzylsuccinic acid resembles the collected products of carboxypeptidase A-catalyzed peptide hydrolysis. This is illustrated diagrammatically in Fig. 5. For both carboxypeptidase A and thermolysin, two of the principal determinants in substrate binding are the interaction of the carboxyl oxygen of the scissile peptide with the zinc ion, and the interaction of the $R_I$ side chain with the specificity pocket of the enzyme. In Fig. 5 we show the approximate distance between the carboxyl oxygen and the $R_I$ side chain, with the specificity pocket of the enzyme. From comparison of the electron density of the inhibitor with that of the protein, the occupancy of L-benzylsuccinic acid in crystalline thermolysin is estimated to be about 70%.

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The observed mode of binding of L-benzylsuccinic acid to crystalline thermolysin, shown in detail in Figs. 2 and 3, is sketched diagrammatically in Fig. 6. For comparison, Fig. 6
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Fig. 4. Portion of section z = 1/8 of the map showing the difference in electron density between benzylsuccinic acid (BZSA) bound to thermolysin and β-phenylpropionyl-L-phenylalanine (β-PPP) bound to thermolysin. Coefficients (F_{BZSA} - F_{β-PPP}). The section is through the thermolysin hydrophobic pocket, and intersects the side chains of Leu 202 and Val 139, and the benzyl groups of the respective inhibitors. The concerted movement of these three groups in going from β-phenylpropionyl-L-phenylalanine:thermolysin to benzylsuccinic acid:thermolysin is indicated by the arrows.

This also shows the presumed mode of binding of an extended substrate inferred from the observed binding of dipeptides (5) and of the transition state analog, phosphoramidon (6). The binding of R_1' is inferred from model building.

It would be expected that the nitrogen of the scissile bond, in going from planar to tetrahedral during hydrolysis, would cause a movement of the R_1' side chain. L-Benzylsuccinic acid contains a tetrahedral center at a position equivalent to that of the nitrogen of a scissile bond, although not at the carbonyl carbon. When the dipeptide Ala-L-Phe is bound to thermolysin (5), the phenyl ring is located close to Leu 202 (this side chain rotates 120° about its Cα-Cβ bond to relieve close contacts with the inhibitor). In contrast, in the case of L-benzylsuccinic acid, the benzyl group is about 0.7 Å further away from Leu 202, and closer to Val 139, which in turn moves slightly away from the inhibitor to avoid unacceptably close approaches (Fig. 4). This suggests that, as one proceeds from the Michaelis enzyme-substrate complex through the transition state to the products of catalysis, there is a movement of the R_1' side chain within the hydrophobic specificity pocket. These adjustments presumably contribute to the different

Fig. 5. Comparison of the geometry of a peptide, the products of hydrolysis, and benzylsuccinic acid.

(a) Peptide

(b) Substrate

Fig. 6. a, schematic diagram illustrating the mode of binding of an extended substrate to thermolysin; b, observed mode of binding of benzylsuccinic acid (BZSA).
binding affinities of the respective species.

As was pointed out above, L-benzylsuccinic acid resembles the collected products of peptide hydrolysis. To the extent that this is a legitimate resemblance, the location of binding of the inhibitor can be taken as an indication of the location of the products immediately following hydrolysis. In particular, the fact that the C1 carboxyl oxygen binds to the zinc in approximately tetrahedral coordination, i.e. at the same location as occupied by the carbonyl oxygen of the substrate, is consistent with the position of this oxygen remaining essentially fixed throughout catalysis, as would be anticipated for general base catalysis. Byers and Wolfenden (11, 12) have argued that the structure of L-benzylsuccinic acid suggests a possible orientation of the incipient products (substrate for the reverse reaction) on the enzyme. Products formed from an acyl-enzyme type intermediate might in principle occupy almost any relative orientation, whereas, if carboxypeptidase (or thermolysin) acts as a general base catalyst for water attack on the bound substrate, the immediate products of hydrolysis would be expected to have a relative configuration as observed for the inhibitor. The observed mode of binding of benzylsuccinic acid to thermolysin, and, by analogy, carboxypeptidase A (see below), strengthens the argument made by Byers and Wolfenden, and can be taken as additional evidence supporting the general base mechanism for peptide hydrolysis.

Unfortunately, it has not been possible to determine crystallographically the mode of binding of benzylsuccinic acid to carboxypeptidase A, as crystals of both native and cross-linked carboxypeptidase A were disordered by a 1 mM solution of the inhibitor (12). Nevertheless, the mode of binding of benzylsuccinic acid to thermolysin determined here, coupled with the close similarity between elements of the active sites of thermolysin and carboxypeptidase A (4), permits us to make a reasonable presumption as to the mode of binding of benzylsuccinic acid to carboxypeptidase A.

The previously determined transformation relating the active sites of thermolysin and carboxypeptidase A (4) was used to transfer the benzylsuccinic acid coordinates from the thermolysin to the carboxypeptidase A active site (9, 10, 21, 22), and the results are shown in Figs. 7 and 8. Obviously, the validity of this transformation relies entirely on the supposition that benzylsuccinic acid binds to both enzymes in a similar manner, but, noting the close correspondence in the binding of dipeptide inhibitors to the respective enzymes, and other structural similarities (4), this assumption seems justified. As can be seen, the benzylsuccinic acid model occupies the carboxypeptidase A active site in a very reasonable manner. In fact, the calculated close approach distances between the transposed benzylsuccinic acid model and carboxypeptidase A (Table III) are remarkably close to the actual values which might be expected for a bound inhibitor. The benzyl group occupies the active site pocket, the C4 carboxyl group forms a salt link with Arg 143 (the characteristic interaction for a carboxypeptidase A substrate), and one of the Cl carboxyl oxygens is liganded to the zinc ion. Because benzylsuccinic acid has a methylene group at C2 rather than a peptide nitrogen, Tyr 248 would not be expected to interact with the inhibitor as it does with glycyl-L-tyrosine (9, 10, 22, 23). The fact that crystals of carboxypeptidase A crack in the presence of benzylsuccinic acid suggests that a conformational change takes place. Also Byers and Wolfenden (12) present spectro-

![FIG. 8. The inferred mode of binding of benzylsuccinic acid to carboxypeptidase A.](http://www.jbc.org/)

**TABLE III**

| Close approach | BZSA* | Distance | Presumed interaction |
|----------------|-------|----------|---------------------|
| Zn O1          |       | 2.3      | Zinc ligand         |
| Tyr 248 OH O2  |       | 3.9      | Hydrogen bond       |
| Glu 270 OE2 O2 |       | 2.9      | Possible hydrogen bonds |
| Arg 145 NH1 O3 |       | 2.2      | "Specificity" hydrogen bonds |
| Arg 145 NH2 O4 |       | 2.0      |                      |
| Ile 243 CD CE1 |       | 3.0      |                      |
| Ile 247 CD CB1 |       | 4.9      |                      |
| Ile 247 CD CD1 |       | 4.8      | Interactions within hydrophobic pocket |
| Ala 250 CB CD1 |       | 4.6      |                      |
| Ile 255 CB CZ  |       | 4.4      |                      |
| Thr 268 CG CE2 |       | 3.0      |                      |
| Thr 268 CG CZ  |       | 3.4      |                      |

* BZSA, benzylsuccinic acid.
scopic and other evidence supporting such a change. Therefore, Tyr 248 might well be in the “down” rather than “up” (away from the zinc) position (24-26), possibly interacting with the Cl carboxyl oxygen of bound benzylsuccinic acid, as suggested in Fig. 8 and in Table III.

The postulated mode of binding of benzylsuccinic acid to carboxypeptidase A shown in Figs. 7 and 8 is consistent with the chemical data presented by Byers and Wolfenden (11, 12). As predicted, the two carboxyl groups of the inhibitor contribute to binding, but only one of them acts as a zinc ligand. Also, the spacing between the two carboxyls (or the distance, d, between the carboxyl oxygen and the R'i a-carbon, in Fig. 5) which gives maximum inhibition, is consistent with that expected from the presumed mode of binding to carboxypeptidase A. Introduction of an additional methylene group, as in benzylgutaric acid, increases the distance d to about 4.5 Å, which is somewhat greater than that for the products shown in Fig. 5, and the binding is weakened about 5-fold. On the other hand, shortening d to 2.3 Å, as in benzyalminic acid, weakens the binding 50-fold.

Dimethylbenzylsuccinate is a very poor inhibitor (K_i > 10^-2 m) presumably because the methyl ester sterically interferes with Arg 145 and prevents tight binding. In contrast, the half-ester L-2-benzyl-3-benzyl-3-carboxypropionic acid binds well (K_i = 9.6 × 10^-6 m), only 1 order of magnitude weaker than the free acid, consistent with the free carboxyl group interacting with Arg 145, and the methyl ester (which could also be a larger group) occupying the R'_i binding site without steric hindrance.

There are two reasons why benzylsuccinic acid does not bind as tightly to thermolysin (K_i = 3.8 mm) as it does to carboxypeptidase A (K_i = 0.45 µm). First, benzylsuccinic acid has a free carboxyl at the R'_i position, which presumably interacts optimally with Arg 145 of carboxypeptidase A, resembling a good substrate. In contrast, thermolysin, an endopeptidase, is presumably because the methylene group at C2 will have different consequences for the two enzymes. For thermolysin, peptides with an amide group will bind very poorly (Kr > 10^-12 M), while an amino group at this position form a hydrogen bond (3.0 Å) to the Ala carbonyl oxygen. In the case of carboxypeptidase A, the favorable interaction between Tyr 248 and the amide nitrogen of a peptide substrate will again not be possible for benzylsuccinic acid but, in this case, because of the flexibility of Tyr 248 adjustment of this residue will not be energetically costly and, as mentioned above, Tyr 248 could potentially interact favorably with the second oxygen of the Cl carboxyl liganded to the zinc.

The binding of benzylsuccinic acid to thermolysin, and, by inference, carboxypeptidase A, might also be taken as an indication of the potential mode of binding of ester substrates to the respective enzymes. Although benzylsuccinic acid is not an ester, its binding does not depend on the presence of a peptide nitrogen, indicating that similar binding could be reasonably expected for an ester. This suggests that esters may bind analogously to peptides, i.e. with the carbonyl oxygen of the scissile bond liganded to the zinc.

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