Expression of Functionality of α-Chymotrypsin

AN ALTERNATE BINDING MODE IN THE SUBSTRATE SPECIFICITY SITE*

(Received for publication, December 9, 1976, and in revised form, August 19, 1977)

ALEXANDER TULINSKY, IRENE MAVRIDIS, AND ROBERT F. MANN

From the Department of Chemistry, Michigan State University, East Lansing, Michigan 48824

Three-dimensional 2.8 Å resolution x-ray crystallographic studies show that toluenesulfonamide and pipsylamide bind isomorphously in the aromatic specificity binding site of α-chymotrypsin. However, their orientation differs by about 90° from that usually associated with substrate-like molecules, suggesting a nonproductive binding mode. A secondary binding site is also operative in one molecule of the dimer of the pipsylamide derivative and it is located some 22 Å from the active site; however, this site is not operative in the toluenesulfonamide derivative. Binding of toluenesulfonamide and pipsylamide in the specificity site occurs without inducing any significant changes in the native enzyme structure, in contrast to the behavior observed upon tosylation or upon transition state analogue binding of phenylethaneboronic acid. The structural changes accompanying the formation of the latter derivatives are generally asymmetric with respect to the dimeric structure of α-chymotrypsin and are generally confined to the binding domain or cylinder 2 of the enzyme (sequence >122). These changes are displayed in a new way via diagonal distance map representation.

Early in our work on the structure determination of α-chymotrypsin we investigated the possibility of preparing a heavy atom derivative by selectively alkylating histidine 57 (1) with PPCK' and thereby also labeling the active site region of the enzyme. When Birktoft et al. (2) reported that the tosyl analog of PPCK (TPCK) does not react with α-chymotrypsin via diffusion into crystals, we were puzzled, since we had already observed changes in the intensities of the x-ray diffraction pattern of crystals we were using with loss of pipsylamide.1 This prompted us to investigate the possible interaction of toluenesulfonamide with α-chymotrypsin. We would now like to summarize these studies and their relevance to the apparent PPCK anomaly.

EXPERIMENTAL PROCEDURES

Crystals of α-chymotrypsin were soaked in 30 mM toluenesulfonamide and PPCK solutions of 75% saturated ammonium sulfate at pH 3.6. These showed pronounced changes in the intensities of the x-ray diffraction pattern. The unit cell dimensions are compared in Table I, from which it can be seen that they are quite isomorphous with those of α-chymotrypsin. Three-dimensional sets of 2.8 Å resolution intensity data were collected from these crystals and converted to "best" difference electron density maps in a manner similar to that described elsewhere (3, 4).

RESULTS AND DISCUSSION

The toluenesulfonamide difference electron density map displayed only two prominent regions which were related by the approximate local 2-fold symmetry axis of the dimeric α-chymotrypsin molecule (Fig. 1b) and these regions were located in the aromatic specificity binding sites of the independent molecules. Although the PPCK difference map displays a marked lack of 2-fold symmetry in this region (Fig. 1a), it proved to be very similar to that of the toluenesulfonamide derivative for one of the molecules of the asymmetric unit, suggesting that the original PPCK degraded during prolonged soaking of crystals (months) with loss of pipsylamide and subsequently bound in the specificity site. From Table II and Fig. 1, it can be seen that assuming the pipsylamide and toluenesulfonamide derivatives to be different occupancy isomorphs permits the location of the iodine position of pipsylamide definitively in one of the molecules of the asymmetric unit and the position of the sulfonamide by inference. Moreover, the latter is found to correspond to the larger difference electron density (positions 2 and 2') of the toluenesulfonamide map (Fig. 1c and Table II). Finally, although the toluenesul-
TABLE I

| Crystal                  | a-A    | b-A    | c-A    | β-deg |
|-------------------------|--------|--------|--------|-------|
| α-Chymotrypsin          | 49.24  | 67.20  | 65.94  | 101.79|
| Toluenesulfonamide      | 49.40  | 66.99  | 65.99  | 101.74|
| Pipsylamide             | 49.31  | 67.12  | 65.92  | 101.81|

Fig. 1. Difference electron density between toluenesulfonamide, pipsylamide, and α-chymotrypsin viewed down local 2-fold axis. (a) pipsylamide derivative: solid positive contours at 0.05 eÅ⁻³ beginning with 0.15 eÅ⁻³ in section z ~ 0.59; local 2-fold designated by cross at y = 0.297, z = 0.473; iodine position at center of Peak 1; (b) toluenesulfonamide derivative: same as pipsylamide. Dotted contours: native a-chymotrypsin electron density at ±z = 0.59, beginning with 0.5 eÅ⁻³ (2.8 σ) in intervals of 0.25 eÅ⁻³; probably corresponds to water molecules which are displaced (see text).

Fig. 2. Binding of toluenesulfonamide in specificity site of α-chymotrypsin. Toluenesulfonamide shown shaded; cross designates midpoint between two displaced, hydrogen-bonded water molecules; sulfonamide group shown in arbitrary orientation; local 2-fold axis shown appropriately.

Fig. 3. Binding of phenylethaneboronic acid in specificity site of α-chymotrypsin. Phenylethaneboronic acid shown shaded; borate shown in tetrahedral configuration, one oxygen corresponding to that of a shifted Ser 195 O₂⁻; otherwise, as in Fig. 2.

The phenyl group is practically the same in both, it will be seen that the orientation of the two differs: the sulfonamide of toluenesulfonamide is oriented about 90° from the direction of the borate group of 2-phenylethaneboronic acid; in addition, the planes of the phenyl rings of the two appear to be perpendicular to each other, although this result is not as certain. Whereas the 2-phenylethaneboronic acid stretches to serine 195 in an extended configuration and apparently forms a tetrahedral intermediate at higher pH values, the sulfonamide group of toluenesulfonamide does not interact with the enzyme in any obvious specific way. Since the former has been implicated in the catalytic mechanism of serine proteases...
Alternate Binding Mode in α-Chymotrypsin Specificity Site

(8), while the latter has not, the toluenesulfonamide binding mode probably represents an example of nonproductive binding. The toluenesulfonamide simply binds with the enzyme in a noncovalent way with its functional or polar end directed toward the dimer interface. In the case of a monomeric molecule, the sulfonamide group would be exposed to solvent.

During the course of the binding, both reagents displace, at least partially, two electron density peaks (about 0.88 and 0.62 eÅ⁻³) from each specificity site of the native enzyme (dotted contours, Fig. 11). These peaks probably represent water molecules, since they do not exchange in SO₃⁻-SeO₃⁻ exchange experiments (9), even though one is inordinately large for a solvent oxygen. The two peaks are within hydrogen-bonding distance of each other and the oxygen atoms of serine 190. The midpoint between the two is indicated by a cross in Figs. 2 and 3.

Another point of interest concerns the pipsylamide derivative, which displays an additional binding site some 22 Å from the active site. This secondary site is intramolecular and resides on the surface of the molecule and is in the vicinity of a cluster of 3 tryptophan residues (Trp 27, Trp 29, Trp 207) and proline 28 and is generally covered by part of the A chain, particularly by residue proline 8 (10). The difference electron density reaches a peak height of about 0.30 eÅ⁻³ in this region of the enzyme. Interestingly, the sequence Trp 27-Pro 28-Trp 29 is approximately positionally palindromic to a known crucial portion of the specificity site of α-chymotrypsin (Ser 214-Trp 215-Gly 216) (11). Evidence for the existence of presumed general nonspecific binding or possibly binding at a secondary site has also been reported in solution studies (12, 13). The crystallographic site does not show local 2-fold symmetry and is confined in one molecule of the pipsylamide derivative. This is probably due to the closer approach of the A chain to the tryptophan cluster in one of the molecules of the dimer, thus restricting access to the site. Surprisingly, binding in this secondary site does not occur in the toluenesulfonamide derivative.

A final and most important point of interest concerns the small structural changes which generally accompany derivative formation. In irreversible inhibition with tosyl and in transition state analog formation with 2-phenylethaneboronic acid, we have observed about 25 to 30 small structural changes accompanying the substitution. In these cases and also in competitive inhibition with Form-Trp and Form-Phe, the structural changes are generally confined to one of the two folding domains of α-chymotrypsin (cylinder 2, discovered by B. W. Matthews (2) and elaborated upon by others (14, 15)). This behavior can be seen from Figs. 4 and 5, which display a heretofore unreported way of representing certain important features of a difference electron density map in diagonal.

5 Toluenesulfonamide can be washed out of derivative crystals with 75% ammonium sulfate, pH 3.6, soaking solution as evidenced by the regeneration of the native enzyme diffraction pattern.

6 Thus the difference density in these regions is not a true representation of the substitution and the displaced electron density of the native structure should be added to the difference density.

7 Steitz et al. (7) have reported four interstitial binding sites for Form-Trp in crystals of α-chymotrypsin, but did not elaborate further since they were presumed to be artifacts of the crystalline state. However, since the pH of these crystals was 5.7 (4.2 for their native state) and since it is known that structural transitions occur among pH conformers with increasing pH (4), some of the difference density interpreted as interstitial binding sites might actually have been due to structural changes. Our own work indicates that there is only one additional binding site for Form-Trp at pH 3.6 and that it occurs in the vicinity of NH₂-terminal cysteine 1 of the A chain of α-chymotrypsin.
Alternate Binding Mode in \(\alpha\)-Chymotrypsin Specificity Site

The maps show a plot of the following relationship for all \(ij\) on a square matrix

\[
|C_{ij} - \delta_i| + |C_{ij} - \delta_j| \leq 9.0 \text{ Å}
\]

distance map representation (15, 16). The maps show a plot of the following relationship for all \(ij\) on a square matrix

\[
|C_{ij} - \delta_i| + |C_{ij} - \delta_j| \leq 9.0 \text{ Å}
\]

where \(C_{ij}\) and \(C_{ji}\) are the positions of the \(C^\alpha\) atoms of the \(i^\alpha\) and \(j^\alpha\) residues of \(\alpha\)-chymotrypsin and \(\delta_i\) is the position of the \(k^\alpha\) peak of the difference electron density map of the derivative (17). Since the matrix is symmetrical about the diagonal, we have taken advantage of that fact and plotted the behavior of the independent molecules of the dimer of \(\alpha\)-chymotrypsin on either side of the diagonal. Thus, any lack of mirror symmetry across the diagonal represents asymmetrical behavior of the independent monomers. The two cylinders (2, 14) or independent folding domains of \(\alpha\)-chymotrypsin appear as structurally palindromic counterparts with respect to residue 122 in more conventional distance diagonal map representation, where the distance between \(C_{ij}\) and \(C_{ji}\) atoms is plotted and contoured (15).

From Figs. 4 and 5, it can be seen that: (a) features arising from the substitution proper (tosyl and 2-phenylethaneboronic acid) occur in a fairly similar and symmetrical way in both derivatives (enclosed by broken ellipses); (b) the other features associated with small structural changes accompanying substitution are generally different in the different derivatives and markedly asymmetric; and (c) most of the features corresponding to structural changes are confined to cylinder 2 of \(\alpha\)-chymotrypsin (sequence >122) or the interface between the two cylindrical domains. The specificity site is contained in cylinder 2 or the binding domain, and the latter appears to be more subject to structural changes upon substitution in the active site region than the other domain. Such behavior is compatible with enzyme models alternately described by "induced fit" (18) or as "the fluctuating enzyme" (19) or "mobile defects" (20). The asymmetry of the response of the independent molecules of the dimer is consistent with the observed variability in the tertiary structure of the dimer (10). In the case of the toluenesulfonamide and pipsylamide derivatives, however, substitution occurs without inducing any changes in the native structure (Fig. 6), that is, no significant peaks occur in the difference electron density other than at the sites of substitution. Such behavior is consistent with: (a) the enzyme remaining structurally dormant in nonproductive binding or (b) weak binding which leads to only a few small changes, while strong binding leads to larger and more structural changes. On the other hand, \(K_i\) of the toluenesulfonamide-\(\alpha\)-chymotrypsin complex is about 3.6 mm at 25\(^\circ\) with a \(\Delta H^o\) of -10.3 kcal/mol\(^2\); moreover, the quantities are similar in sign and magnitude to those of the Ac-Trp-\(\alpha\)-chymotrypsin complex (21). Since the enthalpy of binding in these two derivatives is comparable, but since one shows structural changes while the other does not, the structurally dormant enzyme alternative might apply in the toluenesulfonamide case.

From Fig. 6, it can be seen that the interactions arising from the substitution of toluenesulfonamide are grossly similar to those observed with tosyl and 2-phenylethaneboronic acid (Figs. 4 and 5) and that the toluenesulfonamide substitution shows excellent 2-fold symmetry. The latter is not the case with the secondary substitution site of pipsylamide (Fig. 6). Although the secondary site appears to be in the vicinity of residues interacting with alanine 158 (proline 8, glutamic acid) (Figs. 4 and 5) and that the toluenesulfonamide substitution proper enclosed by broken ellipse blocked at \(\leq 9.0\) Å; other interactions due only to pipsylamide secondary site blocked at \(\leq 15.0\) Å; no other difference density peaks with \(\Delta_\rho > 0.12\) eÅ\(^{-3}\) in either map.

20, threonine 138), it is actually located considerably closer to proline 8 and the indole side chains of tryptophan 27 and tryptophan 207, which leads to some interesting observations.

In Figs. 4 and 5, there are features near 138 and 158 corresponding to the secondary site of the pipsylamide substitution (Fig. 6). These are the result of local interactions with the substitution in the case of pipsylamide but "induced fit" for tosyl and 2-phenylethaneboronic acid. Since the members of the catalytic triad (histidine 57, aspartic 102, serine 195) of \(\alpha\)-chymotrypsin occur in the two different structural domains and their close spatial proximity arises from a close interdomain contact, the two-domain palindromic structure of \(\alpha\)-chymotrypsin has one catalytic site. However, the same does not necessarily apply to the specificity site region (Ser 214-Trp 215-Gly 216). Since this region resides wholly in cylinder 2, the palindromic implication is that there might be another similar site in the region of Trp 29-Gln 30-Val 31, which is astonishingly close to the secondary binding site region of pipsylamide. Notwithstanding such a coincidence, the full and exact significance of the secondary site remains obscure.

Acknowledgments—We would like to thank Dr. D. J. Duchamp, The Upjohn Co., Kalamazoo, Mich., for the use of a computer graphics facility and L. D. Weber and L. S. Hibbard for perfecting the diagonal distance representation of a difference electron density.

REFERENCES
1. Schellmann, G., and Shaw, E. (1963) Biochemistry 2, 252-255
2. Birkoft, J., J., Blow, D. M., Henderson, R., and Steitz, T. A.
Alternate Binding Mode in α-Chymotrypsin Specificity Site

(1970) Philos. Trans. R. Soc. Lond. B235, 67-76
3. Tulinsky, A., Mani, N. V., Morimoto, C. N., and Vandlen, R. L. (1973) Acta Crystallogr. Sect. B 29, 1309-1322
4. Vandlen, R. L., and Tulinsky, A. (1973) Biochemistry 12, 4193-4200
5. Ford, L. O., Johnson, L. N., Maehin, P. A., Phillips, D. C., and Tjien, R. (1974) J. Mol. Biol. 88, 349-371
6. Henderson, R. (1970) J. Mol. Biol. 54, 341-354
7. Stitz, T. A., Henderson, R., and Blow, D. M. (1969) J. Mol. Biol. 44, 357-348
8. Matthews, D. A., Alden, R. A., Birktoft, J. J., Freer, S. T., and Kraut, J. (1973) J. Biol. Chem. 248, 7120-7126
9. Tulinsky, A., and Wright, L. H. (1973) J. Mol. Biol. 81, 47-56
10. Tulinsky, A., Vandlen, R. L., Morimoto, C. N., Mani, N. V., and Wright, L. H. (1973) Biochemistry 12, 4185-4192
11. Segal, D. M., Powers, J. C., Cohen, G. H., Davies, D. R., and Wilcox, P. E. (1971) Biochemistry 10, 3728-3738
12. Fersht, A. R., and Requena, Y. (1971) J. Mol. Biol. 69, 279-290
13. Blair, T. T., Marini, M. A., and Martin, C. J. (1972) FEBS Lett. 20, 41-43
14. Birktoft, J. J., and Blow, D. M. (1972) J. Mol. Biol. 68, 187-240
15. Nishikawa, K., and Ooi, T. (1974) J. Theor. Biol. 51, 351-374
16. Phillips, D. C. (1972) in British Biochemistry, Past and Present (Goodwin, T. W. ed) pp. 11-28, Academic Press, London
17. Liebman, M. N. (1977) Ph. D. thesis, Michigan State University
18. Koshland, D. E. (1958) Proc. Natl. Acad. Sci. U. S. A. 44, 98-104
19. Careri, G. (1975) in Quantum Statistical Mechanics in Natural Sciences (Kursunoglu, B. K., Mintz, S. L., and Widmayer, S. D., eds) pp. 15-35, Plenum Publishing Co., New York
20. Lumry, R., and Rosenberg, A. (1976) Colloq. Int. Cent. Natl. Rech. Sci. 246, 53-62
21. Schultz, R. M., Kounovski-Panayiotatos, A., and Peters, J. R. (1977) Biochemistry 16, 2184-2202
Expression of functionality of alpha-chymotrypsin. An alternate binding mode in the substrate specificity site.
A Tulinsky, I Mavridis and R F Mann

J. Biol. Chem. 1978, 253:1074-1078.

Access the most updated version of this article at http://www.jbc.org/content/253/4/1074

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/253/4/1074.full.html#ref-list-1
Additions and Corrections

Vol. 252 (1977) 1099–1101

A soybean trypsin inhibitor. Crystallization and x-ray crystallographic study.

David L. Hwang, Donald E. Foard, and Chin Hsuan Wei

The space group and unit cell dimensions were in error, owing in part to the extremely small size of the crystals then available.

With an improved method of crystallization, much larger crystals have been obtained, and it was found that the true space group is P2₁2₁2₁, with unit cell parameters a = 52.39, b = 43.25, and c = 28.67 Å (measured at 4° and refined by the least squares method). Regrettably, the space group was reported as P2₁ with unit cell parameters a = 25.92, b = 43.23, c = 19.91 Å, and β = 103.63° (measured at 24°). The correct monoclinic axes A, B, and C are related to the incorrect orthorhombic axes a, b, and c by the transformation

\[
A = a - 2c  \\
B = b  \\
C = a + c.
\]

The precession photograph (Fig. 2) of the hkl zone based on the incorrect choice of axes therefore corresponds to the HKH zone based on the correct choice of axes.

By this transformation, the correct orthorhombic cell parameters can be calculated as \(A = 52.37\), \(B = 43.23\), and \(C = 28.72\) Å which are in close agreement with the newly observed values. The unit cell volume for the orthorhombic cell is thrice that for the monoclinic cell.

The revised \(V_M\) value is 2.39 Å³/dalton on the basis of the assumption that the asymmetric unit contains 1 molecule. The solvent content of the crystal was re-estimated to be 51% by volume. The diffraction data extend to well beyond 2 Å spacings.

Three heavy atom derivatives have been obtained with the use of uranium, platinum, and gold reagents, and x-ray data sets have been collected to 2.8 Å for the native crystals and the uranium derivative, and to 5.6 Å for the platinum and gold derivatives. The determination of the three-dimensional structure is currently under way in our laboratory.

Vol. 253 (1978) 1074–1078

Expression of functionality of α-chymotrypsin. An alternate binding mode in the substrate specificity site.

Alexander Tulinsky, Irene Mavridis, and Robert F. Mann

Page 1076, Fig. 4 legend

Line 5 should read "\(|Δρ| > 0.16 \, \text{eÅ}⁻³\)" instead of "\(Δρ > 0.16 \, \text{eÅ}⁻³\)."

Page 1077, Line 3

"\(|C_i' - δ_i\) + \(|C_j' - δ_j\| < 9.0 \, \text{Å}\" should read "\(|C_i' - δ_i| + |C_j' - δ_j| ≤ 9.0 \, \text{Å}\)."

Page 1077, Fig. 6 legend

Line 7 should read "\(|Δρ| > 0.12 \, \text{eÅ}⁻³\)" instead of "\(Δρ > 0.12 \, \text{eÅ}⁻³\)."

Vol. 253 (1978) 2501–2503

Inactivation of uridine nucleosidase in yeast. Purification and properties of an inactivating protein.

Giulio Magri, Giancarlo Pallotta, Paolo Natalini, Silvestro Ruggieri, Ivano Santarelli, and Alberto Vita

Page 2502, Fig. 1 legend

The sentence "Activity and pH determination was performed immediately." should be changed as follows: pH determination was performed immediately, whereas activities were measured after dialysis of each fraction against 0.02 M sodium phosphate buffer, pH 7.1.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.