Kinetic analyses led to the discovery that N-acetylated tripeptides with polar residues at P$_2$ are inhibitors of porcine pancreatic elastase (PPE) that form unusually stable acyl-enzyme complexes. Peptides terminating in a C-terminal carboxylate were more potent than those terminating in a C-terminal amide, suggesting recognition by the oxy-anion hole is important in binding. X-ray diffraction data were recorded to 0.95-Å resolution for an acyl-enzyme complex formed between PPE and N-acetyl-Asn-Pro-Ile-CO$_2$H at pH 5. The accuracy of the crystallographic coordinates allows structural issues concerning the mechanism of serine proteases to be addressed. Significantly, the ester bond of the acyl-enzyme showed a high level of planarity, suggesting geometric strain of the ester link is not important during catalysis. Several hydrogen atoms could be clearly identified and were included within the model. In keeping with a recent x-ray structure of subtilisin at 0.78 Å (1), limited electron density is visible consistent with the putative location of a hydrogen atom approximately equidistant between the histidine and aspartate residues of the catalytic triad. Comparison of this high resolution crystal structure of the acyl-enzyme complex with that of native elastase at 1.1 Å (2) showed that binding of the N-terminal part of the substrate can be accommodated with negligible structural rearrangements. In contrast, comparison with structures obtained as part of “time-resolved” studies on the reacting acyl-enzyme complex at >pH 7 (3) indicate small but significant structural differences, consistent with the proposed synchronization of ester hydrolysis and substrate release.

Because of the historical importance of the serine proteases in studies on enzyme catalysis and continuing medicinal interest in their inhibition, the details of their catalytic mechanism remain of interest. For some time there has been a consensus on the overall sequence of steps and key residues involved (4, 5). Catalysis is initiated by the noncovalent binding of the polypeptide substrate to an active site cleft. After attack by a nucleophile, the scissile amide bond, the acylation phase of the reaction proceeds via the formation of a tetrahedral oxy-anion intermediate that collapses to form an acyl-enzyme (ester) complex with concomitant release of the C-terminal product fragment. In the decylation phase of catalysis, attack of a water molecule onto the ester bond results in a second tetrahedral intermediate that collapses, releasing the N-terminal product fragment and regenerating the vacant enzyme.

Pioneering work on the structural biology of the serine protease family (4, 6–10) led to the concept of a conserved active site catalytic triad formed by active site serine, histidine, and aspartate residues. A crucial role as a general base was postulated for the conserved histidine, i.e. it deprotonates both the nucleophile serine in the acylation phase and the “hydrolytic” water during the deacylation phase. A hydrogen bond between the active site histidine and a negatively charged aspartate was proposed to stabilize the positive charge on the histidine that arises during catalysis. In addition to the identification of the catalytic triad, the early x-ray structures also indicated that the peptide amides of the active site serine and a neighboring glycine could act as H-bond donors, which create an “oxy-anion” hole serving to stabilize the two tetrahedral intermediates formed during catalysis.

The availability of cryo-cooling techniques and synchrotron radiation sources has recently enabled significant improvements in the resolution of crystal structures to be obtained for several members of the serine protease family. X-ray structures of subtilisin (1), trypsin (11), elastase (2), and proteinase K (12) have all recently been reported at atomic resolution (13, 14). In addition to improving the accuracy of the atomic coordinates of the native enzymes, multiple occupancies for a number of residues have been revealed (1, 2, 11, 12), as have details of substrate binding in the S$_1$ specificity pocket of trypsin (11). From the highest resolution structure, that of subtilisin at 0.78-Å resolution (1) obtained at pH 6, crystallographic evidence was presented for an enhanced H-bond between the active site histidine and aspartate, for which the hydrogen was located approximately equidistant between the two residues (1.2 Å from His-57 N$_{31}$ and 1.5 Å from O$_{62}$ of Asp-102). This observation supported an earlier suggestion deriving from NMR studies (15). The precise nature of this hydrogen bond, which has been termed a “low barrier hydrogen bond” (15) or a “catalytic hydrogen bond” (1), has been controversial (2, 16).
Analysis of Ac-XPI

| Inhibitor         | $K_i$  | $IC_{50}$ |
|-------------------|--------|-----------|
| Ac-Glu-Pro-Ile    | 0.97 ± 0.36 | 3.1 ± 0.5 |
| Ac-Asp-Pro-Ile    | 3.3 ± 0.4  |           |
| Ac-Ser-Pro-Ile    | 0.34 ± 0.10 | 1.2 ± 0.1 |
| Ac-Hse-Pro-Ile    | 0.11 ± 0.04 | 0.42 ± 0.05 |
| Ac-Asn-Pro-Ile    | 0.89 ± 0.12 | 1.2 ± 0.2 |
| Ac-Gln-Pro-Ile    | 0.095 ± 0.009 | 0.34 ± 0.08 |
| Ac-Orn-Pro-Ile    | 0.018 ± 0.001 | 0.24 ± 0.06 |

Analysis of Ac-VXPi

| Inhibitor         | $K_i$  | $IC_{50}$ |
|-------------------|--------|-----------|
| Ac-Val-Glu-Pro-Ile| 0.76 ± 0.03 | 2.4 ± 0.3  |
| Ac-Val-Tyr-Pro-Ile| 6.4 ± 0.2  |           |
| Ac-Val-His-Pro-Ile| 1.7 ± 0.8  | 2.6 ± 0.5  |
| Ac-Val-Lys-Pro-Ile| 1.3 ± 0.3  |           |
| Ac-Val-Met-Pro-Ile| 2.4 ± 0.1  |           |
| Ac-Val-Gln-Pro-Ile| 0.42 ± 0.03 | 1.1 ± 0.2  |
| Ac-Val-Asp-Pro-Ile| 2.5 ± 1.6  |           |
| Ac-Val-Hse-Pro-Ile| 2.4 ± 0.2  |           |
| Ac-Val-Ser-Pro-Ile| 2.7 ± 0.2  |           |
| Ac-Val-Asn-Pro-Ile| 0.87 ± 0.30 | 2.0 ± 0.9  |
| Ac-Val-Orn-Pro-Ile| 0.72 ± 0.08 |           |

Analysis of other inhibitors

| Inhibitor         | $K_i$  | $IC_{50}$ |
|-------------------|--------|-----------|
| Ac-Gln-Pro-Ile-NH2| 0.69 ± 0.08 | 1.2 ± 0.2  |
| Ac-Gln-Pro-Ile-Ala-Ala- Ala-NH2 | 0.40 ± 0.06 | 1.0 ± 0.4 |

The unusual combination of a structure of native elastase at atomic resolution (2), the ability of PPE to form crystals of a catalytically relevant acyl-intermediate (17), and the availability of a high resolution structure of the tetrahedral intermediate of the deacylation pathway (3) motivated attempts to improve the resolution of the x-ray structures of acyl-enzyme complexes formed between PPE and peptide substrates. Of particular interest was the planarity of the ester group in the acyl-envelope complex, and the hydrogen bond between Asp-102 and His-57. The original PPE-BCM7 structure (17) showed a H-bonding interaction up to the $P_g$ glutamic acid residue and Gln-192 of PPE, whereas the remaining inhibitor residues were disordered and possibly interfered with crystal contacts. Recent kinetic studies on BCM7 analogues (19) with a truncated N terminus indicated that significant inhibition of PPE could be effected by simple tri-, tetra-, and pentapeptides terminating in a carboxylic acid. We therefore prepared a series of tri- and tetrapeptide analogues of BCM7 with the $P_g$ glutamic acid replaced by a range of other amino acids (N-acetyl-X-Pro-Ile-CO$_2$H and N-acetyl-Val-X-Pro-Ile-CO$_2$H) and screened these for inhibition of PPE. Co-crystallization of PPE with the N-acetyl-Asn-Pro-Ile-CO$_2$H inhibitor proved successful, and an x-ray diffraction data set to 0.95Å resolution of this complex with good inhibitor occupancy was obtained. Here we report the atomic resolution structure of this acyl-envelope complex and discuss structural issues concerning the mechanism of serine protease catalysis, which can be addressed as a result of the improvements in the accuracy of the atomic coordinates as well as from other features of the electron density map.

** MATERIALS AND METHODS **

**Kinetic Assays**—Kinetic assays and numerical analyses were performed as described previously (19).

**Substrate Preparation**—Human BCM7 and its N-terminally truncated derivatives were synthesized, deprotected, and purified as described previously (19).

**Cryocrystallization**—The PPE-N-acetyl-Asn-Pro-Ile-CO$_2$H complex was crystallized via hanging drop vapor diffusion. A filtered (0.22-μm) solution of 50 mg ml$^{-1}$ PPE and 35 mg ml$^{-1}$ N-acetyl-Asn-Pro-Ile-CO$_2$H in 100 mM sodium acetate (pH 5.0) was mixed with 50 mM Na$_2$SO$_4$ well solution (3 μl). Data were collected at beamline ID14/EH3 of the European Synchrotron Radiation Facility (Grenoble, France) tuned to a wavelength of 0.93 Å. Data were collected at 100 K using a MarCCD detector (MarResearch) to a resolution of 0.95 Å in two passes. For the high resolution pass, the detector was offset to a 29° angle of 20°. Data were processed with programs MOSFLM and SCALA (CCP4 suite) (20). $R_{merge}$ was 4.3% for all data and 34.7% for the highest resolution shell (0.95–0.97 Å). Data were 92.7% complete (60.9% in the highest resolution shell) with 1,038,838 observed reflections, of which 128,641 were unique. The overall $R_{free}$ was 5.9 (2.1 in the highest resolution shell). A total of 5% of the reflections (6,432) were randomly selected to provide a test set for $R_{free}$ calculations.

**Structure Refinement**—The PPE-BCM7 structure (Protein Data Bank identification code 1qix) was used as the starting model (excluding the peptide). The protein moiety of the initial model was subjected to one round of initial refinement by both simulated annealing and conjugate gradient minimization methods. The peptide (N-acetyl-Asn-Pro-Ile-CO$_2$H), the conserved calcium and sulfate ions, and 154 well defined waters (4 σ) were added with CNS. The waters and the protein model were checked and rebuilt manually using O (22). Subse-
sequent refinement steps reduced the $R$-factor and $R_{\text{free}}$ to 21.9 and 22.2%, respectively. At this point, restrained anisotropic $B$ factors were introduced and the structure was refined using conjugate-gradient least-squares minimization with SHELXL-97 (13). The highest resolution limit was extended to 0.95 Å. Intensities were used instead of $F$ values, but care was taken to preserve the integrity of the $R_{\text{free}}$ set. In this step alone, the $R$-factor and $R_{\text{free}}$ were reduced by 5.0 and 4.2%, respectively. The improved phases allowed better modeling of the dis-

![Figure 1](http://www.jbc.org/)
ordered parts of the molecule and the extension of water structure. In particular, 19 residues (23, 58–61, 83, 93, 101, 110, 161, 174, 188A-189, 206, 217A-218, and 244-245) were modeled as disordered and 331 water molecules, of which 117 had an occupancy of 0.5, were included. Within the substrate binding cleft, the peptide inhibitor was incorporated into the model and its occupancy was refined as an additional parameter. To avoid the distortion of anisotropic displacement parameters of the peptide atoms, five water molecules and a sulfate ion were included within the model with a complementary occupancy. Refinement converged to a final occupancy of 60% for the bound inhibitor. At this step the $R$-factor and $R_{free}$ were 13.2 and 15.8%, respectively.

During the final stages of refinement, all riding hydrogens except for hydroxyl groups, histidine N$_{\text{H9254}}$ and N$_{\text{H9280}}$, and the hydrogens of His-57 were added to the model to give a final $R$-factor of 12.3% and free $R$-factor of 14.8%. Inclusion of all data in the refinement did not change the $R$-factor. The ester linkage between the peptide and Ser-195 was simulated unrestrained in the SHELXL refinement steps. Additionally an unrestrained full matrix inversion was performed with the least squares command of SHELXL-97 (BLOC 1, DAMP 0 0) to obtain estimated standard deviations for the atom coordinates.

Quality Check and Analysis of the Structure—The quality and stereochemistry of the model was monitored during the refinement with PROCHECK and WHATCHECK (23, 24). The analysis of anisotropic displacement parameters was performed with PARVATI (25). ESCET 0.1h1 was used (26) to identify regions of the protein that did not change structure upon the binding of the peptide. Additionally an unrestrained full matrix inversion was performed with the least squares command of SHELXL-97 (BLOC 1, DAMP 0 0) to obtain estimated standard deviations for the atom coordinates.

RESULTS AND DISCUSSION

Kinetic Assays—A series of tri- and tetrapeptides based on N-terminally truncated β-casomorphin-7, N-acetyl-X-Pro-Ile- CO$_2$H, were synthesized. The P$_1$ residue was targeted for variation because the original BCM7 peptide contained a polar residue at this position (Glu) and an analogue with alanine at this position did not display significant inhibitory activity (17). These results contrasted with most previous proposals, which
analyzed: N-acetyl-Val-Gln-Pro-Ile-CO₂H derived from BCM7 with IC₅₀ inhibitors were all similar to that of the parent tetrapeptide and peptides terminating in a C-terminal amide rather than a carboxylate. The two smaller peaks at 25,916 and 24,144 Da gave a major peak at 26,284 Da, corresponding to the formation of an acyl-enzyme complex. Two smaller peaks at 25,916 and 24,144 Da were also apparent, and these correspond to native PPE with one or two noncovalently bound molecules of N-acetyl-Asn-Pro-Ile-CO₂H, respectively. When the cone voltage was raised from 75 to 115 V, this second smaller peak at 26,688 Da disappeared, indicating a nonspecific interaction. This noncovalent binding of small peptides within the active site is preceded, because previous crystallographic studies using acetylated tripeptides have identified the ability of small peptides to bind in multiple conformations within the PPE active site (18).

X-ray Structure of the Acyl-Enzyme Complex at 0.95-Å Resolution—As described under “Materials and Methods,” co-crystallization of N-acetyl-Asn-Pro-Ile-CO₂H in complex with PPE yielded crystals after 24 h of growth at pH 5. These crystals diffracted to 0.95-Å resolution at beamline ID14/EH3 of the European Synchrotron Radiation Facility (Grenoble, France). The crystal structure was refined with 330 water molecules, and the root mean square deviation error in the atomic coordinates was 0.067 Å. The complex was refined with an occupancy of 60% for the covalently bound peptide analogue. The remaining occupancy of 40% was refined with an additional five ordered water molecules located in the peptide binding cleft. These five water molecules were located 0.54 Å from C₅₋₆ of Ile-7 (Wat-310), 2.32 Å from O of Pro-6 (Wat-311), 0.66 Å from C₅₋₆ of Pro-6 (Wat-312), 0.65 Å from O of Asn-5 (Wat-313), and 1.99 Å from the methyl carbon of the terminal acetyl group (Wat-314). In addition, a sulfate molecule was modeled with one of its oxygens located in the oxy-anion hole and another in a position to form a hydrogen bond with N₆ of His-57. A sulfate in an analogous position has been reported in all native PPE structures (2, 30). Crystallographic refinement converged to a final R_w = 12.3% and R_free = 14.8% (Table II).

![Image](http://www.jbc.org/)

Fig. 1A shows the 2Fₒ – F_c calc SIGMA A weighted electron density map (31) for the peptide analogue bound within the binding site cleft contoured at 1.7 σ (blue) and 4.0 σ (yellow). At this resolution the electron density is continuous between covalently bonded atoms at the lower contour level, and individual atoms of PPE are well resolved at the higher contour level. Similarly, electron density peaks at 1.7 σ are localized about the positions of the individual atoms of the inhibitor. Despite the inhibitor being present with an occupancy of only 60%, the positions of all its atoms (except the side chain of Asn-5) could be defined with confidence, having a root mean square coordinate error of 0.050 Å. Also clearly visible in the electron density is a water molecule (Wat-305) located in the same position as that previously assigned (3, 17) as the hydrolytic water, because this water molecule is positioned to form a H-bond with His-57, which is presumably protonated on its N₆₋₇ (at pH 5.0; Fig. 2A).

As with the binding of the BCM7 heptapeptide (17), the N-acetyl-Asn-Pro-Ile-CO₂H inhibitor is bound in a productive manner extending a six-stranded antiparallel β-barrel structure by one additional strand. The backbone nitrogen of Val-216 is in position to serve as a H-bond donor, and the carboxyl oxygens of Val-216 and Ser-214 are in position to serve as H-bond acceptors, for the bound inhibitor (Fig. 1). The only distinction between this H-bonding pattern and that for the PPE-BCM7 complex (17) is that the carbonyl oxygen of Val-216 forms a H-bond to the nitrogen of amide backbone of the Glu...
The 2P residue has been mutated to an B, the x-ray structure of BCM7 complex (3), indicating a relatively high flexibility PPE was also observed during the deacylation reaction of the conformation. A change in the conformation of this side chain necessary to model the side chain of Arg-217 with a dual .

Local density map can be seen adjacent to .

The 2102. 

mediate vicinity of His-57 and Asp-

tron density map can be seen adjacent to .

The PPE-inhibitor complex at 0.95 Å resolution. The backbone atoms of elastase overlay almost perfectly in this region, with a root mean square deviation of 0.102 Å for these three residues, whereas the overall deviation is 0.102 Å for the entire PPE. The high level of similarity for the backbone atoms between the two structures suggests the structure of the PPE binding cleft is pre-tuned to accommodate binding of the N-terminal region of substrates, but does not preclude movement of side chains during substrate binding or product release.

Geometry of the Ester Bond—An issue, which can be addressed by the 0.95-Å resolution structure of the acyl-enzyme complex, is the extent to which the ester [C-C(=O)-O] deviates from planar geometry. It has been speculated that, during serine protease catalysis, the formation of H-bonds between the ester carbonyl oxygen and the oxy-anion hole induces strain on the ester-bond, resulting in distortion of the ester toward sp3 hybridization, thereby enhancing the rate of formation of the second tetrahedral intermediate. The x-ray structure of the PPE-BCM7 complex at 1.9-Å resolution suggested that the ester [C-C(=O)-O] might be distorted from planarity by -24°, showing a flattened pyramidal structure (17). At 0.95-Å resolution, however, it is apparent that there is virtually no distortion of the ester from planarity in this regard (Fig. 2A), with the carbonyl carbon lying only 0.05 Å above the plane formed by Oγ of Ser-195, and Cα, and the carbonyl oxygen of Ile-7. The conformation of the ester in the acyl-enzyme complex is clearly in a cisoid conformation as preferentially adopted by small molecule esters, presumably because of secondary orbital interactions (n to σ*), with a O–C–O-C torsion angle of 18°, i.e. close to planarity. As was stated in the earlier work, it appears that the pyramidal distortion lay within the experimental error of the refined structure at 1.9-Å resolution. Thus, based on the available evidence, albeit not accrued at catalytic pH, the acyl enzyme intermediate does not appear to be geometrically strained with respect to distortion from planarity of the ester.

As shown in Fig. 2B and Table III, the length of the C=O ester bond in the PPE:Ac-NPI complex is 1.19 ± 0.05 Å (Fig. 2B), which is the same as that observed (within error) for small molecule structures for esters, e.g. in the case of ethyl acetate (Cambridge Crystallographic Data Centre identification code KEFNUE). In the x-ray structure of ethyl acetate taken from the 0.94-Å resolution structure of actinomycin D (entry 1a7y in the Protein Data Bank), the distance is also 1.2 Å (Fig. 2C). In the PPE:N-acetyl-Asn-Pro-Ile-CO2H structure, a small perturbation of the bond angles of the ester bond is apparent relative to small molecule esters, with the angle from C=O to C-O being 129° (122° in ethyl acetate); that from C=O to C-C is 116° (126° in ethyl acetate), and that from C=C to C-O is 115° (112° in ethyl acetate). This perturbation may be in part caused by the H-bonds of the ester carbonyl oxygen in the oxy-anion hole (Fig. 2A). Although there is no direct evidence from this atomic resolution analysis for polarization of the ester

![Table IV: Distances between selected atoms of the catalytic site](image)

| Interaction | Distance Å |
|-------------|------------|
| Ile-7 N–Ser-214 O | 3.04 ± 0.03 |
| Asn-5 O–Val-216 N | 2.97 ± 0.03 |
| Asn-5 N–Val-216 O | 2.71 ± 0.03 |
| His-57 Nδ–Asp-102 Oδ | 2.67 ± 0.02 |
| His-57 Nγ–Asp-102 Oδ1 | 3.47 ± 0.02 |
| His-57 Nδ–Wat-305 | 2.65 ± 0.06 |
| Wat-305–Ile-7 C | 2.78 ± 0.07 |
| Gly-193 N–Ile-7 O | 2.85 ± 0.03 |
| Gly-195 N–Ile-7 O | 2.72 ± 0.03 |
| Ser-214 O–Asp-102 Oδ2 | 2.68 ± 0.02 |
| Ser-214 Oα–His-57 Cα | 3.10 ± 0.03 |
carbonyl, IR studies suggest that it may be a factor in catalysis (32).

Observation of Hydrogen Atoms at 0.95-Å Resolution—

Atomic resolution x-ray diffraction data offer the possibility of observing hydrogen atoms in the $F_{\text{obs}} - F_{\text{calc}}$ electron density map (1) and thereby enlightening the catalytic mechanism. Hydrogen bonds, including those formed in the oxy-anion hole (Fig. 2, A and B), play key roles in catalysis by serine proteases. However, as with other atomic resolution structures of serine proteases reported to date (1, 2, 11), the amide hydrogens of the oxy-anion hole could not be resolved. Nevertheless, in one particularly well ordered region of the electron density illustrated...
in Fig. 3, positive $F_{\text{obs}} - F_{\text{calc}}$ electron density peaks (green) are visible at 2.5 \( \sigma \) immediately adjacent to the amide nitrogens of Val-67, Gly-69, and Ser-32. These atoms serve as H-bond donors for an antiparallel \( \beta \)-sheet, and it can be seen directly that these protons lie closer to the amide nitrogen of H-bond donors (Val-67, Ser-32, and Gly-69) than to the carbonyl oxygen H-bond acceptors (Ser-32, Val-67, and Wat-301, respectively), and therefore represent normal H-bonds in all cases. An illustration of how the hydrogen atoms lie closer to the donor than acceptor is provided by the region around Wat-301, which serves as both a H-bond acceptor to the amide nitrogen of Gly-69, and a H-bond donor to the carbonyl oxygen of Gln-30.

A debate has recently emerged as to the precise nature of the H-bond between the histidine (His-57) and aspartate (Asp-102) of the catalytic triad. A low energy barrier for this H-bond was implied by NMR studies at low pH on serine proteases (15), which revealed an unusually low field NMR signal for this proton. It was proposed that a catalytic role for this putative low barrier H-bond could be to increase the basicity of the side chain of His-57 and thereby lower the energy barrier for its acceptance of a proton from the active site serine. In turn, this would enhance the nucleophilicity of Ser-195. However, the initial interpretation of NMR spectra has been challenged (16, 33). Recent atomic resolution crystallographic results from a number of serine proteases have not resolved the issue. At pH 5.8 and 0.78-Å resolution, Kuhn et al. (1) reported a H-bond distance of 2.62 Å between His-64 (equivalent to His-57) and Asp-32 (equivalent to Asp-102) of subtilisin, and electron density was observed for a proton approximately equidistant between the donor and acceptor. These results would indicate an unusually strong H-bond between the two residues. In the recently reported 0.98-Å structure of proteinase K from Trichinella spiralis (12) at pH 6.5, an $F_{\text{obs}} - F_{\text{calc}}$ difference density peak was observed between N$_{61}$ of His-69 (equivalent to His-57) and O$_{22}$ of Asp-39 (equivalent to Asp-102) at a significance level of 3 \( \sigma \). In that case the density assigned to a hydrogen atom was associated with the His side chain, being located 1.0 Å from N$_{61}$ of His-69 and 1.6 Å from O$_{22}$ of Asp-39. However, at pH 7–8 and at 1.1-Å resolution, Wurtele et al. (2) reported a H-bond distance between His-57 and Asp-102 of native elastase of 2.70 ± 0.04 Å with the proton (when N$_{61}$ of His-57 was refined with isotropic displacement parameter) being observed to lie 0.8 Å from N$_{61}$ of His-57, thus suggesting a normal H-bond.

In Fig. 4, the $2F_{\text{obs}} - F_{\text{calc}}$ electron density map contoured at 3.5 \( \sigma \) (blue) is shown in the immediate vicinity of His-57. Because the position of the N$_{61}$ and O$_{22}$ can be identified directly by their higher electron density relative to C$_{41}$, this 0.95-Å atomic resolution structure implies that, for this intermediate at pH 5, the catalytic histidine does not adopt a “flipped” conformation as has been suggested (33). Also shown in Fig. 4 is the $F_{\text{obs}} - F_{\text{calc}}$ electron density map, contoured to 1.4 \( \sigma \) (brown). At this level of significance, the electron density map displays considerable noise, and therefore hydrogen atoms cannot be assigned unambiguously. Nevertheless, it is reasonable to interpret electron density as either supporting or opposing other crystallographic results at comparable resolution. As with the work of Kuhn et al. (1), electron density is seen adjacent to C$_{41}$ (although not adjacent to C$_{42}$) of the catalytic histidine, which would indicate a normal position for a hydrogen atom. Furthermore, a weaker peak is seen adjacent to N$_{21}$ of His-57, which is presumably predominantly protonated at pH 5.0 (17). In addition, although weak, a well localized electron density peak arises approximately equidistant between His-57 and Asp-102. Although this peak is too weak to interpret unambiguously, its position correlates almost exactly with that assigned as a proton forming an unusually strong catalytic H-bond in the x-ray structure of subtilisin at 0.78-Å resolution (1).

A possible explanation for the difference between the location of this peak in our work and that assigned as a hydrogen atom by Wurtele et al. (2) in the 1.1-Å resolution structure of native elastase is the difference in pH of the analyses, i.e. our work (like the NMR studies of Frey et al. (Ref. 15)) was performed at acidic pH, whereas the native elastase structure was determined at near neutral pH. The difference between the analyses is also reflected in the crystallographic coordinates, because the structure reported here shows the H-bond distance from N$_{61}$ of His-57 to O$_{22}$ of Asp-102 to be 2.67 ± 0.02 Å (Table IV), which is 0.03 Å shorter than that reported for native elastase (2), but 0.05 Å longer than that reported for the corresponding H-bond in subtilisin (1). As such the present work is consistent with (but does not conclusively establish) an unusual equidistant location for a hydrogen atom in the hydrogen bond between Asp-102 and His-57 under certain conditions. The significance of this in enzymatic catalysis is still a matter of debate. However, it is notable that evidence consistent with the formation of the unusual hydrogen bond is reported only at low pH (i.e. in the present work and that of subtilisin (Ref. 1)), at which His-57 is presumably protonated. With regard to the protonation state of His-57, this structure may better reflect the tetrahedral intermediate rather than the acyl-enzyme intermediate because, at catalytic pH, His-57 is protonated at that stage of enzyme catalysis. Thus, if a specific hydrogen bond involving an “equidistant hydrogen” is involved in catalysis, it may be most relevant at the stages of the two tetrahedral intermediates. Further experimentation or modeling studies may be enlightening with respect to this matter, and theoretical experiments have been initiated (34).

Another type of unusual hydrogen bond has also been proposed in serine proteases, namely a CHO hydrogen bond between the C$_{41}$ of His-57 and the backbone carbonyl O of Ser-214 (33, 35). The high resolution structure of Ac-NPI reveals a distance of 3.10 ± 0.03 Å for C$_{41}$ of His-57 from the carbonyl O of Ser-214, which is within the sum of van der Waals radii of the two atoms. Unfortunately, no electron density is detectable for the corresponding hydrogen atom. Assuming an ideal hydrogen position, bonding angles would be $\xi = 123^\circ$ and $\zeta = 140^\circ$ according to the definition of Derewenda et al. (35), indicating that this interaction may be characterized as a weak hydrogen bond.

**Structural Rearrangements during Catalysis**—The x-ray structure of native elastase has recently been reported to 1.1-Å resolution (2), and that assigned as predominantly reflecting a tetrahedral intermediate along the deacylation pathway to 1.4-Å resolution (3). The atomic resolution structure of the acyl-enzyme complex presented here enables detailed structural comparisons to be performed. Fig. 5A shows an ESCET error-scaled distance difference matrix (26), illustrating differences in the conformation of the catalytic residues, which arise when comparing native elastase (entry 1qnj in the Protein Data Bank) with the PPE-\@acyt-Esn-Pro-Ile-CO$_2$H. The only feature to arise above a 3 \( \sigma \) confidence level (\( \sigma \) is the estimated error of the distances) is a reorientation of the side chain of Ser-195 (−0.2-Å movement of the hydroxyl oxygen) resulting from formation of a covalent bond to the inhibitor. In this context it may be noted that the conformation of the serine side chain in the native structure may be slightly perturbed relative to that in solution by the presence of a sulfate ion. It is striking that the \( \beta \)-strand to which the peptide is “zipped” remains virtually unchanged upon formation of the acyl-enzyme complex (Fig. 1B). Indeed, in all regions of the protein where
structural changes may be anticipated as necessary to accommodate enzyme catalysis, namely for those residues associated in substrate binding and those forming the oxy-anion hole/catalytic triad, the structure is almost indistinguishable from that of native elastase. Furthermore, even the ester bond, which itself is to be cleaved, shows little indication of geometric strain.

In contrast to the structural comparison between the acyl-enzyme and native elastase (Fig. 5A), several structural rearrangements of the catalytic residues are apparent when comparing the structure of the acyl-intermediate with that assigned as predominantly reflecting a tetrahedral intermediate (Fig. 5B). These movements are illustrated in Fig. 5C, which overlays the structures of the acyl- and tetrahedral-intermediates (Protein Data Bank entry 1haz). Because the time-resolved structure was refined at lower resolution (1.4 Å), the accuracy of its atomic coordinates are somewhat reduced, yet significant movements are clearly visible above noise in the error weighted ESCET plot. Probably because of the change in bonding from a planar sp² geometry to a tetrahedral sp³ bonding arrangement, the ester carbonyl carbon of the inhibitor becomes translated in the tetrahedral intermediate. It was proposed that this motion weakens the H-bonds, which zip the inhibitor to the enzyme (Fig. 1, A and B), such that hydrolysis and product release are synchronized (17). Concomitant with the formation of the tetrahedral intermediate, a rotational movement of the side chain of His-57 about Cß-Cγ is apparent. This motion presumably occurs because of deprotonation of Wat-305 (the hydrolytic water) by His-57. Furthermore, in the difference Fourier map calculated between the acyl and tetrahedral intermediates of the PPE-BCM7 complex (Fig. 1B), small but significant movements of the catalytic His-57 and Asp-102 were apparent, and this movement of Asp-102 away from the oxo-anion hole stands out in the ESCET plot (Fig. 5B). Oₓ of Asp-102 is connected by a H-bond to the side chain of Ser-214, and it was previously suggested that this movement of His-57 and Asp-102 could be relayed directly to this serine. Because the carbonyl oxygen of Ser-214 is a H-bond acceptor for the bound inhibitor (Fig. 1, A and B), this movement may also assist with synchronization of the peptide release with the deacylation reaction (3).

CONCLUSIONS

Serine protease catalysis represents one of the best structurally characterized of all enzymatic reactions. Several atomic resolution structures have been reported from this family, as has the tetrahedral intermediate been described at slightly lower resolution. The x-ray structure of an acyl-enzyme complex at 0.95 Å helps to complete the picture of the deacylation pathway at very high resolution. In combination with an atomic resolution structure of the native enzyme (2), it is apparent that there is virtually no geometric strain, either within the protein or the substrate, at this point along the reaction coordinate. By contrast, comparison with the high resolution structure of the tetrahedral intermediate (3) reveals a number of subtle structural rearrangements and supports previously postulated mechanisms for synchronizing the ester bond cleavage with product release. In general, however, it is our view that the combination of structural results are consistent with Warshel’s suggestion that the (prime) driving force for serine protease catalysis is achieved via a “reorganized polar environment,” which provides more electrostatic stabilization to the transition state than occurs in water (36). This proposal does not, of course, preclude catalytic roles for subtle hydrogen transfers and conformational changes, which occur during catalysis.

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