Re-examination of the Charge Relay System in Subtilisin and Comparison with Other Serine Proteases*

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The structure of native subtilisin has been partially refined at 2.0 Å to an R index of 0.23 (unconstrained). During refinement it became clear that the originally assigned side chain dihedral angle, \( \chi_1 = 98^\circ \), for the catalytic Ser-221 was incorrect. A new value for \( \chi_1 = -100^\circ \) was deduced by inspection of a "partial structure" difference-Fourier map, and the new location for O\(_{\gamma}(221)\) is now consistent with difference-Fourier maps obtained previously for boronic acid-subtilisin adducts.

A pH difference-Fourier map of native subtilisin shows no side chain movement at either Ser-221 or His-64 on going from pH 5.9 to 7.5, but the side chain of the buried Asp-32 appears to move about 0.1 Å closer to that of His-64 at the higher pH. These groups compose the catalytic charge relay system.

A fixed solvent molecule, W-202, has been identified as a sulfate ion hydrogen-bonded to components of the catalytic site. Removal of this bound sulfate ion does not affect the positions of the catalytic groups.

An important and unexpected conclusion resulting from partial crystallographic refinement is that there is no hydrogen bond between Ser-221 and His-64 in native subtilisin, or at least that any such hydrogen bond is severely distorted. Indeed O\(_{\gamma}(221)\) is 3.7 Å from N\(_{\varepsilon2}(64)\) and, more significantly, O\(_{\gamma}(221)\) is displaced 2.5 Å from its ideally hydrogen-bonded position. Comparison with the most reliable currently available coordinates of the charge relay residues in trypsin, \( \alpha \)-chymotrypsin, \( \gamma \)-chymotrypsin, and elastase confirms that the same distortion of the Ser-His hydrogen bond is a common feature of serine proteases in general. In contrast, the His-Asp hydrogen bond is in all cases a strong bond with normal geometry. A leaving-group proton-receptor atom in the tetrahedral intermediate is much more nearly in an ideal position to form a hydrogen bond with the catalytic histidine, as deduced from previously determined boronic acid-subtilisin adduct structures. We believe the simplest view of the catalytic function of the His-Asp couple is that it acts as the binding site for a proton in the tetrahedral transition state, or equivalently, as a proton relay station. A moving-histidine mechanism, however, cannot be excluded.

The serine proteases, by definition, contain a unique, unusually reactive serine side chain that forms a transient covalent linkage with the carbonyl carbon atom in the susceptible ester or amide bond of substrates. This serine side chain thus appears to possess an abnormally high intrinsic nucleophilicity, presumably because of some special structural feature of the enzyme. Even before the first x-ray structure determination of any serine protease had been carried out, ample evidence also had accumulated to implicate a histidine side chain in the enzymic mechanism as well (Schoellmann and Shaw, 1963). It was encouraging, therefore, when Blow and co-workers (Matthews et al., 1967; Sigler et al., 1968) reported that the reactive serine side chain in native \( \alpha \)-chymotrypsin, Ser-195, is in a conformation consistent with the existence of a hydrogen bond between O\(_{\gamma1}(195)\) and N\(_{\varepsilon2}\) of His-57. Shortly thereafter, Blow et al. (1969) determined that residue 102 was not an asparagine, as previously believed, but was instead an interior aspartic acid hydrogen-bonded to N\(_{\varepsilon1}\) of His-57. They proposed that such a hydrogen bond system, dubbed a "charge relay system," is polarized because of the buried negative charge of Asp-102 which thereby makes the serine oxygen strongly nucleophilic. This view of the catalytic mechanism received apparently decisive support when the x-ray structure determination of subtilisin revealed an almost identical geometrical arrangement of the Ser-221, His-64, and Asp-32 side chains attached to a main chain which is, however, folded in a totally different way from that of \( \alpha \)-chymotrypsin (Wright et al., 1969; Alden et al., 1970). Subsequently the same geometrical arrangement of Ser-195, His-57, and Asp-102 was also found in other chymotrypsin-homologous enzymes including elastase (Shotton and Watson, 1970), trypsin (Stroud et al., 1974; Bode and Schwager, 1975), and Streptomyces griseus protease B (Coddington et al., 1974).

Even from the beginning, however, it was obvious that the original view of the charge relay system presented some curious problems. Blow et al. (1969) themselves mentioned that the apparent pK\(_a\) of about 7 observed for the pH dependence of enzyme activity would be reasonable for a simple imidazole but that "it is less easy to see what would be the pK\(_a\) of the system" they had proposed. Moreover, one would...
anticipate very little proton transfer to the His-Asp couple from the serine, with a pKₐ of about 14, and thus very little tendency for the serine to exist in the required form of a highly nucleophilic alkoxide ion. Polgár (1972) also pointed out that the charge relay system, as originally formulated, would only promote the first proton transfer, i.e., the general base-catalyzed removal of the proton from serine, but that the second proton transfer, i.e., general acid-catalyzed donation of a proton to the leaving group, would actually be inhibited.

Certain x-ray crystallographic data were also apparently inconsistent with the view that the reactive serine is rendered highly nucleophilic by hydrogen bonding to the His-Asp couple. Chymotrypsinogen, the zymogen precursor of chymotrypsin, was found to contain a hydrogen-bonded charge relay system almost identical in geometry with that of the enzyme (Freer et al., 1970; Kraut, 1971), yet the zymogen is only about 10⁻²₅ as active (Gottlieb et al., 1974). The converse problem is raised by the properties of methyl chymotrypsin, in which His-57 is methylated at Ne2 (Nakagawa and Bender, 1970; Henderson, 1971; Wright et al., 1972). Although it was expected that the modified enzyme would have no activity at all, Henderson (1971) reported that catalytic rates were simply reduced by factors ranging from 2 × 10⁻⁴ to 5 × 10⁻⁴ for specific substrates. Moreover, difference-Fourier maps showed only very slight shifts in the positions of the His-57 and Ser-195 side chains (Wright et al., 1972).

To what extent did the subtilisin structure actually confirm the idea that the catalytic serine side chain is hydrogen-bonded to the catalytic histidine? During the course of examining difference-Fourier maps of certain covalent adducts of subtilisin, it eventually became apparent that our initial assignment for the orientation of the side chain of Ser-221 was probably wrong. Specifically, we could not find the expected holes corresponding to the presumed location from which Oy(221) had been displaced in either phenylmethanesulfonyl subtilisin or the boronic acid derivatives (Wright et al., 1969; Matthews et al., 1975). In addition, the coordinates of Oy(221) tended to shift excessively during our first attempts at crystallographic refinement. Re-examination of the original electron density map, calculated from isomorphous replacement phases, showed that the side chain of Ser-221 was represented by a rather ill-defined blob of electron density. In retrospect, it was evident that we had in fact arbitrarily selected a side chain dihedral angle to agree more or less with that reported for α-chymotrypsin.

**PARTIAL CRYSTALLOGRAPHIC REFINEMENT OF SUBTILISIN**

**Method of Refinement**—In view of the foregoing, an immediate objective was to refine the structure of the native, inhibitor-free enzyme to a sufficient extent that the true position of Oy could be specified with some confidence. In this section we briefly outline the refinement procedure employed.

The starting point was a set of atom coordinates derived from a model that had been built into our original 2.5 Å electron density map obtained with multiple isomorphous replacement phases (Wright et al., 1969; Alden et al., 1971). Structure factor calculations based on this model yielded an R index of 0.44 with newly measured intensity data, now extended to 2.0 Å for the native enzyme. The new data were obtained from crystals grown at pH 5.9 and were extensively replicated both by screenless precession film techniques (Xuong and Freer, 1971) and by diffractometer. In excess of 175,000 individual intensity observations were made on 15,236 symmetry-independent reflections. About half the measurements were made by the film technique.

An initial $F_o - F_i$ difference map was calculated to reveal any gross errors in the starting model (none were found) and to permit some revision of the 150 solvent molecules tentatively located in the original electron density map. Subsequent refinement was carried out by an iterative semiautomated difference-Fourier procedure closely similar to that described by Freer et al. (1975) for the high potential iron protein. The adjustable parameters consisted of three positional coordinates and an isotropic temperature factor for each non-hydrogen atom. Small changes in these parameters were calculated from the slopes and magnitudes of the $F_o - F_i$ difference density at currently assigned atom positions. A total of 11 cycles of refinement have been calculated thus far. Periodically interspersed among these cycles of parameter adjustment were occasional visual inspections of $F_o - F_i$ maps. Much of the manual editing resulting from the latter consisted of adding and deleting solvent molecules. Bond length and angle constraints have not yet been applied.

The present model for native subtilisin arrived at in this manner gives an R index of 0.23 for 2.0 Å data. We estimate, on the basis of our experience in refining other structures, that imposition of bond length and bond angle constraints would probably raise the R index to about 0.30.

**Position of Ser-221 Oy in Native Enzyme**—During the early stages of refinement, when the side chain dihedral angle $\chi_1$ for Ser-221 was assigned its original value of 98°, the Oy atom position suffered large and unreasonable shifts which shortened the Cβ-Oy distance to 0.7 Å (expected value 1.43 Å), further reinforcing our belief that Oy was misplaced. In contrast, Oy behaved normally upon continued refinement when $\chi_1$ was reset to −105°, a value arrived at by inspection of a "partial structure" difference map, to be described next.

A relatively unbiased portrayal of the electron density associated with the Ser-221 side chain was obtained as follows. Contributions from Ca, Cβ, and Oy were subtracted from the calculated structure factors, $F_o$, of the $R = 0.23$ model. Then an $F_o - F_i$ Fourier summation was calculated at 2.0 Å resolution using as observed structure amplitudes, $F_o$, data from intensity measurements made on native subtilisin crystals at pH 5.9. Interpretation of this map with the aid of Kendrew-Watson model parts clearly favored a location for Oy that was only about 1 Å from its position in the covalent boronic acid enzyme complexes (Matthews et al., 1975) where a $\chi_1$ value of about −60° was observed. Several sections of this map showing difference density corresponding to the Ser-221 side chain are presented in Fig. 1. The best choice of position for Oy is labeled OG and the original incorrect position for Oy is labeled OACT. The latter corresponds approximately to the Ser-195 side chain orientation given for α-chymotrypsin (Dickerson and Blow, 1970).

This interpretation is now also consistent with previously calculated difference maps for both phenylmethanesulfonyl subtilisin (Wright et al., 1969) and for the boronic acid subtilisins (Matthews et al., 1975). In these derivatives, a covalent bond is formed between Oy and the sulfur or boron atom of the corresponding inhibitor, necessitating a change in $\chi_1$. If in the native enzyme $\chi_1$ had actually been +98°, with Oy at the position labeled OACT in Fig. 1, a rotation of −158° or movement of Oy by 2.1 Å would have been required. However, in neither case was the expected hole observed at the OACT position. Moreover, the boronic acid adducts yielded particularly clear difference maps, at least partly
owing to the fact that they had been computed with semireefined phases, and in these maps a small hole was actually observed instead at the location labeled OG.

In light of the foregoing, we are now convinced that the true orientation of the Ser-221 side chain in native, active subtilisin crystals at pH 5.9 is characterized by a $\chi_1$ value of about $-100^\circ$ and that formation of a covalent bond to Oy involves rotation of $\chi_1$ by about $+40^\circ$, corresponding to movement of Oy by about 1 Å.

EFFECT OF PH ON THE CHARGE RELAY SYSTEM

Before going on to consider the implications of this new location for Oy of Ser-221, two possible complications must be disposed of. One has to do with whether there is any effect of pH upon the structure of the catalytic site; the other concerns binding of a sulfate ion near the catalytic site and how that may influence its structure. We shall take up the pH question first.

As noted above, one indication that the native Oy position corresponds to $\chi_1 = -100^\circ$ instead of $+98^\circ$ came from interpretation of boronic acid subtilisin difference-Fourier maps (Matthews et al., 1975), in which holes at the former position but not the latter were observed. However, the boronic acid subtilisin adducts had been crystallized at pH 7.5 and so difference maps had been calculated with data obtained from native enzyme crystals at that same pH. On the other hand, crystallographic refinement had been carried out with intensity data obtained from native enzyme crystals at pH 5.9, and strictly speaking, therefore, our conclusion that the Ser-221 side chain angle is $\chi_1 = -100^\circ$ arrived at in this way applies only to the native enzyme at pH 5.9. To establish consistency, it was necessary to calculate a pH difference-Fourier map with coefficients $(F_{\text{obs}} - F_{\text{calc}})$, as already described by Matthews et al. (1975). This map is almost entirely flat, with a root mean square absolute difference density of $\sigma = 0.025$ e/Å$^2$. For present purposes, the most significant conclusion is that the map contains no indication of movement by the side chains of Ser-221 or His-64 caused by changing the pH from 5.9 to 7.5. This observation is entirely in accord with the finding by Mavridis et al. (1974) that little structural change occurs in the $\alpha$-chymotrypsin charge relay system residues Asp-102, His-57, and Ser-195 from pH 2.0 to 9.0.

The pH difference map does, however, contain two features of density that can be interpreted. One is relatively unimportant; it is an area of positive difference density coinciding with several atoms of the Tyr-217 side chain and is probably due to a lower thermal parameter for this group at the higher pH, perhaps because of solvation changes. The more interesting interpretable feature indicates slight movement of the charge relay side chain Asp-32. Evidence for this is visible as a broad negative depression below the Asp-32 side chain and extending down toward Cyl of Val-30 (with which Asp-32 is in van der Waals contact). About 3.5 Å away on the opposite side of the Asp-32 side chain is a set of positive contours of nearly the same density which partially overlap the His 61 side chain. The vector moment (Henderson, 1970) of this feature is approximately 3.1 eÅ in a direction which would represent movement of the Asp-32 side chain toward N61 of His-64. Thus, the side chain of Asp-32 is roughly 0.1 Å closer to N61 of His-64 at pH 7.5 than at pH 5.9. Though small, the effect is probably significant. One possible interpretation is that a stronger and, therefore, shorter hydrogen bond is present between the two side chains at the higher pH. Although it can be argued that such a result favors the proposal of Hunkapiller et al. (1973) concerning the protonation state of the charge relay system, the argument is somewhat tenuous and we shall not further pursue it here.
BINDING OF SULFATE ION NEAR THE CHARGE RELAY SYSTEM

During the course of crystallographic structure refinement on subtilisin, the adjustable isotropic temperature factor of external solvent molecule W-202 decreased rapidly toward a value of zero, and a positive residual difference density peak was observed at the corresponding location in $F_o - F_c$ maps. Such behavior would be understandable if W-202 is actually a sulfate ion instead of a water molecule as we had hitherto assumed. Moreover, observed distances between the center of this object and nearby groups are too large for it to be a hydrogen-bonded water molecule, but are consistent with identification of the object as a bound sulfate ion: 3.7 Å to Ne2 of His-64, 3.4 Å to Oy of Ser-221, and 3.3 Å to Nε2 of Asn-155.

The importance of W-202 is, as these distances indicate, that it is hydrogen bonded to components of the catalytic machinery. Since presumably, if it really is a sulfate ion, W-202 is present only because subtilisin is crystallized from 2.1 M ammonium sulfate and would not be present under physiological conditions, a question arises as to its possible influence upon the three-dimensional arrangement of the catalytic residues as observed in the crystal. In this section we describe experiments showing that W-202 is indeed most probably a sulfate ion and, what is more important, that its removal has no detectable influence upon the geometry of the enzyme.

Replacement by Selenate – The selenate ion SeO$_4^{2-}$ is nearly isostructural with sulfate and therefore readily replaces the latter when protein crystals are transferred into a high selenate medium. Then, because of selenate’s greater electron density, the positions of any locally bound sulfate ions can be readily identified in an appropriate difference-Fourier map (Tulinsky and Wright, 1973).

Ammonium selenate was purified by filtration of an aqueous solution through animal charcoal and subsequent recrystallization. Subtilisin crystals were transferred from their original growth medium of 2.1 M ammonium sulfate buffered at pH 5.9 by 0.05 M acetate into a fresh solution containing 1.9 M ammonium selenate. After a soaking period of 24 h this artificial mother liquor was replaced with a fresh solution. Following a second 24 h soak, a selected protein crystal was mounted for x-ray data collection in the usual manner on a Hilger-Watts automatic diffractometer.

A difference-Fourier map was computed at 2.5 Å resolution with $(F_{nc} - F_{n,o})$ as coefficients and calculated phases obtained from the semirefined model with $R = 0.23$. This map has a root mean square difference density of $\sigma = 0.029$ e/Å$^3$, and its most prominent feature is a peak of height 7σ about 1 Å from the present location of W-202. We interpret this result as confirming identification of W-202 as a bound sulfate ion.

The second largest peak on the difference map has a height of 5σ and is coincident with solvent molecule W-132, which lies on the surface of the protein molecule about 3.2 Å from Nε2 of Lys-136. Thus this solvent molecule is now also identifiable as a weakly bound sulfate ion. A few smaller peaks occurring elsewhere throughout the difference map were not considered significant at this stage of refinement.

Replacement by Polyethylene Glycol – Identification of W-202 as a sulfate ion bound close to the side chains of His-64, Ser-221, and Asn-155 immediately raises a question as to its possible influence on the positions of these key catalytically functional groups. More specifically, one would like to be sure that their relative geometries would not be different in a biochemically more realistic low sulfate medium. Fortunately we have been able to replace the original crystallizing medium with one containing polyethylene glycol instead while maintaining the subtilisin crystals in good condition. Then, with the aid of difference-Fourier methods, we have confirmed that removal of the sulfate ion has no visible structural effect.

An artificial mother liquor was prepared by dissolving 10 g of 6000 to 7000 molecular weight polyethylene glycol in 20 ml of 0.05 M Tris/HCl buffer, pH 7.6. Native subtilisin crystals were transferred into this solution and allowed to soak for 48 h, following which the soaking medium was replaced by a fresh solution and soaking continued for a second 48 h period. Crystals treated in this way appeared to diffract slightly less well than untreated subtilisin crystals but were otherwise seemingly unaffected.

IntENSITY data were collected and a $(F_{PEG} - F_{n,o})$ difference-Fourier map (where PEG is polyethylene glycol) was calculated following usual procedures. This map is slightly more noisy than either the pH difference or SeO$_4^{2-}$-difference map already described. It has a root mean square difference density of $\sigma = 0.039$ e/Å$^3$. The increased noise level probably arises because solvent exchange resulted in some slight disordering or nonisomorphism with respect to the parent structure. However, in accordance with expectation, the most prominent feature in the map is a hole of magnitude 7σ coincident with the position of W-202. This feature is not perfectly spherical but has a distinct "bite" missing from the side closest to Oy of Ser-221, suggesting that in the absence of high sulfate ion concentrations a water molecule is bound at this site instead.

A $(2F_{PEG} - F_o)$ Fourier map provided confirmation for this suggestion by showing an extra peak of positive density at this site, about 3.0 Å from both Oy of Ser-221 and Ne2 of His-64.

A second significant hole in the $(F_{PEG} - F_{n,o})$ difference map, of depth 5σ, is coincident with solvent molecule W-132, further confirming its identification as another bound sulfate ion.

The important conclusion to be drawn from both the selenate and the polyethylene glycol difference maps is that no detectable change in position occurs for the His-64, Ser-221, or Asn-155 side chains upon replacement of bound sulfate W-202 by either selenate or by water. Thus, we can consider these catalytic residues to have the same geometric relationship in the native sulfate-free enzyme as in the parent high sulfate crystals.

Other Serine Proteases Also Bind Sulfate – It is noteworthy that subtilisin is far from unique in having a sulfate ion bind at its catalytic site. Tulinsky and Wright (1973) reported that in α-chymotrypsin a pair of sulfate ions is bound between pairs of molecules making up the dimeric asymmetric unit. As in subtilisin, each sulfate is hydrogen-bonded to the catalytic serine side chain and to the backbone amido group of Gly-193 (functionally analogous to the side chain amide of Asn-155 in subtilisin) of 1 enzyme molecule. In chymotrypsin Sloutin et al. (1971) have ascribed a peak observed in a native – tosyl difference map to displacement of a sulfate ion bound near the side chains of His-77 and Ser-195 and the backbone amido group of Gly-193 in the native enzyme. Earlier, Matthews et al. (1968) had tentatively identified a similarly bound sulfate ion in γ-chymotrypsin by virtue of its displacement upon tosylation of that enzyme. Bode and Schwager (1975) have partially refined the structure of benzamidine-inhibited trypsin, pH 8, at 1.8 Å resolution, and observed that a solvent molecule bound near Oy(195), Ne(257), and N(193) has an unusually high density and large radius.
They too suggest it is a sulfate ion. Moreover, this solvent molecule disappears in native, inhibitor-free trypsin when the pH is raised to 8 without significantly affecting the orientation of the Ser-196 side chain. Thus in this respect the situation is the same for trypsin as for subtilisin.

Protease B from _S. griseus_, a microbial serine protease homologous with the trypsin family, is crystallized from a medium high in phosphate rather than sulfate (Coomb et al., 1974). Nevertheless, an extramolecular peak of electron density is also seen near the catalytic serine side chain and is identified by Delbaere et al. (1975) as two phosphate ions bound in a manner similar to that in which a sulfate ion binds to o-chymotrypsin.

The functional significance, if any, of anion binding at the catalytic site in all serine proteases remains obscure. It should not be surprising, however, to find the same anion-binding geometry in subtilisin as in the trypsin-homologous group since we already know that the catalytic machinery is geometrically very similar in the two families of enzymes (Alden et al., 1970; Robertus et al., 1972). Largely on the basis of this anion-binding phenomenon, Brinigar and Chao (1974) have raised a question as to whether the charge-relay system might in fact carry a net positive charge at pH values between approximately 4 and 6 and accordingly be electrically neutral at higher pH values where enzymic activity is maximal. Though implausible at first glance, this suggestion deserves at least to be ruled out experimentally.

One remaining point concerning the sulfate-binding phenomenon deserves comment. As noted above, raising the pH to 8 in native trypsin crystals caused loss of the bound sulfate (Bode and Schwager, 1975), presumably due to deprotonation of the charge relay system. Similarly, crystalline native elastase in 2 M potassium phosphate at pH 8.5 has no bound anion at the catalytic site (Shotton et al., 1971). Because of the close structural analogy between the catalytic sites in subtilisin and the members of the trypsin family, one might have expected to see at least a partial loss of the bound sulfate in the subtilisin pH 7.5 versus pH 5.9 difference map described in the previous section. However, no such effect was observed. This negative result suggests that the sulfate in crystalline subtilisin is somewhat more firmly bound than in the trypsin family. Several possible reasons why this might be so come to mind. One is that the geometries at the catalytic sites of subtilisin and trypsin, though similar, are not identical. Specifically, the function of the side chain amide of Asn-155 in subtilisin is performed by the backbone NH of Gly-193 in trypsin, both with respect to catalysis (Robertus et al., 1972) and sulfate binding. A second possible cause for a slight difference in sulfate binding might be differences in crystal packing geometries. However, in the absence of more complete experimental data on this point further speculative discussion is unwarranted.

**Structural Review System and Implications for Function**

Structure of Charge Relay System in Subtilisin – An important and somewhat surprising result emerges from our partial crystallographic refinement of the native subtilisin structure; there is no hydrogen bond between the catalytic side chains of Ser-221 and His-64, or at least it is severely distorted. Such a hydrogen bond has long been supposed to play a key role in the charge relay system of serine proteases.

Two aspects of hydrogen bond geometry must be considered in this discussion. First, and most obviously, in order for a hydrogen bond to exist the distance between electronegative donor and acceptor atoms should be suitably short. Although known examples of hydrogen bonds of the type we are concerned with here, namely —OH . . . N, are more scarce than those of the type —NH . . . O (Hamilton and Ibers, 1968; Donohue, 1968), and thus generalizations concerning them are correspondingly less secure, it is probably safe to assign an expected value of 2.8 Å and an upper limit of about 3.1 Å for the O . . . N distance in the former. In comparison, the distance between Oy(221) and Ne2(64) observed in native subtilisin at the present stage of refinement is 3.7 Å. However, because the estimated probable error of our atom coordinates is around 0.2 Å, this distance by itself cannot be considered conclusively to rule out the existence of a hydrogen bond between Oy and Ne2.

The second aspect of hydrogen bond geometry that requires consideration concerns placement of the donor Oy(221) with respect to the axis of the sp² orbital occupied by the electron lone-pair of the acceptor Ne2(64). In general, when the acceptor atom has two or more lone-pair orbitals placement of the donor is not very restrictive, as observed experimentally for numerous small molecule crystal structures (Donohue, 1968). However, when the acceptor has only one lone-pair orbital it is expected that the donor will have a strong tendency to lie along the axis of that orbital (Kollman and Allen, 1972). Such is the situation applying to any hydrogen bond of the type OH . . . N that might exist between Oy(221) and Ne2(64), and it may be noted parenthetically, to the NH . . . N hydrogen bonds in the Watson-Crick base-pairing scheme for nucleic acids as well.

This point is illustrated by Fig. 2, a stereoscopic representation of our current, semirefined coordinates for the charge relay residues in subtilisin. In this figure a dummy atom labeled X and shown in dashed outline has been placed on the axis of the lone-pair orbital of Ne2(64), in the plane of the imidazole ring, and at a distance of 3.8 Å from it, or in other words, at the expected location for Oy(221) if it were an ideally positioned hydrogen bond donor. The most striking and immediately obvious peculiarity of Fig. 2 is the large displacement of Oy(221) from its ideally hydrogen-bonded position X. This displacement is about 2.5 Å, far larger than could be explained away by any reasonable assumption about errors in the coordinates. It is also evident from Fig. 2 that Oy(221) is about 0.8 Å above the plane of the imidazole ring of His-64, but perhaps this effect cannot be regarded as significant at the present stage of refinement.

Consider next the hydrogen bond between His-64 and the buried Asp-32. Fig. 2 clearly shows that the Asp-32 side chain carboxylate oxygen atom lying to the right (O62) is in an ideal location to hydrogen-bond with N61(64). That is, O62(32) is almost precisely in line with a hydrogen atom or with an sp² lone-pair orbital on N61(64). The N . . . O distance 2.4 Å is in fact rather short. No suggestion of a bifurcated hydrogen bond is evident in the geometry of these side chains, and the distance between N61(64) and the left carboxylate oxygen of Asp-32 (O61) is 3.2 Å.

Our main conclusion then, based both on the large Oy-Ne2 distance of 3.7 Å and more particularly on the 2.5 Å displacement of Oy(221) from its ideal position as a hydrogen bond donor to Ne2(64), is that no hydrogen bond of this type is present, or at least that it is extremely weak and distorted. In contrast, the hydrogen bond between N61(64) and O62(32) is normal and perhaps even unusually strong.
Comparison with Other Serine Proteases—Such an unorthodox assertion must be regarded with some skepticism. Possibly, for example, we have somewhat misplaced the imidazole side chain of His-64. We have, therefore, examined the best currently available coordinates for the charge relay residues in four serine proteases of the trypsin family in an attempt to determine whether they exhibit the same distortion. All are at present still undergoing crystallographic refinement and so reliable error estimates are not yet in hand, but these coordinates are probably comparable in accuracy to those of subtilisin. In each case the residues making up the charge relay system were visually oriented by means of an Evans-Sutherland Picture System to coincide with the orientation of the equivalent residues in subtilisin, depicted in Fig. 2. The corresponding ORTEP stereo-pair drawings are shown in Fig. 3, a to d.

It is clear from Fig. 3 that Oy(195) is in all cases much too far to the left (that is, in the direction of the Asp-102 side chain) to donate a good hydrogen bond to Nε2(57). This aspect of the charge relay geometry is thus very much like that seen in subtilisin. Quantitatively, the distances of Oy(195) from their ideally hydrogen-bonded positions (corresponding to dummy atom X in Fig. 2) are 1.4 Å in trypsin, 2.5 Å in α-chymotrypsin, 1.8 Å in γ-chymotrypsin, and 2.6 Å in elastase. Also, as in subtilisin, the distances between Oy(195) and Ne2(57) are slightly too large: 3.1 Å in trypsin, 3.2 Å in α-chymotrypsin, 3.5 Å in γ-chymotrypsin, and 3.2 Å in elastase.

It may be appropriate at this point to emphasize that all four of the structures depicted in Fig. 3 were originally believed by the investigators involved to contain a Ser-His hydrogen bond, as we did in the case of subtilisin. Presumably, therefore, any systematic bias in these structures would be toward standard hydrogen-bonded geometry. Nevertheless, the unambiguous verdict of the actual coordinates with regard to the existence of a Ser-His hydrogen bond is the same as for subtilisin.

A special problem is presented by α-chymotrypsin. The coordinates of Birkoft and Blow (1972) indicate a χ1 value of +90° for the side chain of Ser-195, with Oy in the "up" orientation shown in Fig. 3b. In the other homologous serine proteases, χ1(195) is in the neighborhood of −90° with Oy in the "down" orientation as in subtilisin. However, we have pointed out in a previous paper (Birkoft et al., 1976) that no alternative choice of χ1(195) in α-chymotrypsin is capable of producing a more favorable geometry for hydrogen bonding between Ser-195 and His-57. In addition, on the basis of further crystallographic refinement it now seems likely that the side chain of Ser-195 in α-chymotrypsin actually is in the same orientation as that observed in the other homologous enzymes.

Also to be compared with subtilisin are the observed displacements of Oy(195) from the plane of the imidazole ring of His-57. These are −0.5 Å for trypsin, +0.5 Å for γ-chymotrypsin, and +0.2 Å for elastase, with positive values signifying Oy lying above the plane as viewed in Fig. 3 and negative values the reverse. It will be recalled that the corresponding displacement in subtilisin is 0.8 Å, but apparently this particular effect is not consistently observed in the trypsin family, and it therefore cannot be considered a significant structural feature of serine proteases generally.

Finally, we examine the His-Asp hydrogen bond as observed in the trypsin family. Unfortunately, it is not immediately obvious by inspection of Fig. 3, a to d which of the two carboxylate oxygen atoms of Asp-102 is actually hydrogen-bonded to Nε1(57). However, suitable rotations of the four models on the Evans-Sutherland Picture System together with distance calculations show that the lower left oxygen is clearly the one involved in each case. The N···O distances were calculated to be 2.8 Å for trypsin, 2.6 Å for α-chymotrypsin, 2.5 Å for γ-chymotrypsin, and 3.0 Å for elastase, with an overall mean of 2.7 Å. Also, the hydrogen bond geometries show no evidence of bifurcation between the two carboxylate oxygen atoms. Thus, with respect to the His-Asp hydrogen bond as well, the trypsin-homologous structures confirm our conclusion about the charge relay system in subtilisin; the His-Asp interaction has the geometry expected for a normal unbifurcated, perhaps even unusually strong hydrogen bond.

By this time the reader may have already noticed that in subtilisin Ne2(64) is hydrogen-bonded to the right-hand oxygen atom of the buried aspartic acid side chain as viewed in Fig. 2, whereas in the trypsin family Ne2(57) is, on the contrary, hydrogen-bonded to the left-hand oxygen atom as viewed in Fig. 3. Correspondingly, the direction of the polypeptide chain containing the aspartic acid residue is also reversed between the two structures. If this correlation is more than coincidental, its significance is not yet clear.

Functional Implications—If indeed the reactive serine side chain is not, after all, hydrogen-bonded to the His-Asp couple, why then does the serine appear to act as though it were strongly nucleophilic? We believe the explanation lies in the stereoechemistry of substrate binding. The structural evidence now seems compelling that catalysis of the general acyl transfer reaction by serine proteases is largely due to the enzyme's ability to bind substrate preferentially in a tetrahedron.

1 A. Tulinsky, personal communication.
drally distorted conformation, thereby stabilizing the activated transition state complex. A synopsis of this hypothesis and the evidence supporting it is presented in a recent review by Kraut (1977). According to this view, the catalytic serine reacts with a tetrahedrally distorted and therefore electrophilic carbonyl carbon atom of the substrate's susceptible bond simply because the serine hydroxyl is initially poised in the ideal position to do so. The favorable positioning of the serine Oy atom for such a reaction in trypsin was pointed out by Huber et al. (1974). Thus, the serine itself need not be
intrinsically an especially powerful nucleophile.

What then is the function of the His-Asp couple? A crucial event in the acylation step of the catalytic reaction is transfer of a proton from the attacking side chain amine atom to the amide nitrogen or ester oxygen atom of the substrate’s leaving group. Conversely, the reverse proton transfer, from an attacking nucleophile (normally water) to the serine Oy atom must occur in the deacylation step. Such proton transfers are often rate-limiting (Satterthwait and Jencks, 1974), and it is not surprising that the enzyme should provide a mechanism for facilitating the process. Evidently the catalytic His-Asp couple serves this function. In other words, the His-Asp couple may be regarded as a site for proton binding in the transition state.

Very relevant to this discussion is the location in the transition state of the leaving group proton donor atom during the acylation step, or equivalently, of the attacking nucleophile proton donor atom during the deacylation step. Matthews et al. (1975) have determined the structure of two aromatic boronic acid complexes with subtilisin and shown that these adducts are tetrahedral, with just the geometry expected for a transition state complex. Because these structures were determined from difference maps calculated with semirefined phases, it was possible to define the geometry of the tetrahedral boronic acid group quite accurately and, specifically, to locate Oy (Table I in Matthews et al., 1975) within about 0.3 Å. This atom must occupy the position of the leaving or entering nucleophile atom in the transition state complex; it is represented in Fig. 2 by the atom labeled P. Fortunately, formation of the boronic acid adducts at Ser-221 did not noticeably perturb the positions of other nearby groups, most importantly of His-64, so that the location of P with respect to the native enzyme structure is unambiguous.

It will be noticed immediately that P is much closer to the idealized location, labeled X in Fig. 2, for hydrogen bonding to Ne2(64) than is Oy(221). The distance P to X is 0.8 Å, and the distance P to Ne2(64) is 2.7 Å, to be compared with the corresponding distances of 2.5 Å and 3.7 Å for Oy(221) mentioned earlier.

From the point of view of transition-state stabilization this is precisely what would be expected. In other words, His-64 in the native enzyme is poised to make a relatively good hydrogen bond with the leaving (or entering) nucleophile in the transition state geometry but not with Oy(221) as we had originally supposed.

It is noteworthy that in chymotrypsinogen, the relatively inactive proenzyme for chymotrypsin, the catalytic Ser-195 makes an ordinary hydrogen bond with His-57 which is disrupted upon activation. In chymotrypsinogen the Oy(195) to X distance is only about 0.7 Å, compared with 2.5 Å in a chymotrypsin (Birktoft et al., 1970). The corresponding structural alteration is much less dramatic on activation of trypsinogen to trypsin according to Kossiakoff et al. (1977). They report an Oy(195) to X distance of 0.67 Å in trypsinogen and an Oy(195) to X distance of 1.15 Å in diisopropylphosphoryl trypsin (compare with 1.4 Å for the Oy(195) to X distance in native or benzamidine trypsin mentioned above). We nevertheless believe the effect is significant in the trypsinogen to trypsin conversion as well.

It is tempting to speculate further about the mechanistic details of the proton transfer step. An attractive possibility is that the histidine side chain can act somewhat like a bistable flip-flop with the two oxygen atoms of the buried aspartate side chain furnishing the two "stops." One stable state might be as depicted in Figs. 2 and 3 and the second might be an intermediate in which the histidine is instead hydrogen-bonded via Ne2 to the other aspartate oxygen atom and via Ne2 to the serine Oy atom. Obviously such a device should facilitate the required proton transfer between Oy and the leaving or entering nucleophile; it corresponds to mechanism 1 of the six possible distinct mechanisms enumerated by Satterthwait and Jencks (1974, Equation 9). Unfortunately, in the opinion of those authors the presently available kinetic data are inadequate to distinguish among the six possible mechanisms. The X-ray data are not of much help either, but there are numerous examples of serine protease derivatives in which the catalytic histidine has altered its position somewhat, lending support to the concept of a mobile histidine. Indeed Huber and co-workers (Rühlmann et al., 1973; Bode et al., 1975) report that in the trypsin-pancreatic trypsin inhibitor complex, which is also a tetrahedral adduct somewhat analogous to the subtilisin-boronic acid complexes, Ne2 of the catalytic His-57 is in fact directed toward Oy(195) and not toward the leaving group (N of Ala-161) as in the boronic acid complexes.

There is, however, a slight difficulty inherent in this idea; if the specific motion described above for the histidine is an important feature of the catalytic process, it seems reasonable to suppose that such a motion would result from a simple rotation about one or two bonds. From examination of the available models this does not appear to be possible. Rather, any such movement appears to require concerted small perturbations of the main chain. On the other hand a moving histidine mechanism certainly cannot be eliminated on these grounds, as it is conceivable that the main chain geometry has evolved to facilitate precisely such a movement.

An alternative view is that the His-Asp pair acts as a stationary proton relay. Wang (1970) has emphasized that optimal geometry for proton transfer enzymes would involve bent hydrogen bonds, and a molecular orbital calculation by Ingraham (1973) appears to confirm this qualitative idea. Indeed Wang (1970) has actually postulated the existence of a bent hydrogen bond between Ser-195 and His-57 in the α-chymotrypsin-substrate complex. A major point of the present report is that Wang’s postulate is evidently correct insofar as one can tell from careful examination of the structures of the native serine proteases. Of course it must be kept in mind that the several individual catalytic events may well involve slight perturbations of the native enzyme structure, and that it may be hazardous to place too much reliance on details of the native structure when attempting to postulate mechanisms.

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Charge Relay System in Subtilisin and Other Serine Proteases

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