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## Asymmetrical Changes in the Tertiary Structure of $\alpha$ -Chymotrypsin with Change in pH<sup>†</sup>

Aristides Mavridis, Alexander Tulinsky,\* and Michael N. Liebman

ABSTRACT: Changes in the structure of  $\alpha$ -chymotrypsin ( $\alpha$ -CHT) with change in pH of the surrounding medium have been investigated from pH 1.0 to 10.0 using single crystal Xray diffraction methods. This pH range is subdivided into seven regions corresponding to distinct and differing structural stability (pH conformers). Transitions between pH conformers occur at pH values close to known pK values of different ionizable groups present in proteins. The pH 8.3 conformer shows large asymmetrical changes in tertiary structure in the active site region of  $\alpha$ -CHT dimer, where one molecule undergoes a severe reorganization while the other remains essentially unaltered with the pH change. The asymmetrical change is apparently induced near pH 8.0 by the deprotonation of one of the B

We have already reported indirect evidence for asymmetrical changes in tertiary structure within the dimer interface region of  $\alpha$ -chymotrypsin ( $\alpha$ -CHT) upon dimerization (Tulinsky *et al.*, 1973a) and similar observations have been made on insulin dimer by Hodgkin and her collaborators (Blundell *et al.*, 1972). We have also reported observing asymmetrical changes in tertiary structure to a lesser extent accompanying a change in pH of the soaking solution of crystals (Vandlen and Tulinchain N-terminal amino groups (IIe-16), thus disrupting the active site IIe-16-Asp-194 internal ion pair and causing a major reorganization of other residues in the near vicinity. These residues include the following peptide segments: IIe-16-Asn-18, Leu-143-Tyr-146 (carboxyl terminal, B chain), and Ser-189-Ser-195. The observed difference electron density in the active site regions between the structure of  $\alpha$ -CHT at pH 8.3 and 3.6 has been related to changes in tertiary structure on a molecular level. A probable source of the asymmetrical changes is the variability in the tertiary structure previously observed at pH 3.6. The slightly different environments lead to slightly different pK values for the two independent IIe-16 residues.

sky, 1973) and accompanying the exchange of localized sulfate ions in the crystal structure with selenate ions of a soaking solution (Tulinsky and Wright, 1973). We would now like to report observing fairly spectacular asymmetrical changes in the tertiary structure of the active site region of  $\alpha$ -CHT dimer upon further change in pH, where one molecule of the dimer undergoes a severe reorganization within the active site region while the other remains essentially unaltered with the pH change.

## Experimental Section

The pH of the soaking solution above crystals of  $\alpha$ -CHT was changed gradually in a series of steps by exchanging an aliquot

<sup>&</sup>lt;sup>†</sup> From the Department of Chemistry, Michigan State University, East Lansing, Michigan 48824. *Received March 18, 1974*. This work was supported by the National Science Foundation, Molecular Biology Section, Grant No. GB-6586, GB-7399, and GB-15402.



FIGURE 1: Effect of change of pH on lattice parameters of  $\alpha$ -chymotrypsin. Subdivision (cross-hatches) into seven pH conformer ranges accomplished with additional aid of axial intensity distributions: (---) denote two pH states in reversibility experiment; (x) denote parameter after reversibility experiment.

of the 75% saturated ammonium sulfate soaking solution at a given pH with a similar one at the desired pH. The pH range examined in this way was from pH 1.0 to 10.0. Beyond these limits, the single crystals, which are already cracked by this time, disintegrate into a finely divided solid material unsuitable for X-ray diffraction experiments. The pH of 75% saturated ammonium sulfate is about 4.5; more acidic solutions were made by the addition of 18 M  $H_2SO_4$ , while more alkaline solutions were obtained with the addition of 14.8 M ammonium hydroxide. The 75% saturated ammonium sulfate solution exhibits buffer properties with respect to these reagents near the pH extremities of this work (1.5 < pH < 3.0 for  $H_2SO_4$  and 7.8 < pH < 9.0 for NH<sub>4</sub>OH). Such solutions standing over crystals for periods of months did not show any change in pH. The only weakly buffered pH region is that from about 5.0 < pH < 7.0. However, in what follows, it will be seen that no pH-induced structural changes occur within this region which might be somewhat sensitive to changes in pH.

The pH of the soaking solution was measured at room temperature to  $\pm 0.1$  of a pH unit with a Leeds and Northrup 7411 temperature compensated pH meter equipped with a 117202 Combined Ag|AgCl electrode. The pH meter was calibrated with Mallinckrodt standard buffer solutions at pH 4.0 and 7.0. Since the liquid junction potential of 75% ammonium sulfate is considerably different from that of the more dilute standard buffer solutions, the pH values of the ammonium sulfate solutions will be affected to this extent. However, the relative pH values of the various solutions remain indicative and since the 75% ammonium sulfate solution is not expected to change the liquid junction potential by more than 30 mV, the absolute pH values of these solutions are probably in error by less than 0.5 of a unit (Bates, 1973). The effect of pH on the unit cell pa-



FIGURE 2: Axial intensity distributions of the pH conformers of  $\alpha$ -chymotrypsin.

rameters of  $\alpha$ -CHT is summarized in Figure 1. During the course of these experiments, it was discovered that our original pH electrode was defective giving readings that were high by about 0.7 of a pH unit. Thus, all pH values previously reported by us are high by this amount (Tulinsky *et al.*, 1973a,b; Vandlen and Tulinsky, 1971, 1973; Tulinsky and Wright, 1973).<sup>1</sup> The native structure of  $\alpha$ -CHT discussed by us now corresponds to the pH 3.6 range of Figure 1 where the *b* axis, the  $\beta$  angle, and the unit cell volume all tend to be a minimum. From Figure 1 and with the aid of the comparison of the relative intensity distributions of the principal axes at the various pH values (Figure 2), the 1.0-10.0 pH range can be subdivided

<sup>&</sup>lt;sup>1</sup> The conclusions drawn in these are not thereby affected since they were independent of the absolute pH values and were generally confined to pH regions.

into seven regions corresponding to distinct and differing structural stability.<sup>2</sup> We will hereafter refer to these structural regions as pH conformers with the pH value of the conformer being the mean value of the range. It can be noted from Figure 1 that the transitions from one pH conformer to another are generally sharp and that they occur at pH values corresponding close to well-known pK values of different ionizable groups present in proteins. The effect of pH on the axial intensity distributions is shown in Figure 2, where the logarithm of the intensity is shown as a function of Bragg scattering angle. The intensity changes seen in Figure 2 are readily observable, persistent, and extend to high scattering angles. All the pH conformers except one (low pH extreme) have been found to be reversible with respect to structural changes leading to neighboring conformers. The pH 1.6 conformer shows drastic changes in intensities of reflections in addition to unit cell parameters, both of which persisted even though the pH of the soaking solution of the crystals was adjusted back to pH 2.6. Since the pH 9.0 conformer also displays similar severe changes, it was somewhat of a surprise when both relative intensities and cell parameters reverted close to those of the pH 8.3 conformer when the pH was changed back to pH 8.1. The pH 8.3 conformer shows large asymmetrical changes in tertiary structure in the active site region and it is with these that we would like to address ourselves in this communication.

Before proceeding, it would seem appropriate to compare the foregoing results with those previously reported by others (Table I). From Table I, it can be seen that unit cell dimensions of the classical early work of Bernal et al. (1938) do not correspond closely with any subsequent work. This is most likely due to the more superior methods currently being used to make the corresponding measurements. Furthermore, these authors do not report the nature of and the pH of the solution in contact with the crystals. In the case of the dimensions reported by Corey and his coworkers (1965) it is clear that the crystals are probably those of the pH 5.4 conformer. The crystals were grown at pH 4.0, which is precisely where we report the transition from the pH 3.6 to the pH 5.4 conformer. The unit cell dimensions reported by Sigler et al. (1966) are more difficult to reconcile. Although the reported values correspond fairly closely to those of our pH 3.6 conformer, the crystals are buffered with 0.1 M citrate at pH 4.2; however, the crystals were grown in a slightly different way: from solutions containing 2-4% (by volume) dioxane buffered at pH 4.2 with 0.1 M citrate. We have exchanged our soaking solution at pH 3.6 with one buffered at pH 3.6 with 0.1 M citrate without affecting the unit cell parameters or the relative axial intensity distributions of the pH 3.6 conformer. Therefore, the discrepancy (unit cell parameters, pH) is probably associated with the dioxane treatment at the time of crystal growth, since dioxane binds in the substrate binding pocket (Steitz et al., 1969). We have diffused 3% dioxane (by volume) into crystals of the pH 3.6 conformer and this produced fairly large changes in the unit cell dimensions and in the relative intensities of reflections  $(a = 48.9, b = 68.3, \text{ and } c = 65.8 \text{ Å}, \beta = 101.8^{\circ}; \text{ unpublished}$ results).

The differences can be interrelated in a number of ways, none of which are completely satisfactory, since all are speculative. It is conceivable that crystals grown in the presence of di-

TABLE 1: Comparison of Unit Cell Dimensions of Several  $\alpha$ -CHT Studies.<sup>*a*</sup>

pН	a (Å)	b (Å)	c (Å)	$\beta$ (deg)	Ref
?	49,6	67.8	66.5	102	b
4.0	49.4	68.1	65.9	102.2	С
4.2	49.1	67.4	65.9	101.7	d
3.6	49.3	67.3	66.0	101.8	Present
5.4	49.3	68.0	66.0	102.1	Present

<sup>*a*</sup> Axial lengths are about  $\pm 0.1$  Å; interaxial angles about 0.1°. <sup>*b*</sup> Bernal *et al.* (1938). <sup>*c*</sup> Corey *et al.* (1965). <sup>*d*</sup> Sigler *et al.* (1966).

oxane at pH 4.2 possess a structure which is slightly different from that of the pH 5.4 conformer and the similarity in unit cell dimensions between the former and the pH 3.6 conformer is simply fortuitous. This is somewhat supported by the fact that dioxane has been shown to affect the pH 3.6 structure and has been reported to affect the position of the Met-192 side chain in the dioxane-citrate-pH 4.2 crystals of  $\alpha$ -CHT (Steitz *et al.*, 1969). On the other hand, the presence of substituents in the substrate binding pocket might act to stabilize a given structure and render it relatively insensitive to pH changes (Birktoft and Blow, 1972) or at least, sensitive to pH changes in a different way.<sup>3</sup>

## Results and Discussion

Two sets of three dimensional X-ray diffraction intensity data at 2.8-Å resolution of two independent preparations of pH 8.3 crystals were collected, processed, and converted to figure of merit weighted "best" difference electron density maps with respect to native  $\alpha$ -CHT (pH 3.6) in a manner similar to that described elsewhere (Vandlen and Tulinsky, 1973; Tulinsky et al., 1973b). These maps were based upon the phase angles of native enzyme reflections with a figure of merit greater than 0.7 (about 6300 reflections). A second independent structure determination was carried out because the fall-off of the intensities of the original data set with X-ray exposure was large (about 40% for 80 hr of exposure). The second set of intensity data was collected about 1 year later employing a hardware renovated X-ray diffractometer which featured the repositioning of the balanced Ni/Co filter pair to between the X-ray source and the crystal and the intercession of a 60-cm He-tube tunnel between the crystal and the scintillation counter detector; the fall-off of this data set was only about 15% for 80 hr of X-ray exposure. The two independent difference electron density maps proved to be identical in all principal features and practically so for most of the lesser ones. Two independent sets of intensity data were also used in the pH 5.4 structure determination; in this case, the second set was collected to simply verify the results of the first set.

When the pH of the soaking solution above crystals is increased, the crystals first develop a small number of fine cracks which increase in extent as the pH is increased further. Comparison of precession photographs of cracked pH changed crystals and uncracked native crystals showed only that the pattern of the higher pH crystals decreases faster with scattering angles than that of native crystals; this is also evident from a

 $<sup>^2</sup>$  The pH 6.7 structure previously reported by us (Vandlen and Tulinsky, 1973) now corresponds to the pH 5.4 range. The reason the midpoint of the new range is not 6.0 is because an additional pH-induced structural change (pH 6.8-8.0) has been uncovered in the range. Thus, the original range is narrower than previously reported.

 $<sup>{}^{3}</sup>$ We would like to thank Dr. David M. Blow for pointing out this alternative.

TABLE II: Distribution of Observed Differences in Structure Amplitude,  $||F(8.3)| - |F(3.6)||^{a}$ 

$ \Delta F $ (e/unit cell)	Ν	%
0-33	1790	28.9
33-67	1598	25.8
67–134	1854	30.0
134-200	680	11.0
200-267	189	3.1
267-334	50	0.8
334-400	17	0.3
400-500	7	0.1

comparison of the axial intensity distributions obtained using diffractometric methods (Figure 2). A comparison of the threedimensional radial intensity distributions of the pH 8.3 and 3.6 conformers gives an isotropic  $\Delta B$  factor of about +5.0 Å<sup>2</sup>, which when applied to the structure amplitudes of the higher pH data, bring it congruent with that of the native data for higher order reflections (the Wilson B of the native data is about 27 Å<sup>2</sup>). Similar values have been used by us to correlate uncracked crystals of other derivatives of  $\alpha$ -CHT (irreversible, competitive reversible inhibitors, substrate analogs, etc). The native enzyme scattering has invariably proved to be better and derivative formation always seems to lead to lesser scattering ability. Derivative formation, including the  $\Delta pH$  studies, is also generally accompanied by greater sensitivity to X-ray damage. Finally, the  $\omega$  spreads of selected reflections of native crystals, measured from background-to-background, are generally 0.2-0.3°, while those of derivatives and pH conformers appear to be slightly larger (0.3-0.4°). In any case, no particular problems were experienced in collecting reliable intensity data from the cracked crystals.

The distribution of the observed differences in structure amplitudes between pH 8.3 and 3.6 is given in Table II, where differences less than 33 e/unit cell were considered unobserved. The distribution of the original data set is similar. For comparison, the unobserved reflections of the native data are about <33 e/unit cell, which is slightly smaller than the average lack of closure error of the heavy atom isomorphous replacement phase determination (Tulinsky et al., 1973b) and the largest structure amplitudes of the native data are about 2000 e/unit cell. The difference map between the structures of  $\alpha$ -CHT at pH 8.3 and pH 3.6 possesses the principal features observed for the pH 5.4 conformer (Vandlen and Tulinsky, 1973) and also most of the smaller details of the pH 5.4 map which were not discussed previously. The latter have now either increased very significantly in magnitude or have at least persisted at their previous level. In addition, a number of new large differences in electron density corresponding to additional structural changes appear in the pH 8.3 difference map. All these combine to form a rather complicated and extended picture of tertiary structural changes in the dimeric molecule. One of the new features, which we would like to detail here because of its possible bearing on other, more complicated oligomeric systems, is a massive electron density change in the active site region of one molecule but not in the other of the dimer. This change is apparently provoked near pH 8.0 by the deprotonation of the N-terminal amino group of Ile-16 (B chain), which



FIGURE 3: Difference electron density between pH 8.3 and pH 3.6 conformers of  $\alpha$ -chymotrypsin in vicinity of active site viewed down local twofold axis; contours at 0.05 e Å<sup>-3</sup>(2 $\sigma$ ) beginning with 0.15 e Å<sup>-3</sup>(6 $\sigma$ ); solid contours, positive; broken, negative; 6Å thickness shown; position of local twofold axis indicated by cross.

simultaneously leads to the disruption of the active site Ile-16-Asp-194 ion pair (Matthews et al., 1967) and is accompanied by a major reorganization of some of the other residues in the near vicinity. The changes in the electron density can be seen in Figure 3 which shows about a 6-Å thickness of the pH 8.3 difference electron density map in the vicinity of the active site viewed down the local twofold axis which approximately relates the independent molecules of the asymmetric unit. From Figure 3, it can be seen that the changes do not occur in a twofold way suggesting that the pK values of the independent Ile-16 terminal residues must be different and that the pKvalue of Ile'-16 has not vet been attained in this pH range (8.0-8.6). The pH 9.0 conformer probably corresponds to the deprotonation of the other B-chain terminal of the dimeric molecule (Ile'-16). In addition, it can be seen from Figure 3 that the changes at pH 8.3 are extensive and quite substantial in magnitude ( $\pm 0.4-0.5$  e Å<sup>-3</sup> for most of the changes, which corresponds approximately to a peptide grouping). These values can be compared to the computed root-mean-square error of the difference density (Henderson and Moffat, 1971) which is about 0.02 e  $Å^{-3}$  and to the native electron density proper, where the largest features, such as disulfide bridges, appear at 1.8-1.9 e Å<sup>-3</sup>. These changes would be expected to be even larger if phasing could be accomplished based upon phase angles of the pH 8.3 structure (Luzzatti, 1953).

The difference electron density of Figure 3 is confined to the region of the active site which includes the following peptide segments: Ile-16-Val-17-Asn 18 (amino terminal, B chain); Leu-143-Thr-144-Arg-145-Tyr-146 (carboxyl terminal, B chain); Ser-189-Ser-190-Cys-(191-220)-Met-192-Gly-193-Asp-194-Ser-195. From Figure 3, it can be seen that the difference electron density representing the structural changes in this region between the two pH values is extremely complicated and generally difficult to interpret in detail. For one thing, groups of the structure occasionally appear to move into regions vacated by others causing the difference density to then be somewhat ambiguous. However, the gross aspects of the changes are fairly clear and certain. For instance, the regions marked Tyr-146 correspond to the movement of the terminal carboxylate groups of Tyr-146 and Tyr'-146 upon ionization near pH 4.0. This particular region of difference density is very similar for the pH 5.4 and pH 8.3 conformers indicating that

the ionization occurs in going to the pH 5.4 conformer and that little movement of the group occurs with the subsequent increase in pH. However, the pH 8.3 maps (including electron density proper) display additional features that suggest the plane of the phenyl group of Tyr'-146 reorients about 90° by simply accompanying the carboxylate movement (counterclockwise rotation of  $C_{\alpha}$  around NH- $C_{\alpha}$ ) to conspicuously remove itself from the middle of the dimer interface region toward the more hydrophobic bulk of its monomeric unit.<sup>4</sup>

The movements of Tyr-146 are different because the terminal tetrapeptide segment of chain to which this terminal residue is linked undergoes movements which accompany the deprotonation of Ile-16 of this particular monomeric unit. This segment (Leu-143-Tyr-146) of the pH 3.6 structure is located in large negative difference electron density at pH 8.3. Thus, being in the vicinity of the deprotonation, it is somehow affected by it. The segment of chain can be visualized as possessing a fairly sharp turn between residues Thr-144-Arg-145 to give it a U-like configuration (Figure 4, bold feature). With a change in pH, the U tightens at the bend (clockwise rotation around Thr-144  $C_{\alpha}$ -CO) and undergoes a general translation toward the Ile-16 changes from a small counterclockwise rotation around NH-C<sub> $\alpha$ </sub> (Leu-143). This amounts to a net movement of about 1.5 Å for the Arg-145-Tyr-146 arm. The motion could be a simple rigid body type as described or it could be a much more complicated series of movements of the various individual components of the chain leading to the same resultant change. The different possibilities cannot be differentiated at present.

The dipeptide segment of Ile-16-Val-17 pivots toward Cys-(191-220) which also shows a large movement (Figure 4). The amino group of the Ile-16 terminal moves about 1.5-2.0 Å in the direction of Ser-190 by apparently rotating around either its  $C_{\alpha}$ -CO or the  $C_{\alpha}$ -N bond of Val-17. If the side chain of Ser-190 also reorients slightly, a hydrogen bond might form between  $O_{\gamma}$  of Ser-190 and the amino group of Ile-16. The observed electron densities are consistent with such a formulation. The aliphatic side chain of Ile-16 moves in the opposite direction toward the general direction of the main chain of Gly-140-Trp-141 via a compensating clockwise rotation of about 40-50° around the  $C_{\alpha}$ -C<sub>b</sub> bond.

The side chain of Asp-194 of the Ile-16-Asp-194 active site ion pair at pH 3.6 also undergoes a movement. The two residues readjust so as to move away from each other (Figure 4) but remain internal in the molecule. A twist of the Asp-194 main chain carboxyamide between Gly-193 and Ser-195 and a rotation (either direction) around the  $C_{\alpha}$ - $C_{\beta}$  bond of Asp-194 move the carboxylate ion of the side chain toward the amido group of Trp-141 with which a carboxylate oxygen atom might form a hydrogen bond.<sup>5</sup> This interaction may also be the cause of the apparently isolated movement of the tetrapeptide segment of the carboxylate B chain terminal which begins near Gly-142; conversely, the interaction might be terminating the



FIGURE 4: Drawing depicting the changes of the structure of  $\alpha$ -chymotrypsin at pH 3.6 with change in pH to 8.3 in vicinity of active site. Viewed perpendicular to local twofold axis; dyad axis denoted appropriately; tetrapeptide B chain carboxylate terminus shaded bolder; Arg-145 side chain omitted for clarity; arrows indicate directions of shifts (see text for details); broken lines denote position of Met-192 at pH 8.3; bridging Tyr-146-Ser'-195 localized sulfate ion also shown.

observed movement from propagating further into the interior of the molecule along this chain. The chain from Gly-193 to Ser-189, including the disulfide bond of Cys-(191-220), lies in large negative difference density at pH 8.3. The movements of Glv-193-Met-192 are probably related to the changes occurring at Asp-194, while the remainder of the changes obviously correspond to the movement of the remainder of the segment away from the encroaching terminal amino of Ile-16. The changes are particularly severe around Gly-193 ( $-0.4 \text{ e } \text{Å}^{-3}$ ). practically removing it from the electron density at pH 8.3 (peak height = 0.8 e Å<sup>-3</sup> at pH 3.6). The side chain of Met-192, which is in a crowded and probably strained position at pH 3.6, has undergone a large reorientation (see Figure 4). The  $C_{\alpha}$ - $C_{\beta}$  bond moves about 90° from its original position of pointing toward the local twofold axis but remains approximately perpendicular to it. The side chain relieves its crowded condition by reorienting around the  $C_{\alpha}$ - $C_{\beta}$  bond by about 180° so as to ultimately run parallel to the twofold axis in an approximately extended configuration toward the general direction of His-40. Although Met-192 has been reported to migrate from a buried position in the interior of chymotrypsinogen out to the surface upon activation to the enzyme (Freer et al., 1970), the movements observed at pH 8.3 do not correspond to the reverse of this process as Met-192 remains on the surface throughout the movement (in the dimer interface).

The structural changes shown by the pH 8.3 conformer are probably related to the decrease in catalytic activity of  $\alpha$ -CHT at higher alkaline pH values. The rate of  $\alpha$ -CHT catalyzed reactions increases with pH up to about pH 8.0 and then it decreases at more alkaline pH (Himoe *et al.*, 1967). This behavior has implicated an ionizing group of the enzyme with a pK

 $<sup>^{4}</sup>$ When a rotation around a bond is discussed, the bond is taken as the rotation axis and the sense of the axis is taken from the first to the second atom of the bond.

<sup>&</sup>lt;sup>5</sup> Birktoff and Blow (1972) report a water molecule (W6) hydrogen bonding to the NH of Trp-141. Although the electron density of the amide group of Trp-141 is slightly elongated in the pH 3.6 conformer, there is no distinctly resolved peak in the vicinity that could possibly be assigned as a water molecule. The discrepancy could be the result of the slightly different nominal resolutions of the two structure determinations (2.0 and 2.8 Å). If a water is present, it probably would be displaced by the Asp-194 movement.

of about 8.5 and it has been identified as the N-terminal B chain  $\alpha$ -amino group of Ile-16. The pH dependence appears to be a consequence of the activation mechanism of chymotrypsinogen with a charged Ile-16 maintaining the active site conformation as a partner of an internal ion pair interaction with the side chain of Asp-194: a neutral conformation containing a charged Ile-16 is active and a high pH conformation containing a deprotonated Ile-16 is inactive. Himoe et al. (1967) have also shown that with a neutral amide substrate at alkaline pH,  $k_{cat}$ is pH independent while  $K_{\rm m}$  is pH dependent. This is consistent with our own observations which indicate little structural change in the charge-relay system of Asp-102-His-57-Ser 195 from pH 2.0 to 9.0. Thus, it has been concluded that the ionization of Ile-16 affects the enzyme-substrate dissociation constant but not the bond-cleaving step of the reaction. Heretofore, the inactive conformation has been considered to resemble the conformation of chymotrypsinogen. However, the conformation observed in the present study appears to be one that is inherent of the  $\alpha$ -CHT structure rather than that of the zymogen. In fact, it is difficult to see how the immediate changes of the Ile-16 can grossly affect the substrate binding properties of the enzyme in going to the pH 8.3 conformer. The movements are only of the order of 1-2 Å and produce no obviously unfavorable structural change in the binding region. This could conceivably be due to the restrictions imposed upon the molecules by the molecular packing in the crystals, which of course is maintained, so that the situation in solution might be quite different, where a greater degree of freedom is possible. Of the pH 8.3 changes, the most likely candidate for producing an effect on  $K_m$  of the enzyme is the relatively large movement, as a group, of the side chain of Met-192 which somehow results indirectly from the Ile-16 deprotonation. It has been suggested that Met-192 might function as a flexible hydrophobic lid on the binding pocket since binding in the specificity pocket usually causes a slight repositioning of Met-192 (Blow and Steitz, 1970). Moreover, the selective oxidation of Met-192 to the sulfoxide with trichloromethanesulfonyl chloride enhances the stability of the active substate and reduces the autolysis rate of the enzyme (Taylor et al., 1973). In addition, the derivatized enzyme exists entirely in the active substate until at least pH 9.1, while the native enzyme is known to equilibrate with 15-20% inactive conformer even at neutral pH (Fersht and Requena, 1971). We are presently investigating the implications of these interesting and suggestive observations by determining the structures of the oxidized enzyme at pH 3.6 and at alkaline pH values.

Positive difference electron density in the vicinity of Ser-195 between pH 8.3 and 3.6 implies a small shift in the position of this residue away from the plane of the imidazole of His-57; however, no changes appear to be associated with the latter residue. As already mentioned, this has generally been true of the charge-relay system from the pH 2.5 (unpublished results) to the pH 8.3 conformer. Since the pK of His-57 has surely been attained by pH 8.3, the foregoing observations suggest that deprotonation of the imidazolium ion must be accomplished with little or no movement of the groups in the chargerelay system. Thus, enhancement of catalytic activity with increasing pH up to pH 8.0 must result from a change in the chemical nature of the groups involved (e.g., nucleophilicity or some facsimile) and not result from any structural rearrangements. On the other hand, our present results cannot in themselves differentiate between the pK alternatives proposed for His-57 by competitive labeling studies on  $\alpha$ -CHT, pK = 6.8 (Cruickshank and Kaplan, 1972) and <sup>13</sup>C magnetic resonance studies on  $\alpha$ -lytic protease and imidazole model systems, pK =

3-4 (Hunkapiller *et al.*, 1973). Fersht and Renard (1974) have suggested that the first ionization of the charge-relay system is below pH 2. This might correspond to the irreversible transition to the pH 1.6 conformer which we observe at about pH 2.

In conclusion, it would seem appropriate to consider possible sources for the asymmetrical changes in tertiary structure that have been observed in this experiment. An obvious one is the variability observed in the tertiary structure at pH 3.6, where it has been shown that differences exist between the structures of the independent molecules of the asymmetric unit (Tulinsky et al., 1973a). If the structures of the monomeric units are not the same in the first instance, then there is no compelling reason to expect symmetrical changes in structure in response to a symmetrical external stimulus. This is precisely the observation accompanying the present pH change. The asymmetrical changes occur in the active site region and this region has already been reported to possess slightly different structures in the two molecules. We intend to pursue the matter further by determining the structure of the pH 9.0 conformer, which probably corresponds to the deprotonation of the other B-chain terminal of the dimeric molecule (Ile'-16) and in this way establish that the pK values of these residues are slightly different.

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