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A Detailed Structural Comparison between the Charge Relay System in Chymotrypsinogen and in α-Chymotrypsin†

Jens J. Birktoft,‡ Joseph Kraut,* and Stephan T. Freer

ABSTRACT: An improved 2.5-Å electron density map of chymotrypsinogen was calculated by incorporating heavy-atom anomalous scattering effects and a new model of the molecule was constructed. Phases from x-ray structure factors (R = 0.43) computed from this model were then used in the calculation of another electron density map against which the model was further refined. The catalytic Ser-195 side chain in the new model is in the "down" or "acyl" orientation and its Oγ atom is in position to form a normal hydrogen bond with Nϵ2 of His-57. In contrast, the corresponding hydrogen bond in α-chymotrypsin (Birktoft, J. J., and Blow, D. M. (1972), *J. Mol. Biol. 68,* 187) is severely distorted, probably as a consequence of a 1.5-Å shift in the relative positions of the two cylindrical folding domains composing most of the molecule. We suggest that this activation induced distortion of the charge-relay, hydrogen-bonding system plays an important role in the genesis of enzymic activity, in accord with an earlier proposal by Wang concerning the role of bent hydrogen bonds in enzyme catalysis (Wang, J. H. (1970), *Proc. Natl. Acad. Sci. U.S.A. 66,* 874).

Bovine chymotrypsinogen A[1] is the zymogen precursor of the pancreatic serine protease chymotrypsin A_π, and of the other A-type chymotrypsins, notably A_δ, A_α, and A_γ, in which further autocatalytic peptide cleavages have occurred. The key chemical event responsible for activation of the zymogen is a single tryptic cleavage at the peptide bond Arg-15–Ile-16.

In earlier publications from this laboratory (Freer et al., 1970; Kraut, 1971), the crystal structure of chymotrypsinogen at a nominal 2.5-Å resolution was described and compared with the 2.0-Å structure of tosylchymotrypsin (Sigler et al.,

† From the Department of Chemistry, University of California at San Diego, La Jolla, California 92093. *Received March 31, 1976.* This work has been supported by research grants from the National Institutes of Health (GM 10928 and RR 00757) and the National Science Foundation (BMS 71-01301 and BMS 74-12245) and by a United States Public Health Service Research Career Development Award to S.T.F. (GM 70140).

‡ Present address: Department of Biology, Gilmer Hall, University of Virginia, Charlottesville, Virginia 22901.

[1] The terms chymotrypsinogen or zymogen will hereinafter be used to designate bovine chymotrypsinogen A; bovine chymotrypsin A_α will be called chymotrypsin or simply "the enzyme".

1968; Birktoft et al., 1969; Birktoft and Blow, 1972). As noted at that time, the chymotrypsinogen model was still in a relatively unrefined state, having been constructed from an electron density map calculated without incorporating anomalous scattering data and without the aid of an optical comparator. Nevertheless we were able to present the following main conclusions concerning the structural consequences of chymotrypsinogen activation. (1) The chain segment 187–194 alters its conformation somewhat (the α-carbon atom of Met-192 moves by about 8 Å) so as to place the segment 189–192 in position to form the top of the specificity crevice, thereby completing an important structural feature of the enzyme which is only partially preformed in the zymogen. (2) The hydrogen-bond network linking Ser-195, His-57, and Asp-102 is evidently already preformed in the zymogen. It has been proposed that this catalytic triad constitutes a "charge relay system" responsible for the characteristic enhanced nucleophilicity of the Ser-195 side chain (Blow et al., 1969). Thus, at this stage of the investigation it was not at all clear why the zymogen should not be catalytically active, though perhaps without the well-known specificity for aromatic side chains exhibited by chymotrypsin itself.

Subsequently it was proposed that a crucial feature of the general serine protease mechanism is the enzyme's ability to stabilize a tetrahedral transition state and that an important contribution to this stabilization is provided by formation of a pair of hydrogen bonds between the enzyme and the substrate's carbonyl oxygen atom when the substrate's carbonyl carbon atom assumes a tetrahedral configuration (Henderson, 1970; Robertus et al., 1972; Matthews et al., 1975). For convenience of discussion Robertus et al. (1972) referred to the site where this pair of hydrogen bonds is formed as the "oxyanion hole." In the case of chymotrypsin the two hydrogen bonds in question are donated by the backbone NH groups of Gly-193 and Ser-195, and so it became evident that, in addition to the specificity cavity, the oxyanion hole is also completed by the activation induced movement of chain segment 187–194 referred to above. Thus, there was now at least some structural rationale for the inactivity of the zymogen: its oxyanion hole is incomplete and open to the surrounding solvent, and so it cannot as effectively stabilize a tetrahedral transition state.

Meanwhile, it was being demonstrated that both trypsinogen and chymotrypsinogen do after all possess low but easily measureable levels of intrinsic enzyme-like activity (Kay and Kassell, 1971; Morgan et al., 1972). More recently Neurath, Walsh, and co-workers have shown that a number of active site titrants for trypsin and chymotrypsin react with their respective zymogens (Kerr et al., 1975). Evidently the reaction involves formation of acyl zymogens at rates 10^{-7} to 10^{-3} of those observed for the corresponding enzymic reaction. Thus the initial question of why the zymogen is inactive now becomes a more quantitative problem of accounting in structural terms for an increase in activity by a factor of from 10^3 to 10^7 upon zymogen activation. Kerr et al. (1975) interpreted comparative circular dichroic spectra for the acyl enzymes and acyl zymogens as "consistent with the idea that the diminished activity of the zymogens is due primarily to their distorted substrate binding sites", but did not rule out the open oxyanion hole in the zymogen as an important factor.

To complete this historical perspective on chymotrypsinogen, we should also mention the work of Wright (1973a,b), who directly compared our original 2.5-Å electron density map with the chymotrypsin model constructed by Blow and coworkers in Cambridge, England, and rebuilt the chymotrypsinogen model in the same orientation as the former. This work resulted in some minor revisions and additions to the roster of differences between the two structures, but left intact the main conclusions cited above.

This report is an attempt further to define and assess some of the structural changes occurring upon activation of chymotrypsinogen. Significant improvement in our model has now been afforded both by including anomalous scattering effects in calculation of MIR[2] phases and by utilizing calculated structure factors to prepare new electron density maps. Thus, a somewhat more detailed structural interpretation is now possible and a new improved model of the molecule has been constructed. As a result, we can now see a subtle and hitherto overlooked difference between zymogen and enzyme at the charge relay system. We feel this difference is important for understanding zymogen activation, and its existence is the principal message of this report.

Improvement and Reexamination of Electron Density Maps

The first step in refinement of the chymotrypsinogen structure was improvement of the 2.5-Å MIR phases by utilizing the anomalous scattering component of the uranyl and mercury derivatives (Freer et al., 1970). The resulting electron density map was calculated on a finer grid than before (0.68 × 1.06 × 1.07 Å) and contoured at levels of 0.1, 0.2, 0.4, 0.6 . . . e/Å^3 on sections of constant X. Preliminary visual comparison of a small-scale plot of selected regions of this map with the original electron density map indicated that the quality of the new map was considerably improved. Consequently the new map was plotted on the customary scale of 1 Å = 2.0 cm and a new Kendrew–Watson model was constructed with the aid of a modified optical comparator (Rudko and Low, 1970). As is usual for proteins, the electron density in the interior of the molecule was very clear, with carbonyl groups appearing as obvious protrusions from the polypeptide backbone and side chains showing their characteristic shape, but at the surface of the molecule the density was in general weaker and details often were less easily distinguishable. However, interpretation was straightforward in most areas and it was possible to construct an unambiguous model for most of the polypeptide backbone and side chains, except for two regions comprising residues 70–77 and 143–152 where the density was rather diffuse and contained several breaks. Consequently a unique interpretation of these regions was not possible, and no model was constructed for these parts of the polypeptide chain.

In the next refinement step model coordinates were used to calculate conventional **Fc** structure factors. The usual crystallographic R factor was 43% ($R = \Sigma|F_o - F_c|/\Sigma F_o$, where F_o are the observed structure amplitudes, and F_c are the amplitudes of the calculated structure factors, **Fc**). On the basis of their experience in refining rubredoxin, Watenpaugh et al. (1973) have reported that the first set of such calculated **Fc** phases are clearly superior to the initial MIR phases. This observation is confirmed by our own experiences with refinement of four protein structures currently being undertaken in this laboratory (Freer et al., 1976).

In the case of chymotrypsinogen being considered here, the new set of phases was subsequently used to generate an improved electron density map with coefficients ($2F_o - F_c$) and an electron-density difference map with coefficients ($F_o - F_c$). The model was then checked against these maps for any gross errors, but no changes of more than 1 Å were required in any atomic positions. We estimate that presently assigned atomic positions are accurate to within 0.5 Å or better. Also, the sus-

[2] Abbreviations used are: MIR, multiple isomorphous replacement.

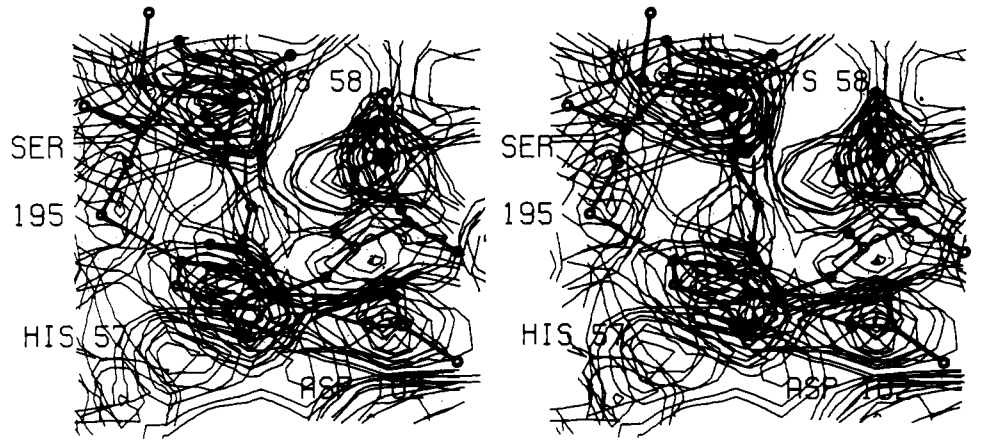

FIGURE 1: Stereoscopic view of the $2F_o - F_c$ map of chymotrypsinogen near the incipient charge relay system. The following residues are included: 42 (side chain only), 55–58, 102 (side chain only), 195, and 196 (N and Cα only). Hydrogen bonds between Oγ(195) and Nε2(57) and between Nδ1(57) and Oδ1/Oδ2(102) are also indicated by thinner lines. Contour levels are at 0.1, 0.2, 0.4, 0.6 . . . e/Å^3.

pected locations of solvent molecules, eight of which were found inside the molecule, were further confirmed by inspection of these maps. Density associated with the two backbone segments 70–77 and 143–152 that had been omitted from the previous model, and consequently from the **Fc** calculations as well, appeared somewhat improved, although several ambiguities still exist. Possibly these two parts of the molecule may actually be somewhat disordered in the crystal. For the purposes of this report, however, it is important to emphasize that the new electron density map appears to be quite clear and unambiguous in the vicinity of the charge relay system (see Figure 1).

Structural Changes Affecting the Charge Relay System

As is the case for chymotrypsin, the overall folding of chymotrypsinogen can best be described as consisting of two internally hydrogen-bonded cylinders, each formed by six strands of antiparallel β sheet and each enclosing a hydrophobic core (Birktoft and Blow, 1972). The point to be noted here is that, although noncovalent interactions within each cylinder are essentially the same in zymogen and enzyme, significant differences are found in interactions between the two cylinders themselves and between the cylinders and noncylinder segments of the polypeptide chain. These differences result primarily from the activation-induced movements of residues 187–194 and 16–20 and lead to a slight shift in the relative position of the two cylinders with respect to each other. This shift is most clearly manifested in the region where Gln-30, His-40, Cys-42, His-57, and Cys-58 in cylinder 1 (residues 25–118) are in contact with Met-192, Gly-193, Asp-194, and Ser-195 in cylinder 2 (residues 125–230) because a number of *inter*cylinder hydrogen bonds in this region change as a result of zymogen activation and the accompanying conformation changes in backbone segment 187–194.

More precisely, in the zymogen the side chain of Asp-194 is hydrogen bonded to Nε2 of the His-40 side chain, to the backbone amido group of Trp-141 and to an internal water molecule W-7 (see Figure 2a, but note that Trp-141 is not depicted). These hydrogen bonds appear to hold cylinder 2 up against cylinder 1 at this point. Upon activation the side chain of Asp-194 swings away from His-40 in order to form an internal ion pair with the α-amino group of Ile-16 and these interactions are broken (see Figure 2b). It is probably fruitless to speculate about cause and effect relationships among the activation induced changes, but their net result in this area is that the two cylinders move apart by about 1.5 Å.

A further indication that there is greater separation between cylinders in the enzyme than in the zymogen is the observation that the number of solvent molecules trapped in the interior of the protein molecule between the two cylinders increases from 8 or 9 in the zymogen to 12 in chymotrypsin. Specifically W-6, W-11, and W-13 are *not* found in the zymogen, and the status of W-17 is still unclear owing to weakness of the electron density association with residue 70 mentioned above. On the other hand W-8, W-9, W-10, W-14, W-39, W-41, and W-50 are found in equivalent positions in both zymogen and enzyme. W-7 is found in approximately the same place in the two structures, but it is involved in somewhat different interactions in the two (compare Figures 2a and 2b).

The main point to be emphasized in this report, however, concerns a slight alteration in the geometry of the charge relay system that occurs as a consequence of the relative shift of the two cylinders just described. It is to this point we now turn our attention.

As will be seen from Figure 2b, in native chymotrypsin the side chain of Ser-195 is in the "up" orientation, with a χ_1 value of approximately +90°. In this orientation, the distance between Oγ of Ser-195 and Nε2 of His-57 is about 3.2 Å, too long by about 0.3 Å to make a good hydrogen bond but perhaps within the tolerance imposed by the present resolution and state of refinement of the chymotrypsin structure. However, a more striking indication that a good hydrogen bond cannot be present is the location of Oγ(195) with respect to the sp^2 orbital occupied by the unshared electron pair of Nε2(57). For a good hydrogen bond it would be expected that the Oγ(195) atom should lie in the plane of the His-57 imidazole side chain and at a distance of about 2.8–2.9 Å from Nε2(57) in the direction of the aforementioned sp^2 orbital. In fact Oγ(195) is actually 2.5 Å from this expected ideal hydrogen-bonding position, much greater than any reasonable estimate of error in the present chymotrypsin model. This displacement can easily be observed in Figure 2b where Oγ(195) is clearly well above the plane of the His-75 imidazole side chain and too far to the right with respect to Nε2(57). Distortion of the presumed hydrogen bond between these two atoms has already been noted by Birktoft and Blow (1972, p 233).

But there is a further complication to be dealt with. Huber et al. (1974) have raised a question concerning the orientation of the Ser-195 side chain in native chymotrypsin at its pH of optimal activity. They suggest it may be in an orientation other

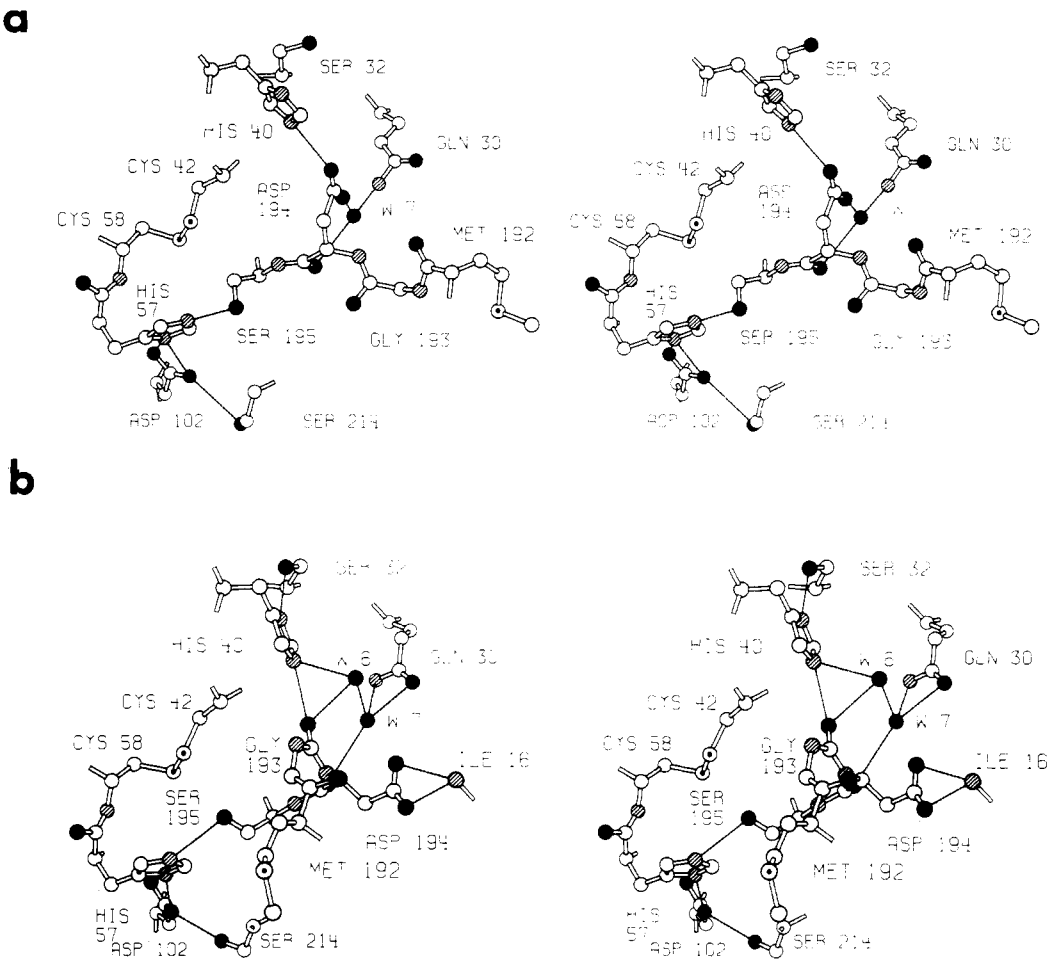

FIGURE 2: (a) Contact region between cylinders 1 and 2 near the incipient catalytic site in chymotrypsinogen. W-7 is an internal water molecule. (b) Contact region between cylinders 1 and 2 near the catalytic site in chymotrypsin. W-6 and W-7 are internal water molecules.

than that observed in the crystal structure. Is it possible, then, that a good hydrogen bond can be made between Nϵ2(57) and Oγ(195) when the latter is in some other orientation, as, for example, in the "down" or "acyl" orientation? In short, the answer is apparently not. We have examined a display of the model with an Evans & Sutherland Computer-Graphics System while freely rotating the Ser-195 side chain about its Cα–Cβ bond and found that: (1) the closest we could bring Oγ(195) to the ideal hydrogen-bonded position described above was 2.2 Å, and when it was in this position its distance to Nϵ2(57) had increased to 3.8 Å; and (2) the closest we could bring Oγ(195) to Nϵ2(57) was 2.6 Å, but it was then 2.9 Å from the ideal hydrogen-bonded position. At no intermediate rotation about the Cα–Cβ(195) bond is the situation any better. Similarly, it is not possible to improve the hydrogen-bonding geometry by any simple manipulation of the His-57 side chain. Our tentative conclusion, then, is that at best only a severely distorted hydrogen bond can exist between Oγ(195) and Nϵ2(57), and that this is a consequence of the local geometry of the backbone chain itself.

This structural detail in the enzyme is to be contrasted with that found in chymotrypsinogen. Inspection of Figure 2a shows that in the zymogen, the side chain of Ser-195 is in the "down" orientation, with a χ_1 value of about $-60°$. But more importantly Oγ(195) is in position to form a normal hydrogen bond with Nϵ2(57). That is, the Oγ to Nϵ2 distance is 2.7 Å and Oγ is only 0.7 Å from the ideal hydrogen bonded location described above. Moreover, it can also be seen by inspection of Figure 2a that Oγ(195) could not be rotated into the "up" orientation (as it is in the chymotrypsin model) because it would then penetrate the van der Waals sphere of Sγ(42). In fact, the distance between Sγ(42) on cylinder 1 and Cβ(195) on cylinder 2 is 3.5 Å in chymotrypsinogen; that is, these atoms are in van der Waals contact, whereas in chymotrypsin the corresponding distance is 4.7 Å. Thus in the zymogen Oγ(195) would be physically prevented from assuming the position between Sγ(42) and Cβ(195) that it occupies in the enzyme where free rotation about the Cα–Cβ(195) bond is evidently permitted.

The other hydrogen bond in the charge relay system, that between His-57 and Asp-102, is apparently not significantly different in the zymogen and the enzyme at present levels of resolution and refinement.

Conclusions

In light of the foregoing our conclusions concerning the structural effects of chymotrypsinogen activation upon the incipient charge relay system are as follows. An important consequence of activation is a shift in the relative positions of the two cylinders that constitute the principal folding domains of both zymogen and enzyme. This shift disrupts a normal hydrogen bond preexisting in the zymogen between Nϵ2(57) on cylinder 1 and Oγ(195) on cylinder 2 and moves the latter atom about 2.5 Å away from its ideal hydrogen-bonded location with respect to Nϵ2(57).

If it can be established that such a distorted hydrogen bond is a generally observed feature of the charge relay system in all the known serine proteases, the shift in relative position of

the two cylinders that causes the distortion of this hydrogen bond would then have to be included along with (1) completion of the specificity crevice and (2) closure of the oxyanion hole as one of the crucial events responsible for zymogen activation. In fact, preliminary examination (Kraut and Matthews, 1976) of the partially refined structures of both subtilisin (Matthews et al., 1976) and of β-trypsin (W. Bode, personal communication) strongly suggests that the hydrogen bond between the serine and histidine side chains of the charge relay system is distorted in all serine proteases. It is especially interesting to note that Wang (1970) has proposed that just such a distorted hydrogen bond in the charge relay system would facilitate the required transfer of a proton from the catalytic serine side chain to the leaving group of the substrate.

Acknowledgments

We thank Mr. Stephen Dempsey for programming the Evans & Sutherland Computer-Graphics System to permit display and manipulation of protein models.

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