finement programs, and to D. A. Waller for help with the use of the graphics programs.

References

Hodsdon, J. M., Sieker, L. C., & Jensen, L. H. (1975) *ACA Abstr. 3*, 16.

Jones, T. A. (1978) *J. Appl. Crystallogr. 11*, 268–272.

Koester, L., & Rauch, H. (1981) *Summary of Neutron Scattering Lengths*, IAEA-Contract 2517/RB, International Atomic Energy Agency, Vienna.

Lehmann, M. S. (1985) Report 85LE11T, Institut Laue-Langevin, Grenoble.

Lehmann, M. S., Mason, S. A., & McIntyre, G. J. (1985) *Biochemistry 24*, 5862–5869.

Mason, S. A., Bentley, G. A., & McIntyre, G. J. (1984) *Neutrons in Biology* (Schoenborn, B. P., Ed.) pp 323–324, Plenum Press, New York.

Thomas, M., Stansfield, R. F. D., Berneron, M., Filhol, A., Greenwood, G., Jacobé, J., Feltin, D., & Mason, S. A. (1983) *Position-Sensitive Detection of Thermal Neutrons* (Convert, P., & Forsyth, J. B., Eds.) pp 344–351, Academic Press, New York.

Thomas, R., Schoemaker, C. B., & Eriks, K. (1966) *Acta Crystallogr. 21*, 12–20.

Wilkinson, C., Khamis, H. W., Stansfield, R. F. D., & McIntyre, G. J. (1988) *J. Appl. Crystallogr. 21*, 471–478.

Wlodawer, A., & Hendrickson, W. A. (1982) *Acta Crystallogr., Sect A: Found. Crystallogr. A38*, 239–247.

Is γ-Chymotrypsin a Tetrapeptide Acyl-Enzyme Adduct of α-Chymotrypsin?†

M. M. Dixon and B. W. Matthews*

Institute of Molecular Biology and Department of Physics, University of Oregon, Eugene, Oregon 97403

Received March 3, 1989; Revised Manuscript Received May 9, 1989

ABSTRACT: Refinement of the structure of γ-chymotrypsin based on X-ray crystallographic data to 1.6-Å resolution has confirmed the overall conformation of the molecule as reported previously [Cohen, G. H., Silverton, E. W., & Davies, D. R. (1981) *J. Mol. Biol. 148*, 449–479]. In addition, the new refinement suggests that γ-chymotrypsin, which is operationally defined by its crystalline habit, may not be the free enzyme but rather a complex, possibly an acyl-enzyme adduct, with the tetrapeptide Pro-Gly-Ala-Tyr (or a close homologue). The crystallographic refinement provides a detailed geometrical description of the enzyme–substrate–solvent interactions that occur in the presumptive adduct.

γ-Chymotrypsin (γ-CHT) is a variant of α-chymotrypsin (α-CHT) originally identified by its different crystalline habit (Kunitz, 1938). Conversion of α-CHT to γ-CHT occurs at a pH (~7.0–8.0) at which the enzyme is active and was initially thought to be irreversible, suggesting that γ-CHT might be an autolytically degraded form of α-CHT. It was subsequently shown, however, that α-CHT can be obtained from γ-CHT, indicating that the two forms of the enzyme are covalently identical (Corey et al., 1965). Crystallographic studies have also shown that the structures of α- and γ-CHT are very similar (Matthews et al., 1967; Cohen et al., 1981).

The prior crystallographic refinement of γ-CHT by Davies and co-workers (Cohen et al., 1981) was based on 15 240 intensities to 1.9-Å resolution. Here we have extended the resolution to 1.6 Å and included 23 255 reflections. The new refinement confirms the overall conformation of the molecule as reported previously (Cohen et al., 1981). In addition, the refinement suggests that the form of crystalline γ-chymotrypsin used in the present study is not the free enzyme but is a complex, possibly an acyl-enzyme adduct, with the tetrapeptide Pro-Gly-Ala-Tyr. The crystallographic refinement provides a detailed geometric description of the enzyme–substrate–solvent interactions that occur in this complex.

The present study was prompted by the observation that crystals of γ-chymotrypsin are exceptionally resistant to changes in pH, the pregrown crystals being stable from pH 2 to pH 11 [B.W.M., reported in Cohen et al. (1981)]. This provided an unusually favorable opportunity to study the behavior of a crystalline protein and its bound solvent as amino acid side chains were titrated. The present paper derives from the high-resolution refinement of the structure at pH 7.0. Comparisons of this structure with the protein at pH 2.0 and pH 10.5 will be reported elsewhere.

Experimental Procedures

γ-Chymotrypsin was crystallized essentially as described (Cohen et al., 1981). The enzyme (Worthington CDG 6204-5) was dissolved to 25 mg/mL in water. To 0.5-mL aliquots were slowly added equal volumes of saturated ammonium sulfate, adjusted to pH 7.0, and the mixture was left at room temperature. (In the standard procedure, unbuffered ammonium sulfate, pH 5.5, is used.) Seeding with microcrystals is often helpful to promote nucleation, but was not used in the present instance.

X-ray data were measured to 1.6-Å resolution by oscillation photography (Schmid et al., 1981) (Table I).

Refinement was commenced with the previous model (Cohen et al., 1981) for the protein but with all water molecules deleted. Cycles of automatic refinement with the TNT system (Tronrud et al., 1981) were alternated with inspection of difference maps on an interactive graphics system in the usual manner. Solvent molecules were added conservatively. They were required to have density of at least 5σ in a difference map and to be located within H-bonding distance of appropriate donors or acceptors.

Results

As the refinement progressed it became obvious that there was significant unexplained electron density in the vicinity of

† This work was supported in part by the National Institutes of Health (GM20066), by the National Science Foundation (DMB8611084), and by a grant from the Lucille P. Markey Charitable Trust.

0006-2960/89/0428-7033$01.50/0

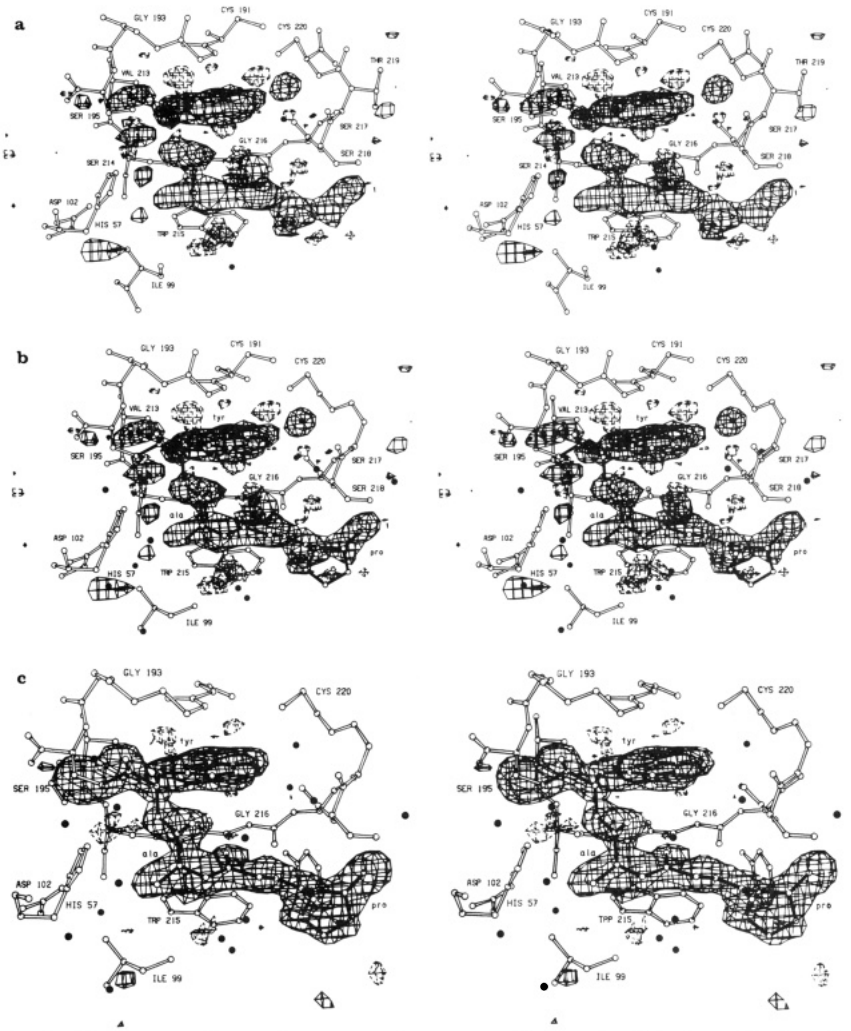

FIGURE 1: Maps showing the density corresponding to the presumptive tetrapeptide bound within the active site of γ-chymotrypsin. (a) Map with coefficients $(F_o - F_c) \exp(i\alpha_c)$, where F_o is the observed structure amplitude and F_c and α_c were calculated from the refined model for γ-chymotrypsin prior to inclusion of the presumptive tetrapeptide. The model used to calculate F_c and α_c is that shown in the Figure. It includes some water molecules (solid circles), but these do not coincide with the presumptive tetrapeptide. Contours are drawn at 3σ (solid) and -3σ (broken), where σ is the root mean square density throughout the unit cell. (b) The electron density map is the same as that shown in (a); i.e., it was calculated prior to inclusion of the presumptive tetrapeptide in the refinement. The superimposed structure corresponds to the final refined model of the protein–adduct complex. The presumptive tetrapeptide is drawn with solid bonds and is labeled with lower case lettering. Solvent atoms are indicated by solid circles. (c) Map with coefficients $(F_o - F_c) \exp(i\alpha_c)$, where F_o is the observed structure amplitude and F_c and α_c were calculated from the final refined γ-chymotrypsin–tetrapeptide complex with the tetrapeptide and the side chain of Ser 195 removed. Contours drawn at 3σ, where σ is the root mean square density throughout the unit cell.

the active site. This density appeared to correspond to, and could be satisfactorily modeled by, an extended polypeptide. The active-site density is shown in Figure 1. Figure 1a is a difference electron density map calculated with phases and amplitudes from the partially refined protein model prior to the inclusion of any "extra" atoms in the active site. Because this electron density map was calculated before any attempt was made to model the active-site density, or to include it in the refinement, the representation seen in Figure 1a is free of any possible bias concerning the identity of the bound moiety.

In terms of the subsequent crystallographic refinement, the most plausible explanation for the active-site density is that it corresponds to a bound tetrapeptide of sequence Pro-Gly-Val-Tyr. Furthermore, the refinement also suggests that this tetrapeptide may be covalently linked to Ser 195. In the refinement the interatomic distance between Ser 195 (O^{γ}) and the presumed tetrapeptide was unconstrained and assumed a final value of 1.7 Å. The final refined model for the presumed tetrapeptide adduct, superimposed on the same (unbiased) density shown in Figure 1a, is displayed in Figure 1b.

Difference maps in which the side chain of Ser 195 and the presumed tetrapeptide were deleted from the final refined model seem to be fully consistent with a tetrapeptide bound to Ser 195 as an acyl-enzyme adduct (Figure 1c). Note that density connecting the presumptive tetrapeptide to Ser 195 is seen in Figure 1c but is neither seen nor anticipated in Figure 1a,b because in these cases the side chain of Ser 195 is included as part of the model and so is "subtracted out" from the difference density map. Although the active-site density appeared to connect to the side chain of Ser 195, alternative models were explored and refined in which the presumed polypeptide was not covalently linked to the serine. It is difficult to rule out the chemically most plausible possibility, namely, that the active-site density corresponds to a product of hydrolysis analogous to those seen for tetrapeptides bound to the bacterial serine proteases (James et al., 1980). The evidence against such an enzyme–product complex in the present instance is twofold. First, a product does not explain the observed electron density as well as a covalent adduct. Second, the attempted refinement of an enzyme–product complex resulted in steric interference between the "product"

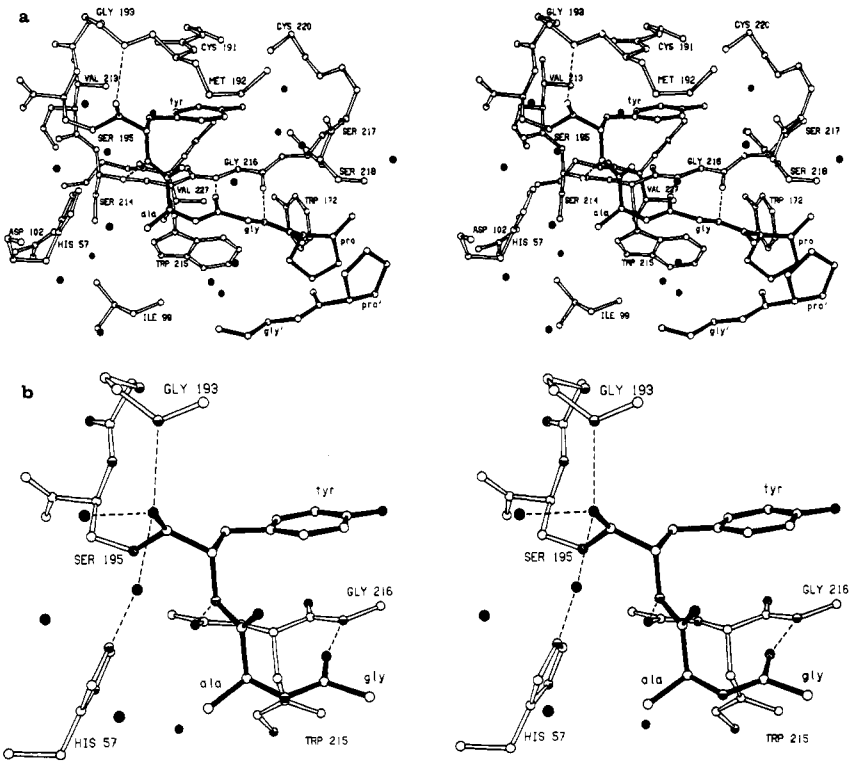

FIGURE 2: Stereoviews of the presumptive tetrapeptide (solid bonds; names in lower case) bound to crystalline γ-chymotrypsin. (a) Extended view showing the bound tetrapeptide (Pro-Gly-Ala-Tyr) together with part of the twofold-related tetrapeptide (Pro′-Gly′). The twofold symmetry axis extends from left to right between the two bound tetrapeptides. (b) Close-up view showing the detailed geometry, as suggested by the crystallographic refinement, in the vicinity of Ser 195 and His 57. Oxygen atoms, including bound solvent, are drawn solid, nitrogen atoms are drawn half solid, and carbon atoms are shown as open circles.

carboxylate and the side chain of Ser 195.

Statistics for the final refinement are included in Table I. The coordinates will be deposited in the Brookhaven Data Bank.

Discussion

Reliability of the Crystallographic Refinement. The inference that crystalline γ-chymotrypsin is an acyl-enzyme adduct is consistent with, and helps to explain, a number of properties of γ-chymotrypsin reported previously (see below). On the other hand, such an acyl-enzyme adduct would be expected to be very short-lived, at least in solution (Bender et al., 1964; Ingles & Knowles, 1968). It is therefore appropriate to discuss the reliability of the crystallographic analysis.

Overall, the refined structure of the protein obtained here agrees well with that reported previously (Cohen et al., 1981). The α-carbon atoms in the respective refined models have a root mean square discrepancy of 0.21 Å. The value for all atoms is 0.48 Å.

It is always of concern that errors in a crystallographic refinement introduce "bias" and are difficult to correct. As discussed above, parts a and b of Figure 1 show the active-site density prior to any attempt to model it and to include it in the refinement process. In an attempt to remove bias that may have entered subsequently, the coordinates for the presumptive tetrapeptide and the side chain of Ser 195 were deleted and the remaining structure refined for 14 additional cycles. A difference map, $(F_o - F_c)$ (not shown), with amplitudes and phases from this model gave virtually the same result as that shown in Figure 1c.

It may be asked why the previous refinement did not detect the postulated tetrapeptide. In the first place the prior work was to lower resolution. In the second place the previous refinement did suggest atoms other than solvent occupying the active-site region (personal communication from G. Cohen). It should also be noted that the crystals used by Cohen et al. (1981) were grown at pH 5.5, whereas the present experiments were at pH 7.0, where the enzyme is more active. Nevertheless, the two crystal forms seem to be virtually identical. Dr. Cohen kindly provided his observed structure amplitudes, permitting a direct comparison. A map using, as coefficients, the differences between the two sets of observed structure amplitudes did not suggest any significant difference between the two crystals.

It has been shown that some serine protease inhibitors will react with pregrown crystals of γ-CHT [e.g., Cohen et al. (1969) and Segal et al. (1971)]. This indicates that the presumptive tetrapeptide either can be displaced from the active site or was present in relatively low levels in the crystals used for the inhibitor-binding studies.

Origin of the Presumptive Tetrapeptide. The crystallographic refinement and difference electron density maps are consistent with a bound tetrapeptide of sequence Pro-Gly-Ala-Tyr (Figures 1 and 2). The identification of this sequence is subject to the same uncertainties that occur in the determination and refinement of protein structures of unknown amino acid sequence. Such studies suggest that in well-determined cases up to 70–80% of an amino acid sequence can be correctly identified, but success rates in the range 50–60% are more typical (Matthews, 1977). Inspection of the amino acid sequence of α- (or γ-) chymotrypsin (Hartley, 1964; Blow et al., 1969) reveals that residues 225–228 have the sequence Pro-Gly-Val-Tyr. Could this be the same tetrapeptide as seen in the active site? Although residue 3 of the tetrapeptide adduct was identified as alanine, inspection of the refined crystal structure shows that a valine side chain could be accommodated at this site without steric hindrance (Figure 2). If a valine were at this location and its side chain were in free rotation, it could appear as an alanine. We therefore assume,

Table I: Data Collection and Refinement Statistics

intensity statistics	
film packs	40
average R_{sym}[a] (%)	3.1
average R_{sca} (%)	3.7
R_{merge} (%)	6.6
total reflections measured	58 476
unique reflections	23 255
resolution (Å)	1.6
refinement statistics	
initial R factor (with solvent)[b] (%)	27.6
initial R factor (without solvent)[b] (%)	31.4
cycles	153
final R factor (%)	17.3
rms deviations from ideal stereochemistry	
bond lengths (Å)	0.019
bond angles (deg)	3.1
torsion angles (not restrained) (deg)	17.5
trigonal planarity (Å)	0.021
other planar groups (Å)	0.022

[a] R-values as defined by Schmid et al. (1981). [b] Coordinates from Cohen et al. (1981).

as a working hypothesis, that the presumed tetrapeptide seen in the γ-chymotrypsin active site is generated by autoproteolytic cleavage.

In the crystal structures of α- and γ-chymotrypsin, residues 221–225 (Ser-Thr-Ser-Thr-Pro) form a mobile, solvent-exposed, loop that could be a site of autoproteolytic cleavage. Because the last residue that participates in a disulfide bridge is Cys 220, cleavage beyond this point would tend to allow the polypeptide segment from the cleavage site to the C-terminus (residue 245) to dissociate from the remainder of the molecule, in which case the Tyr 228–Ala 229 bond would be a prime target for cleavage, giving a peptide consistent with that observed in the crystal structure. Although the crystallographic refinement reveals electron density that corresponds to a tetrapeptide, the bound species could actually be a pentapeptide or longer. Additional amino acid(s) beyond the proline could extend into the solvent and not be visible because of disorder. In the crystal the proline is, in part, held rigidly because pairs of prolines that are related by crystallographic symmetry contact each other (Figure 2a). Throughout this paper we refer to the presumptive peptide as Pro-Gly-Ala-Tyr, which corresponds to the density seen crystallographically, but it should be understood that X-Pro-Gly-(Val/Ala)-Tyr is equally consistent with the X-ray data.

Attempts were made to displace the presumed adduct from crystalline γ-CHT and to determine its composition directly. In one series of experiments the inhibitor L-1-(tosylamino)-2-phenylethyl chloromethyl ketone (TPCK; Schoellmann & Shaw, 1963) was used as follows: Crystals of γ-Chymotrypsin were dissolved in various solutions of TPCK consisting of 0.1 M KH_2PO_4, pH 6.3, 0.5–1.0 mM TPCK (Aldrich 85G725-4), and 2% methanol or 20% acetonitrile, with the final concentration of chymotrypsin being approximately 0.5–1.0 mM. The protein solution was incubated for 1.5–2 h at room temperature and submitted to HPLC analysis. Similar reactions were carried out using lyophilized protein. (The concentration of TPCK was limited to ≤1.0 mM due to the conditions required by the HPLC column.) In another set ot experiments crystals of γ-chymotrypsin were dissolved in 1 M hydroxylamine to a final protein concentration of 0.2–0.5 mM and submitted directly to HPLC analysis. HPLC analysis was also carried out with unreacted γ-chymotrypsin by using either dissolved crystals or lyophilized protein. In each case HPLC yielded a number of different peptides, the most abundant of which were sequenced and found to correspond to chymotrypsin autolysis products, although not the one presumed to occupy the active site (data not shown). These experiments suggest that crystalline γ-chymotrypsin is partially autolyzed. Lumry and co-workers (Yapel et al., 1965) found that all commercial preparations of α-chymotrypsin contained contaminants including presumed autolysis products as well as a tightly bound moiety that prevented substrate binding.

Relation between γ- and α-Chymotrypsin. The presumption that γ-chymotrypsin is a complex of chymotrypsin with a product of its own autolysis would help to explain much of the early literature on the relation between the α and γ enzymes. The conversion of α- to γ-chymotrypsin requires exposure to pH at which the enzyme is active (Kunitz, 1938; Corey et al., 1965). γ-Chymotrypsin would be seen to result from the partial autolysis of α-CHT, as was at one time surmised, but would require the presence of intact α-CHT. This would also imply that the α → γ → α interconversion is not truly reversible, but consumes part of the enzyme molecules during the α → γ transition, as was, in fact, observed in Kunitz's (1938) original experiments.

We presume that the growth of crystals of γ-CHT does not require full occupancy of all the the active sites in the crystal with the presumptive tetrapeptide. In the present case the crystallographic refinement indicates that the occupancy is approximately 0.94. Also the γ-CHT crystal form can, for example, be obtained with enzyme inhibited with diisopropyl fluorophosphate (DFP) (Corey et al., 1965) and toluenesulfonyl fluoride (Sigler et al., 1964).

The above assumptions are consistent with the observation that α-CHT can be readily converted to γ-CHT. However, once the active site of the enzyme becomes occupied with bound tetrapeptide, reversion to α-CHT would be hindered because the close apposition of pairs of active sites in the α-CHT crystals would necessitate prior removal of the tetrapeptide. This could explain the difficulty of reconverting γ-CHT into α-CHT (Kunitz, 1938; Corey et al., 1965). An exception occurs for chymotrypsin inhibited with DFP, in which case the interconversion from α to γ and from γ to α is facile (Massey & Hartley, 1956; Corey et al., 1965). DFP is small enough to be accommodated in both the α and γ crystal forms, but at the same time would prevent the binding of other larger inhibitors, thus removing potential obstacles to the α → γ → α transition.

It is well-known that α-CHT readily dimerizes in solution at low pH but γ-CHT does not (Miller et al., 1971; Gorbunoff et al., 1978). There is also strong evidence that the dimer of α-CHT formed in solution is the same as that seen in the crystals (Hexter & Westheimer, 1971; Birktoft & Blow, 1972). As noted above, the presence of a tetrapeptide bound in the active site of the enzyme would prevent the formation of such a dimer. The presumption that γ-CHT is an enzyme–product complex is therefore also consistent with the differences in aggregation of α-CHT and γ-CHT.

Implications for the Mechanism of Action. The interaction of the presumed tetrapeptide adduct with γ-CHT displays many of the enzyme–substrate interactions that have been inferred from prior studies of serine protease–inhibitor complexes [e.g., see Segal et al. (1971), Blow (1976), Bode et al. (1986), and Fujinaga et al. (1987) and references cited therein]. These interactions include hydrophobic and hydrogen-bonding interactions of the Tyr side chain in the S_1 specificity pocket as well as backbone hydrogen bonds between the tetrapeptide and the protein (Gly 216) in the S_3 subsite (Figures 2 and 3).

This distance from His 57 ($N^{\epsilon 2}$) to O^{γ} of Ser 195 is 3.09 Å, which is substantially less than the value of 3.8 Å found

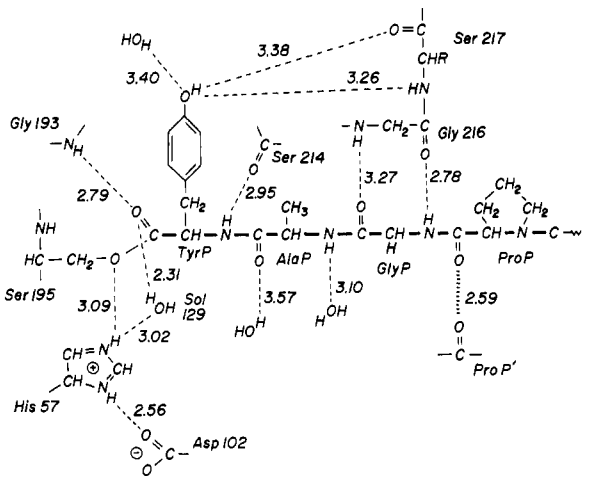

FIGURE 3: Schematic illustration showing the hydrogen-bonding interactions (distances in angstroms) between the presumptive tetrapeptide and γ-chymotrypsin.

by Cohen et al. (1981) but is in line with values reported for other serine proteases (Marquat et al., 1983; Tsukada & Blow, 1985; Read & James, 1988). It will be noted, however (Figures 2 and 3), that His 57 does not appear to be hydrogen-bonded to Ser 195 but to a water molecule (Sol 129). This has been observed in other acyl-enzyme complexes (Henderson, 1970) and is as expected for a presumptive acyl-enzyme adduct in which the presumed role of His 57 in deacylation is to promote the attack of a water molecule, not to help activate Ser 195.

The atoms involved in the apparent covalent link between Ser 195 and the tetrapeptide (Figures 2 and 3) were not constrained during the refinement to be coplanar, although this turned out to be the case (rms deviation from planarity of 0.08 Å). With one exception, all the atoms in the apparent protein–tetrapeptide linkage have low crystallographic thermal factors ($12\ Å^2 < B < 24Å^2$), suggesting that their positions are well determined. The exception is the carbonyl oxygen of the tetrapeptide tyrosine (hereafter O,TyrP), which appears to be quite mobile ($B = 46.5\ Å^2$). Interestingly, this oxygen is *not* located in the "oxyanion hole" (Henderson, 1970; Robertus et al., 1972). In the fully developed tetrahedral intermediate, stabilization of the oxyanion is thought to be provided by two hydrogen bonds, one from the amide of Gly 193 and the other from the amide of Ser 195 (Figure 2b). In the present complex the first of these is observed (distance of 2.79 Å) (Figure 3) but the second is not (distance of 3.54 Å). Oxygen O,TyrP appears to occupy a position between that of the oxyanion hole and the position corresponding to the leaving nitrogen of a peptide substrate. Indeed, the high thermal factor of the oxygen suggests that it may, to some degree, be sampling these two alternative positions. Full penetration of the oxygen into the oxyanion hole would be expected to provide geometry appropriate for deacylation, and this is exactly as suggested by the bound adduct. There is a water molecule (Sol 129) that appears to accept a hydrogen bond from His 57 (distance of 3.02 Å) and, in the refined model, is 2.40 Å[1] from the carbonyl carbon C,TyrP. This water molecule appears to be poised to attack the carbonyl carbon, except that the angle Sol 129–C,TyrP–O,TyrP is 71°, whereas the optimal angle of attack is about 107° (Burgi et al., 1973). If oxygen O,TyrP were to move into the oxyanion hole, the above angle would approach its optimum value for deacylation. If, indeed, γ-CHT crystals do contain a covalently linked acyl-enzyme adduct, then one would have to argue that the crystal geometry favors a configuration that approaches toward, but is not optimal for, deacylation. In a variation on this scenario one could imagine that there is an equilibrium in the crystals between the acyl-enzyme adduct and the products, with the equilibrium favoring the former.

[1] Solvent atom Sol 129 has a crystallographic thermal factor of 39 Å^2 and an occupancy of 0.73. Its position is therefore not defined as well as the more rigid parts of the structure. This could contribute to the anomalously short contact of 2.4 Å. It is also possible that the crystal structure represents an average of two or more conformers.

Acknowledgments

We are pleased to acknowledge the help of Drs. L. Weaver and D. Tronrud with data collection and refinement and Dr. M. Lindorfer for expert help in attempting to separate and identify the presumptive tetrapeptide. We thank Drs. G. H. Cohen and D. R. Davies for providing structure amplitudes and refined coordinates for γ-chymotrypsin. We are grateful for helpful discussions concerning γ-chymotrypsin and the serine proteases with Drs. G. H. Cohen, D. R. Davies, R. Henderson, H. Neurath, P. B. Sigler, and D. Teller.

Registry No. γ-Chymotrypsin, 9004-07-3.

References

Bender, M. L., Kezdy, F. J., & Gunter, C. R. (1964) *J. Am. Chem. Soc. 86*, 3714–3721.

Blow, D. M. (1976) *Acc. Chem. Res. 9*, 145–152.

Blow, D. M., & Birktoft, J. J. (1972) *J. Mol. Biol. 68*, 187–240.

Blow, D. M., Birktoft, J. J., & Hartley, B. S. (1969) *Nature 221*, 337–340.

Bode, W., Wei, A. Z., Huber, R., Meyer, E., Travis, J., & Neumann, S. (1986) *EMBO J. 5*, 2453–2458.

Bürgi, H. B., Dunitz, J. D., & Shefter, E. (1973) *J. Am. Chem. Soc. 95*, 5065–5067.

Cohen, G. H., Silverton, E. W., Matthews, B. W., Braxton, H., & Davies, D. R. (1969) *J. Mol. Biol. 44*, 129–141.

Cohen, G. H., Silverton, E. W., & Davies, D. R. (1981) *J. Mol. Biol. 148*, 449–479.

Corey, R. B., Battfay, O., Bruekner, D. A., & Mark, F. G. (1965) *Biochim. Biophys. Acta 94*, 535–545.

Fujinaga, M., Sielecki, A. R., Read, R. J., Ardelt, W., Laskowski, M., Jr., & James, M. N. G. (1987) *J. Mol. Biol. 195*, 397–418.

Gorbunoff, M. J., Fosmire, G., & Timasheff, S. N. (1978) *Biochemistry 17*, 4055–4065.

Hartley, B. S. (1964) *Nature 201*, 1284–1287.

Henderson, R. (1970) *J. Mol. Biol. 54*, 341–354.

Hexter, C. S., & Westheimer, F. H. (1971) *J. Biol. Chem. 246*, 3928–3933.

Ingles, D. W., & Knowles, J. R. (1968) *Biochem. J. 108*, 561–569.

James, M. N. G., Sielecki, A. R., Brayer, G. D., Delbaere, L. T. J., & Bauer, C.-A. (1980) *J. Mol. Biol. 144*, 43–88.

Kunitz, M. (1938) *J. Gen. Physiol. 22*, 207–237.

Marquat, M., Walter, J., Deisenhofer, J., Bode, W., & Huber, R. (1983) *Acta Crystallogr. B39*, 480–490.

Massey, V., & Hartley, B. S. (1956) *Biochim. Biophys. Acta 21*, 361–367.

Matthews, B. W. (1977) *Proteins (3rd Ed.) 3*, 403–590.

Matthews, B. W., Sigler, P. B., Henderson, R., & Blow, D. M. (1967) *Nature 214*, 652–656.

Miller, D. D., Horbett, T. A., & Teller, D. C. (1971) *Biochemistry 10*, 4641–4648.

Read, R. J., & James, M. N. G. (1988) *J. Mol. Biol. 200*, 523–551.

Robertus, J. D., Kraut, J., Alden, R., & Birktoft, J. J. (1972) *Biochemistry 11*, 4293-4303.
Schmid, M. F., Weaver, L. H., Holmes, M. A., Grütter, M. G., Ohlendorf, D. H., Reynolds, R. A., Remington, S. J., & Matthews, B. W. (1981) *Acta Crystallogr. A37*, 701-710.
Schoellmann, G., & Shaw, E. (1963) *Biochemistry 2*, 252-255.
Segal, D. M., Powers, J. C., Cohen, G. H., & Davies, D. R. (1971) *Biochemistry 10*, 3728-3738.
Sigler, P. B., Skinner, H. C. W., Coulter, C. L., Kallos, J., Braxton, H., & Davies, D. R. (1964) *Proc. Natl. Acad. Sci. U.S.A. 51*, 1146-1151.
Tronrud, D. E., Ten Eyck, L. F., & Matthews, B. W. (1987) *Acta Crystallogr. A43*, 489-503.
Tsukada, H., & Blow, D. M. (1985) *J. Mol. Biol. 184*, 703-711.
Yapel, A., Han, M., Lumry, R., Rosenberg, A., & Schiao, D. F. (1966) *J. Am. Chem. Soc. 88*, 2573-2584.

A Nuclear Overhauser Effect Investigation of the Molecular and Electronic Structure of the Heme Crevice in Lactoperoxidase†

V. Thanabal and Gerd N. La Mar*
Department of Chemistry, University of California, Davis, California 95616
Received October 21, 1988; Revised Manuscript Received April 28, 1989

ABSTRACT: The proton homonuclear nuclear Overhauser effect, NOE, in conjunction with paramagnetic-induced dipolar relaxation, is utilized to assign resonances and to probe the molecular and electronic structures of the heme cavity in the low-spin cyanide complex of resting-state bovine lactoperoxidase, LPO-CN. Predominantly primary NOEs were detected in spite of the large molecular weight ($\sim 78 \times 10^3$) of the enzyme, which demonstrates again the advantage of paramagnetism suppressing spin diffusion in large proteins. Both of the nonlabile ring protons of a coordinated histidine are located at resonance positions consistent with a deprotonated imidazole. Several methylene proton pairs are identified, of which the most strongly hyperfine-shifted pair is assigned to the unusual chemically functionalized 8-(mercaptomethylene) group of the prosthetic group [Nichol, A. W., Angel, L. A., Moon, T., & Clezy, P. S. (1987) *Biochem. J. 247*, 147-150]. The large 8-(mercaptomethylene) proton contact shifts relative to that of the only resolved heme methyl signal are rationalized by the additive perturbations on the rhombic asymmetry of the functionalization of the 8-position and the alignment of the axial histidyl imidazole projection along a vector passing through pyrrole A and C of the prosthetic group. Such a stereochemistry is consistent with the resolution of only a single heme methyl group, 3-CH_3, as observed. A pair of hyperfine-shifted methylene protons, as well as a low-field hyperfine-shifted labile proton signal, exhibit dipolar connectivities similar to those previously reported for the distal arginine and histidine, respectively, of horseradish peroxidase [Thanabal, V., de Ropp, J. S., & La Mar, G. N. (1988) *J. Am. Chem. Soc. 110*, 3027-3035], suggesting that these catalytically relevant residues may also exist in LPO.

Heme peroxidases share the common property of reaction with peroxides to yield a pair of reactive intermediates 1 and 2 oxidizing equiv above the high-spin ferric resting state (Dunford & Stillman, 1976; Morrison & Schonbaum, 1979; Dunford, 1982). These oxidizing equivalents are stored on the iron (Fe^{IV}=O for compound II), with the second one residing largely as a cation radical [porphyrin for horseradish peroxidase, HRP,[1] (Dolphin et al., 1971); amino acid side chain in cytochrome *c* peroxidase, C*c*P (Yonetani & Ray, 1965)]. On the basis of the detailed X-ray structural features determined for the various derivatives of C*c*P (Poulos & Kraut, 1980; Finzel et al., 1984; Edwards et al., 1987), it has been proposed that a general feature of the catalytic site of heme peroxidase would include a distal His to act as a base to transfer a proton and a distal Arg to provide stabilization for the heterolytic cleavage of the O-O bond. While similar crystallographic data are not yet available for HRP, partial sequence homology (Welinder, 1979; Takio et al., 1980) and computer modeling (Sakurada et al., 1986) have indicated the presence of these catalytically relevant residues, and detailed solution ^{1}H NMR studies of HRP-CN have unequivocally established not only the presence of both distal His and Arg but with detailed stereochemistry virtually identical with that of C*c*P (Thanabal et al., 1987a,b, 1988a,b).

Lactoperoxidase, LPO, present in mammalian milk, saliva, and tears, is involved in bacterial defense through the oxidation of thiocyanate ion (Hamon & Klebanoff, 1973). The glycoprotein possesses a heme which is extraordinarily tightly bound to a single polypeptide chain with $M_r \sim 78 \times 10^3$ (10% carbohydrate) (Carlstrom, 1969; Sievers, 1981). Originally, partial extraction led to small yields of a porphyrin which appeared to be normal, noncovalently bound protoporphyrin, suggesting that the heme resides in a buried pocket (Sievers, 1979, 1980). More recent work based on reductive cleavage with mercaptoethanol has resulted in a structure for the extracted heme prosthetic group as shown in Figure 1A, where

† This research was supported by a grant from the National Institutes of Health (GM 26226).

[1] Abbreviations: C*c*P, cytochrome *c* peroxidase; HRP, horseradish peroxidase; LPO, lactoperoxidase; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; ppm, parts per million; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

0006-2960/89/0428-7038$01.50/0