Properties of the Histidine Residues of Indole-Chymotrypsin

IMPLICATIONS FOR THE ACTIVATION PROCESS AND CATALYTIC MECHANISM

By WILLIAM H. CRUICKSHANK and HARVEY KAPLAN

Department of Biochemistry, University of Ottawa, Ottawa, Ont. K1N 6N5, Canada

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The use of a linear free-energy relationship shows that both histidine residues of α -chymotrypsin and chymotrypsinogen are super-reactive toward 1-fluoro-2,4-dinitrobenzene. The binding of indole to the specificity site of α -chymotrypsin causes both histidine residues to become less reactive. On the basis of these results and those from X-ray-crystallographic studies, the following conclusions are made. (1) The super-reactivity of the catalytic-site histidine-57 is due to charge transfer from aspartic acid-102 by means of hydrogen bonding. (2) The aspartic acid-102–histidine-57–serine-195 ‘charge-relay’ system is not complete in the zymogen or native enzyme, and only on binding of a suitable substrate or ligand to the specificity site of the enzyme is the charge transfer to serine-195 completed. (3) The lack of substantial enzymic activity in the zymogen is due to the absence of a completed specificity site, and therefore it cannot bind suitable substrates or ligands to induce completion of the charge-relay system.

The aspartic acid-102–histidine-57–serine-195 ‘charge-relay system’ proposed by Blow *et al.* (1969) for chymotrypsin has been the focus of considerable attention. This proposal was novel in that the catalytic efficiency was in part attributed to a specific protein structure at the catalytic site, which conferred an enhanced reactivity of the serine hydroxyl group towards substrates or substrate-like compounds. It was postulated that a portion of the charge from a buried aspartic acid residue was transferred by means of a hydrogen-bonded network to the serine-195 hydroxyl group, thereby making it a more powerful nucleophile. The evidence for such a system has come from X-ray-crystallographic studies (Blow *et al.*, 1969), but unfortunately these studies only indicate the possibility of hydrogen bonding and cannot unequivocally establish the extent to which it occurs in the protein free in solution (Birktoft & Blow, 1972). Nevertheless, there is little doubt as to the catalytic importance of this arrangement of groups, since it is maintained within the family of pancreatic serine proteinases (Shotton & Watson, 1970; Krieger *et al.*, 1974) and is also present in subtilisin, which is not at all closely related to the pancreatic series (Alden *et al.*, 1970).

The ionization constants and reactivities of the histidine residues of α -chymotrypsin (Cruickshank & Kaplan, 1972) and chymotrypsinogen (Cruickshank & Kaplan, 1974) have been determined in previous studies. Although we consider that a knowledge of these properties is essential for an understanding of the activation process as well as the catalytic mechanism,

there is no reason to assume that these properties remain unaltered throughout the catalytic process. For example, there is a growing amount of evidence that conformational changes induced by substrates or ligands (Koshland & Neet, 1968) are an important aspect of catalysis by many enzymes, and it is implicit in such mechanisms that the binding of a substrate will alter the chemical properties of the catalytic groups. Moreover, it is the chemical properties in the presence of substrates or ligands that ultimately determine the efficiency of the catalytic process. For these reasons we have undertaken, in this study, to determine the properties of the histidine residues, and in particular histidine-57, of indole-chymotrypsin. The knowledge of these properties, together with those in the free enzyme and zymogen, should provide a basis for understanding the relation between structure and chemical properties in producing efficient catalysis.

Materials and Methods

Materials

α -Chymotrypsin (3 $\times$ crystallized, salt-free) and pepsin (2 $\times$ crystallized, salt-free) were purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. 1-Fluoro-2,4-dinitro[3,5- 3 H]benzene (specific radioactivity 24.1 Ci/mmol) and Aquasol scintillation fluid were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. α -N-Acetyl-L-histidine was supplied by Nutritional Biochemicals Corp.,

Cleveland, Ohio, U.S.A., and the indole used was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A. All other compounds and reagents were high-purity preparations obtained from commercial sources.

Methods

Trace-labelling procedure. The trace-labelling procedure was identical with that described previously for α -chymotrypsin (Cruickshank & Kaplan, 1972) except that indole was added to the reaction mixture. α -Chymotrypsin (25mg) was dissolved in 2.5ml of 0.10M-KCl containing 1.0 μ mol of α -N-acetyl-L-histidine, and to this was added 50 μ l of a 0.52M solution of indole in acetonitrile, yielding a final indole concentration of 10mM. The reaction mixture was maintained at 20.0°C under a N₂ atmosphere and adjusted to the desired pH value by the addition of 5M-KOH. The labelling was initiated by the addition of 1-fluoro-2,4-dinitro[³H]benzene ([³H]Dnp-F) (0.030 μ M, specific radioactivity 24.1 Ci/mmol) and was allowed to proceed for 45min at pH 5.12, 5.45, 6.09, 6.55, 6.80, 7.15, 7.61, 8.01, 8.52 and 9.10, or for 30min at pH 9.71 and 10.50. The reaction was stopped by lowering the pH of the reaction mixture to 1.5 with the addition of several drops of concentrated HCl, and the unchanged Dnp-F was extracted with ether. The solution of trace-labelled protein and internal standard was then freeze-dried.

Determination of specific radioactivities. The procedures used for making the samples of trace-labelled protein and internal standard chemically homogeneous and the subsequent steps involved in separation, purification and determination of specific radioactivity of the internal standard and histidine peptides A1 (histidine-57) and A2 (histidine-40) have been described in detail previously (Cruickshank & Kaplan, 1972).

Determination of the reactivity of standard imidazole compounds with Dnp-F. The second-order rate constant, k , for the reaction of imidazole, 4-hydroxymethylimidazole, imidazolylacetic acid, imidazolyl- β -lactic acid and α -N-acetyl-L-histidine with Dnp-F was determined on a pH-stat at pH 9.5 and 20.0°C under a N₂ atmosphere under conditions in which the imidazole compound was in large excess over the Dnp-F; the reaction was thus pseudo-first-order. A solution (10ml of 0.10M) of the imidazole compound in 0.1M-KCl was placed in the reaction vessel of the pH-stat and the pH adjusted to 9.5 by the addition of 10M-KOH. The reaction was initiated by the addition of Dnp-F (20 μ l of 0.10M) in acetonitrile and the rate of addition of 0.02M-NaOH required to maintain the pH constant was recorded. The pseudo-first-order rate constant, $k_{\text{obs.}}$, was

determined from the initial slope of the pH-stat tracing. The spontaneous rate of hydrolysis of Dnp-F, k_w , was measured under identical conditions but in the absence of any imidazole-containing compound. The second-order rate constant, k , for the reaction of Dnp-F with the deprotonated imidazole compound, was calculated from the relationship

$$k = (k_{\text{obs.}} - k_w)/[\text{Im-R}]$$

where [Im-R] is the concentration of the imidazole compound in the reaction mixture.

The pK_a values for all the imidazole compounds were determined by potentiometric titration at 20.0°C under an N₂ atmosphere. An accurately weighed amount (approx. 20 μ mol) of the compound was dissolved in 10.0ml of 0.10M-KCl and the pH adjusted to approx. 3. The compound was then titrated to pH 10.5 by the addition of 0.10M-NaOH from an Agla micrometer-syringe. The pK_a of the imidazole moiety was determined from a plot of volume of base added versus pH after correcting all volumes for the blank value obtained from the titration of 10.0ml of 0.10M-KCl over the same pH range.

Results

The primary data for the trace-labelling of indole-chymotrypsin with [³H]Dnp-F are summarized in Table 1. The degree of dissociation of the internal standard, α_s , at each experimental pH was calculated assuming a pK_a of 7.20 (Cruickshank & Kaplan, 1972). The pH-dependent second-order relative rate constant, αr , was calculated for each histidine residue by using the expression:

$$\alpha r = \alpha_s \times \frac{\text{sp. radioactivity of Dnp-histidine peptide}}{\text{sp. radioactivity of internal standard}}$$

Figs. 1(a) and 1(b) show a plot of the pH-dependent second-order relative rate constant versus pH for the trace-labelling of histidine-57 and -40 of indole-chymotrypsin (open circles) with Dnp-F. The closed circles represent data previously obtained (Cruickshank & Kaplan, 1972) in a study of the native enzyme and are presented here to illustrate the low reactivity of the histidine residues in indole-chymotrypsin at acid pH values. Over the pH range studied (5–10.5), both histidine residues in indole-chymotrypsin do not react in a manner expected for groups which are simply interacting with solvent. Below pH 8, both histidine residues in indole-chymotrypsin are essentially non-reactive. At alkaline pH values these residues become reactive, but not in a manner consistent with a simple ionization process.

The second-order rate constants, k , for the reaction of Dnp-F with a series of imidazole compounds, and

Table 1. Summary of data for competitive labelling of the histidine residues of indole-chymotrypsin

Specific radioactivities were determined by amino acid analysis and scintillation counting. The α - values were calculated by assuming a pK_a of 7.2 for the imidazole moiety of α -N-acetylhistidine. The αr values for the histidine residues of indole-chymotrypsin were calculated by using the expression given in the Results section.

pH	α -N-Acetyl-L-histidine		Peptide A1, histidine-57		Peptide A2, histidine-40	
	$10^{-5} \times$ Radioactivity (c.p.m./ μ mol)	α_s	$10^{-5} \times$ Radioactivity (c.p.m./ μ mol)	αr	$10^{-5} \times$ Radioactivity (c.p.m./ μ mol)	αr
5.12	0.479	0.0104	0.577	0.0125	0.588	0.0127
5.45	2.45	0.0175	24.7	0.176	20.2	0.144
6.09	0.591	0.0720	0.337	0.0410	0.276	0.0336
6.55	1.42	0.183	0.806	0.104	0.581	0.0751
6.80	3.01	0.285	3.11	0.294	1.88	0.178
7.15	3.84	0.471	0.851	0.104	0.547	0.0670
7.61	5.11	0.720	1.60	0.225	12.1	1.70
8.01	6.92	0.866	8.56	1.07	38.7	4.84
8.52	6.35	0.954	6.90	1.04	27.8	4.17
9.10	3.52	0.988	5.56	1.56	3.19	0.894
9.71	3.31	0.997	8.05	2.43	3.48	1.05
10.50	1.22	0.999	4.68	3.83	0.525	0.429

Fig. 1. pH-reactivity profiles for (a) histidine-57 and (b) histidine-40 of indole-chymotrypsin (O) and native α -chymotrypsin (●)

The αr values for the histidine residues of indole-chymotrypsin were calculated by using the data in Table 1. The solid lines are the titration curves fitted to the corresponding data for the native enzyme.

Table 2. Reactivity of imidazole compounds and histidine residues of α -chymotrypsin and chymotrypsinogen

The ionization constants of the imidazole compounds were determined by titration and their second-order velocity constants (k) for the reaction with Dnp-F were determined by measuring initial rates. The ionization constants and reactivities of the histidine residues of α -chymotrypsin and chymotrypsinogen were determined by competitive labelling (Cruickshank & Kaplan, 1972, 1974).

Imidazole compound	pK_a	$10^2 \times k$ ($M^{-1} \cdot min^{-1}$)
4-Hydroxymethylimidazole	6.30	2.57 ± 0.11
α -N-Acetyl-L-histidine	7.20	4.35 ± 0.22
L- β -Imidazolyl-lactic acid	7.27	4.43 ± 0.08
Imidazolylacetic acid	7.31	4.93 ± 0.21
Imidazole	6.93	5.20 ± 0.28
Histidine-57, α -chymotrypsin	7.0	56
Histidine-57, chymotrypsinogen	7.5	19
Histidine-40, α -chymotrypsin	6.8	30
Histidine-40, chymotrypsinogen	8.0	49

the histidine residues of α -chymotrypsin and chymotrypsinogen, are summarized in Table 2. The values reported for the imidazole compounds are the average of two determinations and those for the histidine residues were obtained in earlier studies (Cruickshank & Kaplan, 1972, 1974). The data contained in Table 2 are represented on a Brønsted plot (Brønsted & Peterson, 1924) shown in Fig. 2. The series of standard imidazole compounds lie along a straight line; however, the points for the histidine residues of both α -chymotrypsin and chymotrypsinogen lie well above this line, indicating an enhanced reactivity over that expected on the basis of their pK_a values.

Fig. 2. Brønsted plot for the reaction of imidazole compounds with 1-fluoro-2,4-dinitrobenzene: (1) 4-hydroxymethyl-imidazole; (2) imidazole; (3) α -N-acetyl-L-histidine; (4) L- β -imidazolyl-lactic acid; (5) imidazolylacetic acid

The positions of the histidine residues of native α -chymotrypsin (CT) and chymotrypsinogen (CTG) in relation to this plot are based on the competitive labelling data summarized in Table 2.

Discussion

Indole is known to bind to the specificity site of α -chymotrypsin with a K_i of $5 \times 10^{-4} M$ (Garel & Labouesse, 1970), so that at the concentration used in the present study (10mM) the enzyme is essentially saturated. The results show that the binding of indole to α -chymotrypsin has a marked effect on the reactivity of its two histidine residues towards Dnp-F (Figs. 1a and 1b). At acidic and slightly alkaline pH values both histidine residues of indole-chymotrypsin are for all practical purposes non-reactive, whereas over the same pH range in the native enzyme these residues are extremely reactive. At alkaline pH values the histidine residues become reactive; however, the binding efficiency of indole decreases above pH8 (Garel & Labouesse, 1970), so that the enzyme is no longer saturated and the reactivity is therefore at least in part due to the presence of some free enzyme. Chymotrypsin and indole-chymotrypsin are also known to undergo major conformational changes at alkaline pH values (McConn *et al.*, 1969; Garel & Labouesse, 1970). In addition, other more subtle conformational changes appear to be occurring at alkaline pH values, as indicated by the abrupt decrease in the reactivity of the histidine residues of α -chymotrypsin (Cruickshank & Kaplan, 1972), by variations in thermodynamic parameters (Rajender *et al.*, 1971) and by the conversion of α -chymotrypsin into

γ -chymotrypsin (Kraut, 1971). From these considerations and the data obtained in the present study, it is obvious that the reactivities of the histidine residues of indole-chymotrypsin at alkaline pH values are governed by a complex set of equilibria. On the other hand, below pH8, X-ray crystallography (Steitz *et al.*, 1970) and other physical techniques (Garel & Labouesse, 1969) do not detect any conformational change on binding of indole, yet the reactivity of both histidine residues is decreased dramatically.

The X-ray-crystallographic structure of α -chymotrypsin (Birktoft & Blow, 1972) has histidine-57 in a position to interact with aspartic acid-102 and serine-195. If these interactions actually take place when the enzyme is in solution, then the histidine would be expected to have unusual chemical properties. On the basis of the Brønsted plot (Fig. 2) that we obtained, histidine-57 of α -chymotrypsin is indeed very unusual in that its reactivity is an order of magnitude greater than one would expect from its ionization constant. The most likely explanation for this phenomenon is similar to that originally offered by Blow *et al.* (1969), in which aspartic acid-102 and histidine-57 can form a hydrogen-bonded network whose chemical properties will be intermediate between the various canonical forms that can be drawn. The ionization scheme for such a system is shown in Fig. 3. Deprotonation of the system represented by canonical forms (I) and (II) gives rise to the system represented by forms (III) and (IV), and this system has an ionization with an apparent pK_a 7.0. The increased nucleophilicity of histidine-57 is explained by the contribution of structure (IV) to the overall properties.

The observation that histidine-57 of α -chymotrypsin is super-reactive strongly suggests that its low reactivity in indole-chymotrypsin is of particular mechanistic significance. In view of the fact that indole does not occlude the catalytic site and that no observable conformational changes take place on binding of indole, the only reasonable explanation for these observations is that histidine-57 has formed an additional hydrogen bond with serine-195 (Fig. 4) and therefore no longer has a lone pair of electrons available for a nucleophilic attack. Since we have shown that in α -chymotrypsin a portion of the charge from aspartic acid-102 is transferred to histidine-57, this charge in the hydrogen-bonded structure shown in Fig. 4 must be transferred to serine-195, making it a more powerful nucleophile. Our observations therefore support the proposal of a charge-relay mechanism, but this mechanism is only operative when the substrate or a suitable ligand is bound at the specificity site and is not operative in the free enzyme, as originally proposed by Blow *et al.* (1969).

Fig. 3. Ionization scheme for the aspartic acid-102-histidine-57 system in native α -chymotrypsin

(I) and (II) represent canonical forms of the protonated system. (III) and (IV) represent canonical forms of the deprotonated system.

Fig. 4. Proposed hydrogen bonding of serine-195 in the presence and absence of a substrate or ligand at the specificity site of chymotrypsin

Histidine-57 of chymotrypsinogen is also super-reactive (Fig. 2), which indicates that the charge-relay system involving serine-195 is not present in the zymogen. Since the specificity site is not complete in chymotrypsinogen (Freer *et al.*, 1970), it cannot bind substrates or ligands and therefore the charge-relay mechanism involving serine-195 is inoperative. Hydrolysis of the arginine-15-isoleucine-16 peptide bond in the zymogen permits the formation of the specificity site, and the activation process is complete by the binding of the substrate, which induces the formation of the complete charge-relay system.

An unexpected result of this study is that histidine-40, which is also super-reactive in native α -chymotrypsin and chymotrypsinogen (Figs. 1b and 2), probably as a result of interaction with the carbonyl group of glycine-193 and aspartic acid-194 respectively (Birktoft & Blow, 1972), becomes less reactive in indole-chymotrypsin. This is particularly surprising, because this residue is not believed to have a direct role in the catalytic process. However, this result substantiates our previous contention (Cruickshank & Kaplan, 1974) that the properties of histidine-57 and histidine-40 are linked, i.e. histidine-40 is an essential structural element, and suggests that the formation of new hydrogen bonds on binding of a suitable substrate or ligand extends beyond the immediate vicinity of the catalytic site.

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