A Competitive Labelling Method for Determining the Ionization Constants and Reactivity of Individual Histidine Residues in Proteins

THE HISTIDINES OF α -CHYMOTRYPSIN

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A competitive labelling method (Kaplan *et al.*, 1971), using tritiated 1-fluoro-2,4-dinitrobenzene as the labelling reagent, is described for determining the ionization constants and reactivities of individual histidine residues in proteins. When this method was applied to the two histidines of α -chymotrypsin, histidine-57 was found to have pK_a 6.8 and a reactivity ten times that of α -N-acetyl-L-histidine. Histidine-40 had pK_a 6.7 and a reactivity approximately six times that of α -N-acetyl-L-histidine. Between pH 7.5 and 8 the reactivities of both histidines decrease simultaneously to approximately that of α -N-acetyl-L-histidine. The high reactivities of the histidines are attributed to hydrogen bonding, which increases the nucleophilicity of the imidazole ring. The sharp decrease in reactivity between pH 7.5 and 8 is attributed to a conformational change that disrupts the hydrogen bonding by these residues. The reactivity data support the proposal of a charge-relay mechanism involving histidine-57 (Blow *et al.*, 1969), which makes serine-195 more nucleophilic but indicates that this system is fully operative only in the enzyme–substrate complex.

An important aspect in the study of structure–function relationships in proteins is the determination of the properties of individual ionizable groups within the native protein. Until the development of the method of competitive labelling (Kaplan *et al.*, 1971) no general method was available by which the pK_a and reactivity of a specific functional group in the protein could be determined. By the use of this method with tritiated acetic anhydride as the labelling reagent, it was possible to determine and unequivocally assign a pK_a and reactivity to specific amino groups in native elastase (Kaplan *et al.*, 1971) and native α -chymotrypsin (Kaplan, 1971, 1972). This information was useful in establishing the role of these groups in the catalytic properties of the enzyme and also in providing information about the dynamic properties of the tertiary structure. Since histidine is very often directly involved in the catalytic process of enzymes, the extension of this approach to histidine residues should provide the means for studying structure–function relationships as they apply to the catalytic mechanism.

The evidence that histidine-57 [residues are numbered according to the sequence of chymotrypsinogen A (Hartley, 1970)] of α -chymotrypsin is involved in the catalytic mechanism is extremely well documented (Bender & Kézdy, 1965; Ong *et al.*, 1965). The mechanisms that have been proposed (Bender & Kézdy, 1965; Cunningham, 1957) have this residue functioning as a general base catalyst, which accepts a proton

from serine-195 during the catalytic process. The X-ray-crystallographic structure (Sigler *et al.*, 1968) is consistent with this proposal and further indicates that aspartate-102, histidine-57 and serine-195 are closely associated and may form a charge-relay system (Blow *et al.*, 1969), which enhances the apparent nucleophilicity of serine-195. The pK_a (app.) of ~ 7 determined from kinetic studies (Bender & Kézdy, 1965) has been attributed to the ionization of histidine-57; however, there is no unequivocal evidence to corroborate this assignment.

The primary objective of the present study was to develop a general method for determining the ionization constants and reactivities of histidine residues in proteins. The histidines of α -chymotrypsin appear to be an appropriate choice for this study since histidine-57 may have unusual properties and the other histidine residue, histidine-40, which is not believed to have a direct role in the catalytic mechanism, can serve as a basis for comparison.

Experimental

Materials

α -Chymotrypsin ($3 \times$ crystallized, salt-free) and pepsin ($2 \times$ crystallized, salt-free) were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. 1-Fluoro-2,4-dinitrobenzene-3,5,6-T, specific radioactivity 17.8 Ci/mmol, was purchased from

Amersham-Searle Corp., Arlington Heights, Ill., U.S.A. and α -N-acetyl-L-histidine was a product of Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Aquasol scintillation liquid was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. All other compounds and reagents were high-purity preparations obtained from commercial sources.

Methods

Trace-labelling procedure. α -Chymotrypsin (25 mg) was dissolved in 2.5 ml of 0.10M-KCl containing 1.0 μ mol of α -N-acetyl-L-histidine and the solution placed in a reaction vessel maintained at $20.0^\circ \pm 0.05^\circ\text{C}$ under an atmosphere of N_2 . The pH of the solution was adjusted to the desired value by the addition of 1M-KOH (for pH values up to 7) or 5M-KOH (for pH values above 7) from a pH-stat and 0.014 μ mol of tritiated 1-fluoro-2,4-dinitrobenzene (specific radioactivity 17.8 Ci/mmol) dissolved in 50 μ l of acetonitrile was added. The reaction was allowed to proceed for 45 min at pH 4.99 and 5.45, 30 min at pH values 6.00, 6.50, 7.05, 7.42, 8.05, 8.45, 9.15 and 9.30, and 15 min at pH 9.95 and 11.10. At the end of the desired reaction time, the pH was adjusted to approx. 1.5 by the addition of a drop of conc. HCl and the solution was extracted with three 4 ml portions of diethyl ether to remove any unchanged fluorodinitrobenzene (Dnp-F). The solution of trace-labelled protein and internal standard was then freeze-dried.

Citraconylation and dinitrophenylation of trace-labelled protein. The residue of trace-labelled protein and internal standard was dissolved in 4 ml of 8M-urea at pH 2.4. Citraconylation followed by dinitrophenylation with unlabelled Dnp-F was done at pH 8 as described by Cruickshank *et al.* (1971).

Isolation and purification of internal standard (α -N-acetyl-imidazolyl-Dnp-histidine). The Dnp-F reaction mixture was acidified with conc. HCl and then extracted with diethyl ether until the ether extract no longer became yellow when made basic. The precipitated protein was separated by centrifugation. The supernatant containing the internal standard was adjusted to pH 7 and applied to a column (0.9 cm $\times$ 10 cm) of IR-45 ion-exchange resin in the acetate form. The column was washed with 75 ml of water and then eluted with 1M-acetic acid. The first 40 ml of eluent was collected and evaporated to dryness on a rotary evaporator at a temperature no greater than 45°C . Final purification of internal standard was achieved by electrophoresis at pH 6.5 and a potential gradient of 60 V/cm for 30 min. The α -N-acetyl-imidazolyl-Dnp-histidine was located by its u.v. absorption on the electrophoretogram. This band was cut from the electrophoretogram, eluted with 20% acetic acid and evaporated to dryness.

Isolation and purification of imidazolyl-Dnp-histidine peptides. The precipitated protein separated from the Dnp-F reaction mixture was washed with four 10 ml portions of water and the wet precipitate then freeze-dried. The derived protein was redissolved in 10% formic acid and digested with pepsin (2.5 mg) at 37°C for 6 h. The peptic digest was then oxidized with performic acid according to the procedure of Hirs (1956).

The peptic-digested, performic acid-oxidized protein was subjected to electrophoresis at pH 3.5 and a potential gradient of 60 V/cm for 1 h. The neutral band, which was known to contain the imidazolyl-Dnp-histidine peptides (Cruickshank *et al.*, 1971), was cut from the electrophoretogram and chromatographed with butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.) as solvent. The position of the imidazolyl-Dnp-histidine peptides was determined by thiolysing a guide-strip and spraying with Pauly reagent (Dent, 1947). Two Pauly-positive peptides with R_F values 0.38 (A1) and 0.70 (A2) were obtained. A final purification of these peptides was done by high-voltage electrophoresis at pH 2.1 at a potential gradient of 60 V/cm for 1 h. The peptides were eluted with 20% (v/v) acetic acid and the eluate was evaporated to dryness.

Determination of specific radioactivities. The samples of α -N-acetyl-imidazolyl-Dnp-histidine were dissolved in 150 μ l of a solution containing 152.3 nmol of 2-amino-3-guanidopropionic acid. A portion (25 μ l) was taken for scintillation counting and another portion (35 μ l) was thiolysed (Shaltiel, 1967) before acid hydrolysis for quantitation by amino acid analysis.

The samples of imidazolyl-Dnp-histidine peptides were dissolved in 200 μ l of a solution containing 108.8 nmol of norleucine. A portion (25 μ l) was taken for scintillation counting. Another portion (50 μ l) was prepared for quantitation in the same manner as the internal standard.

Amino acid analysis. Internal standard and peptide samples were hydrolysed with 6M-HCl in sealed evacuated tubes at 110°C for 24 h. Amino acid analysis was performed by the method of Spackman (1963) on a Beckman 120C automatic analyser.

Scintillation counting. The samples of peptide and internal standard solution were dissolved in Aquasol scintillator fluid (10 ml) and scintillation counting was done on a Beckman LS-250 liquid-scintillation counter.

Determination of the rate of reaction of α -N-acetyl-L-histidine with Dnp-F. The second-order rate constant, k_H , for the reaction of Dnp-F with the deprotonated form of α -N-acetyl-L-histidine was determined at pH 9.5 and 20.0°C . Dnp-F (200 μ l of 0.10M) in acetonitrile was added to 10.0 ml of 50 mM- α -N-acetyl-L-histidine in 0.10M-KCl and the rate was measured by means of a pH-stat apparatus with

0.02M-KOH as titrant. The rate of hydrolysis of Dnp-F was determined under identical conditions in the absence of α -N-acetyl-L-histidine. Under these conditions the rate was pseudo first-order and the second-order rate constant, k_H , was calculated by using the expression $k_H = (k_{obs} - k_w)/[\alpha\text{-N-acetyl-L-histidine}]$ where k_{obs} is the observed first-order rate constant and k_w is the first-order rate constant for the reaction with water.

Results

Table 1 gives the amount of incorporation of radioactive label into the protein and internal standard under the experimental conditions employed. Depending on the pH of labelling, the amount of label incorporated into the internal standard is

lower by two to three orders of magnitude. The greatest incorporation (51%) of label into the protein occurs at pH 9.95 and since $0.014\mu\text{mol}$ of labelled reagent was added to $1\mu\text{mol}$ of protein on the average only one in every 140 molecules carries a label. Therefore the condition that the fraction of any group that reacts is negligible in comparison to the total amount of group present is easily met.

Peptide A1 was found after thiolysis (Shaltiel, 1967) to contain histidine-57 in the peptide Ala-Ala-His-CysO₃H-Gly-Val-Thr-Thr-Ser-Asp, which had been characterized by Cruickshank *et al.* (1971). Similarly peptide A2 was found to contain histidine-40 in the peptide His-Phe-CysO₃H-Gly-Gly-Ser-Leu. Table 2 summarizes the primary data obtained for the labelling of histidine-57 (peptide A1), histidine-40 (peptide A2) and α -N-acetyl-L-histidine. The degree

Table 1. Extent of labelling of α -chymotrypsin and internal standard by [^3H]Dnp-F at 20.0°C in 0.10M-KCl

pH	Percentage of label in internal standard	$10^6 \times (\text{Dnp/internal standard})$ molar ratio	Percentage of label in protein	$10^6 \times (\text{Dnp/protein})$ molar ratio
4.99	0.014	2.0	1.4	200
5.45	0.010	1.4	1.1	160
6.00	0.024	3.4	7.4	1000
6.50	0.024	3.4	12	1600
7.05	0.047	6.6	12	1700
7.42	0.055	7.7	15	2100
8.05	0.027	3.8	21	2900
8.45	0.021	2.9	41	5800
9.15	0.0086	1.2	43	6100
9.30	0.0099	1.4	43	6000
9.95	0.0052	0.73	51	7100
11.10	0.012	1.7	39	5400

Table 2. Competitive labelling with [^3H]Dnp-F for the histidine residues of α -chymotrypsin and internal standard

pH	α -N-Acetyl-L-histidine		Peptide A1 Histidine-57		Peptide A2 Histidine-40	
	$10^{-5} \times \text{Radioactivity}$ (c.p.m./ μmol)	α_r	$10^{-5} \times \text{Radioactivity}$ (c.p.m./ μmol)	α_r	$10^{-5} \times \text{Radioactivity}$ (c.p.m./ μmol)	α_r
4.99	0.289	0.00613	1.42	0.0302	1.05	0.0223
5.45	0.195	0.0175	0.735	0.0658	0.532	0.0477
6.00	0.476	0.0594	5.75	0.717	0.394	0.492
6.50	0.474	0.166	9.19	3.23	5.73	2.01
7.05	0.940	0.415	16.0	7.04	10.4	4.60
7.42	1.10	0.624	16.2	9.19	9.03	5.13
8.05	0.547	0.876	0.700	1.12	0.565	0.905
8.45	0.429	0.947	0.428	0.945	0.204	0.450
9.15	0.173	0.989	0.256	1.46	0.0957	0.547
9.30	0.197	0.992	0.328	1.65	0.150	0.755
9.95	0.103	0.998	0.231	2.24	0.130	1.26
11.10	0.236	1.00	0.234	0.989	0.338	1.43

of dissociation of internal standard, α_s , at each experimental pH was calculated by using pK_a 7.20, which had been determined by potentiometric titration in 0.10M-KCl at 20.0°C. The pH-dependent relative rate constants, αr , were calculated by using eqn. (1). (Kaplan *et al.*, 1971).

Figs. 1 and 2 are plots of the relative pH-

$$\alpha r = \alpha_s \times \frac{\text{sp. radioactivity of imidazolyl-Dnp-histidine peptide}}{\text{sp. radioactivity of internal standard}} \quad (1)$$

dependent second-order velocity constant, αr , versus pH for the reaction of Dnp-F with histidine-57 and histidine-40. For histidine-57 the data up to pH 7.5 follow a titration curve with pK_a 6.8 and a pH-independent second-order velocity constant, r , of 10 relative to α -N-acetyl-L-histidine. Between pH 7.5 and 8 there is a discontinuity in the data with the reactivity of histidine-57 decreasing sharply to that of the internal standard. The reaction with histidine-40 has a very similar pH profile with pK_a 6.7 and a relative pH-independent rate constant, r , of 5.7. As with histidine-57, the pH profile for histidine-40 has a sharp discontinuity between pH 7.5 and 8.

Fig. 3 is a plot of αr versus pH for histidine-57 with histidine-40 used as the internal standard. The pK_a of histidine-40 was taken as 6.7 and the pH-dependent second-order velocity constant for histidine-57 relative to histidine-40 was calculated from the data in Table 2 and eqn. (1). Up to pH 7.5 the data precisely fit a titration curve; however, above pH 8 the reactivities vary randomly relative to one another. The linear plot in Fig. 4 emphasizes the fact that the

data above pH 8 (closed circles) do not fit a titration curve. A pK_a of 6.8 is obtained again for histidine-57 and the relative pH-independent second-order rate constant is 1.8.

The absolute rate of reaction of Dnp-F with α -N-acetyl-L-histidine was measured by using a pH-stat. The reaction was carried out with a large excess of

Fig. 2. pH profile for the reaction of histidine-40 with Dnp-F

○, Experimental results; —, theoretical titration curve for a group with pK_a 6.7 and a pH-independent second-order velocity constant of 5.7 relative to α -N-acetyl-L-histidine.

Fig. 1. pH profile for the reaction of histidine-57 with Dnp-F

○, Experimental results; —, theoretical titration curve for a group with pK_a 6.8 and a pH-independent second-order velocity constant of 10 relative to α -N-acetyl-L-histidine.

Fig. 3. pH profile for the reaction of histidine-57 with Dnp-F relative to histidine-40 as internal standard

Experimental results obtained up to pH 7.5 (○) and above pH 8 (●) are shown; —, theoretical titration curve for a group with pK_a 6.8 and a pH-independent second-order velocity constant of 1.8 relative to histidine-40.

α -N-acetyl-L-histidine over Dnp-F so that the rate of production of H^+ would be first-order with respect to the concentration of Dnp-F. The observed first-order constant, $k_{obs.}$, and the first-order rate constant for the reaction with water, k_w , were determined by the method of Guggenheim (1926) and a standard first-order plot respectively. The values obtained for the rate constants at pH 9.95 in 0.10M-KCl and 20.0°C are: $k_{obs.} = 3.14 \times 10^{-3} \pm 0.16 \times 10^{-3} \text{ min}^{-1}$; $k_w = 1.00 \times 10^{-3} \pm 0.21 \times 10^{-3} \text{ min}^{-1}$; $k_H = 43.5 \times 10^{-3} \pm 3.7 \times 10^{-3} \text{ M}^{-1} \text{ min}^{-1}$.

The parameters and absolute reactivities obtained for the two histidine residues of α -chymotrypsin are summarized in Table 3.

Discussion

Several considerations led to the choice of tritiated Dnp-F as the labelling reagent for the study of the properties of individual histidine residues in proteins.

Fig. 4. Linear plot for the data from Fig. 3

α , Data up to pH 7.5; —, least-squares fit to the data, which gives pK_a 6.8 and a pH-independent second-order velocity constant of 1.8 for histidine-57 relative to histidine-40; β , data above pH 8.

Dnp-F is more reactive towards the imidazole moiety of histidine than activated halomethyl compounds such as bromoacetone or iodoacetic acid and, unlike the more reactive acylating reagents such as diethyl-pyrocabonate (Melchior & Fahrney, 1970), it forms a derivative that is stable at neutral and moderately alkaline or acid pH values. Dnp-F is also uncharged, thus avoiding electrostatic interactions with the protein. A most useful property of Dnp-imidazole derivatives is that they are easily converted into the free imidazole by thiolysis (Shaltiel, 1967), which provides a convenient method for locating imidazolyl-Dnp-histidine peptides by means of a histidine diagonal (Cruickshank *et al.*, 1971) and identification and quantitation of α -N-acetyl-imidazolyl-Dnp-histidine and imidazolyl-Dnp-histidine peptides by standard amino acid analysis. Its low reactivity is compensated for by the availability of tritiated Dnp-F of high specific radioactivity, which permits the quantitation of a very low degree of labelling of the protein and internal standard and the removal of unchanged radioactive Dnp-F before complete reaction with unlabelled Dnp-F is easily accomplished by extraction with ether. Another useful property of Dnp-F is that its rate of reaction with α -N-acetyl-L-histidine is easily measured and the absolute rate of reaction of Dnp-F with individual histidine residues in proteins can be determined. A most important consideration is that the low degree of labelling that can be achieved by the use of tritiated Dnp-F is a necessary condition for the application of eqn. (1) and this condition ensures that the label reacts predominantly with the native protein so that objections to the effect that the reaction of one group alters the reactivity of other groups are not applicable.

The pH-dependent second-order velocity constants αr , relative to α -N-acetyl-L-histidine as a function of pH, display similar features for both histidine-57 and histidine-40. In comparison with the points at pH 6.50, 7.05 and 7.42, the low-pH experimental points of the αr versus pH plots for both histidine residues lie somewhat below a theoretical titration curve. Since α -chymotrypsin is known to dimerize at acid pH (Egan *et al.*, 1957), it was suspected that this might be the cause of the low values observed for αr at acid pH. To compensate for the effects due to dimeriza-

Table 3. Summary of the parameters obtained for the reaction of the two histidines of α -chymotrypsin with Dnp-F

The solvent was 0.10M-KCl, at 20.0°C.

	Internal standard	...	α -N-Acetyl-L-histidine		Histidine-40		$10^2 \times k \text{ (M}^{-1} \cdot \text{min}^{-1})$
			pK_a	r	pK_a	r	
Histidine-57			6.8	10	6.8	1.8	45 ± 4
Histidine-40			6.7	5.7	6.7	1	25 ± 2

tion, the properties of histidine-57 were determined relative to histidine-40, and as may be seen in Figs. 3 and 4 the results obtained up to pH 7.5 give a precise fit with the theoretical curve for a group with pK_a (app.) 6.8 and a pH-independent second-order velocity constant of 1.8 relative to histidine-40. The pK_a (app.) of 6.8 determined for histidine-57 is in exact agreement with the value determined previously by kinetic studies (Hammond & Gutfreund, 1955; Bender & Kézdy, 1965) and tentatively assigned to histidine-57.

The present study also permits the specific assignment of pK_a (app.) 6.7 to histidine-40 of α -chymotrypsin. The imidazolyl moiety of this residue is 5.7 times as reactive as that of α -N-acetyl-L-histidine up to approx. pH 8. Above this pH, however, there is a rapid decrease in the reactivity to a value approximating to that of the internal standard. X-ray-crystallographic investigations (Freer *et al.*, 1970) have shown that the N^{ϵ} [the nomenclature for the amino acid side chains is as proposed by Edsall *et al.* (1966)] of the imidazolyl moiety of histidine-40 is hydrogen bonded to the carbonyl group of glycine-193.

Because of this hydrogen bonding, an increase in the nucleophilicity of the imidazolyl moiety of histidine-40 would be expected and this would account for its increased reactivity towards Dnp-F. Previous studies (Henkart, 1971) have shown that dinitrophenylation of the imidazolyl moiety of histidine occurs at the N^{ϵ} position. With histidine-40 of α -chymotrypsin this position is hydrogen-bonded, which should preclude any reaction with Dnp-F. Therefore in accepting the X-ray-crystallographic structure one is forced to the conclusion that the $N^{\delta 1}$ position of the imidazolyl moiety of histidine-40 is reactive as a result of some conformational feature of the protein, which makes this position readily accessible. To achieve the abnormally high reactivity we observe, the imidazolyl moiety would have to be locked in one tautomeric form by the hydrogen bonding, which would prevent any free rotation. This conclusion is in agreement with n.m.r. studies (Bradbury & Wilairat, 1967), which show that no $C^{\epsilon 1}$ proton resonance is observed for the histidines of α -chymotrypsin.

The results for histidine-57 are similar in nature to those of histidine-40 and are of considerable interest in view of the proposed role of this residue in the catalytic mechanism of the enzyme. The reactivity of the imidazolyl moiety of histidine-57 is an order of magnitude greater than that of a histidine free in solution and is approximately twice as reactive as that of histidine-40. It is unlikely that this high reactivity results from a specific orientation of Dnp-F on the protein surface since the sharp decrease in reactivity observed just below pH 8 is uncharacteristic of such reagents. It has been proposed (Blow *et al.*, 1969) that histidine-57 is involved in a charge-relay

mechanism with aspartic-102 and serine-195. The X-ray-crystallographic structure shows that the $N^{\delta 1}$ of histidine-57 is hydrogen bonded to the β -carboxyl group of aspartate-102. One consequence of such a system would be an increase in the nucleophilicity of the $N^{\epsilon 2}$ of histidine-57, which would account for the high reactivity we observe. Our results therefore support the proposal that the hydrogen bonding could contribute to a charge relay mechanism which increases the nucleophilicity of serine-195. However, the high reactivity observed for $N^{\epsilon 2}$ of histidine-57 would indicate that in the native enzyme no extensive hydrogen bonding occurs between the deprotonated form of histidine-57 and serine-195 and that this mechanism is probably only fully functional in the enzyme-substrate complex.

A notable feature in the properties of the histidine residues is the sharp decrease in reactivity, which occurs between pH 7.5 and 8 for both histidine-40 and histidine-57. Such a decrease in the reactivity of histidine-57 in this pH range has previously been observed during study of the inhibition of activity of α -chymotrypsin with benzyl chloromethyl ketone (Stevenson & Smillie, 1970). Although no particular significance was attached to the observation, it seems likely that the decrease in inhibition observed with benzyl chloromethyl ketone and the decrease in reactivity towards Dnp-F observed in these studies could result from the same cause. Lumry and his co-workers (Rajender *et al.*, 1971) have also observed from a study of thermodynamic properties that α -chymotrypsin undergoes a conformational change in the range pH 7.5–8.0 and it is very likely that this is the same conformational change that is responsible for the sudden decrease in the reactivity of the histidine residues. It appears therefore that the active site is somehow altered in this pH range in the absence of substrate, such that the $N^{\delta 1}$ of the imidazolyl moiety of histidine-57 no longer hydrogen bonds to the β -carboxyl group of aspartate-102. Since this conformational change occurs near the optimum pH for activity, it appears that the binding of the substrate is necessary to bring the catalytic groups into proper alignment and reconstitute the charge-relay system. The fact that the sudden decrease in reactivity of both histidine residues occurs simultaneously indicates that their properties are linked to the same structural factors that determine the conformation of the active site and that histidine-40 may have an essential role in establishing the catalytically active conformation.

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