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Robillard, George; Shulman, R.G.

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High Resolution Nuclear Magnetic Resonance Study of the Histidine—Aspartate Hydrogen Bond in Chymotrypsin and Chymotrypsinogen

A high resolution proton nuclear magnetic resonance study of chymotrypsin A$_\delta$ and chymotrypsinogen A in water has shown a single resonance at very low magnetic fields ($-$ 18 to $-$ 15 p.p.m. relative to dimethyl-silapentane-sulfonate). From its pH dependence (pK = 7·2) and response to chemical modification the resonance has been assigned to the hydrogen-bonded proton between His-57 and Asp-102.

The catalytic centers of the serine esterases, chymotrypsin (Sigler, Blow, Matthews & Henderson, 1968), trypsin (Stroud, Kay, Cooper & Dickerson, 1970, *Abst. 8th Internat. Cong. Biochem.*), subtilisin (Wright, Alden & Kraut, 1969) and elastase (Watson, Shotton, Cox & Muirhead, 1970) are known to contain invariant histidine, serine and aspartic acid residues. Chemical modifications and kinetic studies have shown that the histidine and serine are accessible to solution and are essential for catalytic activity but the role of the aspartate, buried in the hydrophobic interior, is less certain. From the three-dimensional arrangement of these residues Blow, Birktoft & Hartley (1969) proposed that the buried aspartate is hydrogen–bonded to the histidine (Fig. 1) and further suggested that this structure might account for the catalytic activity.

Several observations of nuclear magnetic resonance peaks from hydrogen-bonded protons in proteins and nucleic acids dissolved in water have been reported (Kearns, Patel, Shulman & Yamane, 1971; Patel, Woodward & Bovey, 1972). These resonances are sometimes resolved from the large number of other proton resonances because they are moved to lower fields by the chemical shifts of their hydrogen bonds. They are also distinct from the resonances of the water solvent because of the slow chemical exchange rates with solvent created by the hydrogen bonds. By searching at very low magnetic fields in water solutions of chymotrypsin A$_\delta$ and chymotrypsinogen A we have been able to observe a single resonance in each compound attributable to the proton in the hydrogen bond between the active site histidine-57 and the buried aspartic acid-102.

Measurements were made at 220 mHz with a Varian Associates HR220 nuclear magnetic resonance spectrometer, and magnetic fields for resonance are reported in terms of parts per million downfield from the fields required for the resonance of 2,2-dimethyl-2-silapentane-5-sulfonate. Chymotrypsin A$_\delta$ was used rather than the more intensively studied chymotrypsin A$_\alpha$ because of its lower tendency to aggregate (Miller, Horbett & Teller, 1971). Samples were prepared from Worthington bovine chymotrypsinogen A (code CGC) and bovine chymotrypsin A$_\delta$ (code CDD). The enzyme concentrations were 2 to 4 mM in buffer solutions of ionic strength 0·4.
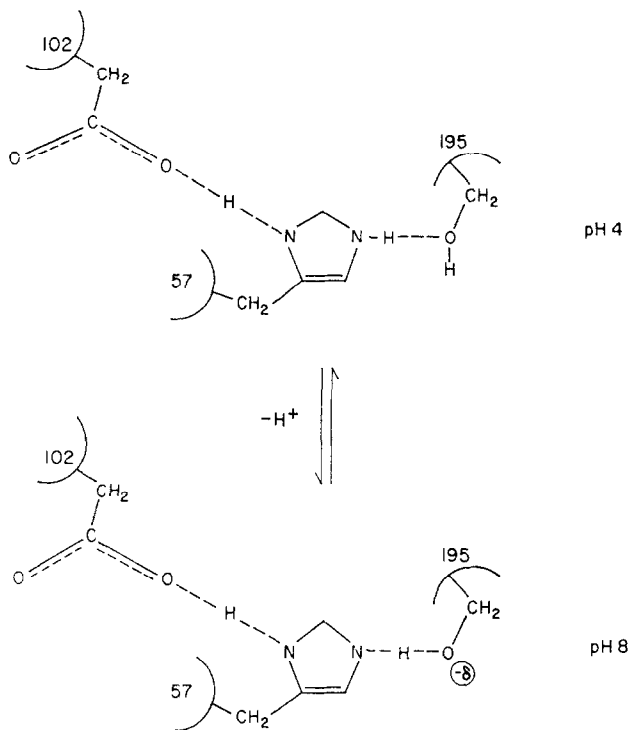
FIG. 1. Proposed hydrogen bond structure at active site of chymotrypsin.

Figure 2(a) illustrates the very low-field spectral region of chymotrypsinogen A as a function of pH. The resonance seen is relatively narrow at the pH extremes but broadens in the intermediate pH region. Figure 2(b) is its titration curve. Super-imposed on this curve at the extreme pH values are the positions of the resonance of chymotrypsin A_δ. The resonance positions of the active enzyme in the neutral pH range are not included because of inaccuracies present in these preliminary measurements by the extreme breadths. However, this resonance, like the similar resonance in chymotrypsinogen A, does move with pH and has an apparent pK near 7·2. In addition to its unusual breadth and extremely low-field position this resonance has the following characteristics:

(1) the resonance disappears when the protein is dissolved in deuterium oxide, showing that it comes from an exchangeable proton.

(2) In chymotrypsinogen A and in chymotrypsin A_δ the resonance moves upfield with increasing pH showing a pK of 7·2. Because of this pK the most obvious assignment would be to the NH of a histidine residue. The proton on one nitrogen, presumably immobilized by a hydrogen bond, would respond to the protonation of the other nitrogen and thereby follow the pK of protonating histidine. To assign the histidine we note that chymotrypsinogen A and chymotrypsin A_δ contain two histidines. The resonance is assigned to the active site histidine-57 because it is observed at identical positions in chymotrypsinogen A and chymotrypsin A_δ. The X-ray structure analysis (Freer, Kraut, Robertus, Wright & Xuong, 1970) has shown
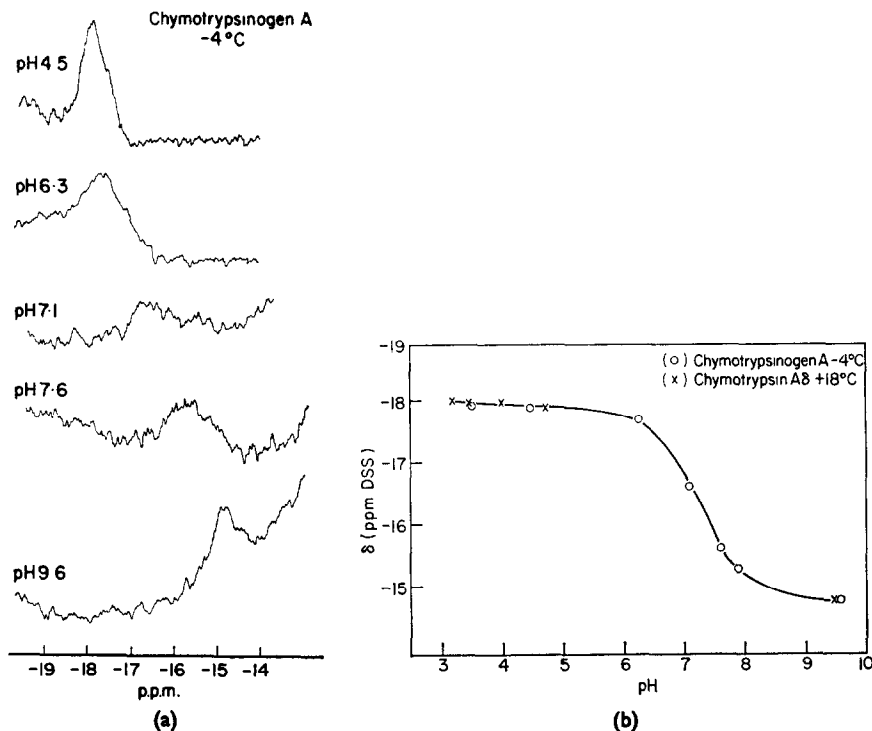
FIG. 2. (a) High resolution nuclear magnetic resonance spectra of bovine chymotrypsinogen A. Spectra were taken with a Varian HR220 spectrometer. All spectra were time-averaged using a Fabri-Tek computer with 1024 channels. Enzyme concentrations were 2 to 4 mM in buffered solutions at $I = 0.4$. Temperature was $-4°C$ maintained with a variable temperature probe.
(b) Titration curve for the resonance peaks illustrated in (a).

that the environment of histidine-57 is exactly the same in these two proteins while that of the other possibility, histidine-40, changes considerably.

(3) Further confirmation that this resonance is associated with the His-57—Asp-102 hydrogen-bonded proton, rather than the His-57—Ser-195 proton (see Fig. 1) was obtained by chemical modification of the active site. Peptide chloromethyl ketones have been shown to inhibit chymotrypsin by alkylation of His-57 (Schoellman & Shaw, 1963). The alkylation occurs at the N^{e2} nitrogen (Segal, Powers, Cohen, Davies & Wilcox, 1971), thereby breaking the His-57—Ser-195 hydrogen bond, while leaving the His-57—Asp-102 bond intact. Two alkylated derivatives were prepared, tosyl phenylalanyl chloromethyl ketone—chymotrypsin A_δ (Schoellmann & Shaw, 1963) and N-benzyloxycarbonyl-L-glycyl, leucyl phenylalanyl chloromethyl ketone-chymotrypsin A_δ (Segal et al., 1971).

In these derivatives the low-field resonance persists, is considerably narrower and does not show a titration at pH $\lesssim$ 8, although a small shift is noted with an apparent pK around 8·3. These results clearly show that the resonance cannot be assigned to the His-57—Ser-195 hydrogen-bonded proton. However, this is the behavior expected for a resonance assigned to the His-57—Asp-102 hydrogen bond, since alkylation would prevent the histidine from being protonated.

In two other derivatives studied, tosyl-chymotrypsin (Sigler *et al.*, 1968) and methyl-chymotrypsin (Henderson, Wright, Hess & Blow, 1971; Wright, Hess & Blow, 1972) this resonance was not observed. X-ray crystallographic data show that in both derivatives His-57 moves outwards into the solvent by approximately 0·3 Å from its position in the native enzyme. An even larger movement away from aspartic-102 may occur in the methylated enzyme in solution, as has been suggested to explain its residual activity (Henderson *et al.*, 1971; Wright *et al.*, 1972). This motion in solution presumably breaks or seriously weakens the hydrogen bond between histidine and the aspartic acid, and is consistent with the disappearance of the nuclear magnetic resonance peak in these derivatives.

(4) The unusually low-field position of this resonance is itself informative about the assignment and the charge state of the proton. Exchangeable imidazole and carboxylate protons are the lowest field resonances which might be observed in these proteins and in the absence of hydrogen bonding are observed near -13 and -11 p.p.m., respectively. Strong hydrogen bonds, such as found in water and hydrogen fluoride, shift proton resonance peaks downfield by about 5 p.p.m. while weaker hydrogen bonds, such as between carbonyls and amino protons, only shift the resonances downfield by approximately 1 p.p.m. The experimental results of a resonance between -16 and -18 p.p.m. once again are consistent with the proposed assignment.

The large downfield shift of this resonance suggests that the diamagnetic shielding is not very effective presumably because the hydrogen-bonded proton is quite positive. A bare proton resonates at $-30·9$ p.p.m., and the hydrogen molecule at $-4·34$ p.p.m. from dimethyl-silapentane-sulfonate. Since to a first approximation the diamagnetic shielding should be proportional to the electron density, one can estimate that -18 p.p.m. corresponds to an effective charge on the proton of about $+0·5$. This highly positive charge on the proton suggests that the charge distribution of the imidazole ring must indeed be altered by the aspartic-102 bond.

Polgar & Bender (1969) have argued that the histidine, if involved in the charge relay system, would be more acidic than a normal histidine, yet the histidine in these serine esterases has a normal pK of 6·5 to 7·5. On the basis of this and other evidence they suggested that the function of the buried aspartate is to stabilize the protonated histidine by a hydrogen bond during the transition state but not in the free enzyme. According to the charge relay hypothesis (Blow *et al.*, 1969), however, this proton should be in a hydrogen-bonded state between histidine and the aspartate in the free enzyme, which is indeed found in the present experiments. Furthermore, since this histidine aspartate hydrogen bond does exist, the histidine—serine hydrogen bond must also exist in the neutral pH region otherwise the histidine would be more basic and have a higher than normal pK.

The present experiments confirm the hydrogen-bonded structure proposed by Blow *et al.* (1969) for the active site, and are consistent with the net negative change found at the active site (Johnson & Knowles, 1966). They also offer some support to the charge rearrangement process which has been invoked to account for the unusual nucleophilicity of the active serine. To the extent that this resonance reflects the condition of the catalytic site, the similarity between chymotrypsin and chymotrypsinogen is noteworthy. However, before drawing any firm conclusions about the role of the hydrogen bond structure in the catalytic mechanism, we must await more quantitative information. We hope that studies underway on the titration of the histidine C(2) proton nuclear magnetic resonance peaks will provide such information.

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Department of Physics, Princeton University   GEORGE ROBILLARD
Princeton, N.J. 08540, U.S.A. and
Bell Laboratories
Murray Hill, N.J. 07974, U.S.A.

Bell Laboratories   R. G. SHULMAN
Murray Hill, N.J. 07974, U.S.A.

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