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Ultrasonic studies of proton-transfer reactions at the catalytic site of α-chymotrypsin

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Ultrasonic relaxation measurements for α-chymotrypsin in phosphate, sulfite and arsenate buffers exhibit a high peak of absorption at neutral pH. The analysis is based on: (i) comparison of the relaxation measurements for the enzyme and for the zymogen and inhibited enzyme; (ii) X-ray and neutron diffraction data, and high-resolution NMR data. The ultrasonic relaxation is shown to result mainly from a proton-transfer reaction that involves the histidine at the catalytic site (His-57). The question is raised of whether the enhanced ultrasonic effect observed in the enzyme is indicative of a property that plays a part in the catalytic activity.

Ultrasonic relaxation spectrometry; Serine protease; α-Chymotrypsin; Catalytic histidyl residue; Proton transfer

1. INTRODUCTION

In proteins, those amino acid residues whose side chains bear carboxyl or amino groups can exchange protons with water by way of protolysis or hydrolysis reactions, respectively. At constant frequency, when the pH is changed, the ultrasonic absorption excess $\Delta\alpha$ associated with those reactions may exhibit a peak at acidic or basic pH, respectively [1]. On the other hand, certain end groups, as well as the imidazole ring of a histidyl residue, may produce an absorption peak at neutral pH that is associated with a proton-transfer reaction:

$$AH + B \rightleftharpoons BH + A \qquad (1)$$

where AH and BH are acids and A and B are bases. At low protein and buffer concentrations, and when the pK_a values of AH and BH are equal, the dependence of $\Delta\alpha$ on pH for reaction 1 is entirely contained in the factor $K_aC_H/(K_a + C_H)^2$, where K_a is the acid dissociation constant of AH and BH, and C_H is the H^+ concentration. When the frequency N is kept constant, $\Delta\alpha$ thus goes through a maximum $\Delta\alpha_{max}$ at $pH_{max} = pK_a$.

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2. MATERIALS AND METHODS

α-Chymotrypsin A (α-CHT), chymotrypsinogen A and DFP (diisopropyl fluorophosphate)-treated α-CHT were purchased from Sigma and used without further purification. α-CHT inhibited by TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone) was prepared according to Schoelmann and Shaw [2]. Protein concentrations were determined from absorbance measurements at 280 nm. The pH was measured to ±0.02 pH units, and adjusted using either HCl or KOH.

Ultrasonic absorptions α of solutions and α_0 of solvents were measured using an Eggers resonator between 0.5 and 5.5 MHz, and a pulse technique between 25 and 95 MHz. Ultrasonic velocities were measured between 0.5 and 70.2 MHz using a differential phase comparison method. All solutions

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were degassed prior to measurement. Chymotryptic activity was checked after each ultrasonic measurement by following the course of *N*-succinyl-L-phenylalanine *p*-nitroanilide hydrolysis, according to Bieth et al. [3].

3. RESULTS

We observed high absorption peaks for α-CHT at pH values close to neutrality in sulfite, phosphate and arsenate buffers. In fig.1, α/N^2 is shown for different values of the pH and frequency N, for a 2×10^{-4} M solution of α-CHT in a 1/15 M sulfite buffer at 40°C. Frequencies ranged from 0.52 to 5.52 MHz. The broken line represents the values of α/N^2 for the buffer at 0.52 MHz. The value pH_{max} at which the measured α is highest was close to 7.1, and differed from that value by less than $\pm$ 0.1 pH unit over the whole frequency range. pH_{max} depended slightly more on N at 25°C. In phosphate buffers the dependence of pH_{max} on N was noticeable even at 40°C, but did not exceed $\pm$ 0.4 pH units. The peak of α vs pH may be ascribed to the reactions:

$$-IH^+ + SO_3^{2-} \rightleftharpoons -I + HSO_3^- \quad (2)$$

$$-IH^+ + HPO_4^{2-} \rightleftharpoons -I + H_2PO_4^- \quad (3)$$

in the two buffers, respectively. I is the imidazole of a histidyl residue.

Fig.2 shows, for several frequency values, the dependence on phosphate concentration of the term $(\alpha_{max} - \alpha_0)/N^2$, where α_{max} is the peak value

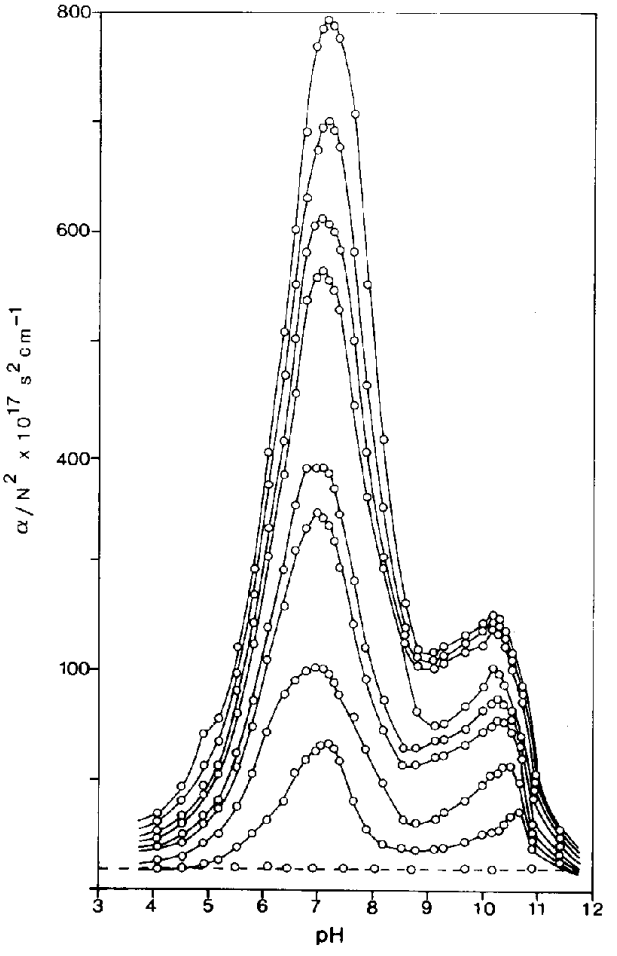

Fig.1. α-CHT, 2×10^{-4} M in 1/15 M sulfite buffer, T = 40°C. Values of α/N^2 (solution's ultrasonic absorption α divided by squared frequency) vs pH for several values of N between 0.52 and 5.52 MHz. (– – –) α_0/N^2 for buffer at 0.52 MHz.

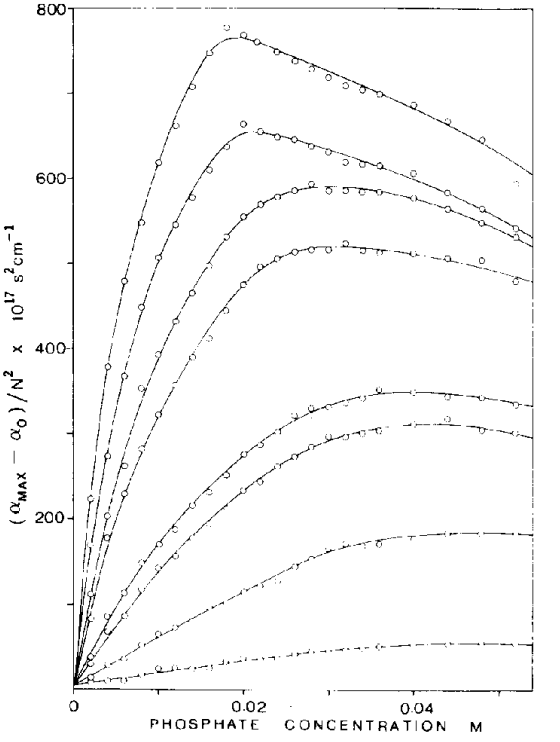

Fig.2. α-CHT, 2×10^{-4} M, T = 40°C. Values of $(\alpha_{max} - \alpha_0)/N^2$ (α_{max} = peak value of α at pH = pH_{max}) vs buffer concentration at different frequencies: (from top to bottom) 0.527, 0.694, 0.862, 1.03, 1.53, 1.70, 2.75, 5.52 MHz.

of α. Temperature and enzyme concentration are as in fig.1. If one tentatively assumes that the value of α at neutral pH is entirely associated with proton-transfer reactions involving histidyl residues, it is found that the contribution per histidyl residue is less than in α-CHT both in bovine serum albumin and in imidazole, by factors of 5.5 and 10, respectively. The question therefore arises as to whether in α-CHT the ultrasonic relaxations associated with reactions 2 and 3 primarily involve His-57 of the catalytic triad (Ser-195, His-57, Asp-102), rather than the sole other histidyl residue, i.e. His-40.

(i) A double-logarithmic plot of $(\alpha - \alpha_\infty)\lambda$ vs N is shown in fig.3 for a 2×10^{-4} M solution of α-CHT in a 1/15 M phosphate buffer at pH 7.3 and 25°C; α_∞ is the α value of the solution as determined by the pulse technique; λ is the ultrasonic wavelength. The absorption per wavelength $(\alpha - \alpha_\infty)\lambda$ shows a maximum in the MHz range, but the spectrum is not compatible with single relaxation behavior. The effect of high-frequency relaxations may be seen in fig.3, which includes data from the pulse technique. On the other hand, comparison (not made here) of the data from absorption and velocity measurements demonstrates that relaxation processes of frequencies lower than those investigated must also exist. Velocity measurement data for conditions similar to those in fig.3 are shown in fig.4.

(ii) In fig.5, plots of $(\alpha_{max} - \alpha_0)\lambda$ are shown for

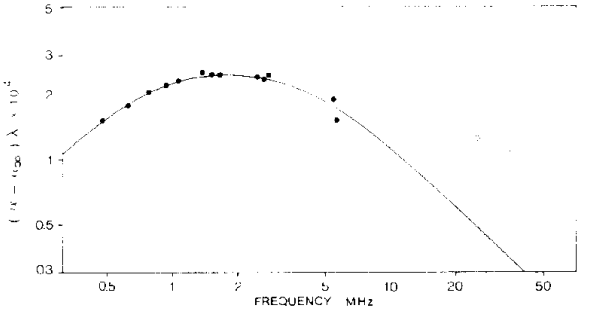

Fig.3. α-CHT, 2×10^{-4} M in 1/15 M phosphate buffer, pH 7.3, $T = 25$°C. Double-logarithmic plot of $(\alpha - \alpha_\infty)\lambda$ vs N; α_∞ = high-frequency value of solution's α; λ = ultrasonic wavelength. Filled and empty circles are data obtained with the Eggers resonator and the pulse technique, respectively. The continuous line is fitted to the resonator data for two relaxation processes.

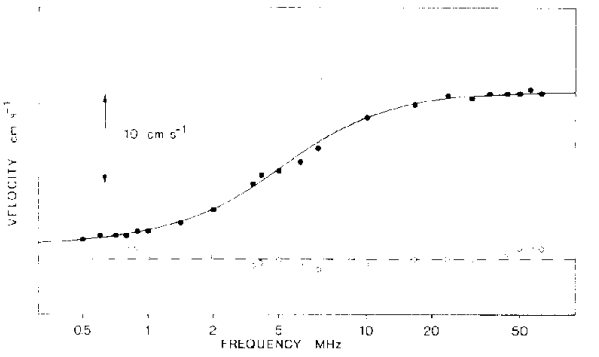

Fig.4. α-CHT, 2×10^{-4} M in 1/15 M phosphate buffer, pH 7.3, T = 25°C. Ultrasonic velocity vs frequency. Filled and empty circles are data for the solution and the buffer, respectively. The continuous line is fitted for two relaxation processes.

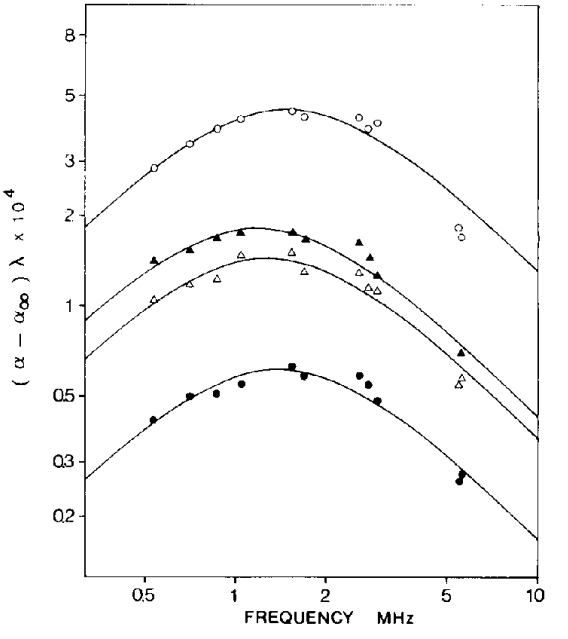

Fig.5. Enzyme, zymogen and inhibited enzyme. Protein concentration, 2×10^{-4} M; 1/15 M phosphate buffer, T = 40°C. Double-logarithmic plot of $(\alpha_{max} - \alpha_0)\lambda$ vs frequency. (○) α-CHT, (●) zymogen, (△) DFP-treated enzyme, (▲) TPCK-treated. Continuous lines are each fitted for a single relaxation process.

the enzyme, the zymogen, and the DFP- and TPCK-inhibited enzyme in a phosphate buffer, under conditions similar to those of fig.3, but at 40°C; α_∞ here is very close to α_0. At 40°C and pH

7.9, at all frequencies the value of $(\alpha_{max} - \alpha_0)\lambda$ was less than in the enzyme both in the zymogen and in the inhibited enzyme, by factors of about 8 and 3, respectively. At 25°C in a 1/15 M sulfite buffer, $(\alpha_{max} - \alpha_0)\lambda$ was less than in the enzyme by a factor of about 5.5 in both the zymogen and DFP-treated enzyme. No absorption peak was observed in the zymogen at pH values around neutrality. At 25°C, $(\alpha_{max} - \alpha_0)\lambda$ was practically unchanged when TPCK was bound to the enzyme.

4. DISCUSSION AND CONCLUSIONS

An estimate of the possible contributions of the amino-end groups and of His-40 to the absorption peak observed at neutral pH in α-CHT was obtained by comparison of the ultrasonic data with those from three other techniques, namely:

(i) The X-ray structures of α-CHT and of the zymogen to 2.8 and 2.5 Å resolution, respectively (Protein Data Bank, File A 127.BRK and File A 129.BRK); (ii) the high-resolution NMR data for a series of tetrahedral intermediate (TI) analogues of Robillard and Schulman [4]; and (iii) the neutron structure analysis to 2.2 Å resolution of Kossiakoff and Spencer [5] for a TI analogue in bovine trypsin. The data (ii) and (iii) show that in the TI the pK_a of the catalytic site region is raised, so that the N^{τ}-nitrogen of His-57 remains protonated at pH values up to 9.5.

An appreciable contribution of Cys-1, Ile-16 and Ala-149 to the absorption peak of α-CHT at neutral pH can be excluded:

(i) For Cys-1, because the X-ray data show no significant difference in environment of this residue in the enzyme and in the zymogen.

(ii) For Ile-16, because its pK_a value is known to be of the order of 8.5 [6], and its absorption peak, therefore, cannot be observed in a buffer of pK_a value close to 7. Confirmatory evidence is provided by the fact that at 40°C in a 0.1 M borate buffer of $pK_a \simeq 9$ an ultrasonic absorption peak was observed at pH 8.5, that stems from Ile-16. This peak, indeed, does not exist in the zymogen and disappears in the DFP-treated enzyme, in which the pK_a value of Ile-16 is raised to above 10 [6].

(iii) For Ala-149, because the terminal α-amino group of an alanine in a protein can be modeled by the terminal α-amino group of tetra-L-alanine. However, at 25°C in a 1/15 M phosphate buffer this compound exhibited an absorption peak 6-times smaller than that of α-CHT at equimolar concentrations, thus excluding Ala-149 as the main source of the ultrasonic absorption in α-CHT at neutral pH.

The preceding conclusion for Ala-149 is confirmed by the following argument. Since Ala-149 is located at the protein surface, its environment is mainly unaffected when the enzyme is inhibited. If this residue were involved in the ultrasonic absorption of α-CHT at neutral pH, the strength of absorption would not be lowered at neutral pH in the inhibited enzyme, in contradistinction to our observations.

The absorption peak observed in α-CHT at neutral pH is therefore mainly due to a proton-transfer reaction between HSO_3^- (or $H_2PO_4^-$, respectively) and either His-40 or His-57.

However, in the DFP-treated enzyme α decreased at neutral pH (see fig.5) and increased at pH ≃ 10 (not shown) in comparison with the corresponding values for the enzyme. Therefore, the detected proton transfer occurs at the histidine whose pK_a value is raised in the DFP complex, i.e. the catalytic His-57. The largest contribution to α at neutral pH must then occur from a proton transfer with the protonable N^{τ}-nitrogen of the imidazole of His-57.

In the TPCK-inhibited enzyme, TPCK is covalently bound to the N^{τ}-nitrogen of His-57. Under conditions where α remains high when TPCK is bound, as in a sulfite buffer at 25°C (see section 3), proton transfer must occur at the N^{π}-nitrogen.

The fact that a proton transfer involving the catalytic histidine produces an ultrasonic absorption higher in the enzyme than in the zymogen is likely to result from small structural differences of the catalytic site in the two proteins. This raises the question of whether the enhanced ultrasonic effect observed in the enzyme is indicative of a property that plays a part in the catalytic activity.

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