Short Communications

Tyrosyl-carboxylate ion hydrogen bonding in ribonuclease

Three of the six tyrosyl residues in ribonuclease appear to be involved in interactions that modify their ultraviolet light absorption[1,2] and ionization behavior[3]. Since the ionization of all six tyrosyl groups is normal in concentrated urea[4], a hydrogen bond-breaking solvent, it seems that the modified reactivity of three tyrosyls in the native protein is due to tyrosyl hydrogen bonds rather than to hydrophobic[3] bonds. It becomes of interest to learn the nature of the acceptor groups interacting with these tyrosyls. In the case of ovalbumin, CRAMMER AND NEUBERGER[5] suggested that one of the likely acceptor groups might be the carboxylate ion since the salicylate ion was "one of the few examples of hydrogen bond formation in water of phenols with groups other than those of the solvent molecules". HARRINGTON AND SCHELLMAN[2], using this hypothesis of CRAMMER AND NEUBERGER, postulated that the acceptor group in ribonuclease was also a carboxylate ion. The present communication provides experimental evidence to support the suggestion that tyrosyl-carboxylate ion hydrogen bonding exists in ribonuclease.

The experimental technique involves the use of differential ultraviolet spectrophotometry previously applied to insulin[6]. If ribonuclease at pH 1.91 is compared in the Beckman DU spectrophotometer with ribonuclease at pH 6.94, the curves of Fig. 1 are obtained. Each curve corresponds to a different protein concentration in the pair of solutions being compared. The peaks near 280 mμ and 287 mμ in the differential spectrum were previously observed for insulin[6], and seem

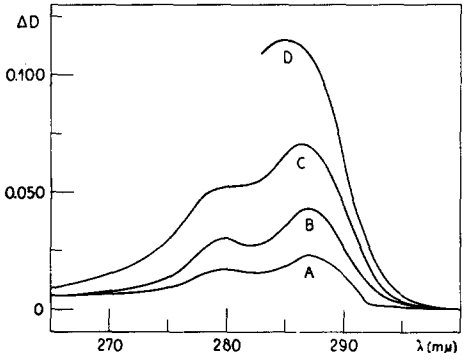

Fig. 1. Difference in optical density at room temperature between ribonuclease solutions at pH 1.91 and pH 6.94. Curves A, B, C, D correspond to experiments carried out with concentrations of 0.59, 1.18, 1.96 and 2.80 mg protein/ml, respectively.

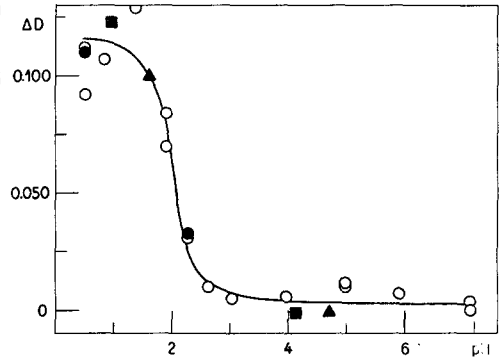

Fig. 2. Difference in optical density at 287 mμ between a solution of ribonuclease at pH 6.95 and at the pH indicated. The pairs of shaded points correspond to the reversibility tests discussed in the text. The protein concentration in all solutions was 1.94 mg/ml.

TABLE I

CONCENTRATION DEPENDENCE OF DIFFERENTIAL ULTRAVIOLET SPECTRUM OF RIBONUCLEASE AT 287 mμ

(pH 1.91 *vs.* pH 6.94)

Ribonuclease concn., c (mg/ml)	ΔD_{287}	$\Delta D/c$
0.59	0.023	0.039
1.18	0.043	0.036
1.96	0.070	0.036
2.80	0.115	0.041

characteristic of the shift of the tyrosyl spectrum toward longer wavelengths when this residue becomes hydrogen-bonded in a protein. This hydrogen bond presumably exists at pH 6.94 where the acceptor group is ionized but not at pH 1.91 where this group has acquired a proton.

Since ribonuclease does not show evidence of aggregation[7], such a hydrogen bond would be an internal rather than an intermolecular one. The constancy of the ratio $\Delta D/c$, shown in Table I, indicates that Beer's law holds, in agreement with this expectation.

The spectrum has the same appearance as that of Fig. 1 at other pH values, where the acceptor group is partially ionized. The pH dependence of the optical density difference at 287 mμ is shown in Fig. 2, which is essentially a titration curve for the acceptor group. The three pairs of shaded points correspond to results obtained from experiments in which the solution was brought from the lower to the corresponding higher pH. Since the points all fall on the same curve, the formation and rupture of the hydrogen bond is reversible.

Because of the high optical densities of the solutions, and the small values of ΔD, it is difficult to obtain good precision. Hence, whereas the apparent pK of the acceptor group appears to be approximately 2, the slopes of the curve of Fig. 2 are not unambiguously established. The observed pK value would identify the acceptor group as a carboxylate ion. The value of 2 is lower than the pK values tabulated by COHN AND EDSALL[8] for carboxyl groups in proteins. Since the isoionic point of ribonuclease in 0.15 M KCl is 9.6[9], one would expect the observed pK to be lower than the intrinsic pK° because of electrostatic effects in the region of carboxyl ionization; the extent of the lowering would depend on the net charge and protein size and shape. An additional lowering of about one pK unit can arise from hydrogen bonding[10]. However, in order to obtain as much as one pK unit from hydrogen bonding, the latter cannot be of the heterologous single-bond type[10]. A lowering of this size could, however, be obtained from a cooperative hydrogen bond involving two tyrosyls and one carboxylate ion, the decrease in pK being equal to $\log_{10}(1+K_{rs})$, where K_{rs} is the equilibrium constant for the formation of such a hydrogen bond[10]. Previous estimates[10] of $K_{rs} \sim 10$ would account for the observed enhanced acidity of the carboxyl group. It is also possible to incorporate steepness into the titration curve by assuming[10,11] that two un-ionized carboxyl groups are involved in a COOH$\cdots$HOOC acetic acid dimer-type hydrogen bond at low pH. Detailed calculations for the changes in thermodynamic parameters accompanying the conversion from a COOH$\cdots$HOOC bond to a cooperative tyrosyl-carboxylate ion bond have been given previously[11]. The essential point here is that the curve of Fig. 2 indicates that a carboxylate ion is the acceptor group in a hydrogen bond with a tyrosyl group. The low pK and possibly steep slope are compatible with the more complicated kind of tyrosyl-carboxylate ion hydrogen bonding situation mentioned above.

Experiments on the effects of proteolytic enzymes on the differential spectrum may aid in the location along the ribonuclease chain of the tyrosyl and carboxyl groups involved in the hydrogen bond(s).

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