BIOORGANIC CHEMISTRY 3, 249–259 (1974)

Reactions of the Amino Groups in Ribonuclease A
I. Kinetic Studies[1]

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Received October 3, 1973

A kinetic analysis has been made of the reaction of the amino groups of ribonuclease A with trinitrobenzene-sulfonic acid. The number of reactive groups and the number of subsets were markedly dependent on the nature and concentrations of the buffer and the pH. Apparent values of pK_a, calculated from the variation of the velocity constants with pH, could, in general, be obtained only for pH values above 7.4. Below this pH the velocity constants were greater than the values calculated from the intrinsic constants. The values of pK_a were in the range of 7.9–9.0, which are somewhat smaller than those derived from titration data.

The change of behavior of the amino groups with pH is confirmed by a study of the effects of ionic strength on the reactions.

The velocity constants generally appear to decrease with increasing concentration of protein.

It is shown that there is a close correlation between the pH region in which large changes occur of the reactivities of the amino groups of RNase and the kinetics of the enzyme reaction.

INTRODUCTION

Kinetic investigations concerning the reactivity of functional groups of proteins have been undertaken in the hope that they would serve as probes of the microstructure of protein and thus reveal hitherto unrecognized properties. A kinetic study of the reaction of the amino groups of human serum albumin (*1*, *2*) with trinitrobenzene–sulfonic acid clearly indicated that they were grouped in subsets each of which was described by an average velocity constant. From the effects of pH and temperature, it was suggested that there were alterations in the microenvironments of the amino groups which might result from local alterations in the protein. Since we wished to see more specifically the kind of information which could be obtained from such kinetic studies, we chose to continue the investigations by studying ribonuclease A, the structure of which is known in detail (*3*). In general, the findings with RNase confirmed those with serum albumin, egg albumin, (*4*), gamma globulin (*4*), chymotrypsinogen (*4*), and glutamate dehydrogenase (*5*), in that the amino groups, depending on the pH range studied, occurred in subsets. In this paper, it will be shown that the amino groups of RNase react in the form of nonprotonated bases with apparent values of pK_a somewhat smaller than has been

[1] Abbreviations. RNase, ribonuclease; k_i, velocity constant of ith subset; k_{oi}, intrinsic velocity constant of ith subset; K_{ai}, dissociation constant for protonated amino groups in "i"th subset; TNBS, trinitrobenzene–sulfonic acid; FDNB, fluorodinitrobenzene; RNA, ribonucleic acid; CCP, cytidine 2′, 3′ cyclic phosphate; TEA, triethanolamine.

reported from titration data. It will also be shown that the qualitative change of buffer affects some amino groups differently and that the velocity constants depend on the RNase concentration.

The variation of reactivities of the amino groups with pH were of such a nature as to lead us to wonder whether there was a parallel change of enzyme activity. Although the effect of pH on the enzyme activity has been studied exhaustively, the results could not be used to make a direct comparison with our kinetic studies because of the interrelated effects of pH, buffer type and ionic strength (*3, 6, 7*). Therefore, a study was undertaken of the enzyme activity of RNase under the same conditions as were used for the kinetic studies. It will be shown that the changes of enzyme activity with pH are parallel to the alterations of reactivity of the amino groups.

MATERIALS AND METHODS

Ribonuclease A was obtained as a phosphorus-free product from Worthington Biochemicals (RAF) and was found to contain less than 5% of polymers by analysis on Sephadex G75. Stock solutions (400–800 μM) were prepared in buffer solutions of pH 6.5 and depolymerized at 60°–65°C for 10 min. Pure monomers and polymers of RNase were prepared as described by Crestfield et al. (*8*). It was found that pure monomers gave identical kinetic data as did depolymerized RNase. Protein concentrations were determined at 280 nm (ε, 0.1% = 0.715).

RNA was the type-VI RNA supplied by Sigma Chemical Company. All samples of RNA were exhaustively dialyzed against distilled water before use. Cytidine 2',3' cyclic monophosphate was obtained as the sodium salt from Aldrich Organic Chemicals. All other chemicals were reagent grade.

The general methods for making the kinetic studies have been described (*1, 2, 9*). The rates of trinitrophenylation of the amino groups were followed by recording the changes of absorbance at 345 nm. In most cases the concentrations of RNase were between 4.5 μM and 5.5 μM and the concentrations of TNBS were between 1500 μM and 2500 μM and the reactions were followed in 10-mm stoppered cells. When higher concentrations of RNase were studied similar molar ratios of RNase to TNBS were used, and cells of suitably shorter path length were used. The analyses of the data were facilitated by programming the various steps on a Digital PDP-15 computer.

The reaction was studied in phosphate buffer as well as in TEA buffer since it had been shown that, in the presence of phosphate and polyanions, there was an alteration of the relative reactivity of the lys-41, ε-amino group with FDNB (*10, 11*) and with the carbonic anhydride of DL alanine (*12, 13*). It soon became apparent in TEA buffers that the fastest reactions (subsets three and four) were so rapid at a pH in excess of 7.5 that the calculated values of k_3 and k_4 which were obtained under our standard conditions, may have been suspect. Therefore, in order to obtain more reliable values, the reactions were run on 5 μM solutions of RNase at TNBS concentrations between 250 μM and 350 μM for about 2 hr. Analysis of these partial curves soon indicated that these subsets alone contained a total of three amino groups as found in the data obtained at pH 7.65, 7.95, and 8.40 (Table 2).

The values of k_{fd}, which are recorded in the tables are the averages of two or three experiments at the same pH, and the individual values were less than 7% from these

means. When the experimental values of k_t and n_t were used to reconstruct the experimental curves the agreement was well within 2%. When the values of k_t were used which were calculated from K_a and k_0 along with values of n_t which were rounded out to the nearest whole number, the calculated points were within 5% of those observed on the experimental curves.

The rate of hydrolysis of CCP by RNase was followed spectroscopically at 290 nm as described by Irie (*6*) and by Herries et al. (*7*). At CCP concentrations of 0.167×10^{-3} *M* to 2×10^{-3} *M* the initial increases of absorbance with time were linear. The enzyme concentrations were 0.8 μM in 0.1 *M* TEA buffer. The difference ($\Delta\varepsilon$) between the molar extinction for CCP and the hydrolysis product was determined in the pH range 6.8–8.0 by observing the changes of absorbance at 290 nm. The absorbances reached maximum values in 4 hr and were constant for another 20 hr. The value of $\Delta\varepsilon$ was 1050, did not vary over the pH range which was studied, and was comparable with the value reported by Hammes and Walz (*14*).

The nonhydrolytic reaction of RNA with RNase was followed spectroscopically at 300 nm by Kunitz's method (*15*, *16*). In RNA solution (0.05% to 0.2%) containing 1 μg RNase per 3.0 ml mixture the reaction was not zero order. The rate of decrease of absorbance was analyzed by a method described by Goldfarb (*1*). Under all conditions the curves could be reproduced only as the sum of two reactions as described in Eq. (1), where Δa is the change of absorbance, and A_1 and A_2 are the change of absorbances at

$$\Delta a = -A_1(1 - e^{-k_1 t}) - A_2(1 - e^{-k_2 t}) \tag{1}$$

infinite time for the velocity constants k_1 and k_2. The values of $A_1 + A_2$, calculated by the method which has been previously described (*1*), were found to be the same as the Δa found experimentally after 2–6 hr. The curves, calculated with the determined constants k and A, fit the experimental curves within 5%.

All studies were carried out at 15.5° ± .1°C.

RESULTS

It had been shown that simple primary amines and acetyl lysine reacted with TNBS in the form of nonprotonated bases which gave identical intrinsic constants, k_0, at pH 7.9 when the known dissociation constants were substituted in Eq. (2) (*2*). Kinetic studies were made

$$k = k_0 K_a/([\mathrm{H}+] + K_a) \tag{2}$$

of the reactions of α-acetyl lysine, diglycine, and triglycine and the values of pK_a were determined, from the variations of k with pH, as being 10.8, 8.25, and 7.90, respectively. These compared favorably with values which have been reported in the literature as being 11.00, 8.13, and 8.11, respectively (12).

The changes of k_t with pH for the reaction of RNase with TNBS are summarized in Tables 1 and 2. On the assumption that the amino groups of RNase also reacted in the form of the free base, the kinetic parameters, K_a and k_0, were calculated with the use of Eq. (2) by a method of least squares. These values were placed at the heads of the columns in which the values of k_t for each subset are compared with those which were found experimentally. In the presence of phosphate buffer (Table 1) the data of the 3rd and 4th subsets fit Eq. (2) from pH 7.00 to 8.50, whereas the 1st and 2nd subsets had applicable

TABLE 1

KINETIC PARAMETERS FOR THE REACTION OF RNase A WITH TRINITROBENZENE SULFONIC ACID (0.1 *M* PHOSPHATE BUFFER, [RNase] = 5 μM)

pH	1st Subset $pK_{a1} = 8.13$ $k_{01} = 2.24$ $n_1 = 7$		2nd Subset $pK_{a2} = 8.64$ $k_{02} = 26.0$ $n_2 = 2.0$		3rd Subset $pK_{a3} = 8.56$ $k_{03} = 158$ $n_3 = 1$		4th Subset $pK_{a4} = 8.03$ $k_{04} = 240$ $n_4 = 1$	
	k_{fd}[a]	k_{calc}	k_{fd}[a]	k_{calc}	k_{fd}[a]	k_{calc}	k_{fd}[a]	k_{calc}
7.00	0.4 (5.5)[b]	0.15	—	0.58	4.4	4.23	19.4	20.5
7.29	0.52 (5.7)[b]	0.28	—	1.11	7.2 (2)[b]	8.05	44.0	37.0
7.50	0.46	0.43	1.60	1.76	16.0	12.7	60.0	55.0
7.70	0.60	0.61	2.60	2.67	19.0	19.2	70.0	76.0
7.92	0.88	0.85	4.3	4.16	26.0	29.4	110.0	105.0
7.95	0.94	0.89	4.8	4.41	31.0	31.1	106.0	109.0
8.29	1.25	1.32	9.5	8.00	52.0	55.0	165.0	156.0
8.50	1.57	1.57	11.8	10.9	76.0	73.0	185.0	179.0

[a] These are average values obtained from two or three experiments at the same pH.

[b] The numbers in parentheses represent the number of reactive groups in this subset when it differs from the value at the top of the column.

TABLE 2

KINETIC PARAMETERS FOR THE REACTION OF RNase A WITH TRINITROBENZENE SULFONIC ACID (0.1 *M* TRIETHANOLAMINE·HCl [RNase] = 5 μM)

pH	1st Subset $pK_{a1} = 8.15$ $k_{01} = 2.44$ $n_1 = 5.5$		2nd Subset $pK_{a2} = 7.90$ $k_{02} = 10.9$ $n_2 = 1.0$		3rd Subset $pK_{a3} = 8.32$ $k_{03} = 120$ $n_3 = 2.0$		4th Subset $pK_{a4} = 9.03$ $k_{04} = 6609$ $n_4 = 1.0$	
	k_{fd}[a]	k_{calc}	k_{fd}[a]	k_{calc}	k_{fd}[a]	k_{calc}	k_{fd}[a]	k_{calc}
7.00	0.43 (5.0)[b]	0.16	3.3 (2)[b]	1.22	27.3 (1)[b]	5.5	63.8 (1.5)[b]	61.0
7.39	0.51 (5.3)[b]	0.36	2.68	2.56	13.2	12.6	140.0	147.0
7.52	0.48 (5.8)[b]	0.46	2.90	3.20	15.8	16.4	210.0	198.0
7.65	n.d.[c]		n.d.[b]		20.7	15.4	266.0	264.0
7.78	0.68	0.73	4.5	4.7	26.7	26.9	336.0	351.0
7.92	0.95	0.91	4.8	5.6	37.5	34.2	480.0	476.0
7.95	n.d.		n.d.		37.6	35.9	508.0	507.0
8.01	0.99	1.03	6.2	6.1	34.0	39.5	560.0	576.0
8.40	n.d.		n.d.		61.9	65.5	1273.0	1249.0
8.50	1.70	1.68	7.0 (2.5)[b]	8.7	75.0	72.0	1514.0	1492.0

[a] These are average values obtained from two or three experiments at the same pH.

[b] The numbers in parentheses represent the number of reactive groups in this subset when it differs from the value at the top of the column.

[c] n.d. stands for the fact that only subsets 3 and 4 could be analyzed in these experiments.

data only at a pH equal to, and greater than 7.5. In the presence of TEA buffer (Table 2) the fit over the whole pH range occurred only in the 4th subset. For the 2nd and 3rd subsets the fit was good from pH 7.39 to 8.5 and for the 1st subset at a pH greater than 7.39. The total number of reactive groups (n_T found in the presence of phosphate increased from 7½, below pH 7.5 to 11 at higher pH values. In TEA buffer the value of n_T rose with increasing pH from 8 to 11.

It has been reported that the optimum pH for the enzyme activity of RNase was dependent on the ionic strength (*6*) and it was thought possible that a similar effect may have contributed to our results. It was, therefore, considered desirable to obtain some indication whether a variation of buffer concentration had any effect on the reactivities of the amino groups. For this purpose a preliminary study was made at pH values of 7.0 and 8.0 in buffer concentrations of 0.02 *M*, 0.10 *M*, and 0.30 *M*; the results are reported in Table 3. In TEA buffer at pH 7.0 there were no differences in k_i between 0.1 *M* and

TABLE 3

EFFECT OF BUFFER CONCENTRATION ON THE REACTION OF RNase WITH TNBS[a]

Concn buffer	k_1 (n_1)	k_2 (n_2)	lys 1α (n)[b]	lys 41 (n)[b]
TEA (pH = 7.00–7.02)				
0.02 *M*	0.55 (6.0)	4.2 (1.0)	48 (1.0)	371.0 (0.8)
0.10 *M*	0.43 (5.0)	3.3 (2.0)	27 (1.0)	60.0 (1.0)
0.30 *M*	0.45 (5.0)	3.0 (1.8)	25 (0.9)	50.0 (0.9)
Phosphate (pH = 6.99–7.01)				
0.02 *M*	0.37 (5.0)		19 (1.0)	5.0 (1.2)
0.10 *M*	0.40 (5.0)		19 (1.0)	4.4 (1.0)
0.30 *M*	0.38 (6.0)		14 (0.8)	4.1 (0.8)
TEA (pH = 8.03–8.04)				
0.02 *M*	0.78 (5.4)	5.0 (1.7)	29 (1.6)	600.0 (1.2)
0.10 *M*	1.10 (5.5)	6.4 (1.0)	42 (2.0)	620.0 (1.0)
0.30 *M*	0.93 (5.3)	5.3 (3.3)	31 (1.2)	620.0 (1.0)
Phosphate (pH = 7.98–8.03)				
0.02 *M*	0.58 (5.4)	4.2 (1.9)	45 (1.1)	245.0 (0.8)
0.10 *M*	0.95 (7.0)	4.8 (2.0)	34 (1.0)	116.0 (1.0)
0.30 *M*	1.02 (8.0)	5.3 (1.0)	22 (1.0)	76.0 (1.0)

[a] All values are the averages of at least two runs.

[b] The assignments were made as discussed in the text.

0.3 *M* solutions. However, in 0.02 *M* TEA, there was a small increase in k_3 and a large increase in k_4. In phosphate buffer at pH 7.0, the constants of all of the amino groups appeared to change slightly, if at all. At a pH of 8.0, the values of k_1 and k_2 were the same in 0.1 *M* and 0.3 *M* solutions in both TEA and phosphate buffers and were smaller in 0.2 *M* solutions. At pH 8.0, k_3 and k_4 varied only slightly with concentration of TEA whereas, in phosphate, there were regular increases with decreasing buffer concentration.

As part of this study, it was desired to prepare larger amounts of trinitrophenylated

Since both gentisaldehyde and *m*-hydroxybenzaldehyde absorb at 340 nm $\varepsilon_{340}^{pH\,7.5}$ 2.7×10^3 and $\varepsilon_{340}^{pH\,7.5}$ 1.6×10^3, respectively, the sum of the extinction coefficients was used in calculating the amount of NADPH (ε_{340} 6.22×10^3) oxidation from the change in optical density.

(II) Enzyme Assay of Patulin Synthesis

Thin layer chromatography of radioactive patulin. The incubation mixture contained in a volume of 1.0 ml: 0.2 μM [^{14}C]-gentisaldehyde (0.088 mCi/mM) or [^{3}H]-gentisaldehyde (1.04 mCi/mM) 1.0 μM NADPH, 1.0 μM ATP–Mg^{2+}, 1.0 μM patulin, 1.0–2.5 mg of protein in the enzyme extract, and H_2O. The incubations were started by the addition of enzyme and continued for 30 min at 28°C. The incubations were stopped by the addition of 3 drops of 60% perchloric acid, 0.25 mg of carrier patulin was added and the mixture extracted with 3 × 5 ml of ether (approximately 75% recovery of patulin). The ether extracts were combined, dried with Na_2SO_4, evaporated under a stream of N_2, and chromatographed on silica gel thin layer plates (Brinkman F254, 20 × 20 cm, 0.2 mm thick) developed with benzene: dioxane: acetic acid (90:25:4). The patulin was detected by uv light, recovered, and assayed for radioactivity in Bray's solution (*12*). R_f values: gentisaldehyde 0.60, patulin 0.38.

RESULTS

Purification Procedure for Aromatic Dehydrogenase(s)

All procedures were carried out at 4°C.

Crude extract. 8 day old mycelium was filtered, washed with 0.25 M NaCl solution, dried between filter papers and weighed. 30 g of cells were suspended in 100 ml of 0.05 M K-phosphate buffer, pH 7.6 containing $1 \times 10^{-3} M$ DTT and passed through a French press at 20,000–10,000 psi.

Polyclar AT treatment. To the French press extract 30 g (wet wt) of the Polyclar AT slurry was added and the suspension stirred for 10 min. The suspension was then centrifuged at 10,000*g* for 15 min and then at 100,000*g* for 1 hr. The supernatant was saved.

40–55% ammonium sulphate fractionation. Solid ammonium sulphate was added to the 100,000*g* Polyclar treated crude extract to 40% saturation. The extract was spun 45,000*g* for 10 min and the pellet discarded. Solid ammonium sulphate was again added to 55% saturation, and the centrifugation carried out as above. The resulting pellet was dissolved in 8.0 ml of 0.05M K-phosphate buffer pH 7.6 containing $1 \times 10^{-3} M$ DTT.

Dialysis. The 40–55% ammonium sulphate pellet solution was dialyzed for 3 hr against one change of buffer in the above K-phosphate buffer.

DEAE cellulose column. The DEAE column (2 × 15 cm) was equilibrated with 0.05M K-phosphate buffer pH 7.6 with 1×10^{-3} DTT. The dialyzed enzyme was placed on the column and eluted with a linear gradient 0.05M → 0.50M K-phosphate pH 7.6 with $1 \times 10^{-3} M$ DTT. 1.0 ml fractions were collected and the protein was monitored continuously by a Uvicord II analyzer. The flow rate was 60 ml/hr. The enzyme assay used to follow the purification was the optical estimation using gentisaldehyde and NADPH as the substrates.

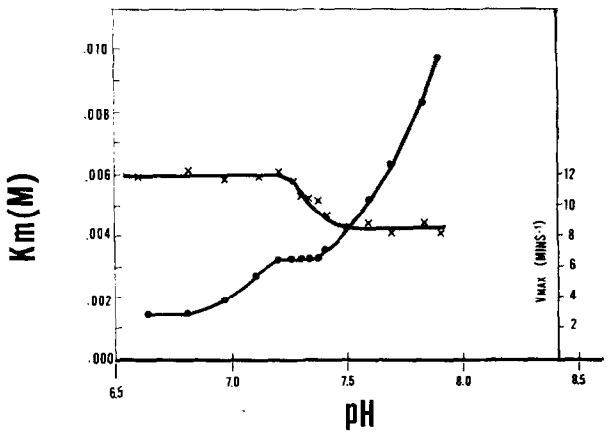

FIG. 1. Reaction of CCP with RNase. Variation of Michaelis constants with pH, 0.1 *M* TEA buffer (×) = V_{max}, (·) = K_m.

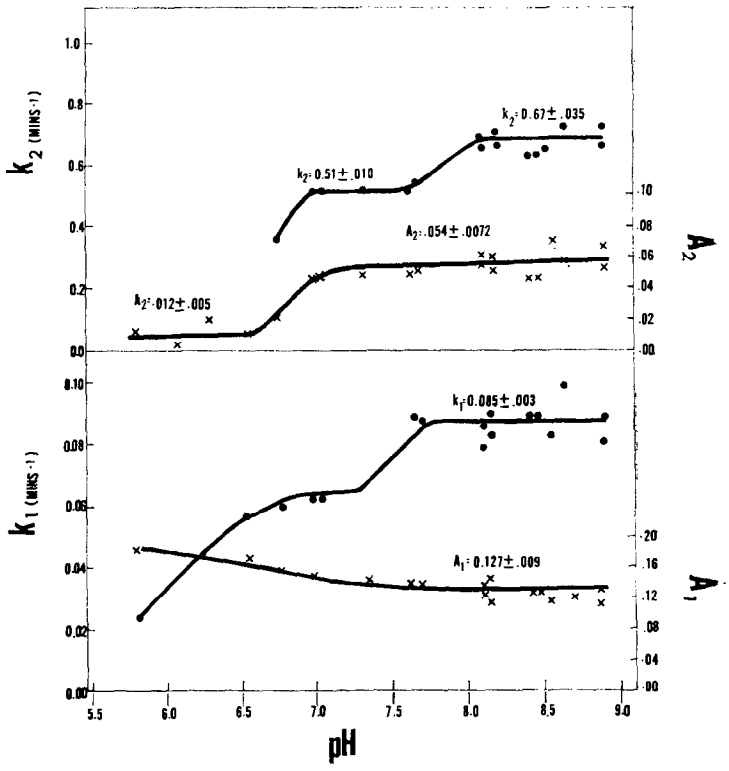

FIG. 2. Reaction of RNA (0.2%) with RNase (1 μg/3.0 ml) in 0.1 *M* TEA buffer. Variation of kinetic constants with pH. (×) = A_1 and A_2; (·) = k_1 and k_2.

From pH 5.8, the velocity constants increased to pH 7.0 for k_1 and to pH 7.3 for k_2. This was followed by a flat region up to pH 7.3 for k_1 and 7.6 for k_2. After transitions between pH 7.3 and 7.7 for k_1 and 7.6 to 8.0 for k_2, both k_1 and k_2 had constant values

to a pH of 8.9. The curve for k_2 was not continued to pH values below 6.7 because the values of A_2 were too small to make possible the calculation of reliable values of k_2. The values of A_1 and A_2 were affected by pH as is indicated in Fig. 2. The occurrence of two velocity constants could have been due to either the presence of two substrates in the sample of RNA which was used for the study, or the occurrence of two simultaneous reactions having two significantly different rates. As partial evidence for either of these alternatives it was desirable to test the homogeneity of the RNA. For this pur-

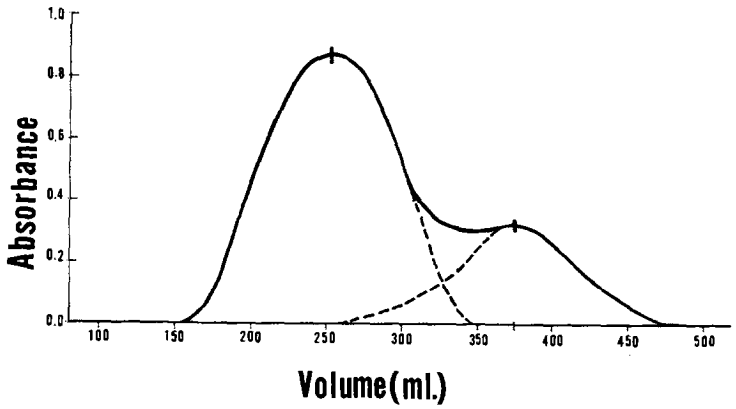

FIG. 3. Chromatography of RNA on a Sephadex G 100 column. For details see the text.

pose the RNA was chromatographed on a Sephadex G 100 (3 × 75 cm) with .05 *M* Tris buffer, pH 7.0. It is clear in Fig. 3 that the RNA used in these experiments contained two components. The ratio of the areas under the separate curves (after they had been made symmetrical) was 2.8 and this compared to the ratios for A_1/A_2 of 3.0 at the pH of 7.0. There is reason, therefore, to suspect that the different velocity constants corresponded to simultaneous reactions with different substrates at different rates. In the usual Kunitz assay of RNase activity with RNA as the substrate (*16*) no mention has ever been made of more than one reaction. However, this was usually performed with 0.05 % RNA and at pH of 5.0. Our data clearly showed that A_2 was so small at a pH of 5.8 that it was impossible to analyze this reaction. The total effect here would be to make it seem that only the reaction involving k_1 and A_1 was present. A limited study was also made of the effect of the concentrations of RNA and RNase on the enzyme kinetics in 0.1 *M* TEA buffers at pH 7.68 and 8.38. As was to be expected, the velocity constants, k_1 and k_2, were proportional to the concentration of RNase without affecting the absorbances A_1 and A_2, and the absorbances were proportional to the concentration of RNA with no effect on the velocity constants.

DISCUSSION

In the derivation of the analysis of kinetic curves, it has been assumed only that individual functional groups had individual rates of reaction and that the absorbance observed at any time was the sum of the individual absorbances due to the reaction of each group (*1*, *9*). No assumption had been made concerning the factors which determined the

individual rates of reaction, and, therefore, the velocity constants and the derived constant k_0 and pK_a could only be "catch-all" values that might include both the properties of the amino group in the "native state" of the protein or an "altered state." It would be a reasonable presumption that previous substitutions at one position might affect the properties at another position. Evidence in respect to this is found in the study by Hirs et al. on the reaction of RNase A with FDNB (10) and in the comparison, by Allwell et al. of the crystalline structures of lys-41DNP RNase and RNase (*22*). The extent to which substitution would affect distant parts of the molecule in solution was, however, considered to be uncertain (*22*).

The fact that the kinetic studies of simple amines and peptides gave apparent values of pK_a which were close to those obtained from titration data made it reasonable to assume that similarly dependable values of pK_a should have been found for the amino groups of RNase. When the data of this paper were combined with those of the following report (*19*), it was found that in 0.1 *M* TEA buffer, the first and second subsets contain most of the amino groups on the surface of the protein, the third subset contained the α-amino group of lys-1, and the fourth subset, the ε-amino group of lys-41. In 0.1 *M* phosphate buffer, the first and second subsets also contained the surface amino groups, the third subset contained lys-41 at pH 7 and lys-1α at pH 8, and the fourth subset contained lys-1α at pH 7.0 and lys-41 at pH 8.0. For obvious reasons, only the values of pK_a derived from experiments in TEA can be compared with values obtained by Tanford and Hauenstein from titration data (*17*) and in Hirs' laboratory from kinetic data (*10*, *18*). Our value for the lys-1α amino group, 8.32, while it is larger than the value of 7.8 obtained from titration data (*17*), falls near the values reported from kinetic studies at 28°C, i.e., 8.14.

From their titration data Tanford and Hauenstein assumed that all of the ε-amino groups in RNase A had the same values of pK_a i.e., 10.2. However, Hirs et al. suggested values for the ε-amino group of lys-41 of 8.8 (*10*) and 9.07 (*18*) from kinetic studies at 28°C and 9.48 at 16°C. The value 9.03, which we report, is somewhat different from the values suggested by Hirs et al., but in common with these earlier studies, has lower values than that suggested by Tanford and Hauenstein. The pK_a values for the other amino groups, which we have found, are very much smaller than those estimated by both Tanford and Hauenstein (*17*) and by Carty and Hirs (*18*). It is clear that the values of pK_a reported in this paper cannot be used to reconstruct the titration curve for RNase. When, however, the isoelectric pH was calculated with our constants, a value of 8.0 was obtained compared to one of 9.5 from titration data and an intermediate value from the constants proposed by Hirs et al. However, there are two other determinations, the first by Barnett and Bull (*20*), who reported an isoelectric range between pH 8.2 and 9.6 (*20*), and a second one by Sasakawa and Walter, who also indicated a range between pH 7.2 and 9.6 (*21*). Both of the suggested ranges included the points calculated from this report as well as those of Tanford and Hauenstein and Hirs et al. In passing it should be pointed out that our constants have been obtained with 5 μ*M* protein solutions, whereas those of other workers were obtained from experiments with very much greater concentrations of RNase, and we have shown a fairly large effect of protein concentration. It is, of course, possible that the differences also may be due to other differences in the experimental conditions under which the data were obtained. It might also be suggested that, in titrating amino groups with pK_a values near 10, reliable data would be

obtained only at pH values above 9. In view of the fact that six phenolic groups are present, with pK_a between 9.6 and 9.95, it is possible to raise the question whether the presence of phenoxide anion at the higher pH values may alter the protein so as to change the properties of some or all amino groups.

On the basis of the variations of k_i with pH and the differences in the effects of the concentration of TEA and phosphate buffer at pH 7 and 8, there appears to be a pH-dependent sensitivity of the properties of the amino groups in RNase, with a "transition" occurring somewhere below pH 7.5. It is also to be noted that, in general, the variation, with pH, of the constants derived from enzyme studies with two kinds of substrates run parallel to the variation of reactivities of the amino groups. Since the optimum pH for the catalytic properties of RNase has been shown to be dependent on ionic strength (*6*), it is possible that the transition could also have been due to a general ionic strength effect. However, the surface amino groups of RNase had the same k_1 in both 0.1 *M* TEA and 0.1 *M* phosphate at all pH values and these solutions certainly had different ionic strengths. Further, the constant for lys-41 amino group in 0.1 *M* TEA did not vary over a 15-fold buffer concentration. A simple rationale for the "transition" of amino group properties is not readily available. If one concedes that the properties of the amino groups are dependent on the microenvironment of these groups, it might be suggested that the changes might arise from a pH-dependent rearrangement of the molecule of unknown magnitude. The fact that the change occurs below pH 7.5 might be correlated with the presence of four imidazol side chains with pK_a values between 6 and 7 (23).

The persistently occurring decrease of the velocity constants with increasing RNase concentration was unexpected. It seemed unlikely that there would be any protein–protein interaction at 50 μM concentration of RNase. Andrews (*24*), in fact, reported that although several proteins showed concentration effects in gel column chromatography, RNase did not. We repeated this on Sephadex G75 and G100 columns and obtained values of V_e for 5 μM and 50 μM solutions within 5% of each other. It was then considered that the apparent change may have been due to differences of association of RNase with TNBS. The association of the closely related, but nonreacting compound, 1,3,5-trinitrobenzoic acid (TNB), was studied by the equilibrium dialysis technique. It was found that there was no apparent difference in the amount of TNB bound per mole of RNase between 5 μM and 50 μM concentration of protein. Parallel concentration effects have been reported by Sasakawa and Walter (*21*) and by Bernfield and Rottman (*25*). The former authors showed that at pH 8, the partition coefficient of RNase fell steeply with concentrations to a minimum value near 8 μM and then increased with concentration, and the latter authors reported a marked concentration effect on the ability of RNase to form oligonucleotides from simpler molecules. Although we are not able to supply a reasonable explanation for this effect, it may play a part in explaining the differences between the pK_a values obtained in our and other studies which have been discussed above.

ACKNOWLEDGMENTS

The author acknowledges the technical assistance during summer fellowships of Messrs. Mark Rottenberg and Howard Liss. Supported by Grants No. GM 15219 and RR 05641 U.S. Public Health Service.

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