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Reactions of the Amino Groups of RNAse A

IV. The Effects of Protein Concentration

A. ROBERT GOLDFARB[1] AND PHILIP D. MARTIN[2]

Department of Research, Sinai Hospital of Detroit, Detroit, Michigan 48235

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A study has been made of the effect of ribonuclease (RNAse) concentration on the properties of the amino groups. The biphasic dependence of pK on pH which has been established (Goldfarb and Martin, *Bioorg. Chem.* **5**, 137 (1976)). for 5 μM solutions of RNAse also have been shown to occur for 50 μM solutions. In the lower pH range (7.5–8.5) the values of pK obtained with 50 μM solutions were similar to those obtained with 5 μM solutions (p$K = 7.5$) but the intrinsic constants were smaller. In the higher pH range (8.5–10) the pKs in the more concentrated solutions were larger than those found at the smaller concentration and the intrinsic constants were generally smaller. A quantitative study of the concentration vs k_i relation at pH 7.5 indicated a sigmoid relationship for all of the subsets with a constant maximum value equal to, and less than that at 5 μM RNAse and a constant minimum value above that at 20 μM. Parallel studies with oxidized RNAse gave parallel, although not identical, results from which it is proposed that the concentration effect does not arise totally from the three-dimensional structure of native RNAse.

INTRODUCTION

During the course of an investigation of the trinitrophenylation of the amino groups of ribonuclease A with trinitrobenzene sulfonic acid, it had been found that the velocity constants for 50 μM solutions were somewhat smaller than those obtained with 5 μM solutions (*1*, *2*). With 5 μM solutions it had been shown also that values were obtained at 16°C which were considerably smaller than the values obtained by titrimetry (*3*). It was suggested that the discrepancy might have been due, in part, to the difference in the concentrations used in our kinetic study compared to that used in the titrimetric studies (*1*). In order to duplicate the experimental conditions under which the titrimetric experiments were performed we repeated our studies on 50 μM solutions of RNAse,[3] at 27°C, and from a pH of 7 to about 10. Under these conditions, as was found for 5 μM solutions, the 50 μM solutions gave data from which were calculated three sets of pK values that were markedly dependent on the pH range for which the data had been collected. As we had surmised, with 50 μM solutions of RNAse, values of pK were obtained which were consistent with those reported from titrimetric experiments, but

[1] Deceased January 27, 1976.

[2] To whom correspondence should be sent. Present address: Department of Biochemistry, Wayne State University School of Medicine, 540 E. Canfield, Detroit, Michigan 48201.

[3] Abbreviations: RNAse, ribonuclease A; OxRNAse, oxidized RNAse; k_i, velocity constants of ith subset; k_{oi}, intrinsic velocity constant of ith subset; K_i, dissociation constant for the protonated amino group in ith subset; TNBS, trinitrobenzene sulfonic acid; TEA, triethanolamine.

only in the pH range 8.5–10. A comparable study with OxRNAse resulted in the finding that only five of the amino groups had pK values which were sensitive to pH.

A quantitative study of the variation of the velocity constant (k_t) with protein concentration, at pH 7.5, resulted in a sigmoid plot of k_t vs [RNAse] with a maximum value below 5 μM and a minimum values above 20 μM.

MATERIALS AND METHODS

Ribonuclease A was obtained as a phosphorus-free product from Worthington Biochemical Corporation. About 1% stock solutions were prepared in water, adjusted to pH 6.5 and depolymerized at 60–65°C for 10 min. The TNBS was obtained from Nutritional Biochemicals, Inc., and was recrystallized from 5 M HCl. The buffers were triethanolamine at a pH between 7 and 9 and triethylamine for a pH greater than 9. The buffers were used in 0.1 M concentrations, in which case the pH changed by less than 0.02 from the start to the completion of the reactions.

All other chemicals were c.p. grade.

The methods for studying the kinetics of the trinitrophenylation have been adequately described (*1*, *2*). The concentration of RNAse was between 49 and 55 μM. The concentration of TNBS was close to 0.02 M. The experiments were run in stoppered 1 mm cells. For lower concentrations of RNAse, stoppered cells of suitable path length were used (*1*). The change of k_i with protein concentration was only studied at pH 7.5 with RNAse concentrations from 3 to 66 μM.

The assignment of values of k_{0i} and pK_i to individual amino acids was based on a comparison of the calculated fractional reaction of a group in a given subset with the fractional reaction as determined by the isolation procedure as already described (*2*). The isolation procedures were carried out at pH 8.0, which represented the pH range 7.5–8.5, and at 9.3 for the pH range 8.5–10.

As we have indicated in Part III (*4*), the experimental curves were fit with an accuracy of better than 3%. The experimental values of k_i were within 7% of the values calculated from pK_i and k_{0i}.

RESULTS

Table 1 contains the kinetic constants which were calculated from the variation of k_t with pH as has been described (*4*). All of the amino groups of native RNAse in subsets 1 and 2 showed a biphasic nature, i.e., gave kinetic constants which depended on the pH range in which measurements were made. In the lower pH range (7.5–8.5) six to eight groups in subsets 1 and 2 had pK_i values of 7.53 or 7.77 with k_{0i} of 0.6 or 3.3 The remainder of the amino groups either showed no regularity of behaviour (subsets 3 and 4) or had reacted so rapidly as to make it impossible to obtain velocity constants (subset 5). In the upper pH range (8.5–10) subset 1 reacted uniformly up to pH 9.4 but, above this, the plot of k_1 vs k_1 [H^+] fit on the curve for subset 2. This was accompanied by the increase of n_2 from a value of 3.5 below pH 9.4 to 5.0 above pH 9.4, and no amino group could be fit into the first subset. The values of pK_i varied somewhat

TABLE 1

KINETIC PARAMETERS FOR NATIVE RNAse (~50 μM)

Subset	pK_i (k_{oi})	n_i	Positions of amino groups[a]
pH range 7.5–8.5			
1	7.77 (0.6)	4.5–3.5	31, 1:23, 4:35, 6:39
2	7.53 (3.3)	3.5–2.5	7, 66, 104
3	— (—)[b]	1.5–2.0	—
4	— (—)[b]	1–2	—
5	— (—)[c]	1	—
pH range 8.5–10[d]			
1	10.4 (55)	3.5	31, 104, 1:23, 6:39
2	9.9 (79)	3–5	7, 66, 98
3	9.39 (227)	2–2.5	61, 4:45
4	— (—)[c]	2.5–3.5	—

[a] The numbers represent the positions of the lysine side chains. 1:23, 4:45, and 6:39 represent the positions of TNP-peptides which were not equivocally identified (Ref. *2*).

[b] The points in the k vs k [H^+] plot were scattered and the least-square calculation was unreliable (cf. text).

[c] The reactions were so rapid that no value of k_i could be determined.

[d] See text.

(9.4–10.4) as did those of k_{oi} (55–227). Below pH 7.5, i.e., at a pH of 6.5 and 7, the velocity constants were invariably greater than at pH 7.5.

In contrast to native RNAse the amino groups of OxRNAse showed a whole spectrum of properties (Table 2). With this material as indicated by the variations of n_i, the amino

TABLE 2

KINETIC PARAMETERS FOR OxRNAse (~50 μM)[a]

	pH range 7–8.5			pH range 8.5–9.5		
Subset	pK_i	(k_{oi})	n_i	pK_i	(k_{oi})	n_i
1	—	(—)	3	—	(—)	0
2	8.05	(3.46)	5	8.90	(9.1)	5
3	8.30	(27.2)	2	8.30	(27.2)	3.5
4	7.67	(137)	1	7.67	(137)	2.5

[a] See footnote *b*, Table 1.

groups shifted with pH from subset to subset. Only three amino agroups in subsets 3 and 4 gave linear plots of k_i vs k_i [H^+] over the whole pH range. On the other hand, the three amino groups of subset 1 in the lower pH range showed only a random plot and therefore could not be assigned reliable kinetic constants. In contrast to these,

subset 2 showed the presence of five groups for which the intrinsic constants depended on the pH range.

The assignment of the kinetic constants at pH 7.5 to the individual amino groups of native RNAse is given in Table 1. The groups in the 1α, 41, 61, and 98 positions could not be assigned values of pK or k_0 for reasons indicated in Table 1. In spite of this it could be determined from the isolation experiments that, at the indicated pH, the rates of reaction decreased in the order 41, 98, 1α, and 61, whereas at pH 9.3 the order was 41, 1α, and then the others.

The quantitative study of the variation of k_i with concentration of native RNAse was only done at pH 7.5 for the reason that in the pH range 7.5 to 8.5 the values of pK

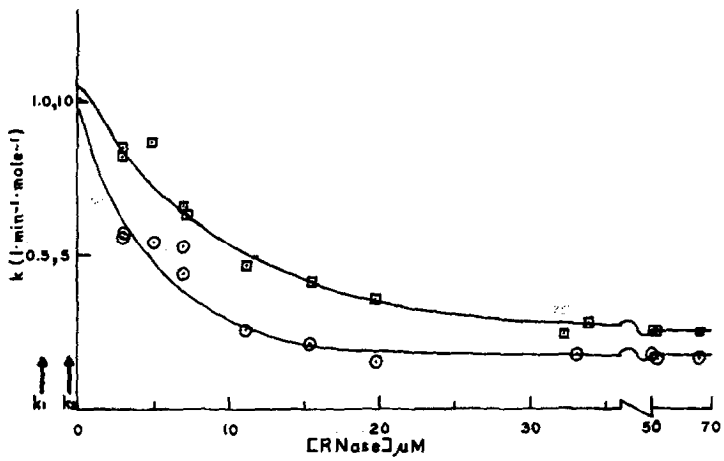

FIG. 1. Variation of k_1 and k_2 with RNAse A concentration. (□) k_1; (○) k_2.

were essentially constant so that the variations of k_i were directly related only to the intrinsic constant, k_{0i}. At pH greater than 8.5 the values of pK varied somewhat with pH and we felt that any study at higher pH values would not serve any useful purpose at this time. The results of the studies of the variation of k_i with concentration of protein are indicated in Figs. 1 and 2. The studies could only be extended to 3 μM solutions of RNAse since the absorbances which were obtained at lower concentrations were too small to enable us to make any reliable calculations of k_i. However, the values of k_i near 3 μM were close to the values for 5 μM solutions. With the more concentrated solutions, k_i was essentially constant from 30 to 70 μM. The points are experimental and the curves were calculated from Eq. (1), where k is the velocity constant obtained at an RNAse concentration equal to R.

$$k = k_{\max} e^{-\alpha R} + k_{\min}(1 - e^{-\alpha R}) \tag{1}$$

The values of $k_{\max}$ and $k_{\min}$ were obtained from the data at the lowest and highest concentrations and that of α by a least-squares method, and these are recorded in Table 3. The experimental values of k_i were within 10% of that calculated from Eq. (1). The appearance of "bad" points in subset 4 was to be expected from the nature of our calculations (*1*, *2*, *4*).

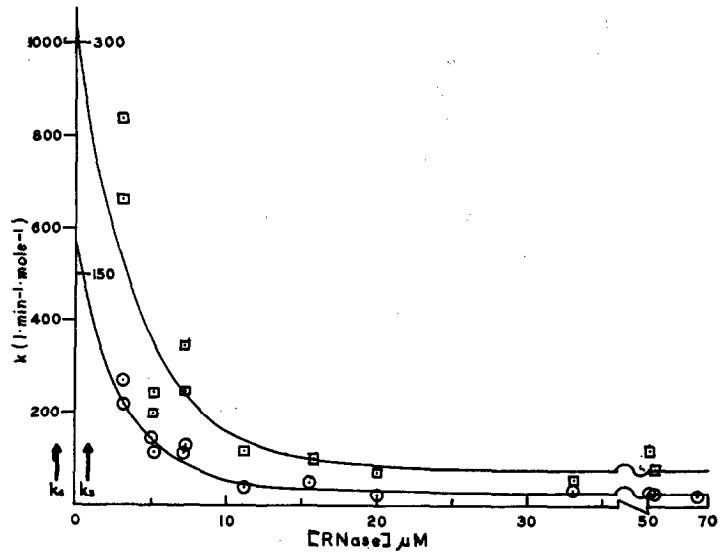

FIG. 2. Variation of k_3 and k_4 with RNAse A concentration. (⊙) k_3; (⊡) k_4

TABLE 3

VALUES OF k_{max}, k_{min} AND α

Subset	k_{max}[a]	k_{min}[a]	α[b]
1	1.05	0.25	1.04×10^5
2	7.75	1.71	1.39×10^5
3	174	9.8	3.16×10^5
4	1010	77	2.44×10^5

[a] k_{max} and k_{min} are given in liters per mole per minute.

[b] α is given in molar^{-1}.

DISCUSSION

The dependence of pK and k_0 on pH, which we have shown to apply for 5 μM solutions (Part III, Ref. *4*) clearly holds for 50 μM solutions of RNase. In the low pH range the values of pK at 50 μM (7.5–7.8) are close to those which had been obtained in the same pH range with 5 μM solutions (about 7.5). In the higher pH range the values of pK in 50 μM solutions are larger than those which were found in 5 μM solutions. Indeed, the larger values of pK (9.9–10.4) for the 50 μM solutions correspond to the value 10.2 which had been reported for relatively concentrated solutions of RNAse by Tanford and Hauenstein from titration data (*3*) and 10.1 by Carty and Hirs (*5*) from kinetic studies of the reaction of RNAse with 4-sulfonoxy-2-nitro-fluorobenzene. It must be taken into consideration that titrations from which pK values of 10.2 have

been obtained must have required the collection of data above pH 9. It is then apparent that our conjecture was fully substantiated that, in order to obtain comparable values of pK by the kinetic method as had been obtained by the titrimetric method, it was not only necessary to reproduce the conditions of temperature and protein concentration but also a similar pH range. It seems to be also a reasonable assumption that in 50 μM solutions of RNAse there are, as suggested for 5 μM solutions (*4*), three pH-dependent states of RNAse which occur below pH 7, between a pH of 7.5–8.5 and one from 8.5–10.

One significant generalization arose out of the studies with 50 μM solutions, i.e., the variability of the numbers of amino groups in a subset. As an example, within the pH range 7.5 to 8.5, the numbers of amino groups in subsets 1 and 2 decreased with increasing pH, in subsets 3 and 4 they increased with increasing pH, and in subset 5 there was no change. If the reasonable assumption is made, that changes in the properties of amino groups represent changes in the vicinity of these groups, it becomes clear that various regions of the surface of the protein are quite labile.

Equation 1 is an expression which describes a system in which there are two limiting conditions, one of which corresponds to k_{max} and the other to k_{min}. One is tempted to correlate these with "states" of RNAse. We have not, as yet, any clear picture concerning the nature of such concentration-dependent states. It surely does not correspond to any association phenomenon since all the evidence indicates that, at the higher concentrations at which all previous studies had been made, RNAse was shown to be a single molecule of molecular weight near 13 700. Indeed, Andrews (*6*) demonstrated that, although several proteins showed concentration effects on gel column chromatography, RNAse did not. We compared the chromatographic behavior of 5 and 50 μM solutions of RNAse on Sephadex G-75 and G-100 and could see no difference. This concentration effect does not stand alone since parallel effects have been reported for RNAse by Sasakawa and Walter (*7*) and by Bernfield and Rottman (*8*). The former authors showed that partition coefficients fell sharply with increasing protein concentration to 8 μM solutions and then rose slowly. The latter authors reported a marked concentration effect on the ability of the enzyme to catalyze the formation of oligonucleotides from simpler molecules, with the largest variations occurring between about 1 and 3 μM RNAse. It is clear that at relatively low concentrations of RNAse there are concentration effects which apparently arise from effects on the surface of the protein.

Possible explanations for the effects of concentration may lie in the assumptions that, at limiting concentrations: (1) association complexes may form which have very short lifetimes, or (2) that intermolecular forces exist which may alter surface structures on the protein, or (3) that similar forces may affect the environment near the protein, e.g., "bound water and ionic shields." In any case, the data on the variations of the properties of the amino groups with pH (*4*) and concentration of proteins emphasize the relative flexibility of the molecule, i.e., its ability to show local fluctuations wthin the limits of constraint set by the primary structure.

The experiments with OxRNAse were incorporated in order to determine how much of the concentration-dependent effect may have been determined by the "native" structure. It is clear that the "concentration effect" is operative since the properties of most of the amino groups in 50 μM solutions differ greatly from those in 5 μM, e.g., in regard to the number of groups which showed biphasic pH–pK relationships and to the values of pK. It would appear then that the effect appears to be somewhat independent

of the gross structure of the molecule and may, in fact, be a general intermolecular reaction which is not easily defined at this time.

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REFERENCES

1. A. R. GOLDFARB, *Bioorg. Chem.* **3**, 249 (1974).
2. A. R. GOLDFARB, D. N. BUCHANAN, AND D. F. SUTTON, *Bioorg. Chem.* **3**, 260 (1974).
3. C. TANFORD AND J. D. HAUENSTEIN, *J. Amer. Chem. Soc.* **78**, 5287 (1956).
4. A. R. GOLDFARB AND P. D. MARTIN, *Bioorg. Chem.* **5**, 137.
5. R. D. CARTY AND C. H. W. HIRS, *J. Biol. Chem.*, **243**, 5254 (1968).
6. P. ANDREWS, *Biochem. J.* **91**, 222 (1964).
7. S. SASAKAWA AND H. WALTER, *Biochemistry* **11**, 2760 (1972).
8. M. R. BERNFIELD AND F. M. ROTTMAN, *J. Biol. Chem.* **242**, 4134 (1967).