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Reactions of the Amino Groups of RNAse A

III. The Existence of pH-Dependent Values of p*K*

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The rates of the trinitrophenylation of the amino groups of ribonuclease A (RNAse) with the specific reagent trinitrobenzene sulfonic acid have been studied at 27°C, between pH 7.0 and 9.9. From the variation of the velocity constants with pH it has been shown that the reaction is biphasic in the sense that for each amino group two p*K*s have been found: one (p$K = 7.3$–7.52) in the range of pH between 7.0 and 8.3 and the other (p$K = 9.28$–9.69) in the pH range 8.5–9.9. It is pointed out that when the experimental conditions approached one another, there was agreement between the p*K* values obtained from titrimetric and kinetic studies. Evidence is presented from the literature concerning the validity of the p*K* value near 7.5 for the ε-amino groups in RNAse. The studies were repeated with performic acid oxidized RNAse and the 10 ε-amino groups were found to be monophasic with p*K* values between 8.01 and 8.10. The α-amino group of the N-terminal lysine was biphasic with a p*K* of 7.26 (pH range 7–8) and 8.13 (pH range 8.2–9.5).

INTRODUCTION

Kinetics of the reactions of the amino groups of proteins have been under study in this laboratory in the hope that the procedure would serve as a specific probe of changes in the microstructure of proteins in the regions of these groups (*1*). The reagent which was used for the study was trinitrobenzene sulfonic acid since it did not react with any other functional group in proteins. From the study with human serum albumin it was apparent that the amino groups of this protein could be grouped into several subsets each of which was described by an average velocity constant and which contained one or more amino groups (*2*). It was further demonstrated that the reactivities of the groups were very sensitive to pH and temperature changes and showed marked, and unexpected, effect of urea (*2*).

In order to correlate, if possible, the properties of the amino groups with the structure of a protein we chose to continue with a study of RNAse A since its structure and properties were well known. It had been shown that amino groups reacted with TNBS[3] in the unprotonated form and it was possible to associate each subset with a dissociation

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[3] Abbreviations: RNAse, ribonuclease A; OxRNAse, oxidized RNase; k_i, velocity constants of *i*th subset; k_{oi}, intrinsic velocity constant of *i*th subset; K_i, dissociation constant for protonated amine in *i*th subset; TNBS, trinitrobenzene sulfonic acid; TEA, triethanolamine.

constant, K, from a study of the variation of the velocity constant, k, with pH by the use of Eq. [1].

$$k = k_0 K/([H^+] + K) \quad [1]$$

Finally a procedure was developed for assigning values of K and the intrinsic constants k_0 to specific amino groups in RNAse by comparing the degree of substitution which had been calculated from velocity constants of the subsets with those determined experimentally by an isolation procedure (*3*). In these studies it was determined that the pK values for the amino groups were in the range 7.9 to 8.32 (*4*) and these were somewhat lower than the values near 10.2 which had been reported from titration data (*5*).

In the reactions of simple primary amines, amino acids, and peptides with TNBS, the kinetic studies resulted in values of pK which were in good agreement with those reported from titration data. Therefore, the low values could not have been attributed to some unrecognized property of the reactions of TNBS with amino groups. On the other hand, our studies were performed at 16°C compared to 25°C for the titration studies. A further consideration was the fact that, in order to obtain suitable titration data for the determination of a pK near 10.2, it would have been necessary to use information obtained above pH 9, whereas we had no data above pH 8.6. Since it had been shown that the reactivities of the amino groups of human serum albumin were very sensitive to both pH and temperature (*2*), we felt that the discrepancy noted above may have been due to the difference in conditions. We have therefore continued the studies at 27°C and have extended the pH range to a region near 10. We have found that the pK was, indeed, very sensitive to a pH range; i.e., the variation of pK with pH was "biphasic" in the sense that two values of pK were derived, one near 7.5 and the other near 9.6. The former held only in the pH range 7.3 to 8.3 and the latter between 8.5 and 9.9.

In view of the unexpected biphasic behavior of native RNAse we thought it would be useful to compare it with an "unstructured" molecule. For this purpose we chose performic acid oxidized RNAse. Under the identical conditions which had been used for native RNAse we found that OxRNAse showed a biphasic property for only the α-amino group of the N-terminal lysine. All of the ε-amino groups had essentially the same pK (8.01–8.13) over the same pH range.

MATERIALS AND METHODS

RNAse A was obtained as a phosphate-free product from Worthington Biochemicals. Although it contained less than 5% polymers, it was completely depolymerized by heating stock solutions (400–800 μM) in buffer at pH 6.5 at 60–65°C for 10 min. Protein concentrations were determined at 280 nm ($\varepsilon_{\text{molar}} = 9781$). Oxidized RNAse was prepared by performic acid oxidation of RNAse as described by Hirs (*6*).

Trinitrobenzene sulfonic acid was purchased from Nutritional Biochemicals, Inc., and recrystallized from 5 M HCl. This reagent is specific for primary and secondary amines. Triethylamine contained traces of primary and secondary amines which were removed by repeated reaction with TNBS and distillation. All other chemicals were c.p. grade.

The methods for making the study have been described (*1–4*). The rates of trinitrophenylation of the amino groups were followed by continuously recording the changes of absorbance with time at 345 nm. The concentrations of RNAse and OxRNAse were between 4.5 and 5.7 μM and of TNBS between 1700 and 2300 μM. Anionic buffers such as phosphate and bicarbonate interact with RNAse and these were avoided. Triethanolamine was used as a buffer between a pH of 6.5 and 9.0 and triethylamine at pH between 9 and 10. At 0.1 M concentrations of buffer the pH at the end of the reactions was within 0.02 unit of the starting pH.

All of the experiments were performed at 27 ± 0.1°C.

RESULTS

As was indicated in the Introduction, the amino groups react with TNBS in nonprotonated form and k_i should vary with $[H^+]$ according to a modified form of Eq. (1), i.e., $k_i[H^+] + k_i K = k_0 K$. A least-squares plot of k_i vs $k_i[H^+]$ is linear with a slope of $-K$, and $k_0 K$ is the value of $k_i[H^+]$ where $k_i = 0$. The lines in all of the figures were "least-squares lines." When the values of k_i were calculated from k_{0i} and K and were used along with the experimentally determined numbers of reactive groups, n_i, the reconstructed kinetic curves fit the experimental curves within 3%.

The results of the studies with the model compounds di- and triglycine (for the α-amino group) and α-acetyl lysine (for the ε-amino group) are summarized in Fig. 1

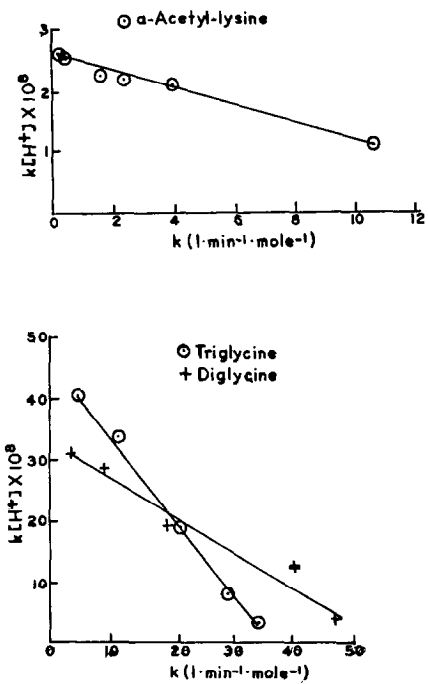

FIG. 1. Reaction of model compounds with TNBS. (+) G_2; (⊙) G_3; (⊙) α-acetyl lysine.

TABLE 1

KINETIC CONSTANTS OF MODEL COMPOUNDS

Compound	pK	k_0	pK (titration)[a]
G_2	8.21	54	8.13
G_3	7.9	36	7.91
AcLys	8.9	20	9.4–10.6

[a] See Refs. *7*, *8*, and *9*.

and Table 1. All studies were carried out in the pH range 7.0 to 9.5, over which all the model compounds gave good linear plots. From these data, values of pK and k_0 were calculated giving the values indicated in Table 1. The values of pK of the α-amino groups which had been calculated from the kinetic studies agreed very well with those reported from titration data. On the other hand, the pK of 8.9 for the ε-group of α-acetyl lysine is a bit lower than values for ε-amino groups of lysine peptides (*7–9*). The experimental values of k, at all pH values, were within 1% of the values calculated by the use of Eq. [1].

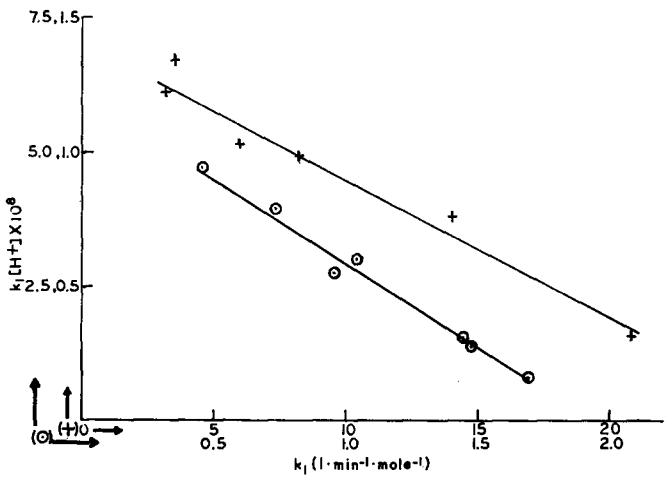

FIG. 2. Reaction of RNAse with TNBS. Subset 1: (⊙) pH 7.0–8.31; (+) pH 8.48–9.92.

The results of the studies with native RNAse solutions are summarized in Figs. 2–4 and the calculated values of pK and k_0 for the amino groups are given in Table 2. The plots of k vs $k[H^+]$ were linear in two portions, one of which held in the pH range from approximately 7 to 8.3 and the other from 8.45 to 9.92 for subsets 1, 2, and 3. More precisely, in the lower pH range, the linearity held down to pH 7.0 only for the first subset, whereas it held down to pH 7.3 for subsets 2 and 3. The calculated values of pK for each of the amino groups were close to each other (7.3–7.5). On the other hand, the intrinsic constants, k_0, differed greatly. In the pH region above 8.3 the values of pK for all the amino groups were near 9.5 and, again, with relatively large differences

TABLE 2

KINETIC CONSTANTS OF THE AMINO GROUPS OF RNAse

Subset	pK (k_0)	Amino groups[a]
pH range 7.0–8.31		
1	7.50 (1.9)	31, 66, 1:23, 6:40
2	7.30 (11.0)	7, 61, 104
3	7.52 (76)	1α, 98
4	—[b]	41
pH range 8.45–9.92		
1	9.28 (87)	1:23, 6:40
2	9.69 (380)	7, 31, 98, 104
3	9.60 (1850)	1α, 61
4	—[b]	41

[a] The whole numbers represent the positions of the lysine residues containing these ε-amino groups. 1α represents the α-amino group of the N-terminal lysine. 1:23 and 6:40 are the coordinates for TNP-peptides for which structures are not determined unequivocally. The assigned constants all belonged to the slowest reacting groups and therefore were assigned to the first subsets. See Ref. *4*.

[b] The velocity constants were very large and at pH above 7.5 could not be obtained. It was therefore not possible to calculate the values of pK and k_0.

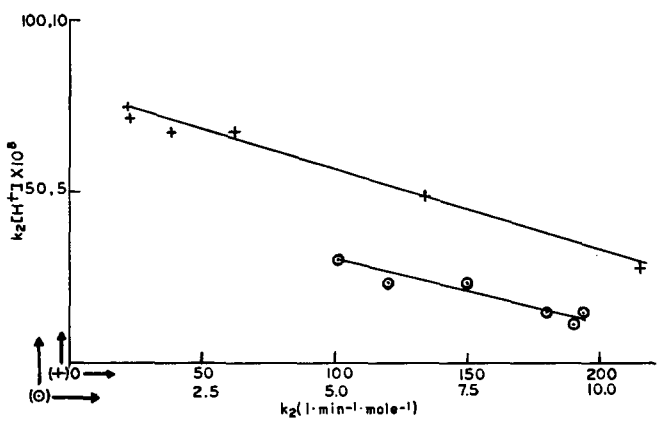

FIG. 3. Reaction of RNAse with TNBS. Subset 2: (⊙) pH 7.25–8.31; (+) pH 8.48–9.92.

of k_0. It will be noted that the amino group of Lys-41 was in subset 4, which was assigned no constants since its rate of reaction was too large at and above a pH of 7.5. In view of the importance of Lys-41 in the enzymic activity of RNAse we are examining this further. The experimental values of k_1 for the first subset were within 2% of those

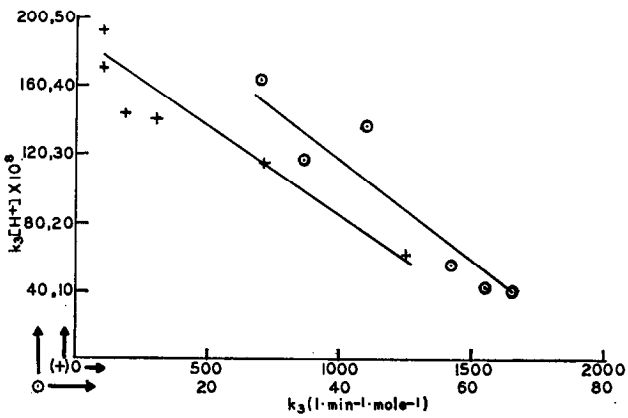

FIG. 4. Reaction of RNAse with TNBS. Subset 3: (⊙) pH 7.25–8.31; (+) pH 8.48–9.92.

calculated from K and k_0. Those for subsets 2 and 3 were within 5 and 7% of the calculated values, respectively.

The kinetic runs for OxRNAse are summarized in Fig. 5 and in Table 3. Three subsets were obtained of which only the third subset showed two pH ranges in which two different values of pK were obtained (Table 3). The most reactive group (subset three) was Lys-1α which had pKs of 7.26 and 8.13 in the pH ranges 7–8 and 8–9.5, respectively. In general, the value of k_0 were somewhat smaller than those in native RNAse. All of the other groups had essentially the same pK values (8.01–8.10) which were associated with varying k_0 (0.06–16.4).

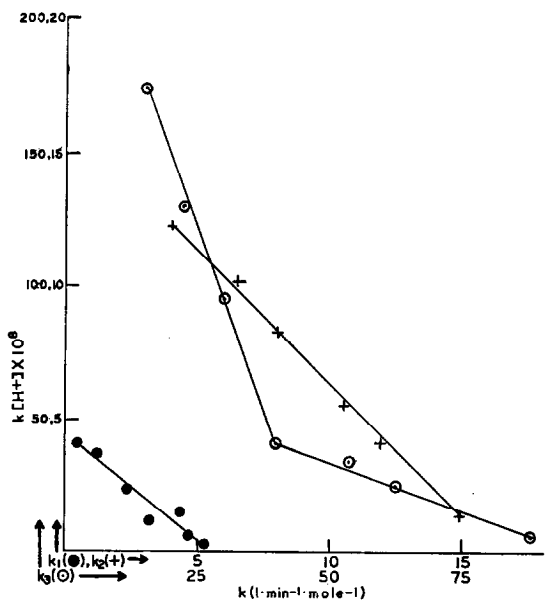

FIG. 5. Reaction of OxRNAse with TNBS. Subset 1: (●); subset 2: (+); subset 3: (⊙).

TABLE 3

THE CONSTANTS OF THE AMINO GROUPS OF OxRNAse[a]

Subset	pK (k_0)	Amino groups
pH range 7–8		
1	8.10 (0.06)	7, 41, 66, 98, 104, 1:23, 4:37
2	8.01 (16.4)	31, 61
3	7.26 (48)	1α
pH range 8.2–9.5		
1	8.10 (0.06)	66, 1:23, 4:37, 6:40
2	8.01 (16.4)	7, 31, 41, 98, 104
3	8.13 (101)	1α

[a] For explanation see Table 2.

DISCUSSION

In the Introduction it has been suggested that the relatively low pK values which were obtained at 16°C with 5 μM solutions of RNAse (*4*) may have arisen from differences in the temperature and protein concentrations which had been used in our study, as compared with those used in the titrimetric experiments. We further considered the need for extending our studies to higher pH ranges. For the moment, we shall consider only the data obtained in the pH range 8.5 to 9.9, since this is the only region from which titrimetric data could have been obtained to give a pK of 10.2. In this case, the pK value of 9.6 approached the titrimetric value. The final proof of the validity of our conjecture will be found in the companion report (Part IV) where it will be shown that in a higher pH range and in 50 μM RNAse, pKs of 9.9 and 10.4 are found. Considering the approximations made in fitting the titration curves of proteins (*10*) the agreement is reasonably good.

We are faced with the fact that a pK of 7.5 for the 10 ε-amino groups of RNAse cannot be used to match the titration curves reported by Tanford and Hauenstein (*5*) or its associated isoelectric pH near 9.5. Contrariwise, there is a body of data which is certainly inconsistent with the titrimetric values of pR and pI cited above. Thus, Barnett and Bull (*11*) and Sasakawa and Walter (*12*) reported isoelectric regions, rather than points. The former gave a region between pH 8.2 and 9.6 and the latter between pH 7.2 and 9.6. The kinetic pK of 7.5, while not consistent with the titration data, is necessary for the lower range of isoelectric pH values obtained from electrophoretic and distribution studies.

It is to be finally noted that Rasper and Kauzmann (*13*) suggested that anomalous specific volume changes with pH could be explained on the assumption of pK values for the ε-amino groups of RNAse much smaller than the value assumed from titrimetric data. From these considerations we suggest that both of the values of pK have equal validity under the appropriate experimental conditions.

The existence of two "forms" or "states" for RNAse, with a transition near pH 6 has been suggested (*14*, *15*). In our studies at both 16 and 27°C there appeared sharp

deviations in the behavior of amino groups at pH values below pH 7.3 and we feel that this is in agreement with the other evidence for the existence of a transition in this region. By the same token, the sharp difference of properties of the amino groups above and below pH about 8.4 suggests still another transition between two states. From ORD studies (*16*) and measurements of optical rotation (*17*) there is no indication of large conformational changes corresponding to this "transition." It would seem that the changes with pH may be due to small alterations in the environments of the amino groups which may involve either microspatial displacements, local alterations of bound water or such changes in environment which lead to local variations of the concentrations of TNBS, or a combination of these.

It has been reported by Harrington and Sela (*18*) that, contrary to the structured configuration of native RNAse, OxRNAse had a random chain configuration. The fact that all of the ε-amino groups in OxRNAse are monophasic whereas the same groups were biphasic in native RNAse suggested that the sensitivity of the amino groups in RNAse was related to the existence of the secondary and tertiary structures of the folded protein. The reverse conjecture, that the existence of a biphasic reaction may be associated with structures of preferred orientation would lead to the possible conclusion that the N-terminal end of OxRNAse has some preferred structure. This might serve as a rationale to explain the directed combination or S-protein with S-peptide to give an active enzyme. It also would serve to explain the importance of the N-terminal end in the aggregation of the protein (*19*).

Note added in proof. One of us (P. D. M.) has crystallized RNAse A and RNAse S from ammonium sulfate-cesium chloride at pH 9 in a form suitable for structure determination by X-ray crystallography. The space group is $P3_121$ and both are isomorphous with RNAse S crystallized at pH 6.5. Initial crystallographic data at 6 Å resolution indicate there are intensity changes between the low and high pH crystal forms which cannot be accounted for by the break between residues 20 and 21 of RNAse S (paper submitted April 1976).

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REFERENCES

1. A. R GOLDFARB, *Biochemistry* **5**, 2574 (1966).
2. A. R. GOLDFARB, *Biochim. Biophys. Acta* **200**, 1 (1970).
3. A. R. GOLDFARB, D. N. BUCHANAN, AND D. F. SUTTON, *Bioorg. Chem.* **3**, 260 (1974).
4. A. R. GOLDFARB, *Bioorg. Chem.* **3**, 249 (1974).
5. C. TANFORD AND D. HAUENSTEIN, *J. Amer. Chem. Soc.* **78**, 5287 (1956).
6. C. H. W. HIRS, *J. Biol. Chem.* **219**, 611 (1956).
7. C. LONG (Ed.), "Biochemists' Handbook," p. 45 ff. Van Nostrand, Princeton, N.J., 1961.
8. D. D. PERRIN (Ed.), "Dissociation Constants of Organic Bases in Aqueous Solutions." Butterworths, London.
9. E. J. COHEN AND J. T. EDSALL (Eds.), "Proteins, Amino Acids and Peptides," p. 445. Rheinhold, New York, 1943.
10. C. TANFORD, "Physical Chemistry of Macromoles," p. 554 ff. Wiley, New York, 1965.
11. L. B. BARNETT AND H. B. BULL, *Arch. Biochem. Biophys.* **89**, 167 (1960).
12. S. SASAKAWA AND H. WALTER, *Biochemistry* **11**, 2760 (1972).

13. J. RASPER AND W. KAUZMANN, *J. Amer. Chem. Soc.* **84**, 1771 (1962).
14. T. C. FRENCH AND G. G. HAMMES, *J. Amer. Chem. Soc.* **87**, 4669 (1965).
15. E. A. BARNARD, *Annu. Rev. Biochem.* **38**, 720 (1969).
16. R. E. CATHOU, G. G. HAMMES, AND P. R. SCHUMMEL, *Biochemistry* **4**, 2687 (1965).
17. B. JIRGENSONS, *Arch. Biochem. Biophys.* **74**, 70 (1958).
18. W. F. HARRINGTON AND M. SELA, *Biochim. Biophys. Acta* **31**, 427 (1959).
19. A. M. CRESTFIELD, W. H. STEIN, AND S. MOORE, *Arch. Biochem. Biophys. Suppl.* **1**, 217 (1962).