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Reactions of the Amino Groups in Ribonuclease A
II. Identification of Reactive Amino Groups in Ribonuclease

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Ribonuclease A has been trinitrophenylated to varying degrees by reaction with trinitrobenzenesulfonic acid. The reactive amino groups were identified by use of the peptides obtained from the oxidized TNP–RNase by tryptic and chymotryptic hydrolysis. From a quantitative study of the TNP-peptides it was possible to associate each amino group with values of pK_a. It was shown that the lys-41 amino group had a pK_a of 9.03 in TEA buffer. The pK_a values of all of the other amino groups were dependent on the nature of the buffer (triethanolamine and phosphate) and on the pH.

INTRODUCTION

In the previous paper (*1*) it was shown that the kinetics of the reactions of the amino groups of RNase A were dependent on pH, the nature of the buffer, and the concentration of protein. In this report the reactive amino groups are identified and the kinetic parameters are assigned to them.

EXPERIMENTAL SECTION

Materials. Phosphorus-free bovine ribonuclease A (RAF) was obtained from Worthington Biochemical Corp. Trypsin, 2× crystallized, was obtained from Sigma Chemical Co. Alpha chymotrypsin 3× crystallized, TNBS,[1] and dansyl chloride were obtained from Nutritional Biochemicals, Inc. Silica Gel G was obtained from Brinkmann Instruments. All other chemicals were C.P. grade.

Methods. The procedure for the study consisted of the isolation and quantification of the TNP peptides obtained from TNP-RNase which has been prepared under precise conditions so that the relative amount of each TNP peptide could be compared with the amount calculated from the kinetic data (*1*).

Preparation of TNP-RNase in phosphate and TEA buffer. The conditions for the preparation of trinitrophenylated RNase paralleled the kinetic experiments (*1*), except that

[1] *Abbreviations.* RNase = ribonuclease A, TNBS = trinitrobenzenesulfonic acid, TNP = trinitrophenyl, TEA = triethanolamine, TNP–RP = trinitrophenyl ribonuclease prepared in phosphate buffer, TNP–RT = trinitrophenyl ribonuclease prepared in TEA buffer, TCA = trichloroacetic acid, R = ratio of the amount of TNP groups in a peptide spot to the total, DNS = (*dansyl*) = 1-dimethylamino naphthalene-5-sulfonyl group.

they were prepared in larger volumes (up to 1 liter). In view of the results of the kinetic studies, the preparations were made using both 0.1 *M* phosphate and 0.1 *M* TEA buffers at pH values of 7.0 and 8.0. The concentrations of RNase were 5 μM for analytical studies and 50 μM for the preparative studies. The concentrations of TNBS were about .002 *M* for the 5 μM preparations. Aliquots were withdrawn at suitable time intervals and the reactions were stopped by the addition of 1 ml of concentrated formic acid per 100 ml of reaction mixture. Ten grams of TCA were added to each 100 ml of the acidified reaction mixtures and, after centrifugation, the precipitates were transferred into 15-ml Pyrex centrifuge tubes and washed free of TNBS with 10% TCA. The precipitates were washed with an ethanolic–ether solution (1:4) and were dried in vacuo.

Oxidation and hydrolysis of trinitrophenylated RNase. The dried precipitates were dissolved in 97–100% formic acid and were oxidized with performic acid (*2*). At this stage over 95% of the TNP groups were recovered. The oxidation mixtures were diluted with water, frozen, and lyophylized. The solids were dissolved in 0.1 *M* $(NH_4)_2CO_2$ and the pH was adjusted between 7.3 and 7.5. The mixture was digested at 37°C for 45 min, first with trypsin (1% w/w) and then with α-chymotrypsin (1% w/w) (*3*). The digestion mixtures were lyophylized. For studies of the *N*-terminal end of the TNP–RNase, some samples of TNP-RNase were reacted with CNBr (*4, 5*) and the fragments were separated on Sephadex G 25 columns with 0.2 *M* acetic acid as the eluting solution. The fragments obtained from CNBr treatment were oxidized and hydrolyzed as previously described for TNP–RNase.

Separation of TNP–peptides. For quantitative analyses of the relative amounts of TNP groups in the peptides, the solid peptide mixture was dissolved in 50% formic acid since this was the only solvent that completely dissolved the solid at high concentrations. The mixture was spotted on Whatmann 3 MM paper saturated with 50% formic acid (pH = 0.6). Electrophoresis was performed at 4°C at a field strength of 7 V per cm for 15–18 hr or at a field strength of 14 V per cm for 7–9 hr. At this stage three strong yellow spots appeared as did one moderately colored and one faint spot. After drying at room temperature, chromatography was performed at right angles using the organic phase of a mixture of *n*-butanol + acetic acid + water (4:1:5). The paper was dried overnight at room temperature and the R_f values were measured. Each spot was assigned a "coordinate" value of the form a.b where "a" was the electrophoretic position and "b" was the calulated R_f. The values of "a" were integers between "1" and "5", where "1" is closest to the anode and "5" closest to the cathode. The yellow spots were cut out, as were adjacent "blanks," and were extracted with 2.5 ml 50% formic acid by standing overnight. The solutions were read at 345 nm. The absorbances of the blanks were usually close to the absorbance of 50% formic acid. The amount, in nanomoles, of TNP-peptide which had been extracted from any given spot was determined by multiplying the absorbance of the extract by 172. The absorbances were in the range of .003–.06 (.5–10 nmoles) and, of course, the smaller values were less reliable.

In order to prepare larger amounts of the TNP-peptides for studies of structure it was found convenient to remove the large mass of unsubstituted peptides by treatment with acid-washed talc in the manner described for use with DNP-peptides (*6*). The recovery of TNP-peptides from the talc treatment was between 90% and 95%. The TNP-peptides were separated from the residual, small amounts of nonsubstituted peptides by repeated HVE in 50% formic acid. Final separation was accomplished by

chromatography on Whatmann 3 MM paper using the organic phase of a mixture of *n*-butanol + acetic acid + water (4:1:5) as the developing solution.

Identification of TNP-peptides. The yellow peptides were generally identified by a combination of N-terminal amino acid determination and qualitative amino acid analysis. The peptides were dansylated (*7*) and the reaction mixture was separated by chromatography on Whatmann 3 MM paper using *n*-butanol + acetic acid + water (8:2:2) as the developing solution. In each case a single yellow fluorescent peptide traveled further than the blue fluorescent dimethyl-amino-naphthalene sulfonic acid which arose from hydrolysis of the danysl chloride. The DNS–TNP peptides were extracted from the paper with 50% formic acid, dried in vacuo, and heated with constant boiling HCl in a sealed tube under N_2 at 105°C for 15–18 hr. The dried residues were dissolved in acetone + 0.1 *M* acetic acid (3.2) and developed on tlc plates coated with .25-mm layers of Silica Gel G. The developer solvents were $CHCl_3 + CH_3OH + 37\%$ NH_3 (4:4:2) in the first dimension and, at right angles, *n*-butanol + acetic acid + water (8:2:2) in the second dimension. The yellow fluorescent spot was marked and the plate was sprayed with ninhydrin to develop the free amino acids.

It was frequently observed that TNP–peptide 5.25, and only this peptide, turned from yellow to orange upon standing on paper. It had been observed earlier that TNP–glycine showed a similar change, and the suspicion arose that a TNP group in the α-position was unstable. Experiments with TNP–diglycine and triglycine, ε-acetyl-α-TNP lysine and TNP–leucine showed similar color changes on paper whereas ε-TNP-α-acetyl lysine did not. We were, therefore, led to suspect that peptide 5.25 was an α-substituted derivative or the N-terminal peptide of RNase. Dansylation and hydrolysis of this peptide gave only ε-dansyl–lysine and the amino acids lys, glu, thr, and ala. This corresponded to the peptide lys 1–lys 7. This was confirmed by treating TNP–RNase with CNBr and separating the small peptide. This small peptide, on acid hydrolysis gave the same amino acids as did peptide 5.25 plus cysteic acid and asp. Finally, hydrolysis of the small CNBr peptide with trypsin yielded peptide 5.25. Acid hydrolysis of an α-TNP–lysine derivative resulted in a complete split of the TNP group from lysine whereas, with the ε-TNP derivative, there was only a partial split. These data were consistent with the conclusion that this peptide contained the TNP group on the α-position of lys-1.

Peptide 2.42 was also present in relatively large amounts (Tables 2 and 3). This peptide had an N-terminal cysteic acid group for which only two TNP-peptides were possible, i.e., cys 26–lys 31 and cys 40–phe 46. The other theoretical possibility was the dipeptide cys 40–lys 41 which, however, does not occur since it is known that trypsin does not cleave at the lys-41 position of oxidized RNase (*3*). Qualitative amino acid analysis showed the presence of pro and val both of which are present in cys 40–phe 46 but not in cys 26–lys 31, therefore, this peptide was substituted on the ε-amino group of lys-41. Six of the remaining possible peptides were identified by means of the N-terminal group and the constituent amino acids. The results are incorporated in Table 1.

The conclusions concerning the nature of the TNP-peptides were based, in part, on the work of Anfinsen et al. (*3*) and the specificity of action of the enzymes. It well known that substitution of the ε-amino groups of the lysine side chain, blocks the specific hydrolysis at this residue and this fact was taken into consideration in judging which the TNP-peptides were formed. However, the theoretical possibility exists that the presence of TNP-groups may alter the enzyme specificities. However, this did not seem likely since,

in the first place, hydrolysis of the TNP-protein gave only such ninhydrin reacting spots as were present in the unsubstituted protein. In the second place, when the lys-41 was completely reacted, Anfinsen's peptide 9 disappeared and similarly for lys-1 α and Anfinsen's 3b. This was as was to be expected.

RESULTS AND DISCUSSION

There are 11 amino groups present in RNase from which it is possible to theoretically account for 14 TNP peptides, at the most. As is shown in Table 1, there have been distinguished 12 spots of varying intensity. During the fractionation of the peptide mix-

TABLE 1

TNP-PEPTIDES ISOLATED FROM OXIDIZED TNP-RNase BY TRYPTIC AND CHYMOTRYPTIC HYDROLYSIS

Coordinate	Structure of TNP-peptide[a]
2.42	cys 40—(T41)—phe 46
5.25	lys 1—(T1α)—lys 7
3.56	lys 1—(T1α, T7)—phe 8
2.80	cys 26—(T31)—arg 33
3.16	val 47—(T61)—lys 66
2.27	val 47—(T61, T66)—tyr 72
5.35	lys 98—(T98)—lys 104
3.40	thr 99—(T104)—tyr 115
4.49	—[b]
1.37	—[b]
3.22	—[b]
3.08	—[b]

[a] The structure is indicated as being the peptide present in RNase. The numeral after T represents the position of substitution.

[b] These peptides were not separated in adequate amounts for structure determination.

ture, it was not possible to obtain sufficiently large amounts of the pure TNP peptides in spots 1.37, 3.08, 3.22, and 4.49 for studies of structure. On the other hand, it was difficult to discern the peptide at spot 2.27 on the maps, but it was obtained in this position during the preparative runs.

A correlation between the ratio (R) of the amount of TNP group in a given spot to the total amount present on the map with the values to be expected from kinetic data (α/n_T) would make it possible to place the amino groups into a subset and thus identify the intrinsic constants for each amino group. The relative amount of the TNP group ex-

tracted from each spot in the analytical maps depended on the fraction of the group which had reacted, $\alpha_i = (1 - e^{-k_i t T})$, where k_i is the velocity constant for the ith subset, T is the concentration of TNBS, and t is the time. If this fraction is multiplied by the total number of amino groups in the subset, the result represents the number of amino groups of this subset which had reacted. The summation of the number of reacted groups over all the subsets is $\sum n_i \alpha_i = n_T$. From these figures it is possible to obtain a fraction α_i/n_T and this can be compared to the fraction R.

TABLE 2

VARIATION OF R^a WITH THE DEGREE OF SUBSTITUTION AT pH 7.0

	TNP–RP peptides			TNP–RT peptides		
Spot no.	$n_T{}^a = 1.59$	$n_T{}^a = 2.17$	$n_T{}^a = 2.70$	$n_T{}^a = 1.00$	$n_T{}^a = 2.10$	$n_T{}^a = 3.08$
1.37	None	None	None	None	None	0.03
2.80	0.04	0.02	0.05	0.04	0.02	0.03
2.42	0.27	0.35	0.33	0.59	0.50	0.34
3.56	0.07	0.16	0.19	—[c]	0.10	0.19
3.40	0.04	0.05	0.06	0.04	0.05	0.03
3.16	0.01	0.03	0.03	—[c]	—[c]	0.02
4.49	—[c]	—[c]	0.02	0.01	—[c]	Trace
5.35	0.05	0.03	0.05	0.07	0.05	0.16
5.25	0.50	0.40	0.28	0.25	0.30	0.21
Subset		$\alpha/n_T{}^b$			$\alpha_1/n_T{}^b$	
1	0.03	0.04	0.06	0.006	0.01	0.03
2	None	None	None	0.05	0.07	0.18
3	0.26	0.31	0.31	0.34	0.38	0.33
4	0.56	0.46	0.37	0.59	0.50	0.33

[a] cf. Discussions.
[b] cf. Discussion. The calculations were made from the kinetic constants obtained in Ref. 1.
[c] Not seen.

The values of R, as they changed with increasing time, i.e., increasing n_T, was recorded in Tables 2 and 3. The values of α/n_T were calculated for the different subsets and placed at the bottom of each table. There was very good agreement between R for the lys-41 amino group (peptide 2.42) under all conditions. The values of R for the α amino group of lys-1 (peptide 5.25) ran parallel to, but smaller than α/n_T in all cases except for the reaction in TEA buffer at pH 8.0. The smaller values are accounted for, in the main, by the isolation of peptide 3.56 which contained substituents on the α amino group of lys-1 and on lys-7. The larger values were not as easily accounted for except by assuming the transient occurrence of a TNP peptide which confused the analyses, the transient nature of which was indicated by a reversal of the relationship at longer time intervals.

Examination of the other peptides show that the amino groups in peptides 2.80, 2.27, 3.40, 3.16, and 4.49 all belong in the first subset under all conditions. Peptide 3.56 has values of R nearly twice that for α_2/n_T in almost all the experimental conditions which

TABLE 3

VARIATION OF R^a WITH THE DEGREE OF SUBSTITUTION AT pH = 8.0

	TNP–RP peptides			TNP–RT peptides			
Spot no.	$n_T{}^a = 1.75$	$n_T{}^a = 2.62$	$n_T{}^a = 4.3$	$n_T{}^a = 1.93$	$n_T{}^a = 2.53$	$n_T{}^a = 2.96$	$n_T{}^a = 4.73$
2.80	—[c]	—[c]	0.04	0.005	0.01	0.03	0.065
2.42	0.50	0.40	0.23	0.53	0.47	0.35	0.26
3.56	0.06	0.17	0.25	0.07	0.12	0.19	0.23
3.40	—[c]	0.15	0.14	0.07	0.03	—[c]	0.07
3.22	0.03	0.02	0.01	—[c]	0.02	0.06	0.06
3.16	0.01	0.03	0.06				
3.08	—[c]	—[c]	0.04	—[c]	—[c]	—[c]	0.07
4.49	—[c]	—[c]	0.03	0.06	0.04	0.06	0.10
5.35	0.04	0.03	0.04	0.06	0.05	0.05	0.05
5.25	0.28	0.23	0.16	0.25	0.29	0.23	0.14
Subset		$\alpha_i/n_T{}^b$			$\alpha_i/n_T{}^b$		
1	0.01	0.02	0.04	0.007	0.01	0.01	0.04
2	0.05	0.08	0.13	0.04	0.06	0.07	0.15
3	0.26	0.32	0.23	0.20	0.25	0.26	0.21
4	0.57	0.38	0.23	0.52	0.40	0.34	0.21

[a] cf. Discussion.
[b] cf. Discussion. The calculations were made from the kinetic constants obtained in Ref. 1.
[c] Not seen.

TABLE 4

VALUES OF pK_a FOR THE AMINO GROUPS OF RNase

Amino group	P-7[a]	P-8[a]	T-7[a]	T-8[a]
1α	8.13	8.56	X[b]	8.32
7	X[b]	8.64	X[b]	7.90
31	X[b]	8.13	X[b]	8.15
41	8.56	8.13	9.03	9.03
61	X[b]	8.13	X[b]	8.15
66	X[b]	8.13	X[b]	8.15
98	X[b]	8.13	X[b]	7.90
104	X[b]	8.64	X[b]	8.15
37	Not isolated			
91	Not isolated			
1ε	Not isolated			

[a] P-7 is value in phosphate buffer at pH 7; P-8 is value in phosphate buffer at pH 8; T-7 is value in TEA buffer at pH 7; T-8 is value in TEA buffer at pH 8.
[b] X stands for the fact that no pK_a could be derived under these conditions, cf. Discussion.

is what it should have for a disubstituted peptide. Finally, peptide 3.08 is only apparent at pH 8 in both buffers and 3.13 is only seen at pH = 8 in phosphate buffer. Combining the findings of this paper with the data of the preceding report (1), it is possible to assign values of pK_a to most of the amino groups of RNase and this tabulation is found in Table 4.

If the reasonable assumption is made that the reactivity of the amino groups in a protein (as given by the intrinsic constants) is determined by the immediate environment, the reverse would probably follow, i.e., that an observed change in properties of the group would suggest a change of environment. The evidence presented above shows that the properties of the amino groups are very sensitive to pH changes, with a transition pH range somewhere below pH 7.5. This is true not only of the slowest reacting groups of subsets one and two, but also of the two most reactive groups (lys 1α and lys 41). For example, whereas in TEA buffer the lys-41 amino group reacts uniformly over the whole pH range in subset four, the lys 1α amino group reacts uniformly in subset three only above pH 7. This would suggest that in this buffer the change in pH does not influence the region of the cleft near the active site but does affect the remainder of the molecule. On the other hand, in phosphate buffer, there is a greater sensitivity to pH change of both of these amino groups since, in going from pH 7 to pH 8, lys-41 moved from subset 3 to subset 4 and lys 1α moved from subset 4 and to subset 3, thereby changing their kinetic constants.

A small change in the RNase molecule has been inferred to occur between pH 7.0 and 7.5 (*1*). This did not include any inference for reaction below pH 7.0, where it has been reported there exists a transition state near pH 6 (*10*). If the transition near pH 7 is real, it may be suspected that enzyme studies should show parallel results. In the companion report (*1*), it was shown that between pH 7.0 and 7.5 there is a discontinuity in the pH–enzyme activity curves. Such a change could serve to account, in part, for the marked differences of pH optima for enzyme activity when substrates are changed (*10*).

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