The Carboxymethylation of Crystalline Ribonuclease*

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ABSTRACT: Crystals of ribonuclease A were treated with bromoacetic acid at pH 5.5 in 75% 2-methyl-2,4-pentanediol and the products compared with those of carboxymethylation of ribonuclease dissolved in water. 1-Carboxymethylhistidine-119-ribonuclease and 3-carboxymethylhistidine-12-ribonuclease were obtained in a ratio of 60:1. Carboxymethylation in aqueous solution under comparable conditions of phosphate concentration yields these products in a ratio of 15:1. It is suggested that the alkylation site in the crystals is similar to, and perhaps identical with, that of at least one conformation in solution. 1-Carboxymethylhistidine-119-ribonuclease and 3-carboxymethylhistidine-12-ribonuclease (both prepared by reaction in aqueous solution) were crystallized, the former in the standard space group, and the latter in a different form.

In a previous communication, we reported that the activity of a molecule of crystalline ribonuclease A (polyribonucleotide 2-oligonucleotidotransferase, cyclizing) toward cytidine 2′,3′-phosphate was of the same order of magnitude as that of a molecule of ribonuclease A in aqueous solution (Bello and Nowoswiat, 1965). This evidence, while it suggests that the conformation of the enzyme in the crystal is similar to that in solution, is not sufficient to form any definite conclusions. It is necessary to compare a variety of chemical reactions of ribonuclease dissolved in water with those of crystals in 75% 2-methyl-2,4-pentanediol–25% water (v/v), the medium in which they are equilibrated for crystallographic studies, before any valid statements can be made regarding the similarity of this enzyme in the two states.

A reaction which is well suited for such comparative studies is the alkylation of ribonuclease with bromo- or iodoacetic acid at pH 5.5. The reasons are: (a) the crystals are equilibrated at a pH (5.0) close to that of the reaction prior to their use for crystallographic studies. (b) The reaction was shown to affect histidines involved in the active center of the molecule (Barnard and Stein, 1959; Stein and Barnard, 1959; Gundlach *et al.*, 1959; Crestfield *et al.*, 1963a,b). (c) The products are easily separated by column chromatography.

Experimental Section

Materials

Ribonuclease A was obtained from the Worthington Biochemical Corp., Freehold, N. J., and Mann Research Laboratory, New York, N. Y. Ribonuclease crystals of form II, space group $P2_1$, were used (King *et al.*, 1956). DFP-carboxypeptidase A was purchased from the Worthington Biochemical Corp. Cleland's reagent, dithiothreitol (Cleland, 1964), and trypsin, pretreated with tosyl-L-phenylalanylchloromethane to rid this enzyme of chymotrypsin activity, was purchase from Calbiochem, Los Angeles, Calif. Iodoacetic acid, bromoacetic acid, and 2-methyl-2,4-pentanediol were obtained from Eastman Organic Chemicals, Rochester, N. Y. The acids were crystallized once from chloroform and stored at −40°. The 2-methyl-2,4-pentanediol was purified as described previously (Bello and Nowoswiat, 1965).

The carboxylic acid resin (corresponding to Amberlite IRC-50), Bio-Rex 70, −400 mesh, and Bio-Gel P-2, 100–200 mesh, used for desalting of peptides were purchased from Bio-Rad Laboratories, Richmond, Calif. Bio-Rex 70 was conditioned as described by Hirs *et al.* (1953). Twice recrystallized Tris was purchased from the Sigma Chemical Co., St. Louis, Mo.

Methods

Enzyme assays were performed by a modification of the titrimetric procedure of Davis and Allen (1955), described in the previous communication (Bello and Nowoswiat, 1965).

Amino acid analyses were performed by Dr. A. Grossberg and Mr. R. Chrzanowski with the Technicon Autoanalyzer using a technique which is slightly modified from that outlined in the Technicon Manual. It is possible to resolve 1-carboxymethylhistidine without sacrificing resolution of the other amino acids when their modification is employed. They kindly allow us to describe the procedure which is as follows. The procedure, buffers, and the quantities used are the same as those recommended in the Technicon manual with the exception that gradient chamber 4 of the Autograd[1] contains 40 ml of pH 2.90 buffer and 35 ml of pH 3.80 buffer, instead of the usual 75 ml of pH 3.80 buffer. The hydrolysate is placed on the 6-mm column which has been equilibrated with pH 3.10 buffer. This buffer is pumped for 2 min at a flow rate of 1.42 ml/min before starting the gradient. The 1-CM-histidine[2] peak lies well resolved

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[1] "Gradient for 133-cm column, 6-hr protein hydrolysate analysis," Technicon Instruction Manual AAA-1, p 38.

between alanine and valine.

Hydrolysis of peptides and proteins was done with constant-boiling HCl at 110° for 22 hr. The removal of dissolved oxygen from the acid was accomplished by bubbling nitrogen for 3 min, partially withdrawing the nitrogen bleeder, and then sealing the hydrolysis tube. Removal of the acid was achieved by allowing nitrogen to flow over the hydrolysate which was immersed in a water bath at 35°.

Carboxymethylation of RNase in water at pH 5.5 was done according to the method of Crestfield *et al.* (1963-a–c) with the exception that bromoacetic acid was used for the alkylations and the total reaction time was 4 hr.

Some experiments were also done at 60% RNase concentration with 1 mole of phosphate/mole of protein and a ratio of protein to bromoacetic of 10:1. To 300 mg of ribonuclease A was added to 0.5 ml of bromoacetic acid solution (60 mg/ml in 0.06 M phosphate buffer, pH 5.5). The pH was readjusted to 5.5. After 2 hr, 0.1 ml was withdrawn and added to 1 ml of 0.2 M phosphate (pH 6.45); this was chromatographed on Bio-Rex 70 as described below.

In experiments where the ratio of protein to bromoacetic acid was 30:1, the same procedure was followed with the exception that the concentration of bromoacetic acid solution was 20 mg/ml in 0.06 M phosphate buffer (pH 5.5).

Carboxymethylation of Crystals of RNase in 75% 2-Methyl-2,4-pentanediol at pH 5.5. Reactions were carried out in test tubes containing 10–15 mg of crystals. The crystals were not weighed, but a rough estimate could be made since a 1-mm^3 crystal weighs about 1 mg and consists of about 60% protein. The supernatant, 75% 2-methyl-2,4-pentanediol, was drained from 17 test tubes and 2 ml of bromoacetic acid solution (1 mg/ml in 75% 2-methyl-2,4-pentanediol) (pH 5.5) was added to each test tube; no other pH adjustment was made and no buffer was used. The tubes were covered and allowed to stand in the dark for 24 hr at 25°. The supernatant was then decanted; the crystals were suspended and pooled into one centrifuge tube. The pooled crystals were washed three times with 75% 2-methyl-2,4-pentanediol, by suspending them in the medium each time and centrifuging. The crystals were dissolved finally in 2 ml of 0.2 M phosphate buffer (pH 6.47 ± 0.02) which is the eluent for subsequent column chromatography and which inhibits further alkylation of the enzyme (Crestfield *et al.*, 1963b). One milliliter was placed on a column (0.9 × 150 cm) of Bio-Rex 70 (Na^+) −400 mesh, maintained at 30°, and equilibrated with the phosphate buffer. The flow rate was 30 ml/hr and 5-ml fractions were collected (Crestfield *et al.*, 1963c). Fractions corresponding to peaks 1, 2, and 3 were saved and lyophilized (Figure 1). These were desalted on a column (3.4 × 70 cm) of Sephadex G-25, medium grade, using 0.2 N acetic acid as the eluent. The amount recovered

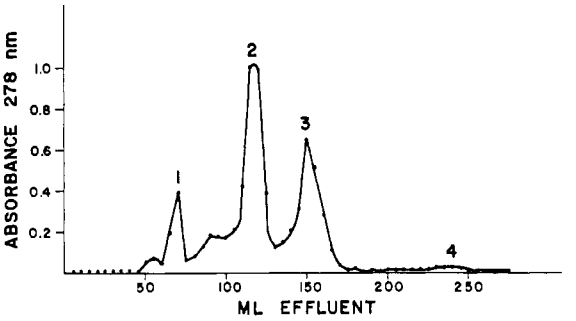

FIGURE 1: Chromatographic separation of products from the reaction of bromoacetic acid and crystals of ribonuclease A in 75% 2-methyl-2,4-pentanediol, pH 5.5. Column: 0.9 × 150 polymethacrylate resin (Bio-Rex 70). Load: 55 mg. Eluent, 0.2 M phosphate, pH 6.47; peak 2, unreacted RNase; peak 3, 1-CMHis-119-RNase; peak 4, 3-CMHis-12-RNase.

from fraction 3 was 46 mg. The material balance was not determined routinely since in the two instances where this was done the recovery was at least 90%. The material balance was obtained from the integrated optical density–volume curve of the chromatogram from the Bio-Rex 70 column, compared with that of a diluted aliquot of the solution of the carboxymethylated crystals.

Reduction of RNase with Cleland's Reagent. The protein, 27.4 mg (2 μmoles), was dissolved in 3 ml of deionized 8 M urea, and to it was added 17 mg (0.11 mmole) of Cleland's reagent in 0.5 ml of 8 M urea. The pH was adjusted to 8.3 with 0.1 N NaOH and maintained there during the course of the reaction. After 2.5 hr, 43 mg (0.23 mmole) of iodoacetic acid in 0.5 ml of 8 M urea was added and the pH quickly readjusted to 8.3. After 30 min, the reaction mixture was placed on a column (1.2 × 60 cm) of Bio-Gel, P-2 100–200 mesh, and eluted with 5% acetic acid. The flow rate was 120 ml/hr. The effluent was read at 278 nm; the protein fraction was pooled and lyophilized.

Digestion with DFP-carboxypeptidase A. A 0.05-ml aliquot of the carboxypeptidase suspension (50 mg/ml) was placed into a 5-ml volumetric flask and adjusted to the mark with 10% LiCl. After the enzyme dissolved, the solution was centrifuged and the concentration of the enzyme was estimated from the optical density at 278 nm, $E^{1\%}_{1\,cm}$ 19.6 (Folk and Schirmer, 1963). An aliquot equal to 25 μg of enzyme for each mg of substrate to be digested was withdrawn and added to 3–4 mg of RCM-RNase contained in 0.8 ml of 0.1 M Tris–0.2 M NaCl buffer (pH 7.9). Digestion was done in a water bath at 37–39° for 4 hr. Following digestion, 0.08 ml of 70% trichloroacetic acid was added and the contents were centrifuged at 15,000 rpm for 15 min. The supernate was decanted and a 1-ml aliquot withdrawn and brought to dryness by allowing nitrogen to flow over it. It was then submitted for amino acid analysis.

Results

To obtain authentic 1-CMHis-119-RNase and 3-CMHis-12-RNase, ribonuclease A was carboxymeth-

[2] Abbreviations used: the derivatives of ribonuclease carboxymethylated (cm) on given residue are abbreviated as follows: 3-CMHis-12-RNase; 1-CMHis-119-RNase; RCM, reduced, carboxymethylated on cysteine.

TABLE I: Amino Acid Analyses of Products of Figure 1.[a]

Amino Acid	Amino Acid Composition of RNase A			
	Theory	Peak 1	Peak 2	Peak 3
Aspartic acid	15	14.8	15.0	15.3
Threonine	10	9.3	9.2	9.3
Serine	15	13.4	13.8	12.4
Glutamic acid	12	12.0	12.6	11.7
Proline	4	4.2	3.8	3.9
Glycine	3	3.0	3.0	3.0
Alanine	12	12.0	12.0	12.0
Valine	9	8.4	8.9	10.4
Cystine	4	3.9	3.9	4.3
Methionine	4	3.7	3.6	3.5
Isoleucine[b]	3	2.0	2.1	3.0
Leucine	2	2.0	1.9	2.2
Tyrosine	6	5.7	5.8	5.8
Phenylalanine	3	3.0	2.9	3.4
Lysine	10	10.2	9.8	10.0
Histidine	4	3.5	3.6	2.8
Arginine	4	4.0	3.8	3.9
1-Carboxymethyl-histidine[c]				1.0

[a] Amino acid analyses of products from the reaction of crystals of ribonuclease A with bromoacetic acid in 75% 2-methyl-2,4-pentanediol, 24 hr (pH 5.5) at 25°. Each column represents the results of a single analysis. The results are not corrected for decomposition during hydrolysis. The molar ratios were calculated relative to alanine = 12.0. [b] The isoleucylisoleucyl bond in ribonuclease is not completely hydrolyzed in 22 hr. The high value of 3.0 for peak 3 is not understood. [c] We did not isolate 1-carboxymethylhistidine; hence, we are not able to determine the color constant of this derivative on the Technicon Autoanalyzer. Peak 3 however was analyzed on the Spinco amino acid analyzer by the method of Crestfield *et al.* (1963a) and the number of residues of 1-CMHis based on the average of two runs was 1.0. When the same hydrolysate was analyzed on the Technicon Autoanalyzer the number of residues found was 0.68 using the glycine color constant (see above reference). All analyses reported in this communication were obtained on the Technicon Autoanalyzer and we have set the value 0.70 to represent 1.0 residue of 1-carboxymethylhistidine.

ylated with iodoacetic acid using conditions described by Crestfield *et al.* (1963a) in their Figure 1, and the products of the reaction were chromatographed. Once the positions of unreacted RNase, 1-CMHis-119-RNase, and 3-CMHis-12-RNase were established, the products of the reaction of RNase crystals and bromoacetic acid were chromatographed (Figure 1). At the beginning of this investigation, a time study of the reaction of crystalline RNase with bromoacetate was made. Chromatographic analysis of the products on Bio-Rex 70 after periods of 1, 2, 4, 8, 16, and 32 days indicated that the yield of 1-CMHis-119-RNase did not increase if the reaction were allowed to proceed for more than 1 day; rather, new products were formed at the expense of the 119 derivative and unreacted RNase. All results described, therefore, refer to a 1-day reaction time. The results of amino acid analysis of the three major peaks are shown in Table I. Peak 1 is inherent in crystals of RNase since it is present when a tube of untreated crystals is chromatographed. Its amino acid composition is similar to that of native RNase A and further characterization of it was not made. Peak 2 is 100% active toward cytidine 2′,3′-phosphate and emerges at the same position as unreacted RNase A; its amino acid composition is the same as that of native ribonuclease A. Peak 3 is not active toward cytidine 2′,3′-cyclic phosphate and emerges at the same position as 1-CMHis-119-RNase; its amino acid composition is normal except that it contains 2.8 residues of histidine instead of 4. On amino acid analysis, a peak appeared between alanine and valine which was presumed to be 1-carboxymethylhistidine. To verify this, bromoacetic acid and L-histidine were reacted as described by Heinrikson *et al.* (1965). The reaction mixture was adjusted to pH 2.2 and a 0.15-ml portion was analyzed. The chromatogram showed three peaks in addition to unreacted histidine. The first peak emerged before carboxymethylcysteine, the second peak emerged between alanine and valine, while the third peak emerged at the same place as cystine. The ratio of the integrated color values of peaks 1:2:3 was about 1:3:6. Crestfield *et al.* (1963a) have identified the products of the reaction of histidine with iodoacetic acid to be 1,3-dicarboxymethylhistidine, 1-carboxymethylhistidine, and 3-carboxymethylhistidine, emerging in the order listed. The 3 isomer was obtained in the greatest

TABLE II: Amino Acids Released by Carboxypeptidase A.[a]

Amino Acid	RCM-RNase	RCM-1-CMHis-119-RNase	
		H_2O	Crystals
Valine	0.7	1.0, 1.1	1.1
Serine	0.8	1.0, 1.0	1.1[b]
Alanine	0.5	1.4, 1.7	0.9
Aspartic acid	0.5	0.6, 0.6	0.5
Phenylalanine	0.6	0.6, 0.8	0.6
Histidine	0.7	0.1, 0.1	0.1
1-CM-Histidine	0.0	0.0, 0.0	0.0
Tyrosine	0.2	0.1, 0.1	0.4
Methionine	0.1	0.0, 0.0	0.1
Isoleucine	0.1	0.0, 0.0	0.4
Threonine	0.0	0.0, 0.1	0.2[b]

[a] Moles of amino acid per mole of protein. [b] Resolution of threonine from serine was poor; consequently, the values reported are approximate values.

TABLE III: Results of Edman Degradation of the Peptide Containing Residues 105–124.[a]

Amino Acid	From Treatment of Crystals of Ribonuclease A, at pH 5.5, 24 hr at 25°				From Treatment of Ribonuclease A, in Water, pH 5.5, 5 hr at 25°	
	As Isolated		After Edman		As Isolated	After Edman
	Found	Theory	Found	Theory	Found	Found
Aspartic acid	2.0	2	2.0	2	1.8	2.0
Threonine	0.4	0	0.2	0	0.0	0.1
Serine	1.0	1	1.0	1	0.9	0.9
Glutamic acid	1.2	1	1.2	1	1.1	1.2
Proline	1.6	2	1.9	2	2.0	2.0
Glycine	0.9	1	0.9	1	1.0	1.2
Alanine	2.0	2	2.0	2	2.0	2.0
Valine	3.3	4	3.3	4	3.8	3.8
Cystine	0.0	0	0.0	0	0.0	0.0
Isoleucine[b]	0.8	2	0.7	2	1.2	1.0
Tyrosine	0.9	1	0.8	1	1.0	0.9
Phenylalanine	0.9	1	0.9	1	1.1	1.0
Lysine	0.2	0	0.1	0	0.1	0.1
Histidine	0.8	1	0.2	0	0.9	0.2
S-Carboxymethylcysteine	1.0	1	1.0	1	1.1	0.8
1-Carboxymethylhistidine[c]	0.8	1	0.7	1	1.0	1.0

[a] Amino acid analyses of peptide containing 1-carboxymethylhistidine. Each column represents the results of a single analysis which is not corrected for decomposition during hydrolysis. The molar ratios were calculated by assuming that the number of micromoles found for alanine was equal to 2.0 residues. The yield of this peptide was 60–70% for solution carboxymethylated, and 70% for crystal carboxymethylated RNase, in good agreement with that of Fruchter and Crestfield (1965). [b] The isoleucylisoleucyl bond contained in this peptide is not completely hydrolyzed in 22 hr. [c] See *c* in Table I.

yield and the ratio of the latter to the 1 isomer was 2:1. By analogy, we concluded that the derivative obtained in greatest yield and emerging last was 3-carboxymethylhistidine. The derivative emerging first was 1,3-dicarboxymethylhistidine while the peak between alanine and valine, which was one-half the size of the 3 isomer, must be 1-carboxymethylhistidine.

Peak 3, the presumed 1-CMHis-119-RNase and its water counterpart, was reduced with Cleland's reagent and the liberated sulfhydryl groups were carboxymethylated with iodoacetic acid.

Two convenient alternatives were available for verifying that histidine 119 was the modified residue. One was to digest the protein with DFP-carboxypeptidase A to see if the digest contained 1-carboxymethylhistidine. The other alternative was to digest the protein with trypsin, isolate the RCM-16 peptide[3] (residues 105–124), perform a one-stage Edman degradation, and by amino acid analysis verify if the amino-terminal histidine was removed while the value for 1-carboxymethylhistidine remained constant (Fruchter and Crestfield, 1965).

[3] The tryptic peptides of ribonuclease were designated by Hirs *et al.* (1956). The prefix RCM is used to indicate that the peptides were obtained from protein which was reduced and carboxymethylated on cysteine.

The first alternative appeared to be the simplest, since Fujioka and Scheraga (1965) have shown that DFP-carboxypeptidase A removes residues 124–119 from oxidized RNase. This procedure was carried out on RCM-RNase as well as on RNase carboxymethylated in solution and in crystal. The amino acids liberated are shown in Table II. The striking difference between RCM-1-CM-His-119-RNase and RCM-RNase is that the action of the exopeptidase on the latter liberates 0.7 residue of histidine whereas 0.1 residue of histidine and 0.0 residue of 1-carboxymethylhistidine were liberated in the former case (Table II). These results indicate that the histidine at position 119 is modified but that a 1-CMHis terminus is not susceptible to hydrolysis by carboxypeptidase A.

The second approach was then used to establish that histidine 119 was carboxymethylated. The RCM-16-peptide was isolated according to the procedure of Fruchter and Crestfield (1965). Amino acid analyses were obtained before and after a one-stage Edman degradation which was performed according to the modified method of Konigsberg and Hill (1962). From the amino acid analyses in Table III, it appears that RCM-16-peptide from carboxymethylated crystals was less pure than that obtained from its water counterpart. Nevertheless, the data clearly show that in both cases the CM-His

residue was in an interior position (119), not accessible to Edman degradation, while the noncarboxymethylated histidine was accessible to the Edman degradation and must have been terminal (position 105).

It is clear that the major product of carboxymethylation of crystalline RNase is the same as the product of carboxymethylation in aqueous solution; namely, 1-CMHis-119-RNase. A small proportion of another product was obtained at the chromatographic position expected for 3-CMHis-12-RNase (peak 4). The integrated intensity of peak 4 is about 1.5% of that of peak 3; however, in some cases it was barely detectable. Carboxymethylation in water yields a ratio of about 9:1 for the CMHis-119 to CMHis-12 products (Crestfield *et al.*, 1963a; Glick *et al.*, 1967; also our work). Fruchter and Crestfield (1965) reported that the proportion of 3-CMHis-12-RNase can be increased at higher temperature. Carboxymethylation of crystals at 35° instead of the usual 25° gave rise, not to more 12-CM product, but to less 119-CM and the appearance of three new products. Chromatographic comparison of the products formed at 35° with those formed at 25° but at longer reaction times suggests that the secondary products of the alkylation are not all the same for two reaction conditions; but this is not yet confirmed.

Is the smaller proportion of 3-CMHis-12-RNase obtained with crystals the result of the presence of phosphate in the active site of the crystalline RNase? Glick *et al.* (1967) showed that carboxymethylation of ribonuclease in water in the presence of phosphate results in a diminution of the quantity of 1-CMHis-119-RNase relative to that of the 3-CMHis-12-RNase.

Since the composition of the crystal is about 0.06 M in both RNase and phosphate, based on the protein content of 60% (M. V. King, unpublished), molecular weight (13,700 g), and density of 1.4 (based on a value of 0.7 for partial specific volumes typical of many proteins), we carried out carboxymethylations in water at the same concentrations, using the same ratio of RNase to bromoacetate, 10:1 by weight, as was used for crystal reactions. The ratio of 1-CMHis-119-RNase to 3-CMHis-12-RNase was about 15:1 under these conditions. However, these conditions are not entirely equivalent to those in the crystal, because in the latter case not all of the bromoacetate is likely to be in the crystal; much of it must be in the supernate. When the reaction was carried out in 0.06 M RNase with a ratio of RNase to bromoacetate of 30:1, the ratio of 1-CMHis-119-RNase to 3-CMHis-12-RNase was again about 15:1. In the use of the RNase:bromoacetate ratio of 10:1, an additional product was formed, emerging from the column as a small peak on the leading slope of the 1-CMHis-119-RNase peak; this has not been identified. This product was not observed at the 30:1 RNase:bromoacetate ratio. When carboxymethylations were carried out in 0.06 M RNase in aqueous solution without added phosphate, the ratio of 1-CMHis-119-RNase to 3-CMHis-12-RNase was 11:1. In the absence of phosphate the reaction is faster leading to nearly complete disappearance of RNase A; in the presence of phosphate, about 40% of the RNase A was recovered at a RNase:bromoacetate ratio of 10 and 60% at a ratio of 30. From the data obtained in concentrated solutions of RNase we cannot conclude that the presence of phosphate in the crystal is the cause of the very low yield of 3-CMHis-12-RNase.

We also considered the possibility that pH 5.5 in 75% 2-methyl-2,4-pentanediol may not be equivalent to pH 5.5 in water. However E. A. Barnard (personal communication) has studied the product composition over the pH range of 3–7, and found that the ratio of 1-CMHis (from 1-CM-119-RNase) to 3-CMHis from (3-CM-12-RNase) was not greater than 10 at any pH. Therefore, our small yield of 3-CMHis-12-RNase cannot be attributed to the true value of the pH in 75% 2-methyl-2,4-pentanediol being different from the measured value.

We have crystallized 1-CMHis-119-RNase and obtained crystals closely isomorphous with native crystals. A crystal structure analysis is in progress. The crystals of 119-CMHis-RNase are not stable in form II unless at least 1 mole of phosphate ion is present per mole of RNase; otherwise, a spontaneous change to a new form occurs. Crystals of native RNase cannot be formed in the absence of phosphate, or other anion such as sulfate, arsenate, or citrate. The formation of metastable form II crystals of phosphate-free CM-RNase suggests that the carboxymethyl group of 1-CMHis-119-RNase can perform the function of phosphate ion, although less effectively. Crystallization of 3-CMHis-12-RNase succeeded only after extensive efforts, and gave rise to a new crystal form even though phosphate was present. An electron density map of this form cannot be obtained directly.

Discussion

From the fact that the major product of carboxymethylation of crystalline RNase is the same as that obtained from carboxymethylation in aqueous solution, we can infer with some confidence that the conformation around the active site histidines is very similar in the two states. There are two differences that require consideration. The first is the difference in product composition, *i.e.*, the higher proportion of 1-CMHis-119-RNase obtained from crystals. While this may be the result of a conformational difference between the two states, there are other possibilities. Bromoacetate may react with either His-119 or His-12 depending on the relative degrees of protonation of the imidazoles at the two positions. The differently protonated forms may have different or essentially the same conformations. If the conformations are different, the crystal may have selected almost exclusively one of these (the His-119-reactive form). If so, then the conformation in the crystal may be essentially identical with at least one conformation that is present in solution. Whether this is the predominant form or the most reactive form is not clear. If, however, the differently protonated forms have essentially the same conformations in solution, the altered product mix in the crystal may be the result of an altered ratio of protonated forms arising from differential pK changes caused by the mixed solvent; but the conformation in the crystal could be the same as in solution. It is also possible that bromoacetic acid can select or induce two different conformations leading to alkyl-

ation either at His-119 or His-12. In this case, it is probable that the conformation in the crystal is the same, or is induced by bromoacetate to be the same, as that of the His-119-reactive form in solution.

The second difference is that raising the reaction temperature produces an increase in the proportion of 3-CMHis-12-RNase in solution, but in the crystal produces new products. This suggests that there is a conformation change in the crystal between 25 and 35°. We plan crystal structure analyses at several temperatures.

Yang and Hummel (1964) reported that CMHis-119-RNase does not bind anions. The fact that phosphate, at a phosphate to protein ratio of 2:1, stabilized CMHis-119-RNase crystals in form II suggests that phosphate is bound. X-Ray diffraction may resolve this question.

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Kinetic Studies of Yeast Nucleoside Diphosphate Kinase*

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ABSTRACT: Initial velocity, product inhibition, and isotopic exchange studies have been carried out on crystalline nucleoside diphosphate kinase from yeast, with the magnesium complexes of adenine and uridine nucleotides as reactants.

The kinetic mechanism is Ping-Pong (the first product dissociates before the second substrate combines with the enzyme), and all reactants give competitive substrate inhibition by combining with the improper stable enzyme form. A stable phosphoenzyme was isolated which may contain a phosphohistidine linkage. All of the kinetic constants have been determined and shown to be consistent with the Haldane relationships. An analysis of the kinetic constants suggests that the turnover numbers are the maximum possible for the physiological conditions.

Nucleoside diphosphate kinase catalyzes the phosphorylation of nucleoside diphosphates by nucleoside triphosphates, and the substrate specificity is broad, with all nucleotides tested generally acting as substrates. With the availability of the crystalline yeast enzyme (Ratliff *et al.*, 1964), kinetic studies were undertaken to elucidate the mechanism of the reaction, employing the reaction between adenine and uridine nucleotides[1] (eq 1).

$$MgATP^{2-} + MgUDP^{-} \rightleftharpoons MgADP^{-} + MgUTP^{2-} \quad (1)$$

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