THE SYNTHESIS OF 2'-BROMOACETAMIDO-2'-DEOXYURIDINE AND ITS REACTION WITH RIBONUCLEASE A

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Summary: Bovine pancreatic ribonuclease A reacts with 2'-bromoacetamido-2'-deoxyuridine at pH 5.5 and 25°C rapidly and with absolute specificity. The single product formed has been tentatively identified as N-3-(2'-carboxamidomethyl-2'-deoxyuridinyl)-histidine-12-ribonuclease A. The reaction exhibits binding kinetics, K_{m} being equal to 0.11 M for the nucleoside derivative and k_3 equal to 57 x 10^{-4} sec^{-1}. The reaction of 2'-bromoacetamido-2'-deoxyuridine with ribonuclease A occurs at a rate which is approximately 4000 times faster than its corresponding reaction with L-histidine.

Previous studies (Pincus and Carty, 1970) indicate that 2'(3')-O-bromoacetyluridine reacts rapidly and selectively with RNase A* at pH 5.5. The preponderant alkylated product is 3-carboxymethylhistidine-12-RNase A. Attempts to isolate a protein derivative to which the uridine moiety was bound covalently to the protein in ester linkage under a variety of conditions were unsuccessful. In addition, uncertainty arose as to the exact nature of the specificity since it is possible that 1-carboxymethylhistidine-119-RNase A is formed by reaction of the enzyme with bromoacetic acid generated in the reaction mixture via hydrolysis of the α-haloacetyl nucleoside ester. To overcome the effects of these shortcomings in the nucleoside ester reagent, the corresponding amide analog, 2'-BAMU, was prepared and allowed to react with RNase A at 25°C and pH 5.5. The experiments described in this report provide information on the nature of the reaction and the physical and chemical properties of the protein product.

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* The following abbreviations are used: RNase A, bovine pancreatic ribonuclease A; 2'-BAMU, 2'-bromoacetamido-2'-deoxyuridine; 3'-(CAMUr)-His-12-RNase A, N-3-(2'-carboxamidomethyl-2'-deoxyuridinyl)-histidine-12-ribonuclease A.

EXPERIMENTAL

2'-Bromoacetamido-2'-deoxyuridine - Dry 2'-amino-2'-deoxyuridine (500 mg, 2 mmoles) (Verheyden et al., 1971) was suspended in 25 ml of dry, redistilled p-dioxane and cooled with stirring to 11°C. Bromoacetic anhydride (Brauns, 1925), freshly redistilled, was melted over a small flame and 0.75 ml (ca. 5.8 mmoles) transferred to the suspension which immediately clarified. The resulting clear solution was stirred at 11°C for 10 minutes and then at room temperature for 60 minutes. Thin-layer chromatography in 20% CH_3OH-$CHCl_3$ indicated the absence of starting material. The reaction mixture was treated with 0.2 ml (11.1 mmoles) of water and after 2 hours evaporated under reduced pressure to an oil. The residue was dissolved in 50 ml of ethanol and coevaporated under reduced pressure. The product was triturated with ether to give a white solid. The ether was removed with suction and the solid washed twice more with ether to remove bromoacetic acid. The sample was chromatographed on a 20 x 1.9 cm column of silica gel using a gradient consisting of 1850 ml of chloroform followed by 2000 ml of 10% CH_3OH-$CHCl_3$ (v/v). The effluent was analyzed for 280 nm absorbance and the fractions corresponding to the major peak which eluted last from the column were pooled and evaporated to a crystalline solid. The compound was recrystallized from hot methanol to give 356 mg of product (48%) in 2 crops. The compound gave a negative test for inorganic halogen and a positive test for organic halogen. m.p. 202.5-204.5°C, UV max (H_2O) 261 nm (ε 9500), 203 nm (ε 14,400).

Anal. Calcd. for $C_{11}H_{14}N_3O_6Br$: C, 36.28; H, 3.88; N, 11.54; Br, 21.95

Found: C, 36.28; H, 3.76; N, 11.40; Br, 22.23

RNase A activity was measured with cytidine-2',3'-cyclic phosphate (Murdock et al., 1966) and analytical protein chromatography was carried out on columns of Amberlite CG-50, Type III, 400-600 mesh (Hirs et al., 1965). UV spectra were obtained on a Cary Model 14 spectrophotometer. Amino acid analyses were performed on columns of Aminex A-4 and A-5 (Spackman et al., 1958).

Analytical Experiments - RNase A (0.0021 M) was allowed to react with 2'-BAMU concentrations ranging from 0.0041 M to 0.047 M in 0.2 M sodium acetate buffer, pH 5.5, at 25°C in a total volume of 0.5 ml. Aliquots of 0.05 ml were removed at zero time and at suitable intervals thereafter and inactivated by addition to 4 ml of 0.02 M HCl. Each inactivated sample was assayed in triplicate for residual enzymatic activity.

Preparative Experiment - 2'-BAMU (7.3 mg) and RNase A acetate (31.1 mg) were dissolved in 1 ml of 0.2 M sodium acetate buffer, pH 5.5, at 25°C. After 4 hours, (ca. 18 half-lives) 0.6 ml of reaction mixture was added to 0.9 ml of 0.3 M HCl. Proteins were separated from unreacted 2'-BAMU and buffer salts on a column of Sephadex G-25, fine, bead form, equilibrated in 0.2 M sodium phosphate buffer, pH 6.02. Appropriate dilutions of the protein containing fraction were utilized for spectrophotometric analysis and analytical chromatography on Amberlite CG-50. A 1 ml portion of this protein fraction was desalted and subjected to amino acid analysis before and after oxidation with performic acid (Hirs, 1956).

RESULTS

The reactants and products of the reaction leading to the specific inactivation of RNase A are illustrated in Figure 1. The reaction indicated is an alkylation by the bromoacetamidonucleoside at the N-3 of the imidazole ring of the histidine-12 residue of RNase A displacing bromide ion. The variation in k'_{obs}, the first-order rate constant for inactivation of RNase A, as a function of the initial concentration of 2'-BAMU is depicted in Figure 2 as a double reciprocal plot which is derived from a mechanism formally identical to the classical Michaelis-Menten scheme. The data are plotted according to the rate law, $1/k'_{obs} = K_m/k_3(2'\text{-BAMU}) + 1/k_3$. The value of K_m, a constant analogous to the Michaelis constant, is 0.11 M, while that for k_3, the constant describing the covalent decomposition of the enzyme-nucleoside complex, is 57×10^{-4} sec^{-1}. The purity of the covalent protein-nucleoside product was

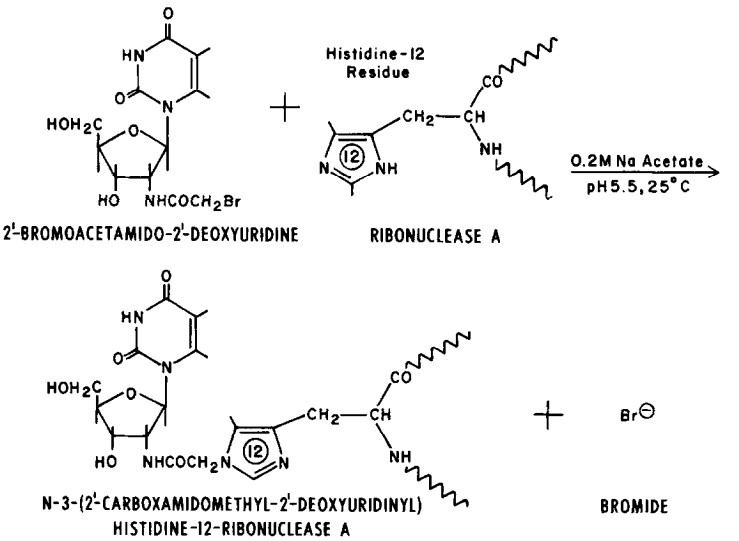

Figure 1 Reactants, products and reaction conditions involved in the preparation of the covalent nucleoside-enzyme compound, 3'-(CAMUr)-His-12-RNase A.

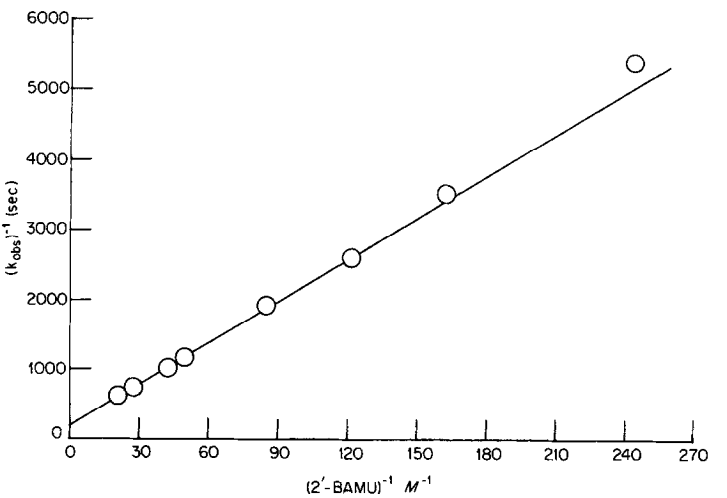

Figure 2 A double reciprocal plot depicting the variation of k'_{obs}, the first-order rate constant for the enzymatic inactivation of RNase A, as a function of the concentration of 2'-BAMU. The line through the points was fitted to the data by a weighted, least-squares procedure (Wilkinson, 1961).

established through analytical chromatography on Amberlite CG-50 which is depicted in Figure 3. The product, which does not bear an additional negative

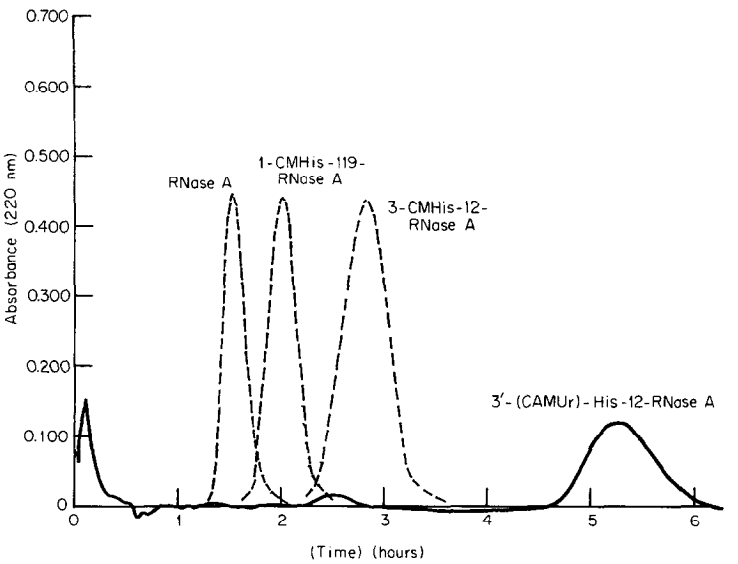

Figure 3 220 nm absorbance profile (solid line) of the effluent from an analytical Amberlite CG-50 chromatogram of the products of the reaction of 2'-BAMU with RNase A. The column dimensions were 35 x 0.9 cm, the eluent was 0.2 M sodium phosphate buffer, pH 6.38, and the flow rate was maintained at 20 ml/hr using a constant volume pump. The expected positions of RNase A and the derivatives, 1-CMHis-119-RNase A and 3-CMHis-12-RNase A in a hypothetical elution pattern are indicated (dashed line) for comparative purposes.

charge as in the carboxymethyl derivatives of RNase A, is substantially retarded relative to the native protein and its carboxymethyl derivatives which are exhibited in Figure 3 for comparative purposes. Analysis of the effluent profile of absorbance in Figure 3 indicates that the product is 95% pure and, therefore, suitable for structural analysis. Table 1 indicates the relevant amino acid and aminonucleoside composition and chromatographic mobilities of the modified RNase A derivatives. 2'-Amino-2'-deoxyuridine elutes as a slightly broadened peak between ammonia and arginine when chromatographed on the basic column of the amino acid analyzer. The color constant is 80% of that expected for glycine. The recovery of 2'-amino-2'-deoxyuridine after acid hydrolysis of the nucleoside-protein derivative is corrected for loss. 2'-BAMU served as a model compound and 83% of 2'-amino-2'-deoxyuridine was recovered after treatment at 110°C for 22 hours with 6 M HCl.

TABLE 1

Partial Amino Acid and Aminonucleoside Analyses and Chromatographic Mobilities of Alkylated Derivatives of RNase A

	RNase A	1-CMHis-119-RNase A[a,b]	3-CMHis-12-RNase A[a,b]	3'-(CAMUr)-His-12-RNase A
LYSINE	10	10.10	10.0	10.09
METHIONINE	4	3.90	3.71	3.69[c]
1-CM-HISTIDINE		0.99	0.00	0.00
3-CM-HISTIDINE		0.00	1.01	0.92[c]
2'-AMINO-2'-DEOXYURIDINE				0.92
CHROMATOGRAPHIC MOBILITY	1	1.32	1.84	3.40

a Data obtained from Crestfield et al., 1963
b CM, carboxymethyl
c Analytical data obtained from an acid hydrolysis of a sample of performic acid oxidized 3'-(CAMUr)-His-12-RNase A

Analysis of the UV spectrum of purified 3'-(CAMUr)-His-12-RNase A indicates that the 280/260 ratio is 0.985 and that the peak is shifted from 278 nm in the native RNase A to 269.5 nm in the derivative where ε_m is 15,450 $\underline{M}^{-1}cm^{-1}$. The molecular extinction at 280 nm is 12,850 $\underline{M}^{-1}cm^{-1}$. Table 2 provides an interesting comparison of kinetic data for the formation of alkylated products of the histidine-12 residue of RNase A by various reagents such as 2'-BAMU, 2'(3')-O-bromoacetyluridine, iodoacetate and bromoacetate.

DISCUSSION

The reaction of 2'-BAMU with RNase A is a specific, accelerated alkylation of the N-3 of the histidine-12 residue at the active site of RNase A. Support for the structural identity of the product includes the finding of one mole each of 2'-amino-2'-deoxyuridine and 3-carboxymethylhistidine upon acid hydrolysis. Lack of substitution at the histidine-119 residue is suggested by

TABLE 2

Partial Amino Acid and Aminonucleoside Analyses and Chromatographic Mobilities of Alkylated Derivatives of RNase A

	RNase A	1-CMHis-119-RNase A[a,b]	3-CMHis-12-RNase A[a,b]	3'-(CAMUr)-His-12-RNase A
LYSINE	10	10.10	10.0	10.09
HISTIDINE	4	2.96	2.84	2.84
METHIONINE	4	3.90	3.71	3.69[c]
1-CM-HISTIDINE		0.99	0.00	0.00
3-CM-HISTIDINE		0.00	1.01	0.92[c]
2'-AMINO-2'-DEOXYURIDINE				0.92
CHROMATOGRAPHIC MOBILITY	1	1.32	1.84	3.40

a Data obtained from Crestfield et al., 1963
b CM, carboxymethyl
c Analytical data obtained from an acid hydrolysis of a sample of performic acid oxidized 3'-(CAMUr)-His-12-RNase A

the absence of 1-carboxymethylhistidine in the acid hydrolyzate. Further evidence supporting the assignment includes the close kinetic analogy to the reaction of 2'(3')-O-bromoacetyluridine with RNase A. The reaction of 2'-BAMU with RNase A is remarkably accelerated relative to the reaction of the bromoacetamidonucleoside with L-histidine. Preliminary data indicate that the rate with the enzyme is 4000 times greater than that with the free amino acid. This rate enhancement must be due in part to binding and in part to the special reactivity of an active-site imidazole group towards alkylating agents. Analysis of the data in Figure 2 substantiates the ability of 2'-BAMU to behave like a substrate and bind to the active site. An advantage to studies with 2'-BAMU resides in the formation of a covalent nucleoside-enzyme compound in which the nucleoside is bound at the active site in a conformation similar or identical

to that of the nucleoside portion of substrates. Such a result would facilitate studies of the role played by the active site in such phenomena as pH and thermal denaturation and the role of ligands in protecting or sensitizing the enzyme to the effects of acid and heat, respectively.

Reliable estimates for the constants, K_m and k_3, for 2'-BAMU are hampered by the relatively low solubility of 2'-BAMU which is 0.05 M at 25°C. The fact that concentrations well below K_m, 0.11 M, are necessarily used introduces relatively large errors in k_3, and hence K_m, since the slope of the plot in Figure 2 is K_m/k_3. It is of interest, however, that, if K_m is a binding constant, the binding capacity is reduced by a factor of 10 relative to uridine where K_i is 0.013 M (Ukita et al., 1961). This suggests that the bulky bromoacetamido group reduces the binding capacity, but also possibly that the 2'-OH group of the sugar portion of the nucleoside facilitates binding. To study the possible role of the 2'-OH group in nucleoside binding, we are currently preparing 3'-bromoacetamido-3'-deoxyuridine and intend to study its reactivity with RNase A. If the 2'-OH group is important in binding of nucleosides to the active site of the enzyme, then it is plausible to expect more favorable binding parameters for the 3' isomer.

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