THE REACTION OF 2'(3')-O-BROMOACETYLURIDINE WITH RIBONUCLEASE A

Matthew Pincus and Robert P. Carty
State University of New York, Downstate Medical Center
450 Clarkson Avenue, Brooklyn, New York 11203

Received February 6, 1970

Summary: Reaction of bovine pancreatic ribonuclease A with 2'(3')-O-bromoacetyluridine at pH 5.5, 25°C, leads to a rapid and stereospecific inactivation of the enzyme with formation of 3-carboxymethylhistidine-12 RNase A and 1-carboxymethylhistidine-119 RNase A in the ratio of 7:1. The product distribution is the reverse of that obtained for the reaction of bromoacetate with RNase A. The results suggest that the imidazole group of histidine-12 is favorably disposed to interact with the phosphate residues of cyclic nucleotide substrates and 2'- and 3'-nucleotide inhibitors.

RNase A* reacts rapidly with α-haloacetates at pH 5.5 exclusively at N-1 of histidine-119 and N-3 of histidine-12 in the ratio of 9:1, respectively (Heinrikson et al., 1965). Alkylation at a single imidazole residue prevents reaction at the other imidazole within the same molecule, no dicarboxymethyl RNase A derivatives being found. The rate accleration compared with the reaction of N-1 or N-3 of free histidine is over 2000 times. The relative rate enhancement appears to be due in part to a specific binding effect of the haloacetate anion to the positively-charged site on the enzyme responsible for nucleotide binding and catalysis. The exclusion of reactivity of the second imidazole is attributed to charge repulsion between the α-haloacetate anion and the anion of the carboxymethylhistidyl residue of the monoalkylated RNase A. Alkylation at either residue is inhibited by polyvalent anions (Crestfield et al., 1963). These findings suggest that the imidazoles of histidine-12 and histidine-119 are near to each other at the active site, a fact corroborated by X-ray analysis of RNase S crystals (Wyckoff et al., 1967).

No explanation is apparent for the peculiar product distribution

---

*The following abbreviations are used throughout: RNase A, bovine pancreatic ribonuclease A; BAU, 2'(3')-O-bromoacetyluridine.

although the inaccessibility of N-1 of histidine-12 is documented by X-ray studies. In order to examine the stereospecificity of the product distribution of the alkylation reaction and the relationship of the nucleoside binding site to the imidazoles at the active center, BAU has been allowed to react with RNase A at 25°C and pH 5.5. The experiments reported here provide information concerning the kinetics and product distribution of this reaction.

EXPERIMENTAL

BAU was synthesized from uridine and trimethylorthobromoacetate (Fromageot et al., 1967). The product, R_f 0.16, showed a faint trace of uridine, R_f 0.02, upon thin-layer chromatography in 10% methanol-chloroform and gave a positive ester test with hydroxylamine and ferric chloride. The compound gave a negative inorganic and positive organic bromide test. Combustion and UV absorption analysis indicated that the BAU preparation was 90% pure and that the major part of the impurity was not nucleoside material. RNase A activity was measured with cytidine-2',3'-cyclic phosphate (Murdock et al., 1966) and protein chromotography was carried out on columns of Bio-Rex 70 (Hirs et al., 1965). Amino acid analyses were performed on columns of Aminex A-4 and A-5 (Spackman et al., 1958).

Analytical Experiments

1.) BAU/RNase A 7:1 Molar Ratio

RNase A (0.0023 M) was allowed to react with BAU (0.016 M) in 0.2 M sodium acetate buffer, pH 5.5, at 25°C. The reaction mixture was 10% ethanol by volume. Aliquots were removed at suitable time intervals* and examined for residual enzymatic activity and the formation of protein reaction products.

2.) BAU/RNase A 2:1 Molar Ratio

Experiments were carried out as above except that BAU was 0.0041 M. Reaction mixtures were 10% acetone by volume. Aliquots were removed at suitable time intervals and examined for residual enzymatic activity and RNase A disappearance as well as the formation of alkylated protein products.

Preparative Experiment

1.) BAU/RNase A 2:1 Molar Ratio

A large scale reaction was carried out in which 157 mg (11.5 umoles) of RNase A were allowed to react with 7.45 mg (20.4 umoles) of BAU for 10.75 hours under the conditions specified above for the analytical reaction conducted at a 2:1 molar ratio. Proteins were separated from unreacted BAU and buffer salts on a column of Sephadex G-25 medium. The protein fraction was chromatographed on a 36 X 3.75 cm column of Bio-Rex 70, -400 mesh, in 0.2 M sodium phosphate buffer, pH 6.45, at 4°C. Effluent fractions were analyzed for 280 and 260 mu absorbance and enzymatic activity. Fractions containing protein were pooled, desalted and subjected to amino acid analysis.

RESULTS

The loss of enzymatic activity in the 7:1 BAU/RNase A reaction is first-order for over 80% of the reaction. The calculated second-order rate constant based upon inactivation is 1.69 X 10^{-2} $M^{-1}sec^{-1}$. The second-order rate constant for the formation of the major protein product, identified as 3-carboxymethylhistidine-12 RNase A, was 1.42 X 10^{-2} $M^{-1}sec^{-1}$. The carboxymethyl derivative is apparently derived from the rapid hydrolysis of the parent uridine carboxymethyl RNase A ester. The lability of amino acyl nucleoside esters to base hydrolysis has been well documented (Zachau and Feldmann, 1965). The rate of loss of enzymatic activity and RNase A disappearance were shown to be identical in the 2:1 BAU/RNase A reaction which was second-order over 60% of the reaction. The second-order rate constants for the loss of RNase A and formation of 3-carboxymethylhistidine-12 RNase A are 3.48 X 10^{-2} $M^{-1}sec^{-1}$ and 2.94 X 10^{-2} $M^{-1}sec^{-1}$, respectively. In both cases, for the 7:1 and the 2:1 BAU/RNase A reactions, the rate of appearance of the 12-derivative represents 85% of the total rate of RNase A modification. Table 1 illustrates comparative kinetic data for the formation of 3-carboxymethylhistidine-12 RNase A from BAU, iodoacetamide and bromoacetate.

Figure 1 indicates the 280 and 260 mu absorbance profiles of the effluent from a preparative chromatogram of BAU plus RNase A reaction products. Peak II was identified as unreacted RNase A from its specific activity which was the same as that of a stock RNase A solution. Peaks III and IV were inactive against cytidine-2',3'-cyclic phosphate and were identified from amino acid analyses and their relative chromatographic mobilities on analytical Bio-Rex 70 columns. Peaks III and IV are 1-carboxymethylhistidine-119 RNase A and 3-carboxymethylhistidine-12 RNase A, respectively. Table 2 illustrates the relevant amino acid composition and chromatographic mobilities of the modified proteins.

DISCUSSION

The reaction of BAU with RNase A is a highly specific, acclerated alkylation which is mediated by the attachment of the reactive bromoacetyl residue to the nucleoside portion of the molecule. The inhibitor induced alkylation produces rates of reaction comparable to those found for the reaction of bromoacetate with RNase A where the value of the second-order rate constant is $2.05 \times 10^{-2}\ \underline{M}^{-1}sec^{-1}$ (Heinrikson et al., 1965). The rate enhancement compared to the reaction of iodoacetamide is of the order of 200-350 fold. This relative

TABLE 1

Comparison of the Second-Order Rate Constants for the Formation of 3-Carboxymethylhistidine-12 RNase A in the Reaction of RNase A with α-Haloacetyl Derivatives at 25°C, pH 5.5

Reaction	Molar Ratio (α-haloacetyl/RNase A)	Second-Order Rate Constant ($\underline{M}^{-1}sec^{-1}$)
RNase A + BAU	7	1.42×10^{-2}
RNase A + BAU	2	2.94×10^{-2}
RNase A + Iodoacetamide*	148	0.11×10^{-3}
RNase A + Bromoacetate**	3 - 26	0.21×10^{-2}

* Data obtained from Fruchter and Crestfield, 1967
**Data obtained from Heinrikson et al., 1965

TABLE 2

Partial Amino Acid Analyses and Chromatographic Mobilities of Carboxymethyl Derivatives of RNase A

	RNase	Peak III	Peak IV	1-CM-119 RNase[a,b]	3-CM-12 RNase[a,b]
LYS	10	10.45	9.98	10.10	10.0
MET	4	3.61	3.89	3.90	3.71
HIS	4	3.00	3.03	2.96	2.84
1-CM-HIS[b]	-	0.86	0.00	0.99	0.00
3-CM-HIS[b]	-	0.00	1.02[c]	0.00	1.01
CHROMATOGRAPHIC MOBILITY	1	1.30	1.84	1.32	1.98

[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-carboxymethylhistidine-12 RNase A

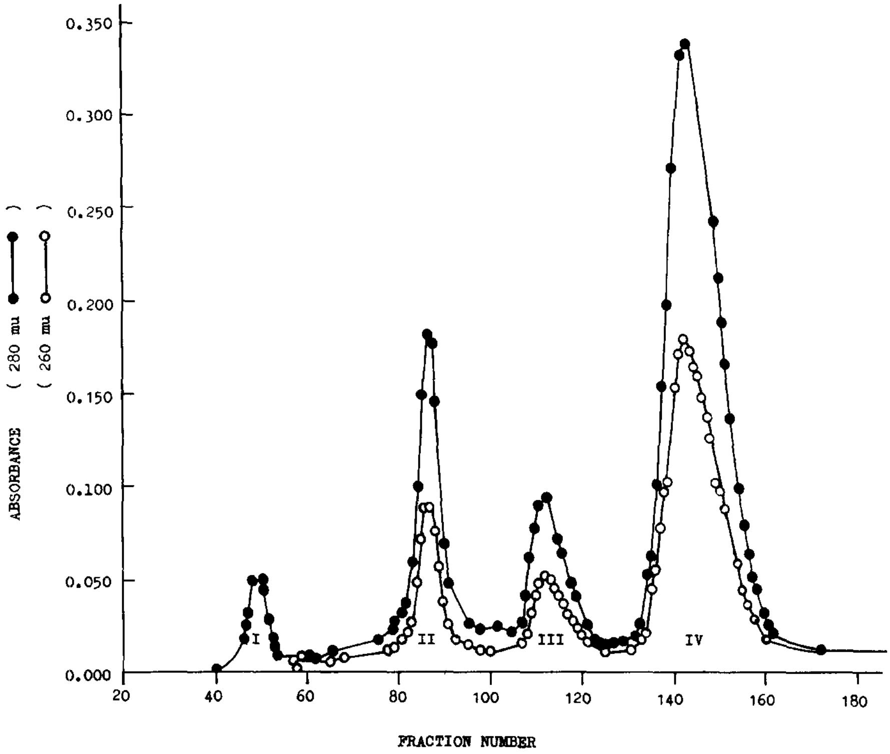

Figure 1. 280 and 260 mu absorbance profile of the effluent from a preparative chromatogram of the products of the reaction of BAU and RNase A at a molar ratio of 2:1

rate enhancement is attributed in part to the binding affinity of the uridine moiety of BAU. In addition, the binding of the nucleoside portion of BAU may orient the alkylating moiety such that it lies in close proximity to histidine-12, thus adding to the rate accleration with respect to iodoacetamide.

Uridine has a dissociation constant of 1.3 X 10^{-2} $\underline{M}$ (Ukita et al., 1961), and assuming BAU to have a similar binding affinity for RNase A, 58% and 21% of the total initial RNase A in the 7:1 and 2:1 BAU/RNase A reactions, respectively, are in the form of a BAU-RNase A complex. That a mechanism might exist where a binding step preceeds alkylation is suggested by the non-linear dependence of the first-order rate constant for enzymatic inactivation upon BAU concentration. This is reflected in the different values for the second-order rate constants calculated from the 7:1 and 2:1 BAU/RNase A reactions. That the higher BAU/RNase A ratio gives a lower value of the second-order rate constant suggests that saturation kinetics may be operative.

It might be expected from the stereospecific binding of the uridine portion of the BAU molecule that the product distribution should reflect the proximity and specific orientation of imidazole groups at the active site. Studies with models indicate that the distance of the active α-carbon of the bromoacetyl residue from the 2' or 3' oxygen in the BAU molecule is approximately the same as the distance between the anionic phosphoryl oxygen and the phosphoryl ester oxygen in 2'- or 3'-uridylic acid. NMR spectra of RNase A complexes of 2'-, 3'- and 5'-cytidylic acids suggests specific interactions of the phosphates of the 2'- and 3'-nucleotides with the imidazolium ion of histidine-119 (Meadows et al., 1969). Therefore, the location of the α-haloacyl group at the 2' or 3' position of the ribose ring of uridine should constitute a powerful stereospecific inhibitor.

The product distribution of carboxyalkylated derivatives from the BAU-RNase A reaction indicates the reversal of the distribution observed with bromoacetate (Heinrikson et al., 1965). Alkylation with BAU leads to the formation of 3-carboxymethylhistidine-12 RNase A and 1-carboxymethylhistidine-119 RNase A in the ratio of 8:1. A ratio of 1:9 for these products was found with bromoacetate. The distribution of carboxymethylated products found in a control experiment in which RNase A was treated with a 7 molar excess of uridine and bromoacetate, which could theoretically arise from a very rapid hydrolysis of BAU, indicated that the product distribution observed with BAU was a unique feature of the covalent interaction of this α-haloacylnucleoside with RNase A, and not due to alkylation with bromoacetate in the presence of uridine. The specificity of the BAU alkylation is not incompatible from a stereochemical viewpoint with the specificity of the bromoacetate alkylation, because of the presence of a different orientation factor in each reagent. The carboxylate anion of bromoacetate could bind to the imidazolium ion of histidine-12 directing the α-bromomethylene residue away from histidine-12. If the stereospecificity of the bromoacetate reaction depends heavily on this orientation factor, then histidine-119 would be alkylated to a greater extent, because at pH 5.5, the imidazolium ion of histidine-12 is present in a 2.5 fold greater concentration than the corresponding ion of histidine-119. This is due to the small difference in their pK_a values. Histidine-12 has a pK_a of 6.2 and histidine-119 has a pK_a of 5.8 (Meadows and Jardetzky, 1968). On the other hand, alkylation of histidine-12 by BAU would be favored if nucleoside binding conferred on the orientation of the bromoacetyl residue a geometry which facilitated reaction at histidine-12. It is clear that an alkylating function such as α-bromomethylene can have entirely different geometries relative to the active site imidazole groups depending on whether an anion or nucleoside provides the orienting force. In addition, it is clear that nucleoside binding can also force a specific orientation of a phosphate group esterified at one of the hydroxyl residues of the ribose ring. It is quite probable that different binding orientations exists for inorganic phosphate and the phosphate residue of ribonucleotides as indicated by differences in the NMR chemical shifts and pK_a alterations associated with ligand-RNase A complex formation (Meadows et al., 1969).

The reaction of BAU with RNase A does not lead to the isolation of uridine carboxymethyl RNase A esters. The 280/260 ratios for peak III and IV are 1.90 and 1.86, respectively, indicating the absence of covalently bound nucleoside. A calculated 280/260 ratio for a 1:1 molecular compound of uridine and RNase A should be 0.85 based on the summation of the extinctions for uridine and RNase A at these wavelengths. Experiments are now being carried out to determine the optimal conditions for the isolation of a covalent nucleoside-protein compound.

REFERENCES

Crestfield, A.M., Stein, W.H. and Moore, S., J. Biol. Chem., 238, 2413 (1963)

Fromageot, H.P.M., Griffin, B.E., Reese, C.B. and Sulston, J.E., Tetrahedron, 23, 2315 (1967)

Fruchter, R.G. and Crestfield, A.M., J. Biol. Chem., 242, 5807 (1967)

Heinrikson, R.L., Stein, W.H., Crestfield, A.M. and Moore, S., J. Biol. Chem., 240, 2921 (1965)

Hirs, C.H.W., Halmann, M. and Kycia, J.H., Arch. Biochem. Biophys., 111, 209 (1965)

Meadows, D.H., Roberts, G.C.K. and Jardetzky, O., J. Mol. Biol., 45, 491 (1969)

Meadows, D.H. and Jardetzky, O., Proc. Natl. Acad. Sci., 61, 406 (1968)

Murdock, A.L., Grist, K.L. and Hirs, C.H.W., Arch. Biochem. Biophys., 114, 375 (1966)

Spackman, D.H., Stein, W.H. and Moore, S., Anal. Chem., 30, 1190 (1958)

Ukita, T., Waku, K., Irie, M. and Hoshino, O., J. Biochem. (Japan), 50, 405 (1961)

Wyckoff, H.W., Hardman, K.D., Allewell, N.M., Inagami, T., Johnson, L.N. and Richards, F.M., J. Biol. Chem., 242, 3984 (1967)

Zachau, H.G. and Feldmann, H., in J.N. Davidson and W.E. Cohn (eds.), Progress in Nucleic Acid Research and Molecular Biology, Vol. 4, Academic Press, New York, 1965, p. 217