Reaction of (Bromoacetamido)nucleoside Affinity Labels with Ribonuclease A: Evidence for Steric Control of Reaction Specificity and Alkylation Rate†

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Received April 8, 1986; Revised Manuscript Received August 14, 1986

ABSTRACT: Four new bromoacetamido pyrimidine nucleosides have been synthesized and are affinity labels for the active site of bovine pancreatic ribonuclease A (RNase A). All bind reversibly to the enzyme and react covalently with it, resulting in inactivation. The binding constants K_b and the first-order decomposition rate constants k_3 have been determined for each derivative. They are the following: 3′-(bromoacetamido)-3′-deoxyuridine, $K_b = 0.062$ M, $k_3 = 3.3 \times 10^{-4}$ s^{-1}; 2′-(bromoacetamido)-2′-deoxyxylofuranosyluracil, $K_b = 0.18$ M, $k_3 = 1700 \times 10^{-4}$ s^{-1}; 3′-(bromoacetamido)-3′-deoxyarabinofuranosyluracil, $K_b = 0.038$ M, $k_3 = 6.6 \times 10^{-4}$ s^{-1}; and 3′-(bromoacetamido)-3′-deoxythymidine, $K_b = 0.094$ M, $k_3 = 2.7 \times 10^{-4}$ s^{-1}. 3′-(Bromoacetamido)-3′-deoxyuridine reacts exclusively with the histidine-119 residue, giving 70% of a monoalkylated product substituted at N-1, 14% of a monoalkylated derivative substituted at N-3, and 16% of a dialkylated species substituted at both N-1 and N-3. Both 2′-(bromoacetamido)-2′-deoxyxylofuranosyluracil and 3′-(bromoacetamido)-3′-deoxyarabinofuranosyluracil react with absolute specificity at N-3 of the histidine-12 residue. 3′-(Bromoacetamido)-3′-deoxythymidine alkylates histidines-12 and -119. The major product formed in 57% yield is substituted at N-3 of histidine-12. A monoalkylated derivative, 8% yield, is substituted at N-1 of histidine-119. A disubstituted species is formed in 14% yield and is alkylated at both N-3 of histidine-12 and N-1 of histidine-119. A specific interaction of the "down" 2′-OH group, unique to 3′-(bromoacetamido)-3′-deoxyuridine, serves to orient the 3′-bromoacetamido residue close to the imidazole ring of histidine-119. The 2′-OH group of 3′,5′-dinucleoside phosphate substrates may serve a similar role in the catalytic mechanism, allowing histidine-119 to protonate the leaving group in the transphosphorylation step. (Bromoacetamido)nucleosides are bound in the active site of RNase A in a variety of distinct conformations which are responsible for the different specificities and alkylation rates.

Haloacetyl derivatives of pyrimidine nucleosides react rapidly and selectively at the active site of RNase A.[1] 2′-(3′)-*O*-(Bromoacetyl)uridine alkylates the N-3 atom of His-12 exclusively (Pincus et al., 1975; Pincus & Carty, 1970) at a rate that exceeds alkylation by bromoacetamide at the same N-3 atom by a factor of 210. The product is N^3-Cm-His-12-RNase A, which results from the rapid hydrolysis of the labile ester bond in the covalent enzyme–nucleoside derivative. Small amounts of N^1-Cm-His-119-RNase A are sometimes detectable in alkylation reaction products (Machuga & Klapper, 1975) as a result of hydrolysis of 2′(3′)-*O*-(bromoacetyl)uridine, generating bromoacetate which preferentially carboxymethylates N-1 of His-119 (Crestfield et al., 1963; Heinrikson et al., 1965). The bromoacetyl group of 2′(3′)-*O*-(bromoacetyl)uridine rapidly isomerizes between the 2′- and 3′-positions of the ribofuranosyl ring (Griffin et al., 1966) in aqueous solution, and it was not possible to account for the specificity of alkylation at N-3 of His-12 in terms of reaction with either the 2′- or 3′-isomer. 2′-(Bromoacetamido)-2′-deoxyuridine reacts likewise with absolute specificity for N-3 of His-12 (Lan & Carty, 1972). This amide analogue of 2′(3′)-*O*-(bromoacetyl)uridine does not undergo hydrolysis at pH 5.5 and 25 °C, and the bromoacetyl residue is incapable of isomerizing to the 3′-position of the sugar ring. It reacts with RNase A at a rate essentially the same as that for 2′-(3′)-*O*-(bromoacetyl)uridine, suggesting that it is the 2′-isomer which reacts specifically with N-3 of His-12. 2(3′)-(Bromoacetyl)uridine reacts with RNase A 3100 times faster than with L-histidine. Using the reaction of bromoacetamide with

† A preliminary report of this work has appeared in abstract form (Carty et al., 1984).

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; Cm, carboxymethyl; N^3-(2′-CAMUrd)-His-12-RNase A, [N^3-[[(2′-deoxyuridinyl-2′-amino)carbonyl]methyl]histidine-12]ribonuclease A; N^1-(3′-CAMUrd)-His-119-RNase A, [N^1-[[(3′-deoxyuridinyl-3′-amino)-carbonyl]methyl]histidine-119]ribonuclease A; N^3-(3′-CAMUrd)-His-119-RNase A, [N^3-[[(3′-deoxyuridinyl-3′-amino)carbonyl]methyl]-histidine-119]ribonuclease A; N^1,N^3-bis(3′-CAMUrd)-His-119-RNase A, [N^1,N^3-bis[[(3′-deoxyuridinyl-3′-amino)carbonyl]methyl]histidine-119]ribonuclease A; N^3-(3′-CAMaraU)-His-12-RNase A, [N^3-[[(3′-deoxyarabinofuranosyluracilyl-3′-amino)carbonyl]methyl]histidine-12]-ribonuclease A; N^3-(2′-CAMxylU)-His-12-RNase A, [N^3-[[(2′-deoxyxylofuranosyluracilyl-2′-amino)carbonyl]methyl]histidine-12]ribonuclease A; N^3-(3′-CAMT)-His-12-RNase A, [N^3-[[(3′-deoxythymidinyl-3′-amino)carbonyl]methyl]histidine-12]ribonuclease A; N^1-(3′-CAMT)-His-119-RNase A, [N^1-[[(3′-deoxythymidinyl-3′-amino)-carbonyl]methyl]histidine-119]ribonuclease A; bis[N^3-(3′-CAMT)-His-12,N^1-(3′-CAMT)-His-119]-RNase A, bis[N^3-[[(3′-deoxythymidinyl-3′-amino)carbonyl]methyl]histidine-12,N^1-[[(3′-deoxythymidinyl-3′-amino)carbonyl]methyl]histidine-119]ribonuclease A; UpcA, dinucleoside phosphonate analogue of uridylyl(3′,5′)adenosine in which the 5″-ribosyl oxygen is replaced by a methylene group.

0006-2960/87/0426-0135$01.50/0

RNase A as a determinant of the inherent nucleophilic reactivity of N-3 of His-12, it has been possible to separate the overall rate acceleration of 3100 into contributions of nucleoside binding (a factor of 25) and increased nucleophilic reactivity (a factor of 125) (Pincus et al., 1975). Also, reaction of bromoacetate with N-3 of His-12 occurs 120 times faster than with the imidazole nitrogens of histidine hydantoin (Lennette & Plapp, 1979a,b).

In this paper the syntheses of four new bromoacetamido pyrimidine nucleosides, 3′-(bromoacetamido)-3′-deoxyuridine, 3′-(bromoacetamido)-3′-deoxyarabinofuranosyluracil, 3′-(bromoacetamido)-3′-deoxythymidine, and 2′-(bromoacetamido)-2′-deoxyxylofuranosyluracil, are described. The specificity and rate of the alkylation reaction of each derivative with RNase A are determined and compared to previous results with 2′(3′)-*O*-(bromoacetyl)uridine and 2′-(bromoacetamido)-2′-deoxyuridine. This study demonstrates that (1) 3′-(bromoacetamido)-3′-deoxyuridine alkylates histidine-119 exclusively, (2) the specificity of 3′-(bromoacetamido)-3′-deoxyuridine is determined by a specific interaction of the enzyme with the 2′-OH group in the ribo configuration, (3) the 2′-deoxyribofuranosyl ring of 3′-(bromoacetamido)-3′-deoxythymidine is mobile within the enzyme–nucleoside complex, and (4) variations in alkylation rates of bromoacetamido pyrimidine nucleosides at N-3 of His-12 are due to differences in the steric placement of the bromoacetamido residue near the active-site imidazole groups. This steric disposition is influenced by the conformation of the nucleoside derivative in the binary complex of enzyme and affinity label, as well as specific interactions of furanose ring hydroxyl groups with the active site.

Materials and Methods

RNase A (Sigma, type IIA, lot no. 32F-0282) was purified by the method of Taborsky (1959) and desalted by passage over Sephadex G-25 2 times. The affinity labels 2′(3′)-*O*-(bromoacetyl)uridine (Pincus et al., 1975) and 2′-(bromoacetamido)-2′-deoxyuridine (Lan & Carty, 1972) were synthesized and purified as described previously. The protein derivative N^3-(2′-CAMUrd)-His-12-RNase A was prepared by using the procedure of Lan and Carty (1972).

Amberlite CG-50, type III, −400 mesh, was obtained from Rohm and Haas. The resin was fractionated (Hirs, 1955) and then sized hydraulically (Hamilton, 1958). Fractions having diameters of 51 ± 13 μm were used for protein chromatography. Sephadex G-25 (fine beads) was a Pharmacia product, and (carboxymethyl)cellulose (CM-52) was purchased from Whatman Ltd. AG 50W-X2 (200–400 mesh) was purchased from Bio-Rad. Tris (free base), sodium cytidine 2′,3′-cyclic monophosphate, uridylyl(3′,5′)adenosine, and trypsin (type XI, diphenylcarbamyl chloride treated) were from Sigma. α-Chymotrypsin was a Worthington Biochemical product. All other chemicals were reagent grade.

Synthesis of New Affinity Labels. (A) Aminodeoxynucleosides. 3′-Amino-3′-deoxyuridine was prepared from 3′-azido-3′-deoxyuridine 2′,5′-di-*O*-benzoate (Vorbrüggen & Bennua, 1978). The azido derivative was debenzoylated (Niedballa & Vorbrüggen, 1974) and then reduced to the aminonucleoside (Verhayden et al., 1971). 3′-Amino-3′-deoxyuridine was purified by ion-exchange chromatography on Amberlite CG-50 using 0.2 M sodium phosphate, pH 6.37, as eluent (Hummel & Carty, 1983). The aminonucleoside was desalted and crystallized from 95% ethanol. The preparation gave a melting point, specific rotation, and infrared spectra identical with those reported in the literature (Kissman & Weiss, 1958; Codington et al., 1962).

3′-Amino-3′-deoxyarabinofuranosyluracil and 2′-amino-2′-deoxyxylofuranosyluracil were prepared as described previously (Hummel & Carty, 1983).

3′-Amino-3′-deoxythymidine was prepared by catalytic reduction of 3′-azido-3′-deoxythymidine synthesized by the method of Glinski et al. (1970). The azidonucleoside was dissolved in absolute MeOH and hydrogenated according to the procedure of Glinski et al. (1973) for the reduction of 3′-azido-3′-deoxythymidine 5′-phosphate.

(B) Synthesis of Affinity Labels. 3′-(Bromoacetamido)-3′-deoxyuridine. Dry 3′-amino-3′-deoxyuridine (0.5 g, 2.1 mmol) was suspended in 25 mL of Na-dried, freshly distilled *p*-dioxane at 25 °C. After stirring for 10 min, 0.26 mL (2.2 mmol) of bromoacetic anhydride was added. After 45 min, 0.2 mL of water was added to the homogeneous reaction mixture. Solvents were removed by vacuum evaporation, and the oily residue was dissolved in a mixture of *p*-dioxane and $CHCl_3$, adsorbed to 5 g of silica gel, and dried to a free-flowing powder. The powder was deposited on a 30 × 2 cm column of silica gel and eluted with a gradient containing $CHCl_3$ (2 L) and 20% MeOH–$CHCl_3$ (2 L) as limiting solutions. The major 280-nm-absorbing fractions were pooled and concentrated to a white powder. The yield was 0.6 g (80%). The powder was crystallized from warm absolute EtOH to give the product: mp 188–189 °C; UV_{max} (H_2O) 261 nm (ε 9980); IR (KBr) 1695, 1664, and 1634 cm^{-1} (C=O); NMR (CD_3SOCD_3) δ 11.21 (br s, 1, N_3H), 8.25 (br d, 1, $J_{3',NH}$ = 7 Hz, amide NH), 7.88 (d, 1, $J_{5,6}$ = 8 Hz, C_6H), 5.67 (d, 1, $J_{1',2'}$ = 3 Hz, $C_{1'}H$), 5.55 (dd, 1, $J_{5,6}$ = 8 Hz, J_{5,N_3H} = 1 Hz, C_5H), 3.85 (s, 2, $COCH_2Br$), 3.52 (m, 2, $C_{5'}H$). A sample was recrystallized from absolute EtOH and gave the following analytical data. Anal. Calcd for $C_{11}H_{14}N_3O_6Br$: C, 36.28; H, 3.87; N, 11.54; Br, 21.95. Found: C, 36.41; H, 3.86; N, 11.35; Br, 22.15.

2′-(Bromoacetamido)-2′-deoxyxylofuranosyluracil. Dry 2′-amino-2′-deoxyxylofuranosyluracil (0.49 g, 2 mmol) and 0.2 g (2 mmol) of triethylamine were mixed with 25 mL of Na-dried, freshly distilled *p*-dioxane and stirred for 10 min at 25 °C. Freshly distilled bromoacetic anhydride (0.52 g, 2 mmol) was added and the mixture stirred for 4 h, after which 250 μL of water was added. After being stirred an additional 30 min, the solution was evaporated to an oil, dissolved in MeOH, and adsorbed on 5 g of silica gel. The dried powder was added to a 29 × 2 cm column of silica gel equilibrated in $CHCl_3$. The product was eluted with a gradient containing $CHCl_3$ (1.7 L) and 20% MeOH–$CHCl_3$ (1.7 L) as limiting solutions. The (bromoacetamido)nucleoside eluted between 860 and 1250 mL and was evaporated to a white powder. Attempts to crystallize the product from MeOH failed, and the derivative was rechromatographed on a 24 × 0.9 cm column of silica gel employing a gradient of $CHCl_3$ (0.5 L) and 18% MeOH–$CHCl_3$ (0.5 L). Fractions corresponding to the major peak were pooled and evaporated to a white powder which was dried over P_2O_5 in vacuo. The yield was 0.16 g (22%): mp 118–120 °C; UV_{max} (H_2O) 261 nm (ε 10000); IR (KBr) 3175 (NH), 1695, 1681, and 1653 cm^{-1} (C=O); NMR (D_2O) δ 7.92 (d, 1, $J_{5,6}$ = 8 Hz, C_6H), 5.90 (d, 1, $J_{1',2'}$ = 4.5 Hz, $C_{1'}H$), 5.87 (d, 1, $J_{5,6}$ = 8 Hz, C_5H), 4.36 (d, 2, $C_{5'}H$), 3.87 (s, 2, $COCH_2Br$). Anal. Calcd for $C_{11}H_{14}N_3O_6Br$: C, 36.28; H, 3.87; N, 11.54; Br, 21.95. Found: C, 36.39; H, 3.79; N, 11.42; Br, 22.00.

3′-(Bromoacetamido)-3′-deoxyarabinofuranosyluracil. Dry 3′-amino-3′-deoxyarabinofuranosyluracil (0.73 g, 3 mmol) was suspended in 35 mL of Na-dried, freshly distilled *p*-dioxane. After 15 min bromoacetic anhydride (0.37 mL, 3 mmol) was

added and the mixture stirred at 25 °C for 20 min. Then 0.2 mL of H_2O was added, and the solvents were removed by evaporation. The residue was dissolved in MeOH and added to 5 g of silica gel. After the mixture was dried, the free-flowing powder was added to a 30 × 2 cm column of silicic acid equilibrated in $CHCl_3$. The separation was developed with a gradient containing $CHCl_3$ (1.5 L) and 20% MeOH–$CHCl_3$ (1.5 L) as limiting solutions. The effluent was monitored by 280-nm absorption, and fractions corresponding to the (bromoacetamido)nucleoside were pooled and evaporated to a white solid. The yield was 0.45 g (41%). A portion of the product was rechromatographed on a 24 × 0.9 cm column of silica gel with a gradient containing $CHCl_3$ (0.5 L) and 20% MeOH–$CHCl_3$ (0.5 L) as limiting solutions. Effluent fractions corresponding to (bromoacetamido)nucleoside were pooled and cooled at 4 °C for 2 days and then at −10 °C for 2 days. The resulting crystals were filtered and dried and weighed 92 mg. The mother liquor was evaporated at low temperature in vacuo, dissolved in dry dioxane, and lyophilized to give the product: mp 168–170 °C; UV_{max} 262 nm (ϵ 10 300); IR (KBr) 1695 (C=O), 1550 and 1471 cm^{-1} (NH); NMR (CD_3SOCD_3) δ 11.25 (br s, 0.7, N_3H), 8.70 (d, 0.7, $J_{3',NH}$ = 7 Hz, amide NH), 7.73 (d, 1, $J_{5,6}$ = 8 Hz, C_6H), 6.06 (d, 1, $J_{1',2'}$ = 4.5 Hz, C_1H), 5.60 (d, 1, $J_{5,6}$ = 8 Hz, C_5H), 3.88 (s, 2, $COCH_2Br$), 3.62 (d, 2, C_5H). Anal. Calcd for $C_{11}H_{14}N_3O_6Br$: C, 36.28; H, 3.87; N, 11.54; Br, 21.95. Found: C, 36.57; H, 3.96; N, 11.24; Br, 21.84.

3'-(Bromoacetamido)-3'-deoxythymidine. 3'-Amino-3'-deoxythymidine (3 g, 12.5 mmol, dried over P_2O_5 at 78 °C) was suspended in 200 mL of Na-dried *p*-dioxane at 11–12 °C. Bromoacetic anhydride (3 mL, 2-fold molar excess) was added to the suspension, which was stirred for 5 h whereupon all solids dissolved. Water, 1.75 mL, was added, and the solution was stirred for an additional 1 h. The reaction mixture was concentrated to an oil, dissolved in 10 mL of MeOH, and adsorbed onto 20 g of dry silicic acid and dried. The resulting powder was added to the top of a 40 × 3.75 cm column of silica gel equilibrated in $CHCl_3$. The column was eluted with a gradient of $CHCl_3$ (2 L) and 10% MeOH–$CHCl_3$ (2 L). Fractions corresponding to the major 280-nm-absorbing band, which eluted last from the column, were pooled and evaporated to a solid at 30 °C. The crystalline mass was recrystallized from 70 mL of hot EtOH to give 2.7 g (60%) in two crops: mp 171.5–173 °C; UV_{max} (H_2O) 267 nm (ϵ 9950); IR (KBr) 1717 and 1669 (C=O), 1560 and 1484 cm^{-1} (NH); NMR (CD_3SOCD_3) δ 11.15 (s, 0.9, N_3H), 9.00 (d, 0.7, $J_{3',NH}$ = 8 Hz, amide NH), 7.72 (s, 1, C_6H), 6.25 (t, 1, $J_{1'2'}$ = 3.5 Hz, C_1H), 3.80 (s, 2, $COCH_2Br$), 2.23 (q, 2, C_2H), 1.77 (s, 3, CH_3). Calcd for $C_{12}H_{16}N_3O_5Br$: C, 39.79; H, 4.45; N, 11.60; Br, 22.07. Found: C, 39.83; H, 4.37; N, 11.61; Br, 22.00.

RNase A Assay. Hydrolysis of cytidine 2',3'-cyclic phosphate was measured by using a difference spectrophotometric technique (Murdock et al., 1966). Transphosphorylation activity was determined with uridylyl(3',5')adenosine as substrate (Ipata & Felicioli, 1968).

Protein Chromatography. Columns of Amberlite CG-50 (type III, particle size 51 ± 13 cm) were filled and operated as previously described (Carty & Hirs, 1968). Preparative reaction mixtures were desalted, lyophilized, dissolved in the eluting buffer, heated at 65 °C for 15 min (Crestfield et al., 1962), diluted 1:3 with H_2O, and then applied to the column. Fractionation of protein derivatives from the reactions of 3'-(bromoacetamido)-3'-deoxyuridine and 3'-(bromoacetamido)-3'-deoxythymidine was achieved on 120 × 1.9 cm columns of resin. All other nucleoside–RNase A derivatives were purified on 30 × 0.9 cm columns. The eluting buffer was 0.2 M sodium phosphate, pH 6.37. Nucleoside–RNase A derivatives eluted from Amberlite CG-50 were sufficiently pure for structural identification with the exception of N^3-(3'-CAMUrd)-His-119-RNase A and N^1,N^3-bis(3'-CAMUrd)-His-119-RNase A, which were obtained as a mixture after chromatography on Amberlite CG-50 using 0.2 M sodium phosphate, pH 6.37 (cf. Figure 2). The mixture was desalted, lyophilized, dissolved in 0.2 M sodium phosphate, pH 6.02, heated for 15 min at 65 °C, diluted 1:3 with H_2O, and then applied to a 2.5 × 110 cm column of Amberlite CG-50 equilibrated with 0.2 M sodium phosphate, pH 6.02. Elution with this solvent separated the two derivatives completely.

The determination of residual hydrolytic and transphosphorylation activities was achieved after further purification of derivatives by ion-exchange chromatography on CM-52 (Lin et al., 1984). Analytical protein chromatography was performed as previously described (Pincus et al., 1975) except that 50 μL of reaction mixture containing 0.12 μmol of protein was loaded directly onto the column.

Peptide Fractionations. Separation of C-Peptide and C-Protein. All purified RNase A derivatives containing nucleoside substitution at His-12 were identified by cleavage with CNBr (Gross & Witkop, 1962). The C-peptide was separated from C-protein on a 120 × 3.75 cm column of Sephadex G-25 using 0.1 M acetic acid as eluent.

Separation of Peptides in Which Histidine-119 Contains Covalently Bound Nucleoside. Nucleoside–RNase A derivatives were oxidized with performic acid (Hirs, 1956). Performic acid oxidized proteins were treated sequentially with trypsin and chymotrypsin (Carty & Hirs, 1968) and then fractionated on columns of AG 50W-X2 by the procedure of Schroeder et al. (1962).

Amino Acid Analyses. Hydrolyses were carried out in sealed, evacuated tubes on 0.15–0.2 μmol of protein or peptide derivative, using 2 mL of constant-boiling HCl at 110 °C for 22 h. Amino acid analyses were performed by ion-exchange chromatography (Spackman et al., 1958) using ninhydrin solutions prepared according to Moore (1968).

Analytical Reactions of (Bromoacetamido)nucleosides with RNase A. Determination of K_b and k_3. Reaction mixtures were prepared that contained initially 4–117 mM concentrations of (bromoacetamido)nucleoside and 2.4 mM RNase A in 0.5 mL of 0.2 M sodium acetate buffer, pH 5.5, at 25 °C. At zero time and various times thereafter, 50-μL aliquots were withdrawn and pipetted into 3 mL of 0.2 M sodium phosphate, pH 6.37, which stops the alkylation reactions. The extent of reaction was determined by analysis of residual enzymatic activity using cytidine 2',3'-cyclic phosphate as substrate. Linear least-squares analysis of the log of the residual activity vs. time gave values for slopes from which first-order rate constants for inactivation were calculated. At low concentrations of alkylating agent, second-order rate constants were estimated by graphical analysis and first-order rate constants computed from these by multiplying by the initial alkylating agent concentration. Values of K_b and k_3 were estimated by using the direct linear method of Eisenthal and Cornish-Bowden (1974). Errors in the values of K_b and k_3 were estimated by the rank correlation methods described by Porter and Trager (1977).

Preparative Reactions of (Bromoacetamido)nucleosides with RNase A. (A) 2'-(Bromoacetamido)-2'-deoxyxylofuranosyluracil and RNase A. The reaction mixture contained 4.48 mg (12.3 μmol) of nucleoside and 34 mg (2.5 μmol) of

RNase A in 1.0 mL of 0.2 M sodium acetate buffer, pH 5.5, at 25 °C. After 6 min, the entire sample was added directly to a 30 × 0.9 cm column of Amberlite CG-50.

(*B*) *3′-(Bromoacetamido)-3′-deoxyuridine and RNase A.* A reaction mixture containing initially 70.4 mg (193 μmol) of nucleoside and 267 mg (19.5 μmol) of RNase A in a total volume of 7.5 mL of 0.2 M sodium acetate buffer, pH 5.5, was stirred at 25 °C for 5 h. The mixture was fractionated directly on a 120 × 1.9 cm column of Amberlite CG-50.

(*C*) *3′-(Bromoacetamido)-3′-deoxyarabinofuranosyluracil and RNase A.* A reaction mixture containing 5.7 mg (15.65 μmol) of nucleoside and 34 mg (2.5 μmol) of RNase A was prepared in 0.2 M sodium acetate, pH 5.5, up to 1 mL and stirred at 25 °C for 5 h. The mixture was fractionated directly on a 34 × 0.9 cm column of Amberlite CG-50.

(*D*) *3′-(Bromoacetamido)-3′-deoxythymidine and RNase A.* A reaction mixture containing 181 mg (500 μmol) of nucleoside and 329 mg (23.5 μmol) of RNase A was stirred in 0.2 M sodium acetate, pH 5.5, at 25 °C for 6 h. The total volume was 10 mL. The mixture was loaded directly onto a 120 × 1.9 cm column of Amberlite CG-50 and eluted with 0.2 M sodium phosphate, pH 6.37, at a flow rate of 20 mL/h.

Reaction of (Bromoacetamido)nucleosides with L-*Histidine.* Reaction mixtures, 0.3-mL total volume, were prepared containing 80 mM nucleoside and 10 mM L-histidine in 0.2 M sodium acetate, pH 5.5. They were incubated at 25 °C, and 50-μL aliquots were removed at 0, 24, 48, 72, and 96 h, added to 2 mL of constant-boiling HCl, and hydrolyzed for 22 h. The rate of alkylation of L-histidine was followed by measuring residual L-histidine on the short column of the amino acid analyzer. First-order rate constants were calculated from the slopes of plots of the log of L-histidine remaining vs. time.

Model Studies. A framework and partially space filled molecular model of RNase A was constructed by using the Academic Press/Molecular Design Inc. (AP/MDI) molecular model system. The model is built to a scale of 12.5 mm/Å and is based on the coordinates for the least-squares refined structure at 1.45-Å resolution (Borkakoti et al., 1982). Atomic coordinates, supplied with the model, were obtained from the Protein Data Bank at Brookhaven National Laboratory. Nucleosides were constructed from pieces also available in the AP/MDI system.

The search for the optimally reactive nucleoside conformation involved a systematic variation of the following parameters: (1) the glycosidic torsion angle defined as the dihedral angle O(1′)–C(1′)–N(1)–C(6) (Sundaralingham, 1969); (2) the variation of the sugar ring pucker (only C-(2′)-endo, C(3′)-endo, C(2′)-exo, and C(3′)-exo conformations were examined); (3) the dihedral angles C(1′)–C(2′)–N(2′)–C′ in 2′-(bromoacetamido)nucleosides and C(2′)–C(3′)–N(3′)–C′ in 3′-(bromoacetamido)nucleosides; and (4) the dihedral angles N(2′)–C′–C_α–Br in 2′-(bromoacetamido)nucleosides and N(3′)–C′–C_α–Br in 3′-(bromoacetamido)nucleosides. The rationale for these variations was, first, to provide a reasonably proximate and well-aligned bromomethylene carbon suitably placed for the backsided, in-line, nucleophilic attack of the N atom of the appropriate side chain and, second, to search for a conformation that might be stabilized by a H bond in which the donor was the OH hydrogen vicinal to the bromoacetamido group on the pentofuranose ring and the acceptor was a suitable group on the enzyme.

The following parameters were not allowed to vary: (1) the side-chain dihedral angles for His-12; (2) the requirement for two H bonds between the O(2) and N(3)H of the pyrimidine base of the bound nucleoside and the backbone amide H and side-chain O of Thr-45; (3) the side-chain dihedral angles of all active-site residues with the exception of those for His-119 [the side chain of His-119 exists in at least two conformations (Borkakoti et al., 1982; Richards & Wyckoff, 1973)]; and (4) gross movement of polypeptide segments within the active site that would alter the steric relationship of residues lining the cleft.

Given the above conditions, conformations of the nucleosides in the active site were judged optimal if they made no bad contacts with the enzyme residues of the active site and were involved in a number of favorable H bonds with the enzyme.

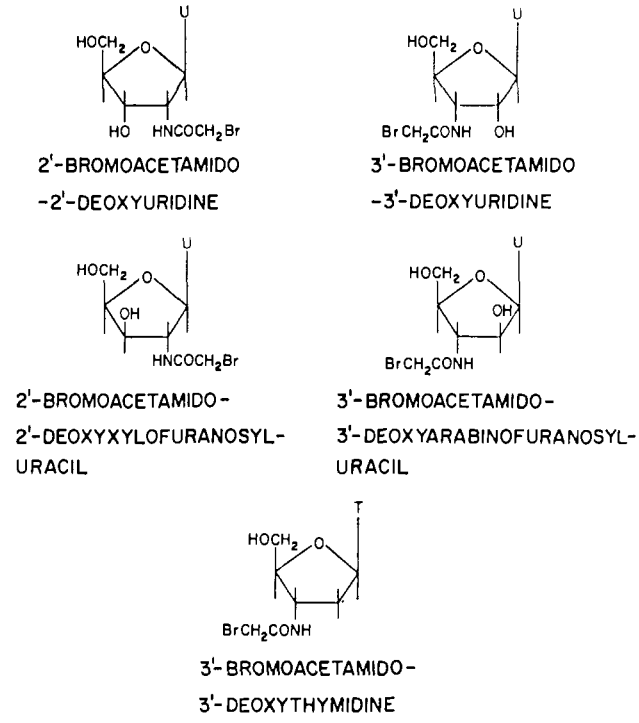

FIGURE 1: Nucleoside affinity labels for RNase A.

RESULTS

Figure 1 indicates the structures of the four newly synthesized bromoacetamido pyrimidine nucleosides and 2′-(bromoacetamido)-2′-deoxyuridine. Of the four uracil derivatives all possible stereochemical configurations of the pentofuranosyl sugar ring at the 2′- and 3′-positions in which the bromoacetamido group is "down" are represented. Common features include the β-configuration at the anomeric carbon of the sugar, which ensures that all four compounds possess a trans relationship between the pyrimidine base and the bromoacetamido group; that is, the bromoacetamido group and the pyrimidine base project from opposite sides of the sugar ring. The differences include the substitution of the bromoacetamido group at either the 2′- or 3′-position of the sugar, and within each of these groups, the neighboring vicinal hydroxyl group is either cis or trans to the bromoacetamido group. The single exception to the above isomers is 3′-(bromoacetamido)-3′-deoxythymidine in which the 2′-hydroxyl is replaced by a hydrogen.

Reaction of 3′-(Bromoacetamido)-3′-deoxyuridine with RNase A. The protein products formed in the reaction of 3′-(bromoacetamido)-3′-deoxyuridine with RNase A are depicted in the preparative chromatogram of Figure 2. The major derivative eluting after RNase A is N^1-(3′-CAMUrd)-His-119-RNase A, and it represents 70% of all alkylated proteins. Amino acid analysis of the purified protein

Table I: Amino Acid Analysis of Peptide Fragments Derived from Nucleoside-Substituted RNase A Derivatives

amino acids	peptide segment[a] A	B	C	D	E	F	G	H	I	J
Lys				2.10	1.99			2.01	2.03	2.02
His				0.11	0.08		0.22	0.05	0.06	0.06
Arg				0.97	0.97			1.00	1.04	1.01
Cys										
Asp										
Thr				1.02	1.00			0.92	1.02	1.05
Ser										
Glu				3.08	3.06			3.02	2.96	3.10
Pro	0.97	1.04	0.97			0.89	1.01			
Gly										
Ala				2.94	2.99			2.85	2.95	3.04
$^1/_2$-Cys										
Val	2.04	1.90	1.92			1.98	1.94			
Met										
Ile										
Leu										
Tyr										
Phe	1.01	1.01	1.07	0.99	0.98	1.01	1.11	1.04	1.03	0.95
N^3-Cm-His		0.90		0.90	1.08			0.97	0.87	0.91
N^1-Cm-His	0.78					0.73	0.69			
N^1,N^3-bis(Cm)-His			0.84							
Met sulfone										
Hse + Hse lactone				0.73	0.76			0.79	0.92	0.72
aminonucleoside[b]	0.96	1.30	2.3						1.21	0.77
yield (%)	59	70	33	69	62	38	76	63	66	57

[a] Relevant protein fragments isolated from cyanogen bromide (Gross & Witkop, 1962) or tryptic–chymotryptic digestion (Carty & Hirs, 1968) of modified proteins. Each column is lettered alphabetically as follows: A, peptide 116–120 from performic acid oxidized N^1-(3′-CAMUrd)-His-119-RNase A; B, peptide 116–120 from performic acid oxidized N^3-(3′-CAMUrd)-His-119-RNase A; C, peptide 116–120 from performic acid oxidized N^1,N^3-bis(3′-CAMUrd)-His-119-RNase A; D, C-peptide from N^3-(3′-CAMT)-His-12-RNase A; E, C-peptide from bis[N^3-(3′-CAMT)-His-12,N^1-(3′-CAMT)-His-119]-RNase A; F, peptide 116–120 from bis[N^3-(3′-CAMT)-His-12,N^1-(3′-CAMT)-His-119]-RNase A; G, peptide 116–120 from N^1-(3′-CAMT)-His-119-RNase A; H, C-peptide from N^3-(3′-CAMaraU)-His-12-RNase A; I, C-peptide from N^3-(2′-CAMxylU)-His-12-RNase A; J, C-peptide from N^3-(2′-CAMUrd)-His-12-RNase A. The theoretical composition of C-peptide derived from unmodified RNase A is Lys (2), His (1), Arg (1), Thr (1), Glu (3), Ala (3), Phe (1), and Hse plus Hse lactone (1). The theoretical composition of peptide 116–120 derived from unmodified RNase A is His (1), Pro (1), Val (2), and Phe (1). [b] The aminonucleoside content is corrected for loss during acid hydrolysis.

showed the loss of 1 mol of histidine and the appearance of approximately 1 mol of N^1-Cm-histidine per mole of protein. 3′-Amino-3′-deoxyuridine elutes between ammonia and arginine on the basic column of the amino acid analyzer. Only 22% of the aminonucleoside survives the hydrolytic conditions of constant-boiling HCl at 110 °C for 22 h in contrast to 2′-amino-2′-deoxyuridine, where 83% of the aminonucleoside was recovered (Lan & Carty, 1972). Definitive proof of structure was provided by isolating the nucleoside-substituted peptide represented by residues 116–120 in the native structure following ion-exchange chromatography of a peptide mixture formed from the successive tryptic–chymotryptic digest of the performic acid oxidized protein. The amino acid composition and yield of this peptide are found in Table I (cf. column A).

The double peak migrating more rapidly than RNase A contains N^3-(3′-CAMUrd)-His-119-RNase A formed in 14% yield and N^1,N^3-bis(3′-CAMUrd)-His-119-RNase A with a yield of 16%. These derivatives were completely separated by rechromatography on Amberlite CG-50 using 0.2 M sodium phosphate, pH 6.02, as eluting buffer. Amino acid analysis of the performic acid oxidized proteins showed the loss of 1 mol of histidine per mole of protein in each case. For N^3-(3′-CAMUrd)-His-119-RNase A, approximately 1 mol each of N^3-Cm-histidine and 3′-amino-3′-deoxyuridine is found per mole of protein derivative while for the disubstituted product, 1 mol of N^1,N^3-bis(Cm)-histidine and 2 mol of aminonucleoside are observed. The amino acid compositions of the substituted 116–120 peptides purified by ion-exchange chromatography are given in Table I [cf. column B for N^3-(3′-CAMUrd)-His-119-RNase A and column C for N^1,N^3-bis-(3′-CAMUrd)-His-119-RNase A]. All substituted peptides migrate in a position normally occupied by the segment Val-Pro-Val-His-Phe, residues 116–120, in the elution profile of tryptic–chymotryptic peptides of performic acid oxidized RNase A.

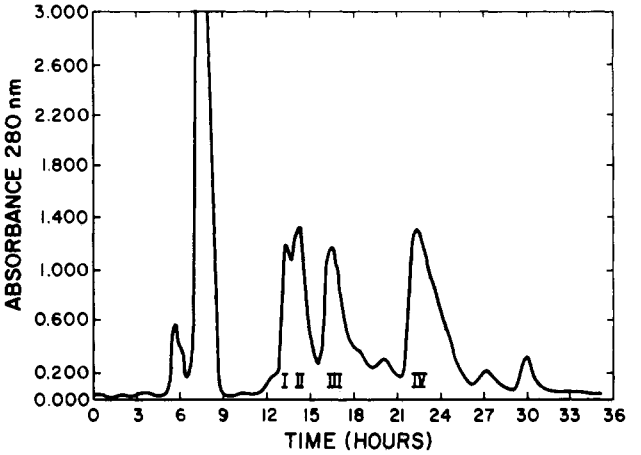

FIGURE 2: Amberlite CG-50 chromatography of products obtained on reacting 25.7 mM 3′-(bromoacetamido)-3′-deoxyuridine with 2.6 mM RNase A at pH 5.5 and 25 °C for 5 h. The zones are identified as follows: I, N^3-(3′-CAMUrd)-His-119-RNase A; II, N^1,N^3-bis-(3′-CAMUrd)-His-119-RNase A; III, RNase A; IV, N^1-(3′-CAMUrd)-His-119-RNase A.

The ratio of substitution at N-1 of His-119 to that at N-3 of His-119 by 3′-(bromoacetamido)-3′-deoxyuridine in all derivatives is ca. 3:1. No substitution at His-12 could be detected. The hydrolytic activity toward 2′,3′-cyclic CMP and transphosphorylating activity toward UpA of all three nucleoside–protein compounds are listed in Table II.

Table II: Residual Enzymatic Activities of Protein Derivatives Formed in Reactions of Affinity Labels with RNase A

protein derivative	relative activities (%)[a] trans-phosphorylation	hydrolysis
RNase A	100	100
N^3-(2′-CAMUrd)-His-12-RNase A	0.14	0.03
N^3-(3′-CAMaraU)-His-12-RNase A	0.31	0.18
N^3-(2′-CAMxylU)-His-12-RNase A	0.09	0.03
N^3-(3′-CAMT)-His-12-RNase A	0.22	0.28
N^1-(3′-CAMUrd)-His-119-RNase A	0.98	0.66
N^3-(3′-CAMUrd)-His-119-RNase A	4.39	5.28
N^1,N^3-bis(3′-CAMUrd)-His-119-RNase A	0.14	0.15
N^1-(3′-CAMT)-His-119-RNase A	1.21	1.26
bis[N^3-(3′-CAMT)-His-12,N^1-(3′-CAMT)-His-119]-RNase A	0.10	0.03

[a] Enzymatic activities are expressed as the percent of activity of the unmodified native enzyme. Concentrations of alkylated derivatives used in the assay were ca. 100–1000 times the concentration of the native enzyme.

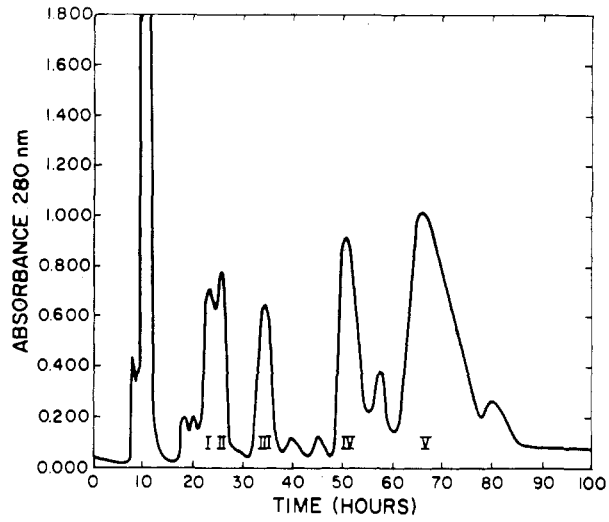

FIGURE 3: Amberlite CG-50 chromatography of products obtained on reacting 50 mM 3′-(bromoacetamido)-3′-deoxythymidine with 2.35 mM RNase A at pH 5.5 and 25 °C for 6 h. The zones are identified as follows: I, RNase A; II, N^3-(3′-CAMT)-His-119-RNase A; III, N^1-(3′-CAMT)-His-119-RNase A; IV, bis[N^3-(3′-CAMT)-His-12,N^1-(3′-CAMT)-His-119]-RNase A; V, N^3-(3′-CAMT)-His-12-RNase A.

Reaction of 3′-(Bromoacetamido)-3′-deoxythymidine with RNase A. The elution profile of protein derivatives formed in the preparative reaction of a 20-fold molar excess of 3′-(bromoacetamido)-3′-deoxythymidine with RNase A is exhibited in Figure 3. Four major derivatives are observed. The most retarded species (peak V, Figure 3), formed in 57% yield, is N^3-(3′-CAMT)-His-12-RNase A. The purified protein showed the loss of 1 mol of histidine and the appearance of 1 mol of N^3-Cm-histidine per mole of protein upon amino acid analysis. Treatment of the derivative with CNBr followed by gel chromatography gave the C-peptide in 69% yield. The composition of this peptide is given in column D of Table I and establishes the structure of this nucleoside–enzyme derivative as the species alkylated at N-3 of His-12.

3′-Amino-3′-deoxythymidine elutes between ammonia and arginine on the basic column of the amino acid analyzer; however, no aminonucleoside could be recovered in hydrolysates of peptide or proteins derived from the reaction of 3′-(bromoacetamido)-3′-deoxythymidine with RNase A.

Eluting slightly ahead of N^3-(3′-CAMT)-His-12-RNase A (peak IV, Figure 3) is a disubstituted protein formed in 14% yield that shows the loss of 2 mol of histidine and the appearance of 1 mol each of N^1-Cm-histidine and N^3-Cm-histidine per mole of protein upon amino acid analysis. This suggested that both His-12 and His-119 were substituted. Separate CNBr and tryptic–chymotryptic digestions revealed that the C-peptide contained 1 mol of N^3-Cm-histidine (cf. column E, Table I) and that fragment 116–120 contained ca. 1 mol of N^1-Cm-histidine (cf. column F, Table I), respectively, establishing the identity of this fraction as bis[N^3-(3′-CAMT)-His-12,N^1-(3′-CAMT)-His-119]-RNase A.

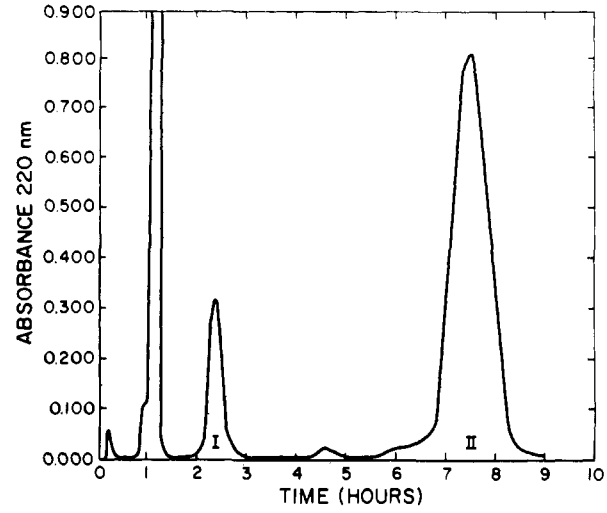

FIGURE 4: Amberlite CG-50 chromatography of products obtained on reacting 15.7 mM 3′-(bromoacetamido)-3′-deoxyarabinofuranosyluracil with 2.5 mM RNase A at pH 5.5 and 25 °C for 5 h. The zones are identified as follows: I, RNase A; II, N^3-(3′-CAMaraU)-His-12-RNase A.

The fraction that appears in the elution profile at 35 h (peak III, Figure 3) in a yield of 8% is a monosubstituted species which shows the disappearance of 1 mol of histidine and the appearance of ca. 1 mol of N^1-Cm-histidine per mole of protein upon amino acid analysis. The amino acid composition of peptide fragment 116-120 isolated from a tryptic–chymotryptic digest of the performic acid oxidized protein, indicated in column G of Table I, establishes the structure as N^1-(3′-CAMT)-His-119-RNase A.

The fourth derivative, which eluted as a band overlapping with RNase A, appeared at 26 h (peak II, Figure 3). It represents 9% of the substituted proteins and was tentatively identified from amino acid analysis of the tryptic–chymotryptic peptide, segment 116–120, as N^3-(3′-CAMT)-His-119-RNase A. The ratio of substitution at His-12 compared to that at His-119 is 2.3 to 1 while the ratio of substitution at N-1 of His-119 relative to that at N-3 of His-119 is 1.3 to 1. No derivative substituted at both N-3 of His-12 and N-3 of His-119 appears to form in major amounts. The enzymatic activities of the CM-52-purified fractions appear in Table II.

Reaction of 3′-(Bromoacetamido)-3′-deoxyarabinofuranosyluracil with RNase A. Figure 4 demonstrates the product distribution obtained from the reaction of a 6-fold molar excess of 3′-(bromoacetamido)-3′-deoxyarabinofuranosyluracil over enzyme. The sole derivative formed is N^3-(3′-CAMaraU)-His-12-RNase A. Amino acid analysis of the purified protein revealed the loss of 1 mol of histidine per mole of protein. 3′-Amino-3′-deoxyarabinofuranosyluracil chromatographs on the short column of the amino acid analyzer between ammonia and arginine. However, no aminonucleoside could be detected in chromatograms of hydrolysates of the alkylated protein or C-peptide. CNBr treatment followed by isolation of the C-peptide gave a fragment containing

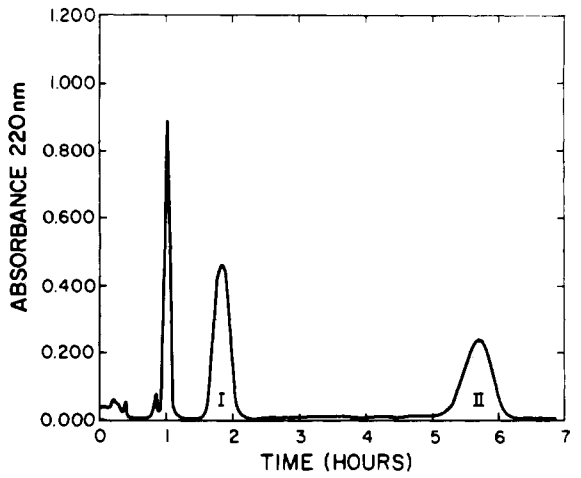

FIGURE 5: Analytical Amberlite CG-50 chromatography of products obtained on reacting 27.6 mM 2′-(bromoacetamido)-2′-deoxyxylofuranosyluracil with 2.3 mM RNase A at pH 5.5 and 25 °C for 2 min. RNase A was added as a marker to the 2-min sample and chromatographed along with the nucleoside–RNase A derivative. The zones are identified as follows: I, RNase A; II, N^3-(2′-CAMxylU)-His-12-RNase A.

1 mol of N^3-Cm-histidine (cf. column H, Table I), establishing alkylation at His-12. N^3-(3′-CAMaraU)-His-12-RNase A possesses very low transphosphorylating and hydrolytic activities, similar to what is observed for other His-12 derivatives (cf. Table II).

Reaction of 2′-(Bromoacetamido)-2′-deoxyxylofuranosyluracil with RNase A. N^3-(2′-CAMxylU)-His-12-RNase A is the only alkylation product formed in the reaction of a 5-fold molar excess of 2′-(bromoacetamido)-2′-deoxyxylofuranosyluracil over RNase A (cf. Figure 5). The structure of the alkylated derivative was revealed from amino acid analysis of the purified protein and the C-peptide fragment obtained from CNBr cleavage. The protein showed the loss of 1 mol of histidine and the appearance of 1 mol of N^3-Cm-histidine. The C-peptide contained no histidine, and 1 mol of N^3-Cm-histidine was observed (cf. column I, Table I). 2′-Amino-2′-deoxyxylofuranosyluracil chromatographs between ammonia and arginine on the amino acid analyzer. When the aminonucleoside is treated for 22 h at 110 °C in constant-boiling HCl, 63% is destroyed. The C-peptide derived from N^3-(2′-CAMxylU)-His-12-RNase A analyzes for 1.2 mol of aminonucleoside. The high value may be due to the significant overlap between the homoserine lactone and aminonucleoside peaks. The CM-52-purified alkylated RNase A possesses less than one-tenth of 1% of the transphosphorylating and hydrolytic activity of the native enzyme (cf. Table II).

Reaction Kinetics of the Affinity Labels. The reactions of all bromoacetamido pyrimidine nucleosides with RNase A at pH 5.5 and 25 °C exhibit either pseudo-first-order or second-order kinetics. For all nucleoside derivatives, calculated first-order rate constants were nonlinearly dependent on affinity label concentration. The kinetic behavior is expressed in the model (Gold & Fahrney, 1964; Pincus et al., 1975)

$$E + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EB \xrightarrow{k_3} P \qquad (1)$$

where K_b, equal to k_2/k_1, is the dissociation constant of the RNase A–affinity label complex. Values of K_b and k_3 for the reaction of each bromoacetamido pyrimidine nucleoside with RNase A are given in Table III. Values of K_b and k_3 for 2′-(bromoacetamido)-2′-deoxyuridine and 2′(3′)-*O*-(bromoacetyl)uridine are included for comparative purposes. Values of K_b for all nucleosides range from 0.038 to 0.18 M, a factor of 5. K_b is relatively insensitive to the structure of the nucleoside. Values of k_3 range from 2.7×10^{-4} s^{-1} for 3′-(bromoacetamido)-3′-deoxythymidine to 1700×10^{-4} s^{-1} for 2′-(bromoacetamido)-2′-deoxyxylofuranosyluracil, a factor of 630. Values of k_3/K_b, which provide an estimate of the overall reaction rate and include contributions from nucleoside binding as well as the k_3 step [cf. Pincus et al. (1975)], vary from 28 $\times 10^{-4}$ M^{-1} s^{-1} for 3′-(bromoacetamido)-3′-deoxythymidine to 9100×10^{-4} M^{-1} s^{-1} for the xylofuranosyl nucleoside, a factor of 330. Variations in k_3/K_b follow variations in k_3. Table III also includes the value of the second-order rate constant for the inactivation of RNase A by bromoacetamide at pH 5.5 and 25 °C.

Table III: Kinetic Constants for the Interaction of Bromoacetamido Pyrimidine Nucleosides with RNase A

nucleoside	affinity label concn (mM)	K_b (M)	$10^4 k_3$ (s^{-1})	$10^4 k_3/K_b$ or $10^4 k''_{obsd}$[a] (M^{-1} s^{-1})
2′-(bromoacetamido)-2′-deoxyuridine	4.1–47	0.14[b]	58	410
2′-(bromoacetamido)-2′-deoxyxylofuranosyluracil	16–87	0.18	1700	9100
3′-(bromoacetamido)-3′-deoxyuridine	8.7–78	0.062	3.3	54
3′-(bromoacetamido)-3′-deoxyarabinofuranosyluracil	16–117	0.038	6.6	170
3′-(bromoacetamido)-3′-deoxythymidine	15–105	0.094	2.7	28
2′(3′)-*O*-(bromoacetyl)-uridine[c]	12–303	0.087	35	400
bromoacetamide[c]				1.9

[a] Entries are given as k_3/K_b for affinity labels and k''_{obsd} for the alkylating agent, bromoacetamide. [b] Median and 95% confidence limits are as follows: 2′-(bromoacetamido)-2′-deoxyuridine, K_b $p(0.10 < 0.14 < 0.29) > 0.95$, k_3 $p(0.0021 < 0.0058 < 0.012) > 0.95$; 2′-(bromoacetamido)-2′-deoxyxylofuranosyluracil, K_b $p(0.10 < 0.18 < 0.26) > 0.95$, k_3 $p(0.10 < 0.17 < 0.22) > 0.95$; 3′-(bromoacetamido)-3′-deoxyuridine, K_b $p(0.039 < 0.062 < 0.084) > 0.95$, k_3 $p(0.00025 < 0.00033 < 0.00040) > 0.95$; 3′-(bromoacetamido)-3′-deoxyarabinofuranosyluracil, K_b $p(0.022 < 0.038 < 0.092) > 0.95$, k_3 $p(0.00048 < 0.00066 < 0.0015) > 0.95$; 3′-(bromoacetamido)-3′-deoxythymidine, K_b $p(0.068 < 0.094 < 0.17) > 0.95$; k_3 $p(0.00022 < 0.00027 < 0.00046) > 0.95$. [c] Data taken from Pincus et al. (1975).

Table IV: Second-Order Rate Constants for Reaction of L-Histidine with Bromoacetamido Pyrimidine Nucleosides and Rate Acceleration Factors for Alkylation of RNase A Relative to L-Histidine

nucleoside	$10^4 k''$ (M^{-1} s^{-1}) (L-His)	$(k_3/K_b)/k''$ or k''_{obsd}/k''	rate enhancement at His-12 due to nucleoside binding
2′-(bromoacetamido)-2′-deoxyuridine	0.113	3600	29
2′-(bromoacetamido)-2′-deoxyxylofuranosyluracil	0.084	110000	850
3′-(bromoacetamido)-3′-deoxyuridine	0.074	730	
3′-(bromoacetamido)-3′-deoxyarabinofuranosyluracil	0.122	1400	11
3′-(bromoacetamido)-3′-deoxythymidine	0.107	260	1.5
2′(3′)-*O*-(bromoacetyl)uridine[a]	0.129	3100	25
bromoacetamide[a]	0.0155	125	

[a] Data taken from Pincus et al. (1975).

Reaction of Affinity Labels with L-Histidine. L-Histidine was allowed to react with an 8-fold molar excess of each of the bromoacetamido pyrimidine nucleosides at pH 5.5 and 25 °C. Second-order rate constants, k'', are given in Table IV.

Values range from 0.074×10^{-4} to 0.122×10^{-4} M^{-1} s^{-1}, a factor of only 1.65. Values for 2′-(bromoacetamido)-2′-deoxyuridine, 2′(3′)-*O*-(bromoacetyl)uridine, and bromoacetamide are also given for comparison. Overall rate-acceleration factors are calculated as the ratio, k_3/K_b, for each nucleoside divided by k''. These factors vary from 260 for 3′-(bromoacetamido)-3′-deoxythymidine to 110000 for 2′-(bromoacetamido)-2′-deoxyxylofuranosyluracil.

Discussion

General Considerations. The alkylation of RNase A by bromoacetamido pyrimidine nucleosides includes several important features. 2′-(Bromoacetamido)nucleosides react exclusively with N-3 of His-12, irrespective of the configuration of the adjacent 3′-OH group. 3′-(Bromoacetamido)nucleosides react at both active-site histidine residues. Only 3′-(bromoacetamido)-3′-deoxyuridine reacts exclusively with His-119. Epimerization of the "down" 2′-OH group of 3′-(bromoacetamido)-3′-deoxyuridine to give 3′-(bromoacetamido)-3′-deoxyarabinofuranosyluracil reverses the specificity of active-site alkylation so that only His-12 is modified. Removal of the 2′-OH group as in 3′-(bromoacetamido)-3′-deoxythymidine results in alkylation predominantly at His-12 but also in some modification of His-119. Alkylation rates of 2′-(bromoacetamido)nucleosides are substantially greater than those of 3′ derivatives, and finally, the 3′-epimers of the 2′-(bromoacetamido)nucleosides also show large differences in reaction rate.

To explain these results, the following assumptions are made: (1) The uracil or thymine portion of all (bromoacetamido)-nucleosides binds in the active site in a similar manner (Richards & Wyckoff, 1971, 1973; Pavlovsky et al., 1978). Groups on the surface of the enzyme that bind the pyrimidine to the B_1 site are not significantly repositioned subsequent to interaction with the base (Borkakoti, 1983). (2) The bromoacetamido pyrimidine nucleosides can adopt a variety of conformations in the active site. (3) Selected conformations of (bromoacetamido)nucleosides may be stabilized by specific interactions of sugar ring OH atoms with groups at the active site (Walz, 1971; Pavlovsky et al., 1977). (4) The His-119 side chain but not the His-12 side chain is capable of rotation about the C_α–C_β and C_β–C_γ bonds (Wyckoff et al., 1970; Borkakoti, 1983; Richards & Wyckoff, 1971; Wlodawer et al., 1982; Wlodawer & Sjolin, 1983).

Factors Contributing to Specificity and Reactivity. Several factors may contribute to the differences in specificity and reaction rate of (bromoacetamido)nucleosides with RNase A. First, 2′- and 3′-bromoacetamido substituents may influence the location of the $-CH_2Br$ group in the active site. Second, differences in the conformations of bound nucleoside alkylating agents may alter the position of the bromoacetamido group. Third, intrinsic differences in the reactivity of (bromoacetamido)nucleosides may arise as a result of interaction of vicinal OH groups with the bromoacetamido group. Fourth, bound nucleosides may interact with active-site histidine residues, resulting in modification of the intrinsic nucleophilic reactivity of the imidazole groups.

It is assumed that the electrophilic character of the bromomethylene carbon is unaffected by the neighboring vicinal OH group. Potentially, interactions such as a O—H···O=C H bond between the bromoacetamido carbonyl O and a *cis*-hydroxyl H could activate the electrophilic center to attack by a nucleophile. Such a H bond would not form in the *trans*-OH epimer. Alternatively, a H bond such as H–O··· H–N between the amide H of the bromoacetamido group and a *cis*-hydroxyl O atom might deactivate the bromomethylene center compared with the *trans*-OH epimer. Evidence supporting the absence of these effects is embodied in the similarity of the reaction rates of L-histidine with all (bromoacetamido)nucleosides. It is further assumed that the intrinsic nucleophilicity of N-3 of His-12 is unmodified by the presence of bound nucleosides. While no definitive proof exists for this assumption, it is supported circumstantially by the observation that the pK_a of this residue is unaffected by the binding of UpcA (Griffin et al., 1973; Blackburn & Moore, 1982). Furthermore, the reaction rate of His-12 with a variety of electrophiles such as the anionic bromo- and iodoacetate, as well as the uncharged bromo- and iodoacetamides, shows the same relative rate enhancement with respect to the reaction of each of these compounds with L-histidine or histidine hydantoin (Pincus et al., 1975; Fruchter & Crestfield, 1967; Lenette & Plapp, 1979a,b). The rate increase is a factor of approximately 120 for each alkylating agent. The anionic alkylating agents bind to the active site prior to reaction (Lenette & Plapp, 1979b; Plapp, 1973). Yet the rate enhancements for all derivatives, irrespective of charge, prior binding, or leaving group (I^- and Br^-), are essentially identical. While these agents are not nucleosides, the similarity of rate enhancements of a spectrum of different alkylating agents suggest the absence of special effects activating or deactivating N-3 or His-12. Thus, the difference in specificity and alkylation rate is attributed to the bromoacetamido substitution site on the pentofuranosyl ring and also on intrinsic conformational differences in the bound nucleosides at the active site. These structural effects position the bromoacetamido group near either His-12 or His-119 and control the orientation of the reactive groups.

Specificity and Reactivity of 2′-Substituted Nucleosides. For 2′-(bromoacetamido)-2′-deoxyuridine (Lan & Carty, 1972) and 2′-(bromoacetamido)-2′-deoxyxylofuranosyluracil, the specificity is absolute for alkylation of N-3 of His-12. The stereochemistry of the OH group adjacent to the 2′-bromoacetamido moiety affects only the rate. It appears likely that the "up" 3′-OH group of the xylofuranosyl isomer forms an interaction with the enzyme that stabilizes a particularly reactive conformation. This conformation would be accessible to 2′-(bromoacetamido)-2′-deoxyuridine in which the 3′-OH is "down"; however, due to the absence of any interaction of the 3′-OH group, it would not be as stable as in the xylofuranosyl isomer.

Through use of the model-building procedures it was possible to construct an optimally reactive nucleoside conformation which provides the necessary in-line geometry and distance for nucleophilic attack on the bromomethylene carbon by the N-3 of His-12 (cf. Figure 6). The uracil base makes the assumed stable contacts with the enzyme (Richards & Wyckoff, 1971). In this conformation, the bromomethylene carbon is close to N-3 of His-12. A H bond exists between the "up" 3′-OH hydrogen and the N-1 of His-119, which can also hydrogen bond with the carboxyl group of Asp-121. In addition, the bromoacetamido carbonyl O atom is within hydrogen-bonding distance of the side-chain amido group of Gln-11 and sufficiently close to the ϵ-amino group of Lys-41 to form a favorable ion–dipole interaction. The Lys-7 ϵ-ammonium ion may stabilize the departing Br^- ion during nucleophilic attack on the $-CH_2-$ group. There is an unrestricted path in the active site for Br^- to leave.

It would appear that the considerable number of favorable interactions of the bromoacetamido residue and the strong H bond formed by the "up" 3′-OH group and the His-119 side chain "freeze" the bromoacetamido group in the optimally

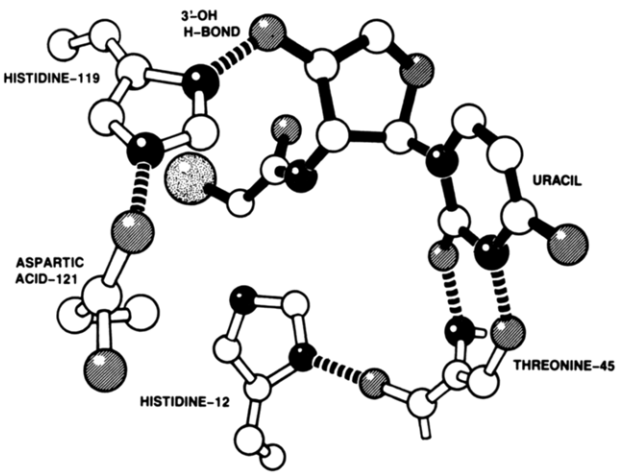

FIGURE 6: Proposed structure of optimally reactive complex of 2′-(bromoacetamido)-2′-deoxyxylofuranosyluracil with RNase A. The atom code is as follows: open circles represent carbon, solid circles nitrogen, cross-hatched circles oxygen, and stippled circles bromine. Nucleoside atoms are connected by solid bars and enzyme atoms by open bars. H bonds are represented by segmented bars. The critical H bond partly responsible for the specificity and high alkylation rate of 2′-(bromoacetamido)-2′-deoxyxylofuranosyluracil is located in the top central portion of the figure where it joins N-1 of His-119 to the 3′-OH of the nucleoside.

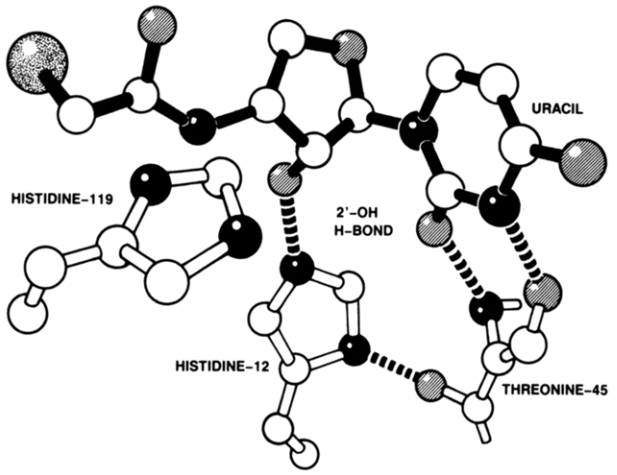

FIGURE 7: Proposed structure of optimally reactive complex of 3′-(bromoacetamido)-3′-deoxyuridine with RNase A. Atom and bond representations are identical with those in Figure 6. The critical H bond responsible for the alkylation specificity of this (bromoacetamido)nucleoside is located in the center of the figure where it connects N-3 of His-12 to the 2′-OH oxygen of the nucleoside.

reactive conformation and accounts for the unusually high reactivity of the xylofuranosyl isomer. In 2′-(bromoacetamido)-2′-deoxyuridine the optimally reactive conformation is stabilized only by the bromoacetamido group interactions. This suggests that the reduction in k_3, a factor of 30 relative to the xylofuranosyl isomer, is due to the existence of other conformations of the 2′-bromoacetamido group that have a greater probability because the 3′-OH cannot form a H bond with His-119 and these conformations do not optimally align the N-3 of His-12 for in-line attack on the bromomethylene carbon. The putative H bond between the "up" 3′-OH group and the N-1 of His-119 therefore stabilizes a major "productive" binding mode.

Evidence that the "up" 3′-OH group of the xylofuranosyl isomer can interact with the enzyme comes from inhibitor studies comparing the competitive inhibition constant for uridine, 9–13 mM (Ukita et al., 1961; Pincus et al., 1975; Lindquist et al., 1973), with that for 1-β-D-xylofuranosylthymine, 1 mM (Ukita et al., 1961), a factor of 10 favoring the xylofuranosyl derivative.

Specificity of 3′-Substituted Nucleosides. 3′-(Bromoacetamido)-3′-deoxyuridine. With 3′-(bromoacetamido)-3′-deoxyuridine not even small quantities of His-12 substitution products could be detected in the minor peaks in preparative chromatograms. The structural feature that distinguishes this (bromoacetamido)nucleoside from all the others is the "down" 2′-OH group. It is proposed that the unique alkylation specificity is associated with a specific interaction of the "down" 2′-OH group with the N-3 atom of the imidazole ring of His-12. This interaction is strong and may weaken the nucleophilic reactivity of His-12, but it stabilizes the position of the furanose ring in the active site.

Application of the model-building search method resulted in one structure in which the $-CH_2Br$ group is in an appropriate position to react with the N-1 of His-119 (cf. Figure 7). The bromoacetamido group does not make specific favorable contacts with groups on the enzyme and appears to be more "flexible" in that a number of conformations for this group other than the "reactive" one can exist. The absence of these stabilizing interactions may explain the lower rate of reaction compared with that of the 2′-substituted derivatives.

Implications for Catalysis. The critical feature of the reaction of 3′-(bromoacetamido)-3′-deoxyuridine with RNase A is the absolute specificity of reaction for His-119. The orientation of the 3′-substituent toward His-119 appears to depend upon the interaction of the "down" 2′-OH group with His-12. In the first step (transphosphorylation) of RNase A catalyzed hydrolysis of 3′,5′-ribonucleotides such as UpA, His-12 is thought to act as a general base with respect to the nucleophilic 2′-OH group while His-119 protonates the 5″-oxygen of the leaving nucleoside (Blackburn & Moore, 1982). If this mechanism is correct, then the 5″-oxygen of the departing nucleoside must lie close to the N-1 (or, less likely, the N-3) of His-119. From the results obtained with 3′-(bromoacetamido)-3′-deoxyuridine it is suggestive that in addition to increasing its nucleophilicity the interaction of the "down" 2′-OH group of nucleotide substrates with His-12 may orient the 3′-substituent, particularly the 5″-oxygen of the departing nucleoside, toward His-119. Absence of the "down" 2′-OH group results in loss of specificity of reaction of (bromoacetamido)nucleosides with His-119 (cf. Figures 3 and 4).

Crystallographic studies on the structures of a number of different nucleotide inhibitors of RNase A including UpcA (Richards & Wyckoff, 1971), 2′,5′-CpA (Wodak et al., 1977), and 3′-CMP (Richards & Wyckoff, 1971) reveal that the phosphate moiety binds in the same position. The P atom lies approximately equidistant between N-1 of His-119 and N-3 of His-12. In the UpcA–RNase A complex, the phosphonate methylene carbon (replaced by an oxygen in the substrate, UpA) is within H-bond distance of the N-1 of His-119. The distance from the 2′-OH oxygen to the N-3 of His-12 is somewhat greater, but the distance from the 2′-oxygen to the side chain of Asn-44 is sufficiently close to form a favorable H bond. The latter residue is thought to be involved in catalysis, possibly aiding in proton transfer (Blackburn & Moore, 1982; Gutte, 1975).

Placement of the 3′-(bromoacetamido)-3′-deoxyuridine in the same position as that for the Up portion of UpcA resulted in a structure with favorable interactions. However, the $-CH_2Br$ group could not be appropriately oriented for attack by the N-1 of His-119. A favorable alignment of the alkylating group with N-1 of His-119 could be achieved by altering the glycosidic torsion angle from 0°, that found in the UpcA–RNase A complex, to 25°.

If the conformation of UpcA is changed so that the uridine portion occupies the same position as the one proposed for 3′-(bromoacetamido)-3′-deoxyuridine, the same favorable contacts occur as in the original X-ray structure except that now the 2′-oxygen lies closer to the N-3 of His-12, while the 5″-oxygen ($-CH_2$ group in the inhibitor) lies closer to the N-1 of His-119. The P atom and attached oxygens move slightly upwards toward the surface of the active-site cleft. The extent of this movement may be governed in part by the nature of the 5′-nucleoside and may partly explain differences in rates of transphosphorylation by UpX substrates where X is A, G, U, or C.

It is possible that the conformation of the inhibitor, UpcA, bound to RNase A is a catalytically active one for substrates such as UpA. In this structure the 2′-OH group can hydrogen bond to the carboxamido group of Asn-44 but not to the N-3 of His-12. The carboxamido group is not a likely candidate as a general base compared with the imidazole group. In view of the proposed mechanism of catalysis, it seems more likely that, in the approach to the transition state, the structure of the complex may change minimally to the one proposed to explain the specificity of reaction of 3′-(bromoacetamido)-3′-deoxyuridine.

Mechanism of Formation of Disubstituted His-119 Derivatives. The existence of alkylation of both N-1 and N-3 of His-119 fortifies the notion of rotation about the C_α–C_β bond in the side chain of this residue. The ratio of substitution at N-1 relative to that at N-3 is ca. 3. This is close to the site occupancy ratio of 4:1 observed in the crystal structure of RNase A (Borkakoti et al., 1982). Binding of 2′-CMP does not alter this ratio (Borkakoti, 1983). The close similarity of these ratios suggests that it is the imidazole N-1 which faces predominantly into the active site and that both N-1 and N-3 may be capable of protonating the leaving group oxygen in catalytically sensitive dinucleoside phosphates.

Of interest is the detection of substantial quantities of an N-1,N-3-disubstituted His-119 alkylation product. A dialkylated product could arise from one of three mechanisms. In the first mechanism, a second alkylation of His-119 might occur through a bimolecular collision process without prior binding. However, the absence of secondary alkylation products in the reactions of 2′-(bromoacetamido)nucleosides with RNase A, particularly 2′-(bromoacetamido)-2′-deoxyuridine in which the 3′-OH group does not interact with His-119, points strongly to the requirement for a prior binding step in all alkylations. A second mechanism invokes the existence of two nucleoside binding sites that do not overlap and that direct the alkylation to either N-1 or N-3 of His-119. Richards and Wyckoff (1971) postulate the existence of a B_2'–R_2 site capable of binding the uridine portion of cytidylyl(3′,5′)uridine.

The third mechanism for alkylation of both N-1 and N-3 of His-119 in the same molecule supposes binding to a single site prior to the first alkylation followed by movement of the covalently bound nucleoside out of this site and concurrent rotation of the imidazole ring of His-119 as a prerequisite to binding the second nucleoside molecule in the same site. Support for this mechanism comes from the observation that the imidazole ring of His-119 occupies one of two subsites in the free enzyme as well as in RNase A–nucleotide complexes (Borkakoti et al., 1982; Borkakoti, 1983).

Effect of the "Up" 2′-OH Configuration on the Specificity of Reaction. The most unusual alkylation involves the reaction of 3′-(bromoacetamido)-3′-deoxyarabinofuranosyluracil with RNase A. The derivative lacks the "down" 2′-OH group that orients 3′-(bromoacetamido)-3′-deoxyuridine to react exclusively with His-119. The only product is the N^3-(3′-CAMaraU)-His-12-RNase A derivative.

Model-building studies reveal that a structure exists in which the $-CH_2Br$ group lies close to the N-3 of His-12 while the "up" 2′-OH group hydrogen bonds to the N-3 of His-119. The H bond to the "up" 2′-OH group is made possible by an unusual rotation of the imidazole side chain of His-119. While the nucleoside may be considerably immobilized in this conformation, the bromoacetamido group may have substantial flexibility due to the absence of specific interactions between atoms of the enzyme and the bromoacetamido group. The absence of these interactions may account for the low alkylation rate of His-12 compared with the 2′-substituted xylofuranosyl and ribofuranosyl derivatives. The ratio of k_3 values for 2′-(bromoacetamido)-2′-deoxyxylofuranosyluracil to 3′-(bromoacetamido)-3′-deoxyarabinofuranosyl is 250 to 1.

The reactions of arabinofuranosyl and xylofuranosyl nucleosides involve specific secondary interactions of their respective trans "up" OH groups with His-119. Evidence supporting this view shows that the 3′-phosphate ester of arabinofuranosyluracil binds RNase A 3 times more tightly than 3′-UMP (Pollard & Nagyvary, 1973). The dinucleoside phosphate arabinofuranosyluracilyl(3′,5′)uridine binds to RNase A with a dissociation constant, 0.48 mM, nearly 8 times lower than the K_m for uridylyl(3′,5′)uridine (Gassen & Witzel, 1967). Of all five (bromoacetamido)nucleosides, the arabinofuranosyl derivative has the lowest value of K_b, 0.038 M.

Reaction of 3′-(Bromoacetamido)-3′-deoxythymidine. The reaction of 3′-(bromoacetamido)-3′-deoxythymidine with RNase A is characterized by substitution at both His-12 at N-3 and His-119 at N-1 and N-3. Because of the absence of steric hinderance at C(2′) and any hydroxyl-mediated, furanose ring stabilizing interaction with the enzyme, the glycosidic torsion angle can assume a wide range of values in the C(3′)-endo ring pucker. This allows the nucleoside to adopt the conformation favored by 3′-(bromoacetamido)-3′-deoxyuridine, accounting for alkylation at either N-1 or N-3 of His-119. In the C(2′)-endo ring pucker mode (Hruska, 1973), the glycosidic torsional angle is even less restricted, suggesting that the nucleoside can adopt a conformation similar to that which leads to exclusive reactivity of 3′-(bromoacetamido)-3′-deoxyarabinofuranosyluracil with N-3 of His-12. The ratio of substitution at N-3 of His-12 to that at both N atoms of His-119 is 2.3:1, which is almost identical with the ratio of k_3 values for alkylation of RNase A by 3′-(bromoacetamido)-3-deoxyarabinofuranosyluracil and 3′-(bromoacetamido)-3′-deoxyuridine, 2:1 (cf. Table III). This strongly supports the notion that 3′-(bromoacetamido)-3′-deoxythymidine can alkylate RNase A from the optimally reactive conformation of either the 3′-ribofuranosyl or 3′-arabinofuranosyl isomer and that the effect of the "up" or "down" hydroxyl group at the 2′-position is to stabilize one or the other conformer.

An unusual product of the 3′-(bromoacetamido)-3′-deoxythymidine reaction is bis[N^3-(3′-CAMT)-His-12,N^1-(3′-CAMT)-His-119]-RNase A, formed in 14% yield. The nucleoside portion of N^3-(3′-CAMT)-His-12-RNase A, unlike His-119 derivatives, cannot rotate out of the B_1–R_1 site (Borkakoti, 1983). If the monoalkylated His-12 derivative is the substrate for the second alkylation at N-1 of His-119, then a second binding site is required. If the binding to the B_1–R_1 site is a prerequisite for the alkylation of N-1 of His-119 by 3′-(bromoacetamido)-3′-deoxythymidine, then N^3-(3′-CAMT)-His-12-RNase A is not a precursor of the disubsti-

tuted product. In this case the alkylation sequence would involve monoalkylation of N-1 of His-119 from an enzyme–nucleoside complex involving the B_1–R_1 site. Next the nucleoside moves out of the B_1–R_1 site, a second (bromoacetamido)nucleoside molecule binds, and alkylation proceeds at N-3 of His-12. These two alternative pathways can be distinguished by analyzing the product distribution of mixtures of 3′-(bromoacetamido)-3′-deoxythymidine and N^3-(3′-CAMT)-His-12-RNase A as well as the nucleoside and N^1-(3′-CAMT)-His-119-RNase A.

Activities of Enzyme Derivatives. All derivatives substituted at N-3 of His-12 and N^1,N^3-bis(3′-CAMUrd)-His-119-RNase A are considered to be inactive. N^1-(3′-CAMUrd)- and N^1-(3′-CAMT)-His-119-RNase A each have ca. 1% transphosphorylating and 1% hydrolytic activity. N^3-(3′-CAMUrd)-His-119-RNase A has ca. 5% transphosphorylating and 5% hydrolytic activity. Inactivity of all His-12-modified derivatives and N^1,N^3-bis(3′-CAMUrd)-His-119-RNase A suggests that the nucleoside portions of each of these products occupies the B_1–R_1–p_1 subsite where transphosphorylating and hydrolytic activity alike would be blocked with equal effectiveness. The simplest explanation for the appearance of similar values of the relative transphosphorylating and hydrolytic activities in N-1 or N-3 His-119 derivatives is that these alkylations must have occurred subsequent to prior binding in the B_1–R_1–p_1 subsite. Residual activity is associated with mobility of the nucleoside moiety which would carry it out of the B_1–R_1–p_1 subsite but not into the B_2–R_2 subsite. This would leave the entire active site free to bind UpA and 2′(3′)-cyclic CMP. The difference between the derivatives substituted at N-1 of His-119 (1% activity) and those at N-3 (5% activity) may reflect the tendency of the His-119 imidazole chain to occupy the A or B sites (Borkakoti, 1983).

In Table IV are included the rate acceleration factors for reaction at N-3 of His-12 attributable to nucleoside binding. The value for 3′-(bromoacetamido)-3′-deoxythymidine is based on the product distribution, there being 71% overall substitution at the His-12 position. With the inherent nucleophilicity of N-3 of His-12 accounting for a rate acceleration of 125 in all derivatives, the order of decreasing rate enhancement due to orientation effects in nucleoside binding is as follows: 2′-(bromoacetamido)-2′-deoxyxylofuranosyluracil ≫ 2′-(bromoacetamido)-2′-deoxyuridine = 2′(3′)-*O*-(bromoacetyl)-uridine > 3′-(bromoacetamido)-3′-deoxyarabinofuranosyluracil > 3′-(bromoacetamido)-3′-deoxythymidine. The low contribution of nucleoside binding to the rate enhancement in the thymidine derivative is consistent with a very mobile sugar ring with extensive flexibility about the torsional glycosidic bond. Enhanced mobility in the thymidine derivative implies the absence of selected interactions with the enzyme that would serve to anchor the reactive bromoacetamido group in an orientation favorable for reaction. Obviously, 2′-bromoacetamido derivatives have a more favorable orientation relative to 3′ derivatives. However, in both 2′ and 3′ derivatives the orientation of the vicinal OH group, or even whether it is present or absent, plays a pivotal role in determining the conformational accommodation of the nucleoside at the active site.

Acknowledgments

We thank Dr. Leon Lerner for helpful discussions concerning the synthesis of various nucleoside derivatives.

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Energetics of α-Chymotrypsin-Mediated Hydrolysis of a Strained Cyclic Ester†

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Received April 17, 1986; Revised Manuscript Received September 15, 1986

ABSTRACT: Hydrolysis of *o*-hydroxy-α-toluenesulfonic acid sultone (sultone II) is mediated by α-chymotrypsin. Sultone II is a highly strained cyclic ester substrate that forms a covalent intermediate with the enzyme and is therefore expected to release ring-strain energy upon formation of the sulfonyl enzyme species. It is found that the equilibrium constant for forming the covalent intermediate from the Michaelis complex is quite modest (K_2 = 16.4), suggesting that perhaps the strain energy is not released in the ring-cleavage event. The implied retention of chemical (strain) energy by the sulfonyl enzyme species raises the question of the means by which the enzyme avoids expression of strain energy and the implications of this effect in the catalytic sequence. High-pressure liquid chromatography (HPLC) rate data demonstrate facile reversion of sulfonyl enzyme to the Michaelis complex, and that reversion is preferred over hydrolysis of the covalent intermediate. pH-independent rate and equilibrium constants are derived for the α-chymotrypsin-mediated hydrolysis of sultone II, and pK_a values for groups on the enzyme are reported that are consistent with literature values obtained from analysis of nonspecific substrate hydrolysis by the enzyme.

In numerous cases, the $\Delta G°$ for binding a transition-state analogue to an enzyme has been found to be far more favorable than the $\Delta G°$ for binding the corresponding substrate (Wolfenden, 1972, 1978; Lienhard, 1973). On the basis of these observations as well as others, it has been proposed that the difference in free energy between the $\Delta G°$ values for transition-state analogue–enzyme and enzyme–substrate complex formation is representative of the free energy that can be utilized in bringing about rate acceleration (Jencks, 1975). More specifically, Jencks suggested that the full complement of binding energy is prevented from being realized as observed binding in the ES complex because of requirements for distortion, desolvation, and perhaps other energy-requiring factors (Jencks, 1960, 1975). These destabilizing effects are ultimately removed on reaching the transition state, which is presumed to be the state of most favorable interaction between the enzyme and the species being catalyzed.

The principle behind the proposal is that the potential or expected change in free energy from one part process (e.g., binding) is fully realized only in the subsequent transition state and a portion of the expected free energy does not appear in the observed $\Delta G°$ for binding. Transition-state analogue binding has provided much of the support for this concept with the resulting tendency to focus on noncovalent binding as the source of energy to be utilized. The principle is a general one and is not necessarily restricted to bimolecular (binding) events in enzyme catalysis. All that is necessary for application of

† Supported by U.S. Public Health Service Research Grant GM22300 from the National Institutes of Health.
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0006-2960/87/0426-0146$01.50/0