Inactivation of the *Lactobacillus leichmannii* Ribonucleoside Triphosphate Reductase by 2′-Chloro-2′-deoxyuridine 5′-Triphosphate: Stoichiometry of Inactivation, Site of Inactivation, and Mechanism of the Protein Chromophore Formation†

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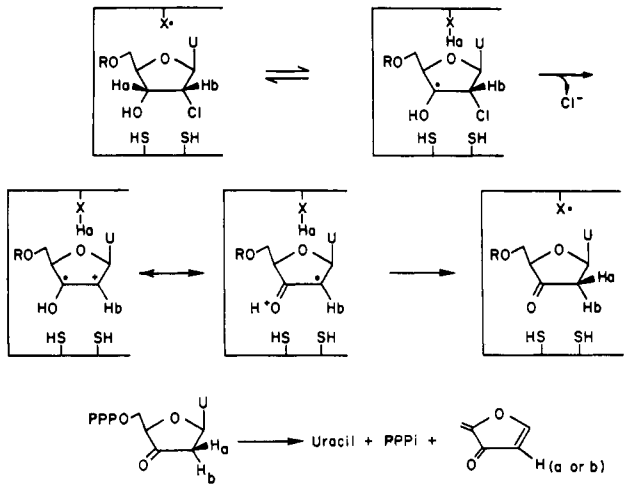
ABSTRACT: The ribonucleoside triphosphate reductase (RTPR) of *Lactobacillus leichmannii* is inactivated by the substrate analogue 2′-chloro-2′-deoxyuridine 5′-triphosphate (ClUTP). Inactivation is due to alkylation by 2-methylene-3(2*H*)-furanone, a decomposition product of the enzymic product 3′-keto-2′-deoxyuridine triphosphate. The former has been unambiguously identified as 2-[(ethylthio)methyl]-3(2*H*)-furanone, an ethanethiol trapped adduct, which is identical by ^{1}H NMR spectroscopy with material synthesized chemically. Subsequent to rapid inactivation, a slow process occurs that results in formation of a new protein-associated chromophore absorbing maximally near 320 nm. The terminal stages of the inactivation have now been investigated in detail. The alkylation and inactivation stoichiometries were studied as a function of the ratio of ClUTP to enzyme. At high enzyme concentrations (0.1 mM), 1 equiv of [5′-^{3}H]ClUTP resulted in 0.9 equiv of ^{3}H bound to protein and 83% inactivation. The amount of labeling of RTPR increased with increasing ClUTP concentration up to the maximum of approximately 4 labels/RTPR, yet the degree of inactivation did not increase proportionally. This suggests that (1) RTPR may be inactivated by alkylation of a single site and (2) decomposition of 3′-keto-dUTP is not necessarily enzyme catalyzed. The formation of the new protein chromophore was also monitored during inactivation and found to reach its full extent upon the first alkylation. Thus, out of four alkylation sites, only one appears capable of undergoing the subsequent reaction to form the new chromophore. While chromophore formation was prevented by $NaBH_4$ treatment, the chromophore itself is resistant to reduction. Model studies suggest that the new chromophore is due to addition of an amino group to the 5-position of enzyme-bound furanone, followed by ring opening and tautomerization to give a β-aminoenone structure. When inactivation by [2′-^{3}H]ClUTP is performed in the presence of $NaBH_4$ in order to stabilize the label and the protein is treated with iodoacetamide and then digested with trypsin, three very closely eluting peptides are observed by high-performance liquid chromatography. Sequencing of the first of these peptides demonstrated that the label was present on one of the active-site thiols of RTPR [Lin, A., Ashley, G. W., & Stubbe, J. (1987) *Biochemistry 26*, 6905–6909].

The coenzyme B_{12} (5′-deoxyadenosylcobalamin, AdoCbl[1]) dependent ribonucleoside triphosphate reductase (RTPR) of *Lactobacillus leichmannii* is inactivated by substrate analogues having halogens in place of the 2′-hydroxyl group. Extensive studies in our laboratory on the inactivation of RTPR by 2′-chloro-2′-deoxyuridine 5′-triphosphate (ClUTP) have resulted in the general hypothesis shown in Scheme I (Harris et al., 1984; Ashley & Stubbe, 1985). The initial product of enzyme action on ClUTP is 3′-keto-2′-deoxyuridine triphosphate (3′-keto-dUTP). Decomposition of this unstable product yields the observed products uracil and PPP_i, along with 2-methylene-3(2*H*)-furanone. Alkylation of RTPR by this reactive furanone results in inactivation. Subsequent to inactivation, a much slower process occurs that results in formation of a protein-associated chromophore absorbing maximally near 320 nm (Harris et al., 1984). This chromophore is also noted after inactivation of RTPR by other 2′-halo-2′-deoxynucleotides (Harris et al., 1987).

Similar events have been observed upon inactivation of the ribonucleoside diphosphate reductase of *Escherichia coli*, which does not require AdoCbl. In this case, use of ClUDP

Scheme I



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[1] Abbreviations: RTPR, ribonucleoside triphosphate reductase from *Lactobacillus leichmannii*; AdoCbl, 5′-deoxyadenosylcobalamin; ClUTP, 2′-chloro-2′-deoxyuridine 5′-triphosphate; 3′-keto-dUTP, 1-(2-deoxy-β-D-glyceropentofuran-3-ulosyl)uracil 5′-triphosphate; HPLC, high-performance liquid chromatography; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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results in initial formation of 3′-keto-dUDP, followed by decomposition to 2-methylene-3(2*H*)-furanone and covalent modification of the protein (Harris et al., 1984; Ator & Stubbe, 1985). Intriguingly, the slow formation of the 320-nm chromophore is also observed in this system (Thelander et al., 1976).

We now report the results of experiments designed to probe the stoichiometry of inactivation and labeling of RTPR by ClUTP and also the results of model studies designed to yield information on the nature of the 320-nm chromophore. The results of these experiments provide a more detailed view of the final stages of the interaction between RTPR and ClUTP.

Materials and Methods

General. RTPR was isolated from *L. leichmannii* ATCC 7830, assayed, and prereduced accordingly to Ashley et al. (1986). The following compounds were prepared according to literature procedures: [5′-^{3}H]ClUTP and 2-[(ethylthio)-methyl]-3(2*H*)-furanone (Harris et al., 1984), [2′-^{3}H]ClUTP (G. W. Ashley, G. Harris, and J. Stubbe, unpublished results), 4-(diethylamino)-3-penten-2-one (Leonard & Adamcik, 1959), 4-chloro-3-penten-2-one (Gruber et al., 1975), and 4-imidazolyl-3-penten-2-one (Kashima & Tajima, 1980). SepPak C_{18} cartridges were from Waters Associates, Centricon 30 ultrafiltration devices were from Amicon, and Scint-A scintillation cocktail was from Packard. ^{1}H NMR spectra were recorded at 270 MHz on a Bruker WH-270 spectrometer. Coupling values are in hertz. HPLC was performed with an Altex gradient system and a Vydac 218TP reversed-phase peptide HPLC column at a flow rate of 1.0 mL/min with UV detection at 220 nm. Peptide sequencing was performed by automated Edman degradation at the University of Wisconsin Biotechnology Center. Cycles from the Edman degradation were analyzed for radioactivity by liquid scintillation counting.

4-(Ethylthio)-3-penten-2-one. A solution of 4-chloro-3-penten-2-one (0.20 mg, 1.7 mmol), ethanethiol (0.75 mL), and triethylamine (1.0 mL) in 2.0 mL of ethyl ether was stirred for 12 h at ambient temperature. The solvent was evaporated, and the residue was redissolved in ether and washed with H_2O. The organic phase was dried with $MgSO_4$, filtered, and concentrated. Flash chromatography (Still et al., 1978) on SiO_2 using ethyl acetate/hexanes (1:2) gave 104 mg of product as a maladorous oil: ^{1}H NMR ($CDCl_3$) δ 5.93 (s, 1 H), 2.80 (q, 2 H, J = 7), 2.38 (s, 3 H), 2.18 (s, 3 H), 1.33 (t, 3 H, J = 7); UV (H_2O) λ_{max} 301 nm (ϵ 15 540 M^{-1} cm^{-1}).

1-[(1-Hydroxyethyl)amino]-4-hydroxy-5-(ethylthio)-1-penten-3-one. A solution of 2-[(ethylthio)methyl]-3(2*H*)-furanone (100 mg) and ethanolamine (30 μL) in 0.5 mL of CH_3OH was kept for 10 min at ambient temperature, and the solvent was evaporated. The residue was dissolved in H_2O and applied to a SepPak C_{18} cartridge. After the cartridge was washed with H_2O to remove excess ethanolamine, the product was eluted with CH_3OH: ^{1}H NMR ($CDCl_3$) δ 6.88 (dd, 1 H, J = 7.2, 13.1), 5.11 (d, 1 H, J = 13.1), 4.27 (dd, 1 H, J = 4.2, 6.6), 3.74 (t, 2 H, J = 5.0), 3.38 (dd, 2 H, J = 5.7, 10.7), 2.91 (dd, 1 H, J = 4.2, 13.8), 2.70 (dd, 1 H, J = 6.7, 13.8), 2.62 (q, 2 H, J = 7.2), 1.24 (t, 3 H, J = 7.2).

Trapping of 2-Methylene-3(2H)-furanone by Ethanethiol in the Reaction of RTPR with ClUTP. A solution of 0.9 mM AdoCbl, 0.1 M potassium phosphate (pH 7.3), 1 mM EDTA, 0.1 mM dCTP, and 1.0 mM [5′-^{3}H]ClUTP (1.2 × 10^7 cpm/μmol) in a volume of 10 mL was deoxygenated with argon. Prereduced RTPR (90 nmol) was then added, and the reaction was started by addition of ethanethiol to a final concentration of 0.25 M and incubated for 8 h at 37 °C. After 8 h the reaction mixture was extracted with 6 × 2 mL of $CHCl_3$ and the $CHCl_3$ layer dried over sodium sulfate. The $CHCl_3$ layer was removed in vacuo and redissolved in freshly distilled $CDCl_3$ to give 7.3 μmol of 2-[(ethylthio)methyl]-3-(2*H*)-furanone, whose UV and NMR spectra were identical with those of material prepared chemically (Harris et al., 1984).

Stoichiometry of RTPR Inactivation by ClUTP. Reaction mixtures contained prereduced RTPR (10 nmol), [5′-^{3}H]-ClUTP (10, 15, 20, 50, or 100 nmol, 2.3 × 10^7 cpm/μmol), and AdoCbl (equimolar with ClUTP) in 100 μL of 50 mM potassium phosphate, pH 7.3. RTPR activity was measured before and after reaction by using the ATP assay. After 10 min at 37 °C, the protein was isolated by chromatography on Sephadex G-50 (10 mM potassium phosphate, pH 7.3) and kept for 12 h at 4 °C to allow complete formation of the new chromophore. Protein concentrations were measured spectrophotometrically, with ϵ_{280} = 101 000 M^{-1} cm^{-1} (Blakley, 1978). Portions of the protein solutions were analyzed for ^{3}H by scintillation counting.

Susceptibility of the New Chromophore to Reduction by $NaBH_4$. A mixture of prereduced RTPR (20 nmol), ClUTP (250 nmol), and AdoCbl (100 nmol) in 500 μL of 50 mM potassium phosphate, pH 7.3, containing 1 mM EDTA was kept for 5 min at 37 °C. The mixture was cooled on ice and divided into two equal portions. One portion was treated with 30 μL of a 2 M solution of $NaBH_4$ in 10 mM NaOH, while the second portion was treated with 30 μL of 10 mM NaOH. After 30 min at 4 °C, the samples were chromatographed on a 1.5 × 20 cm column of Sephadex G-50 with 50 mM potassium phosphate, pH 7.3. Protein-containing fractions were pooled and concentrated by Centricon 30. After the fraction stood for 12 h at 4 °C, the spectra of the proteins were measured, and samples were analyzed for ^{3}H by scintillation counting. The sample that had not been treated with $NaBH_4$ and that showed complete formation of the new chromatophore (A_{280}/A_{320} = 3.8) was subsequently dissolved in 200 μL of 0.3 M Tris-HOAc, pH 7.2, and treated with 25 μL of a 2 M solution of $NaBH_4$ in 10 mM NaOH for 30 min at 4 °C. Remeasurement of the electronic spectrum of this sample indicated no loss of the 320-nm absorbance (A_{280}/A_{320} = 4.2).

Inactivation of RTPR by [2′-^{3}H]ClUTP in the Presence of $NaBH_4$. Prereduced RTPR (116 nmol, 1.35 units/mg) was treated with [2′-^{3}H]ClUTP (1500 nmol, 4.0 × 10^6 cpm/μmol) and AdoCbl (1500 nmol) in 1.5 mL of 0.3 M Tris-HCl, pH 7.4, containing 1 mM EDTA at 37 °C. Reaction was initiated by addition of AdoCbl, and 50-μL aliquots of a 2.0 M solution of $NaBH_4$ in 10 mM NaOH were added at 0, 2, and 5 min. After an additional 10 min, the protein was isolated by gel filtration on a 1.4 × 20 cm column of Sephadex G-50 (0.3 M Tris-HCl, pH 8.3) and concentrated by centrifugal ultrafiltration (Centricon 30). The protein had a specific activity of 2.7 × 10^6 cpm/μmol, indicating binding of 0.65 ^{3}H per RTPR. RTPR activity assays indicated that the enzyme had lost 63% of its activity; thus, there was approximately 1.0 alkylation per RTPR molecule inactivated.

Location of the Site of Radiolabeling. The modified protein (106 nmol) was dissolved in 1.0 mL of 0.3 M Tris-HCl, pH 8.3, containing 4 mM EDTA, 30 mM DTT, and 6 M guanidine hydrochloride. After 30 min at 37 °C, iodoacetamide (50 mg, 270 μmol) was added and the mixture was kept for 30 min in the dark. After addition of 30 μL of 2-mercaptoethanol, the solution was dialyzed against H_2O (2 × 500 mL) in the dark. Analysis of the dialysis fluid indicated a maximum loss of 5% of the radiolabel. The precipitated protein was

collected by suspension in H_2O and lyophilized. The protein was redissolved in 300 μL of 0.10 M NH_4HCO_3, pH 8.2. A 0.40-mg portion of TPCK-trypsin (1:20 w/w) was added, and the mixture was kept at 37 °C for 5 h. The resulting peptide mixture was separated by HPLC [Vydac C18 reversed-phase peptide column, H_2O/CH_3CN (0–45% CH_3CN over 90 min) containing 0.1% CF_3COOH, flow rate 1.0 mL/min, detection at 220 nm]. Fractions were collected manually every 1.0 min, and 100 μL aliquots were analyzed for radioactivity by addition to 8 mL of scintillation cocktail (Eco-Scint) followed by liquid scintillation counting. The sole region of radioactivity (eluting between 21% and 23% CH_3CN) was pooled and evaporated. This region was rechromatographed on HPLC with a 10 mM NH_4OAc (pH 6.8)/CH_3CN gradient (0–35% CH_3CN over 90 min). Again, a single region of radioactivity was observed, eluting between 14% and 17% CH_3CN. As several UV-absorbing peaks were observed in this region, however, the radioactive region was pooled, evaporated, and rechromatographed in the same system with a shallower gradient (10–13% CH_3CN over 30 min). This resulted in resolution of three closely migrating radiolabeled regions. The peptide (A_{220}) corresponding to the first radioactive area could be purified by rechromatography with H_2O/CH_3CN containing 0.1% CF_3COOH (10–20% CH_3CN over 30 min), which removed some contaminating peptides. The second and third radioactive regions have not been sufficiently purified to allow sequencing.

Sequencing of the peptide from the first radioactive region by automated Edman degradation gave the sequence D-L-E-L-V-D-Q-T-D-*-E-G-G-A-*-P-I-K (* represents an unusual residue). Analysis of the cycles from the Edman degradation by liquid scintillation counting indicated radioactivity only in cycle 10.

Results and Discussion

Ethanethiol Trapping of 2-Methylene-3(2H)-furanone from the Reaction of RTPR with ClUTP. Studies of Ator and Stubbe (1985) showed that incubation of *E. coli* RDPR with ClUDP in the presence of ethanethiol allowed trapping of 2-methylene-3(2*H*)-furanone, the species responsible for inactivation of RDPR. While previous studies of Harris et al. with RTPR and ClUTP, using DTT as a trap, strongly implicated the 2-methylene-3(2*H*)-furanone as the species responsible for inactivation of RTPR, the DTT adduct(s) of the furanone could not be isolated in good yield and unambiguously identified. These early studies were therefore repeated with ethanethiol as a trap in place of DTT. Incubation of 90 nmol of RTPR with 10 μmol of [5′-^{3}H]ClUTP in the presence of saturating ethanethiol allowed production of 7.3 μmol of a $CHCl_3$-extractable adduct. The adduct isolated had an NMR spectrum identical with that of 2-[(ethylthio)-methyl]-3(2*H*)-furanone synthesized chemically (Ator & Stubbe, 1985).

Variation of Labeling Stoichiometry with Inactivator/Enzyme Ratio. When a constant, high concentration of RTPR (0.1 mM) was treated with increasing amounts of [5′-^{3}H]-ClUTP, the amount of radiolabel covalently attached to the inactivated protein increased (Table I). A 1:1 ratio of ClUTP to RTPR resulted in 83% inactivation and 0.90 equiv of ^{3}H bound, indicating that a single alkylation could result in essentially complete inactivation of the enzyme. Increasing the ClUTP/RTPR ratio led to significant increases in labeling, up to 3.6 labels/RTPR, even though the degree of inactivation did not increase proportionally. This suggests that decomposition of 3′-keto-dUTP need not be enzyme catalyzed. In this situation, the rate of formation of 3′-keto-dUTP from ClUTP would depend on enzyme concentration yet the rate of decomposition of 3′-keto-dUTP would not. The use of higher RTPR concentrations would allow greater quantities of 3′-keto-dUTP to accumulate. Nonenzymic formation of 2-methylene-3(2*H*)-furanone from the excess 3′-keto-dUTP followed by nonspecific labeling could then lead to the observed stoichiometries.

Table I: Stoichiometry of RTPR Inactivation by [5′-^{3}H]ClUTP[a]

I_0/E_0	% inact	^{3}H sp act.[b]	^{3}H/RTPR[c]	A_{280}/A_{320}[d]	ϵ_{320}[e]
1	83	2.0×10^7	0.87	7.6	15 000
1.5	90	2.3×10^7	1.0	5.1	20 000
2	93	3.1×10^7	1.3	6.3	16 000
5	99	5.5×10^7	2.4	5.6	18 000
10	98	8.3×10^7	3.6	5.0	20 000

[a] Prereduced RTPR (100 μM) was treated with [5′-^{3}H]ClUTP (2.3×10^7 cpm/μmol) and AdoCbl (equimolar with ClUTP) at pH 7.3 for 10 min at 37 °C. Proteins were isolated by gel filtration. [b] Specific radioactivity (cpm/μmol) of isolated RTPR. Protein concentration was determined by UV spectrophotometry using ϵ_{280} = 101 000 M^{-1} cm^{-1}, and ^{3}H was determined by scintillation counting. [c] Number of alkylations per RTPR molecule, calculated as the ratio of the protein specific radioactivity to the specific radioactivity of starting [5′-^{3}H]-ClUTP. [d] Observed ratio of protein UV absorbance at 280 nm to chromophore absorbance at 320 nm, measured spectrophotometrically 24 h after initial isolation of protein. [e] Estimated extinction coefficient (M^{-1} cm^{-1}) of the new chromophore at 320 nm, based on protein absorbance at 280 nm (ϵ_{280} = 101 000 M^{-1} cm^{-1}). Assumes formation of one chromophore per RTPR molecule.

Variation of Chromophore Formation with Labeling Stoichiometry. After isolation of the proteins from the above experiments by gel filtration, the extent of formation of the 320-nm chromophore was determined (Table I). The extinction coefficient of the chromophore was estimated from the ratio of the absorbances at 280 and 320 nm, using ϵ_{280} = 101 000 M^{-1} cm^{-1} for RTPR (Blakley, 1978) and assuming that the new chromophore has negligible absorbance at 280 nm. As shown in Table I, formation of the new chromophore is essentially complete after the initial alkylation event. Increasing label stoichiometries did not appreciably increase the absorbance at 320 nm. It is apparent that only the labeling site responsible for inactivation is capable of undergoing the further reaction to form the 320-nm chromophore. On the basis of the maximal formation of the new chromophore, an extinction coefficient of approximately 20 000 M^{-1} cm^{-1} is calculated.

Treatment of the 320-nm Chromophore with $NaBH_4$. To probe the chemical properties of the new chromophore, its susceptibility to reduction by $NaBH_4$ was tested. Enzyme that had been inactivated by ClUTP was divided into two portions, one of which was treated immediately with 0.1 M $NaBH_4$. After 2 h at 37 °C, the proteins were isolated by gel filtration. The $NaBH_4$-treated protein had not developed the 320-nm chromophore, whereas the control protein had a fully developed absorbance at 320 nm (Figure 1). The chromophore-containing protein was subsequently treated with 0.1 M $NaBH_4$. No change in its 320-nm absorbance was noted. Thus, although formation of the new chromophore can be prevented by reduction with $NaBH_4$, the chromophore itself is resistant to reduction.

Model Studies on the Structure of the Protein Chromophore. In order to gain insights as to the structure of the new protein chromophore, a series of model compounds was investigated. As the chromophore appears subsequent to inactivation by 2-methylene-3(2*H*)-furanone and formation occurs even after excess components of the inactivation mixture have been chromatographically removed, the new protein chromophore must be derived from the enzyme-bound furanone.

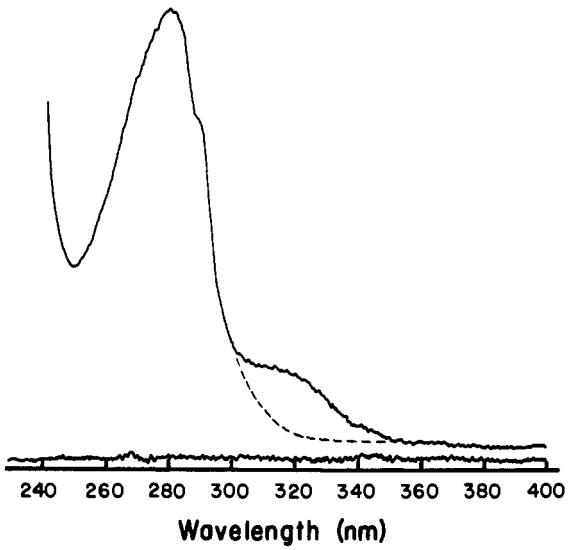
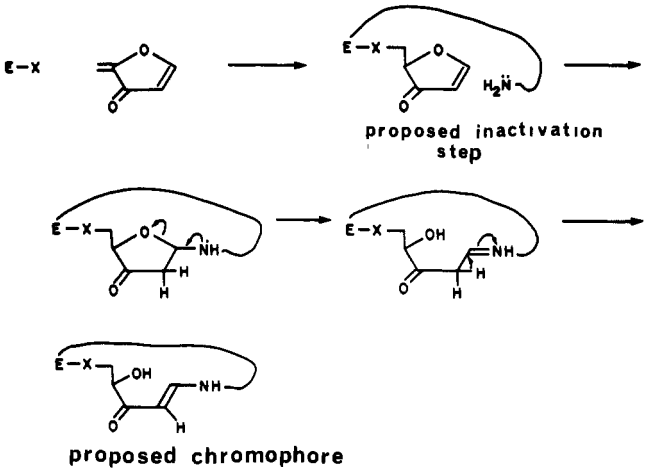


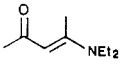
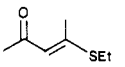
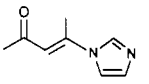
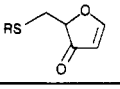
FIGURE 1: Sensitivity of the new protein chromophore and its precursor to reduction by $NaBH_4$. (---) Protein treated with $NaBH_4$ subsequent to inactivation; (—) control protein not treated with $NaBH_4$.

Scheme II



Previous studies indicated that the exocyclic double bond of 2-methylene-3(2*H*)-furanone is significantly more reactive toward addition of nucleophiles than is the endocyclic double bond (Harris et al., 1984). Our assumption was thus that the initial inactivation event would occur by addition of a protein nucleophile to the external double bond, leaving the endocyclic double bond to participate in chromophore formation. A likely reaction pathway would be addition of a nucleophile to the enzyme-bound furanone, followed by ring opening and tautomerization to form a β-hetero-substituted enone system (Scheme II). We therefore examined the spectral properties and chemical reactivities of a series of these compounds (Table II) prepared from 2,4-pentanedione. Both the β-amino analogue (λ_{max} 316 nm) and the β-thio analogue (λ_{max} 301 nm) had absorbances in the correct range. The β-imidazolyl enone (λ_{max} 272 nm) is clearly not a good model; presumably, the lone pair of electrons on nitrogen is involved in the imidazole π system and does not conjugate effectively with the enone system. Investigation of the chemical reactivities of the models allowed a choice between the amino and thiol analogues. Of the analogues listed in Table II, all except the β-aminoenone are rapidly reduced by $NaBH_4$, with concurrent destruction of their chromophores. As the protein-bound chromophore is also resistant to reduction, the β-aminoenone appears to be the best model. The resistance of β-aminoenones toward reduction by $NaBH_4$ has been previously noted (Kashima et al., 1975). It has also been reported that the protein chromophore in RDPR is sensitive to treatment with *N*-bromosuccinimide at pH 4 (Thelander et al., 1976). This is consistent with the known reactivity of β-aminoenones toward electrophilic brominating reagents (Spande & Witkop, 1967). On the basis of these model studies, the mechanism of Scheme II is proposed to account for formation of the 320-nm chromophore.

Table II: Properties of Chromophore Model Compounds

compound	λ_{max} (nm)	ϵ (M^{-1} cm^{-1})	$NaBH_4$ reducible?
protein chromophore	320	20000	no
	316	24800	no
	301	15500	yes
	272	11600	yes
	260	4000	yes

Production of the β-Aminoenone System from Furanone. As a test of the feasibility of Scheme II, a model for the protein-bound furanone, 2-[(ethylthio)methyl]-3(2*H*)-furanone (λ_{max} 260 nm), was treated with ethanolamine in MeOH. This system cleanly produced the expected β-aminoenone system (Scheme II; X = SEt and $NH_2\sim$ = $NHCH_2CH_2OH$), unequivocally identified by NMR. This product showed λ_{max} = 316 nm. Treatment of the model furanone with ethanethiol or imidazole resulted only in loss of the initial 260-nm chromophore. In these cases, nucleophilic addition to the endocyclic double bond occurs, but subsequent ring opening is unfavorable due to the lack of a removable proton on the attacking nucleophile. Ring opening by direct elimination of the furan oxygen from the ketone is disfavored in all cases (Baldwin et al., 1977). A similar formation of the β-aminoenone system has been observed in the thermal decomposition of certain orthoamides (Carpenter et al., 1972).

Inactivation of RTPR by ClUTP in the Presence of $NaBH_4$. As RTPR can be significantly inactivated by 1 equiv of ClUTP, it seemed likely that alkylation of a single reactive residue might be involved. In order to suppress nonspecific alkylation by 2-methylene-3(2*H*)-furanone outside the active site as well as to stabilize the alkylated residue against 4′,5′-elimination, the inactivation reaction was performed in the presence of $NaBH_4$. It was expected that the $NaBH_4$ would trap by reduction the 3′-keto-dUTP dissociated from the active site and 2-methylene-3(2*H*)-furanone formed outside the active site. However, when RTPR is treated with excess [5′-^{3}H]-ClUTP in the presence of 100 mM $NaBH_4$, the enzyme is completely inactivated and 1.1 equiv of ^{3}H is bound to the protein. Use of [2′-^{3}H,U-^{14}C]ClUTP under the same conditions resulted in incorporation of 1.3 equiv of ^{14}C with loss of 15% of the original ^{3}H through exchange with solvent.

In order to determine the specificity and location of labeling, peptide mapping was performed on RTPR that had been inactivated by [2′-^{3}H]ClUTP in the presence of $NaBH_4$ in a large-scale reaction. After this treatment, the RTPR contained 0.65 equiv of [^{3}H]RTPR and was 63% inactive. As up to 15% of the 2′-^{3}H may be washed out during the reaction, this indicates 1.0–1.2 alkylations per enzyme. The labeled protein was denatured, treated with iodoacetamide, and digested with

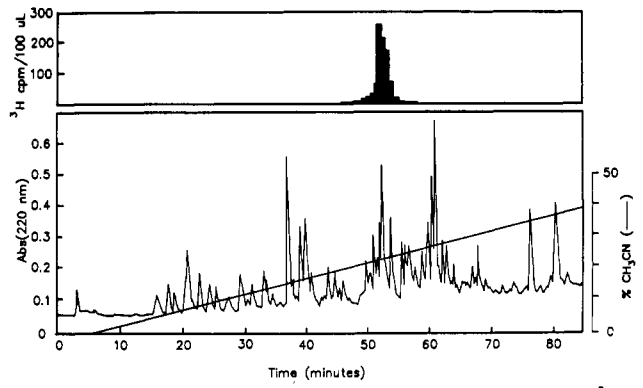


FIGURE 2: HPLC tryptic peptide map of $NaBH_4$-reduced [2′-^{3}H]-ClUTP-inactivated RTPR. The inactivated RTPR was denatured, treated with iodoacetamide, and digested with TPCK–trypsin as described under Materials and Methods. The digest was chromatographed on HPLC with H_2O/CH_3CN containing 0.1% CF_3COOH. Fractions were collected every 1 min, and the ^{3}H content of each fraction (vertical bars) was determined by liquid scintillation counting of a 100-μL aliquot.

trypsin. Initial HPLC separation of the tryptic peptides at low pH (Figure 2) indicated a single region of radioactivity eluting at 22–23% CH_3CN. Rechromatography of this region at pH 6.8 again revealed a single region of ^{3}H eluting at 14–16% CH_3CN. Because this region was somewhat broad and contained several UV-absorbing peaks, the radioactive region was chromatographed a third time, at pH 6.8 but with a very shallow gradient (Figure 3). This began to resolve three distinct areas of ^{3}H. Of these three regions, only the first has been purified sufficiently to sequence, giving D-L-E-L-V-D-Q-T-D-*-E-G-G-A-*-P-I-K. Radioactivity was associated only with the * residue in cycle 10. Fast atom bombardment mass spectral analysis of this peptide gave a mol wt of 2006. This is consistent with the * in cycle 10 being the expected $NaBH_4$-reduced 2-methylene-3(2*H*)-furanone adduct of cysteine and with the * in cycle 15 being unmodified cysteine. As the protein had been treated with iodoacetamide, it is not clear at this time why the cysteine in cycle 15 remained unmodified. We have isolated this same peptide sequence in an experiment designed to locate the active-site thiols of RTPR (Lin et al., 1987), thus confirming the assignment of the * residues as cysteines and suggesting that the inactivation of RTPR by ClUTP occurs by alkylation of an active-site cysteine by 2-methylene-3(2*H*)-furanone. While we may speculate that the lysine residue at the C-terminus of this peptide may be the residue responsible for formation of the new protein chromophore, this is uncertain as the tertiary structure of RTPR is not known. Attempts to isolate a chromophore-containing tryptic peptide have not yet been successful.

While the sequenced peptide contains only 30% of the radioactivity present in the inactivated RTPR, we believe that the remainder of the radioactivity is likely to be on the same peptide. Experiments designed to specifically label the redox-active thiols of RTPR using [1-^{14}C]iodoacetamide result in the labeling of two reactive peptides, the dithiol peptide sequenced above and a second peptide, T-G-D-S-L-N-N-C-W-F (Lin et al., 1987). However, this second peptide migrates substantially later (28% CH_3CN) than the dithiol peptide in the low-pH peptide map, while the 2-methylene-3(2*H*)-furanone peptides all migrate very close to the dithiol peptide. There are several potential causes of the observed heterogeneity in the labeled peptides: (1) incomplete iodoacetamide alkylation of unmodified cysteines; (2) heterogeneity of 2-methylene-3(2*H*)-furanone labeling on either the cycle 10 or cycle 15 cysteine; (3) formation of diastereomers from the

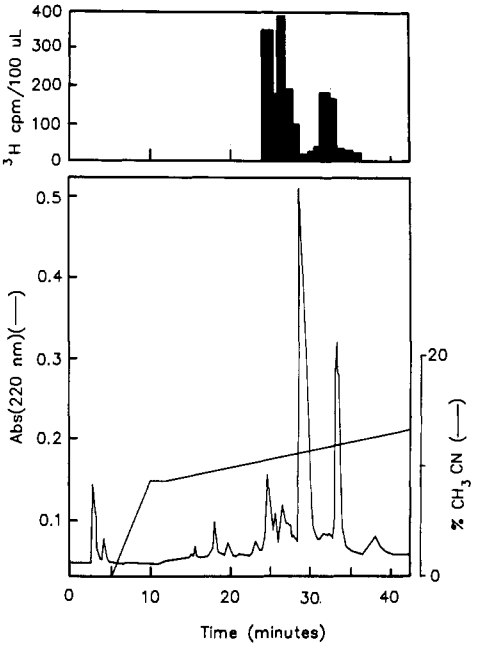


FIGURE 3: Resolution of three radiolabeled regions from the ^{3}H-containing region of Figure 2. The ^{3}H-containing region of Figure 2 was rechromatographed a second time on HPLC with 10 mM NH_4OAc (pH 6.8)/CH_3CN. The ^{3}H-containing region of this second chromatograph was once again evaporated and rechromatographed on HPLC at pH 6.8 with a shallow gradient.

$NaBH_4$ reduction of the modified peptide.

Summary. Results of experiments reported here clarify the terminal events in the inactivation of RTPR by 2′-halonucleotides. The initial action of RTPR on ClUTP results in formation of 3′-keto-2′-deoxyuridine 5′-triphosphate. Decomposition of this species results in production of uracil, PPP_i, and 2-methylene-3(2*H*)-furanone. Alkylation of an active-site cysteine by the more reactive exocyclic olefin of this furanone results in rapid inactivation of the enzyme. Subsequent addition of a primary amino group, probably the ε-amino group of a lysine residue, to the remaining endocyclic olefin of the protein-bound furanone followed by ring opening and tautomerization leads to formation of the new protein chromophore, a β-aminoenone species.

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NMR Studies of Carbonic Anhydrase–4-Fluorobenzenesulfonamide Complexes†

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ABSTRACT: Binding of 4-fluorobenzenesulfonamide to human carbonic anhydrases I and II has been studied by proton, fluorine, and nitrogen-15 nuclear magnetic resonance spectroscopy. All three types of experiments provide evidence that the stoichiometry of the interaction of this inhibitor with both enzymes is 2 mol of inhibitor bound per mole of enzyme. Observations which suggest that the bound forms are involved in an exchange process that is rapid at room temperature but slower at 2 °C are described. Nitrogen-15 shift data show that the bound inhibitors are present at the active site as anions. The proton experiments indicate appreciable reorganization of the tertiary structure of the protein upon binding. Saturation-transfer experiments to determine the rate of dissociation of the inhibitor–enzyme complex lead to the conclusion that the dissociation process is more complicated than a simple free–bound equilibrium.

The presence of carbonic anhydrase in human erythrocytes has been known for over 50 years. Subsequent efforts have shown that the erythrocyte anhydrase activity arises from two distinct zinc-containing metalloproteins, which will be referred to here as CA I and CA II.[1] The molecular masses of these isozymes are nearly identical (30 kDa), although there is only a 60% sequence homology, and both contain a single zinc ion per molecule. CA II has very high activity toward CO_2 hydration and appears to be an almost perfectly evolved enzyme in that turnover is limited primarily by substrate availability (Fersht, 1977; Pocker & Janjic, 1987). The amino acid sequence of CA II has been highly conserved throughout evolution (Tashian et al., 1983). Although significantly more abundant than CA II in the red cell, CA I has an activity toward CO_2 that is about a thousandfold less than CA II. Curiously, CA I is nearly completely inhibited by Cl^- at the concentration of this anion found in the red cell, and a biochemical role for this high-abundance protein is not yet clear (Chapman & Maren, 1978).

Recent work has revealed that virtually all plant and animal tissues contain one or more carbonic anhydrase isozymes. CA II is apparently the more widely distributed of the erythrocyte isozymes, being found in kidney, brain, pancreas, gastric mucosa, skeletal muscle, retina, and the lens. A form of carbonic anhydrase (CA III) has been found at high concentrations in red muscle and in liver, although small amounts of this isozyme are also present in erythrocytes (Deutsch, 1987). A membrane-bound form of carbonic anhydrase (CA IV) has been detected in lung and kidney (Whitney & Briggle, 1982; Henry et al., 1986). This enzyme appears to be similar to the other isozymes but is conjugated to polysaccharide so that the molecular mass is increased to 52–68 kDa. Forms of carbonic anhydrase, also glycoproteins, have been found in saliva of humans and sheep and in sheep parotid glands (Fernley et al., 1984; Murakami & Sly, 1987).

Aromatic and heterocyclic primary sulfonamides are particularly potent competitive inhibitors of carbonic anhydrase (Maren & Sanyal, 1983). A wide variety of structures have been examined for inhibitory activity, and quantitative structure–activity relationships (QSAR) have been developed, these efforts being aided recently by interactive computer graphics (Vedani & Meyer, 1984; Hansch et al., 1985).

NMR studies have demonstrated that sulfonamide inhibitors coordinate to the metal ion at the active site of carbonic anhydrase through the nitrogen atom of the SO_2NH_2 group (Evelhoch et al., 1981; Blackburn et al., 1985) and that the sulfonamide is present at the active site as an anion (Kanamori & Roberts, 1983).[2]

Formation and dissociation of sulfonamide–carbonic anhydrase complexes are kinetically more complicated than a simple bimolecular process (King & Burgen, 1976). The associative step(s) is (are) pH dependent while dissociation of the complexes is pH independent (King & Maren, 1974; Taylor, P. W., et al., 1970). Given the small values of the dissociation constant (K_D), the half-life for dissociation of a sulfonamide complex is expected to be long; values determined for simple benzenesulfonamides range from 40 to 1100 ms. The complexity and slow time course for binding of aromatic sulfonamides is reflected in the leisurely and complex time

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[1] Abbreviations: CA I and CA II, isozymic forms of human erythrocyte carbonic anhydrase, referred to in earlier literaure as the B and C forms; NMR, nuclear magnetic resonance; fid, free induction decay; NOE, nuclear Overhauser effect; ^1H NMR, proton magnetic resonance; kDa, kilodalton(s); QSAR, quantitative structure–activity relationship(s), Tris, tris(hydroxymethyl)aminomethane; TMS, tetramethylsilane.

[2] Other sulfonamides apparently bind as the neutral molecule (Petersen et al., 1977).

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