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Products of the Inactivation of Ribonucleoside Diphosphate Reductase from *Escherichia coli* with 2′-Azido-2′-deoxyuridine 5′-Diphosphate†

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Received September 10, 1986; Revised Manuscript Received December 17, 1986

ABSTRACT: Ribonucleoside diphosphate reductase (RDPR) from *Escherichia coli* was completely inactivated by 1 equiv of the mechanism-based inhibitor 2′-azido-2′-deoxyuridine 5′-diphosphate (N_3UDP). Incubation of RDPR with [3′-^{3}H]N_3UDP resulted in 0.2 mol of ^{3}H released to solvent per mole of enzyme inactivated, indicating that cleavage of the 3′ carbon–hydrogen bond occurred in the reaction. Incubation of RDPR with [β-^{32}P]N_3UDP resulted in stoichiometric production of inorganic pyrophosphate. One equivalent of uracil was eliminated from N_3UDP, but no azide release was detected. Analysis of the reaction of RDPR with [$^{15}N_3$]N_3UDP by mass spectrometry revealed that the azide moiety was converted to 0.9 mol of nitrogen gas per mole of enzyme inactivated. The tyrosyl radical of the B2 subunit was destroyed during the inactivation by N_3UDP as reported previously [Sjöberg, B.-M., Gräslund, A., & Eckstein, F. (1983) *J. Biol. Chem. 258*, 8060-8067], while the specific activity of the B1 subunit was reduced by half. Incubation of [5′-^{3}H]N_3UDP with RDPR resulted in stoichiometric covalent radiolabeling of the enzyme. Separation of the enzyme's subunits by chromatofocusing revealed that the modification was specific for the B1 subunit.

Ribonucleoside diphosphate reductase (RDPR)[1] from *Escherichia coli* catalyzes the conversion of the four common ribonucleoside 5′-diphosphates to the corresponding 2′-deoxyribonucleotides. The enzyme is proposed to be a 1:1 complex of two subunits designated B1 and B2, each of which consists of two polypeptide chains (Thelander & Reichard, 1979; Sjöberg & Gräslund, 1983; Carlson et al., 1984). The B1 subunit ($\alpha\alpha'$, M_r 170 000) contains binding sites for the substrates and nucleoside triphosphate allosteric effectors which control the specificity and activity of the enzyme. Subunit B1 also contains redox-active thiols in each of its two active sites which become oxidized during turnover (Thelander, 1974). Reducing equivalents for the reaction are provided by NADPH through a transport chain consisting of the proteins thioredoxin and thioredoxin reductase, or glutaredoxin, glutathione, and glutathione reductase. The B2 subunit ($\beta\beta$, M_r 87 000) contains the cofactor that is unique to this enzyme, a binuclear iron center and organic free radical arising from the oxidation of a single tyrosine residue of the subunit. The tyrosyl radical has a characteristic visible absorbance at 410 nm and an EPR signal at $g = 2.0047$ that disappear when the radical is reduced by reagents such as hydroxyurea or hydroxylamine (Kjøller-Larsen et al., 1982). Although the tyrosyl radical is essential for enzyme activity, a specific role in catalysis has not been demonstrated.

We have proposed that the chemical mechanism of ribonucleotide reduction involves radical intermediates (Stubbe & Ackles, 1980; Ashley & Stubbe, 1986). The hypothetical radical mechanism has been supported by extensive studies with isotopically labeled substrates (Stubbe & Ackles, 1980; Stubbe et al., 1983) and the mechanism-based inhibitor 2′-chloro-2′-deoxyuridine 5′-diphosphate (ClUDP) (Stubbe & Kozarich, 1980; Harris et al., 1984; Ator & Stubbe, 1985). The first direct evidence for radical involvement in RDPR-mediated reactions, however, has come from studies with 2′-azido-2′-deoxycytidine 5′-diphosphate (N_3CDP) and 2′-

† This research was supported by U.S. Public Health Service Grant GM 29595 from the National Institutes of Health. J.S. is a recipient of an H. I. Romnes Fellowship from the University of Wisconsin and NIH Research Career Development Award AM 01222.

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[1] Abbreviations: RDPR, ribonucleoside diphosphate reductase; EPR, electron paramagnetic resonance; ClUDP, 2′-chloro-2′-deoxyuridine 5′-diphosphate; N_3CDP, 2′-azido-2′-deoxycytidine 5′-diphosphate; N_3UDP, 2′-azido-2′-deoxyuridine 5′-diphosphate; N_3ADP, 2′-azido-2′-deoxyadenosine 5′-diphosphate; NH_2UDP, 2′-amino-2′-deoxyuridine 5′-diphosphate; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IP, ion pairing; HPLC, high-performance liquid chromatography; PEI, poly(ethylenimine); TLC, thin-layer chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FDH, formate dehydrogenase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DEAE, diethylaminoethyl; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/87/0426-3408$01.50/0

azido-2′-deoxyuridine 5′-diphosphate (N_3UDP), novel inhibitors originally reported by Thelander et al. (1976). Inactivation was attributed to destruction of the B2 tyrosyl radical since the characteristic visible absorbance at 410 nm was lost following incubation of N_3CDP with the enzyme. In subsequent studies Sjöberg et al. (1983) examined this reaction by EPR spectroscopy and made the intriguing observation that tyrosyl radical loss was accompanied by the transient formation of a new radical signal with 25-G triplet and 6-G doublet splittings. Since isotopic labeling of the protein with ^{15}N and ^{2}H did not alter the EPR spectrum, they concluded that the apparent hyperfine couplings arose from nuclei of the substrate analogue rather than the enzyme. The first conclusive evidence for the localization of the spin density on the nucleotide was provided by ^{15}N labeling of the azido moiety of N_3UDP, which collapsed the triplet splitting to a doublet (Ator et al., 1984).

The observation of a radical intermediate in the reaction of RDPR with 2′-azido-2′-deoxynucleoside 5′-diphosphates is extremely important since it is the first unequivocal demonstration of radical chemistry catalyzed by the enzyme. We believe that the first evidence for tyrosyl radical mediated production of substrate radical intermediates will arise from a detailed investigation of the mechanism of inactivation of RDPR by N_3UDP. We have utilized isotopically labeled N_3UDP and 2′-azido-2′-deoxyadenosine 5′-diphosphate (N_3ADP) to reexamine the identity and stoichiometry of the products formed in the reaction of these compounds with RDPR. Our experiments have allowed us to propose for the first time a reasonable working hypothesis involving 3′ carbon–hydrogen bond cleavage for the inactivation of RDPR by N_3UDP.

Materials and Methods

Ribonucleoside diphosphate reductase was originally isolated from *E. coli* strain KK546 (Eriksson et al., 1977) by using a dATP–Sepharose affinity resin synthesized by extensive modifications of the procedures of Berglund and Eckstein (1974) as described by Knorre et al. (1976). The specific activities of B1 and B2 were typically 0.35 and 3.3 μmol/(min·mg), respectively. More recently, the individual subunits have been purified from overproducing strains N6405/pSPS2 and C600/pMB1 (Salowe & Stubbe, 1986) with typical specific activities for B1 and B2 of 0.5 and 5.0 μmol/(min·mg), respectively. The enzyme (1:1 subunit ratio) was routinely prereduced with 10 mM dithiothreitol and centrifuged through a 1-mL column of Sephadex G-25 or G-50 (Penefsky, 1977) equilibrated in 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (pH 7.6), 15 mM magnesium sulfate, and 1 mM EDTA (buffer A) prior to use. Enzyme concentrations were determined from the absorbance at 280 nm with published extinction coefficients (Thelander, 1973). Thioredoxin and thioredoxin reductase were isolated by modification of previously described procedures (Laurent et al., 1964; Moore et al., 1964; Pigiet & Conley, 1977). More recently, thioredoxin has been purified from an overproducing strain (Lunn et al., 1984) with a specific activity of 200 A_{412} units/(min·mg). Polynucleotide phosphorylase from *Micrococcus luteus* was obtained from P-L Biochemicals. Yeast formate dehydrogenase was purchased from Boehringer Mannheim. Bovine hemoglobin, yeast inorganic pyrophosphatase, and bovine intestinal alkaline phosphatase were obtained from Sigma.

[3′-^{3}H]- and [5′-^{3}H]uridines were prepared as previously described (Stubbe & Ackles, 1980; Harris et al., 1984). Sodium [^{15}N]azide (99 atom %; Stohler Isotope Chemicals) was exchanged to the lithium salt on Dowex 50 ion-exchange resin. The isotopically labeled or unlabeled 2′-azido-2′-deoxyuridines were synthesized from the corresponding uridines and lithium azide as previously described (Hobbs & Eckstein, 1977), while 2′-azido-2′-deoxyadenosine was the gift of Dr. Morris Robins, University of Alberta. The 2′-azido-2′-deoxyuridine was purified further on a preparative reversed-phase HPLC column in 10% methanol (flow rate 4 mL/min; retention time 18 min). Nucleosides were converted to the mono- and diphosphates by established methods (Yoshikawa et al., 1967; Kozarich et al., 1973). [β-^{32}P]N_3ADP and [β-^{32}P]N_3UDP were obtained from the unlabeled nucleotide by the polynucleotide phosphorylase catalyzed exchange reaction with [^{32}P]P_i (Czarnecki et al., 1982). When necessary, N_3ADP and N_3UDP were further purified by HPLC to remove contaminating 3′-*O*-methylcarbonates which were side products of the second phosphorylation reaction. This problem was eventually remedied by using water instead of methanol for the decomposition of excess carbonyldiimidazole reagent. The diphosphates were chromatographed on a semipreparative column equilibrated in 30% methanol–70% ion-pairing (IP) buffer (50 mM potassium phosphate, pH 4.8, 5 mM tetrabutylammonium bromide) (flow rate 2 mL/min; compound and retention time: N_3UDP, 5 min; N_3ADP, 6 min; 3′-*O*-methylcarbonate of N_3UDP, 12 min; 3′-*O*-methylcarbonate of N_3ADP, 16 min). Catalytic hydrogenation of N_3UDP was used to obtain 2′-amino-2′-deoxyuridine 5′-diphosphate (NH_2UDP) (Hobbs et al., 1973). The compound was purified by reversed-phase HPLC in IP buffer (flow rate 1.5 mL/min; retention time 9.5 min). Extinction coefficients of 10 000 M^{-1} at 261 nm and 15 000 M^{-1} at 259 nm were assumed for the uridine and adenosine nucleotide analogues, respectively.

ATP, NAD, NADPH, dGTP, TTP, CDP, hydroxyurea, 2-deoxyribose 5-phosphate, ammonia standard solution, and Nessler's based ammonia color reagent were obtained from Sigma. Polybuffer 74, Polybuffer Exchanger 94, and Sephadex resins were from Pharmacia. [^{14}C]CDP was purchased from New England Nuclear. [^{15}N]Ammonium sulfate (99 atom %) was purchased from Amersham. Poly(ethylenimine) (PEI) TLC plates from EM Laboratories were prerun in H_2O prior to use. Imidazole was recrystallized from benzene. All other reagents were obtained in the highest quality available.

Absorbance spectra were recorded on a Cary 210 spectrophotometer. A Packard 300C scintillation counter and Scint-A (Packard) scintillation fluid were used. Reversed-phase HPLC was performed on an Altex 110A system using Alltech C_{18} columns. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described by Laemmli (1970).

Stoichiometry of Inactivation. Various amounts of N_3UDP were added to tubes containing buffer A, 0.25 mM TTP, 0.5 mM NADPH, 1.5 μg of thioredoxin reductase, 5 μg of thioredoxin, and 10 μM RDPR in a final volume of 50 μL. After a 3-h incubation at 25 °C, 4 μL of each tube was assayed for residual activity. The assay mixture contained buffer A, 1.6 mM ATP, 1 mM NADPH, 1.25 mM [^{14}C]CDP (specific activity 5 × 10^5 cpm/μmol), 2 μg of thioredoxin, 0.6 μg of thioredoxin reductase, and enzyme in a final volume of 100 μL. After 10 min at 25 °C, the reaction was terminated by incubation in a boiling water bath for 1 min. Each sample was treated with 50 μL of 0.5 M Tris-HCl (pH 8.5) containing 1 unit of alkaline phosphatase for 1 h at 37 °C. Deoxycytidine formation was then determined as previously described (Steeper & Steuart, 1970).

Time-Dependent Inactivation of RDPR by Radiolabeled N_3UDPs. The inactivation mixtures contained buffer A, 0.25

mM TTP, 0.25 mM NADPH, 22–34 μM RDPR, 2 μg of thioredoxin reductase, and 8 μg of thioredoxin in a volume of 1.4 mL. The mixture was divided equally between two cuvettes and placed in the spectrophotometer reference and sample compartments thermostated at 25 °C. Hydroxyurea (20 μL of 1 M) was added to the reference cuvette while an equal volume of water was added to the sample side. After 30 min the difference spectrum of the tyrosyl radical was recorded. The reaction was initiated by the addition of N_3UDP to both cuvettes. The decrease in absorbance at 410 nm was followed for approximately 1 h, at which time no additional loss of tyrosyl radical occurred. Aliquots were removed before and after the reaction for the determination of enzymatic activity as described above. When measuring B1 specific activity, a 40-fold molar excess of B2 was included in the assay. For inactivations examining the release of PP_i, 8 mM sodium fluoride was included to inhibit any contaminating inorganic pyrophosphatase (Josse & Wong, 1971). To examine the requirement for reduced B1 sulfhydryl groups, oxidized RDPR was prepared as previously described (Ator & Stubbe, 1985) and the reducing system was omitted in the reaction. For inactivations using N_3ADP instead of N_3UDP, 0.1 mM dGTP replaced TTP as allosteric effector.

Reaction of [3′-^{3}H]N_3UDP with RDPR: Formation of ^3H_2O. RDPR (22 μM) was incubated with 180 μM [3′-^{3}H]N_3UDP (4.5×10^5 cpm/μmol) as described above. The contents of each cuvette were then transferred to capped plastic tubes and boiled for 1 min. After removal of precipitated protein by centrifugation, the supernatants were shell frozen and bulb-to-bulb distilled. Radioactivity in the distillates was determined by scintillation counting.

Reaction of [β-^{32}P]N_3UDP with RDPR: Detection and Identification of PP_i. RDPR (22 μM) was incubated with 150 μM [β-^{32}P]N_3UDP (1.1×10^7 cpm/μmol) as described above. After inactivation, 100 μL of 0.1 M tetrasodium pyrophosphate was added to each cuvette. The contents were extracted with charcoal and chromatographed on DEAE-Sephadex A-25 as previously described (Harris et al., 1984). Fractions were analyzed for radioactivity and phosphate content (Ames & Dubin, 1960). The region of constant specific activity was pooled and evaporated to dryness in vacuo. In an experiment designed to correlate protein labeling with PP_i release, 29 μM RDPR was inactivated as described above with 213 μM [5′-^{3}H,β-^{32}P]N_3UDP (7.3×10^6 cpm of ^{3}H/μmol; 4.7×10^6 cpm of ^{32}P/μmol). Carrier PP_i was added, and one-fourth of the reaction mixture was analyzed for bound ^{3}H as described below. The remainder was extracted with charcoal and analyzed as above for PP_i.

One portion of radioactive material (3600 cpm, 76 nmol of phosphate) isolated from a DEAE-Sephadex A-25 column was chromatographed on a PEI plate in 4 M ammonium formate (pH 3.5) (system A compound, R_f: PP_i, 0.44; P_i, 0.86; N_3-UDP, 0.88). The unknown was located by scintillation counting of strips of the plate which were each treated with 1 mL of 1 M triethylammonium bicarbonate (pH 7.5). Radioactivity was then eluted from the remainder of the TLC plate, and the specific activity of the reisolated material was determined by phosphate assay and scintillation counting.

A second portion of the unknown (4300 cpm, 100 nmol of phosphate) was treated with yeast inorganic pyrophosphatase in a reaction mixture that contained 0.1 M Tris-HCl (pH 7.2), 0.2 mM $MgCl_2$, and 0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) in a volume of 26 μL. Inorganic pyrophosphatase (0.2 unit) was added, and the mixture was maintained at room temperature for 30 s. The reaction was stopped by incubation for 1 min in a boiling water bath, and the mixture was chromatographed on a PEI plate in 0.5 M $KHSO_3$ (system B compound, R_f: 2-deoxyribose 5-phosphate, origin; PP_i, 0.22; P_i, 0.66). The radioactivity was located on the plate, and the specific activity of the material was measured as described above.

To demonstrate transient association of ^{32}P with protein, an inactivation mixture was allowed to react for only 5 min. It was then chromatographed on a 1 × 24 cm Sephadex G-50 column equilibrated in 50 mM Tris-HCl (pH 7.6) and 8 mM sodium fluoride. Fractions containing protein and radioactivity were pooled and maintained at 4 °C for 1 h. The solution was then filtered through an Amicon Centricon 30 ultrafiltration membrane. The enzyme was held at 4 °C for an additional hour and was then subjected to ultrafiltration a second time.

Reaction of [5′-^{3}H]N_3UDP with RDPR: Labeling of Enzyme. RDPR (34 μM) was incubated with 340 μM [5′-^{3}H]N_3UDP (1.6×10^7 cpm/μmol) as described above. The inactivated enzyme was desalted through a 1.5 × 12 cm column of Sephadex G-50 equilibrated in 25 mM imidazole hydrochloride (pH 7.4), loaded on a 3-mL column of Polybuffer Exchanger 94 resin equilibrated in the same buffer, and eluted with 48 mL of 8-fold diluted Polybuffer 74 hydrochloride (pH 4.0). Subunit B1 appeared approximately two-thirds of the way through the elution as a cloudy suspension that was immediately cleared by neutralization with several drops of 1 M HEPES, sodium salt. Subunit B2 was subsequently eluted with 300 mM NaCl in H_2O and again neutralized as soon as possible. Overall recovery of the proteins was 40–50%. SDS–PAGE of the protein pools indicated B2 to be entirely free of contaminating B1. A very minor amount of B2 was detected in the B1 pool. The amount of radioactivity bound to each subunit was determined by scintillation counting following quantitation of protein by UV absorbance.

Reactivation of Subunit B2. RDPR inactivated by N_3UDP and hydroxyurea-treated subunit B2 (60 nmol of each) were passed separately through Sephadex G-50 equilibrated in 25 mM imidazole hydrochloride (pH 7.4) and subsequently loaded on 3.5-mL Polybuffer Exchanger 94 columns. After elution as described above, the B2 pool from each column was concentrated to approximately 1 mL in a Centricon 30, dialyzed against 50 mM Tris-HCl (pH 7.6), and subjected to the iron removal/reconstitution procedure of Atkin et al. (1973). Specific activity was determined in the presence of excess B1 by using the [^{14}C]CDP assay described earlier, while iron and radical content were determined from the optical spectrum (Petersson et al., 1980).

Analysis of the Reaction of RDPR with N_3UDP for Uracil Formation. The inactivation mixture contained buffer A, 0.1 mM TTP, 0.1 mM prereduced RDPR, and 0.2 mM N_3UDP in a volume of 200 μL. A control run in parallel contained hydroxyurea-treated enzyme. After a 2-h incubation at 25 °C, 50 μL of 0.5 M Tris-HCl (pH 8.5) containing 0.5 unit of alkaline phosphatase was added, and the samples were incubated at 37 °C for 0.5 h. Protein was removed by centrifugation through a Centricon 30. An additional 750 μL of water was centrifuged through the membrane to ensure maximal recovery. The combined filtrates were then lyophilized, redissolved in 250 μL of water, and analyzed on a reversed-phase HPLC column eluted with water (flow rate 1.7 mL/min; uracil, 4.5 min).

Analysis of the Reaction of RDPR with N_3UDP and N_3-ADP for Azide Formation. The inactivation mixture contained buffer A, 1.6 mM ATP, 0.5 mM NADPH, 33 μM RDPR, 4 μg of thioredoxin reductase, 40 μg of thioredoxin, and 670

μM N_3UDP in a volume of 120 μL. An analogous N_3ADP reaction (670 μM) contained 0.1 mM dGTP in place of ATP. Inactivated protein was denatured by several methods. (1) The protein was heat denatured by incubation of a 40-μL aliquot of the inactivation mixture in a boiling water bath for 1 min. (2) The protein was acid precipitated by addition of 5 μL of 30% $HClO_4$ to a 45-μL aliquot of inactivation mixture, followed by neutralization with 5 μL of 5 M KOH. (3) The protein was acid precipitated by addition of 2 μL of 20% trichloroacetic acid, followed by neutralization with NaOH. (4) The protein was denatured by addition of 90 μL of 100% ethanol to 45 μL of inactivation mixture. In each case, the protein was removed by centrifugation and the supernatant was added to one of the assay systems described below.

(A) Azide release was measured by the inhibition of a formate dehydrogenase (FDH) assay system (Blanchard & Cleland, 1980) containing 100 mM HEPES (pH 7.5), 10 mM NAD, 1.7 mM sodium formate, and 0.04 unit of FDH in a volume of 1.0 mL. Aliquots of the inactivation mixture were added to the assay mix, and the rate of change in absorbance at 340 nm was monitored. This was compared to the rate observed with a control containing hydroxyurea-inactivated RDPR.

(B) Azide release was assayed by the shift in the Soret band of hemoglobin (Antonini & Brunori, 1971). The assay contained 0.2 mg/mL bovine hemoglobin, 10 mM HEPES (pH 7.5), and 0.9% NaCl in a total volume of 0.4 mL. Equal volumes of inactivation mixture and hydroxyurea-inactivated control were added to the sample and reference cuvettes, respectively. Azide binding was measured by formation of a difference spectrum with a maximum at 422 nm and a minimum at 404 nm.

Controls were performed to examine whether any loss of azide occurred under the denaturation conditions used in these experiments. Identical treatment of mixtures that contained known qualities of azide and bovine serum albumin in place of RDPR led to detection of the same amount of azide in both assays as when an equal quantity of azide was added directly.

Reaction of [$^{15}N_3$]N_3UDP with RDPR: Detection of Nitrogen Gas Release. Nitrogen gas evolution was detected with a Varian MAT 250 isotope ratio mass spectrometer. A metal U-tube fitted with an end cap containing a rubber septum was attached to the sample inlet portal and submerged in a dry ice–alcohol bath to prevent contamination of the instrument with water vapor. Various quantities (0–50 nmol) of a standardized [^{15}N]ammonium sulfate solution were reacted with alkaline hypobromite (Bremner, 1965) to generate known quantities of m/e 30 dinitrogen for instrumental calibration. Samples were run in 2-mL vials fitted with a screw cap containing a double thickness (6-mm) rubber septum. Each vial containing ammonium sulfate and water in a volume of 250 μL was cooled in ice and degassed with a vacuum pump through a 20-gauge side-hole needle. The reaction was initiated by the addition with a Hamilton gas-tight syringe of 250 μL of argon-purged hypobromite reagent (Hermes et al., 1985). The mixture was incubated for 1 min at room temperature and then frozen in a dry ice–alcohol bath. Gas samples were introduced into the mass spectrometer via a double needle made by fusing together the plastic ends of two 20-gauge hypodermic needles. To avoid plugging with septum material, side holes were drilled into the needle shafts, and the original openings were filled with solder. One end of the double needle was inserted into the septum of the instrument inlet portal while the other was inserted into the vial septum just far enough to cover the side hole. This procedure allowed degassing of the inlet system. The sample was then introduced by pushing the needle completely through the vial septum to allow gas formed in the reaction to enter the mass spectrometer. Accelerating voltage was scanned through m/e 28, 29, and 30 (corresponding to $^{14}N_2$, $^{14}N^{15}N$, and $^{15}N_2$, respectively) while the pen deflection was recorded on a strip-chart recorder. Instrumental background, which was recorded between each sample, remained constant over the day of use.

For enzymatic samples, 500 μL of inactivation solution containing buffer A, 0.25 mM TTP, 0.4 mM NADPH, 4 μg of thioredoxin reductase, 11 μg of thioredoxin, and 40 or 80 μM RDPR (active or preincubated with 50 mM hydroxyurea) in a 2-mL vial was chilled on ice and degassed through a 20-gauge needle. After the solution was warmed to 25 °C, the reaction was initiated by the addition of argon-purged [$^{15}N_3$]N_3UDP (210 μM final) with a gas-tight syringe. After a 45-min incubation the samples were frozen and analyzed as described for the standards above. Afterward, each sample was thawed, and 125 μL was centrifuged through a 1-mL column of Sephadex G-50 in buffer A. The collected protein was scanned to estimate radical loss and assayed for remaining activity.

Results

Time-Dependent Loss of Activity and Tyrosyl Radical. Time-dependent inactivation of RDPR and loss of absorbance at 410 nm due to destruction of the B2 tyrosyl radical was observed upon incubation of N_3UDP or N_3ADP with the enzyme. The amount of radical remaining after complete reaction was somewhat variable, but in most of the experiments reported in this paper, 85–90% of the original A_{410} was lost during the inactivation. In contrast to the original report of Thelander et al. (1976) with N_3CDP, a decrease in the B1 specific activity of approximately 50% was observed at the end of the reaction with N_3UDP. As expected, no inactivation of B1 occurred in the absence of B2. In agreement with the results of Thelander et al. (1976) with N_3CDP, no enzyme inactivation was observed with either N_3UDP or N_3ADP when the negative allosteric effector dATP was substituted for the proper positive effector, nor did inactivation or tyrosyl radical loss occur with N_3UDP when the active-site thiols of B1 were oxidized. Unlike the case of RDPR inactivation by ClUDP (Ator & Stubbe, 1985), the presence of 200 mM ethanethiol did not protect against inactivation by N_3UDP.

Determination of Number of Turnovers per Inactivation. The efficiency of inactivation of RDPR by N_3UDP was determined by partially inactivating the enzyme with various amounts of the inhibitor. The remaining catalytic activity was measured after an extended incubation to ensure complete consumption of the compound. Figure 1 demonstrates that the inactivation was extremely efficient with only 1 equiv of N_3UDP capable of completely eliminating RDPR activity.[2] The lack of multiple turnovers necessitated the preparation of isotopically labeled compounds in order to isolate, identify, and quantitate the very small amount of products formed in the reaction.

Reaction of [3′-^{3}H]N_3UDP with RDPR: Formation of ^3H_2O. Previous studies from this laboratory have demonstrated

[2] Thelander et al. (1976) claimed that 0.2 equiv of N_3CDP was sufficient for total inactivation, but in a later experiment they reported consumption of 1.5 mol of N_3CDP/mol of B2 concomitant with oxidation of 2.8 mol of sulfhydryl groups/mol of 2-deoxyribose 5-diphosphate formed. They noted that their B2 preparations had "variable and low content of free radical". Since information regarding specific activity and radical content of the enzyme in each experiment was not provided, these internal inconsistencies are impossible to reconcile.

Table I: Product Formation from Radiolabeled N_3UDP Reactions

compound	fractional activity remaining	fractional tyrosyl radical remaining	mol/mol of RDPR		
			^3H_2O	$[^{32}P]PP_i$	protein-bound ^3H
$[3'-^3H]N_3UDP$	0.02	0.09	0.2		
$[\beta-^{32}P]N_3UDP$	0.01	0.14		0.8	
$[5'-^3H]N_3UDP$	0.02	0.11			0.9
$[5'-^3H,\beta-^{32}P]N_3UDP$	0.02	0.11		1.1	1.1 (0.9)[a]

[a] After dialysis.

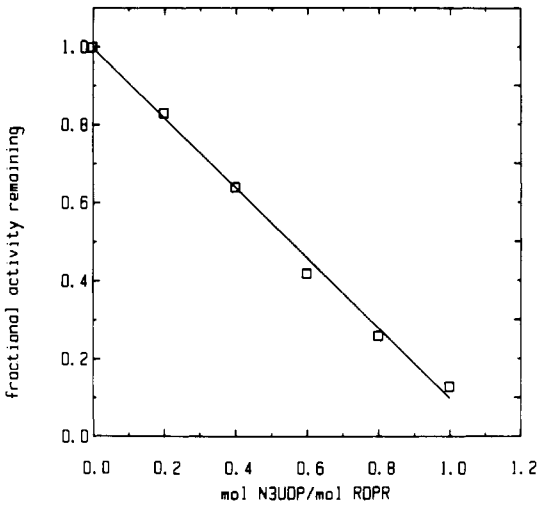


FIGURE 1: Determination of number of turnovers per inactivation. RDPR (10 μM) was incubated for 3 h with different concentrations of N_3UDP as described under Materials and Methods. The fraction of remaining enzyme activity is plotted vs. the initial inactivator to enzyme molar ratio.

that RDPR catalyzes cleavage of the 3′ carbon–hydrogen bonds of UDP, ADP and ClUDP (Stubbe & Ackles, 1980; Stubbe et al., 1983; Harris et al., 1984). The production of ^3H_2O accompanying inactivation of the enzyme by $[3'-^3H]N_3UDP$ (Table I) demonstrates that cleavage of the 3′ carbon–hydrogen bond of N_3UDP is also catalyzed by RDPR. Pretreatment of the enzyme with hydroxyurea to eliminate the B2 tyrosyl radical suppressed >95% of the ^3H_2O formation. The amount of ^3H_2O produced from $[3'-^3H]N_3UDP$ was about 20% of the amount of enzyme inactivated. Since inactivation occurs in one turnover (Figure 1) and the percentage of the total compound consumed was low, this result is consistent with an isotope effect of approximately 5 on 3′ carbon–hydrogen bond cleavage.

Reaction of $[\beta-^{32}P]N_3UDP$ with RDPR: Formation and Identification of PP_i. Thelander et al. (1976) reported that 2-deoxyribose 5-diphosphate was a product of the reactions of both ClUDP and N_3CDP with RDPR. Since the actual product of the ClUDP reaction with the enzyme was later shown to be inorganic pyrophosphate and not 2-deoxyribose 5-diphosphate (Stubbe & Kozarich, 1980), the phosphate-containing product from the azido nucleotide reaction was reexamined.

After reaction of RDPR with $[\beta-^{32}P]N_3UDP$, carrier PP_i was added and nucleotides were removed by treatment with charcoal at low pH. The unadsorbed material was chromatographed on DEAE-Sephadex A-25 with a triethylammonium bicarbonate gradient. Approximately 25% of the ^{32}P loaded onto the column eluted in the P_i region, while the remainder cochromatographed with the added PP_i carrier. The formation of $[^{32}P]P_i$ appears to be the result of phosphatase action on the nucleotide or decomposition during the charcoal extraction, since in the hydroxyurea inactivated enzyme control approximately the same amount of ^{32}P was found in the P_i region but essentially none (<5% of reaction) was found associated with the carrier PP_i. The fractions of constant specific activity in the PP_i region of the experimental sample were combined, and recovery of radioactivity and phosphate were determined. Approximately 1 mol of PP_i was produced per mole of enzyme inactivated (Table I). Similar results were obtained with $[\beta-^{32}P]N_3ADP$ (data not shown). The ^{32}P-labeled compound that comigrated with PP_i was further analyzed to provide conclusive identification. The radioactive material was chromatographed on PEI plates in system A, which separates PP_i from N_3UDP and N_3ADP. The ^{32}P was recovered only in the region of PP_i, and the specific activity of the material was unchanged, consistent with the identification of the material as PP_i.

The possibility that the ^{32}P-containing product of the enzymatic reaction was 2-deoxyribose 5-diphosphate was excluded by treatment with yeast inorganic pyrophosphatase. This enzyme specifically cleaves PP_i to P_i in the presence of Mg^{2+}, although it will also hydrolyze organic pyrophosphates in the presence of Zn^{2+} (Butler, 1971). Metal ions other than Mg^{2+} were removed from the assay through the use of EGTA (Moe & Butler, 1972). The pyrophosphatase-treated ^{32}P-labeled material was chromatographed on PEI TLC plates in system B, which separates P_i from PP_i and retains 2-deoxyribose 5-phosphate at the origin. All of the ^{32}P was recovered in the region of P_i with a specific activity of approximately 50% that of the starting material. This is the expected result for cleavage of $[^{32}P]PP_i$ to P_i, since only the terminal phosphate of the azido nucleotide was ^{32}P labeled. Cleavage of 2-deoxyribose 5-diphosphate to 2-deoxyribose 5-phosphate by pyrophosphatase would have produced P_i with a specific activity equal to that of the starting material. No formation of P_i was observed upon treatment of 2-deoxyribose 5-phosphate with pyrophosphatase, which rules out the production of 2 equiv of P_i from 2-deoxyribose 5-diphosphate. These results conclusively demonstrate that PP_i and not 2-deoxyribose 5-diphosphate is the product of the reaction of RDPR with the azido nucleotides.

RDPR-Catalyzed Release of Uracil. Sjöberg et al. (1983) reported that cytosine is a product of the reaction of N_3CDP with RDPR. The reaction of RDPR with N_3UDP was also examined for base release. When an N_3UDP reaction mixture was chromatographed on reversed-phase HPLC subsequent to RDPR inactivation, a peak was observed that comigrated with uracil and that was not present in the control incubation containing hydroxyurea-treated enzyme. The compound had a λ_{max} at 260 nm that shifted to 284 nm at alkaline pH in a manner identical with authentic uracil. Quantitation of the material from the UV spectrum indicated that 0.9 ± 0.1 mol uracil was formed per mole of RDPR inactivated.

Analysis of the Reaction of RDPR with N_3UDP and N_3ADP for Azide Formation. The production of base and PP_i in the reaction of N_3UDP with RDPR is analogous to the observations made with ClUDP (Thelander et al., 1976;

Table II: Formation of Dinitrogen from [$^{15}N_3$]N_3UDP

sample	nmol of RDPR	nmol of $^{30}N_2$ formed[a]	mol of N_2/mol of RDPR
active RDPR[b]	20	18, 17	0.9
	40	34, 34	0.9
hydroxyurea-treated RDPR	20	<1	0.0
	40	<1	0.0

[a] Values shown for active RDPR are duplicate runs and are ±4 nmol. [b] At the end of the reaction ≤5% enzymatic activity and ≤10% tyrosyl radical remained.

Stubbe & Kozarich, 1980). The elimination of chloride from that analogue suggested that azide might be released from the azido nucleotides. This possibility was tested by using two very sensitive biological assays for azide ion. Blanchard and Cleland (1980) reported that azide is a potent competitive inhibitor (K_i = 7 nM) of yeast formate dehydrogenase. A standard curve for quantitation of azide was constructed by addition of known amounts of azide to FDH assays. The addition of 0.1 μM azide resulted in 45% inhibtion of the enzyme. When reactions containing RDPR inactivated by N_3UDP or N_3ADP were deproteinized and added to FDH assays, <15% inhibition was observed. This corresponds to production of <0.02 mol of azide per mole of enzyme inactivated. The possibility that azide was loosely associated with the protein was excluded by denaturing the enzyme with heat, acid, or ethanol. No additional FDH inhibition was observed following workup of the reactions under these conditions.

A second procedure for the detection of azide involved the measurement of the shift in the Soret band of ferric hemoglobin on the binding of azide (Antonini & Brunori, 1971). A difference spectrum with a minimum at 404 nm and a maximum at 422 nm was formed on addition of azide to the sample cell, with the magnitude of the spectrum being a linear function of azide concentration up to 1 μM. Parallel reactions that contained an azido nucleotide and either RDPR or hydroxyurea-treated RDPR were run. After removal of protein, the experimental and control incubations were added to the hemoglobin-containing sample and reference cuvettes, respectively. No significant difference spectrum was formed, consistent with the conclusion that azide is not a product of the inactivation of RDPR by N_3UDP and N_3ADP.

Reaction of [$^{15}N_3$]N_3*UDP with RDPR: Formation of Dinitrogen.* Since azide is not a product of the N_3UDP reaction with RDPR and only one nitrogen atom is observable in the EPR signal of the new radical species (Ator et al., 1984), production of molecular nitrogen was investigated by exploiting the sensitivity of an isotope ratio mass spectrometer. The azido nucleotide was prepared with ^{15}N isotopic substitution in order to reduce the problems with environmental contamination ($^{28}N_2$:$^{30}N_2$ = 73 000:1 in air) as well as provide unequivocal evidence for the source of any gas detected. Instrumental response was calibrated by generating known quantities of dinitrogen from the reaction of [^{15}N]ammonium sulfate with alkaline hypobromite (Bremner, 1965), and a standard curve for *m/e* 30 was constructed (Figure 2). Masses 28 and 29 were monitored as well for indications of atmospheric contamination. As indicated in Table II, approximately 0.9 mol of *m/e* 30 dinitrogen was formed per mole of RDPR inactivated with [$^{15}N_3$]N_3UDP. No nitrogen above background was detected with the hydroxyurea-inactivated control.

Reaction of [5′-^3H]N_3*UDP with RDPR: Labeling of Protein.* In the reaction of ClUDP with RDPR, the sugar moiety of the compound is converted to 2-methylene-3(2*H*)-furanone, which alkylates protein residues (Harris et al., 1984; Ator & Stubbe, 1985). RDPR was examined for covalent modification

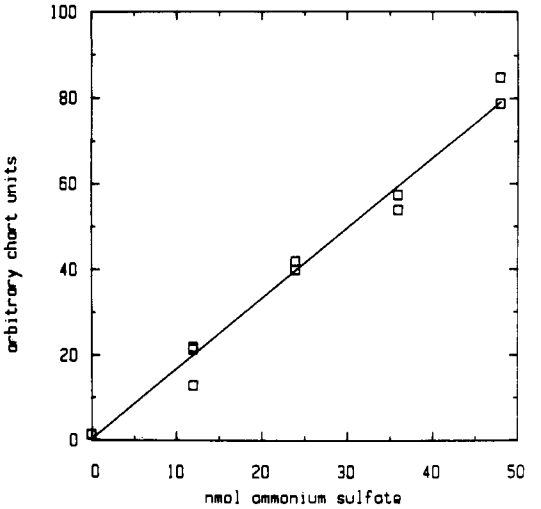


FIGURE 2: Standard curve for nitrogen formation. Various amounts of [^{15}N]ammonium sulfate were oxidized with hypobromite and introduced into the mass spectrometer as described under Materials and Methods. Voltage was scanned, and the pen deflection for *m/e* 30 was noted. The small nonzero intercept reflects instrumental background.

by a similar species after reaction with N_3UDP. Following inactivation with [5′-^3H]N_3UDP and isolation of the protein by gel filtration, 0.9–1.1 mol of ^3H was bound per mole of RDPR inactivated (Table I). RDPR that was inactivated with hydroxyurea prior to the addition of [5′-^3H]N_3UDP contained less than 1% of this amount of ^3H. The label was stable to dialysis against 25 mM imidazole hydrochloride (pH 7.4), with 0.9 mol of ^3H/mol of RDPR remaining bound after 18 h. In an experiment using the double-labeled compound [5′-^3H,β-^{32}P]N_3UDP, the amount of released [^{32}P]PP_i was equivalent to the amount of protein-bound ^3H (Table I). In contrast to the results with [5′-^3H]N_3UDP, RDPR inactivated with [3′-^3H]N_3UDP had <0.01 mol of ^3H/mol of enzyme bound after gel filtration on Sephadex G-50.

In order to determine whether the radiolabeling of the protein by [5′-^3H]N_3UDP demonstrated any selectivity for one subunit over the other, the enzyme was subjected to chromatofocusing to separate the B1 and B2 subunits. SDS–PAGE indicated that the subunits were effectively separated by this procedure. The isolated B1 retained 0.8 mol of ^3H/mol of protein, while <0.01 mol of ^3H/mol of protein was found to be bound to the B2 subunit, indicating a very high selectivity of labeling.

The optical spectrum of the recovered B1 revealed a new absorbance shoulder at approximately 320 nm. The A_{320}/A_{280} ratio of the modified protein was nearly double that of unmodified B1 subjected to the same chromatofocusing conditions. With the amount of bound ^3H as a measure of concentration, and extinction coefficient of approximately 13 000 M^{-1} at 320 nm was calculated for the new chromophore.

The reaction of RDPR with N_3UDP was also examined for evidence of covalent modification of the enzyme by a phosphorylated compound. After a brief incubation with [β-^{32}P]N_3UDP sufficient to destroy 70% of the tyrosyl radical, an amount of radioactivity corresponding to 0.6 mol of ^{32}P/mol of enzyme comigrated with the protein isolated by gel filtration chromatography. After 1 h at 4 °C the protein was subjected to ultrafiltration, resulting in isolation of 84% of the ^{32}P in the filtrate. Ultrafiltration of the protein solution a second time after an additional 1 h at 4 °C allowed recovery of the remainder of the radioactivity in the filtrate. Chromatography of the combined filtrates on DEAE-Sephadex A-25 and ion-

pairing reversed-phase HPLC demonstrated that the radioactive compound cochromatographed with added PP_i carrier. This result contrasts with the lack of ^{32}P bound to RDPR inactivated by [β-^{32}P]N_3CDP reported previously (Thelander et al., 1976). The longer reaction time (20 min) employed in that study may have allowed dissociation of the radioactivity from the enzyme, however.

The above results suggested that the only modification of the B2 subunit during inactivation was tyrosyl radical destruction. Native B2 can be regenerated from radical-free B2 (e.g., from hydroxyurea treatment) by removing the bound Fe^{3+} and aerobically reconstituting with Fe^{2+} (Atkin et al., 1973). N_3UDP-inactivated B2 and native B2 that had been subjected to the same chromatofocusing conditions were carried through the iron removal/reconstitution procedure. Both samples regained 90% of their original specific activity as well as iron and radical content as determined from the visible spectra. Thelander et al. (1976) achieved 79% reactivation in a similar experiment with N_3CDP-inactivated enzyme.[2]

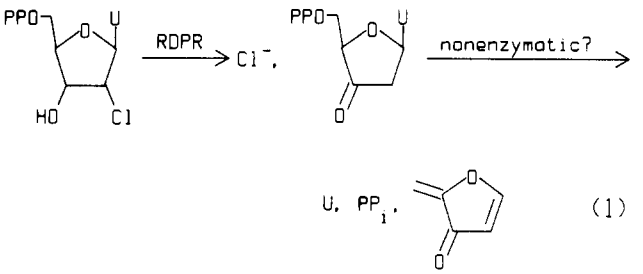
Discussion

As reported previously (Thelander et al., 1976), incubation of N_3UDP with RDPR results in enzyme inactivation and tyrosyl radical loss. The potency of this compound as an inactivator is demonstrated by the fact that 1 equiv can completely eliminate enzymatic activity (Figure 1). Inactivation by N_3ADP extends the list of azido-substituted analogues to include a purine nucleotide. Destruction of the catalytically essential B2 tyrosyl radical is obviously an important contributor to inactivation as was first suggested by Thelander et al. (1976). The initial study by these workers, however, incorrectly concluded that B1 was unaffected during the reaction. Our work clearly demonstrates that stoichiometric covalent modification of this subunit occurs (Table I) along with a reduction in specific activity of approximately 50%. The loss of half of the B1 activity is consistent with one turnover per enzyme inactivation event and 1 equiv of radiolabeling since B1 has two active sites while B2 has only one tyrosyl radical.

Previous studies from our laboratory have demonstrated that cleavage of the nucleotide 3′ carbon–hydrogen bond is a key event in the reaction of RDPR with substrates UDP and ADP (Stubbe & Ackles, 1980; Stubbe et al., 1983) and mechanism-based inactivator ClUDP (Harris et al., 1984). The formation of ^3H_2O from [3′-^{3}H]N_3UDP (Table I) indicates that this bond cleavage is also an essential feature of the reaction of RDPR with N_3UDP. The release of ^3H implies that the 3′ hydrogen is abstracted by a residue that can subsequently exchange with solvent. The production of 0.2 equiv of ^3H in one turnover may be interpreted as an isotope effect on this reaction of approximately 5. We have sought to confirm the existence of an isotope effect by comparing the rates of enzyme inactivation and tyrosyl radical destruction by [3′-^{2}H]N_3UDP and unlabeled N_3UDP. Although we cannot yet extract quantitative data, our preliminary experiments with the deuteriated compound have found significantly slower initial rates of inactivation and tyrosyl radical loss (M. Ator, J. Stubbe, and D. Ballou, unpublished results).

These very important observations suggest that the destruction of the tyrosyl radical, a process that clearly requires one-electron chemistry, may be coupled to cleavage of the 3′ carbon–hydrogen bond of the nucleotide, a common feature of all RDPR-catalyzed reactions that have been examined. This would indicate that the 3′ hydrogen is abstracted as a hydrogen atom to produce a nucleotide radical intermediate. The same step has been hypothesized to occur with all substrates and substrate analogues studied (Stubbe et al., 1983; Harris et al., 1984). In the case of substrates and ClUDP we have proposed that the nucleotide radical exists only transiently, regenerating the tyrosyl radical after each turnover. In the reaction of the azido nucleotides, however, the initially formed nucleotide radical appears to decompose by a different pathway in which the azido group acts as an intramolecular radical trap to form a new, relatively stable radical species. Enzyme inactivation thus occurs in part because the tyrosyl radical is not regenerated at the end of each turnover.

The products of ClUDP-mediated inactivation of RDPR are PP_i, base, Cl^-, and 2-methylene-3(2*H*)-furanone, which alkylates the protein (eq 1). The possibility of analogous



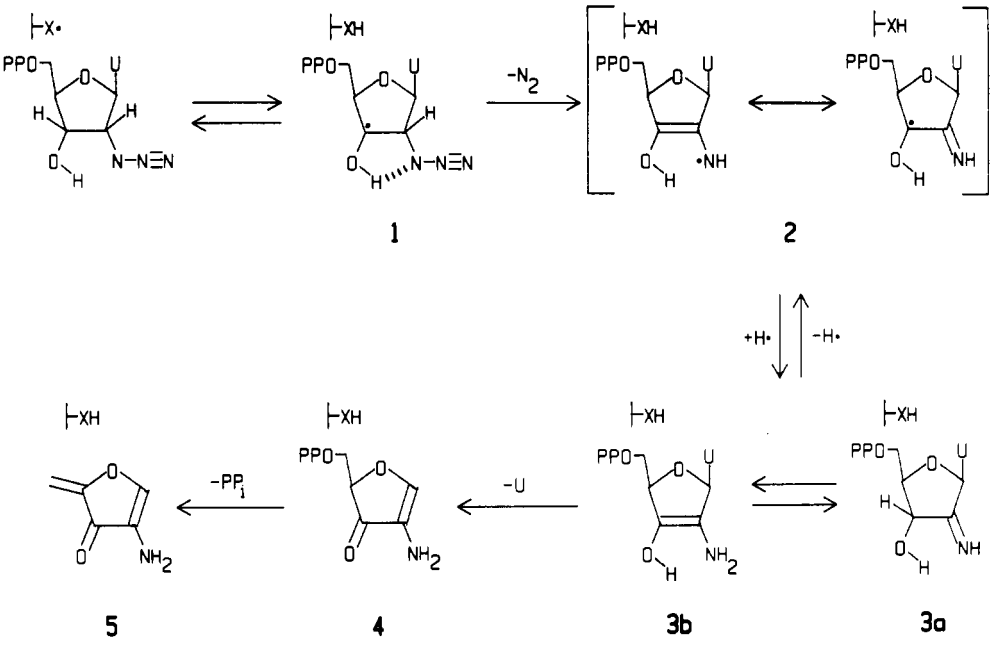
(1)

products in the N_3UDP reaction was therefore investigated with isotopically labeled compounds. Our experiments with [β-^{32}P]N_3UDP demonstrated the production of 1 equiv of inorganic pyrophosphate (Table I) rather than 2-deoxyribose 5-diphosphate, incorrectly identified by Thelander et al. (1976). Uracil was stoichiometrically eliminated from N_3UDP, consistent with the observation of stoichiometric cytosine release from N_3CDP reported by Sjöberg et al. (1983). The 2′ substituent of the azido analogues was not eliminated as azide ion, however. Mass spectrometric analysis of the reaction of RDPR with [$^{15}N_3$]N_3UDP demonstrated that two of the azido nitrogens were stoichiometrically released as nitrogen gas (Table II). The remaining nitrogen atom is observable in the transient EPR signal that accompanies inactivation (Sjöberg et al., 1983; Ator et al., 1984).

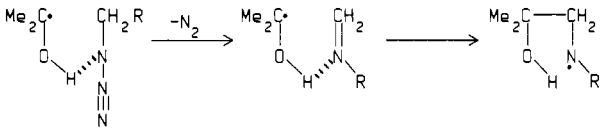
In addition to destroying the tyrosyl radical, reaction of N_3UDP with RDPR also results in covalent modification of the enzyme. Radiolabeling was achieved with [5′-^{3}H]N_3UDP (Table I) with high specificity for the B1 subunit. Modification was accompanied by the formation of a new absorbance at 320 nm similar to the absorbance formed during ClUDP inactivation of RDPR (Thelander, 1976; Harris et al., 1984). Chemical models suggest in that case that the new chromophore is an adduct of 2-methylene-3(2*H*)-furanone (G. Ashley, unpublished work), the alkylating species generated during ClUDP turnover (Harris et al., 1984). A related structure appears likely for the N_3UDP-derived adduct. However, ethanethiol, which protects against inactivation by ClUDP, has no effect upon inactivation by N_3UDP, reflecting the likelihood in the latter case that the putative furanone species is not released to solution. Work is under way to identify the B1 residue(s) that is (are) modified.

The most intriguing product of the reaction of RDPR with azido nucleotides is the remarkably stable new radical observable by EPR spectroscopy (Sjöberg et al., 1983; Ator et al., 1984). While the structure of this species was not directly addressed in these studies, the observation that ^{32}P was tightly, but not covalently, bound to the enzyme following inactivation with [β-^{32}P]N_3UDP suggests that the nucleotide's phosphates are not released to solution prior to generation of the new radical. The elimination of PP_i might occur only when the active site opens, allowing the nucleotide radical to be

Scheme I



Scheme II



quenched. It is not possible to distinguish, however, whether the PP_i is still sugar bound in the new radical or whether it has been eliminated but remains held in the closed active site.

To date no plausible mechanism has been proposed to account for the inactivation of RDPR by azido nucleotides. On the basis of the results presented in this paper, we propose Scheme I as a reasonable working hypothesis for the reaction mechanism. The initial step is proposed to be abstraction of the 3′ hydrogen by the B2 cofactor to generate 3′ nucleotide radical **1**. The protein residue is denoted as "-X·" since the tyrosyl radical may only be a radical initiator rather than the actual abstractor. Compound **1** is hypothesized to decompose with liberation of N_2 to yield the EPR-observable delocalized radical **2**. The protonation states of the 3′ oxygen and 2′ nitrogen are arbitrarily designated in structure **2**. Eventually **2** would abstract a hydrogen atom to form **3a** or **3b**. Abstraction from a component of the reaction mixture is postulated to occur in the majority of turnovers, but an occasional reabstraction from the active-site tyrosine could regenerate a small amount of tyrosyl radical. Tautomerization of **3b** with β-elimination of uracil would then form **4**, which in turn would release PP_i through another β-elimination. The unsaturated ketone **5** would be a Michael acceptor that alkylates the protein.

While the mechanism in Scheme I is consistent with the experimentally observed products of inactivation, we have not found a good chemical precedent in the literature for the decomposition of a β-azidoalkyl radical such as **1**. Roberts and Winter (1979) found that the 1-hydroxy-1-methylethyl radical reacted with primary alkyl azides to form dialkylaminyl radicals (Scheme II). They proposed that hydrogen bonding, or even complete proton transfer, facilitated the expulsion of N_2. Scheme I would then appear to be an intramolecular variation of that reaction. It should be noted, though, that the reaction of 1-hydroxy-1-methylethyl radical with secondary azides produced only iminyl radicals. Although the EPR parameters of iminyl and aminyl radicals do not fit the N_3UDP-derived radical very well, structure **2** in Scheme I has delocalized spin density with closer resemblance to the semiquinonimine radical observed for cysteinyldopa melanin (Sealy et al., 1982). Experiments designed to model this reaction and more definitely assign the new EPR-observable species are in progress.

Scheme I proposes no role for the redox-active thiols of subunit B1. Although our study on N_3UDP and the work of Thelander et al. (1976) on N_3CDP agree that inactivation requires the reduced form of B1, these results do not distinguish between a requirement for actual thiol participation and a requirement for an active protein conformation induced by disulfide reduction. Thelander et al. (1976) claimed that sulfhydryl groups were oxidized during the reaction,[2] but we calculate from their data that an absorbance change of just 8% in the spectrophotometric thiol assay would have been observed between native and inactivated enzyme. The variation in the sulfhydryl determination of native B1 by their cited method (Thelander, 1973, 1974) was about 5%. Furthermore, the loss of titratable thiols does not distinguish between covalent modification of the residues and oxidation to a disulfide. Consequently, we believe that sulfhydryl involvement has not been conclusively demonstrated in the reaction.

An alternative mechanism to that shown in Scheme I can be described that invokes the redox thiols in the reduction of the nucleotide azido group to molecular nitrogen. The reaction of organic azides with dithiothreitol at high pH (Staros et al., 1978) is a reasonable chemical model. After abstraction of the 3′ hydrogen (which could occur before or after the reduction), generation of a nitrogen-centered radical would require homolytic C–N bond cleavage, a step having chemical precedent for a trialkylamine (Gilbert et al., 1972). The resulting aminyl radical, however, is a poor candidate for the EPR-observable nucleotide radical. Furthermore, this mechanism predicts that NH_2UDP might be a time-dependent inactivator of RDPR. No inactivation or destruction of the tyrosyl radical was observed with NH_2UDP (data not shown), arguing against its intermediacy, although alternative explanations can be offered. On balance we currently prefer the mechanism in Scheme I to account for our experimental observations.

In summary, substrate analogue N_3UDP inactivates RDPR in one turnover with stoichiometric formation of uracil, PP_i, and N_2. The 3′ carbon–hydrogen bond is broken during the reaction. The tyrosyl radical on the B2 subunit is destroyed, and the B1 subunit is covalently modified with a loss of half of its specific activity. The inactivation of the enzyme is proposed to occur by a radical mechanism that is closely related to normal substrate turnover.

ACKNOWLEDGMENTS

We thank Professor Morris Robins for the generous gift of 2′-azido-2′-deoxyadenosine and Professor Jeremy Knowles for suggesting the reduction of organic azides by dithiols as a potential model reaction. We greatly appreciate the assistance of Paul Weiss and Professor Robert Burris in the operation of the mass spectrometer.

Registry No. RDPR, 9047-64-7; N_3UDP, 36792-49-1; tyrosyl radical, 34537-57-0; dinitrogen, 7727-37-9; pyrophosphate, 2466-09-3; uracil, 66-22-8.

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