Interaction of C225SR1 Mutant Subunit of Ribonucleotide Reductase with R2 and Nucleoside Diphosphates: Tales of a Suicidal Enzyme

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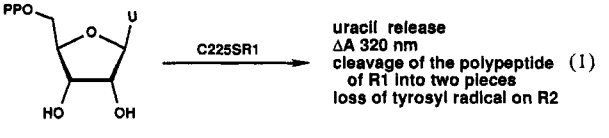
ABSTRACT: Ribonucleotide reductase (RDPR) from *Escherichia coli* is composed of two subunits, R1 and R2, both of which are required to catalyze the conversion of nucleotides to deoxynucleotides. This reduction process is accompanied by oxidation of two cysteines within the active site to a disulfide. One of these putative active site cysteines, C225, has been mutated to a serine, and the properties of this mutant (C225SR1) have been investigated in detail. Incubation of C225SR1 and R2 with [3′-^{3}H,U-^{14}C]UDP results in time-dependent inactivation of the enzyme! This inactivation is accompanied by production of 2.4 uracils, ^3H_2O, and ^{3}H,^{14}C-labeled protein with an absorbance change at 320 nm. There is an isotope effect ($k_H/k_{^3H}$) on uracil production of 3.2. In addition, the tyrosyl radical on R2 is reduced. The observation of ^3H_2O, indicative of 3′ carbon–hydrogen bond cleavage and loss of the tyrosyl radical, provides a direct test of our mechanistic hypothesis that cleavage of this bond occurs concomitantly with tyrosyl radical reduction. Incubation of [3′-^{2}H]UDP with C225SR1 and R2 resulted in a V and V/K isotope effect on loss of the radical of 2.0 and 2.0, respectively. These studies provide the first direct evidence for protein radical involvement in catalysis. Reduction of the tyrosyl radical on R2 is accompanied by a stoichiometric cleavage of the R1 polypeptide into two new polypeptides of 26 and 61 kDa. The 26-kDa polypeptide is the N-terminus of R1, and hence cleavage of the polypeptide is occurring in the region of the mutation. The N-terminus of the 61-kDa polypeptide is blocked. The mutant RDPR is inactivated by three different mechanisms: loss of the tyrosyl radical on R2, alkylation of R1 by 2-methylene-3(2*H*)-furanone, and cleavage of R1 into two polypeptides. This sequence of events is also observed with the purine substrate ADP and requires the presence of the appropriate allosteric effector. Studies with a variety of single and double R1 mutants suggest that the cleavage reaction requires the presence of C462 and C439. Small amounts of dUDP (dADP) are also observed during the interaction of UDP (ADP) with C225SR1R2 and are attributed to the presence of contaminating heterodimer of wt-R1 and mutant. A single mutation of an active site cysteine to a serine has converted the normal substrates into mechanism-based inhibitors. A mechanism to accommodate these amazing results is presented.

Ribonucleotide reductases catalyze the conversion of nucleotides to deoxynucleotides concommitantly with oxidation of two cysteines within the active site (Stubbe, 1990; Eriksson & Sjöberg, 1990; Thelander & Reichard, 1979). The *Escherichia coli* protein (RDPR) is composed of two subunits: R1 and R2. R1, a dimer of M_r = 171.4 kDa, binds the nucleoside diphosphate (NDP) substrates and the allosteric effectors that control the rate and the specificity of substrate reduction. R1 also contains the redox-active cysteines involved in catalysis. R2, a dimer of M_r = 86.5 kDa, contains the unusual tyrosyl radical–dinuclear iron center cofactor required for catalysis. It has been proposed that the active enzyme is composed of a 1:1 complex of these two subunits. Mounting evidence suggests however that R2 might function as a cofactor in catalysis in much the same way that adenosylcobalamin is a cofactor in the *Lactobacillus leichmannii* reductase (Stubbe, 1989, 1990). The details of the dynamic interaction between these subunits remain to be elucidated.

On the basis of studies using isotopically labeled substrates, a complex mechanistic model for RDPR-catalyzed NDP reduction has been proposed [Stubbe (1990), accompanying papers, and Scheme I, preceding paper]. The key initial step is protein radical (X•) mediated homolytic cleavage of the substrate's 3′ C–H bond to produce a substrate radical and a reduced protein radical (XH). Studies using 3′-^{2}H- and ^{3}H-labeled NDPs have demonstrated that this 3′ carbon–hydrogen bond is cleaved during catalysis (Stubbe et al., 1983). All efforts to demonstrate tyrosyl radical reduction during catalysis have thus far proven unsuccessful (Stubbe, 1989).

In the past few years, two complementary approaches have been taken in an attempt to further unveil the mechanistic subtleties of the reductase-catalyzed reaction. The first involves the use of substrate analogs and mechanism-based inhibitors (Thelander et al., 1976; Harris et al., 1984; Sjöberg et al., 1983; Salowe et al., 1987); the second involves the use of protein analogs, that is, site-directed mutants (Mao et al., 1989, 1990; Aberg et al., 1989). In the latter case, the proposal was made that removal of one or both of the cysteines directly involved in substrate reduction might uncouple 3′ carbon–hydrogen cleavage from the complex reduction process. This uncoupling might then allow direct observation using stopped-flow kinetics methods of a correlation between cleavage of the 3′ carbon–hydrogen bond of NDP and reduction of the tyrosyl radical.

Evidence described by Mao et al. (1989) suggests that C225 is one of the thiols directly involved in substrate reduction. Incubation of the C225SR1 with NDP and R2 results in the amazing series of transformations indicated in eq 1.



The observed cleavage of the base (cytosine) from the sugar

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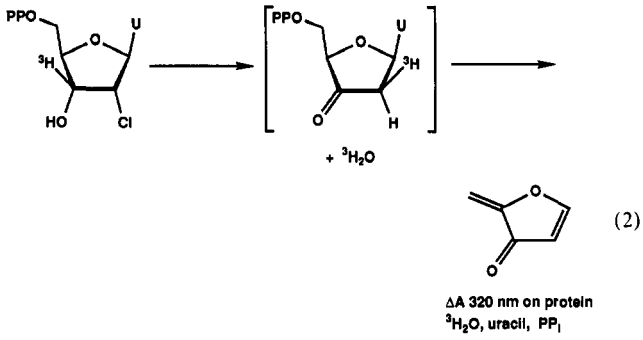
of CDP is reminiscent of the RDPR-catalyzed decomposition of the mechanism-based inhibitor 2′-chloro-2′-deoxyuridine 5′-diphosphate (ClUDP) (eq 2) (Ator & Stubbe, 1985).



(2)

In these studies it was demonstrated that 3′ carbon–hydrogen bond cleavage is required for base (uracil) release. The observation therefore with C225SR1 that cystosine is released suggested that this event might also be a consequence of 3′ carbon–hydrogen bond cleavage. Furthermore, the fact that the tyrosyl radical of R2 is destroyed (eq 1) suggested that detailed studies with this mutant might provide the first direct evidence that the tyrosyl radical, or a protein radical in equilibrium with the tyrosyl radical, initiates catalysis by hydrogen atom abstraction from the 3′ position of NDP. This paper describes in detail the interaction of substrates CDP, UDP, and ADP with C225SR1 and R2. These studies provide the first direct evidence that nucleotide reduction involves 3′ C–H bond cleavage, which is initiated by a protein radical mediated process. The results presented also support the hypothesis that C225 is one of the two cysteines directly involved in substrate reduction.

MATERIALS AND METHODS

C225SR1 (ϵ_{280nm} = 189 000 M^{-1} cm^{-1}) was isolated from the overproducing strain K38/C225SR1 as previously described (Mao et al., 1989). R2 (ϵ_{280nm} = 130 500 M^{-1} cm^{-1}) was isolated from strain N6405/pSPS2 (Salowe et al., 1986). The drop-line-corrected absorbance at 412 nm, [A_{412nm} − ($2A_{406nm}$ + $3A_{416nm}$)/5] (ϵ = 1920 M^{-1} cm^{-1}) (Bollinger et al., 1991) was used to determine the absolute tyrosyl radical content of R2. *E. coli* alkaline phosphatase was purchased from Sigma. [3′-^{3}H]-, [3′-^{2}H]-, and [3′-^{1}H]uridine diphosphate (UDP) were prepared by identical procedures as previously described (Stubbe et al., 1980). [U-^{14}C]uridine (specific activity 521 mCi/mmol) and [8-^{14}C]adenosine diphosphate (ADP) (specific activity 56.3 mCi/mmol) were purchased from New England Nuclear. Dithiothreitol (DTT) was purchased from United States Biochemical. Thymidine triphosphate (TTP) and 2′-deoxyguanosine triphosphate (dGTP) were purchased from Sigma. All other materials were purchased in the highest purity available.

UV/visible absorption spectra were recorded on a Hewlett-Packard 8452A spectrophotometer. All scintillation counting was performed on a Packard 1500 liquid scintillation analyzer with Scint-A XF (Packard) scintillation fluid. HPLC chromatography was performed on an Altex 110A with an Altex 420 controller and a Hitachi 100-40 detector. SDS/PAGE was performed as described by Laemmli (1970). Densitometry of Coomassie blue stained gels was carried out on an LKB 2202 Ultrascan laser densitometer with LKB 2220 recording integrator. Edman peptide sequencing was performed by the Biomolecular Resource Center at the University of California at San Francisco.

Assays with [3′-^{3}H,U-^{14}C]UDP. Mutant C225SR1 was prereduced with 10 mM DTT for 20 min at 25 °C. DTT was removed using a 1-mL G-25 spin column by the procedure of Penefsky (1977). A typical assay contained, in a final volume of 1.2 mL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 0.25 mM TTP, 1 mM UDP (^{3}H, 3.05 × 10^6 cpm/μmol; ^{14}C, 7.30 × 10^5 cpm/μmol; ^{3}H/^{14}C = 4.18), 20 μM C225SR1, and 20 μM R2 (with 1.19 equiv of tyrosyl radical per R2 dimer). All assay components except UDP were mixed, and the solution was equilibrated at 25 °C for 5 min. After the reaction was initiated by the addition of UDP, aliquots of this assay mixture were taken and used to determine the time course of several processes described in sections i–v.

(i) Immediately after initiation, 400 μL of assay mixture was transferred to a cuvette to study loss of the tyrosyl radical and appearance of the absorption feature on the protein at 320 nm. The reaction was monitored spectrophotometrically from 200 to 800 nm for 90 min at 25 °C.

(ii) At various times, a 100-μL aliquot was removed from the assay mixture and quenched by a 1.5-min incubation in boiling water. After the aliquot was cooled on ice, 50 μL of 0.5 M Tris buffer, pH 8.5, containing 1 unit of *E. coli* alkaline phosphatase was added. The aliquot was incubated at 37 °C for 3 h and the reaction stopped by placing the sample in a boiling water bath for 1.5 min. Carrier solution containing 100 μL of 1 mM uracil and 1 mM 2′-deoxyuridine (dUrd) was then added. The aliquot was centrifuged for 5 min to pellet the denatured protein, the supernatant was removed, and the pellet was rinsed with 300 μL of water. The wash was combined with the supernatant, and volatile ^{3}H was quantified by bulb-to-bulb distillation of the combined supernatants, followed by scintillation counting of a portion (300 μL) of the distillate. The residue from the bulb-to-bulb distillation was redissolved in 1 mL of water and concentrated to about 300 μL in vacuo for product analysis by reverse-phase HPLC. An ODS-II reverse-phase C_{18} column (Alltech) was used with water as eluate (flow rate 1 mL/min). Fractions of 1 mL were collected. Fractions containing uracil (retention time 10 min) and dUrd (retention time 35 min) were pooled separately. Deoxyuridine was well separated from uridine (retention time 19 min). UV analysis of the appropriately pooled fractions was used to determine percent recovery of products, and an aliquot of each pooled fraction was counted to quantify the uracil and dUrd produced.

(iii) In addition to the aliquots removed for product analysis, time points were taken for quantitation of the cleavage of C225SR1 into two polypeptides. At various times, aliquots of 5 μL were removed, mixed with 40 μL of 2-fold concentrated Laemmli sample buffer [125 mM Tris–HCL (pH 6.8), 4% SDS, 10% mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue], and incubated in boiling water for 1 min. The samples were analyzed by electrophoresis on a 8% SDS/PAGE gel, which was then stained with Coomassie blue. The intensities of the stained bands were determined by densitometry, with the R2 band used as an internal standard.

(iv) At time 90 min of the reaction, a 200-μL aliquot was loaded onto a Sephadex G-25 column (1.5 × 20 cm) previously equilibrated with 50 mM Hepes buffer (pH 7.6), containing 15 mM $MgSO_4$ and 1 mM EDTA at 4 °C. Fractions of 800 μL were collected, and the protein-containing fractions were pooled. An absorbance spectrum of the pooled fractions was recorded, and an aliquot of these fractions was counted to quantify ^{3}H and ^{14}C bound to the protein.

(v) The selection effects on 3′ carbon–hydrogen bond cleavage associated with uracil production and with dUDP

production were determined independently. In the former case, the assumption was made that cleavage of the 3′ carbon–hydrogen bond which is required for uracil production results in either volatile ^3H (^3H_2O) or ^3H-labeled protein [presumably alkylated by [^3H]-2-methylene-3(2*H*)-furanone]. The selection effect is calculated as the micromoles of uracil produced divided by the sum of micromoles of ^3H_2O and [^3H]protein. In the case of dUDP production, the isotope effect was determined by direct comparison of the $^3H/^{14}C$ ratio of dUrd with the $^3H/^{14}C$ ratio of uridine at time 0. In both cases, only a very small amount of the total UDP pool was converted into product.

Isotope Effect for Tyrosyl Radical Reduction with [3′-^2H]UDP. C225SR1 was prereduced, and DTT was removed as described above. A typical assay contained, in a final volume of 400 μL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 0.25 mM TTP, 20 μM C225SR1, 20 μM R2, and 0.05–4.0 mM [3′-^2H]- or [3′-^1H]UDP. After equilibration of the reaction mixture at 25 °C, the reaction was initiated by the addition of UDP and monitored spectrophotometrically. A drop-line-corrected absorption at 412 nm [$A_{412nm} - (2A_{406nm} + 3A_{416nm})/5$] ($\epsilon$ = 1920 M^{-1} cm^{-1}) was used to determine the absolute tyrosyl radical concentration as a function of time. Initial rates of tyrosyl radical reduction were determined at each concentration of UDP. The K_m and V_{max} for [3′-^1H]- and [3′-^2H]UDP were determined by fitting the data to eq 3 using the programs of Cleland (1979).

$$v = V_{max}[A]/(K_m + [A] + [A]^2/K_i) \quad (3)$$

Interaction of [8-^{14}C]ADP with C225SR1 R2. C225SR1 was prereduced and the reductant DTT removed as described above. A typical assay solution contained, in a final volume of 620 μL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 50 μM dGTP, 0.5 mM [8-^{14}C]ADP (specific activity 1.8×10^6cpm/μmol), 20 μM C225SR1, and 20 μM R2. An aliquot (100 μL) containing everything except R1 and R2 was taken as a zero time point. After equilibration at 25 °C, the reaction was initiated by addition of ADP. At various times, a 100-μL aliquot was removed and quenched by a 1.5-min incubation in boiling water. The aliquot (100 μL) was treated with alkaline phosphatase as described above, and a carrier solution containing 0.5 mM adenine and 0.5 mM 2′-deoxyadenosine (dAd) was added. Subsequent to boiling to kill the phosphatase, the aliquot was centrifuged for 5 min to pellet the denatured protein. The supernatant was then chromatographed on a C_{18} reverse-phase column (Alltech) eluted with 10% methanol (flow rate 1 mL/min). Fractions containing adenine (retention time 7.5 min) and dAd (retention time 19.5 min) were pooled separately. In order to completely remove the contaminating adenosine (retention time 17 min) from the dAd pool, the pool was concentrated in vacuo and chromatographed on a 1 mL Dowex 1–borate column (Cory et al., 1973). The column was eluted with 10 mL of 1 mM sodium borate (pH 9.2), and an aliquot of the effluent was counted to quantitate dAd. The A_{260nm} was used to calculate recoveries from the HPLC column, and scintillation counting was used to determine the amount of adenine and dAd produced.

Kinetics of Cleavage and Tyrosyl Radical Reduction with ADP. C225SR1 was prereduced and DTT removed as described above. The assay contained, in a final volume of 500 μL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 0.1 mM dGTP, 0.5 mM ADP, 20 μM C225SR1, and 20 μM R2 (0.96 equiv of tyrosyl radical/R2 dimer). After equilibration at 25 °C, the reaction was initiated by the addition of ADP and monitored spectrophotometrically from 200 to 800 nm. At various times, 5-μL aliquots were removed, quenched, and analyzed by SDS/PAGE and densitometry as described above for assays with [$^3H/^{14}C$]UDP.

Examination of Additional R1 Mutants for Autocatalytic Cleavage. A typical assay contained, in a final volume of 120 μL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 10 mM DTT, 1 mM CDP, 1.6 mM ATP, 10 μM R1 or R1 mutant, and 10 μM R2. After equilibration at 25 °C, the reaction was initiated by addition of CDP. At various times a 10-μL aliquot was removed, quenched, and analyzed by SDS/PAGE and densitometry as described above. The following R1 mutants were characterized with regard to the cleavage reaction: the single mutants C225S, C230S, C439S, C462S, C754S, and C759S; and the double mutants C225–230S, C225–439S, C225–462S, C225–759S, and C754–759S (preceding paper).

Effect of Allosteric Effectors on Cleavage of Mutant C225SR1. A typical assay contained, in a final volume of 100 μL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1 mM UDP, 20 μM C225SR1, 20 μM R2, and 0 or 0.25 mM TTP. After equilibration at 25 °C, the reaction was initiated by the addition of UDP. At various times, a 5-μL aliquot was removed, quenched, and analyzed by SDS/PAGE electrophoresis and densitometry as described above. In a separate experiment, 0.5 mM ADP and 50 μM dGTP replaced UDP and TTP as substrate and allosteric effector, respectively.

Isolation and Analysis of Peptide Fragments. (1) Blotting on PVDF: A solution containing 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 1 mM CDP, 10 mM DTT, 2.6 μM C225SR1, and 5.9 μM R2 in a final volume of 400 μL was incubated at 25 °C for 60 min. Proteins were separated on an 8% SDS/PAGE (1.5 mm thick) gel, and blotted onto a PVDF membrane (Millipore) (Matsudaira 1987). The membrane was stained with Coomassie blue, and the desired band was cut out. The stained peptide attached to the membrane was used directly for Edman sequencing. (2) Electroelution: A solution containing 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 1 mM CDP, 10 mM DTT, 10 μM C225SR1, and 20 μM R2 in a final volume of 800 μL was incubated at 25 °C for 90 min. An 8% SDS/PAGE gel (3 mm thick) was used to separate the peptide fragments. The desired bands were cut out and the peptide fragments eluted into Centricon 10 filtration units using a Centrilutor microelectroelutor (Amicon). Electroelution was performed at 150 V for 2.5 h at room temperature in standard Laemmli running buffer [25 mM Tris–HCl (pH 6.8), 192 mM glycine, 0.1% SDS] diluted 1:1 with water. Protein eluted into the Centricon was concentrated by centrifugation, 3 mL of 15 mM NH_4HCO_3 (pH 7) was added, and the protein was concentrated again. A solution containing 40 μg of the 61-kDa fragment in a volume of 150 μL was submitted for sequence analysis to the Biomolecular Resource Center at University of California, San Francisco.

RESULTS

In previous studies, Mao et al. (1989) made the observation that incubation of CDP with mutant C225SR1 and R2 resulted in an amazing series of transformations (eq 1). CDP became a mechanism-based inhibitor in which it was converted to cytosine and the putative 2-methylene-3(2*H*)-furanone. Inactivation was the result of destruction of the tyrosyl radical of the R2 subunit, cleavage of the R1 polypeptide into two pieces, and alkylation. These results were observed independent of the presence of an external reductant as might be

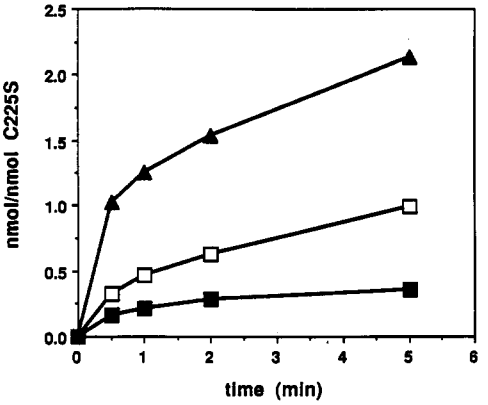


FIGURE 1: Kinetics of product production upon interaction of UDP with C225SR1 and R2: (▲) uracil; (□) tyrosyl radical loss; (■) ^3H_2O.

expected if one of the redox-active cysteines was incapacitated by mutation to a serine. To address mechanistic aspects of these observations and to facilitate quantitative analysis of reaction products, isotopically labeled UDPs, readily available using procedures described in previous studies, have been used to study this process in detail (Stubbe et al., 1983; Ator & Stubbe, 1985).

Quantitation of Products Produced on Interaction of [3′-^{3}H,U-^{14}C]UDP with C225SR1-R2. To identify and quantify the products of this transformation as a function of time, prereduced mutant R1·R2 was incubated with [3′-^{3}H,U-^{14}C]-UDP in the absence of an external reductant. As indicated in Figure 1, uracil was in fact produced as anticipated from the CDP studies. Its production was accompanied by both destruction of the tyrosyl radical, monitored by loss in absorbance at 410 nm, and release of volatile tritium, determined as equivalents of ^3H_2O. These data indicate that at the end of the reaction (90 min) 2.4 uracils are released per tyrosyl radical lost and that the release of volatile tritium is substoichiometric with respect to both uracil production and tyrosyl radical loss.

Observation of the release of both uracil and ^3H_2O suggested that as in the case of ClUDP (eq 2) the protein might be alkylated by the putative 2-methylene-3(2*H*)–furanone produced concomitantly with uracil release. Previous studies with ClUDP also indicated that alkylation of the protein is accompanied by a change in absorbance of the protein at 320 nm (Thelander et al., 1976; Ator & Stubbe, 1985). Protein was, therefore, reisolated at the end of the experiment and analyzed by scintillation counting for covalently bound sugar and for a change in absorption at 320 nm. The protein contained 2.2 sugar residues/RDPR, based on ^{14}C analysis, consistent with the production of each uracil being accompanied by a furanone which alkylated the protein. This calculation assumes that $^5/_9$ of the carbons of uniformly labeled [^{14}C]UDP are bound. Furthermore, alkylation was accompanied by a change in absorbance at 320 nm on the protein as indicated in Figure 2. Our previous model studies have allowed us to propose that this increase in absorption at 320 nm is due to a β-amino-α,β-unsaturated ketone chromophore with an ϵ = 20 000 M^{-1} cm^{-1} (Ashley et al., 1986).

Comparison of the total ^{3}H in the products, determined as volatilized ^3H_2O and ^{3}H-labeled protein, with total uracil produced suggests that there is a selection on 3′ carbon–hydrogen bond cleavage, on the order of 3.2 (average of two determinations), for this process. Observation of tyrosyl radical loss and 3′ carbon–hydrogen bond cleavage now provides a test of our mechanistic proposal that the two events are directly coupled.

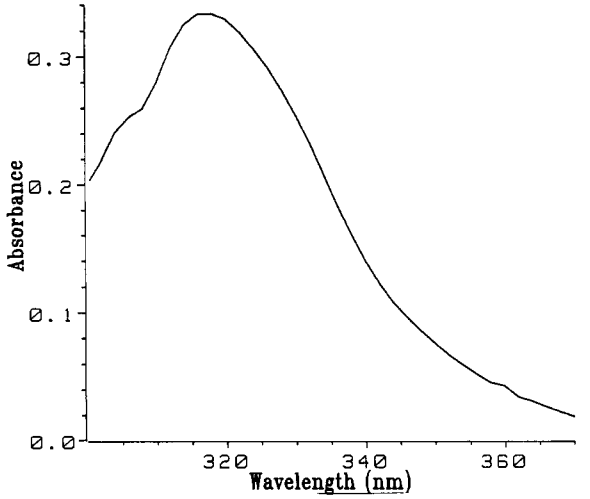


FIGURE 2: Difference spectrum between C225R1·R2 and C225R1·R2 inactivated by UDP. The change in absorbance at 320 nm is indicative of changes previously observed when 2-methylene-3(2*H*)-furanone alkylates wt-R1·R2.

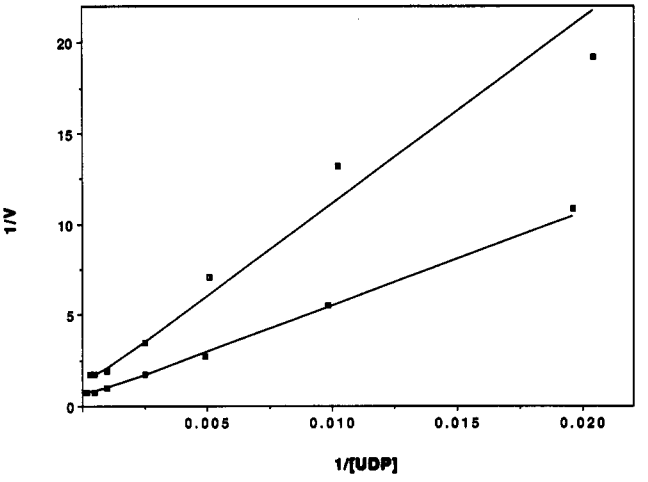


FIGURE 3: Demonstration of an isotope effect on the Y• loss upon interaction [3′-^{2}H]UDP with C225SR1 and R2: A Lineweaver–Burke plot; (■) [3′-^{1}H]UDP; (□) [3′-^{2}H]UDP. Lines derived from a fit to eq 3.

[3′-^{2}H]UDP with C225SR1·R2: Isotope Effect on Tyrosyl Radical Loss. Various concentrations of [3′-^{2}H]UDP or [3′-^{1}H]UDP were incubated with the C225SR1 and R2, and the loss of the absorbance of the tyrosyl radical was monitored as a function of time. Measuring the initial rates of tyrosyl radical loss as a function of UDP concentration gave results indicated in Figure 3. The best fits of these data were obtained with eq 3 describing substrate inhibition. The V_{max} and K_m for each substrate were determined. Isotope effects on V_{max} of 2.0 ± 0.2 and V/K or 2.0 ± 0.4 were obtained from these fits. These results provide the first direct evidence for protein radical initiated chemistry on the substrate and have interesting mechanistic consequences which will be discussed below.

Cleavage of R1 into Two Pieces. Previous studies investigating the interaction of R2·C225SR1 and CDP reported that, concomitantly with tyrosyl radical loss and cytosine production, the R1 subunit was cleaved into two polypeptides (Mao et al., 1989). The kinetics of this reaction with UDP have been investigated in some detail. As shown in Figure 4A, incubation of UDP with R2·C225SR1 results in time-dependent cleavage. These data show that there is a one to one correspondence between tyrosyl radical loss and cleavage. In most preparations of R1·R2 there is ~1.1 tyrosyl radicals/

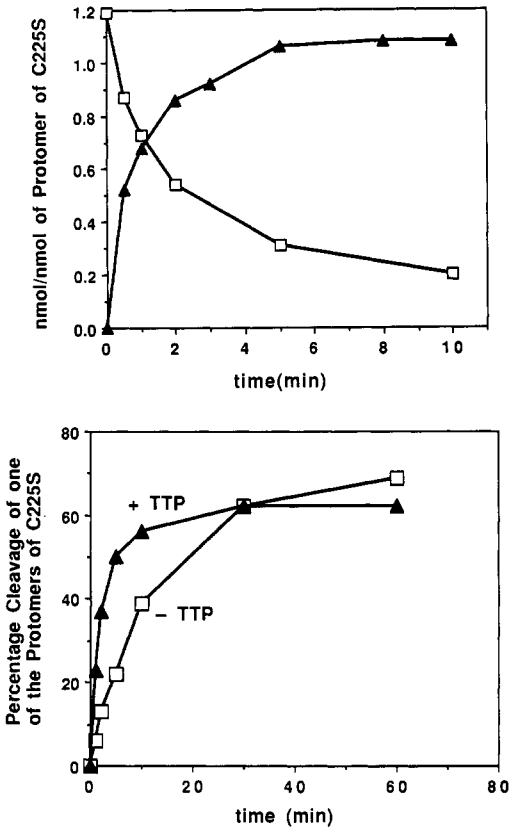


FIGURE 4: (A, top) Correlation of cleavage of the protomers of C225SR1 into two pieces and Y• loss upon interaction of UDP with C225SR1 and R2. The reaction was carried out on prereduced enzyme in the absence of an external reductant and in the presence of allosteric effector TTP: (□) Y• loss; (▲) C225SR1 cleavage. (B bottom) Dependence of cleavage of the protomers of C225SR1 on the presence of substrate and allosteric effector. Conditions identical to those used in (A): (▲) +TTP; (□) –TTP.

Table I: Studies of CDP-Mediated Cleavage of R1 Mutants

	cleavage	no cleavage
single mutants	C225S	wildtype
		C230S
		C439S
		C462S
		C754S
		C759S
double mutants	C225–230S	C225–439S
	C225–759S	C225–462S
		C754–759S

R2. Therefore, only 1.1 cleavage events per R1 are observed. The rate of this cleavage reaction is dependent on the presence of the proper allosteric effector (Figure 4B).

Studies of Mao et al. (preceding and following papers) indicate that two cysteines, 462 and 439, in addition to C225 appear to be crucial for RDPR-mediated catalysis. A variety of double and single mutants of the R1 subunit were examined in an effort to define residues essential for this cleavage process. The results are summarized in Table I. The intriguing observation is made that if one makes the double mutants where, in addition to C225, either C462 or C439 is replaced with serine, then the cleavage reaction no longer occurs. These results are consistent with the postulated role of these thiols in catalysis.

Attempts To Identify the R1 Cleavage Products. Results from analysis of the size of cleaved peptides by SDS gel electrophoresis in comparison with standards of known molecular mass revealed that the two polypeptides were ~26 and 61 kDa regardless of the substrate (UDP, ADP, CDP). The 26-kDa piece was successfully isolated by either electroelution onto PVDF membranes or Vydac HPLC chromatography. Edman sequencing of the purified fragment was complicated due to N-terminal processing, resulting in removal of either M or M and N (the penultimate residue). However, once this processing problem was identified, a peptide sequence that is identical to the amino terminal end of the R1 subunit was deduced. Attempts to obtain information about the C-terminal end of this 26-kDa peptide using carboxypeptidases A and B, and the *o*-phthalaldehyde method of amino acid detection, failed to reveal release of any amino acids.

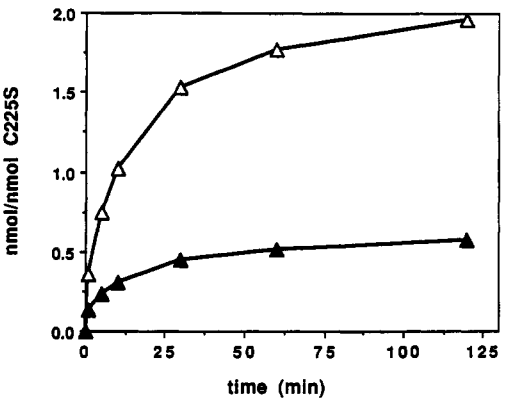


FIGURE 5: Kinetics of product production upon interaction of ADP with C225SR1 and R2: (△) adenine; (▲) dADP.

The 61-kDa polypeptide was isolated by electroelution from an SDS gel, and 130 pmol was submitted for sequence analysis by the Edman degradation procedure. No sequence information was obtained. Given that the method employed is capable of providing information from 1 pmol of material, we conclude that this piece does not contain a free amino terminus.

Quantitation of Products Produced on Interaction of ADP with C225SR1 and R2. Incubation of [^{14}C]ADP with prereduced C225SR1·R2 and analysis of the products produced after 120 min revealed the production of 2 equiv of adenine and 0.6 equiv of dADP per RDPR (Figure 5). The adenine release is accompanied by loss of the tyrosyl radical, which appears to occur concomitantly and stoichiometrically with cleavage of the C225SR1 subunit into two polypeptides of 26 and 61 kDa (Figure 6A). As in the case of UDP, the cleavage is dependent upon the appropriate allosteric effector, in this case dGTP (Figure 6B). The rate of these processes is much slower than that of the corresponding processes observed with the pyrimidine substrates. Even after 120 min, the reaction does not appear to be complete.

Production of dUDP: Mutant or Wild-Type R1? If C225 is actually directly involved in substrate reduction, then no deoxynucleotide should be produced by C225SR1 under conditions required for turnover. However, in addition to the uracil produced on incubation of [3′-^{3}H,^{14}C]UDP with C225SR1·R2, 1.3 equiv of [^{3}H,^{14}C]dUDP/equiv of protein is also observed. An isotope effect of 2.8 on this process was calculated by comparing the ^{3}H/^{14}C ratio of the product dUDP with that of UDP at low extents of reaction. This contrasts with the previously determined isotope effect of 2.0 with wt-R1 measured under similar conditions. These results suggest that dUDP is not due to a contamination of C225SR1 with a homodimer of wt-R1. The implications of these findings will be discussed subsequently.

DISCUSSION

Studies on the wt-RDPR with [3-^{x}H]NDPs (X = 1, 2) and a variety of mechanism-based inhibitors (2′-substituted 2′-

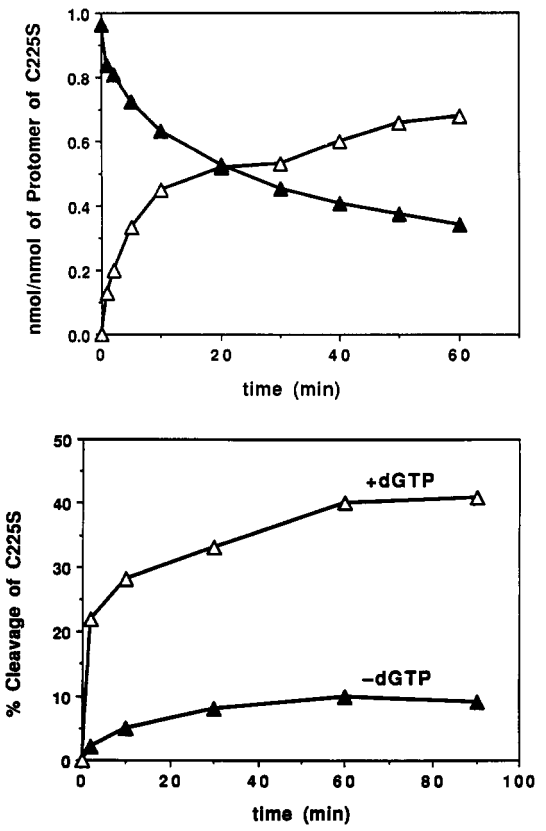


FIGURE 6: (A, top) Correlation of cleavage of the protomers of C225SR1 into two pieces (△) and Y• loss (▲) upon interaction of ADP and C225SR1 and R2. (A, top) The reaction was carried out with prereduced enzyme in the absence of external reductant and in the presence of allosteric effect dGTP. (B, bottom) Cleavage of the protomers C225SR1 into two polypeptides is dependent on the allosteric effector as well as the substrate ADP: (△) +dGTP; (▲) -dGTP.

deoxynucleotides) in addition to a number of model chemical reactions provide the basis for the complex mechanism for reduction of nucleotides to deoxynucleotides [Scheme I, preceding paper; Stubbe (1989, 1990)]. This hypothesis has been further refined on the basis of information obtained from studying the interaction of nucleotides with a variety of mutant R1 subunits in which key cysteine residues have been changed to serines (Mao et al., 1989) and on the basis of information made available from the X-ray structure of the R2 subunit (Nordlund et al., 1990).

The new model predicts that a protein radical X• is generated on the R1 subunit by long-range electron transfer to the tyrosyl radical on the R2 subunit. This putative X• is proposed to mediate 3′ carbon–hydrogen bond cleavage of the normal substrate NDP. Subsequent to loss of H_2O from the 2′ position of NDP, the putative 3′-ketone-2′-radical nucleotide is proposed to be reduced by multiple electron- and proton-transfer processes to ultimately produce the 3′-deoxynucleotide radical. This species is proposed to be reduced concomitantly with regeneration of the putative protein radical X•.

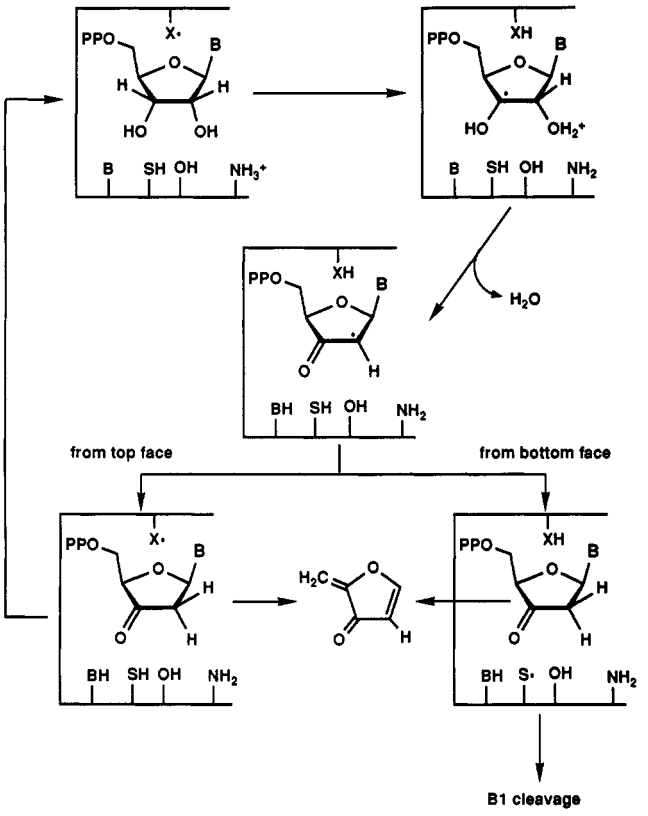
While previous studies using 3′ isotopically labeled nucleotides with the wild-type RDPR provided unambiguous evidence that the 3′ C–H bond is cleaved during turnover, no evidence existed to correlate this process with reduction of a protein radical on R1 and, as a consequence of a rapid equilibrium, reduction of the tyrosyl radical on R2. In an effort to obtain support for this thesis, one of the putative redox active site cysteines of R1 was changed to a serine. It was anticipated that uncoupling of the redox chemistry from the carbon–hydrogen bond cleavage would facilitate detection of protein radical reduction which might occur concomitantly with chemistry at the 3′ position of the substrate.

Incubation of [3′-^{3}H]UDP with C225SR1·R2 and monitoring of the product production with time revealed that production of uracil, reduction of the tyrosyl radical, and release of ^3H_2O all appear to be kinetically correlated. Furthermore, the ^3H_2O is substoichiometric with respect to the amount of tyrosyl radical loss, which suggests that the Y• is directly (or indirectly) responsible for 3′ C–H bond cleavage of the UDP (Scheme I, preceding paper). There is a large isotope effect (3.2) on this process. To address this question more directly, [3′-^{2}H]UDP was prepared synthetically. The proteated compound was prepared by identical procedures. Various concentrations of [3′-^{x}H]UDP (x = 1 or 2) were incubated with C225SR1·R2, and loss of the tyrosyl radical was monitored as a function of time. Analysis of the initial rates of loss of the tyrosyl radical via eq 3 (Figure 3) revealed V/K and V isotope effects of 2.0 and 2.0, respectively, on this process. The best fit to the data was obtained using eq 3 for substrate inhibition. Analysis of the number of turnovers of UDP prior to enzyme inactivation (Figure 1) reveals 2.4 uracils lost for every tyrosyl radical destroyed. Therefore, some step subsequent to 3′ carbon–hydrogen bond cleavage is required to regenerate the tyrosyl radical so that more than one uracil can be produced. Tyrosyl radical regeneration would require X–H bond scission, and hence an isotope effect on this process might also be anticipated. These considerations might therefore lead to the expectation that the V and V/K isotope effects might be inequivalent. Within experimental error however the isotope effects appear to be the same. The origin of these effects cannot at present be evaluated due to the complexity of the mechanism. The observation of the isotope effect on protein radical reduction however provides the first direct evidence that the 3′ C–H bond cleavage and protein radical reduction are coupled.

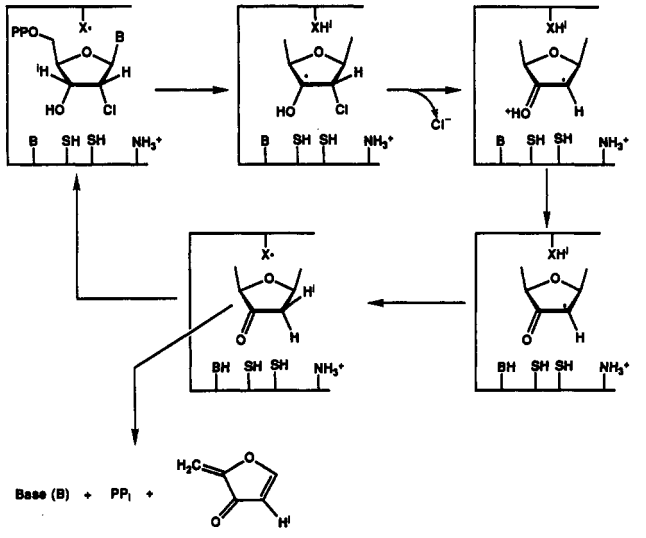
A model to account for the observation that multiple uracils are produced per tyrosyl radical lost is shown in Scheme I. The first few steps through the loss of the OH group from C2′ as H_2O are proposed to be identical to those obtained with the wild-type enzyme. It is proposed that the subsequent reduction steps have been blocked due to replacement of one of the active site cysteines with a redox-inert serine. The postulate for the subsequent steps is based on the extensive investigation of the mechanism of inactivation of RDPR by ClUDP (Scheme II). In this case, the redox thiols were proposed to play no role due to the enhanced leaving group ability of a chloride relative to a H_2O molecule. Reduction of the putative 3′-ketone-2′-radical nucleotide was proposed to occur by hydrogen atom transfer rather than electron transfer (Akhlaq et al., 1987). Model studies indicate that reduction by the former process occurs 3–4 orders of magnitude more slowly than reduction by the latter process.

Thus to account for multiple turnovers observed in the case of the C225SR1, one can postulate that 1.4 out of every 2.4 times, reducing equivalents are provided by the C439 on the top face of the substrate, analogous to the chemistry defined with ClUDP. This reduction process would thus regenerate the X•, allowing an additional turnover. However if the reduction of the intermediate 3′-ketone-2′-radical nucleotide by C462 on the bottom face of the nucleotide occurs, the same 3′-ketone deoxynucleotide would be generated, but the C439 protein radical would not be regenerated. A R1–thiyl radical would be left in the active site. This R1–thiyl radical might lead to peptide bond scission. Interestingly, the C225AR1 does not appear to be capable of catalyzing cleavage. At

Scheme I: Postulated Mechanism for the Interaction of C225SR1·R2 and NDP



Scheme II: Postulated Mechanism for the Interaction of RDPR with ClUDP



present, the mechanism of this cleavage remains a mystery and as described subsequently requires isolation of the altered ends of the peptides to formulate a reasonable hypothesis.

The mechanism postulated in Scheme I makes an additional prediction that has been tested experimentally. Every turnover leading to tyrosyl radical reduction should result in generation of a new protein radical. If this new protein radical can lead to peptide backbone cleavage, then one would expect a one to one correspondence between tyrosyl radical loss of R2 and peptide cleavage of R1. As indicated in Figures 4A and 6A with UDP and ADP as substrates, this prediction is confirmed experimentally. In addition, the model predicts that, regardless of the pathway, formation of each equivalent of uracil is accompanied by production of 1 equiv of 2-methylene-3(2*H*)-furanone. Consistent with this prediction, the protein isolated at the end of the reaction is modified by 2.2 equiv of furanone per 2.4 equiv of uracil produced. The number of 2.2 for the furanone makes the assumption that $^5/_9$ of the carbons of UDP end up covalently bound to the protein. In addition, furanone formation in the case of ClUDP is accompanied by an increase in absorbance at 320 nm on the protein. Model studies have previously suggested that this absorption is due to a chromophore with $\epsilon = 20\,000\ M^{-1}\ cm^{-1}$ (Ashley et al., 1986). Analysis of the visible difference spectrum of the protein before and after inactivation reveals an absorption spectrum (Figure 2) similar to that observed with ClUDP and a stoichiometry of 1:1. Thus only one of every two furanones covalently bound to RDPR appears to undergo a second alkylation at C-1′ by an amino group resulting in chromophore formation. This result is also very similar to those observed earlier with ClUDP.

Identification of the nature of the R1 backbone cleavage products is essential to testing our model (Scheme II). Toward this end both the 26- and 61-kDa peptides have been isolated and submitted for sequence analysis. The 26-kDa peptide is the N-terminus of R1. An estimate of the M_r based on SDS/PAGE and the corresponding M_r based on sequence information would place the cleavage site somewhere between residues 216 and 229. Efforts to sequence the C-terminal end of the small peptide have thus far failed. Attempts to obtain an N-terminal sequence for the 61-kDa polypeptide have also failed, suggesting that the cleavage does not produce an N-terminal amino acid. However, further experimentation is essential. Use of electrospray ionization mass spectrometry ought to define exactly the point of cleavage (Henry et al., 1991). Analytical methods to establish the modified species at the C-terminal end of the 21-kDa protein and "N"-terminal end of the 61-kDa protein are in progress.

The rate of the cleavage process appears to be substrate dependent. UDP (CDP) mediates cleavage at a much faster rate than ADP. In addition, the appropriate allosteric effector appears to modulate the rate of cleavage (Figures 4B and 6B). Finally, a variety of single and double mutants have been examined for the ability to mediate peptide bond cleavage in the presence of substrate (Table I). The interesting observation is made that if a second C-to-S mutation is made in either C462 or C439, no cleavage is observed. These are the two additional thiols thought to be essential for catalysis as outlined in the previous paper. Mutation of a number of additional cysteines of R1 including C754 and C759 did not appear to alter the cleavage process. While further investigation of these mutants (chemically and structurally) is required, their requirement for cleavage is consistent with our model that C439 needs to initiate cleavage of the 3′ C–H bond of the substrate and that C462 is the thiyl radical that initiates the peptide cleavage process.

Formation of dUDP. If C225 is really at the active site and is directly involved in substrate reduction, then one would expect that in the absence of external reductants the C225S mutant would produce *no* dNDP. However, as outlined in the preceding paper, the mutant proteins all contain 1.5–3% contamination with wt-R1, probably as the heterodimer with the mutant R1. This is the result of the fact that *E. coli* deficient in the *nrdA* gene product are nonviable, and thus expression of the mutant proteins is accompanied by production of the wt-R1 from the *E. coli* chromosome. Initially we anticipated that if the analysis of interaction of NDP with R1·R2 were carried out under single-turnover conditions, the presence of contaminating wild-type enzyme at levels corre-

sponding to 1.5–3% of the mutant R1 would not provide significant interference. We did not anticipate, however, that two cysteines, presumably C754 and C759 at the C-terminal end of the R1 subunit, could function as a thioredoxin. This point has been unambiguously demonstrated in the case of C439SR1 mutant (following paper). In analogy with the C439S mutant studies therefore, we propose that C225SR1 can also serve as a thioredoxin equivalent in allowing the contaminating wild-type enzyme to turn over CDP (UDP) to dCDP (dUDP) to the extent of 2 equiv of dNDPs/equiv of mutant R1 at infinite time. Thus, we still believe that the 1.3 equiv of dUDP observed in this reaction is due to the contaminating wild-type enzyme. The isotope effect on uracil production of 3.2 is significantly different from the isotope effect of 2.8 observed on dUDP formation. This latter number is different from the value of 2.0 reported for the reduction of UDP by wt-R1 with TTP as an effector. A direct comparison between these numbers, however, is not possible, as evidence now suggests that the contaminating wt-R1 is present as a heterodimer.

Of course, it is essential to establish whether this hypothesis that C225 is involved directly in nucleotide reduction is in fact valid. Efforts are now focused on using molecular biological methods to remove the contaminating wild-type enzyme. If our thesis about the function of C225 is valid, then *no* dNDP should be produced from the mutant R1 protein.

To summarize, simple mutation of a putative active site cysteine to a serine has converted the normal substrate into a mechanism-based inhibitor in which not only is the substrate dismantled but the R1 subunit is cleaved into two polypeptides and the tyrosyl radical on the R2 subunit is destroyed. These studies provide compelling evidence that C225 is one of the redox-active cysteines within the active site. Uncoupling of the redox chemistry from the C–H bond cleavage reaction has allowed the first demonstration of a correlation between tyrosyl radical reduction and chemistry on the substrate. In addition, the substrate decomposes in a fashion predicted by studies with ClUDP, a mechanism-based inhibitor, where redox chemistry of the thiols is not required. Elucidation of the cleavage products of the R1 subunit will define whether an additional protein radical on R1 is produced concomitantly with loss of the tyrosyl radical on R2.

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Registry No. RDPR, 9047-64-7; dUDP, 4208-67-7; dADP, 2793-06-8; dCDP, 800-73-7; uracil, 66-22-8; cysteine, 52-90-4; tyrosyl radical, 16978-66-8.