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Direct Photoaffinity Labeling of Ribonucleotide Reductase from *Escherichia coli* Using dTTP: Characterization of the Photoproducts†

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ABSTRACT: Subunit B1 of *Escherichia coli* ribonucleotide reductase contains one type of allosteric binding site that controls the substrate specificity of the enzyme. This site binds the allosteric effector dTTP as well as other nucleoside triphosphates. Cross-linking of dTTP to protein B1 by direct photoaffinity labeling, as well as the isolation and sequence determination of the labeled tryptic peptide, has recently been reported [Eriksson, S., Sjöberg, B.-M., Jörnwall, H., & Carlquist, M. (1986) *J. Biol. Chem. 261*, 1878–1882]. In this study, we have further purified the dTTP-labeled peptide and characterized it using UV spectroscopy. Two types of dTTP-cross-linked peptide were found: one having an absorbance maximum at 261 nm typical for a dTTP spectrum, i.e., containing an intact 5,6 double bond, and one minor form with low absorbance at 261 nm. In both cases, the same amino acid composition was found, corresponding to the peptide Ser^{291}-X-Ser-Gln-Gly-Gly-Val-Arg^{299} in the B1 sequence with X being Cys-292 cross-linked to dTTP. Isotope labeling experiments revealed that one proton in the 5-methyl group of thymine was lost during photoincorporation. Therefore, the cross-linking occurs via the 5-methyl group to Cys-292 in a majority of incorporated dTTPs, but a second, possibly 5,6-saturated form of incorporated nucleotide was also detected. The reasons for the high stereospecificity of the reaction and the possible structure of the allosteric site of protein B1 are discussed.

Photochemistry has provided a successful approach to study protein–nucleotide interactions, since proteins and unmodified nucleotides or nucleic acids cross-link covalently when irradiated with ultraviolet light. This form of direct photoaffinity labeling has been used to investigate the properties of nucleic acid binding proteins such as aminoacyl-tRNA synthetase (Schoemaker & Schimmel, 1974; Schoemaker et al., 1975; Yue & Schimmel, 1977; Schimmel, 1977), DNA and RNA polymerases (Markovitz, 1972; Strniste & Smith, 1974; Hillel & Wu, 1978), RNase A (Sperling & Havron, 1976; Havron & Sperling, 1977), ssDNA binding proteins (Paradiso et al., 1979; Merrill et al., 1984), and recA proteins (Banks & Sedgwick, 1986) as well as many other systems [see Shetlar (1980)].

This technique has also been used in recent years to study several nucleotide binding proteins, e.g., myosin (Maruta & Korn, 1981), ribonucleotide reductase (Eriksson et al., 1982, 1986; Caras & Martin, 1982; Caras et al., 1983; Eriksson, 1983), DNA polymerase (Biswas & Kornberg, 1984), deoxycytidylate deaminase (Maley & Maley, 1982), and terminal deoxynucleotidyltransferase (Modak & Gillerman-Cox, 1982), using both purine and pyrimidine nucleotides as photoaffinity ligands.

Many different amino acids including serine, isoleucine, threonine (Havron & Sperling, 1977), tyrosine (Maly et al., 1980), cysteine, lysine, and arginine (Smith & Meun, 1968; Schott & Shetlar, 1974; Shetlar et al., 1975; Varghese, 1973, 1976; Paradiso et al., 1979) have been shown to be covalently cross-linked to nucleotides in direct photoaffinity labeling reactions. Generally, only those amino acids that are in close proximity to the nucleotide in the native protein structure are supposed to be involved in cross-linking (Shetlar, 1980). However, only in very few instances has the chemical nature of the products of direct photoaffinity reactions been identified.

In this investigation, we have characterized the structure of the covalent linkage between *Escherichia coli* ribonucleotide reductase and dTTP that was obtained by using direct photoaffinity labeling. This enzyme is responsible for the reduction of all four ribonucleotides to the corresponding deoxyribonucleotides (Thelander & Reichard, 1979) needed for DNA

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synthesis. The substrate specificity and the overall activity of ribonucleotide reductase are controlled by allosteric effectors. The *E. coli* enzyme is composed of two subunits: protein B1 (M_r 2 × 85 000), which contains binding sites both for the substrates and for the allosteric effectors, and protein B2 (M_r 2 × 43 000), which combines with B1 to form the active enzyme complex. The allosteric binding sites on protein B1 are of two classes: one type, h-sites, binds dTTP, dATP, dGTP, and ATP and governs substrate specificity, while the other type, l-sites, binds only dATP and ATP and regulates the overall activity of the enzyme (Eriksson & Sjöberg, 1988).

Previously, photoaffinity labeling of protein B1 with dTTP led to identification of a tryptic octapeptide which forms part of the h-site (Eriksson et al., 1986). The amino acid that was cross-linked to dTTP could not be directly identified, but the corrected DNA sequence of the gene for protein B1 (*nrdA*) showed that it corresponds to Cys-292 (Eriksson & Sjöberg, 1988; Nilsson et al., 1988). Here we describe spectroscopic studies of the dTTP-labeled octapeptide isolated from photolabeled protein B1. These results together with isotope labeling experiments allow us to define the structure of the major labeling product.

Experimental Procedures

Materials. Radioactive nucleotides were obtained from Amersham and were at least 98% pure as checked by thin-layer chromatography (TLC). Thymidine 5′-triphosphate, guanosine 5′-diphosphate, and dithiothreitol were from Sigma; iodoacetic acid was from Fluka. L-1-(Tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin was obtained from Worthington.

The protein B1 subunit was purified according to described methods (Eriksson et al., 1977) from an overproducing pLSH1 strain of *E. coli* (Larsson, 1984) generously provided by Dr. B.-M. Sjöberg (Department of Molecular Biology, Stockholm University, Stockholm, Sweden). The amount of B1 used in each experiment was calculated from its absorbancy at 280 nm. A value of $E^{1\%}_{1cm}$ = 10.8 at 280 nm and a molecular weight for the dimer subunit of 160 000 were used (Thelander, 1973).

Photoaffinity Labeling. The photochemical reactions of [α-^{32}P]dTTP and [*methyl*-^{3}H]dTTP with 173 pmol of protein B1 subunit were performed in drops (100 μL) on parafilm at room temperature (21 °C). The buffer was 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.6, containing 10 mM $MgCl_2$, 2 mM dithiothreitol, and 10% glycerol. The radioactive nucleotide (24 μM) had specific radioactivities of 5590 cpm/pmol in the case of [α-^{32}P]dTTP and 714 cpm/pmol in the case of [*methyl*-^{3}H]dTTP. Irradiation conditions and the method for detection of photoincorporation were the same as before (Eriksson et al., 1983).

Isolation of Radioactively Labeled Protein. For large-scale photolabeling of the B1 subunit (26.3 mg) with [α-^{32}P]dTTP, the reaction mixture (31 mL) contained additionally 50 μM GDP to enhance photoincorporation (Eriksson, 1983). The reaction mixture was divided in two equal portions and placed in two siliconized petri dishes (10-cm diameter), and then it was irradiated for 30 min at room temperature. The UV irradiation was with a UVS-54 Mineral Light lamp (UV Prodacts, Inc., San Gabriel, CA), giving a dose of 100 erg mm^{-2} s^{-1}. Photoincorporation was 0.42 mol of dTTP/mol of protein. The mixture was dialyzed against saturated ammonium sulfate (15 h at +4 °C), and the precipitated protein was dissolved in 4 mL of 1 M Tris-HCl, pH 8.0, containing 10 mM ethylenediaminetetraacetic acid (EDTA) and 6 M guanidinium chloride, reduced with 10 mM dithiothreitol, and carboxymethylated with 23 mM recrystallized iodoacetic acid.

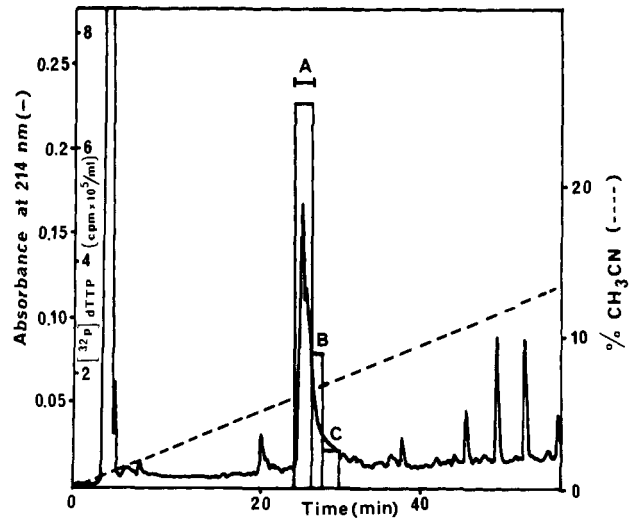

FIGURE 1: Purification by reverse-phase HPLC of the [α-^{32}P]dTTP tryptic peptide. After chromatography on DEAE-Sephadex (A-50), the tryptic peptides of labeled protein B1 were fractionated by HPLC. The buffer employed was 0.1% trifluoroacetic acid with a gradient of acetonitrile (---). The amount of ^{32}P label per milliliter of each fractions is shown with *open boxes*, but only for those containing radioactivity. The total recovery of radioactivity was 60%.

The carboxymethylated B1 subunit was then dialyzed against 0.1 M ammonium bicarbonate at pH 8.0, for 6 h at +4 °C, followed by incubation with trypsin 1:30, by weight, trypsin:protein B1 ratio) for 30 h at 37 °C in 0.1 M ammonium bicarbonate, pH 8.0. The resulting tryptic peptides were separated by DEAE-Sephadex chromatography (Eriksson et al., 1986) using a linear gradient of 0.1–0.8 M ammonium bicarbonate, pH 8.0. Two radioactive pools containing 70% and 2% of the applied radioactive material were collected and analyzed by high-performance liquid chromatography (HPLC).

HPLC and amino acid analyses of the labeled peptides were performed as described earlier (Eriksson et al., 1986).

UV spectra were run on a Beckman DU-50 instrument with a 10-mm light path length of normal cuvettes (200 μL) or microcuvettes (60 μL).

Results

Large-Scale Preparation of dTTP-Labeled Octapeptide. Photoaffinity labeling of protein B1 was performed by using [α-^{32}P]dTTP in the presence of 50 μM GDP as described under Experimental Procedures. After carboxymethylation and digestion with trypsin, the resulting tryptic peptides were separated by DEAE-Sephadedx chromatography (Eriksson et al., 1986). Two pools, one major with 70% and one minor with 2% of the totally applied radioactivity, were collected. The second pool was eluted at 0.35 M ammonium bicarbonate, and it showed a different chromatographic behavior when analyzed on HPLC. Therefore, this material probably represents another labeled peptide, but the low dTTP incorporation prevented further analysis.

The major pool (eluted at 0.25 M ammonium bicarbonate) was concentrated and further purified by reverse-phase HPLC. A typical HPLC chromatogram is presented in Figure 1 using 0.1% trifluoroacetic acid with a gradient of acetonitrile, from 0% to 15%, during 60 min. The peak of radioactivity coincides with the UV-absorbing peak, and both are asymmetric on the left side. For detailed analysis, three pools, denoted as pools, A, B, and C (Figure 1), were collected. Pools A and B were rechromatographed, using the same system but with a gradient of acetonitrile from 0% to 10% in 60 min. Only one major

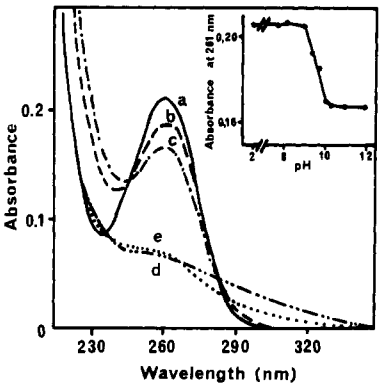

FIGURE 2: UV absorption spectra of pool A (curves a, b, and c), pool B (curve d), and pool C (curve e), 29 μM in aqueous medium at different pHs. (a, d, and e) In 50 mM Tris-HCl at pH 7.7; (b) in 25 mM borate buffer at pH 9.8; (c) in 25 mM sodium bicarbonate buffer at pH 10.9; (insert) spectrophotometric titration of pool A at 261 nm.

Table I: Amino Acid Analysis of Pools A, B, and C of dTTP-Labeled Peptide

	amount of amino acid in HPLC pools[a] (nmol)		
amino acid	A	B	C
Ser	1.00	2.02	1.54
Glx	0.59	1.19	0.90
Gly	1.09	2.23	1.84
Val	0.5	1.0	0.7
Arg	0.4	0.8	0.7

[a] Results of the amino acid analysis were obtained after acid hydrolysis without corrections for impurities or destruction. Pools A, B, and C were collected from the radioactive peaks (Figure 1), and pools A and B were rechromatographed as described in the text. The three fractions analyzed from pools A, B, and C contained 0.5, 1.0, and 0.8 nmol of incorporated dTTP, respectively.

UV-absorbing peak was found in both cases, and this material was lyophilized and used for further analysis. Totally, 23 nmol of pool A, 5.5 nmol of pool B, and 2.2 nmol of pool C were obtained, calculated on the basis of incorporated radioactivity. The total recovery was estimated at 60%.

Structural Analysis. For structural characterization of the dTTP-labeled peptides, the total amino acid composition (Table I) and the UV spectra (Figure 2) were determined.

The result presented in Table I is in full accordance with the amino acid sequence Ser-X-Ser-Gln-Gly-Gly-Val-Arg of the dTTP-labeled tryptic peptide which was determined previously (Eriksson et al., 1986). The labeled amino acid was found in the second position and was identified as Cys-292 by comparison with the primary structure of protein B1 as deduced from the revised nucleotide sequence of the *nrdA* gene (Eriksson & Sjöberg, 1988). The peptides in pools A, B, and C collected from HPLC chromatography (Figure 1) all contained the same amino acids with approximately the same relative amounts.

The UV absorption spectra in aqueous medium of pool A (Figures 2, curves a–c) include one band at 261 nm with an extinction coefficient of 7300 M^{-1} cm^{-1} at acidic and neutral pH. The UV absorption spectra at pH 7.7, 9.8, and 10.9 have an isosbestic point at about 246 nm. The spectral titration of pool A at 261 nm (Figure 2, insert) led to a single pK = 9.6 for deprotonation. This value is very close to the pK's for deprotonation of dTTP (9.8) (Sigel, 1968) and UTP (9.5) (Bock et al., 1956). Since the peptide does not contain any aromatic amino acids, all UV absorption above 220 nm is from the attached dTTP. This result shows that the dTTP-labeled tryptic peptide in pool A contains an intact thymine ring.

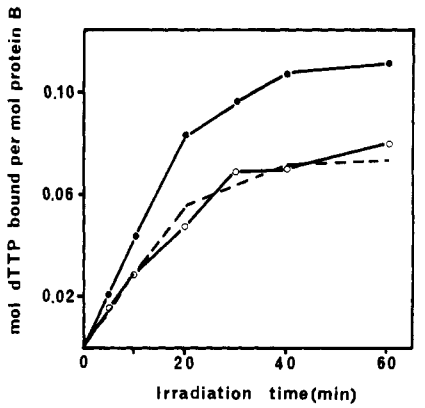

FIGURE 3: Direct photoaffinity labeling of B1 subunit with [α-^{32}P]dTTP (●) or with [*methyl*-^{3}H]dTTP (○) using the same reaction conditions. Each time point is the mean of three determinations. Dashed lines indicate an estimated two-thirds of the incorporation of [α-^{32}P]dTTP at each time point.

The UV absorption spectrum in neutral aqueous medium of pool B (Figure 2, curve d) includes, in addition to the major band at about 260 nm (with an extinction coefficient of about 1300 M^{-1} cm^{-1}), an appreciable absorption in the 290–300-nm range. A very similar UV spectrum was found for pool C in neutral aqueous medium (Figure 2, curve e). Thus, pools B and C show severalfold lower absorption at 260 nm as compared to pool A, although each contains the same relative amount of incorporated dTTP (Table I).

Photoaffinity Labeling of B1 Subunit with [methyl-^{3}H]-dTTP and [α-^{32}P]dTTP. In order to investigate the structure of the dTTP–B1 cross-link, we compared the photoincorporation of [α-^{32}P]dTTP and [*methyl*-^{3}H]dTTP into protein B1. The incorporation of tritium-labeled dTTP per amount of B1 subunit is approximately one-third lower then the incorporation of ^{32}P-labeled dTTP at different times of irradiation (Figure 3). Taking into account that tritium is randomly distributed between three positions of the 5-methyl group (information from Amersham Co.), we have also included a theoretical curve indicating the two-thirds incorporation of [α-^{32}P]dTTP. Thus, our observed values for [*methyl*-^{3}H]dTTP agree very well with the theoretical one.

Furthermore, the tritium released from [*methyl*-^{3}H]dTTP into the medium as ^3H_2O was measured, using charcoal to absorb all aromatic ring containing compounds. As a control, we measured tritium released during a photoaffinity reaction between [*methyl*-^{3}H]dTTP and denatured B1 subunit (boiled for 2 min). The photoincorporation of dTTP into denatured protein B1 was 13-fold lower than the incorporation into the native protein, using the conditions described under Experimental Procedures.

The amount of tritium released during the photoaffinity labeling reaction between native B1 subunit and [*methyl*-^{3}H]dTTP was approximately half of that photoinserted into protein B1. For the calculation, we subtracted the tritium released in the control at different times of irradiation. However, approximately 1% of total nucleotide-bound tritium was released after 20-min irradiation of the control containing denatured protein B1. This is in the same range as that photoincorporated into native B1 subunit and gives very high background. For that reason, we did not perform a detailed study with this method.

Discussion

Direct photoaffinity labeling of subunit B1 of ribonucleotide reductase with dTTP resulted in cross-linking of the nucleotide

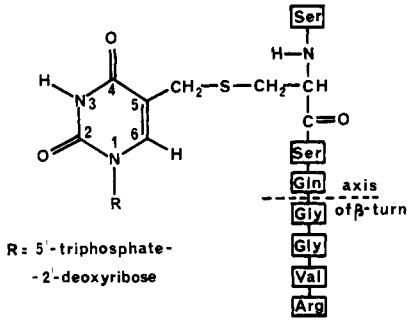

FIGURE 4: Structure of dTTP octapeptide. The axis of the β-turn was obtained as a maximum (approximately 4×10^{-4}) of the β-turn probability curve, calculated by using the Chou and Fasman prediction with a window of four residues.

to essentially only one site on the protein, demonstrating the high specificity of this technique, using dTTP as a ligand. The identification of the tryptic peptide which forms part of this site, i.e., amino acids 291–298 in the revised deduced primary sequence (Eriksson et al., 1983), is clearly confirmed in this study.

No direct demonstration of the presumed labeled amino acid (Cys-292) could be found in the amino acid analysis of the labeled peptide. However, recent protein engineering studies showed a direct involvement of this amino acid in dTTP photolabeling, since replacement of Cys-292 with alanine and subsequent purification of the mutant protein B1 showed that its photolabeling with dTTP was reduced at least 20-fold (Nilsson et al., 1988).

Purification of the dTTP-labeled tryptic peptide by reversed-phase HPLC revealed a heterogeneity in the UV absorption properties of the cross-linked peptide. The major fraction (70%) of the incorporated dTTP showed, after rechromatography, a UV spectrum very similar to that of dTTP. The pH dependence of the spectra of dTTP-labeled peptide was also very similar to that of pyrimidine nucleotides, and the fact that we obtained an isosbestic point demonstrates that we have only one absorbing species. However, the peak of maximal absorbance appears to be shifted about 6 nm to 261 nm compared to 267 nm for dTTP. In conclusion, the major fraction of the dTTP has been cross-linked to Cys-292 without saturation of the 5,6 double bond.

Isotope incorporation experiments using [*methyl*-^{3}H]dTTP and [α-^{32}P]dTTP clearly indicated the loss of one proton from the methyl group of dTTP, since we found 30% less incorporation with the former nucleotide and a corresponding release of ^3H_2O. This fact, together with the UV spectral properties of the major form of dTTP-labeled peptide, strongly suggests that the cross-link is via the 5-methyl group as shown in Figure 4.

The structure of the several photoproducts obtained in photochemical model studies of cysteine and thymine, irradiated with high doses UV light, has been described by Varghese (1973). The most frequent cross-linking was between the thiol group of cysteine and C(6) or C(5) of thymine (see Figure 4 for numbers), with a concomitant saturation of the double bond as a result of free radical formation on the thymine ring. However, coupling via the 5-methyl group of thymine was also detected (approximately 5% of the total photoproducts), and the effect of the 5-methyl substitution on the UV spectrum of thymine was similar to that observed here, which further supports the structure shown in Figure 4.

A second form of cross-linking of dTTP to the same peptide could be demonstrated and further purified although it corresponded to less than 30% of totally incorporated nucleotide. The UV spectrum of this form was clearly different with a broad absorption band ranging up to 310 nm without a maximum around 260 nm. There was an increase in absorbance at alkaline pH, but no indication of transient increase at 240 nm, which could have been indicative of a cleavage of the base ring, as has been found with various dihydropyrimidines (Janion & Shugar, 1960). We cannot at present suggest a likely structure of this other cross-linked dTTP, but the fact that the absorption maximum at 260 nm is lost points to a saturation of the 5,6 bond. However, the effect of increasing pH speaks against this structure and suggests that the base ring is already open and/or chemically modified.

To our knowledge, this is the first detailed characterization of the structure of photoproducts formed by direct photoaffinity labeling between a nucleotide and an intact protein. Paradiso et al. (1979) have described a somewhat analogous situation where *fd* gene 5 protein was cross-linked to DNA. They also defined a tryptic peptide that was attached via a cysteinyl–thymine linkage. However, the linkage was photolabile and acid labile, and the tryptic-labeled peptide showed no absorption maximum at about 260 nm. Therefore, no assignment of the structure of this photoproduct can be made, but it is possible that it may have a structure related to the second minor form of incorporated dTTP–Cys-292.

The high yield and specificity of the photoincorporation of dTTP into protein B1 indicate very strict sterical constraints within the allosteric binding site. This region of primary structure contains only one preserved amino acid (Arg-299) among the five presently sequenced ribonucleotide reductases (Nilsson et al., 1988; Eriksson & Sjöberg, 1988). Only protein B1 contains a cysteine in this region which may explain the high photoincorporation, since in several studies of the reactivity of various amino acids in direct photoaffinity reactions with nucleotides cysteine has been found to be the most reactive (Shetlar, 1974; Varghese, 1976).

The animal virus reductases so far studied lack allosteric regulation by dTTP, and three sequenced viral B1 protein analogues show several differences in this region; i.e., as pointed out by Nikas et al. (1987), they all lack a segment of eight to nine amino acids immediately N-terminal to this region. Furthermore, they also lack the predicted β-turn in this region (see below). In the case of dTTP-controlled subunits, it is clear that they show predicted secondary structure in the form of a β-turn in the corresponding octapeptide, where the sequence Gln-Gly-Gly is conserved (Figure 4). It is likely that this turn is an important feature in the structure of the allosteric site and it may be involved in the high stereospecificity of the photolabeling reaction.

By sequence comparison to other dTTP binding proteins, a second region (corresponding to amino acids 78–84 in the protein B1 sequence) has been suggested to participate in the binding of this nucleotide (McIntosh & Haynes, 1986). We found with direct photoaffinity binding studies no indication of labeling of this region. However, further protein engineering and crystallographic studies will clarify the structure of this allosteric site.

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