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Direct photoaffinity labeling of an allosteric site on subunit protein M1 of mouse ribonucleotide reductase by dTTP

(deoxyribonucleotide/feedback-resistant enzyme/mouse T-lymphoma cell/peptide mapping)

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ABSTRACT **The protein M1 subunit of ribonucleotide reductase contains at least two allosteric nucleotide binding sites that control the capacity of the enzyme to reduce ribonucleotides to the deoxyribonucleotides required for DNA synthesis. Direct photoaffinity labeling of partially purified protein M1 from mouse T-lymphoma (S49) cells was observed after UV irradiation in the presence of dTTP at 0°C. The relative molar incorporation of nucleotide per subunit was 4–8%. Competition experiments showed that the dTTP was bound to an allosteric domain genetically and kinetically defined as the substrate specificity site of the enzyme. An altered protein M1 isolated from a thymidine-resistant mutant cell line showed significantly decreased photoincorporation of dTTP, consistent with the fact that its CDP reductase activity is resistant to feedback inhibition by dTTP. Specific photolabeling of several other proteins with pyrimidine and purine nucleotides was also found, indicating the general usefulness of direct photoaffinity labeling in the study of enzymes involved in nucleotide and nucleic acid metabolism.**

The deoxyribonucleotides required for DNA synthesis are generated by reduction of the corresponding ribonucleotides in a reaction catalyzed by ribonucleotide reductase. A single enzyme, containing two nonidentical subunits, protein M1 and protein M2, reduces all four ribonucleoside diphosphate substrates and the regulation of the enzyme is under strict allosteric control (1). The overall activity of the nucleotide binding subunit, protein M1, is influenced by the effectors ATP (stimulating) and dATP (inhibiting). The substrate specificity is regulated by either ATP (required for pyrimidine reduction), dTTP (required for GDP reduction), or dGTP (required for ADP reduction). This complex pattern of regulation by effectors can be accounted for by postulating the existence of two types of allosteric binding sites. One type binds ATP or dATP and governs the overall activity. The other type binds those NTP that govern the substrate specificity of the enzyme.

Affinity labeling of the ribonucleotide reductase with specific ligands that affect the enzyme reaction would allow one to define and characterize molecularly the nucleotide binding sites of both normal and mutant enzymes. We have explored direct photoaffinity labeling by nucleotides, as recently described (2, 3). Ribonucleotide reductase subunit protein M1, partially purified from cultured mouse T-lymphoma cells (S49) (4), could be specifically photoaffinity labeled by using dTTP as the affinity ligand. Experiments with a mutant protein M1 that is unresponsive to regulation by dTTP (5) confirmed the specificity of the photoaffinity labeling technique and provided information about the mechanism of regulation of ribonucleotide reductase from deoxyribonucleoside-resistant mammalian cells.

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EXPERIMENTAL PROCEDURES

The protein M1 subunit of ribonucleotide reductase was partially purified from mouse T-lymphoma cells (S49) as described (4), using ammonium sulfate precipitation and affinity chromatography on dextran blue-Sepharose. The enzyme subunit was purified 20-fold and was 2–5% pure. The purification of protein M1 from wild-type cells and from the HAT 1.5A mutant cell line using KCl gradient elution from dextran blue-Sepharose has been described (5).

Radioactive nucleotides [*methyl*-^{3}H]dTTP, [α-^{32}P]dTTP, and [α-^{32}P]dCTP were from Amersham and their radiopurities were verified by thin-layer chromatography on polyethyleneimine-cellulose (Baker) developed in LiCl (6).

Photoaffinity labeling was carried out using a protein M1 preparation (0.7 mg of protein per ml) in 50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/2 mM dithiothreitol/2 μM [^{32}P]dTTP (2–4 μCi; 1 Ci = 3.7×10^{10} becquerels) in 60–120 μl. The reaction mixture was placed as a drop on parafilm on ice. The UV source was a General Electric germicidal lamp (G8 shortwave UV lamp) in an MR 4 holder (Gates, New York) 9 cm from the reaction mixture. The UV dose rate was 1.8 mW/cm^2 as determined by an UV meter (black-ray shortwave UV meter J255, Ultraviolet Products, San Gabriel, CA). Aliquots of the reaction mixture were removed after various times of irradiation, 50 nmol of dTTP was added, and the proteins were precipitated with 250 μl of cold 5% trichloroacetic acid. After 15–30 min on ice, the solution was centrifuged and the pellet was washed with two 1-ml portions of cold 5% trichloroacetic acid. The proteins were dissolved in sample buffer and analyzed on 7.5% $NaDodSO_4$ slab gels (7, 8). The dried and stained gels were autoradiographed at −70°C with Kodak X-R5 films and intensifying screens. Solutions of [^{32}P]dTTP containing known quantities of radioactivity were applied to DE-81 paper discs and exposed simultaneously with the gels, allowing approximation of the radioactivity in the protein bands. The protein M1 (89-kilodalton) band was identified by coelectrophoresis with pure protein M1 (4). The band intensities were determined by densitrometric scanning followed by integration of the area under each relevant peak. Due to the nonlinearity of the radiographic method at low levels of radioactivity, our determinations at low band intensities must be regarded as semiquantitative.

The radioactive nucleotides in the trichloroacetic acid supernatants after UV irradiation were analyzed along with known standards on polyethyleneimine-cellulose thin-layer chromatography sheets (6).

[^{35}S]Methionine-labeled protein M1 was prepared from cells grown in [^{35}S]methionine (5 μM, 50 μCi/ml) in medium containing 10% dialyzed horse serum. Cells were harvested after

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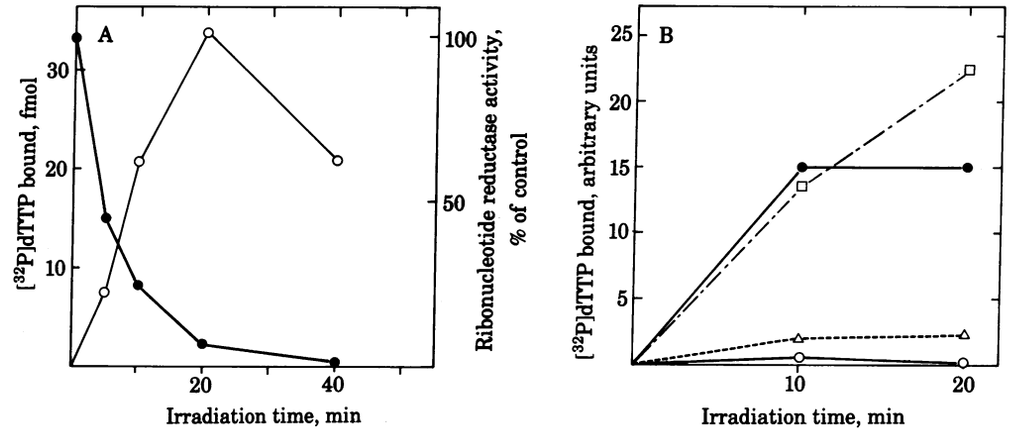

FIG. 1. Time courses of photoaffinity labeling of protein M1 with [^{32}P]dTTP and UV inactivation of CDP reductase activity. (*A*) Mixtures of protein M1 and [^{32}P]dTTP (2 μM, 10 mCi/μmol) were UV irradiated. Aliquots were withdrawn at various times and analyzed by NaDodSO$_4$ gel electrophoresis. ○, Radioactive nucleotide incorporated in the protein M1 band; ●, enzyme activity as percent of control (i.e., unirradiated protein M1). (*B*) [^{32}P]dTTP incorporated into protein M1 under standard conditions (2 μM dTTP) (●), with enzyme previously irradiated for 20 min (○), and in the presence of 12 μM GTP (□) or dGTP (△) was determined.

5 hr and washed with phosphate-buffered saline. [^{35}S]Methionine-labeled protein M1 was purified by chromatography on dextran blue-Sepharose and dATP-Sepharose (4) followed by NaDodSO$_4$ gel electrophoresis.

RESULTS

UV irradiation of mixtures containing [α-^{32}P]dTTP and the partially purified M1 subunit of ribonucleotide reductase from wild-type S49 cells resulted in photolabeling of the protein M1 (Fig. 1*A*) as well as a number of other proteins present in the preparation (Fig. 2). The photoincorporation of [^{32}P]dTTP into protein M1 increased linearly during the initial 10–20 min of irradiation and then decreased (Fig. 1*A*). The decrease was presumably due to breakdown of the M1 protein; longer irradiation times produced concomitant disappearance of the 89-kilodalton polypeptide band detected by Coomassie blue staining and increased low molecular weight radioactive peptides. A similar labeling pattern was observed using [*methyl*-^{3}H]dTTP as the photoaffinity ligand (data not shown), indicating that the intact nucleotide, as opposed to only the phosphate moiety, was co-

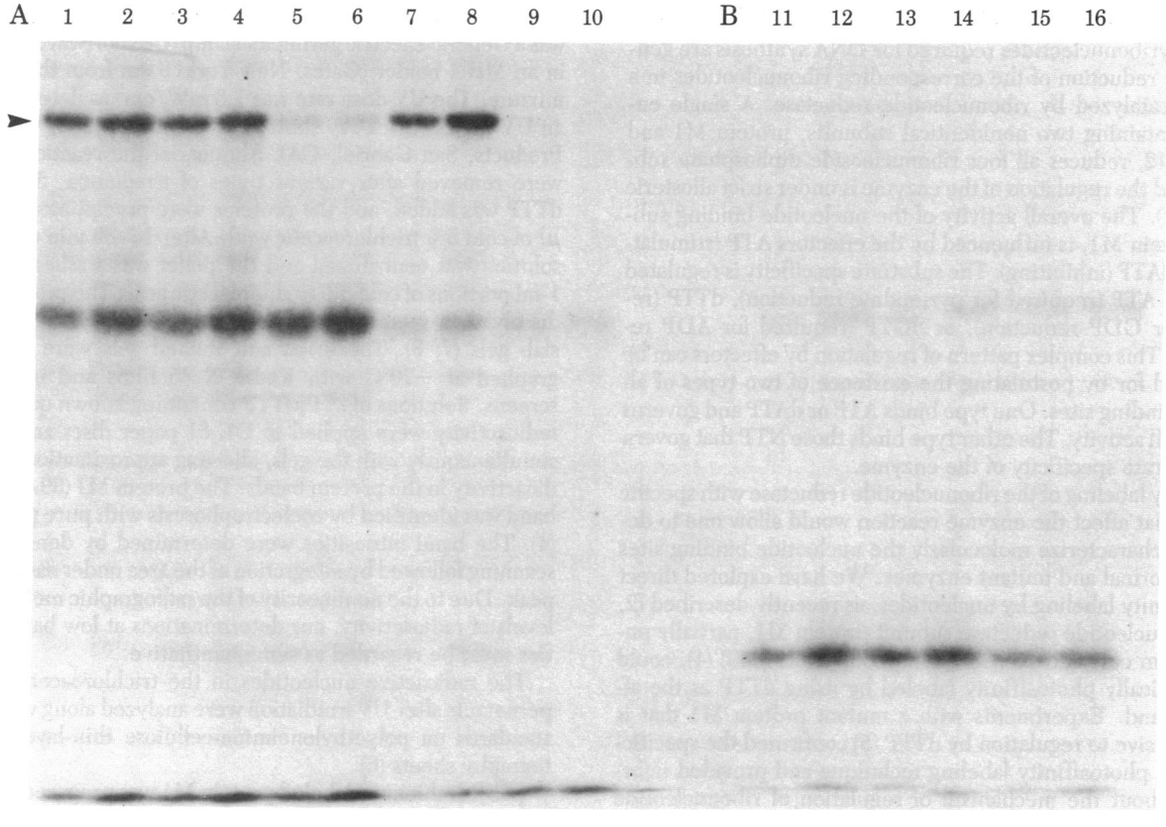

FIG. 2. Photoaffinity labeling of proteins in protein M1 preparation with 2 μM [^{32}P]dTTP (*A*) or [^{32}P]dCTP (2 μCi) (*B*). Radioactive nucleotides were mixed with the protein M1 preparation and irradiated for 10 min (odd-numbered lanes) or 20 min (even-numbered lanes). The labeled proteins were analyzed by NaDodSO$_4$ gel electrophoresis and autoradiography. Lanes: 1, 2, 11, and 12, no addition; 3, 4, 13, and 14, 12 μM deoxyguanosine; 5, 6, 15, and 16, 12 μM dGTP; 7 and 8, 12 μM GTP; 9 and 10, previously irradiated (20 min) enzyme. Arrowhead, protein M1.

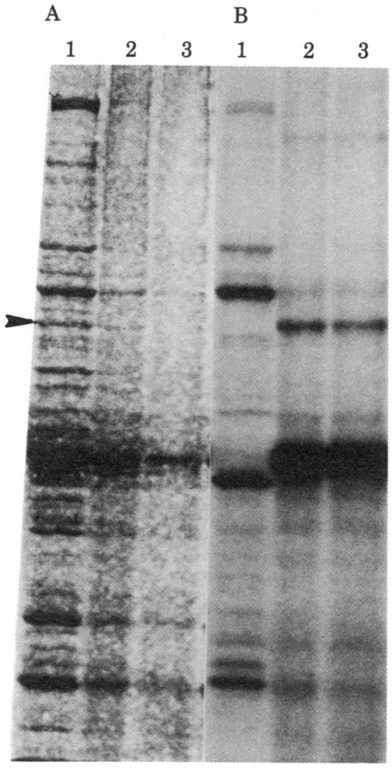

FIG. 3. Photoaffinity labeling of protein M1 preparation with [^{32}P]dTTP under standard conditions or with a previously irradiated nucleotide solution. (*A*) Coomassie blue-stained gel. (*B*) Its autoradiogram. The [^{32}P]dTTP stock solution (200 μM, 4 mCi/μmol) was irradiated for 10 min and then mixed (final concentration, 10 μM) with the protein M1 preparation. This mixture was incubated for 10 min on ice and then analyzed (lane 1). A mixture of the protein M1 preparation and 10 μM (nonirradiated) [^{32}P]dTTP (4 mCi/μmol) was irradiated for 10 min (lane 2) or 20 min (lane 3) and analyzed. Arrowhead, Protein M1.

valently bound to the protein. The reductase activity of protein M1 was inactivated by UV irradiation with a half-life of ≈5 min (Fig. 1*A*). It appeared that only active protein M1 incorporated [^{32}P]dTTP; a protein M1 preparation that had been inactivated by previous irradiation (20 min at 0°C) could not be photolabeled by [^{32}P]dTTP (Figs. 1*B* and 2). The amount of radioactive nucleotide incorporated into protein M1 increased as the concentration of [^{32}P]dTTP increased up to 10 μM and then appeared to saturate. We estimate that, under these conditions and with an irradiation time of 10 min, 4–8% of the protein M1 molecules were labeled with [^{32}P]dTTP. When a previously irradiated solution of [^{32}P]dTTP (20 min at 0°C) was added to the protein M1 preparation, no radioactive nucleotide was incorporated into protein M1 although several other proteins were labeled (Fig. 3). This labeling pattern differed considerably from that observed under standard labeling conditions (presence of dithiothreitol). The addition of dithiothreitol (2 mM) to the [^{32}P]dTTP mixture reduced the level of protein labeling observed with previously irradiated nucleotide solutions, but still no labeling of protein M1 was observed (data not shown). The concentration of [^{32}P]dTTP did not markedly change during the standard photolabeling reaction; ≈10% of the dTTP was hydrolyzed to dTDP after 20 min of UV irradiation (data not shown).

Table 1. Effects of unlabeled nucleosides and nucleotides on photoaffinity labeling of protein M1 by [^{32}P]dTTP

Addition	Unlabeled nucleotide, μM	Relative photoaffinity labeling
None	—	1.0
dTMP	15	1.1
dTDP	15	1.0
dTTP	15	<0.10
CDP	15	1.5
dCTP	15	1.4
Deoxyguanosine	12	1.0
dGTP	6	0.4
dGTP	15	0.1
GTP	15	1.5
Deoxyadenosine	15	1.1
dATP	5	<0.10

Protein M1 fraction (0.7 mg/ml) from wild-type S49 cells was irradiated at 254 nm in the presence of 2 μM [^{32}P]dTTP (10 min at 0°C) and analyzed by NaDodSO$_4$ gel electrophoresis. The level of labeling was determined by densitometric scanning of autoradiograms.

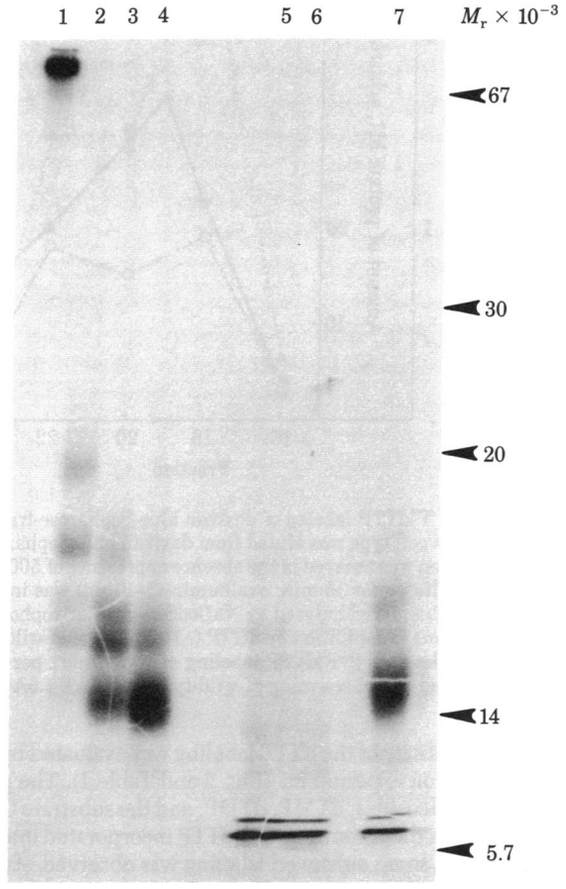

FIG. 4. Peptide mapping of [^{32}P]dTTP-labeled and [^{35}S]methionine-labeled protein M1. [^{32}P]dTTP-photoaffinity-labeled protein M1 and [^{35}S]methionine-labeled protein M1 were purified by NaDodSO$_4$ gel electrophoresis. The gels were fixed, stained, and dried, and the protein M1 bands were identified and excised. The gel pieces were washed with 80% methanol followed by water and then equilibrated with 0.125 M Tris·HCl, pH 6.8/10% glycerol/1 mM 2-mercaptoethanol/1 mM EDTA/0.15% NaDodSO$_4$. *Staphylococcus aureus* V8 protease was added and the gel pieces were incubated overnight at 22°C. The eluted peptides were separated on a 7.5% acrylamide/7 M urea gel having an acrylamide/bisacrylamide ratio of 10:1. Lanes: 1–4, [^{32}P]dTTP-labeled protein M1 after digestion with 0, 2, 10, and 30 μg of V8 protease; 5 and 6, [^{35}S]methionine-labeled protein M1 digested with 10 and 30 μg, respectively, of protease; 7, mixture of [^{32}P]dTTP-labeled and [^{35}S]methionine-labeled protein M1 digested with 30 μg of protease. (The apparent separation in the [^{32}P]dTTP-labeled peptide in lane 7 was due to cracking of the gel after drying.)

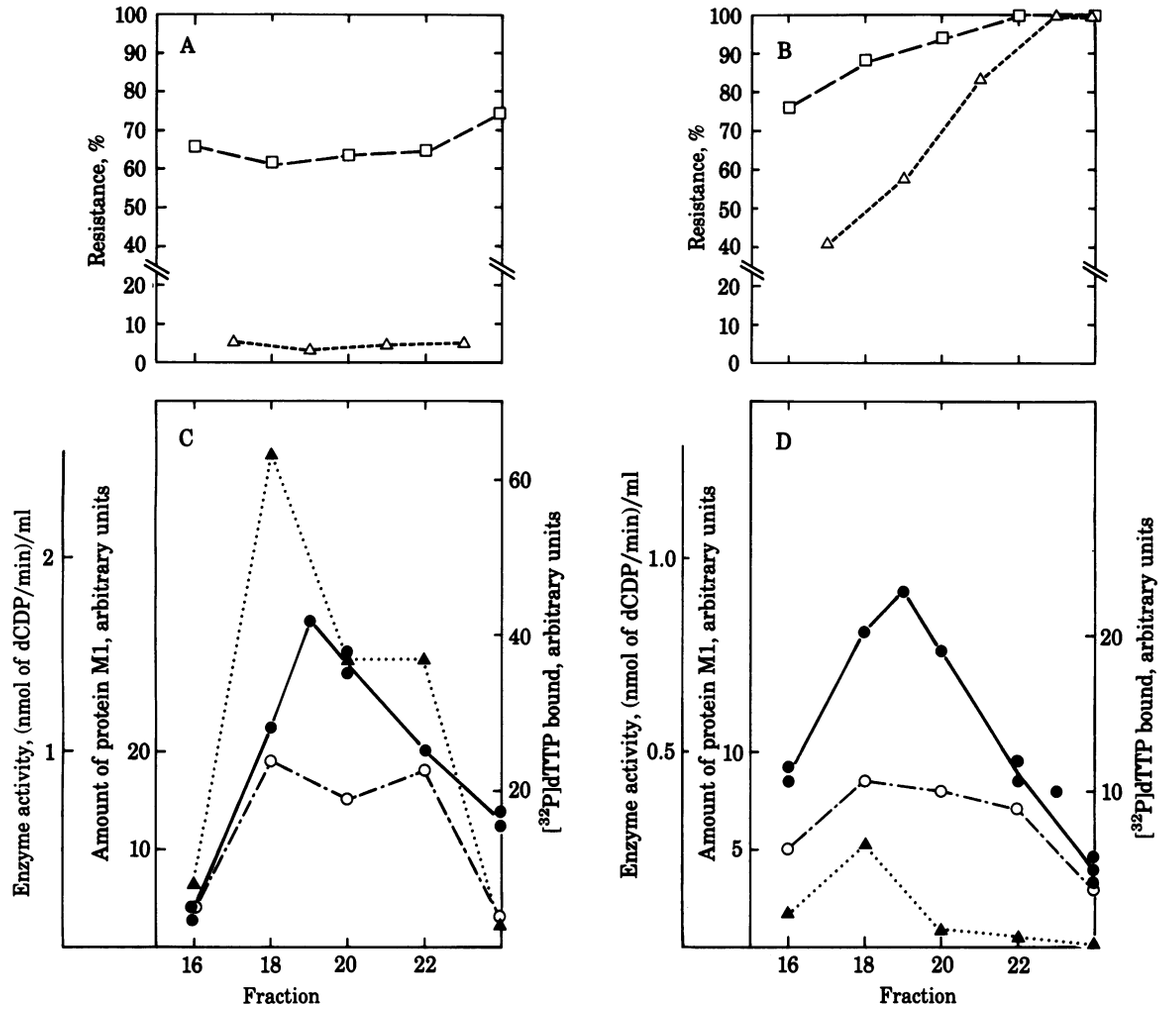

FIG. 5. [^{32}P]dTTP-labeled of dextran blue-Sepharose-fractionated protein M1 from wild-type (*A* and *C*) and HAT 1.5A (*B* and *D*) cells. Protein M1 from each cell type was eluted from dextran blue-Sepharose with a linear KCl gradient (0–0.3 M) as described (5). The CDP reductase activity in each fraction was assayed in the absence or presence of 500 μM dGTP or 400 μM dTTP. An aliquot of each fraction was mixed with 2 μM [^{32}P]dTTP and UV irradiated for 10 min; ovalbumin (5–10 μg) was included to bring the protein concentration to 0.4 mg/ml. The Cl_3CCOOH-precipitated labeled proteins were analyzed by $NaDodSO_4$ gel electrophoresis and autoradiography. (*A* and *B*) Relative resistance of CDP-reductase activity in eluted fractions to inhibition by dGTP (△) or TTP (□) of wild-type and HAT 1.5A preparations, respectively. (*C* and *D*) CDP reductase activity per fraction (●), level of [^{32}P]dTTP labeling of protein M1 per fraction (▲), and amount of Coomassie blue-stained protein M1 present per fraction (○) determined by gel scanning of wild-type and HAT 1.5A preparations, respectively.

The specificity of the dTTP labeling was evaluated by a series of competition experiments (Fig. 2 and Table 1). The presence of deoxynucleosides, dTMP, dTDP, and the substrate CDP, did not decrease the amount of [^{32}P]dTTP incorporated into protein M1. In fact, some enhanced labeling was observed when other nucleotides were present (Fig. 1*B*). However, the level of [^{32}P]dTTP incorporation was drastically reduced in the presence of the allosteric effector nucleotides, dGTP and dATP, both of which compete with dTTP for the regulation of protein M1 at the substrate specificity site (Fig. 1*B* and Table 1) (9, 10). Furthermore, GTP and dCTP, which have no effector role in the regulation of ribonucleotide reductase (1, 9), showed no capacity to compete with [^{32}P]dTTP in the photoaffinity labeling of protein M1 (Table 1 and Fig. 1*B*). Addition of the second ribonucleotide reductase subunit, protein M2 (4), did not alter the labeling pattern or the amount of [^{32}P]dTTP incorporated into protein M1.

UV irradiation of the protein M1 preparation in the presence of [^{32}P]dCTP resulted in the labeling of several proteins (Fig. 2). However, we observed no incorporation of [^{32}P]dCTP into protein M1, consistent with the prediction from kinetic analyses (1, 9) that the protein M1 subunit contains no binding site for dCTP.

To determine whether [^{32}P]dTTP photoderivatized the protein M1 at many or few sites on the subunit, we extensively digested labeled protein M1 with *Staphylococcus aureus* V8 protease and analyzed the radioactive peptides by acrylamide gel electrophoresis (Fig. 4). As the amount of protease increased, the number of labeled peptides decreased to no more than two. [^{35}S]Methionine-labeled protein M1 subjected to identical protease digestion generated several labeled peptides. Thus, the [^{32}P]dTTP appears to photoderivatize protein M1 specifically at one, or at most two, peptide sites.

To provide further proof of the specificity of the dTTP labeling and to gain insight into the properties of a mutant protein M1, the enzyme from the S49 cell line, HAT 1.5A (5), was investigated. HAT 1.5A cells are resistant to the cytotoxic effects of high concentrations of thymidine and deoxyguanosine (5). Ribonucleotide reductase isolated from these cells does not respond normally to regulation by the allosteric effectors dGTP and dTTP. The sensitivity to feedback inhibition of CDP reduction (by dTTP and dGTP) is diminished, as is the activation

of GDP or ADP reduction by dTTP or dGTP, respectively (5, 10). These observations suggest that the protein M1 from HAT 1.5A cells contains an altered substrate specificity allosteric site with decreased affinity for both dGTP and dTTP (5, 10). This hypothesis was tested by comparing the ability of wild-type and HAT 1.5A protein M1 preparations to photoincorporate [^{32}P]dTTP. The HAT 1.5A cell line is apparently heterozygous for a mutation in the protein M1 structural gene, and the cells contain both wild-type and mutant protein M1 molecules (5). The two forms of protein M1 were partially separated by chromatography on dextran blue-Sepharose using KCl gradient elution (5). The mutant protein M1 eluted later, at a higher salt concentration than the wild-type protein M1, as shown by the progressively decreasing sensitivity of CDP reduction to inhibition by dGTP or dTTP (Fig. 5). In contrast, all fractions obtained by similar dextran blue-Sepharose chromatography of a wild-type extract remained fully sensitive to the inhibitory effects of dGTP and dTTP.

We compared the ability of the eluted fractions from both wild-type and HAT 1.5A protein M1 preparations to photoincorporate [^{32}P]dTTP (Fig. 5*B* and *C*). Using fractions derived from wild-type cells, we observed excellent correlation between the level of [^{32}P]dTTP incorporation into protein M1 and the CDP reductase activity. However, fractions derived from HAT 1.5A cells showed reduced ability to incorporate [^{32}P]dTTP relative to both amount of protein M1 present and CDP reductase activity. In addition, we observed an across-the-gradient progressive decrease in [^{32}P]dTTP incorporation relative to catalytic activity. This decrease in labeling correlated with the decreased responsiveness of the CDP reductase activity to inhibition by dTTP.

DISCUSSION

We observed specific and relatively high stoichiometric photodependent incorporation of radioactively labeled dTTP into an allosteric site on ribonucleotide reductase protein M1. A partially purified enzyme preparation containing several other polypeptides could be used. Some of these peptides were also photolabeled and served as controls in competition experiments, showing that decreased labeling of protein M1 was not due to generalized inhibition of the photoreaction. Evidence for the specificity of the [^{32}P]dTTP labeling of protein M1 is as follows: (*i*) only active protein M1 incorporated nucleotide; (*ii*) the addition of known nucleotide effectors of ribonucleotide reductase inhibited the photoaffinity labeling while nucleotides or nucleosides known to have no effect on the activity or to bind only to the catalytic site did not influence photolabeling; (*iii*) a mutant protein M1 with an altered specificity site showed reducted ability to photoincorporate dTTP; and (*iv*) one-dimensional protease mapping of the labeled protein M1 showed that dTTP was incorporated predominately into a single peptide.

Experiments with [^{32}P]dCTP as a photoaffinity ligand showed that, although several proteins in the preparation could be labeled, protein M1 was not. This result is consistent with the fact that dCTP is not an allosteric effector of the enzyme (9). The use of purine dNTPs as photoaffinity ligands resulted in specific but lower incorporations.

Protein M1 from the mutant cell line HAT 1.5A photoincorporated considerably less [^{32}P]dTTP than did its wild-type counterpart. The low photoincorporation of dTTP by HAT 1.5A protein M1 correlated with the lack of responsiveness of the mutant reductase activity to dTTP- and dGTP-mediated regulation. This includes resistance to feedback inhibition of pyrimidine nucleotide reduction and reduced ability to use ADP and GDP as substrates (10).

The chemical reactions leading to crosslinking of nucleotides and nucleic acids to proteins by UV light have recently been reviewed (11). The only amino acid that has been definitely shown to be involved is cysteine, forming an adduct (5-*S*-cysteinyl-6-hydrouracil) with uracil. However, several other adducts have been isolated both with pyrimidine and purine nucleotides and involving several amino acids. Direct isolation of the crosslinked nucleotide-peptide photoproduct is clearly required to define the chemical reaction involved and the structure of the specific nucleotide binding domains of ribonucleotide reductase.

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