Localization and Characterization of Two Nucleotide-binding Sites on the Anaerobic Ribonucleotide Reductase from Bacteriophage T4*

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We have used 8-azidoadenosine 5'-triphosphate (8-N3ATP) to investigate the nucleotide-binding sites on the NrdD subunit of the anaerobic ribonucleotide reductase from T4 phage. Saturation studies revealed two saturable sites for this photoaffinity analog of ATP. One site exhibited half-maximal saturation at approximately 5 μM [γ-32P]8-N3ATP, whereas the other site required 45 μM. To localize the sites of photoinsertion, photolabeled peptides from tryptic and chymotryptic digests were isolated by immobilized Al3+ affinity chromatography and high performance liquid chromatography and subjected to amino acid sequence and mass spectrometric analyses. The molecular masses of the photolabeled products of cyanogen bromide cleavage were estimated using tricine-SDS-polyacrylamide gel electrophoresis. Overlapping sequence analysis localized the higher affinity site to the region corresponding to residues 289-291 and the other site to the region corresponding to residues 147-160. Site-directed mutagenesis of Cys290, a residue conserved in all known class III reductases, resulted in a protein that exhibited less than 10% of wild type enzymatic activity. These observations indicate that Cys290 may reside in or near the active site. High performance liquid chromatography analysis revealed that photoinsertion of [γ-32P]8-N3ATP into the site corresponding to residues 147-160 was almost completely abolished when 100 μM dATP, dGTP, or dTTP was included in the photolabeling reaction mixture, whereas 100 μM ATP, GTP, CTP, or dCTP had virtually no effect. Based on these nucleotide binding properties, we conclude that this site is an allosteric site analogous to the one that has been shown to regulate substrate specificity of other ribonucleotide reductases. There was no evidence for a second allosteric nucleotide-binding site as observed in the anaerobic ribonucleotide reductase from Escherichia coli.

Ribonucleotide reductases catalyze the reduction of ribonucleotides to their corresponding 2'-deoxyribonucleotides. Currently, they are divided into three classes based on differences in cofactor requirements, structural composition, and type of radical employed for catalysis (1, 2). Because of the importance of maintaining a balanced supply of deoxyribonucleotides for DNA synthesis (3, 4), they are enzymes that are subject to complex allosteric regulation (5, 6). Although there may be striking differences in primary sequence, the same general mechanism for allosteric regulation appears to apply to all ribonucleotide reductases described to date, with only subtle differences observed (7). Three different nucleotide-binding sites have been localized on the prototypical class I reductase from Escherichia coli using photoaffinity labeling (8) and x-ray crystallography (9, 10). Two of these sites are allosteric sites that coordinate the reduction of all four ribonucleotide substrates at a single active site. One allosteric site binds only ATP and dATP and regulates the overall activity of the enzyme. The other allosteric site, via its interactions with the effectors dATP, dGTP, dTTP, and ATP, controls the substrate specificity of the active site. A recent kinetic study indicated that the class III anaerobic reductase from E. coli also has two allosteric sites (11). One site regulates pyrimidine ribonucleotide reduction via its interactions with ATP and dATP. The other site binds dATP, dGTP, and dTTP with high affinity and regulates purine ribonucleotide reduction. Unlike the specificity site in the class I aerobic reductase, the latter site has a very low affinity, if any, for ATP.

The bacteriophage T4 genome encodes for two different ribonucleotide reductases. One operates under aerobic conditions and belongs to the class I set of enzymes. The other is a class III reductase consisting of a 68-kDa NrdD subunit and an 18-kDa NrdG subunit that have been proposed to form a tetrameric structure consisting of two homodimers (12-14). Like other class III reductases, it is active only under anaerobic conditions and uses nucleoside triphosphates rather than nucleoside diphosphates as substrates. The requirement for anaerobic conditions is believed to be due to the deleterious effect of molecular oxygen, which reacts with the radical harbored at glycine 580 within the NrdD subunit, resulting in cleavage of the polypeptide backbone at or near this residue (14), as has been demonstrated for the corresponding enzyme in E. coli (15, 16). Formation of the glycy1 radical requires the presence of the NrdG subunit, which contains an iron-sulfur center (16, 14). Whereas the allosteric properties of the aerobic class I enzyme from T4 phage have been characterized (17-19), little is known about the recently discovered anaerobic enzyme. Based on sequence homology (58% identity and 72% similarity) with the anaerobic reductase from E. coli (13) for which the allosteric properties have been described (11), it is postulated that the active site and the allosteric sites reside within the NrdD subunit.

Although kinetic evidence supports the existence of two different allosteric sites and an active site in the class III reductase from E. coli, the location of these nucleotide-binding sites has not been determined. In fact, very little is known about the structure of any of the anaerobic reductases. One difference noted upon sequence alignment of the anaerobic reductases from E. coli and bacteriophage T4 is that the T4 NrdD subunit lacks approximately 100 residues that exist at the N-terminal end of the E. coli enzyme (2) and thus may represent a more
simple protein to examine. Photoaffinity labeling with azido- purine and azido pyrimidine nucleotide derivatives has been a useful technique for the study of nucleotide-binding proteins (20, 21). In this study, we have used the 8-azido derivative of ATP to localize and characterize two different nucleotide-bind- ing sites on the class III ribonucleotide reductase from bacteriophage T4.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]8-N3ATP was synthesized as described previously (20). All other nucleotides were purchased from Amersham Pharmacia Biotech. The concentration and purity of the nucleotides were determined by UV spectral properties and TLC analysis. Sequencing grade modified trypsin was from Promega. Sequencing grade chymotrypsin was from Boehringer Mannheim. Imidodicetic acid epoxy-activated Sepharose 6B resin was from Sigma.

**Overexpression and Purification of T4 NrdD—** Cultures of JM109(DE3) bacteria carrying the pET29T4NrdD plasmid in LB medium supplemented with 30 μg/ml kanamycin were incubated at 37 °C under aerobic conditions. When the cells reached an absorbance (640 nm) of 0.8, the overexpression of NrdD was induced by the addition of 200 μM isopropyl-1-thio-β-D-galactopyranoside. After 3–4 h of induction, the cells were harvested by centrifugation, and the pellet was frozen on dry ice. For purification, the frozen pellet was pressed five times in a Xpress (BIOX) and suspended in 20 mM Tris-HCl, pH 8.0, 1 mM DTT. After centrifugation, nucleic acids were removed from the isolated supernatant by precipitation with 1% streptomycin sulfate. Following fractionation with 40% ammonium sulfate, the pellet was resuspended in 20 mM Tris-HCl, pH 8.0, 1 mM DTT.

The final purification step was hydrophobic interaction chromatography (butyl Sepharose 4 Fast Flow medium from Amersham Pharmacia Biotech) using a gradient of 0.5–5 mM (NH₄)₂SO₄ in 50 mM NaPi, pH 7.0, 2 mM DTT. The NrdD-containing fraction was pooled and then dialyzed against and stored in 50 mM NaPi, pH 7.0, 2 mM DTT. Immediately prior to photolabeling experiments, the DTT was removed by desalting on an Amersham Pharmacia Biotech NAP-5 column equili- brated with 50 mM NaPi, pH 7.0.

**Photoaffinity Labeling—** The standard photoaffinity reaction mixture consisted of NrdD buffered in 10 mM NaPi, pH 7.0, 5 mM MgCl₂ and the appropriate concentration of [γ-32P]8-N3-ATP. Samples were incubated for 60 s and then irradiated for 90 s with a 254-nm UVG-11 lamp (UV Products, Inc.) at a distance of 4 cm. All incubations and irradiations were carried out in microcentrifuge tubes over ice. After photo- labeling, the protein was precipitated in 8% perchloric acid and washed with cold methanol.

**Saturation of T4 Anaerobic Ribonucleotide Reductase—** 1.5 μg of NrdD was incubated with increasing concentrations of [γ-32P]8-N3-ATP in the presence of 10 mM NaPi, pH 7.0, 5 mM MgCl₂ and photolabeled as described above. Following acid precipitation and a cold methanol wash, the pellet was resolubilized in 60 μl of sample buffer and analyzed by SDS-PAGE using a 4% acrylamide stacking gel and a 10% acrylamide separating gel. Gels were stained with Coomasie Brilliant Blue R in 25% isopropanol/10% acetic acid for 1 h, destained with a 10% isopropanol/5% acetic acid solution overnight, and then dried. A Phos- phorImager detector and the ImageQuant program from Molecular Dynamics were used to quantify photoincorporation of [γ-32P]8-N3-ATP. Stoichiometry of photoinsertion of [γ-32P]8-N3-ATP was determined using a NAP-5 column (Amersham Pharmacia Biotech) as described previously (22), except that the desalting was done in the presence of 10 mM Tris-HCl, pH 8.0 instead of DTT.

**Isolation and Identification of Photolabeled Peptides—** 400 μg of NrdD was photolabeled with 100 μM [γ-32P]8-N3-ATP in a 500-μl solution of 10 mM NaPi, pH 7.0, and 5 mM MgCl₂ as described above. Following acid precipitation, the pellet was resolubilized in a solution of 4% acrylamide stacking gel and 10% acrylamide separating gel. Gels were stained with Coomasie Brilliant Blue R in 25% isopropanol/10% acetic acid for 1 h, destained with a 10% isopropanol/5% acetic acid solution overnight, and then dried. A Phos- phorImager detector and the ImageQuant program from Molecular Dynamics were used to quantify photoincorporation of [γ-32P]8-N3-ATP. Stoichiometry of photoinsertion of [γ-32P]8-N3-ATP was determined using a NAP-5 column (Amersham Pharmacia Biotech) as described previously (22), except that the desalting was done in the presence of 10 mM Tris-HCl, pH 8.0 instead of DTT.

**Localization of the Nucleotide-binding Sites—** To localize the sites of photoinsertion of [γ-32P]8-N3-ATP, the NrdD subunit was separated from unmodified peptides by immobilized Al₃⁺ affinity chromatography (IAAC) as described previously (23) and then resolved by reversed-phase HPLC (RP-HPLC) and collected in 0.25-ml fractions. Radioactivity was determined by quantification of Cerenkov radiation. Fractions containing significant radioactivity with a corresponding absorbance at 241 nm were analyzed by a Procise™ 494 Protein Sequencing System from Applied Biosystems or by MALDI-MS using a PerSe- nary Voyager Elite mass spectrometer equipped with delayed extraction. Minor peaks were pooled from two or more separate preparative runs. For MALDI-MS, the samples were prepared as described (24) using 2,5-dihydroxybenzoic acid as the matrix.

**RESULTS**

**Saturation of NrdD with [γ-32P]8-N3-ATP—** Figure 1 illustrates the results from two experiments in which the NrdD subunit from phage T4 was photolabeled with increasing concentra- tions of [γ-32P]8-N3-ATP. As shown by the biphasic saturation curve in Fig. 1A, the NrdD subunit contains at least two sites that are saturable with this photoaffinity analog of ATP. The first site exhibited half-maximal saturation at approximately 5 μM, whereas the second site was maximally saturated at 40 μM [γ-32P]8-N3-ATP. To confirm the presence of the first saturable site, NrdD was photolabeled with a greater number of lower concentrations of [γ-32P]8-N3-ATP (Fig. 1B). The stoichiometry of photoinsertion of 100 μM [γ-32P]8-N3-ATP was determined to be 2.2 ± 0.1 mol of nucleotide bound per mol of NrdD monomer.

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unit was photolabeled with saturating concentrations of photoprobe and then digested with either trypsin or chymotrypsin. Preliminary attempts to isolate and identify the radiolabeled trypsin-generated peptides by directly applying the digest to a reversed-phase HPLC column were unsuccessful because of the inability to resolve them from all of the unmodified peptides. To alleviate this problem, IAAC was used prior to RP-HPLC in order to separate the photolabeled peptides from the unmodified peptides (via the coordination of the phosphate groups to the immobilized \( \text{Al}^{3+} \)). As shown in Fig. 2, the technique was very effective. When the flow-through fractions from IAAC were pooled, dried, and subjected to RP-HPLC analysis, a large number of unmodified peptides with no associated radioactivity were observed (Fig. 2A). In contrast, RP-HPLC analysis of the fractions that eluted from the IAAC column with 10 mM KPi, pH 8.0, revealed only a few peptides (Fig. 2B). All of these peptides had radioactivity associated with them. Fractions corresponding to the radioactive peaks designated T1, T2, T3, T4/5 (a doublet, as determined by both the UV and radioactivity profiles), T6, and T7 were subjected to amino acid sequence and/or MALDI-MS analyses. As shown in Table I, sequence analysis of peaks T1 and T2 revealed the same N-terminal sequence to 277AITGSSVPVSPMGCR 291 in the primary sequence of NrdD assuming cleavage at Arg 291 by trypsin. The molecular ion at \( m/z \) 2005.8 observed upon MALDI-MS analysis of T2 indicates a single modification of Ala277-Arg291 (monoisotopic mass of 1460.71) by the photoreactive species, ATP-N and the adduction of an Al3+ ion. Amino acid sequence analyses of peaks T3, T4/5, and T7 revealed the same N-terminal sequence in all three analyses. Assuming cleavage at Lys146 by trypsin, this sequence corresponds to 147HIE-DAEKWQIALNYAQSK 166. MALDI-MS analysis of fractions T3, T4/5, and T6 confirmed this identity and revealed T6 to be the same modified peptide (His147-Lys166). The observed molecular ions at \( m/z \) 2874 indicate a single modification of His147-Lys166 (monoisotopic mass of 2329.13) by ATP-N and the adduction of an Al3+ ion. It should be noted that no or very little tryptophan was detected in cycle 8 during amino acid sequence analysis of T3, T4/5 or T7. In addition, the analyses revealed that trypsin had failed to cleave photolabeled NrdD at Lys153.

To narrow down the site of photoinsertion within the region of His147-Lys166, chymotrypsin was used to digest NrdD that had been photolabeled with 100 \( \mu \text{M} \) [\( \gamma^{32}\text{P} \)8-N3ATP]. IAAC and RP-HPLC (Fig. 3) were again used to isolate the peptides. The combination of amino acid sequence and MALDI-MS analyses identified the major radiolabeled peptide, C4, as 145AKHIEDAEKWQIADAL 160 (Table II). Edman degradation of the fractions corresponding to peaks C5/6 revealed the same N-terminal sequence as found in C4. As was the case in the sequencing of peaks T3, T4/5, and T7, virtually no tryptophan was detected in the cycle corresponding to Trp154 during the sequencing of C4 and C5/6. In addition, chymotrypsin failed to cleave at Trp154. It was not considered necessary to have any of the other fractions in Fig. 3 analyzed.

To further narrow down the site of photoinsertion within the...
higher affinity site \((277\text{AITGSSVPVMGCR291})\), we took advantage of the difference in the molecular masses of the two possible cyanogen bromide fragments that overlap this region of NrdD (cf. Fig. 9). If the site of photoinsertion was within the region of Ala\(^{277}\)-Met\(^{288}\), the molecular mass of the corresponding cyanogen bromide fragment (Tyr\(^{266}\)-Met\(^{288}\)) would be 2.4 kDa. If the site of photoinsertion was within the region of Gly\(^{289}\)-Arg\(^{291}\), then the molecular mass of the corresponding cyanogen bromide fragment (Gly\(^{289}\)-Met\(^{345}\)) would be 6.5 kDa. The calculated molecular mass of the cyanogen bromide fragment that contains the other site of photoinsertion (Ala\(^{113}\)-Met\(^{209}\)) is 11.0 kDa. Fortunately, the molecular masses of these three fragments (2.4, 6.5, and 11.0 kDa) are very close to the three lower molecular mass standards included in the Peptide Marker Kit from Amersham Pharmacia Biotech (2.5, 6.2, and 10.7 kDa). The autoradiogram in Fig. 4 revealed two radiolabeled bands with apparent molecular masses of 6.6 and 10.4. No differences in cleavage patterns were detected from the two different time points of digestion, as observed in both the autoradiogram and the Coomassie-stained gel (not shown). The observation of a radiolabeled 6.6-kDa fragment indicates that photoinsertion of \(\gamma\text{-}{\text{\textsuperscript{32}P}}\text{-N\textsubscript{3}ATP}\) into the higher affinity site was within the region of Gly\(^{289}\)-Arg\(^{291}\). The observation of only two radiolabeled bands is consistent with the sequencing and MALDI-MS data (Tables I and II) that indicated only two nucleotide-binding sites.

**Characterization of the Nucleotide-binding Sites**—As shown in Fig. 2C, 100 \(\mu\text{M}\) dTTP was very effective at inhibiting the photoinsertion of \(\gamma\text{-}{\text{\textsuperscript{32}P}}\text{-N\textsubscript{3}ATP}\) into the site corresponding to His\(^{147}\)-Leu\(^{160}\) (peaks T3, T4/5, T6, and T7), whereas it had a small effect on the photoinsertion into the site corresponding to Gly\(^{289}\)-Arg\(^{291}\) (peaks T1 and T2). Using peaks T2 and T3 as indicators, it was calculated that dTTP decreased photoinser- tion into the site corresponding to Gly\(^{289}\)-Arg\(^{291}\) by 30%, whereas it almost abolished binding into the His\(^{147}\)-Leu\(^{160}\) site (decrease of 93%). To further characterize the two nucleotide-binding sites, a RP-HPLC radiometric assay was developed in which the effect of various nucleotides on the photoinsertion of a saturating concentration of \(\gamma\text{-}{\text{\textsuperscript{32}P}}\text{-N\textsubscript{3}ATP}\) into the two different sites could be determined more easily by quantitating

**FIG. 3.** RP-HPLC of IAAC-purified peptides from NrdD photolabeled with \(\gamma\text{-}{\text{\textsuperscript{32}P}}\text{-N\textsubscript{3}ATP}\) and digested with chymotrypsin. NrdD was photolabeled with 100 \(\mu\text{M}\) \(\gamma\text{-}{\text{\textsuperscript{32}P}}\text{-N\textsubscript{3}ATP}\), digested with chymotrypsin, and applied to an immobilized Al\(^{3+}\) column as described under “Experimental Procedures.” The 10 \(\mu\text{M}\) KP eluate was then analyzed by RP-HPLC. The solid line indicates absorbance at 214 nm, whereas the dotted line indicates radioactivity.
the radioactivity in the individual HPLC peaks with an on-line scintillation counter. As shown in Fig. 5, ATP had only a minimal effect on the photoinsertion of its 8-azido analog into either site. In the presence of 100 \( \mu M \) ATP (Fig. 5B), the radioactivity detected in peaks T2 and T3 was 83 and 105%, respectively, of the amount detected in the corresponding peaks in the control sample. In the presence of 100 \( \mu M \) dATP (Fig. 5C), the radioactivity detected in peak T2 was reduced by 43% (as compared with the control sample), and the amount in peaks T3, T4/5, T6, and T7 was reduced to near background levels. As shown in Fig. 6, GTP decreased photoinsertion into peaks T2 and T3 by 12 and 23%, respectively, whereas dGTP decreased photoinsertion into T2 and T3 by 34 and 80%, respectively. Like the other substrates tested (ATP and GTP), 100 \( \mu M \) CTP had minimal effect on the photoinsertion of 100 \( \mu M \) \([\gamma-32P]8-N_3ATP\) into any of the peaks (data not shown). 200 \( \mu M \) CTP reduced the photoinsertion of 5 \( \mu M \) \([\gamma-32P]8-N_3ATP\) into peaks T2 and T3 by 36 and 29%, respectively (Fig. 7). Fig. 7A also indicates that the site corresponding to Gly289-Arg291 has the higher affinity for 8-N_3ATP because the relative amount of radioactivity in peak T2 is significantly greater than that of T3 at the lower concentration of 5 \( \mu M \) \([\gamma-32P]8-N_3ATP\) (compare with the relative amounts observed at 100 \( \mu M \) photoprobe in Figs. 5A, 6A, and 8A). This difference was even more pronounced when the relative amounts were compared at a concentration of 1 \( \mu M \) \([\gamma-32P]8-N_3ATP\) (data not shown).

To determine whether the binding of dATP, dGTP, and dTTP to the site corresponding to His147-Leu160 could be attributed solely to the absence of a 2'-hydroxyl group on a nucleotide, we examined the effect of dCTP. As shown in Fig. 8, 100 \( \mu M \) dCTP had minimal effect on photoprobe into either site. The seemingly high background is a consequence of the low specific activity of the probe at the time of the experiment.

**TABLE II**

| Amino acid sequence and MALDI-MS analyses of radiolabeled peptides from NrdD photolabeled with \([\gamma-32P]8-N_3ATP\) and digested with chymotrypsin |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | A               | K               | H               | I               | E               | D               | A               | E               | K               | W               | Q               | I               | A               | D               | A               | L               |
| **Yield (pmol)** | 130             | 171             | 128             | 157             | 70              | 94              | 71              | 48              | 50              | ND              | 7               | ND              | ND              | ND              | ND              | ND              |
| **Mass (Da)**   | Observed: 2382.3 | Calculated: 2381.9 |

- **A** Assigned residue.
- **ND** Not detected.

**Fig. 4.** Tricine-SDS-PAGE of peptides from NrdD photolabeled with \([\gamma-32P]8-N_3ATP\) and digested with cyanogen bromide. NrdD was photolabeled with 100 \( \mu M \) \([\gamma-32P]8-N_3ATP\), digested with cyanogen bromide for 0, 21, or 38 h and then subjected to tricine-SDS-PAGE. Photoinsertion of \([\gamma-32P]8-N_3ATP\) was detected by autoradiography. The positions of the molecular mass standards (in kDa) and of undigested, photolabeled NrdD are indicated.

**Fig. 5.** Effect of ATP and dATP on the photoinsertion of \([\gamma-32P]8-N_3ATP\) into the individual nucleotide-binding sites of NrdD. NrdD was photolabeled with 100 \( \mu M \) \([\gamma-32P]8-N_3ATP\) in the absence of cold nucleotide (A), in the presence of 100 \( \mu M \) ATP (B), or in the presence of 100 \( \mu M \) dATP (C) as described under “Experimental Procedures.” Following digestion with trypsin and IAAC, the KPi eluate from IAAC was analyzed by RP-HPLC and an on-line scintillation counter.

**Site-directed Mutagenesis of Cys290**—Previous sequence alignment of NrdD with other class III reductases revealed the presence of seven conserved cysteines (2). Because we had determined that one of these cysteines, Cys290, was located...
within or immediately adjacent to a nucleotide-binding site that exhibited binding characteristics consistent with those of the active site of the anaerobic ribonucleotide reductase from *E. coli*, it was of interest to examine the effect of mutating it to a serine on enzymatic activity. Because an *in vitro* reconstitution assay has yet to be developed, it was necessary to use anaerobic extracts of bacteria overexpressing either wild type NrdD or the C290S mutant NrdD protein and to determine their activity after mixing with anaerobic extracts of overproduced wild type NrdG. Although able to form a glycyl radical, the C290S mutant (24 nmol/min/nmol of radical) exhibited less than 10% of the activity of the wild type protein (340 nmol/min/nmol of radical). Other cysteine mutants had activities comparable to wild type, showing that the mutation of a cysteine residue per se did not inhibit the enzymatic activity.  

**DISCUSSION**

Using the 8-azido analog of ATP, we have identified and localized two different nucleotide-binding sites within the primary sequence of the NrdD subunit of the anaerobic ribonucleotide reductase from bacteriophage T4. Apparently, the azido group increases the affinity of the ATP analog for both sites, because equimolar concentrations of ATP had little effect on the photoinsertion of the analog into either site (Fig. 5). Previous studies with other adenine nucleotide-binding proteins, such as creatine kinase (29, 23), have demonstrated a similar effect of an azido moiety attached to the purine ring.

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The results from Fig. 2C and the radiometric HPLC analyses (Figs. 5–8) demonstrate that the nucleotide-binding site corresponding to His<sup>147</sup>-Leu<sup>160</sup> binds dATP, dGTP, and dTTP with relatively high affinity but has a much lower affinity, if any, for ATP, GTP, CTP, and dCTP. Thus, this site exhibits nucleotide binding properties very similar to the specificity-determining “purine site” on the *E. coli* anaerobic reductase (11). Except for the lack of significant affinity for ATP, its nucleotide binding properties are also similar to the allosteric site that regulates substrate specificity of the aerobic class I reductase from *E. coli* that was localized by direct photoaffinity labeling with dTTP (8). The inability of dCTP to decrease photoinsertion empha-
sizes the specificity of nucleotide interaction at this site and further substantiates the similarity of this site to an allosteric site because dCTP exerts no significant allosteric effects on other reductases (6, 30).

Three observations indicated that the amino acid residue modified at this site is Trp 154. First, little, if any, tryptophan was detected in the cycles corresponding to Trp 154 during sequence analysis of peptides T3, T4/T5, T7, C4, and C5/6. Second, trypsin failed to cleave photolabeled NrdD at Lys 153, a residue that is immediately adjacent to Trp 154. Third, chymotrypsin failed to cleave at Trp 154. Taken together, it is possible that the covalent attachment of a nucleotide to Trp 154 impeded its detection during sequence analysis. In support of our conclusion that Trp 154 is located within a nucleotide-binding site because dCTP exerts no significant allosteric effects on other class III reductases. This suggests that Trp 154 and its neighboring residues may reside within an α-helix and that the residues on one side of the helix are conserved.

Four observations suggest that the other nucleotide-binding site (Gly 289) modified by 8-N3ATP is the active site. 1) It contains Cys 290, one of seven conserved cysteine among the class III reductases (2). Cysteines have been shown to play an important role in the catalytic mechanism of all ribonucleotide reductases in which it has been studied (31–36). Recent crystallographic studies have confirmed the location of three cysteines within the active site region of the class I reductase from E. coli. (9, 37, 38). 2) The position of Cys 290 in the primary sequence of T4 NrdD aligns with the active site region of other class III reductase sequences described to date. Upon examination of the amino acid sequence around Trp 154, we noticed that every third or fourth amino acid was also conserved when compared with this region within the primary sequence of other class III reductases. This suggests that Trp 154 and its neighboring residues may reside within an α-helix and that the residues on one side of the helix are conserved.

Fig. 9. Amino acid sequence alignment of various class III ribonucleotide reductases within the regions of the two nucleotide-binding sites. The dotted lines depict theoretical molecular masses of cyagen bromide cleavage fragments. The regions enclosed by arrows indicate the observed cleavage sites and photolabeled peptides from chymotryptic and trypsinic digestions of T4 NrdD as determined by sequence and mass spectrometry analyses. Underlined peptide regions are those proposed to contain residues modified by the 8-azido analog of ATP based on the overlapping sequence analysis. The conserved cysteine at position 290 is indicated by boldface type. Residues suggested to make a conserved phase of an α-helix comprising Trp 154 are indicated by asterisks. T4, NrdD from bacteriophage T4; Ec, NrdD from E. coli; Hi, NrdD from Hemophilus influenza; Ll, NrdD from Lactococcus lactis. Sequences and the alignment are from Ref. 2 and references therein.
There are two possible explanations for the ability of dATP, dTTP, and dGTP to cause a slight decrease of photoinsertion of 8-N3ATP into a site that we propose might be the active site. First, these nucleotides may bind to and hence compete with the photoprobe for binding to this site. However, if it is the active site, it would imply that the products of the reaction would bind with higher affinity than the substrates. A second, more likely possibility is that the tight binding of these nucleotides to the other site might decrease the binding of 8-N3ATP to this site.

The reason for the multiple retention times for the peptides 277ATGSSVPVSPMGCR291 (T1 and T2), 147HIEDAEKWQIALNYAQSK166 (T3, T4/T5, T6, and T7), and 149AKHIEQDARQ250 (C4 and C5/6) upon HPLC analysis is unclear, but a similar phenomenon has been observed before in other photoaffinity labeling studies (39). From the mass spectrometry data, one can rule out oxidation, multiple modifications, and reaction of the N terminus of the peptide with urea (further substantiated by the observation of the same chromatographic profile when guanidine hydrochloride was substituted for urea, data not shown). Previous studies have shown that a peptide can exhibit different retention times upon HPLC analysis when it is modified on a different residue (40). Although all indications are that the same residue is modified in our case, it is conceivable that photoinsertion into different regions of a hydrophobic residue like tryptophan could result in significant alterations in exposed hydrophobic surfaces, thereby affecting the retention times for elution from a reversed-phase column.

Our studies provided no evidence for the existence of a second allosteric nucleotide-binding site that has been shown to regulate the overall activity of many reductases or regulate pyrimidine ribonucleotide reduction as observed in the homologous class III anaerobic reductase from E. coli. However, we cannot rule out the possibility that such a site exists but is labile to the conditions of analysis or that the formation of the site requires complexation of NrdD with the NrdG subunit. If such a site does not exist, one might speculate that a constitutively active enzyme is tolerated by or even beneficial to T4 under anaerobic conditions of viral replication, as was shown earlier for aerobic growth of T4 phase (17).

In summary, we have localized two different nucleotide-binding sites within the class III ribonucleotide reductase from bacteriophage T4 and conclude that one of these sites (His147, Leu160) is an allosteric specificity site, whereas the other (Gly280–Arg291) is likely to be the active site.

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