Two Active Site Asparagines Are Essential for the Reaction Mechanism of the Class III Anaerobic Ribonucleotide Reductase from Bacteriophage T4*

Jessica Andersson‡§, Sabrina Bodevin³, MariAnn Westman, Margareta Sahlin, and Britt-Marie Sjöberg**

From the Department of Molecular Biology and Functional Genomics, Stockholm University, SE-10691 Stockholm, Sweden

Class III ribonucleotide reductase is an anaerobic enzyme that uses a glycolaldehyde radical to catalyze the reduction of ribonucleotides to deoxyribonucleotides and formate as ultimate reductant. The reaction mechanism of class III ribonucleotide reductases requires two cysteines within the active site, Cys-79 and Cys-290 in bacteriophage T4 NrdD numbering. Cys-290 is believed to form a thiolate radical that initiates the reaction with substrate and Cys-79 to take part as a transient thyl radical in later steps of the reductive reaction. The recently solved three-dimensional structure of class III ribonucleotide reductase (RNR) from bacteriophage T4 shows that two highly conserved asparagines, Asn-78 and Asn-311, are positioned close to the essential Cys-79. We have investigated the function of Asn-78 and Asn-311 by site-directed mutagenesis and measured enzyme activity and glycyl radical formation in five single (N78(A/C/D) and N311(A/C)) and one double (N78A/N311A) mutant proteins. Our results suggest that both asparagines are important for the catalytic mechanism of class III RNR and that one asparagine can partially compensate for the lack of the other functional group in the single Asn → Ala mutant proteins. A plausible role for these two asparagines could be in positioning formate in the active site to orient it toward the proposed thyl radical of Cys-79. This would also control the highly reactive carbon dioxide radical anion form of formate within the active site before it is released as carbon dioxide. A detailed reaction scheme including the function of the two asparagines and two formate molecules is proposed for class III RNRs.

The building blocks for DNA synthesis, the four deoxyribonucleotides are obtained from ribonucleotides by reduction of ribonucleotides to deoxyribonucleotides. Ribonucleotide reductase (RNR) is the enzyme that catalyzes this reaction in all organisms. This seemingly simple reaction, the exchange of the 2′-hydroxyl group for a hydrogen atom, requires complex free radical chemistry.

Currently, there are three known classes of RNR with a common structural fold (1–3) but little overall sequence similarity (4–7). The classification of the RNRs is mainly based on the different systems developed by these enzymes to generate a transient thyl radical, the formation of which initiates the reaction with the ribonucleotide substrate. The aerobic class I enzymes generate within one subunit (R2) a stable tyrosyl radical that via a long-range radical transfer pathway is delivered at the active site cysteine located in the other subunit (R1). The class II enzymes, indifferent to oxygen, use adenosylcobalamin as a thyl radical generator. The focus of this study, the class III enzymes use a stable glycol radical within the NrdD protein (the reductase) as a thyl radical generator. The glycol radical is formed by homolytic cleavage of the cofactor S-adenosylmethionine (AdoMet), a reaction catalyzed by the oxidation of an [4Fe-4S]−-center in the activase protein (NrdG) (8). The RNR class III activation mechanism, i.e. the AdoMet cleavage and the formation of a stable glycol radical within the NrdD reductase, is similar to the activation of pyruvate formate-lyase (PFL), another anaerobic radical enzyme (9–12). Recently, other proteins have also been suggested to be members of this new growing group of glycol radical enzymes (13–15).

The reaction mechanism has been studied in detail for the class I and II enzymes, and in both cases it involves three cysteines within the active site (16–18). One of the cysteines is proposed to form the thyl radical that initiates the reaction mechanism by transiently oxidizing the substrate. In later steps, the two other cysteines form a redox-active cysteine pair involved in the actual reduction of substrate.

The recently solved crystal structure of the class III NrdD protein from bacteriophage T4 clearly demonstrated that the class III RNR fold is organized around a ten-stranded α/β barrel structure (3) similar to the one previously observed for the class I RNR fold (2). The T4 NrdD structure also revealed important differences in the compositions of the two active sites, where some essential residues in the Escherichia coli R1 subunit are conserved in the T4 NrdD structure and some are not (compare Fig. 1A). This strongly emphasizes important differences in the catalytic mechanisms used by these two classes of RNR. Most prominently, class I RNRs use a redoxin (thioredoxin, glutaredoxin, or NrdH-redoxin) system as reductant (19–21), whereas class III RNRs use formate (22).

We recently demonstrated that the highly conserved Cys-79 and Cys-290 in the active site of T4 NrdD are essential for the reaction mechanism of the class III RNRs (23). As can be seen in Fig. 1A, the position of Cys-290 in the active site of T4 NrdD matches that of Cys-439 in the R1 active site, which has been
proposed to initiate catalysis in the class I RNR by forming the transient thyl radical (24). In analogy to the class I mechanism, we proposed that Cys-290 is the site of the transient thyl radical that initiates the reaction with substrate, and that Cys-79 takes part in later steps of the reaction mechanism (3, 23). The redox-active cysteines, Cys-225 and Cys-462, in the active site of R1 are located close to each other in the vicinity of the 2’-hydroxyl group of the substrate. In the NrdD active site, Cys-79 is found in a location corresponding to that of Cys-225 in the R1 active site. Interestingly, the asparagine residue Asn-311 in NrdD is found at a position corresponding to the site of Cys-225 in the class I RNR. Asn-311 is highly conserved within the NrdD sequences (Fig. 2). The redox-active cysteines, Cys-225 and Cys-462, in the radical that initiates the reaction with substrate, and that carbon dioxide is released during the reaction (22). Similar reactive species have also been proposed to initiate catalysis in the class I RNR by forming the transient thiyl radical, which also seems to be a good candidate for formate reductase (30) was a kind gift from Vera Bianchi and Elisabet Haggard-Ljungquist. The expression plasmid pDH1 for flavoodoxin (31) was a kind gift from Peter Reichard.

Oligonucleotide-directed Mutagenesis—All single mutants were constructed with the Kunkel method (32, 33) using the Mutagen kit (Stratagene) as reagent in denaturating buffer. The primers and the restriction enzymes used were: for N311A: 5’-d(CACCAAGAACACAGTGGTCA-3’; for N311C: 5’-d(CACCAAGAACACAGTGGTCA-3’; for N311D: 5’-d(CACCAAGAACACAGTGGTCA-3’; Mun1; for N78A: 5’-d(TAACAACGGAAACGGTAAATTG)-3’, CassB; for N78C: 5’-d(TAACAACGGAAACGGTAAATTG)-3’, CassB; for N78D: 5’-d(TAACAACGGAAACGGTAAATTG)-3’, CassB. The mismatching bases are shown in bold and the changed codon is underlined.

The double mutant N78AN311A was constructed using the QuikChangeTM site-directed mutagenesis kit from Stratagene, with the mutated N311A plasmid as a template and two complementary oligonucleotides containing the desired mutation at position 78. All the sequences of the mutated plasmids were confirmed by sequencing using a DYE Terminator cycle sequencing kit from Amersham Pharmacia Biotech. The analyzing gel was done at KISEq at the Center for Genomics Research, Karolinska Institute.

Oxygen-dependent Cleavage—The JM109(DE3) strain, containing a wild type or mutant rT299T4ndG (kanamycin) plasmid and a pET21aT4ndG (carbenicillin) plasmid, was grown aerobically in LB medium supplemented with 35 μg/ml kanamycin and 100 μg/ml carbenicillin. The cultures were grown until A600 reached 0.5 and then were induced with IPTG (isopropyl-1-thio-β-D-galactopyranoside) to a final concentration of 1 mM. Samples of 0.5 absorbance units were taken out before and 3 h after induction. The samples were analyzed by SDS-PAGE on a 10.5 or 12.7% gel and stained with Coomassie Brilliant Blue.

Aerobic Expression and Purification of NrdD—Wild type and mutant proteins were expressed aerobically as described in (26). Biochemical data corroborate that formate is the hydrogen donor for the class III RNRs from E. coli and that carbon dioxide is released during the reaction (22). Similar reactive species have also been proposed as reaction intermediates in the catalytic mechanism of PFL (27, 28).

To be an efficient reductant in RNR catalysis, the formate molecule has to be strictly oriented within the active site. Asn-311 is highly conserved within the NrdD sequences (Fig. 1B), and a plausible role for the Asn-311 in the active site of T4 NrdD could be in formate positioning by hydrogen bonding to one of the oxygens of formate. Interestingly, another highly conserved asparagine in the active site of T4 NrdD is Asn-78 (Fig. 1B), which also seems to be a good candidate for formate positioning. We have mutated the highly conserved residues Asn-78 and Asn-311 to several other side chains and constructed the double mutant N78A/N311A. Our results suggest that both asparagines are important for efficient catalysis by class III RNRs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotides used for site-directed mutagenesis were synthesized by TAG Copenhagen A/S. Restriction enzymes were from Amersham Pharmacia Biotech, New England Biolabs, Roche Molecular Biochemicals, or Stratagene. Chemicals were from Sigma, Saveen, Pharmacia Biotech and Amersham.

**Bacterial Strains and Plasmids**—E. coli CJ236 (Δ(lac-proAB), thi-1, relA1/pCJ105), E. coli MV1190 (Δ(lac-proAB), thi, supE, ΔsrI-recaA306::Tn10/F’traD36, proAB, lacYΔZΔM15), E. coli JM109(DE3) endA1, recA1, gyrA96, thi, hsdR17, (rK-, mK+), relA1, supE44, Δ(lac-proAB), [F’, truD36, proAB, lacYΔZΔM15], λ(DE3) were the bacterial strains. Plasmids pET29T4ndD and pET21aT4ndG were described in Ref. 29. Plasmid pEE1010 containing the gene for flavoadoxin (flavoodoxin) NADPH reductase (30) was a kind gift from Vera Bianchi and Elisabet Haggard-Ljungquist. The expression plasmid pDH1 for flavoodoxin (31) was a kind gift from Peter Reichard.

**Reaction Mechanism of Class III Ribonucleotide Reductase**

**SCHEME 1. Proposed catalytic mechanism for class III RNR from Eklund and Fontecave (25).** Numbering refers to phage T4 NrdD.
radical during the activation step, a parallel protein sample frozen before addition of azido-CTP was used as a control.

**EPR Measurements**—X-band EPR measurements were performed at 77 K on an Elexys E500 EPR (Bruker) spectrometer using a cold finger Dewar for liquid nitrogen. Double integrals of the EPR signals were determined using the XpE Bruker software, and radical content was calculated from the spin concentration comparing with a T4 NrdD sample of known glycyl radical content and a Cu^2+-EDTA standard.

**Enzymatic Activity Assays after Anaerobic Activation Procedures**—Purified wild type or mutant NrdD, crude extract of NrdG, the activation mixture, and the substrate mixture were flushed separately with argon for 40 min to remove oxygen before transferring them to the anaerobic box. NrdD, NrdG, and the activation mixture were then mixed and incubated for 10 min. The incubation mixture contained 0.12 μM pure NrdD, NrdG-containing crude extract (10 mg/ml, ~20 μM NrdG), 30 mM Tris-HCl, pH 8.0, 30 mM KCl, 5 mM DTT, 1.25 mM NADPH, 2 μM flavodoxin reductase, 0.5 mM flavodoxin, and 0.5 mM AdoMet in a total volume of 25 μl. Then, 25 μl of substrate mixture was added giving final concentrations of 30 mM Tris-HCl, pH 8.0, 30 mM KCl, 5 mM Na-formate, 5 mM DTT, 20 mM MgCl₂, 1 mM dATP, and 5 μM [³H]CTP. Incubation with substrate was stopped after 20 min by addition of 500 μl of 1% perchloric acid and analyzed as described earlier (23).

**RESULTS**

**Production of Mutant Proteins**—Asn-78 was mutated to an alanine (N78A), a cysteine (N78C), and an aspartic acid (N78D) residue, and Asn-311 was mutated to an alanine (N311A) and a cysteine (N311C) residue. In addition, the double mutant N78A/N311A was also made. The alanine mutants N78A and N311A introduced in each case a residue that lacks the hydrogen bonding possibility. The cysteine mutants were introduced to mimic the active site of the R1 subunit of class Ia RNR from *E. coli* where a redox-active cysteine pair is essential for catalysis (1, 3). With the N78D mutant, we aimed to introduce an acidic group that might destabilize interactions with the acidic formate. All six mutant proteins behaved like wild type NrdD throughout expression and purification.

**All Mutant Proteins Can Form a Glycyl Radical**—The glycyl radical within the NrdD protein of the class III RNR is destroyed upon exposure to oxygen, and this leads to truncation of the polypeptide backbone (29). The glycyl radical formation and the subsequent oxygen-dependent truncation occurs when the wild type proteins NrdD and NrdG are co-expressed aerobically and can be monitored on a SDS-PAGE gel. This cleavage assay is a quick and convenient method to check if a NrdD mutant is able to form a glycyl radical under *in vivo* like conditions. We co-expressed each NrdD mutant protein (N78A, N78C, N78D, N311A, N311C, and N78A/N311A) together with wild type NrdG. Samples were taken before and 3 h after induction. Two other NrdD proteins were also expressed in the presence of NrdG: the wild type NrdD protein as a positive control and as a negative control the NrdD mutant G580A, unable to undergo the truncation since the radical-harboring glycine has been mutated to an alanine (29). When analyzed on SDS-PAGE gels, all asparagine mutants exhibit the same truncation pattern as the one observed for the wild type (Fig. 2, A and B), i.e. all six NrdD mutant proteins can form a transient glycyl radical during aerobic co-expression with NrdG.

**Anaerobic Stability of the Glycyl Radical in the Mutant Proteins**—To study the EPR characteristics of the glycyl radical, each purified NrdD mutant protein was combined during anaerobic conditions with NrdG-containing crude extract and the activation components, as described under “Experimental Procedures” and incubated for 45 min at room temperature. For comparisons, the wild type NrdD protein was incubated identically, and a sample without NrdD but with NrdG extract and the activation mixture served as background control. As can be seen in Fig. 3, A and B, all NrdD mutants, except N311A and the double mutant N78A/N311A, displayed EPR signals with the characteristics of the wild type NrdD glycyl radical signal. When observable, the radical content in the mutants was comparable with that of wild type NrdD (0.2–0.4 glycyl radical per dimeric NrdD). For the mutants N311A and N78A/N311A, the residual EPR signal is reminiscent of the singlet EPR signal seen in the background samples and which originates from the flavodoxin system in the activation mixture. The lack of a glycyl radical signal in N311A and N78A/N311A was unexpected since both mutated proteins could form a glycyl radical in the oxygen-dependent cleavage assay (Fig. 2). One explanation could be that the glycyl radical is unstable in these mutants. An unstable glycyl radical would be easier to trap in the *in vivo* like conditions of the oxygen-dependent cleavage assay compared with the *in vitro* conditions used for the EPR measurements.

**Enzymatic Activity of Mutant Proteins**—The enzymatic activity of the mutated proteins was measured using [³H]CTP as substrate and dATP as allosteric effector (23, 34). As above, we used wild type NrdD as a positive control and a mixture with all components except NrdD as a negative control.

All three single mutations introduced at position 78 retained some enzyme activity (Table I). Most significant is the 7.6% activity of the N78A mutant compared with wild type NrdD, whereas the N78C and N78D mutants only retained about 1% of the wild type activity, a level that is two times the detection limit of the assay (compare nmol of dCTP formed in Table I). For the single mutations at position 311 the results are more disperse. The N311A protein retains close to 18% activity compared with wild type, whereas the N311C mutant is inactive (Table I). The high enzyme activity of the N311A protein is especially interesting considering the lack of observable glycyl radical signal in the *in vitro* EPR experiment and strongly underscores that a glycyl radical forms at least transiently in this mutant, as was also evident from the *in vivo* like oxygen truncation assay. One explanation for the lack of activity in the N311C mutant may be that it perturbs the redox activity of the closely positioned Cys-79 during catalysis (compare Fig. 1A and Scheme 2).

The double mutant with alanines engineered at both positions (N78A/N311A) lacked enzymatic activity (Table I). Theoretically we would expect an enzyme activity of 1.4% (i.e. 7.6% multiplied by 17.9%) for a combination of the N78A and N311A mutations.

**EPR Experiments in Presence of Azido-CTP**—The substrate analogue azido-CDP is a mechanism-based inhibitor of class I RNR, where upon binding, it irreversibly scavenges the tyrosyl

| NrdD protein | "H-dCTP formed | Specific activity | Relative specific activity |
|--------------|----------------|------------------|--------------------------|
| Wild type    | 9.37 ± 1.75    | 1155             |                          |
| N78A         | 0.83 ± 0.11    | 87.6             | 0.076                    |
| N78C         | 0.22 ± 0.14    | 11.8             | 0.010                    |
| N78D         | 0.21 ± 0.19    | 10.5             | 0.009                    |
| N311A        | 1.79 ± 0.75    | 207              | 0.179                    |
| N311C        | 0.17 ± 0.04    | 5.6              | 0.005                    |
| N78A/N311A   | 0.16 ± 0.06    | 3.6              | 0.003                    |
| Background   | 0.13 ± 0.11    | —                | —                        |

* Values are the mean of double samples from 2–4 experiments with 1–3 different batches of purified protein; errors are sample standard deviation.

* The specific activity of wild type NrdD was set to 1.00, and the specific activities of the NrdD mutants were related to this value.

TABLE I

Enzymatic activity of wild type and mutant NrdD proteins

---

Downloaded from http://www.jbc.org/ by guest on July 24, 2018
radical and a new nitrogen-centered radical is formed (35, 36). The corresponding substrate analogue azido-CTP was used with the class III RNR from E. coli where it was shown to destroy the glycyl radical but no new radical species was detected (37). We have recently shown that glycyl radical loss also occurs for phage T4 NrdD in the presence of azido-CTP (38). As the reaction proceeded very fast (complete radical loss in 77 s at room temperature) with the wild type protein (38), we decided to perform the experiments with the mutant proteins on ice to enable detection of intermediate radical species if any. Incubation of the wild type NrdD protein in presence of azido-CTP caused rapid and complete loss of glycyl radical even at low temperature, and we did not observe any intermediary radical species during this process (Fig. 4A). The spectroscopic behavior of the N78A mutant was the same as the one observed for the wild type NrdD (Fig. 4B), indicating that binding of substrate nucleotides is not perturbed by the mutation. For the N311A NrdD protein incubated in presence of azido-CTP, we did not detect any EPR signal (data not shown).

**DISCUSSION**

In this article we present biochemical evidence that Asn-78 and Asn-311 have important roles in the reaction mechanism of the class III RNR from phage T4. These two asparagines are highly conserved among the known NrdD sequences (Fig. 1B).

Both asparagines have also pertinent positions in the active site of T4 NrdD (3). Asn-311 is in a position that corresponds to one of the redox-active cysteines in the active site of the R1 protein of class Ia RNR (1, 3), and the amide O/N is about 4–5 Å from the S/H of Cys-79 (3, 39). Asn-78 is next to Cys-79 that is proposed to take part in the reaction mechanism by protonating the leaving 2’/H and/or reducing the resulting substrate intermediate (23, 25). The amide O/N of Asn-78 is about 4 Å from the 2’/H of the modeled substrate (3, 39).

The enzymatic activity assay showed that both Asn-78 and Asn-311 were clearly important for the activity of T4 NrdD (Table I). While no significant activity could be seen for N311C and the double mutant N78A/N311A and only low activity was observed for N78C and N78D, the two single alanine mutants, N78A and N311A, retained respectively 7.6 and 17.9% of the wild type activity. This suggested that both Asn-78 and Asn-311 separately were important for full enzymatic activity of phage T4 NrdD even though they were not essential residues per se. If the complete loss of catalytic activity observed with the double mutant N78A/N311A and only low activity was observed for N78C and N78D, the two single alanine mutants, N78A and N311A, retained respectively 7.6 and 17.9% of the wild type activity. This suggested that both Asn-78 and Asn-311 separately were important for full enzymatic activity of phage T4 NrdD even though they were not essential residues per se. If the complete loss of catalytic activity observed with the double mutant N78A/N311A is really caused by a more than additive effect of two mutated residues at the active site, it suggests that the two asparagines are needed in combination for the catalytic mechanism of phage T4 NrdD.
indicate that they are both able to partially substitute for each other.

Based on the effects of site-directed mutagenesis at the positions 78 and 311 in the active site of T4 NrdD we can formulate three hypotheses on the putative role of Asn-78 and Asn-311:

**Structural Role**—They can be key residues involved in folding the polypeptide backbone around the active site pocket. A structural role for an asparagine residue has been proposed for the keratinocyte growth factor receptor where the asparagine is involved in the local fold of a loop to allow a proper ligand binding (40).

**Substrate Selectivity**—One or both asparagines can be involved in the selectivity of the enzyme toward a substrate, as has been observed for thymidylate synthase (41) and cis-biphenyl-2,3-dihydrodiol-2,3-dehydrogenase (42).

**Formate Positioning**—The two asparagines can interact with the co-substrate and position formate in the right orientation for it to react with the thiol radical formed at Cys-79. Positioning of formate by amino acid side chains is seen in the active site of formate dehydrogenase where an asparagine together with an aspartic acid orients a formate molecule to allow hydride transfer (43).

The first hypothesis, a major conformational rearrangement is not likely since all the mutant proteins are able to interact with the NrdG protein and form a glycyl radical (Fig. 2). A local structural rearrangement, however, might affect the radical, since the glycyl radical is quite close to the active site (H11011 Å) (3). The failure to detect a glycyl radical in the EPR measurements of the single mutant N311A and the double mutant N78A/N311A is most likely due to an unstable glycyl radical (Fig. 3). Since both these mutants could form a glycyl radical in the oxygen-dependent cleavage assay of the NrdD protein. Samples were taken out and analyzed in panel A on a 10.5% SDS-PAGE gel and in panel B on a 12.7% SDS-PAGE gel. Lanes 1 and 9 in A and lanes 1 and 6 in B show a low molecular weight marker (Pharmacia). A, lane 2 shows a typical background sample taken before IPTG induction. In lanes 3-8, samples of wild type or mutant NrdD after 3 h of induction are shown. The G580A mutant is the negative control. B, samples of the NrdD mutants N311A and N311C are shown before and after 3 h of IPTG induction. NrdD and the truncated form, called NrdD', are indicated by arrows.

The second hypothesis, concerning the selectivity of the enzyme toward substrate is also not likely since there is an allosteric control of substrate specificity in all RNRs (34, 39, 44–46). This means that an allosteric effector, binding to a site different from the active site, decides which of the four ribonucleotide substrates will be reduced in the active site. Also, the three-dimensional structure is an indirect support of this since the substrate GDP of the class Ia RNR from E. coli has been successfully modeled into the active site of T4 NrdD and no interactions between GDP and the asparagines were suggested (1, 3). But most importantly, our result using the substrate analogue azido-CTP provides direct evidence that speaks against this second hypothesis. The same loss of glycyl radical in the NrdD mutant N78A and the wild type NrdD (Fig. 4, A and B) shows that the substrate analogue azido-CTP was able to bind to the active site of the NrdD mutant N78A to destroy...
the glycyl radical. Plausibly this binding ability also reflects the real substrate situation.

Based on our results we favor the third hypothesis, that the asparagines are important for positioning an incoming formate molecule. The enzyme activity detected with both N78A and N311A mutants but not with the double mutant is in good agreement with the existence of a compensating effect when one of these asparagines is mutated to an alanine. The results obtained with N78D, N78C, and N311C are compatible with a distorted compensation when a charged or larger residue than alanine is introduced at any of these positions.

From the three-dimensional structure of phage T4 NrdD, it is clear that the redox chemistry performed by the class I active site cysteine pair, Cys-225 and Cys-462 is not possible for the class III RNRs. Indeed, Asn-311 is found to correspond to one of the cysteines in the active site of T4 NrdD (3). In the class I reaction mechanism, the two redox-active cysteines are oxidized to a disulfide bridge during catalysis and at the end of one turnover they need to be reduced. The physiological hydrogen donor systems for class I are the thioredoxin and glutaredoxin systems (19, 20). No such complicated hydrogen donor system is required for class III RNRs; instead a simple organic molecule, formate, acts as a reducing agent with the formation of carbon dioxide as the end product (22). In the proposed class III RNR reaction mechanism (compare Scheme 1), formate is suggested to interact with a thyl radical formed on Cys-79, which leads to the formation of a carbon dioxide radical anion (25). Since the carbon dioxide radical anion is a highly reactive species, it is very likely that the incoming formate molecule needs to be held in place and properly orientated toward Cys-79. We suggest that this positioning of a formate molecule is maintained by Asn-78 and Asn-311, which hydrogen bond via their amino-hydrogens to the oxygens of the formate (Scheme 2). In fact, this fits very well with our and others’ biochemical data as well as with the proposed reaction mechanism (22, 25).

In addition, we propose a role for a second formate molecule, that acts as a general base in the first steps of the reaction mechanism (Scheme 2), i.e. mimicking the proposed role for Glu-441 in the active site of the R1 protein of the class Ia RNR from E. coli (47, 48). This adds to the similarities of the first steps of the catalytic mechanisms between the different classes of RNR, as do the results with the azido-substituted substrates.

As can be seen in Scheme 2, the second step of the catalytic mechanism, the rearrangement of the substrate to a ketonucleotide and the elimination of water is facilitated by a formate molecule making these first steps even more similar to the class Ia catalytic mechanism. After these steps, the class III mechanism deviates from that of the other RNR classes. The resulting ketosubstrate is reduced at the 2′-position and a transient thyl radical is formed by Cys-79. A formate molecule is then positioned toward Cys-79 by Asn-78 and Asn-311; formate reduces the thyl radical of Cys-79 and forms a carbon dioxide radical anion, which performs the second one-electron reduction leading to carbon dioxide formation. Finally, the hydrogen atom of Cys-290 is reintroduced and the deoxyribo nucleotide product is released.

Recently, the crystallographic structure of PFL was solved (49). The Cα positions of 352 residues of PFL and T4 NrdD can be superimposed with a root mean square deviation of 2.1 Å. The substrate analogue oxamate was co-crystallized with PFL, and found in the active site where its carboxylic group is held in place by an arginine residue. Interestingly, it is the carboxylic group of oxamate (or the real substrate pyruvate) that after homolytic cleavage gives rise to a carbon dioxide radical anion in the active site of PFL. Again, similarities can be seen between PFL and the class III RNRs. In this paper we suggest that two asparagines in class III RNR have the same role as the arginine in PFL, namely to properly orient the formate/pyruvate that in the following step forms a carbon dioxide radical anion.

In summary, we have suggested that two functionally cou-
abled residues, Asn-78 and Asn-311, are important for the reaction mechanism of the class III RNR from phage T4. We propose that they are equally required to position formate toward the essential Cys-79 in the active site of T4 NrdD. The proper orientation of formate is crucial since it forms a highly reactive radical species that reduces the substrate. The role of the asparagines would be dual in orienting formate as well as controlling the highly reactive radical species within the phage T4 NrdD active site. To shed more light on this new intriguing example of two functionally coupled residues we have started to solve the structure of both single Asn → Ala mutants and of the double mutant. The refined preliminary structure of N78A shows no striking differences to the structure of the wild type enzyme.  

Acknowledgment—We thank D. T. Logan for generous help with Fig. 1A.

REFERENCES

1. Uhlin, U., and Eklund, H. (1994) Nature 370, 533–539
2. Uhlin, U., and Eklund, H. (1996) J. Mol. Biol. 262, 358–369
3. Logan, D. T., Andersson, J., Sjöberg, B.-M., and Nordlund, P. (1999) Science 283, 1499–1504
4. Sahlin, M., and Sjöberg, B.-M. (2000) Sabedd. Biochem. 35, 405–443
5. Sjöberg, B.-M. (1997) Struct. Bonding 88, 139–173
6. Jordan, A., and Reichard, P. (1998) Annu. Rev. Biochem. 67, 71–98
7. Stubbe, J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2723–2724
8. Tamarit, J., Mulliez, E., Meier, C., Trautwein, A., and Fontecave, M. (1999) J. Biol. Chem. 274, 31291–31296
9. Knappé, J., and Sauders, G. (1998) FEMS Microbiol. Rev. 6, 383–398
10. Wagner, A. F., Frey, M., Neugebauer, F. A., Schafer, W., and Knappé, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 986–1000
11. Frey, M., Rothe, M., Wagner, A. F., and Knappé, J. (1994) J. Biol. Chem. 269, 12432–12437
12. Broderick, J. R., Henshaw, T. F., Cheek, J., Wojtuszewski, K., Smith, S. R., Trojan, M. R., McGhan, R. M., Kopf, A., Kibbe, M., and Broderick, W. E. (2000) Biochem. Biophys. Res. Commun. 269, 451–456
13. Hesslinger, C., Fairhurst, S. A., and Sauders, G. (1998) Mol. Microbiol. 27, 477–492
14. Leuthner, B., Leutwein, C., Schulz, H., Horth, P., Haehnel, W., Schütz, E., Schagger, H., and Heider, J. (1998) Mol. Microbiol. 28, 615–628
15. Selmer, T., and Andrei, P. I. (2001) Eur. J. Biochem. 268, 1365–1372
16. Åberg, A., Hahné, S., Karlsson, M., Larsson, Å., Ormo, M., Åhgren, A., and Sjöberg, B.-M. (1980) J. Biol. Chem. 265, 12249–12252
17. Mao, S.-S., Holmgren, A., and O’fer, M., Boller, J. M., Booker, S., Johnston, M. I., and Stubbe, J. (1999) Biochemistry 38, 12676–12685
18. Booker, S., Licht, S., Brudierick, J., and Stubbe, J. (1994) Biochemistry 33, 12676–12685
19. Holmgren, A. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2275–2279

2 D. T. Logan, personal communication.
