It has been recently recognized that the class III aerobic ribonucleotide reductase requires the presence of a second activating gene product, NrdG. We have proposed that the role for NrdG involves the generation of an oxygen sensitive glycyl free radical within the NrdD enzyme. In this article we present the generation of such a glycyl free radical within the T4 NrdD subunit and its dependence upon the phage NrdG subunit. Initially, an overexpression system was created that allowed the joint production of T4 NrdD and T4 NrdG. With this system and in the presence of T4 NrdG, an oxygen-sensitive cleavage of NrdD was observed that mimicked the cleavage observed in phage infected Escherichia coli extracts. Under anaerobic conditions the presence of T4 NrdD with NrdG revealed a strong doublet EPR signal (g = 2.0039). Isotope labeling of the NrdD with [2H]glycine and [13C]glycine, respectively, confirmed the presence of a stabilized glycine radical. The unpaired electron is strongly coupled to C-2 in glycine and the doublet splitting originates from one of the α-protons. The glycine residue at position 580 was determined to be the radical containing residue through site-directed mutagenesis studies involving a G580A NrdD mutant. The glycyl radical generation was specific for the T4 NrdG, and the host E. coli NrdG was found to be unable to activate the phage reductase. Finally, anaerobic purification revealed the holoenzyme complex to contain iron, whereas the NrdD polypeptide was found to lack the metal. Our results suggest a tetrameric structure for the T4 anaerobic ribonucleotide reductase containing one homodimer each of NrdD and NrdG, with a single glycyl radical present.

The ability to generate deoxynucleotides for the synthesis of DNA requires the action of ribonucleotide reductase. Although all organisms have in common the requirement to utilize deoxynucleotides generated by the reduction of ribonucleotides, a number of different classes of ribonucleotide reductases have been identified that can carry out this transformation. Currently three distinct classes of ribonucleotide reductases have been recognized. All three classes of ribonucleotide reductase are proposed to share the common feature of using free radical chemistry in the reduction of the ribose sugar but use very distinct components to generate and harness the unpaired electron. The Escherichia coli has encoded within its chromosome the genes for two distinct class I reductases and a single class III enzyme (2, 3). The bacteriophage T4 genome contains genes for a single class I enzyme and a recently identified class III anaerobic ribonucleotide reductase (4, 5).

Both the class I and class III reductase activities are in reciprocal ways affected by the level of oxygen present within the cell. Among the class I enzymes molecular oxygen is essential for generation of a μ-oxo bridged iron center and the stable tyrosyl radical (6). For the class III enzymes, molecular oxygen is a powerful inhibitor and has been proposed to react with a glycyl radical and thereby cleave the polypeptide backbone of the reductase (7). The anaerobic ribonucleotide reductase from E. coli has recently been determined to harbor this oxygen-sensitive radical on Gly-681 (8). This glycine residue aligns with Gly-580 within the bacteriophage T4 anaerobic ribonucleotide reductase and is the basis for selecting Gly-580 for site-directed mutagenesis studies.

The stabilization of a glycyl radical within a protein structure was originally identified within the pyruvate formate-lyase (Pfl) enzyme system (9). The Pfl enzyme is a homodimeric enzyme that also requires anaerobic conditions and converts pyruvate into formate and acetyl-CoA. The generation of the Pfl radical is dependent upon a small protein termed the Pfl activase or activating enzyme (10–12). The Pfl activase has been determined to be an iron-containing protein with a proposed iron-cysteine center. The Pfl activase has been shown to bind and cleave S-adenosylmethionine (13). This cleavage at the sulfur-carbon bond results in the formation of a deoxyadenosyl radical that is proposed to abstract a hydrogen atom from Gly-734 within Pfl, thereby generating a stable glycyl radical within the catalytic subunit of Pfl (14).

Comparison between the class III anaerobic ribonucleotide reductase and Pfl reveals a conserved penta-residue core structure positioned at the two radical containing glycol sites (15). The finding of similarity between the anaerobic ribonucleotide reductase, a proposed model for the original progenote reductase, and pyruvate formate-lyase was unexpected. The limited four out of five amino acid conservation did not rule out the possibility that the observed similarity between the two enzyme systems simply reflected convergent evolution and not resildium from a common ancestral enzyme. Nevertheless, homology of the two systems was bolstered with the findings that the anaerobic ribonucleotide reductase also requires and cleaves S-adenosylmethionine and that a second gene product required for the anaerobic reductase, NrdG, contained clear similarity to the Pfl activase-subunit (16–19). We have recently proposed that the requirement for this newly identified polypeptide involves generation of a stable glycyl radical on Gly-580 of bacteriophage T4 NrdD (17). To confirm this pro...
posed role for NrdG, we have pursued the identification and requirements for the glycol radical within the bacteriophage T4 anaerobic ribonucleotide reductase and demonstrated that a glycine residue at position 580 is an absolute requirement for radical retention and enzyme activity.

**Materials and Methods**

**Generation of a NrdG Overexpression Plasmid System—Plasmids** pET21a (ampicillin) and pET29 (kanamycin) were purchased from Novagen and used for construction of overproducing recombinants. A pET21aT4NrdG plasmid was generated using "polymerase chain overlap extension" (20). A 600-base pair PCR product of the pET29 promoter region was joined to a nrdG PCR fragment that had been previously amplified from T4D wild type phage. The joined PCR fragment was then digested and ligated into the pET29 plasmid using XbaI and SalI restriction enzymes.

**Generation of the G580A Mutation** Single stranded DNA was generated directly in vitro from the pET21aT4NrdD construct by infection with R408 helper phage from Bio-Rad (21, 22). Single stranded DNA was generated directly in vitro from the pET21aT4NrdD plasmid (17) and was generated by transfer of the overexpression plasmid construct was then sequenced using an automated laser fluorescence sequencer. Generation of the E. coli NrdD clone pN9 (ampicillin) was described previously (18). Bacterial strain J M109(DE3) was used for overexpression of the enzymes. The plasmids were co-transformed into the host strain JM109(DE3) and expressed using the expression plasmid pET29 (21) but lack the hyperfine splitting typical of the glycyl radical signal formed in aerobic reductase extracts (9). The overexpression plasmid was co-transformed into the host strain JM109(DE3) and expressed using the expression plasmid pET29 (21) but lack the hyperfine splitting typical of the glycyl radical signal formed in aerobic reductase extracts (9).

**Anaerobic Growth Conditions—Conditions were used as previously reported (17).** For anaerobic expression a l-lactate culture of LB medium supplemented with 2 g/liter glucose was grown at 37°C in a Forma Scientific anaerobic box, and oxygen levels were monitored with a CO2 Laboratories oxygen sensor. We had previously observed that phosphate buffering of the growth medium resulted in early lysis of the isopropyl-1-thio-D-galactopcopyranoside-induced bacteria. The LB medium was therefore unbuffered, and a neutral pH was maintained by the addition of 1-mM aliquots of 1 N NaOH. Overexpression was carried out in the presence of an overproduced host NrdG extract. The activation of the T4NrdD signal closely matches the previously described T4NrdD-NrdG holoenzyme (25) but lacks the hyperfine splitting typical of the glycyl radical signal formed in aerobic reductase extracts (9). The overexpression plasmid was co-transformed into the host strain JM109(DE3) and expressed using the expression plasmid pET29 (21) but lack the hyperfine splitting typical of the glycyl radical signal formed in aerobic reductase extracts (9).

**Assaying for Anaerobic Ribonucleotide Reductase Activity—** For all assays, conditions were used as described previously (17). All assays contained 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM dithiothreitol, 10 mM MgCl₂, 20 mM phosphate, 50 μg/ml pyruvate kinase, 10 mM MgCl₂, 5 mM dithiothreitol, 4 mM ATP, 4 mM formate, 3 mM hydroxyurea, 1 mM NADPH, 0.2 mM S-adenosylmethionine, and 1.2 mM (5'-32P)JCTP (16 Ci/mmol). Assay mixtures were made anaerobic by placement on an argon manifold for approximately 1 h before the addition of reductase. Reactions were carried out at room temperature under normal argon gas that had been passed through an OxiClear gas purifier cartridge purchased from Sigma.

**Aerobic Purification of T4 NrdD—Aerobically Overexpressed** T4 NrdD was initially precipitated by the addition of ammonium sulfate (final concentration, 0.25 g/ml). The pellet was resuspended in 20 mM Tris-HCl, pH 8.5, 20 mM KCl, 1 mM dithiothreitol, 10 mM phenylmeth- ylsulfonyl fluoride and passed over a Superose 12 fast protein liquid chromatography column from Pharmacia Biotech Inc. The T4 NrdD was further purified over a MonoS 1000 DEAE cartridge eluted with a linear gradient of 0–1 M NaCl in 20 mM Tris-HCl, pH 8.5, 20 mM KCl, 1 mM dithiothreitol, 10 mM phenylmethanesulfonyl fluoride. The purified NrdD was concentrated using Centricon-50 from Amicon. The purified enzyme was used to generate a polyclonal antibody against the T4 NrdD subunit.

**Purification of NrdD Associated with NrdG—J M109(DE3) bacteria carrying the pET29T4NrdD, the pET29T4NrdD (GS580A), or the pET21aT4NrdG plasmids were induced and lysed anaerobically as described previously (2, 17). An equivalent amount of protein from crude NrdD extracts was combined with NrdG extracts and incubated at room temperature for 1 h. The overexpressed holoenzyme was precipitated with ammonium sulfate (final concentration, 0.25 g/ml) and dissolved in a 1 M sodium phosphate, pH 7.0, 50 mM ammonium sulfate. The protein was then passed over a Pharmacia HiTrap butyl-Sepharose 4 Fast Flow hydrophobic column. The column was washed with 7 ml of anaerobic 50 mM sodium phosphate, pH 7.0, 100 mM ammonium sulfate. The purified holoenzyme was then eluted with 3 ml of 50 mM sodium phosphate buffer. For the purification of the T4NrdD(GS580A) mutant protein complexed with NrdG, overexpression and purifications were carried out under aerobic or anaerobic conditions. The purified protein fractions were analyzed by SDS-polyacrylamide gel electrophoresis, stained with Coomassie, and scanned by densitometry using a Molecular Dynamics gel scanner. A linear response for the band intensities were determined by loading various amounts of the samples. As a further control, each polyclonal antibody gel was repeatedly scanned at different stages of development. The Coomassie signal strengths from the NrdG and NrdD bands were normalized by dividing by their respective mucous masses (67,862 and 18,248 Da).

**EPR Measurement—** X-band EPR spectra were recorded at 77K on a Bruker E200D or ES300 spectrorometer using a cold finger Dewar for liquid nitrogen. EPR spectra were evaluated using the Bruker software. Spin concentrations were determined from double integrations of the EPR spectra and comparing the integral values with that of a standard Cu²⁺-EDTA sample (1–10 mM). Determination of the g value was performed at 20 K on the ES300 spectrophotometer equipped with an Oxford Instrument cryostat. The amount of radical present within the T4 NrdD dimer was determined on overexpressed anaerobic NrdG extracts in v/v in the presence of excess aerobic T4 NrdG and S-adenosyl methionine. Activation was stopped by freezing aliquots of the mixture in EPR tubes. The amount of NrdG present within the crude anaerobic extracts was determined by competitive enzyme-linked immunosorbent assay carried out with the T4 NrdD polyclonal rabbit antibody. Typically the concentration of purified T4 NrdG was determined by Bradford protein analysis using a Coomassie protein assay kit from Pierce and bovine serum albumin as a protein standard. The NrdD protein concentration determined by Bradford analysis agreed with the concentration obtained by measuring absorption at 280 nm using a calculated molar extinction coefficient of 64,360 for the T4 NrdD amino acid sequence (23).

**Glycine Specific Isotopic Labeling of T4 NrdD—A pET29T4NrdD overexpression J M109(DE3) was grown anaerobically in LB medium (glycine content ~ 3–4 mM) supplemented with 2 g/liter glucose and 30 μg/ml kanamycin. 30 min before induction of the bacteria, [2-13C]glycine (98% isotopic enrichment, from Cambridge Isotope Laboratories) or [2-13C]glycine (99% isotopic enrichment, from Sigma) was added to a final concentration of 50 mM. The labeled extracts were then activated by A T4NrdG extract as described above, and their EPR spectra were recorded.

**Iron Analysis—** To determine the amount of protein bound iron present within the isolated T4 NrdD and the T4NrdD-NrdG holoenzyme complex, an aliquot of purified protein was acid denatured with trichloroacetic acid, and the iron content was determined using the bathophenanthroline assay (24).

**Results**

**Free Radical Generation in and Activation of NrdD Requires NrdG—A series of EPR measurements were carried out to confirm the role of NrdG as the initial generator of the stable free radical on the NrdD subunit. As shown in Fig. 1 (spectrum a), a strong doublet EPR signal, centered at g = 2.0039, was observed in an anaerobic T4 NrdD extract incubated with a T4 NrdG extract. In contrast, Fig. 1 (spectra b and c) demonstrates very low or insignificant EPR signals from the individual NrdD and NrdG anaerobic extracts. The activation appears species-specific because there is no EPR signal formed when the phage NrdD is incubated with an overproduced host NrdG extract. The T4 NrdD signal closely matches the previously identified stable glycyl radical signal of E. coli NrdD (8, 25) but lacks the hyperfine splitting typical of the glycyl radical EPR signal of pyruvate formate-lyase (9).

**To determine the number of radicals present within the phage-encoded class III ribonucleotide reductase, we followed the time-dependent generation of the radical signal within an anaerobic extract of T4 NrdD incubated with T4 NrdG. The**
amount of T4 NrdD present within the crude extract was measured by enzyme-linked immunosorbent assay assays using a polyclonal T4 NrdD antibody. Fig. 2 demonstrates that there is a slow radical generation in the T4 NrdD enzyme over time. A maximum radical content of 0.61 free radicals per NrdD dimer was reached after -90 min and persisted for at least 3 h. The radical content closely agrees with a situation where a single radical is present within the NrdD dimer. Activation of T4 NrdD follows the radical generation, and the specific activity was 180 nmol/min/mg total protein after 25 min of NrdG-dep

The Free Radical Resides on a Glycine Residue—The hyperfine doublet EPR signal of T4 NrdD is split by 1.44 mT by a coupling to a proton. To confirm that the observed radical originates from a glycine residue within the T4 NrdD protein, isotope labeling experiments were carried out using perdeuterated and [2-13C]glycine. The experiments were performed by overproducing T4 NrdD anaerobically in the presence of the labeled isotope. Because the overproducing construct requires rich medium for anaerobic growth, the labeling experiment will result in a mixture of labeled and unlabeled species (ratio of unlabeled to labeled glycine, approximately 1:10). The unmanipulated spectra from growth in protonated, [13C]glycine and [2-13C]glycine are shown in Fig. 3. Even though the two latter spectra (b and d) display a considerable portion of nonlabeled signal, the changes clearly show that the radical originates from a glycine residue. The spectra resulting from subtraction of the unlabeled fraction are also shown in Fig. 3 (spectra c and e). The [13C]glycine signal is broadened from coupling to the 13C (I = 1/2) nucleus (Fig. 3, spectrum c). The outermost lines give an estimate of 6.6 mT for the hyperfine coupling Aπ and the Aα and Aα couplings are also discernible (Table I). Similar spectra have been observed in [13C]glycine labeled E. coli NrdD and Pfl (8, 15). The broad singlet resulting from growth in [2H]glycine (Fig. 3, spectrum d) narrows in the pure [2H]labeled spectrum to a peak-to-peak width of 0.9 mT (Fig. 3, spectrum e, and Table I), comparable with the splitting observed for the deuteron-labeled glycol radical in Pfl (15). The T4 NrdD mixed 2H/1H EPR signal contained at most 65% deuterated species, implying some isotope exchange. However, because the ratio of deuterated versus protonated glycol radical signal did not decrease during 3 h of incubation in H2O buffer at 25°C, it is conceivable that the deuterium-labeled glycine exchanged with solvent protons before incorporation into protein. The retention of the deuterium labeling of the T4 and E. coli NrdD glycol radicals thus differentiate them from the Pfl glycol radical, which exchanges very rapidly with solvent (15, 25).

Glycine 580 Contains the Free Radical—To identify the specific glycine residue responsible for the radical signal in T4 NrdD, site-directed mutagenesis was applied. Sequence alignments between the Pfl enzyme and the NrdD sequences suggested that glycine residue 580 in the T4 reductase exchanged with solvent protons before incorporation into protein. Engineering of glycine residues in general has the disadvantage that any side chain replacement will be a dramatic departure from the original H atom. An alanine substitution was selected to produce the minimal amount of conformational change and with the assumption that the radical would not be formed or stabilized at an alanine residue. The mutant protein T4 NrdD(G580A) was found to lack both glycol radical (Fig. 1, spectrum d) and activity (Table I). The low background activity seen with the mutant NrdD was comparable with background activities seen with the E. coli control extract or the T4 wild type NrdD and NrdG extracts before mixing (Table I).

NrdG-dependent Cleavage of NrdD—In order to test the oxygen sensitivity of the phase-specific anaerobic ribonucleotide reductase, we constructed several different overexpression systems that produced T4 NrdD and NrdG either separately or conjointly. Overexpression of T4 NrdD alone within E. coli cells produces a single full-length NrdD polypeptide chain (Fig. 4A,
was overproduced under anaerobic conditions (Fig. 4). Oxygen-dependent and did not occur when the dual construct due to Gly-580. The cleavage of the T4 NrdD was seen to be cleavage occurred at the the proposed radical containing residues of product previously reported in aerobic bacteriophage T4 infection. The truncated product corresponded in size to the cleavage as was previously shown for the host reductase (2). The stoichiometry of T4 NrdG subunits versus NrdD subunits within the purified holoenzyme complex, determined by densitometry of Coomassie-stained SDS-polyacrylamide gel electrophoresis, was found to be 1:2, indicating a dimer to dimer association of the NrdD-NrdG complex.

The Mutant NrdD(G580A) Component Can Bind the NrdG Component—To confirm that the mutant protein NrdD(G580A) was folded properly and that no drastic rearrangement of the tertiary structure had occurred, a purification of the NrdD(NrdG) complex (Fig. 5). It was found that the retention of T4 NrdG on the column occurred by complex formation with NrdD, because NrdD alone was retained on the column, whereas the isolated T4 NrdG protein eluted in the flow-through. The host NrdD and NrdG components have previously been reported to be tightly associated during purification (18, 19).

Purification and Subunit Composition of the NrdD-NrdG Holoenzyme—Initial attempts at purification of the phage specific NrdD-NrdG holoenzyme complex gave low yields of the NrdG protein. Through a screening of different hydrophobic matrices, it was discovered that a purification of the holoenzyme could be readily accomplished using butyl-Sepharose. Elution of the T4 NrdD-NrdG complex was achieved with 50 mM phosphate buffer as an eluant. This purification method permitted facile anaerobic purification of the wild type NrdD-NrdG complex (Fig. 5). It was found that the retention of T4 NrdG on the column occurred by complex formation with NrdD, because NrdD alone was retained on the column, whereas the isolated T4 NrdG protein eluted in the flow-through. The host NrdD and NrdG components have previously been reported to be tightly associated during purification (18, 19).

Purified T4 NrdD wild type protein was identified as a homodimer using gel exclusion chromatography (data not shown), as was previously shown for the host reductase (2). The stoichiometry of T4 NrdD subunits versus NrdD subunits within the purified holoenzyme complex, determined by densitometry of Coomassie-stained SDS-polyacrylamide gel electrophoresis, was found to be 1:2, indicating a dimer to dimer association of the NrdD-NrdG complex.

The Mutant NrdD(G580A) Component Can Bind the NrdG Component—To confirm that the mutant protein NrdD(G580A) was folded properly and that no drastic rearrangement of the tertiary structure had occurred, a purification of the NrdD(G580A) mutant protein overexpressed in the presence of wild type T4 NrdD was carried out (Fig. 6). As in the wild type case the NrdG was found to co-purify with the NrdD compo-
The isolated T4 NrdD subunit was found to lack protein bound iron (Table III), even if expressed and purified under anaerobic conditions. Yet, when the same crude extract was incubated with an anaerobically overexpressed T4 NrdG extract and purified, the holoenzyme was found to contain iron. A total of approximately 2 iron/hoooloenzyme were found for the purified wild type holoenzyme; similar results were also obtained with the holoenzyme containing the mutant G580A reductase (Table III). Currently we cannot distinguish between the two possibilities that the observed iron is present only within the NrdG polypeptide or that the NrdG transfers and/or promotes the formation of an iron-sulfur center(s) within the NrdD dimer. However, the low microwave power saturation of the radical signals of both T4 and E. coli NrdG complex (Table II) indicates that their glycyl radical relaxes like a free radical and that it is not close enough in space to acquire enhanced relaxation properties from an iron-sulfur center, thereby excluding the adjacent cysteine residue (Cys-579 in T4 NrdD and Cys-680 in E. coli NrdD) as a ligand of the metal center. The iron-sulfur center of the E. coli anaerobic ribonucleotide reducease has recently been proposed to reside in the NrdG component (19).

### DISCUSSION

In this study we show that the radical in bacteriophage T4 anaerobic ribonucleotide reductase is a glycyl radical positioned at Gly-580 in the NrdD polypeptide chain. The glycyl radical, which is essential for enzyme activity, is introduced into the NrdD component by the NrdG component, previously called activase. The NrdG component was found to bind tightly to the NrdD homodimer in a one-to-one complex, suggesting a tetrameric structure of the NrdD-NrdG holoenzyme. Only the NrdD-NrdG complex, but not the isolated NrdD homodimer, contains iron, presumably in the form of an iron-sulfur center.

During the infection of aerobically growing E. coli with bacteriophage T4, a gene product named SunY had been observed to undergo self-cleavage (26). With the identification of SunY as the NrdD subunit of the phage anaerobic ribonucleotide reductase (5), an apparent explanation for the cleavage is that the glycyl radical within the protein undergoes an attack by molecular oxygen with the final product yielding a cleavage at the radical site. The ability to distinguish the presence or the absence of the glycyl radical through the examination of polyacrylamide gel electrophoresis should allow a simple method to screen for the effect upon the glycyl radical of potential enzyme inhibitors or site-directed mutations within the anaerobic ribonucleotide reductase. Recently the genome of the pathogenic bacteria Haemophilus influenzae was sequenced, and the genes for both NrdD and NrdG are present within this facultative anaerobe (GenBank accession numbers H10075 and H11155; Ref. 27). The anaerobic reductase likely plays an essential role during anaerobic infection and would make an excellent target for inhibiting the growth of the bacteria.

A puzzling observation with the aerobically overexpressed T4 NrdG with NrdD is the consistent incomplete cleavage of the T4 NrdD. When aerobically inductions of T4 NrdD along with excess T4 NrdG were carried out and the resulting bacterial pellets were directly lysed in SDS sample buffer, approximately 50% of the NrdD was observed to have undergone cleavage. We therefore considered the possibility that only one monomer was activated within the NrdD homodimer. In the case of the Pfl enzyme, reported to contain a large dimeric protein activated by a monomeric small polypeptide, the Pfl activase, quantitation of the radical content revealed one radical/Pfl homodimer (9). A simple explanation for the single radical observed within the dimeric NrdD would be the interaction of a monomeric NrdG subunit functioning on just one of the two subunits of the NrdD dimer. However, because our data indicate that a dimer-dimer interaction is the likely native conformation for the NrdD-NrdG complex, the single radical generation must have another explanation than a monomer to dimer activation. The tyrosyl radical content of the aerobic class I ribonucleotide reductase has also been consistently observed to have approximately one radical/homodimeric R2 component (28-30), despite the fact that the mechanism for generation of this radical is autocatalytic and understood in considerable detail (31-33).
The function of the glycyl radical observed within the T4 reductase almost certainly involves the generation of a transient radical in the ribose ring of the ribonucleotide substrate (34, 35). However, it is far from clear whether the glycyl radical participates directly or through an intermediate. If the anaerobic ribonucleotide reductase contains an active site similar to the proposed active sites within the class I and class II reductases (36), the glycyl radical would be expected to function as one part of an electron transfer chain to a separate active site containing a potential transient thyl radical (37, 38). In support of such a model are the facts that the E. coli anaerobic ribonucleotide reductase has been observed to be inhibited by class I and class II mechanism-based inhibitors (34) and that the $k_{\text{cat}}$ of 3 s$^{-1}$ obtained in this study for the T4 anaerobic reductase closely matches the $k_{\text{cat}}$ of 4–11 s$^{-1}$ found for the E. coli class I enzyme (30, 39, 40). Recently, the pyruvate formate-lyase enzyme has been identified to contain a thyl radical at its active site (41). We are currently investigating the role of conserved cysteine residues as potential members of an anaerobic ribonucleotide reductase active site that would be homologous to the active site cysteines in the aerobic class I reductase.

How could electron transfer occur within the NrdD subunit? It may occur as has been proposed for the class I ribonucleotide reductase by way of transient passage through side chain residues that are specifically aligned to allow efficient electron transfer (42). Another possibility would be that the electron transfer involves a direct migration of the electron along the peptide backbone. During the early evolution of proteins electron transfer along the peptide backbone may have favored the use of glycyl radicals in that the evolving enzyme would only be required to stabilize the radical and not simultaneously develop aligned amino acid side chains for electron transfer. Finally, the proposed iron-sulfur center may also play a vital role in migration of an electron from the active site to the glycyl radical.

The proposition that the early development of life first passed through an RNA world to today’s current system has been promoted with the idea that molecular processes from this earlier time are still present in today’s biochemistry as molecular fossils (43, 44). Ribonucleotide reductases in general have been characterized as such a molecular fossil representing a late addition to a previous ribonucleotide biochemistry pathway, i.e. deoxynucleotides today are never synthesized de novo but through reduction of RNA nucleotide precursors. Specifically, the class III anaerobic ribonucleotide reductase has been proposed to resemble the original progenote ribonucleotide reductase (1). One of the key steps in the evolution of a ribonucleotide reductase would be the development of a system to harness free radical chemistry. As demonstrated in this paper, the pyruvate formate-lyase and the anaerobic reductase share in general a similar system involving one component (NrdG) that initiates radical generation in another catalytic component (NrdD). The proposed cleavage of a ribonucleotide-based cofactor, S-adenosylmethionine, by the PFi activase or the NrdG component for the initial step of generating the stable glycine radical may in fact represent a remnant from a previous ribonucleotide-based biochemistry shared both with PFi and the first ribonucleotide reductase. Further investigation of the class III reductase should reveal information about the origins of ribonucleotide reductase chemistry in general.

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