The Free Radical of the Anaerobic Ribonucleotide Reductase from Escherichia coli Is at Glycine 681*

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The anaerobic ribonucleotide triphosphate reductase of Escherichia coli is an iron-sulfur protein carrying an oxygen-sensitive organic radical, which is essential for catalysis. The radical was tentatively proposed to be on glycine 681, based on a comparison with the glycylic radical-containing enzyme pyruvate formate-lyase. By EPR spectroscopy of selectively 2H- and 13C-labeled anaerobic ribonucleotide reductase, the radical was now unambiguously assigned to carbon-2 of a glycine residue. The large 1H hyperfine splitting (1.4 millitesla) was assigned to the α-proton. Site-directed mutagenesis was used to change glycine 681 into an alanine residue. In separate experiments, the two adjacent residues, cysteine 680 and tyrosine 682, were changed into serine and phenylalanine, respectively. All mutated proteins were retained on DATP-Sepharose, indicating that the mutant proteins had intact allosteric sites. They also contained amounts of iron comparable with the wild type reductase and showed the same iron-sulfur-related spectrum, suggesting that the mutant proteins were properly folded. Of the three mutant proteins only the G681A protein completely lacked the detectable glycylic radical as well as enzyme activity. Our results identify glycine 681 as the stable free radical site in E. coli anaerobic ribonucleotide reductase.

The anaerobic ribonucleotide reductase, which is encoded by the nrdD gene (Sun et al., 1993), is a homodimer of 160 kDa. This active protein contains a stable organic free radical and a poorly defined iron-sulfur cluster (Mulliez et al., 1993). The radical is introduced into an inactive form of the reductase in a reaction requiring a 17.5-kDa protein (Sun et al., 1995), S'-adenosylmethionin (AdoMet), NADPH (Eliasson et al., 1990; Harder et al., 1992), flavodoxin, and ferredoxin (flavodoxin) NADP⁺ oxidoreductase (abbreviated flavodoxin reductase below) (Bianchi et al., 1993a, 1993b). The organic radical is oxygen-sensitive, and exposure of the radical-containing enzyme to air leads to truncation at Gly-681 (King and Reichard, 1995), accompanied by inactivation (Sun et al., 1993). There is a striking amino acid sequence similarity between a stretch of 5 residues comprising glycine 681 in the anaerobic reductase (Sun et al., 1993) or glycine 580 in the corresponding bacteriophage T4 anaerobic ribonucleotide reductase (Young et al., 1994) and the sequence surrounding glycine 734 at the active site of the glycylic radical-containing enzyme pyruvate formate-lyase (PFL)1 (Wagner et al., 1992). Formation of an oxygen-sensitive radical at Gly-734 in PFL and oxygen-dependent truncation of PFL at this position are analogous reactions to the ones described above for the anaerobic reductase. Glycine 681 in the anaerobic reductase was therefore proposed to be the position of the free radical in this system (Sun et al., 1993). In agreement with this proposition, the EPR spectrum of the radical of the anaerobic reductase shares some common features with that of the glycylic radical of PFL, in particular the large dominant doublet hyperfine splitting (Mulliez et al., 1993).

In this study, the chemical nature of the free radical of the anaerobic ribonucleotide reductase has been investigated by isotopic substitution experiments. The effects of isotopically labeling the glycines of the enzyme on the hyperfine structure of the characteristic EPR spectrum were consistent with the organic radical being on a glycine residue. Moreover we used site-directed mutagenesis to identify the location of the glycylic radical. In addition to the mutation G681A, the adjacent mutations C680S and Y682F were also constructed. Mutant proteins were characterized with regard to the presence of iron-sulfur cluster, glycylic radical, and enzyme activity. Taken together our results demonstrate that the free radical of anaerobic ribonucleotide reductase is located on glycine 681. In a similar study, the stable glycylic radical of the T4 anaerobic ribonucleosidetriphosphatereductase...
Glycyl Radical of E. coli Anaerobic Ribonucleotide Reductase

Ribonucleotide reductase was localized to position 580 in this enzyme.

EXPERIMENTAL PROCEDURES

Materials—E. coli strain J109(DE3) was obtained from Promega. Restriction enzymes were from New England Biolabs and Promega. The PCR kits were from Perkin-Elmer. The PCR purification spin kit and plasmid preparation kit were from Qiagen. The gene clean kit was from Biolabs. The Taq DNA polymerase sequencing kit was from Applied Biosystems. The oligonucleotides used for mutagenesis were synthesized by Scandinavian Gene Synthesis (Köping, Sweden). The Muta-Gen e phagemid mutagenesis kit was from Bio-Rad. The mutagene phagemid mutagenesis kit was from Bio-Rad. The oligonucleotides used for mutagenesis were synthesized by Scandinavian Gene Synthesis (Köping, Sweden). The Muta-Gen e phagemid mutagenesis kit was from Bio-Rad. The mutagene phagemid mutagenesis kit was from Bio-Rad.

Construction of Plasmids—Plasmid pDA carries the nrdG operon under T7 promoter control. It was constructed by insertion of a 673-base pair M6n-KpnI fragment, containing the nrdG gene, from pPX41 into Mua-KpnI-linearized pRSS plasmid, containing the reductase gene, nrdD; pPX41, pREH, and pRSS were constructed in previous work (Sun et al., 1995). Plasmid pAE183 was described by Eliaison et al. (1994). Oligonucleotide-directed Mutagenesis—The primers used for mutagenesis were (mismatches underlined): G681A, 5'-GGGTCGTAAGCCGGTATTTATGAAATCTGAGG-3'; C680S1, 5'-GCGG-3'; and its reverse primer C680S2, 5'-GGCAGGACTTACCAACACATG-3',; and its reverse primer C680S2; 5'-GGCAGGACTTACCAACACATG-3'.

Mutation C680S and G681A was introduced into plasmid pAE183 using the Bioline e phagemid mutagenesis kit. Mutations C680S and G681A were separately introduced into plasmid pRSS by PCR overlap extension as described by Higuchi (1989). All mutations were then achieved by anaerobically growing

E. coli JM109(DE3) containing wild type or mutant plasmid pDA was rescued anaerobically. The downstream gene encodes a 17.5-kDa protein, which is essential for activation of the anaerobic ribonucleotide reductase (Sun et al., 1995). Only the enzyme purified from constructs containing both nrdD and nrdG genes is active, and plasmid pREH (Sun et al., 1995), used for the isotopic labeling experiments, is one such plasmid. In this study, we also constructed plasmid pDA containing both nrdD and nrdG genes. Bacteria carrying plasmid pDA with a wild type or mutant nrdD gene gave good overexpression; extracts contained 6-12% anaerobic ribonucleotide reductase (data not shown).

RESULTS AND DISCUSSION

The E. coli anaerobic ribonucleotide reductase gene, nrdD, has previously been cloned in different plasmids for overproduction of the enzyme. The downstream nrdG gene encodes a 17.5-kDa protein, which is essential for activation of the anaerobic ribonucleotide reductase (Sun et al., 1995). Only the enzyme purified from constructs containing both nrdD and nrdG genes is active, and plasmid pREH (Sun et al., 1995), used for the isotopic labeling experiments, is one such plasmid. In this study, we also constructed plasmid pDA containing both nrdD and nrdG genes. Bacteria carrying plasmid pDA with a wild type or mutant nrdD gene gave good overexpression; extracts contained 6-12% anaerobic ribonucleotide reductase (data not shown).

Evidence through Isotopically Labeled Enzyme That Anaerobic Ribonucleotide Reductase Contains a Glycyl Radical—From cells carrying pREH and grown anaerobically in minimal medium in the presence of isotopically labeled glycine we prepared [13C]- or [2H]glycine-substituted enzyme. Fig. 1 shows the EPR spectra of the native and the two isotopically labeled reductases. The main feature of the EPR spectrum of the unlabeled enzyme (spectrum A) is a large doublet splitting (14-15 G) centered at g = 2.0033. This splitting was originally assigned to hyperfine coupling of the unpaired electron spin to a nitrogen nucleus (Mulliez et al., 1993). The effects of labeling the glycine residues of the reductase on the EPR signal now directly prove that the radical is centered at C-2 of a glycine residue. The EPR features of the [2-13C]glycine-containing enzyme clearly reflect strong coupling of the unpaired electron to the 13C (I = 1/2) nucleus (spectrum B). Moreover, the presence of a singlet signal in the [2H]glycine-substituted enzyme unambiguously demonstrates that the 14 G doublet splitting in the wild type enzyme originates from the hyperfine coupling of the unpaired electron spin to the α-hydrogen of the glycyl radical center (spectrum C). Spectrum B proved to be difficult to simulate, and further studies are required to find out the correct g and A tensors describing the radical. However, from the outermost lines of the signal, it is possible to obtain a value for the hyperfine 13C coupling constant A, in the 46-50 G

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Evidence by Site-directed Mutagenesis That the Essential Radical Is Located on Glycine 681—To map the site of the glycyl radical we mutated Gly-681 in the nrdD gene and used mutations in the adjacent residues, Cys-680 and Tyr-682, as controls. All three mutant proteins (C680S, G681A, and Y682F) behaved like wild type anaerobic ribonucleotide reductase during the purification. An important observation was that all three mutant proteins migrated like wildtype protein during SDS-polyacrylamide gel electrophoresis, indicating that all preparations contained full-length polypeptides. The iron-sulfur center of the anaerobic ribonucleotide reductase is essential for enzyme activity, which was found to correlate linearly with the iron content of the protein (Mulliez et al., 1993). The iron content of the protein preparations purified from the mutants varied between 1.1 and 1.3 atoms of iron/protein dimer (Table I). These values are similar to the iron content of wild type protein preparations from J M109(DE3)/pREH cells, which is typically between 1.2 and 1.5 iron atoms.

### TABLE I

Comparison of wild type and mutant anaerobic ribonucleotide reductases

| Protein           | Iron content \( \text{g atom/mol RR} \) | \( \text{cm}^{-1} \text{ cm}^{-1} \text{ m}^{-1} \text{ mg} \) | Enzyme activity \( \text{units/mg} \) | Radical/RR |
|-------------------|-----------------------------------------|--------------------------------|---------------------------------|------------|
| Wild type         | 1.3                                    | 3100                          | 300                             | 0.42–0.49  |
| G681A             | 1.1                                    | 3100                          | ND                              | ND         |
| C680S             | 1.1                                    | 3000                          | 40                              | 0.11       |
| Y682F             | 1.1                                    | 3100                          | 22                              | 0.06       |

\( ^a \) RR denotes the native, dimeric form of anaerobic ribonucleotide reductase.

\(^b\) Calculated per iron atom.

\(^c\) ND, not detectable.

The glycyl radical of PFL has the same coupling characteristics with a large \( ^1 \text{H} \) hyperfine splitting (15 G) and an estimated \( A_{\text{iso}} \) hyperfine coupling constant also in the range of 16–21 G. A theoretical model study of a dipeptide analog of a glycine radical demonstrated that only planar or nearly planar conformations are energetically accessible due to \( \pi \)-electron delocalization. The hyperfine couplings computed for these conformations (Barone et al., 1995) are in excellent agreement with the experimental values for both glycyl radicals. On the other hand, it is possible to distinguish between the glycyl radicals of the anaerobic reductase and PFL. First, over a 24-h incubation period (Mulliez et al., 1993), the reductase radical does not exchange its \( H_2 \) atom, responsible for the doublet splitting, with the solvent, whereas the PFL radical exchanges rapidly under these conditions. Second, the EPR spectrum of the PFL radical is more complex, with partially resolved subdoublet splitting, arising from two nonexchangeable protons (Wagner et al., 1992).

Therefore, the glycyl radical signal typical of PFL (Knappe and Sawers, 1990) was observed.

The EPR signal of the glycyl free radical also can be recorded directly in bacterial pellets from E. coli J M109(DE3)/pREH cells induced for overexpressing the reductase. The EPR spectra of cells grown in minimal medium in the presence of normal or labeled glycine gave EPR spectra identical to those of the corresponding pure enzyme. This shows that the reductase exists in the radical form within anaerobic cells and that its concentration is such that the endogenous PFL signal is not detectable. On the other hand, in plasmid-free cells only the background glycyl radical signal typical of PFL (Knappe and Sawers, 1990) was observed.

The absorption coefficient at 420 nm for the mutant proteins is essentially the same as that for the active enzyme prepared from plasmid pEH10 (Mulliez et al., 1993), indicating that all preparations contained intact iron-sulfur centers.

Evidence by Site-directed Mutagenesis That the Essential Radical Is Located on Glycine 681—To map the site of the glycyl radical we mutated Gly-681 in the nrdD gene and used mutations in the adjacent residues, Cys-680 and Tyr-682, as controls. All three mutant proteins (C680S, G681A, and Y682F) behaved like wild type anaerobic ribonucleotide reductase during the purification. An important observation was that all three mutant proteins migrated like wild type protein during SDS-polyacrylamide gel electrophoresis, indicating that all preparations contained full-length polypeptides.

The iron-sulfur center of the anaerobic ribonucleotide reductase is essential for enzyme activity, which was found to correlate linearly with the iron content of the protein (Mulliez et al., 1993). The iron content of the protein preparations purified from the mutants varied between 1.1 and 1.3 atoms of iron/protein dimer (Table I). These values are similar to the iron content of wild type protein preparations from J M109(DE3)/pREH cells, which is typically between 1.2 and 1.5 iron atoms. The presence of intact iron-sulfur centers in the preparations from the mutants was verified by UV-visible spectroscopy. Fig. 2 shows that mutant and wild type preparations display a spectrum between 300 and 600 nm, characteristic of iron-sulfur proteins. The small difference between the spectra in the region between 300 and 350 nm is not considered to be significant. The absorption coefficients at 420 nm for the mutant proteins are similar and compare well with those published for the active enzyme prepared from plasmid pEH10 (Mulliez et al., 1993).

If the catalytically essential radical of anaerobic ribonucleo-
otide reductase is located at glycine 681, we would expect the mutant G681A to be devoid of both radical and enzyme activity. Fig. 3 shows the EPR signals recorded from the activated proteins. With the exception of the G681A, which completely lacked a radical EPR signal, all the other proteins showed the typical glycyl radical signal. We also quantified the amount of radical (Table I). In the wild type protein the radical content is between 0.43 and 0.49 radical/reductase enzyme. The mutant protein C680S has about 20% radical and Y682F has about 12% radical/reductase, compared with the wild type protein (Table I). Since all purified enzymes contained substantial amounts of truncated polypeptides (see below), the actual radical content per full-length protein might be significantly higher.

The EPR signal of the glycyl radical also can be recorded directly in bacterial pellets from induced JM109(DE3)/pDA cells, thereby avoiding extensive purification protocols, which may affect the recovery of the radical. Whereas cells carrying the wild type plasmid gave the glycyl radical signal typical of
anaerobic ribonucleotide reductase, cells carrying the G681A plasmid only showed the glycyl radical signal from PFL (data not shown), as expected if the induced mutant anaerobic reductase lacks a glycyl radical. In this case, washing of the cell pellet with anaerobic D2O resulted in reduced linewidth of the EPR spectrum (data not shown), in agreement with the solvent exchangeability of the proton responsible for the dominating doublet hyperfine splitting of the PFL radical.

Enzyme activities of the pure wild type and mutant reductases are shown in Table I. The G681A mutant protein completely lacked enzyme activity, whereas the C680S protein had about 14% activity and the Y682F protein about 7% as compared with the wild type protein. Taken together, these results clearly show that the G681A mutant protein lacks both radical and enzyme activity, whereas the C680S and Y682F mutant proteins still retain some radical and some enzyme activity. The difference between radical content and corresponding enzyme activity of the mutants C680S and Y682F might be caused by experimental variation or by conformational changes introduced by the mutations that influence the enzyme activity and the generation of radical differently.

What are the functions of Cys-680 and Tyr-682? Our results indicate that their presence influences the surroundings of the radical-harboring Gly-681 residue but that neither is essential for formation of the glycyl radical or for activity of the anaerobic reductase. The mutation C680S generates the sequence RVSGY between residues 678 and 682, which in fact is identical to the corresponding sequence surrounding the glycyl radical of PFL (Frey et al., 1994; Sun et al., 1993). In reciprocal experiments in the PFL system, substitution of Ser by Cys in the active site oligopeptide RVSGYLG led to loss of substrate efficiency (Frey et al., 1994), whereas substitution of Ser by Ala in PFL proper gave an enzyme with 34% residual activity (Frey et al., 1994). In addition, substitution of Tyr by Phe in the PFL active site oligopeptide only led to partial loss of substrate efficiency. Thus, mutations of the residues adjacent to the essential glycine residue are tolerated to some extent both by the anaerobic reductase and by PFL.

Conclusion—Our results clearly demonstrate that the presence of Gly-681 is essential for generation of a glycyl radical and concomitant enzyme activity in the anaerobic ribonucleotide reductase, whereas mutations of the adjacent residues result in proteins that still can harbor a glycyl radical and retain some enzymatic activity. Together with the isotope substitution experiments these results demonstrate that the location of the glycyl radical-harboring residue in E. coli anaerobic ribonucleotide reductase is at position 681.

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