Recombinant *Desulfovibrio vulgaris* Rubrerythrin. Isolation and Characterization of the Diiron Domain†

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ABSTRACT: The gene encoding *Desulfovibrio (D.) vulgaris* rubrerythrin (Prickril, B. C., Kurtz, D. M., Jr., LeGall, J., & Voordouw, G. (1991) *Biochemistry 30,* 1118), a protein of unknown function containing both FeS_4 and (μ-oxo)diiron sites, was cloned and overexpressed in *Escherichia coli.* Upon cell lysis, the overexpressed protein was found in an insoluble form deficient in iron. Iron was incorporated *in vitro* by dissolving the protein in 3 M guanidinium chloride, adding Fe(II) anaerobically and diluting the denaturant. This recombinant rubrerythrin was found to have properties very similar to those of rubrerythrin isolated from *D. vulgaris*, except that the recombinant rubrerythrin contained six rather than four (or five) iron atoms per 44 kDa homodimer. Analyses of UV−vis, Mössbauer, and EPR spectra showed that the six iron atoms in recombinant rubrerythrin are organized as two FeS_4 and two (μ-oxo/hydroxo)diiron sites. In order to allow examination of the diiron sites in the absence of the FeS_4 sites, a truncated gene encoding the N-terminal 152 residues of *D. vulgaris* rubrerythrin was also cloned and overexpressed as an insoluble protein in *E. coli*, and iron was incorporated by a procedure analogous to that for recombinant rubrerythrin. This so-called "chopped" rubrerythrin (CRr) was found to consist of an ≈35 kDa homodimer containing four iron atoms. Spectroscopic characterization indicated that the four iron atoms in CRr are organized as two diiron sites, the majority of which closely resemble the (μ-oxo)diiron(III) sites in *E. coli* ribonucleotide reductase R2 protein, and a minor fraction of which resemble the mixed-valent diiron(II,III) site in methane monooxygenase hydroxylase. Both the diiron(III) and diiron(II) sites of CRr were shown by UV−vis and Mössbauer spectroscopies to form complexes with azide. The diiron(II)CRr azide adduct could be detected by a parallel-mode EPR signal at $g \approx 17.5$. A similar parallel-mode EPR signal at $g \approx 19.8$ was detected when excess azide was added to the reduced recombinant rubrerythrin. EPR-based redox titrations of CRr gave diiron(III)-to-mixed-valent and mixed-valent-to-diiron(II) reduction potentials of 215 ± 5 and 154 ± 5 mV, respectively, vs NHE. These potentials were approximately 70 mV more negative than those measured for the corresponding redox couples in recombinant rubrerythrin. Possible activities for rubrerythrin are discussed.

Rubrerythrin (Rr)[1] is a non-heme iron protein found in the anaerobic sulfate-reducing bacterium *Desulfovibrio (D.) vulgaris* (Hildenborough). The as-isolated Rr was characterized as a 44 kDa homodimer containing four iron atoms distributed as two FeS_4 centers and one (μ-oxo/hydroxo)-diiron cluster (LeGall et al., 1988; Dave et al., 1994). The FeS_4 centers bear close spectroscopic resemblances to those in rubredoxins, while the diiron cluster was found to exhibit spectroscopic and magnetic properties similar to those in other diiron proteins, e.g., hemerythrin, ribonucleotide reductase R2 protein (RNR-R2), methane monooxygenase hydroxylase, and stearoyl-CoA Δ^9-desaturase (Sanders-Loehr, 1989; Que & True, 1990; Vincent et al., 1990; Fox et al., 1993a,b; Kurtz, 1994). These spectroscopic resemblances led to the trivial name, rubrerythrin (Rr), a contraction of rubredoxin and hemerythrin, for this protein. An independent group more recently reported approximately five iron atoms in the as-isolated Rr homodimer (Pierik et al., 1993). Rr has no known physiological function. Despite the apparent spectroscopic resemblance to hemerythrin, Rr does not bind O_2 reversibly. Both the FeS_4 and diiron sites in as-isolated Rr were found to have reduction potentials ≥230 mV vs NHE (LeGall et al., 1988; Pierik et al., 1993), which is unusually high when compared to those of rubredoxins (≈0 mV). Such high reduction potentials seem

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[1] Abbreviations: Rr, rubrerythrin; CRr, rubrerythrin minus the C-terminal FeS_4 domain; GuHCl, guanidinium chloride; EPR, electron paramagnetic resonance; RNR-R2, *Escherichia coli* ribonucleotide reductase R2 protein; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; Tris, tris(hydroxymethyl)aminomethane; PCR, polymerase chain reaction; LB, Luria−Bertani medium; EDTA, ethylenediaminetetraacetate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; LMCT, ligand-to-metal charge transfer; IPTG, isopropyl β-D-thiogalactoside.

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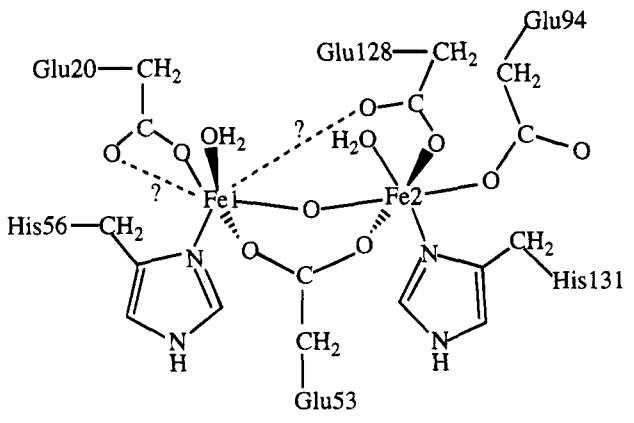
Chart 1



inconsistent with participation of Rr in the electron transport chain of *D. vulgaris* (Odom & Singleton, 1993).

The amino acid sequence of Rr inferred from the nucleotide sequence of the gene (Prickril et al., 1991) and that determined by classical amino acid sequencing (Van Beeumen et al., 1991) are identical to each other, which indicates no post-translational processing and a cytoplasmic localization. The 39-residue C-terminal portion of the Rr subunit shows some sequence homology to rubredoxins, especially the presence of two CXXC pairs. The N-terminal portion contains two EXXH sequences embedded in internally homologous stretches of the polypeptide (Kurtz & Prickril, 1991). Two EXXH sequences provide Glu and His ligands to the diiron clusters in RNR-R2 (Nordlund & Eklund, 1993), methane monooxygenase hydroxylase (Rosenzweig et al., 1993), and probably stearoyl-CoA Δ^9-desaturase (Fox et al., 1993a). These sequence comparisons suggested that each subunit of the Rr homodimer could accommodate one FeS_4 site and one diiron cluster, for a total of six irons per homodimer. This hypothesis was subsequently confirmed by freeing the as-isolated *D. vulgaris* Rr of iron and then reconstituting with ^{57}Fe-enriched iron *in vitro* (Ravi et al., 1993). Both spectroscopic data and sequence comparisons suggested that the diiron clusters of reconstituted Rr more closely resembled those in RNR-R2 than those in hemerythrin. Therefore, a (μ-oxo)diiron(III) cluster structure similar to that in RNR-R2 was proposed for Rr, as shown in Chart 1 (Ravi et al., 1993). However, the implications of this proposed structural resemblance to the function of Rr remain unclear; additional probes of both structure and function of Rr are needed.

Here we report a procedure for cloning and overexpression of the *D. vulgaris* Rr gene in *Escherichia* (*E.*) *coli* and *in vitro* incorporation of iron into the overexpressed protein. In order to allow more detailed characterization of the diiron sites in Rr, we have also overexpressed an artificially truncated gene coding for residues 1−152 of *D. vulgaris* Rr. The resulting protein, referred to as "chopped" Rr (CRr), contains the two EXXH sequences referred to above, but lacks the 39-residue C-terminal FeS_4 domain.

MATERIALS AND METHODS

Cloning and Overexpression of Recombinant Rr and CRr. Molecular biological procedures generally followed those described in Sambrook et al. (1989) or in *Current Protocols in Molecular Biology* (Ausbel et al., 1990). Nucleotide sequencing of Qiagen tip-100-purified plasmids was performed at the Molecular Genetics Instrumentation Facility on the University of Georgia campus. Oligonucleotides were purchased from Integrated DNA Technologies, Inc. Protein overexpression in *E. coli* was monitored by running SDS−PAGE (15% acrylamide; Hames, 1981) on portions of 1 mL aliquots removed from the cultures and on cell lysate fractions.

For overexpression of the *D. vulgaris* Rr gene, a PCR was carried out using *Pst*I-digested pBP96-4 (Prickril et al., 1991) as template and oligonucleotides DMK4 and DMK5 as primers:

NdeI↓
DMK4 5′ TATACATATGAAATCGCTCAAGGGC 3′

*Bam*HI *
DMK5 5′ TAGAGGATCCTTACCAGTTGATGCC 3′

The 7 kb *Pst*I fragment of pBP96-4 contains the *D. vulgaris* Rr gene. Starting with the start and stop codons indicated by the arrow and asterisk, DMK4 and DMK5 duplicate the 5′ and complementary 3′ ends, respectively, of the Rr gene and are designed to add the indicated *Nde*I and *Bam*HI restriction sites onto the 5′ and 3′ ends of the Rr gene. The PCR was carried out using the reagents and recommended conditions in the GeneAmp PCR reagent kit #N801-0055 (Perkin-Elmer Cetus). The PCR product was ligated into plasmid pT7-7 (Tabor, 1990) after *NdeI*/*Bam*HI double digestion of the PCR product/pT7-7 mixture. The ligated product, plasmid pDK3-5, was transformed first into *E. coli* strain 71/18, and then into *E. coli* strain BL21(DE3) (Novagen, Inc.) (Studier et al., 1990). The nucleotide sequence of the Rr gene in pDK3-5 was determined to be identical to the published sequence (Prickril et al., 1991). Induction of Rr gene expression was accomplished as follows: the BL21(DE3)[pDK3-5] strain from either a glycerol stock or a single colony was inoculated into 50 mL of LB (Luria−Bertani medium) containing 100 μg/mL of ampicillin and incubated at 37 °C overnight in an orbital shaker at 250 rpm. The 50 mL culture was used to inoculate 1 L of LB containing 100 mg of ampicillin, and incubation was continued at the same temperature and shaker speed until the OD_{600} reached ≈1.0 (≈2 h), at which point protein expression was induced by addition of IPTG to 0.4 mM. The induced culture was incubated at 37 °C until the OD_{600} reached ≈2.2 (≈2 h). The flask containing the 1 L culture was then placed on ice for 5 min before harvesting the cells by centrifugation at 5000*g* for 5 min. The cells were washed once with 250 mL of 50 mM Tris-HCl and 2 mM EDTA (pH 8.0) and then resuspended in 30 mL of the same buffer. One milliliter of lysozyme (10 mg/mL) and 10 mL of 1% (v/v) Triton X-100 were then added. After incubation at 30 °C for 15 min, the mixture was sonicated on ice using a Branson sonifier cell disruptor 350 with a 0.5-in. probe tip for 2 × 60 s at 7 kHz and 90% output power. The pale pink pellet from the resulting cell lysate was collected by centrifugation at 12000*g* for 30 min at 4 °C. SDS−PAGE analysis (Figure 1) indicated that the majority of this pellet consisted of an overexpressed protein having the same molecular weight as the Rr subunit.

For cloning and overexpression of CRr, a PCR reaction was carried out as described above for recombinant Rr, except using primer NG2 in place of DMK5 for the complementary 3′ end:

BamHI *

NG2 5′ TAGAGGATCCTTACTGTTCGCGCAGGAACAC 3

Primer NG2 was designed so that, when combined with primer DMK4, the DNA encoding the N-terminal diiron domain of *D. vulgaris* Rr, residues 1−152, would be amplified in the PCR. The resulting PCR product was ligated into pT7-7 after *NdeI*/*Bam*HI double digestion of the PCR product/pT7-7 mixture. The resulting plasmid, pNG17, was used to transform *E. coli* 71/18 and then *E. coli* BL21(DE3). The nucleotide sequence of the gene in pNG17 was determined to be identical to that coding for amino acid residues 1−152 of the *D. vulgaris* Rr sequence (Prickril et al., 1991). Induction of protein expression in BL21-(DE3)[pNG17] was accomplished as described above for BL21(DE3)[pDK3-5] and resulted in a white pellet after cell lysis. The majority of this pellet consisted of an over-expressed protein having the molecular weight expected for the CRr subunit (cf. Figure 1).

Isolation and Purification of Recombinant Rr. The procedure described below is for the combined pellet of the cell lysate from two 1 L cultures. Unless otherwise noted, all steps were carried out at room temperature, and buffer is 0.1 M Tris-HCl, pH 7.5. The pale pink pellet (≈10 g wet weight) was resuspended in 6 mL of 3 M GuHCl in buffer while passing argon over the surface of the solution. The pale pink supernatant was freed of membranes by centrifugation at 30000*g* for 30 min. The supernatant was transferred to a 250 mL Schlenk-type flask. Eighty microliters of 2-mercaptoethanol was added, and the solution was degassed and flushed with argon using a vacuum manifold. Five hundred microliters of a 0.14 M ferrous ammonium sulfate solution in buffer (freshly prepared under argon) was added dropwise to the anaerobic solution over a period of 10 min. The solution was allowed to equilibrate for 30 min, after which anaerobic buffer was added dropwise from a dropping funnel to raise the total volume to 40 mL over a period of 2 h. The turbid suspension was left to equilibrate for another 30 min and then centrifuged for 30 min at 30000*g*. The clear supernatant was reduced to a volume of 5 mL in a 50 mL Amicon cell (YM-10 membrane) under argon pressure and then centrifuged for 30 min at 30000*g*. The supernatant was diluted to 50 mL with aerobic buffer, reconcentrated to 5 mL in the Amicon cell, and centrifuged as before. This concentration/redilution process was repeated five more times. Final purification, if necessary, was achieved by gel filtration on a 1.6 × 50 cm Superose-12 FPLC column (PharmaciaLKB) equilibrated with 0.1 M Tris-HCl (pH 8) or 50 mM Hepes and 200 mM Na_2SO_4 (pH 7). The column was calibrated by using standard molecular weight proteins (bovine serum albumin, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome *c*, 12.4 kDa) in the same buffer. Iron incorporation with ^{57}Fe-enriched Fe(II) was carried out as described above except that an ^{57}Fe-enriched stock solution, prepared as described by Ravi et al. (1993), was used in place of the ferrous ammonium sulfate stock solution.

Isolation and Purification of CRr. Incorporation of either ^{57}Fe-enriched or natural isotopic abundance Fe(II) into the white pellet from the induction of BL21(DE3)[pNG17] was accomplished by the procedure described above for recombinant Rr except that 50 mM Hepes (pH 7) was used in place of Tris-HCl. The rate of addition of buffer to dilute the denaturant (particularly the first dilution) should not exceed ≈0.15 mL/min; faster dilution caused protein precipitation and a lower yield of CRr. Final purification, if necessary, was achieved by Superose-12 gel filtration chromatography with 50 mM Hepes plus 200 mM Na_2SO_4 (pH 7). Gel filtration in the same buffer on the Superose-12 column, calibrated as described above, was used to determine the molecular weight of CRr.

Spectroscopies. UV−vis, Mössbauer, and EPR spectroscopies were carried out as described by Ravi et al. (1993). Mössbauer samples were prepared by placing the ^{57}Fe-enriched protein (600 μL of 700 μM Rr homodimer in 0.1 M Tris-HCl (pH 8.1) or 500 μL of 400 μM CRr homodimer in 50 mM Hepes and 200 mM Na_2SO_4 (pH 7)) into a shallow delrin sample cup and freezing with liquid nitrogen.

Redox Titrations. The EPR−spectroelectrochemical cell used in this study was built according to the design reported by Paulsen et al. (1992) with minor modifications. EPR-based redox titrations were performed at room temperature on proteins, 2 mL of 100 μM in homodimer in 50 mM Hepes and 200 mM Na_2SO_4 (pH 7.0). Mediators (70 μM each or saturated for less soluble mediators) present in the solutions were: ferroceneacetic acid (360 mV/NHE), 1,4-benzoquinone (280 mV/NHE), 2,6-dichlorophenolindophenol sodium salt (220 mV/NHE), and 1,2-naphthaquinone (157 mV/NHE). The potentials were adjusted using sodium dithionite and potassium ferricyanide (0.1 M each) as reductant and oxidant, respectively. The solution was allowed to incubate for 20 min in order to allow the poised potential (E) to reach equilibrium. A 200 μL aliquot of the protein solution was then transferred via a gas-tight Hamilton syringe to an EPR tube attached to the electrochemical cell. The sample in the EPR tube was then frozen immediately in liquid nitrogen. The transfer process was performed inside the cell under argon atmosphere, and the EPR tube was detached after the sample was frozen. The oxidation states of the FeS_4 site and diiron cluster were monitored by the $g = 4.3$ and $g = 1.76$ EPR signal intensities, respectively. EPR spectra were recorded at 6 K using nonsaturating power for both signals (20 mW for the diiron cluster signal and 0.2 mW for the FeS_4 signal). Titration points (experimental E vs relative intensity) obtained from both oxidizing and reducing directions were least-squares fitted to either the one-electron Nernst equation in the case of the $g = 4.3$ signal or two sequential one-electron Nernst equations in the case of the $g = 1.76$ signal, and midpoint potentials were obtained from the fitted curves. In the case of the diiron cluster, the maximum fraction of mixed-valent species is related to the difference between the two midpoint potentials in the equation described by Clark (1960).

Iron, Protein, and Extinction Coefficient Determinations. Iron concentrations for recombinant Rr and CRr were determined by the bathophenanthroline method described by Ravi et al. (1993), except that 6 M GuHCl was used in place of 1 M sodium acetate, and the assay mixture was allowed to incubate for 30 min at room temperature before recording absorbance at 535 nm. Protein concentrations for recombinant Rr were determined by quantitative amino acid analysis on aliquots of ≈5 pmol of homodimer/μL samples in 50 mM Hepes and 200 mM Na_2SO_4 at the University of Georgia Molecular Genetics Instrumentation Facility. Corrections due to sample losses were applied on the basis of parallel analysis of a standard bovine serum albumin solution (15 pmol/μL). Recombinant Rr protein concentrations were

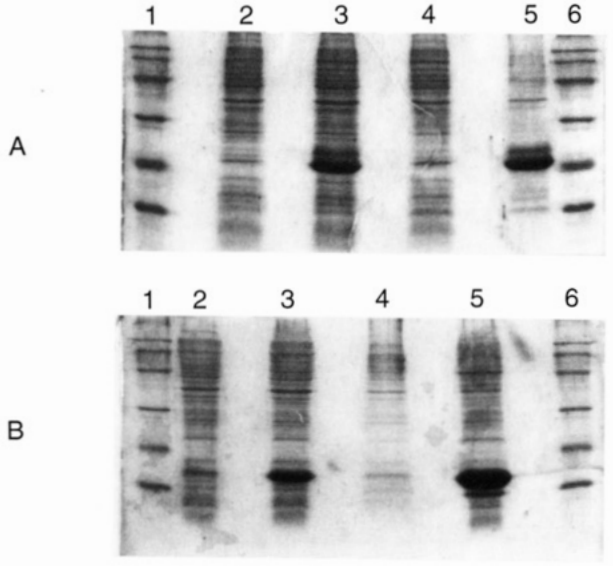


FIGURE 1: SDS−PAGE monitoring of overexpression of (A) recombinant Rr in *E. coli* BL21(DE3)[pDK3-5] and (B) CRr in *E. coli* BL21(DE3)[pNG17]. Lanes 1 and 6 are molecular weight markers (from bottom to top in kDa): 14.4, 21.5, 31, 42.7, 55, 66.2, 97.4. Lane 2, whole cells before IPTG induction; lane 3, whole cells after IPTG induction; lane 4, supernatant from induced cell lysate; lane 5, pellet from induced cell lysate.

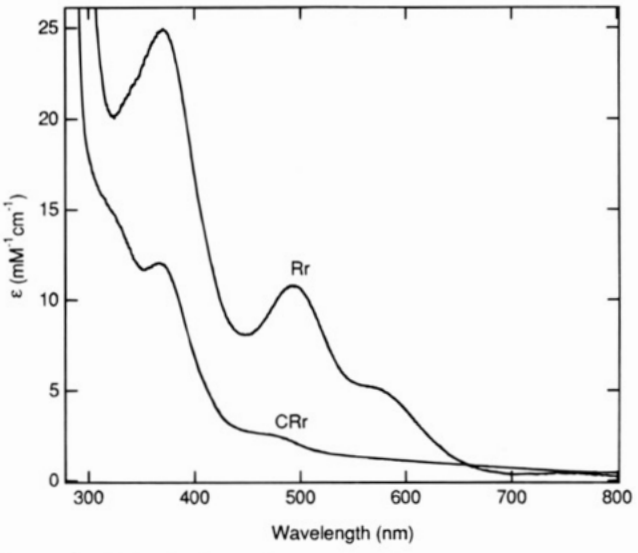


FIGURE 2: UV−vis absorption spectra of recombinant Rr and CRr in 50 mM Hepes and 200 mM Na_2SO_4 (pH 7).

then calculated on the basis of the measured amounts of alanine, valine, arginine, isoleucine, leucine, and proline in the samples and the amino acid composition of recombinant Rr deduced from the gene sequence (Prickril et al., 1991). Samples of recombinant Rr having known A_{492} were analyzed in this fashion, and the data were used to determine a value for ϵ_{492}. The Bio-Rad protein assay was then used to determine protein concentrations of CRr using recombinant Rr as the standard, and extinction coefficients were determined on CRr samples having known A_{365} and A_{474}.

RESULTS AND DISCUSSION

In the following discussion the *D. vulgaris* Rr resulting from expression of the gene in *E. coli* is referred to as "recombinant Rr". Rr previously isolated from *D. vulgaris* (LeGall et al., 1988; Pierik et al., 1993) is referred to as "as-isolated Rr". A third form in which the as-isolated Rr was denatured and reconstituted with iron (Ravi et al., 1993) is referred to as "reconstituted Rr".

Yields and Molecular Properties of Recombinant Rr and CRr. Figure 1 shows the SDS−PAGE analyses of the induction process for protein expression in BL21(DE3)-[pDK3-5], which contains the Rr gene, and in BL21(DE3)-[pNG17], which contains the CRr gene. After induction, the BL21(DE3)[pDK3-5] culture shows an additional protein band migrating at 22 kDa on SDS−PAGE, and this protein comprises the vast majority of the pellet after cell lysis (Figure 1A). The subunit molecular weight of Rr calculated from the known amino acid sequence is 21 544 (Prickril et al., 1991; Van Beeumen et al., 1991); thus, the *D. vulgaris* Rr gene is overexpressed as an insoluble protein in *E. coli* BL21(DE3). After iron incorporation into the denaturant-dissolved pellet, as described in Materials and Methods, dialysis by sequential dilutions and reconcentrations was used to removed the denaturant. This dialysis caused precipitation of nearly all of the denaturant-dissolved proteins other than Rr, and the precipitated contaminants were then readily separated from the recombinant Rr solution by centrifugation. At this stage the recombinant Rr usually gave only a single protein band on SDS−PAGE and eluted as a single symmetrical band (monitoring absorbance at 280 nm) from a Superose-12 gel filtration column. A molecular weight of 44K for recombinant Rr was determined by gel filtration. This molecular weight is consistent with a homodimeric subunit composition and is identical to that determined for the as-isolated Rr homodimer (LeGall et al., 1988). The absorbance ratios determined for the recombinant Rr, $A_{280}/A_{492} = 5.6 \pm 0.7$ and $A_{370}/A_{492} = 1.8 \pm 0.1$, agree with those for the reconstituted Rr (Ravi et al., 1993). The iron content of recombinant Rr determined by wet chemical analysis was found to be 5.9 ± 0.2 Fe/homodimer. The yield of the recombinant Rr having the properties listed above from 2 L of an induced BL21(DE3)[pDK3-5] culture averaged 120 ± 12 mg.

Similarly, the pellet after cell lysis of the induced BL21-(DE3)[pNG17] culture consisted almost entirely of a protein which migrates at a molecular weight of ≈17K on SDS−PAGE (Figure 1B). This molecular weight agrees well with the molecular weight of 17 224 calculated from amino acid residues 1−152 of *D. vulgaris* Rr. Superose-12 gel filtration of CRr after iron incorporation gave a single symmetrical peak (monitoring absorbance at 280 nm) at a molecular weight of ≈35K, indicating that CRr also assembles as a homodimer. A purity ratio $A_{280}/A_{365} = 3.6 \pm 0.3$ was determined for CRr prepared in this fashion. Iron and protein analyses gave 4.0 ± 0.2 iron/homodimer. An average yield of 48 ± 7 mg of CRr having the properties listed above was obtained from 2 L of induced BL21(DE3)[pNG17].

UV−Vis Absorption Spectra. The absorption spectrum of the recombinant Rr has maxima at 750, 492, 370, and 280 nm with shoulders at 570 and 350 nm (Figure 2). This spectrum is essentially identical to that of reconstituted Rr (Ravi et al., 1993), including the absorbance ratios listed above, except for the somewhat less intense shoulder at 350 nm in the recombinant Rr spectrum. An extinction coefficient at 492 nm of 10.8 mM^{-1} cm^{-1} was determined for the recombinant Rr homodimer by amino acid quantitation, which agrees well with the value of 10.6 mM^{-1} cm^{-1}

previously determined for ϵ_{492} of reconstituted Rr (Prickril et al., 1991).

The difference absorption spectrum of as-isolated Rr minus Rd (the latter containing an FeS_4 site as the only chromophore) showed a peak at ≈365 nm, which LeGall et al. (1988) attributed to absorption by the (μ-oxo)diiron(III) unit in Rr. The near-UV−vis absorption spectrum of CRr (Figure 2), which cannot have an FeS_4 site, but which contains the putative diiron domain, completely supports this assignment. The peak at 365 nm with ϵ = 12.0 mM^{-1} cm^{-1} per homodimer (based on protein) in the CRr absorption spectrum is very similar to that in the Rr minus Rd difference absorption spectrum. Furthermore, the 365-nm absorption together with the shoulders at 474 (ϵ = 2.5 mM^{-1} cm^{-1}) and 325 nm in the spectrum of CRr closely resembles the absorption spectrum of metRNR-R2, but does *not* resemble the featureless absorption spectrum of methane monooxygenase hydroxylase between 300 and 500 nm (Que & True, 1990). The spectroscopic, magnetic, and structural properties of this latter protein indicate a hydroxo bridge at its diiron(III) site (Que & True, 1990; Rosenzweig et al., 1993), whereas *E. coli* metRNR-R2 is known to have a (μ-oxo)(μ-carboxylato)diiron(III) site (Sanders-Loehr, 1989; Que & True, 1990; Nordlund & Eklund, 1993; Kurtz, 1994). Considering that the Mössbauer data presented below indicate that nearly all of the iron in CRr is organized as diiron sites, the iron analysis (4.0/homodimer) indicates that CRr must contain two such sites, and the extinction coefficient at 365 nm would then be ≈6.0 mM^{-1} cm^{-1} per diiron(III) site. This extinction coefficient agrees well with ϵ_{370} ≈5.5 mM^{-1} cm^{-1} per diiron(III) site reported for metRNR-R2 by Que and True (1990) and is within the range of other (μ-oxo)diiron(III) complexes and proteins.

EPR Spectra. The EPR spectrum at 4.2 K of recombinant Rr (Figure 3) shows signals at g = 9.4 and 4.3, which are attributable to the ground- and excited-Kramers doublets of the $S = {}^5/_2$ FeS_4 centers, and which are identical to those observed in both as-isolated and recombinant Rr (LeGall et al., 1988; Pierik et al., 1993; Ravi et al., 1993). A relatively weak signal is seen at g = 1.98, 1.76, and 1.57 for recombinant Rr, and an identical signal has been assigned to a minor portion of mixed-valent diiron(II,III) sites in both as-isolated and reconstituted Rr. The EPR spectrum of CRr shows a very similar mixed-valent diiron site signal with slightly different g values: 1.99, 1.76, and 1.62 (Figure 3). The signal at g = 4.3 in the CRr EPR spectrum is assigned to a very minor proportion of iron which is adventitiously bound. The Mössbauer studies described below show that little or no adventitiously bound iron can be detected.

Mössbauer Spectroscopy. The Mössbauer spectrum of ^{57}Fe-enriched recombinant Rr recorded at 4.2 K in a parallel applied field of 50 mT is shown in Figure 4A. The spectrum can be satisfactorily deconvoluted into the following four components: (i) a paramagnetic six-line spectrum (solid line) extending from −5.5 to +6.4 mm/s, which is essentially identical to that assigned to the oxidized FeS_4 center in both as-isolated and reconstituted Rr (LeGall et al., 1988; Ravi et al., 1993), (ii) a minor quadrupole doublet (indicated by arrows) with parameters (ΔE_Q = 3.13 mm/s and δ = 0.70 mm/s) characteristic of a reduced FeS_4 center, (iii) an intense central quadrupole doublet (marked by a bracket) with observed parameters (ΔE_Q = 1.60 mm/s and δ = 0.53 mm/s) similar to those assigned to the (μ-oxo)diiron(III) cluster

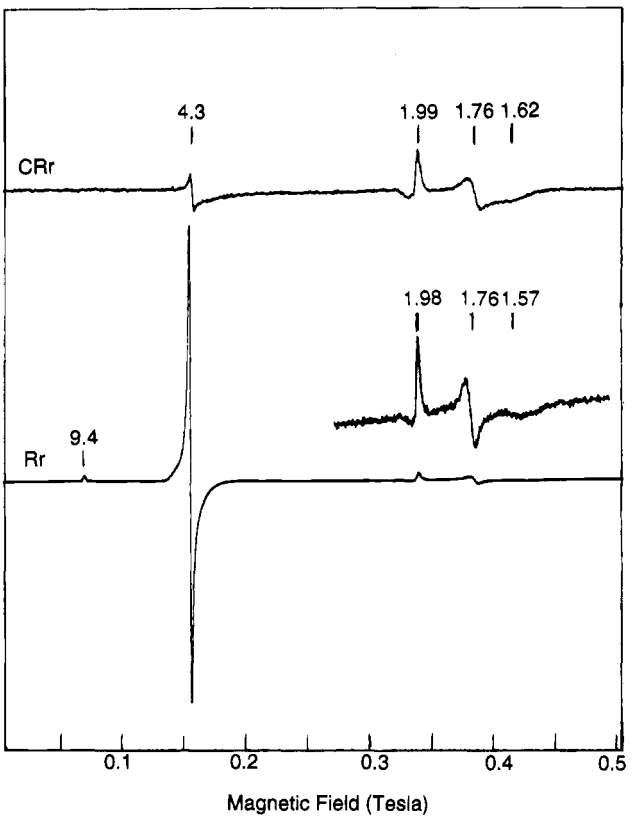


FIGURE 3: X-band EPR spectra of 0.1 mM recombinant Rr homodimer (labeled Rr) and 0.16 mM CRr homodimer (labeled CRr) in 50 mM Hepes and 200 mM Na_2SO_4 (pH 7). EPR conditions: temperature, 4.0 K; microwave frequency, 9.613 GHz; microwave power, 2.0 mW; modulation amplitude, 6.43 G; receiver gain, 1.0 × 10^4.

in reconstituted Rr (Ravi et al., 1993), and (iv) a second six-line spectrum extending from −3.5 to + 5.0 mm/s, which is very similar to that of the mixed-valent diiron site in methane monooxygenase (Fox et al., 1993b). The diiron(III) quadrupole doublet has about the same intensity relative to the oxidized FeS_4 absorption as does that for reconstituted Rr (Ravi et al., 1993), but is relatively more intense than that for as-isolated Rr (LeGall et al., 1988). Quantitative analysis of the spectrum in Figure 4A indicates that 29 ± 4% and ≈3% of the total iron absorption can be attributed to the oxidized and reduced FeS_4 center, respectively, 42 ± 5% to the oxidized diiron(III) cluster, and the remaining 26% absorption the mixed-valent diiron cluster. This analysis indicates that approximately 32% and 68% of total iron are present as FeS_4 and diiron sites, respectively, consistent with 2 and 4 Fe out of a total of 6 Fe in the recombinant Rr homodimer being in the FeS_4 and diiron sites, respectively.

Reduction of recombinant Rr anaerobically with excess ascorbate considerably simplifies the 4.2 K Mössbauer spectrum, as shown in Figure 5, and confirms the above analysis. Two sharp (linewidth ≈0.27 mm/s) quadrupole doublets corresponding to the reduced FeS_4 and diiron(II) cluster are observed. Least-squares fit of the data yielded the following parameters: ΔE_Q = 3.13 ± 0.02 mm/s and δ = 0.70 ± 0.01 mm/s for the minor doublet, characteristic of reduced FeS_4 center, and ΔE_Q = 2.92 ± 0.02 mm/s and δ = 1.27 ± 0.01 mm/s for the major doublet, representing the reduced diiron(II) cluster. The quadrupole doublet of the reduced FeS_4 site is practically identical to that of the as-isolated Rr (LeGall et al., 1988), while the doublet of the

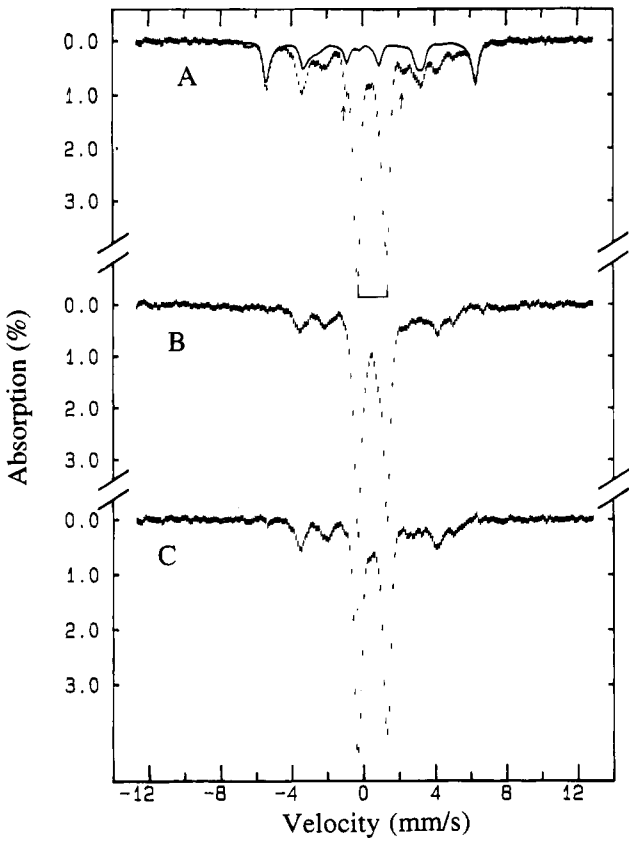


FIGURE 4: Mössbauer spectra at 4.2 K of ^{57}Fe-enriched (A) recombinant Rr, and (B) CRr with a parallel applied field of 50 mT. The spectrum in (C) was prepared from the spectrum in (A) by removing 29% and 3% contributions of oxidized and reduced FeS_4 center, respectively. The solid line in (A) is a theoretical simulation of the oxidized FeS_4 site using parameters from LeGall et al. (1988), and it is normalized to 29% of the total iron absorption. The bracket indicates the doublet assigned to the diiron(III) cluster, and the arrows indicate the positions of the doublet from the reduced FeS_4 center.

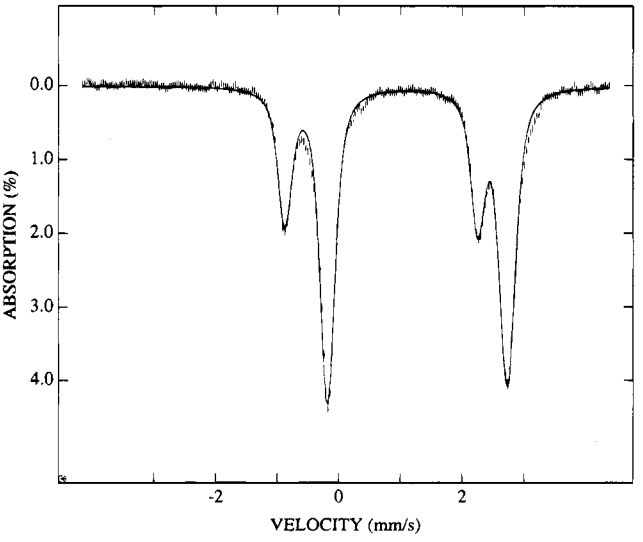


FIGURE 5: Mössbauer spectrum at 4.2 K of the recombinant Rr sample from Figure 4 after reduction with excess ascorbate. The data were recorded in the absence of an applied field. The solid line is a least-squares fit of the data. The minor doublet is assigned to the FeS_4 site and the major doublet to the diiron(II) site following assignments in LeGall et al. (1988).

diiron(II) cluster is sharper and exhibits smaller ΔE_Q in the recombinant protein. The spectrum in Figure 5 is best fitted

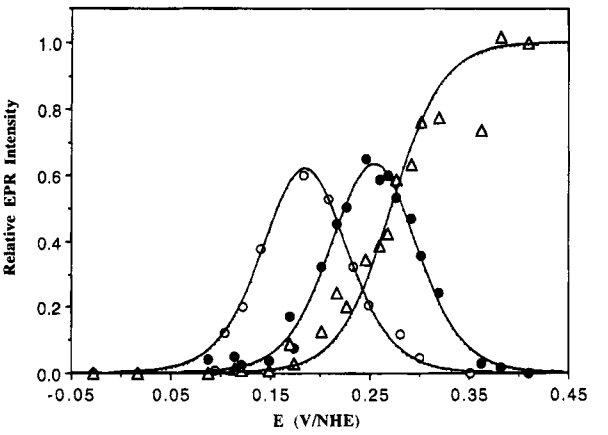


FIGURE 6: EPR redox titration of the FeS_4 (g = 4.3, △) and diiron (g = 1.76, ●) sites of recombinant Rr, and the diiron site (g = 1.76, ○) of CRr. Experimental conditions are described in Materials and Methods.

with Voigt-shape lines, and the relative absorption intensities are 30 ± 4% and 70 ± 4%, respectively, for the minor and major doublets, in agreement with the percents determined for the FeS_4 and diiron sites in recombinant Rr, prior to reduction.

A Mössbauer spectrum of CRr recorded at 4.2 K in a parallel applied field of 50 mT is shown in Figure 4B. In comparison with the spectrum of recombinant Rr recorded under the same conditions (Figure 4A), it is obvious that, in the CRr spectrum, the spectral components corresponding to the FeS_4 site are absent and only those originating from the diiron cluster are present. To illustrate this point, the contributions from the oxidized and reduced FeS_4 site were removed from the spectrum of the recombinant Rr, and the resulting spectrum is shown in Figure 4C. The close resemblance observed between spectra shown in Figure 4, parts B and C, indicates unambiguously that CRr contains only a diiron cluster and that this cluster must be very similar to that in recombinant Rr.

Redox Titrations Monitored by EPR. Redox titrations were performed on the recombinant Rr and on CRr by monitoring EPR signals at g = 4.3 for the FeS_4 site and g = 1.76 for the diiron site at a series of poised potentials. The changes in EPR signal intensity versus equilibrium potentials are plotted in Figure 6. These data were fitted to the Nernst equation for sets of one-electron, noninteracting redox couples, from which the midpoint potentials were determined. For the FeS_4 center of recombinant Rr, the midpoint potential was found to be +260 ± 10 mV vs NHE, which is within the range of potentials previously determined for the FeS_4 center of as-isolated Rr (+230 mV (LeGall et al., 1988) and +281 mV (Pierik et al., 1993)). For the diiron site of recombinant Rr, the midpoint potentials for diiron(III)-to-mixed-valent and mixed-valent-to-diiron(II) couples were determined to be +286 ± 5 and +222 ± 5 mV vs NHE, respectively. Pierik et al. (1993) determined corresponding diiron site midpoint potentials of +339 and +246 mV for as-isolated Rr. The corresponding midpoint potentials for the diiron site of CRr were determined to be +215 ± 5 and +154 ± 5 mV vs NHE, respectively. Thus, the ≈60 mV spread between the diiron(III)-to-mixed-valent and mixed-valent-to-diiron(II) potentials in recombinant Rr is maintained in CRr, and this spread allows a maximum of approximately 60% of the diiron sites to be obtained in the mixed-valent

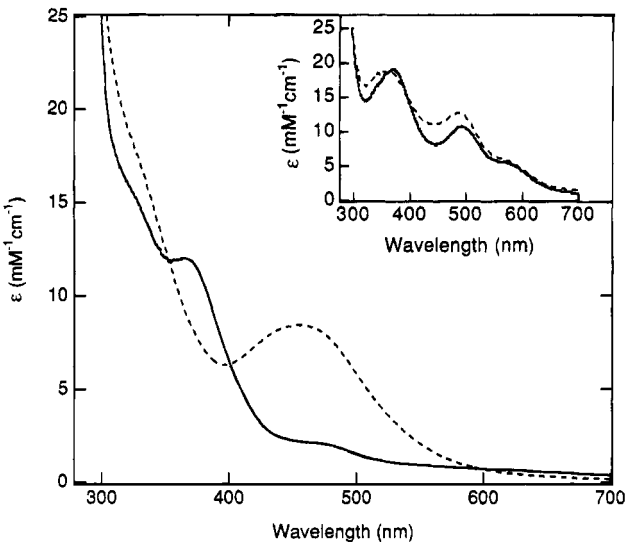


FIGURE 7: UV−vis absorption spectra of CRr in the absence (solid) or presence (dashed) of 0.2 M sodium azide. Inset: recombinant Rr (solid curve) and recombinant Rr plus 0.5 M azide (dashed). Buffer: 50 mM Hepes and 200 mM Na_2SO_4 (pH 7).

state for both proteins. However, there is an ≈70 mV decrease in the corresponding midpoint potentials for the diiron site of CRr vs those of recombinant Rr. Although this difference indicates that the presence of the FeS_4 domain in Rr affects the diiron site redox properties, no FeS_4/diiron site redox cooperativity was needed to fit the EPR titration data for recombinant Rr. Pierik et al. (1993) reached the same conclusion from their EPR titration data on as-isolated Rr, whereas a preliminary analysis by Moura et al. (1989) required such cooperativity to fit their EPR titration data on as-isolated Rr. The diiron site midpoint potentials determined here for recombinant Rr and CRr are considerably more positive than that reported for the two-electron reduction of the diiron(III) site in *E. coli* metRNR-R2 (−115 mV vs NHE at pH 7.6 (Silva et al., 1993)), and those reported for the two sequential one-electron reductions of the diiron(III) site of methane monooxygenase hydroxylase (+48, −135 mV vs NHE (Liu & Lippard, 1991) and +76, +21 vs NHE at pH 7 (Paulsen et al., 1994)). Also, the mixed-valent oxidation level of the diiron cluster is considerably less stable in *E. coli* RNR-R2 (Gerez & Fontecave, 1992; Sahlin et al., 1989; Hendrich et al., 1991a) than in Rr or methane monooxygenase hydroxylase. These observed differences in redox behavior are presumably due to differences in coordination and/or solvent environments among the various diiron clusters, but the exact nature of these differences is currently unknown. The diiron site midpoint potentials of methane monoxygenase hydroxylase change by 50−250 mV upon complexation with other protein components and substrates (Liu & Lippard, 1991; Paulsen et al., 1994). A similar sensitivity of the diiron site in Rr may be indicated by the ≈70 mV difference between the diiron site midpoint potentials of recombinant Rr vs those of CRr.

Diiron Site Reactivities of Recombinant Rr and CRr. In the presence of 80−500 mM sodium azide, the UV−vis absorption spectrum of CRr undergoes the changes shown in Figure 7. The peak at 365 nm is replaced by features with λ_{max} at 453 nm ($\epsilon \approx 8.5$ mM^{-1} cm^{-1} per homodimer) and ≈326 nm (sh). Very similar types of absorption spectral changes have been previously observed upon binding of azide to the diiron(III) sites in methemerythrin (Garbett et al., 1969), and in stearoyl-acyl carrier protein Δ^9-desaturase (Fox et al., 1993a). The feature at 453 nm in the absorption spectrum of the CRr azide adduct is assigned as azide-to-Fe(III) LMCT by analogy to these other proteins. The extinction coefficient at 453 nm for this CRr azide adduct (≈4.2 mM^{-1} cm^{-1} per diiron site) is very similar to the extinction at 447 nm for the azide adduct of methemerythrin (Garbett et al., 1969), in which one azide coordinates to each diiron site in a bent, end-on fashion (Stenkamp & Holmes, 1991). The mixed-valent EPR spectrum of CRr disappears in the presence of excess azide, and the Mössbauer spectrum of CRr plus excess azide obtained at 4 K in the absence of an applied field showed only a sharp symmetrical quadrupole doublet ($\Delta E_Q = 0.83$ mm/s, $\delta = 0.47$ mm/s, and linewdith = 0.30 mm/s), which is characteristic of a strongly antiferromagnetically coupled diiron(III) site. Comparison with the Mössbauer parameters listed above indicates that the (μ-oxo)diiron(III) cluster in CRr was perturbed by addition of azide. Thus, binding of azide apparently stabilizes the diiron(III) relative to the mixed-valent oxidation level of the diiron site in CRr. Relative stabilization of the diiron(III) oxidation level is reasonable if azide coordinates as N_3^- and displaces a neutral ligand such as H_2O; adding a negative charge should make the diiron site harder to reduce. Azide may bind to the diiron(III) site of recombinant Rr, but, perhaps because of the intense absorption band at 492 nm due to the FeS_4 center, no changes could be detected in the optical spectrum of recombinant Rr upon addition of excess azide up to 125 mM. However, addition of 500 mM azide to solutions of 50−100 μM recombinant Rr homodimer caused the spectral changes shown in the inset to Figure 8. The 492-nm peak shifts to 489 nm with an increase in absorbance, and changes are also evident between 400 and 300 nm. Such changes are consistent with formation of an azide adduct at the diiron site in recombinant Rr.

EPR spectra of reduced Rr and CRr (Figure 8) obtained at 4.2 K in parallel mode showed very weak signals at $g \approx 16$. Anaerobic addition of azide to these reduced proteins enhanced the signal intensity approximately 18-fold with minima at $g \approx 19.8$ for recombinant Rr and $g \approx 17.5$ for CRr. Very similar EPR signals have been previously observed for the azide adducts of the diiron(II) sites in hemerythrin (Reem et al., 1987; Hendrich et al., 1991b) and RNR-R2 (Elgren et al., 1993). These low-field signals have been assigned to EPR transitions originating from the ground state of an $S = 4$ spin system, which results from ferromagnetic coupling between the two $S = 2$ Fe(II) centers of the diiron cluster. Elgren et al. (1993) have proposed the presence of an aqua bridge and two carboxylato bridges in the diiron(II) azide adduct of RNR-R2.

In the absence of redox mediators, reduction of the iron centers in as-isolated Rr by stoichiometric amounts of sodium dithionite or sodium ascorbate was reported to be slow and often incomplete (Pierik et al., 1993). We have made similar observations for both recombinant Rr and CRr; under anaerobic conditions both proteins required several minutes to reduce completely with equimolar ascorbate or dithionite, as monitored by disappearance of the visible absorption spectrum. For this reason methyl viologen was added as a redox mediator to the samples of reduced proteins whose EPR spectra are shown in Figure 8. In the absence of redox mediators, reduction of the diiron sites with equimolar ascorbate or dithionite was qualitatively found to be slower

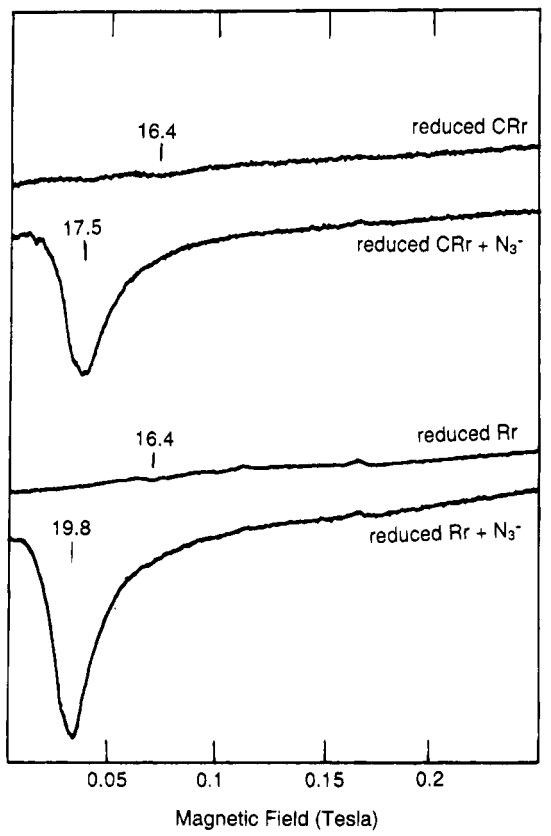


FIGURE 8: Parallel-mode X-band EPR spectra of 0.3 mM reduced recombinant Rr and CRr homodimers in 50 mM Hepes and 200 mM Na_2SO_4 without and with 0.13 M sodium azide as indicated by the labels near each spectrum. Sodium dithionite was 50 mM and methyl viologen was 100 μM in each solution. g values of low-field resonances are indicated. EPR conditions: temperature, 4.0 K; microwave frequency, 9.38 GHz; microwave power, 50 mW; modulation frequency, 100 KHz; modulation amplitude, 6.43 G; receiver gain, 2.5×10^4.

for CRr than for recombinant Rr, an observation consistent with the ≈70 mV more negative diiron site reduction potentials for CRr relative to recombinant Rr cited above.

While we have used CRr to delineate some properties of the diiron site in Rr, the physiological function of Rr remains unclear. *D. vulgaris* is known to possess a catalase and a superoxide dismutase (Hatchikan & LeGall, 1977) and can survive exposure to air for limited periods (Marschall et al., 1993). Therefore, interactions of Rr with O_2 or its immediate reduction products *in vivo* cannot be ruled out *a priori*. However, as-isolated Rr has been tested and found negative for catalytic activities characteristic of two other diiron proteins which utilize O_2 as a substrate, namely, *E. coli* RNR-R2 (Pierik et al., 1993) and methane monooxygenase hydroxylase (Prickril, 1992). No reports of tests for fatty acyl desaturase activity of Rr have appeared, and this activity remains a possibility. Tests of as-isolated Rr for catalase, superoxide dismutase, nitrate reductase, phosphatase, and pyrophosphatase activities were also reported to be negative (Pierik et al., 1993; Kurtz & Prickril, 1991; Prickril, 1992). Recently, a dimetal site in *E. coli* bacterioferritin has been described with a structure and amino acid sequence spacing of the metal ligands which are very similar to that proposed for Rr in Chart 1 (Frolow et al., 1994). No ferritin or ferritin-like protein has been described for anaerobic sulfate-reducing bacteria, although a ferritin-like protein has been described from one "obligately anaerobic" bacterium (Rocha et al., 1992). Thus, an iron storage function for Rr represents another possible *in vivo* activity. Further studies are in progress to delineate the structure and physiological function of Rr and the molecular determinants of its relatively high reduction potentials.

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