Spectroscopic Characterization of ^{57}Fe-Reconstituted Rubrerythrin, a Non-Heme Iron Protein with Structural Analogies to Ribonucleotide Reductase†

Natarajan Ravi,‡ Benet C. Prickril,§,‖ Donald M. Kurtz, Jr.,*,§ and Boi Hanh Huynh*,‡

Department of Physics, Emory University, Atlanta, Georgia 30322, and Department of Chemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602

Received March 31, 1993; Revised Manuscript Received June 15, 1993

ABSTRACT: Rubrerythrin, a contraction of rubredoxin and hemerythrin, is the trivial name given to a non-heme iron protein isolated from *Desulfovibrio vulgaris* (Hildenborough). This protein, whose physiological function is unknown, was first characterized by J. LeGall et al. [(1988) *Biochemistry 28*, 1636] as being a homodimer of subunit M_r = 21 900 with four Fe per homodimer distributed as two rubredoxin-type FeS_4 centers and one hemerythrin-type diiron cluster. Subsequent analysis of the amino acid sequence of the rubrerythrin gene [Kurtz, D. M., Jr., & Prickril, B. C. (1991) *Biochem. Biophys. Res. Commun. 181*, 137] revealed an internal homology which suggested that each subunit can accommodate one diiron cluster. Here, we report a procedure for reconstitution of the as-isolated *D. vulgaris* rubrerythrin with ^{57}Fe. The reconstituted protein was characterized by optical, electron paramagnetic resonance, and Mössbauer spectroscopies. The results indicate successful incorporation of ^{57}Fe into the two types of sites and strongly suggest that each subunit of rubrerythrin can indeed accommodate one diiron cluster as well as one rubredoxin-type center. Combined with amino acid sequence analysis, the spectroscopic characterization further suggests that the rubrerythrin subunit contains a diiron site whose structure is more closely related to that in ribonucleotide reductase than to that in hemerythrin.

Rubrerythrin (Rr)[1] is a non-heme iron protein of unknown function found in the anaerobic sulfate-reducing bacterium *Desulfovibrio* (*D.*) *vulgaris* (hildenborough). Initial characterization indicated that Rr is a homodimer with subunit molecular mass of 21.9 kDa and contains four iron atoms per homodimer (LeGall et al., 1988). Optical, EPR, and Mössbauer spectroscopic data on as-isolated Rr established that the four iron atoms are distributed as two rubredoxin (Rd)-type $Fe(SCys)_4$ sites and one diiron cluster (LeGall et al., 1988). This diiron cluster was found to exhibit spectroscopic and magnetic properties similar to those of the non-heme, non-sulfur (μ-oxo/hydroxo) diiron clusters found in hemerythrin, methane monooxygenase, subunit R2 of *Escherichia coli* ribonucleotide reductase (RNR-R2), and purple acid phosphatases [reviewed in Kurtz (1990), Wilkins (1992), Sanders-Loehr (1989), Que and True (1990), and Vincent et al. (1990)]. Since only one diiron cluster was found per two subunits of Rr, this cluster was proposed to possibly bridge the subunits in the homodimer. Subsequent Mössbauer measurements of samples of different preparations, however, showed a variable FeS_4 site to diiron cluster ratio between 1.4 and 2.0. More recently, Rr has also been found in *D. desulfuricans*, and preliminary Mössbauer measurements suggest that it may contain two diiron clusters per homodimer (P. Tavares, N. Ravi, I. Moura, M. Y. Liu, J. LeGall, J. J. G. Moura, and B. H. Huynh, unpublished data).

Subsequent to the initial characterization, the *D. vulgaris* Rr gene was cloned and sequenced (Prickril et al., 1991), and the amino acid sequence was also determined through classical amino acid sequencing methods (Van Beeumen et al., 1991). The amino acid sequences determined from both methods were identical to each other. Analysis of this sequence revealed two metal-center-binding regions for each subunit of Rr. The C-terminal portion, residues 153–191, shows sequence homology with those of rubredoxins, consistent with the spectroscopic findings. The N-terminal portion, residues 1–152, contains 2 highly homologous 34-residue regions, each of which contains a conserved EXXH sequence (Kurtz & Prickril, 1991). Since a pair of EXXH sequences are known to provide Glu and His ligands to the diiron cluster in RNR-R2 (Nordlund et al., 1990), it was proposed that the N-terminal region of the Rr subunit binds the diiron cluster and that residues Glu-53, His-56, Glu-128, and His-131 provide ligands to this cluster (Kurtz & Prickril, 1991). This sequence analysis strongly suggests that each subunit of Rr is capable of accommodating one diiron cluster, and that the Rr samples used in the initial spectroscopic characterization (LeGall et al., 1988) contained homodimers with unoccupied diiron sites. Recently, a new protein, named "nigerythrin", with spectroscopic properties similar to those of Rr has also been purified from *D. vulgaris* (Pierik et al., 1993). On the basis of results obtained from redox titrations monitored by EPR spin quantification, an approximately 1:1 ratio for the diiron cluster to the mononuclear FeS_4 center was inferred for both Rr and nigerthrin although only five irons per Rr homodimer were reported (Pierik et al., 1993).

This paper reports a procedure for reconstitution of the iron centers in *D. vulgaris* Rr from the apoprotein. The

† This work was supported by National Science Foundation Grant DMB-9001530 (to B.H.H.) and by National Institutes of Health Grants GM 40388 (to D.M.K.) and GM 47295 (to B.H.H.). D.M.K. is an NIH Research Career Development Awardee during 1988–1993 (HL 02077). This research is also supported in part by the University Research Committee of Emory University and by a Biomedical Research Support Grant (to B.H.H.) from the National Center for Research Resources. This paper is dedicated to Richard H. Holm on the occasion of his 60th birthday.

* Authors to whom correspondence should be addressed.

‡ Emory University.

§ University of Georgia.

‖ Present address: NIH/NCI, Building 37, Room 4A13, Bethesda, MD 20892.

[1] Abbreviations: Rr, rubrerythrin; Rd, rubredoxin; GuHCl, guanidine hydrochloride; EPR, electron paramagnetic resonance; RNR-R2, *E. coli* ribonucleotide reductase R2 subunit.

0006-2960/93/0432-8487$04.00/0

reconstituted protein has been characterized by optical, EPR, and Mössbauer spectroscopies. The results indicate successful incorporation of iron into both types of sites. Moreover, each homodimer was found to contain two FeS_4 sites and two diiron clusters, as predicted from the amino acid sequence analysis. Further comparisons of the amino acid sequence of Rr with that of RNR-R2, whose diiron site structure has been solved by X-ray crystallography (Nordlund et al., 1990), show a significant degree of homology between the two proteins and allow a proposed structure for the diiron cluster-binding region in Rr.

MATERIALS AND METHODS

Protein Purification. The growth of *D. vulgaris* (Hildenborough) and the purification of Rr were performed as previously described (LeGall et al., 1988). The as-isolated *D. vulgaris* Rr in 0.1 M Tris-HCl, pH 7.6 (hereafter referred to as buffer), was concentrated at 4 °C to ~11 mg/mL before use.

Reconstitution of D. vulgaris Rr. Reconstitution was carried out using a method very similar to that described by Zhang et al. (1991) for reconstitution of the diiron cluster of hemerythrin. Unless otherwise noted, all manipulations and reactions were carried out at room temperature using 10-mL septum-capped glass vials connected to a vacuum/argon manifold via hypodermic needles. All solutions were made anaerobic by repeated vacuum-degassing/argon-flushing cycles. Argon gas was passed over a heated column of BASF catalyst (Schwarz/Mann) and a column of anhydrous calcium sulfate (Drierite) to remove traces of oxygen and water, respectively. Hamilton gas-tight syringes or sterile plastic syringes were used for all anaerobic transfers. Equilibration and concentration of both apo and reconstituted forms of Rr were performed at 4 °C using either a 10-mL stirred-flow concentrator (Amicon, YM-10 membrane) pressurized with argon or, for aerobic procedures, a Centricon-10 microconcentrator. A Cole–Palmer glass microelectrode or pH paper (for samples of anaerobic solutions) was used for pH determinations.

ApoRr was prepared as follows: 0.6 mL (7 mg) of as-isolated Rr in buffer, 0.1 mL of 0.1 M 2,2′-dipyridyl stock solution, and 0.73 g of GuHCl were combined in a septum-capped 10-mL glass vial and made anaerobic. Ten milligrams of solid sodium dithionite was added directly to the vial under a flow of argon. The solution changed from nearly colorless to a bright red immediately upon addition of dithionite, indicating formation of the tris(bipyridyl)Fe(II) complex (ϵ_{522} = 8.65 mM^{-1} cm^{-1}) (Smith & Wilcox, 1942). The solution was equilibrated under argon for 30 min before exposing it to air, and the subsequent steps were performed aerobically. The solution volume was adjusted to 5 mL with 6 M GuHCl, transferred to a 10-mL stirred-flow concentrator, and concentrated to less than 1 mL. This process of dilution and reconcentration was repeated 3 times to remove excess reagent. The colorless apoRr solution was then transferred to a Centricon-10 microconcentrator, diluted to 2 mL with 6 M GuHCl, concentrated to approximately 0.1 mL, and stored at 4 °C. The apoprotein concentration was estimated using a value of ϵ_{280} = 72.8 mM^{-1} cm^{-1} and 6 M GuHCl as a reference solution. This extinction coefficient for apoRr was assumed to be equal to that of the as-isolated protein based on an A_{280}/A_{492} value of 7 and ϵ_{492} = 10.4 mM^{-1} cm^{-1} for as-isolated Rr (LeGall et al., 1988). (This value of ϵ_{280} for apoRr is probably an overestimate, since the contribution of the iron sites is ignored.)

For the reconstitution of Rr with ^{57}Fe, a solution of ^{57}Fe was made as follows: One milligram of the metal was added to 0.1 mL of concentrated HCl in a 5-mL glass vial and heated at 80 °C on a water bath until dissolved. The solution was then cooled to room temperature and made anaerobic by repeated vacuum/argon cycles. Ten microliters of 2-mercaptoethanol was added, and the solution was then neutralized to pH 7.5 by dropwise addition of an anaerobic solution of 1 M Tris base. Iron(III) was reduced to iron(II) during neutralization as judged by the absence of any $Fe(OH)_3$ precipitate. This solution was used immediately after preparation. Approximately half of this anaerobic ^{57}Fe solution (0.5 mg of iron in 0.7 mL) was added dropwise to a 10-mL vial containing 0.5 mL of the anaerobic apoRr solution over a period of 5 min. The amount of added ^{57}Fe represents a 10-fold molar excess of iron over that in as-isolated Rr. The solution was allowed to equilibrate for 30 min, after which anaerobic buffer was added dropwise with mixing over a period of 30 min to raise the total volume to 5 mL. The diluted solution was then transferred anaerobically to the 10-mL stirred-flow concentrator and concentrated under argon until the volume was less than 1 mL. This concentrated solution was diluted with buffer to 5 mL and reconcentrated to 1 mL 3 times in order to remove GuHCl, excess Fe(II), and 2-mercaptoethanol. The solution was then transferred aerobically to a Centricon-10 microconcentrator and subjected to two additional 30-fold concentration/dilution cycles before a final concentration to approximately 100 μL. A portion of this concentrated sample was removed for iron and protein analyses, and the remainder was diluted to 300 μL for Mössbauer and EPR studies. The reconstituted Rr samples were stored at −20 °C.

Spectroscopic Methods. UV–visible absorption spectra were recorded on a Perkin-Elmer Model 554 spectrophotometer or a Shimadzu UV-210PC spectrophotometer. Electron paramagnetic resonance (EPR) measurements were performed on a Bruker ER 200-SRC spectrometer equipped with an Oxford Instrument continuous-flow cryostat. Mössbauer spectra were recorded on either a strong-field or a weak-field spectrometer operating in a constant acceleration mode in a transmission geometry. The zero velocity of the Mössbauer spectra is referred to the centroid of the room-temperature spectrum of a metallic iron foil. A Mössbauer sample was prepared by placing the ^{57}Fe-reconstituted Rr solution (300 μL in volume and 525 μM in Rr homodimer) into a shallow delrin sample cup and freezing with liquid nitrogen.

Iron and Protein Determinations. Iron concentration was determined by a chemical method (*The Iron Reagents*, 1980) and by inductively coupled plasma emission spectroscopy. For the chemical method, the following reagents were used: 1 M sodium acetate, 10 mg/mL bathophenanthrolinedisulfonic acid (GFS Chemical Co.) in water, and 1 M sodium dithionite (BDH Chemical Co.). The protein sample (1–100 μL containing 0–20 nmol of iron) was placed into a clean glass or plastic tube, and 1 mL of sodium acetate was added. Then, 0.1 mL each of bathophenanthroline and dithionite solutions was added and the tube briefly vortexed. The absorbance at 535 nm was recorded after equilibration for at least 0.5 h at room temperature. The iron concentration was calculated using the extinction coefficient of the Fe(II)–bathophenanthroline complex [ϵ_{535} = 22.14 mM^{-1} cm^{-1} (Kirschenbaum, 1976)]. For the plasma emission method, 1-mL samples of reconstituted Rr in buffer containing 0.5–5 ppm of Fe were analyzed on a Jarrel–Ash Model ICAP-965 instrument. Each set of measurements were performed on three samples with different protein concentrations, and a blank solution of the sample buffer was used as reference.

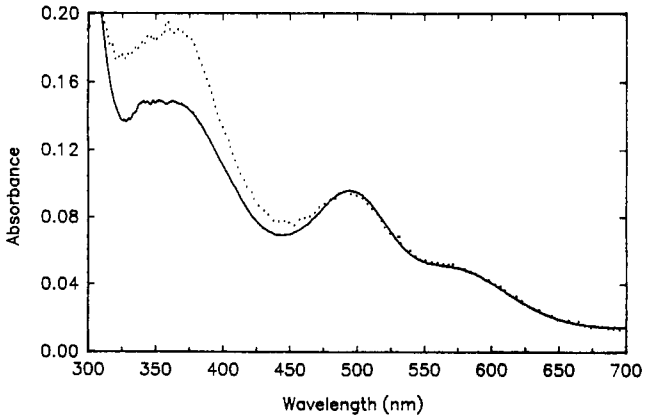

FIGURE 1: Optical absorption spectra of as-isolated (*solid line*) and reconstituted (*dotted line*) *D. vulgaris* rubrerythrin. The spectra are normalized to have the same absorption at 492 nm.

The protein concentration was determined by amino acid composition analysis at the University of Georgia Molecular Genetics Instrumentation Facility. In order to determine losses due to hydrolysis and/or sample manipulation, samples of bovine serum albumin of known concentration were analyzed along with the Rr samples of known A_{492}.

RESULTS AND DISCUSSION

UV/Vis Spectrum and Iron Content. A value of ϵ_{492} = 10.6 mM^{-1} cm^{-1} was determined for the oxidized ^{57}Fe-reconstituted Rr homodimer from the amino acid quantitation. This value is in excellent agreement with the value of 10.4 mM^{-1} cm^{-1} previously determined for as-isolated Rr (LeGall et al., 1988) and was therefore used in estimating the concentration of the reconstituted protein. Figure 1 shows an UV/Vis spectrum of ^{57}Fe-reconstituted Rr in comparison with that of as-isolated Rr. The spectra are normalized to have the same peak intensity at 492 nm. The reconstituted Rr exhibits a UV/Vis absorption spectrum very similar to that of as-isolated Rr with maxima at 492 and 365 nm and shoulders at 570 and 350 nm. However, the reconstituted Rr shows higher absorption below 450 nm. This increase in absorption is not due to a tailing of the protein absorption, since A_{280}/A_{492} was found to be 6.5 for the reconstituted protein, compared to 7.0 for the as-isolated protein. By comparison with the spectrum of rubredoxin which contains only the FeS_4 center, previous analysis of the as-isolated Rr spectrum (LeGall et al., 1988) suggests that the diiron cluster contributes significant absorption below 450 nm with a maximum at approximately 365 nm. The increase in absorption in this region is therefore an indication of an increase in the amount of the diiron cluster relative to that of the FeS_4 center in the reconstituted protein, a result that is in agreement with other data presented below.

Iron analysis has been performed on the as-isolated protein used in the optical measurement presented above. Consistent with the result reported in the initial characterization of Rr (LeGall et al., 1988), a value of 4.0 ± 0.2 Fe per homodimer was found, suggesting the presence of unoccupied iron sites. Iron analysis of the ^{57}Fe-reconstituted Rr gave a value of 7.5 ± 0.4 mol of Fe per mole of Rr homodimer. This value is higher than the value, 6.0, expected for fully occupied iron sites, suggesting that approximately 20% of the iron is adventitiously bound to the reconstituted Rr. This suggestion is supported both qualitatively and quantitatively by the following Mössbauer data, which show iron absorption of unknown origin in addition to those originating from the Rd-type centers and diiron clusters.

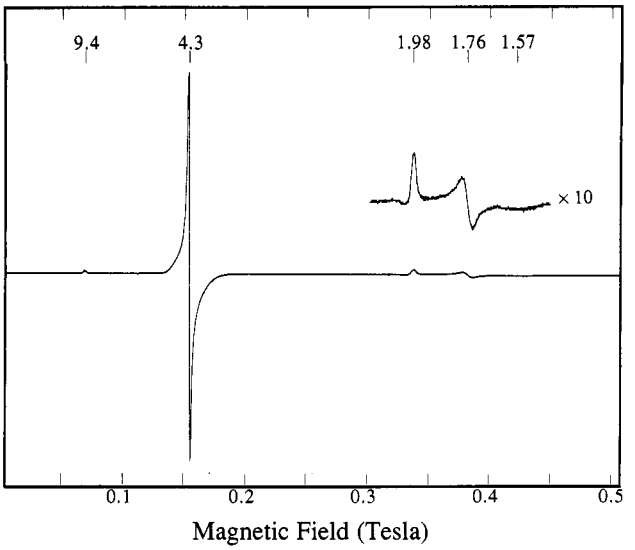

FIGURE 2: EPR spectrum of ^{57}Fe-reconstituted *D. vulgaris* rubrerythrin. Experimental conditions: temperature, 4.2 K; microwave frequency, 9.43 GHz; microwave power, 0.2 mW; modulation amplitude, 1 mT; receiver gain, 4.0×10^3.

EPR and Mössbauer Data. Figure 2 shows a 4.2 K EPR spectrum of the reconstituted Rr. The spectrum is practically identical to that of the as-isolated Rr (LeGall et al., 1988), exhibiting strong signals at g = 9.4 and 4.3 arising from the ground- and excited-Kramers doublets of the Rd-type center, and a weak but characteristic signal for a mixed-valent diiron cluster at g = 1.98, 1.76, and 1.57. Spin quantification of the diiron cluster signal yields 0.5 spin per homodimer. The similarity observed between this EPR spectrum and that of the as-isolated protein strongly indicates that the iron centers are successfully incorporated. In the as-isolated protein, the majority of the diiron clusters were found to be in the EPR-silent diferric state (LeGall et al., 1988). Consequently, the less than stoichiometric amount of the mixed-valent-cluster EPR signal observed for the reconstituted protein does not represent insufficient incorporation of iron into the diiron sites, but rather suggests that the redox properties of the reconstituted diiron cluster are very similar to those of the as-isolated protein.

Figure 3 shows a 4.2 K Mössbauer spectrum of the reconstituted Rr recorded in a parallel field of 50 mT. Spectral components characteristic of the Rd-type FeS_4 center (a six-line magnetic spectrum extending from −5.5 to +6.4 mm/s) and oxo-bridged diiron cluster (the central quadrupole doublet) are observed. This spectrum is very similar to that of the as-isolated Rr except for the increase in intensity of the central quadrupole doublet and the presence of an additional broad magnetic spectrum attributable to adventitiously bound iron.[2] Mössbauer spectra (data not shown) recorded with strong applied fields (2–8 T) further indicate that the electronic state of the reconstituted Rd-type center can be characterized with the same spin-Hamiltonian parameters obtained previously for the FeS_4 center of the as-isolated protein (LeGall et al., 1988) and that the central quadrupole doublet originates from a diamagnetic state, as expected for an antiferromagnetically

[2] We have attempted to remove the "adventitious" iron by applying the gel filtration technique and ion-exchange chromatography (using DE-52), but were unsuccessful. Recently, we have cloned and expressed the gene coding for *D. vulgaris* Rr in *E. coli*. ^{57}Fe reconstitution of this cloned Rr by a procedure very similar to that described here results in a protein whose Mössbauer spectrum does *not* show the absorption attributable to "adventitious" iron, but *does* show the absorptions attributed to FeS_4 and diiron cluster in a 1:2 ratio (D. M. Kurtz, Jr., F. Bonomi, N. Ravi, and B. H. Huynh, unpublished results).

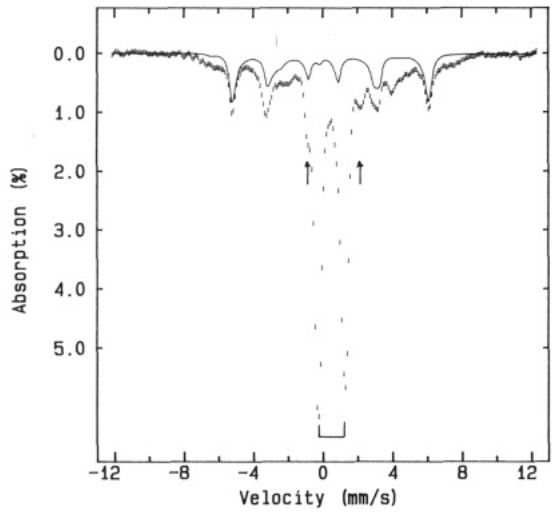

FIGURE 3: Mössbauer spectrum of ^{57}Fe-reconstituted *D. vulgaris* rubrerythrin measured at 4.2 K with a parallel applied field of 50 mT. The solid line is a theoretical simulation of the oxidized Rd-type center and is normalized to 22% of the total Fe absorption. The arrows indicate the positions of the quadrupole doublet originating from the reduced Rd-type center, and the bracket indicates the quadrupole doublet of the diferric cluster.

coupled diferric cluster. In order to estimate the amounts of different types of iron in the reconstituted Rr, the following analysis was carried out.

To estimate the amount of oxidized Rd-type center, a theoretical spectrum was simulated using the parameters previously reported for the as-isolated protein (LeGall et al., 1988). Since the six-line magnetic spectrum of the oxidized FeS_4 center is relatively sharp in comparison with that of the adventitiously bound iron, its percent Mössbauer absorption can be estimated with reasonable accuracy. We found that $22 \pm 2\%$ of the absorption is attributable to the oxidized FeS_4 center. The *solid line* in Figure 3 is the theoretical simulation of Rd-type center normalized to 22% of the total absorption. In Rr, as-isolated from *D. vulgaris*, a small portion of the FeS_4 center was found to be reduced (LeGall et al., 1988), and this reduced FeS_4 center shows a characteristic quadrupole doublet at 4.2 K with parameters $\Delta E_Q = 3.15$ mm/s and $\delta = 0.70$ mm/s. The FeS_4 centers in the reconstituted protein also appear to be partially reduced, as indicated by Mössbauer peak at +2.28 mm/s and a shoulder at –0.88 mm/s. The percent Mössbauer absorption originating from the reduced FeS_4 centers in the ^{57}Fe-reconstituted Rr was estimated to be $3 \pm 1\%$. Consequently, the total contribution to the Mössbauer absorption from the FeS_4 center is 25%. On the basis of the iron determination of 7.5 Fe per molecule, this percentage indicates approximately *two* FeS_4 centers per homodimer in the reconstituted protein. This result is consistent with that of the as-isolated protein (LeGall et al., 1988) and is expected from amino acid sequence analyses (Prickril et al., 1991; Van Beeumen et al., 1991).

To estimate the amount of diiron cluster in the ^{57}Fe-reconstituted protein, we first removed the contributions from the oxidized and the reduced FeS_4 center. The central portion of the spectrum was then least-squares-fitted with two quadrupole doublets of equal intensity. The parameters obtained were $\Delta E_Q = 1.74$ mm/s and $\delta = 0.55$ mm/s for doublet 1, and $\Delta E_Q = 1.38$ mm/s and $\delta = 0.53$ mm/s for doublet 2. These parameters are very similar but not identical to those of the diferric cluster in the as-isolated protein. The percent contribution arising from the differic clusters in the reconstituted protein was estimated to be $42 \pm 4\%$. As evidenced from the EPR data presented above, a fraction of

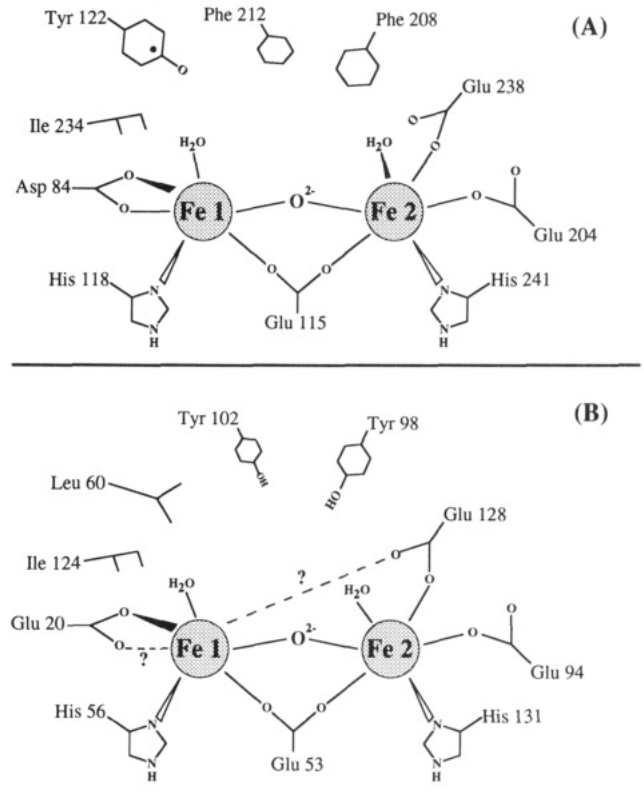

FIGURE 4: Schematic representation of (A) the diiron site in *E. coli* ribonucleotide reductase R2 subunit (Nordlund et al., 1990) and (B) the proposed structure of the diiron site in *D. vulgaris* rubrerythrin.

the diiron clusters are in the mixed-valent Fe(II)–Fe(III) state. Unfortunately, at 4.2 K the Mössbauer spectrum of the mixed-valent cluster cannot be resolved from the spectrum of the adventitiously bound iron. At higher temperatures (above 77 K), however, the electronic relaxation is fast such that the spectrum of the mixed-valent cluster consists of two quadrupole doublets, corresponding to the Fe(II) and Fe(III) ions. The high-energy line of the Fe(II) doublet is well resolved from the rest of the spectrum and can be used for quantification. From a 190 K spectrum (not shown), a contribution of 12% absorption was estimated for the mixed-valent cluster. On the basis of a value of 7.5 Fe/homodimer, 12% absorption for a mixed-valent diiron cluster corresponds to 0.45 spin per homodimer, a value that is in very good agreement with the EPR spin quantification. Consequently, the total contribution to the Mössbauer absorption from the diiron cluster is estimated to be 54%, which yields almost exactly *two* diiron clusters per homodimer. This result is consistent with the amino acid sequence analysis and strongly suggests that half of the diiron sites (presumably one of the two in each homodimer) in the previously reported as-isolated *D. vulgaris* Rr are unoccupied. Note that the Mössbauer absorption quantification of the ^{57}Fe-reconstituted Rr leaves 21% attributable to adventitiously bound iron. This percentage is in excellent agreement with that estimated above from the iron analysis.

Proposed Diiron Site Structure. The stoichiometry of one diiron cluster per subunit in the Rr homodimer demonstrated in this work strongly supports the proposal (Kurtz & Prickril, 1991) that a pair of EXXH sequences provides Glu and His ligands to the diiron cluster. Figure 4 takes this suggestion a step further by comparing the known diiron site structure in RNR-R2 (Figure 4A) with an analogous structure proposed for the diiron site in *D. vulgaris* Rr (Figure 4B). This proposed structure is based on several observations. The inclusion of an oxo bridge is consistent with the absorption in the 365-nm

region referred to above and with an $H_2{}^{18}O$-sensitive vibrational frequency observed in resonance Raman spectra of Rr (Prickril et al., 1989; Czernuszewicz et al., 1993). These spectroscopic features are very similar to those found for the diiron cluster of RNR-R2 (Kurtz, 1990; Wilkins, 1992; Sanders-Loehr, 1989; Que & True, 1990; Vincent et al., 1990). The sequence spacing of key amino acid residues proposed to be at or near the diiron site in Rr is strikingly similar to the spacing of analogous residues in RNR-R2. Thus, the E53-X-X-H56 and E128-X-X-H131 sequences of Rr are proposed to be structurally analogous to E115-X-X-H118 and E238-X-X-H241 sequences, respectively, in RNR-R2. On the basis of the spacing of nearest-neighbor carboxylate ligands in RNR-R2 (E238 – E204 = 34 residues; E115 – D84 = 31 residues) and either the same or similar spacing in Rr (E128 – E94 = 34 residues; E53 – E20 = 33 residues), E94 and E20 are proposed to provide terminal carboxylate ligands in Rr (cf. Figure 4). Relative to the more C-terminal EXXH sequences, residues Y98, Y102, and I124 in Rr are sequentially homologous to F208, F212, and I234 in RNR-R2.

While these similarities are strking, some differences should also be noted. The sequence spacing between the two sets of EXXH sequences in RNR-R2 (119 residues) is considerably longer than that for Rr (72 residues). The mixed-valent oxidation level of the diiron cluster in Rr, as embodied in the EPR spectrum of Figure 2, appears to be considerably more stable than that in RNR-R2 (Gerez & Fontecave, 1992; Sahlin et al., 1989; Hendrich et al., 1991; Bollinger et al., 1991). This difference in stability presumably reflects differences in coordination and/or solvent environments of the diiron clusters in the two proteins. Also, the Mössbauer spectrum of the diferric cluster in RNR-R2 shows two well-resolved sharp quadrupole doublets (Lynch et al., 1989), indicating distinct coordination environments for the two ferric sites. The diferric cluster in Rr, on the other hand, exhibits a broad and unresolved quadrupole doublet similar to that of hemerythrin (Okamura et al., 1969; Kurtz, 1990; Wilkins, 1992), suggesting a more symmetric environment for the ferric ions. A recent X-ray crystallographic study of manganese-substituted RNR-R2 revealed that the dinuclear manganese cluster, which occupies the iron cluster-binding site, is bridged by not one but two carboxylates from E115 and E238 (Atta et al., 1992). On the basis of this observation, and evidence from model compound studies (Rardin et al., 1992), a mechanism involving a shift of the E238 carboxyalte from a terminal ligand to a bridging ligand is proposed for reduction of the diiron cluster in RNR-R2 (Atta et al., 1992). In the case of Rr, it is possible that the diferric cluster is bridged by both E53 and E128 with E20 and E94 as terminal monodentate ligands, forming a more symmetric coordination environment (this alternative proposal is also indicated in Figure 4 by dashed lines).

The sequence comparison predicts that Y122 in RNR-R2, which is known to form a stable tyrosyl radical, and which is ~5 Å from the nearest iron (Nordlund et al., 1990), has L60 as its counterpart in Rr (i.e., four residues C-terminal to the nearest His ligand; cf. Figure 4). The currently available data on Rr provide no evidence for a stable organic radical or for any ribonucleotide reductase activity. Therefore, the apparent structural similarity between the diiron sites in Rr and RNR-R2 may not extend to function. A similar comparison with amino acid sequences of methane monooxygenases led to the proposal of a diiron site structure very similar to that in RNR-R2, but with a cysteine residue occupying the position analogous to Y122 in RNR-R2 (Nordlund et al., 1992). Thus, despite the striking similarities in the diiron sites of the three aforementioned proteins, these differences point to distinct functions for each site. Further studies aimed at delineating the structure and function of the diiron site in Rr are in progress in our laboratories.

ACKNOWLEDGMENT

We thank Dr. Jean LeGall for his generosity for providing the rubrerythrin used in this study and Mr. Ming-Yin Liu for his assistance in purifying the protein.

REFERENCES

Atta, M., Nordlund, P., Aberg, A., Eklund, H., & Fontecave, M. (1992) *J. Biol. Chem. 267*, 20682–20688.
Bollinger, J. M., Edmondson, D., Huynh, B. H., Filley, J., Norton, J. R., & Stubbe, J. (1991) *Science 253*, 292–298.
Czernszewicz, R., Kurtz, D. M., Jr., Johnson, M. K., Prickril, B. C., & LeGall, J. (1993) *J. Biol. Chem.* (submitted for publication).
Gerez, C., & Fontecave, M. (1992) *Biochemistry 31*, 780–786.
Hendrich, M. P., Elgren, T. E., & Que, L., Jr. (1991) *Biochem. Biophys. Res. Commun. 176*, 705–710.
Kirschenbaum, D. M. (1976) in *Handbook of Biochemistry and Molecular Biology* (Fasman, G. D., Ed.) 3rd ed., Vol. 2, p 383, CRC Press, Cleveland, OH.
Kurtz, D. M., Jr. (1990) *Chem. Rev. 90*, 585–606.
Kurtz, D. M., Jr., & Prickril, B. C. (1991) *Biochem. Biophys. Res. Commun. 181*, 337–341.
LeGall, J., Prickril, B. C., Moura, I., Xavier, A. V., Moura, J. J. G., & Huynh, B. H. (1988) *Biochemistry 27*, 1636–1642.
Lynch, J. B., Juarez-Garcia, C., Münck, E., & Que, L., Jr. (1989) *J. Biol. Chem. 264*, 8091–8096.
Nordlund, P., Sjöberg, B.-M., & Eklund, H. (1990) *Nature 3*, 593–598.
Nordlund, P., Dalton, H., & Eklund, H. (1992) *FEBS Lett. 207*, 257–262.
Okamura, M. Y., Klotz, I. M., Johnson, C. E., Winter, M. R. C., & Williams, R. J. P. (1969) *Biochemistry 8*, 1951–1958.
Pierik, A. J., Wolbert, R. B. G., Portier, G. L., Verhagen, M. F. J. M., & Hagen, W. R. (1993) *Eur. J. Biochem. 212*, 237–245.
Prickril, B. C., Kurtz, D. M., Jr., LeGall, J., Johnson, M. K., Huang, H.-Y., & Czernuszewicz, R. (1989) *J. Inorg. Biochem. 36*, 228.
Prickril, B. C., Kurtz, D. M., Jr., LeGall, J., & Voordouw, G. (1991) *Biochemistry 30*, 11118–11123.
Que, L., Jr., & True, A. E. (1990) in *Progress in Inorganic Chemistry* (Lippard, S. J., Ed.) Vol. 38, pp 97–200, Wiley & Sons, New York.
Rardin, R. L., Poganiuch, P., Bino, A., Goldberg, D. P., Tolman, W. B., Liu, S., & Lippard, S. J. (1992) *J. Am. Chem. Soc. 114*, 5240–5249.
Sahlin, M., Graeslund, A., Petersson, L., Ehrenberg, A., & Sjöberg, B.-M. (1989) *Biochemistry 28*, 2618–2625.
Sanders-Loehr, J. (1989) in *Iron Carriers and Iron Proteins* (Loehr, T. M., Ed.) pp 373–466, VCH Publishers, New York.
Smith, G. F., & Wilcox, S. (1942) *Ind. Eng. Chem., Anal. Ed. 14*, 49.
The Iron Reagents (1980) pp 14–15, G. Frederick Smith Chemical Co., Columbus, OH.
Van Beeumen, J. J., Van Driessche, G., Liu, M.-Y., & LeGall, J. (1991) *J. Biol. Chem. 266*, 20645–20653.
Vincent, J. B., Olivier-Lilley, G., & Averill, B. A. (1990) *Chem. Rev. 90*, 1447–1467.
Wilkins, R. G. (1992) *Chem. Soc. Rev.* 171–178.
Zhang, J.-H., Kurtz, D. M., Jr., Xia, Y.-M., & Debrunner, P. G. (1991) *Biochemistry 30*, 583–589.