The Activating Component of the Anaerobic Ribonucleotide Reductase from *Escherichia coli*

AN IRON-SULFUR CENTER WITH ONLY THREE CYSTEINES*

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Class III anaerobic ribonucleotide reductase small component, named protein *b*, contains a (4Fe-4S) center. Its function is to mediate electron transfer from reduced flavodoxin to *S*-adenosylmethionine, required for the introduction of a glycy radical in the large component, named protein *a*, which then becomes active for the reduction of ribonucleotides. By site-directed mutagenesis we demonstrate that the three cysteines of the conserved CXXXCXXC sequence are involved in iron chelation. Such a sequence is also present in the active site of the pyruvate formate-lyase and in the biotin synthase, both carrying an iron-sulfur center involved in reductive activation of *S*-adenosylmethionine. Even though they are able to bind iron in the (4Fe-4S) form, as shown by Mössbauer spectroscopy, the corresponding Cys to Ala mutants are catalytically inactive. Mutation of the two other cysteines of the protein did not result in inactivation. We thus conclude that the (4Fe-4S) cluster has, in the wild type protein, only three cysteine ligands and a fourth still unidentified ligand.

During anaerobic growth, *Escherichia coli* depends on a class III ribonucleotide reductase for the synthesis of the deoxyribo- nucleotides, required for DNA synthesis (1). The enzymatic system catalyzes the reduction of ribonucleoside triphosphates into the corresponding deoxyribonucleotides by formate (2). It is essential during the process that makes it catalytically competent, is the introduction of an air-sensitive glycy radical at position 681 (7). As a consequence, the activated enzyme is active only under strict anaerobic conditions.

Protein *b* is an iron-sulfur protein, also sensitive to oxygen. Under strict anaerobic and reductive conditions it can assemble a (4Fe-4S) center, which can enjoy both (4Fe-4S)$^2^+$ and (4Fe-4S)$^-^1$ redox states (6, 8). Protein *b* is essential during anaerobic activation of protein *a* because it catalyzes the one-electron transfer from reduced flavodoxin to AdoMet required for the formation of the glycy radical. As a matter of fact it has been shown that the reduced (4Fe-4S)$^-^1$ cluster is able to reduce AdoMet, and it is postulated that this reaction results in the homolytic cleavage of its S-(5′-deoxyadenosyl) bond and formation of a 5′-deoxyadenosyl radical, responsible for H atom abstraction at the specific glycine residue (9).

Under exposure to air, protein *b* stabilizes (2Fe-2S)$^2^+$ centers instead, which under anaerobic and reductive conditions are transformed back into active (4Fe-4S) centers (10).

The combination of an iron-sulfur center and AdoMet for generating free radicals appears to be a general strategy in biological systems. It is now quite well established that such a chemistry is indeed utilized also in the pyruvate formate-lyase system and in the biotin synthase (11, 12). Even though there is no amino acid sequence homology between these systems it has been suggested that the cysteines of the CXXXCXXC motif common to biotin synthase and the activating components of pyruvate formate-lyase and ribonucleotide reductase (Fig. 1A) provide a specific metal binding site in each of these enzymes (11). The Fe-S enzyme lipoate synthase also contains this motif but has not been shown yet to require AdoMet for activity (13, 14). The lysine aminomutase belongs to this class of enzymes but is not included in Fig. 1 because its amino acid sequence is unknown (15).

Considering the limited knowledge of the mechanisms involved in the iron-dependent activation of AdoMet and the importance of such a chemistry in the anaerobic ribonucleotide reductase system, we found it crucial to investigate further the structural and reactivity properties of the iron-sulfur center of protein *b*. In this work we changed the five cysteines of protein *b* into alanines in order to identify the ligands for the iron center. We actually demonstrate that only the three cysteines Cys-26, Cys-30, and Cys-33 of the CXXXCXXC motif conserved in all known ribonucleotide reductase sequences, a few of which are displayed in Fig. 1B, are important for activity.

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1 The abbreviations used are: DTT, dithiothreitol; AdoMet, *S*-adenosylmethionine; DAF, 5-deaza-7,8-dimethyl-10-methylisooloxazine; T, tesla.
**Fig. 1.** Panel A, the cysteines motif compared to the activating components of the E. coli anaerobic ribonucleotide reductase (NRDG) and pyruvate formate lyase (PFLA), E. coli biotin synthase (BIOS), and E. coli lipoate synthase (LIPA). Panel B, comparison of the sequences of the terminal half of class III ribonucleotide reductase (T4) and bacteriophage T4 (BPT4). The three conserved cysteines are underlined. In the E. coli sequence, the mutated cysteines are in bold characters.

**Table 1**

**Generation of mutated genes**

| Mutation  | Mutagenic oligonucleotides
|-----------|-----------------------------|
| C19A      | 5′-CAGGGTTGACAGGTTGCCG-3′   | Cs |
|           | 5′-GGCGACCTCTGGCAAAGATG-3′ | Ncs |
| C26A      | 5′-ATGACAGGCAGCAGGAGC-3′    | Cs |
|           | 5′-GTCTCCCGGGGCGTCGATG-3′ | Ncs |
| C30A      | 5′-TACGACGCGCGCGCGCGG-3′   | Cs |
|           | 5′-TGGAGACCGCGCGCGTCC-3′  | Ncs |
| C33A      | 5′-TTTGTATAGGCCGACCACG-3′  | Cs |
|           | 5′-CCCGGCTCTATACAAAC-3′   | Ncs |
| C36A      | 5′-GCTTTACCCCCCGGAGCG-3′   | Cs |
|           | 5′-GAGGCTCCGGGGAATAGG-3′  | Ncs |

Mutations are noted with the one-letter code for amino acids. The first letter indicates the original residue, the following number is its position in the sequence, and the second letter is the substituting residue.

**Mutated bases are underlined.**

**EXPERIMENTAL PROCEDURES**

**Materials**—Enzymes and other components of the anaerobic ribonucleotide reductase system have been obtained as described previously (6). Fe(III)Cl₃ was converted into its chloride form by dissolving it in a hot concentrated (35%) hydrochloric acid of analytical grade (Carlo Erba) and repetitively concentrated in water. Fe(NH₄)₂(SO₄)₂ was from Aldrich. AdoMet was from Aldrich. 7,8-demethyl-10-methylisoalloxazine (DAF) was available in our laboratory.

**Strains and Plasmids**—E. coli strain BL21(DE3) was used as a host for the overexpression of protein β (wild type or mutants). E. coli DH5α was used for routine plasmid manipulations. Plasmid pN9, the pET-3b derivative that contains the nrdG gene (5), was used as a template for site-directed mutagenesis.

**Construction of Mutants**—Site-directed mutagenesis of pN9 was performed using a polymerase chain reaction overlap extension procedure with oligonucleotides synthesized by Eurogentec and listed in Table I (16). Polymerase chain reaction final reaction products, identified and purified by agarose gel electrophoresis, were purified using the QIAEX gel extraction kit from Qiagen, digested with BamHI and SphI, and the second at position 27 (GTT instead of GTC) and the first letter indicates the original residue, the following number is its position in the sequence, and the second letter is the substituting residue.

**Mutations are noted with the one-letter code for amino acids. The first letter indicates the original residue, the following number is its position in the sequence, and the second letter is the substituting residue.**

**Mutated bases are underlined.**

**Ribonucleotide Reductase Activity**—Activity assays were performed under anaerobic conditions as described previously. One unit of enzyme activity is defined as the formation of 1 nmol of dTTP/min (3, 6).

**Binding Experiments**—1 mg of protein α was incubated for 1 h at 4 °C with a stoichiometric amount of the different β mutants in the presence of 2 mM DTT. These preparations were applied to a 4-ml dATP-Sepharose column equilibrated with 0.1 M Tris-HCl buffer, containing 50 mM KCl and 2 mM DTT. The column was submitted to two consecutives washes. During the first one, with 12 ml of the equilibration buffer, the unbound proteins were eluted. The ribonucleotide reductase complex (containing both α and β proteins) was present in the fractions eluted during the second wash, with the eluting buffer containing 1.5 mM ATP. To determine the amount of protein β bound to protein α, concentrated fractions were analyzed by gel electrophoresis, under denaturing conditions. The amount of protein β was determined by densitometry using a Gel Doc 1000 system and the Molecular Analyst 2.1.2 software from Bio-Rad, after calibration with known amounts of pure preparations of protein β loaded on the same gel.

**UV-visible Absorption Spectroscopy**—UV-visible spectra of aerobic and anaerobic ribonucleotide reductases were recorded with a Cary 1 Bio (Varian) spectrophotometer. Spectra could be also recorded inside the glove box using a Hewlett-Packard 8453 diode array spectrophotometer equipped with optical fibers connected to a sample holder inside the box.
**RESULTS**

**Expression and Purification of Mutated Enzymes**

The different mutated plasmids were prepared from plasmid pN9, a derivative of pET-3b, using a polymerase chain reaction-based method (see “Experimental Procedures”). The mutated enzymes, in which one of the five cysteines of protein β, Cys-19, Cys-26, Cys-30, Cys-33, and Cys-96, was changed into alanine, were overexpressed in _E. coli_ BL21(DE3) under conditions similar to the wild type enzyme. As judged from SDS-polyacrylamide gel electrophoresis analysis of both whole cells and soluble extracts, the level of expression and the low amount of inclusion bodies for all the mutants were as for the wild type enzyme (data not shown). Inclusion bodies could be minimized if cells were grown at 25 °C after the addition of isopropyl-1-thio-β-D-galactopyranoside.

The ability of an increasing amount of these extracts to activate a given amount of protein α and thus to complement it during CTP reduction was assayed (Fig. 2). From this experiment it is clear that mutants can be classified in two groups, with C19A and C96A mutant extracts giving high activity, comparable to that of the wild type extract, and C26A, C30A, and C33A mutant extracts giving no activity.

All of the mutant proteins were purified to homogeneity, as shown by SDS-polyacrylamide gel electrophoresis, according to the standard procedures developed for the wild type enzyme (8). Briefly, this implies treatment of the soluble extracts by streptomycin sulfate and DNase, followed by ammonium sulfate precipitation and then filtration on a Superdex 75 column. The elution profiles during chromatography and the purification yields were comparable to the wild type enzyme. In all cases, during filtration the protein appeared as a mixture of monomer and polymeric forms with the monomer being the major species. DTT was added to the buffers to prevent extensive precipitation of the proteins.

That the purification led essentially to apoprotein forms, as in the case of the wild type enzyme, was evident from the near absence of chromophore, as shown from the light absorption spectrum of the purified proteins and the very low level of iron and sulfide bound to the protein.

**Characterization of Reconstituted Mutated Enzymes**

Wild type protein β is able to assemble a (2Fe-2S) or a (4Fe-4S) center depending on the reconstitution procedure (6). A (2Fe-2S) center is assembled when the protein is incubated with a slight molar excess of iron and sulfide, in the presence of DTT, under anaerobic conditions and desalted on an aerobic Sephadex G-25 column (method A). On the other hand, reconstitution of a (4Fe-4S) center requires strict anaerobiosis, which can be achieved inside an anaerobic box, during both incubation and chromatography (method B). This cluster is sensitive to oxygen and degrades to a (2Fe-2S) center, identical to the one produced by method A. However, this degraded form is enzymatically active because it is converted, under the reductive conditions of the assay, back to the (4Fe-4S) form, which is the active form of the cluster (10).

Both methods were used for the reconstitution of mutated enzymes. C19A and C96A mutants had iron and sulfide content, spectroscopic properties, and enzyme activities comparable to those of the wild type enzyme, indicating that they were able to bind both (2Fe-2S) or (4Fe-4S) centers as the wild type enzyme (data not shown). We thus concluded that Cys-19 and Cys-96 were not involved in the chelation of the iron center of protein β. Consequently these mutants will not be discussed further. In contrast, the three other mutants showed significant differences from the wild type enzyme. Because their properties were highly comparable and for the sake of clarity, they will be illustrated with only one mutant in the following. For that purpose the C30A mutant was chosen.

Iron and Sulfide Content—Single mutations had little effect on the amount of iron and sulfide which the protein could bind during reconstitution by method A, as shown in Table II. The C33A mutant contained about 2 iron and 2 sulfur atoms/chain, as did the wild type enzyme, whereas the two other mutants,

**TABLE II**

Characterization of wild type protein β and mutants reconstituted by method A or B (as described under “Experimental Procedures” and after exposure to air

| Mutant   | Method A | Method B | Method B (air exposed) |
|----------|----------|----------|------------------------|
|          | Fe<sup>a</sup> | S<sup>a</sup> | [4Fe-4S]<sup>b</sup> | Activity<sup>c</sup> | Fe<sup>a</sup> | S<sup>a</sup> | [4Fe-4S]<sup>b</sup> | Activity<sup>c</sup> | Fe<sup>a</sup> | S<sup>a</sup> |
| Wild type| 1.9 | 2.0 | 0.4 | 100 | 3.8 | 3.6 | 0.8 | 100 | 1.7 | 1.8 |
| C26A     | 1.4 | 1.3 | 0   | <1 | 2.5 | 2.5 | 0   | <1 | 1.1 | 1.0 |
| C30A     | 1.5 | 1.5 | 0   | <1 | 2.9 | 2.8 | 0   | <1 | 1.7 | 1.6 |
| C33A     | 2.1 | 1.9 | 0   | <1 | 3.2 | 3.2 | 0   | <1 | 1.6 | 1.5 |

<sup>a</sup> Iron and sulfur content are expressed in atoms/β protein (17.5 kDa).

<sup>b</sup> Amount of [4Fe-4S] S = ½ per β protein in samples reduced by dithionite or deazaflavin determined by EPR spectroscopy.

<sup>c</sup> Maximal ribonucleotide reductase activity of α protein activated with increasing amounts of each preparation (wild type and mutants); 100% = 300 units/mg of α.
C26A and C30A, could bind as much as about 1.5 iron and 1.5 sulfur atoms/polypeptide. That an iron-sulfur center, similar to that of the wild type enzyme, thus in all probability a (2Fe-2S) cluster, was assembled in the mutants was supported further from the remarkable similarity of the light absorption spectra, which all displayed a band at 420 nm and an additional broad absorption at 590 nm (Fig. 3). Furthermore, as shown in Fig. 4, despite the mutations, the mutated enzymes had rather stable iron centers because EDTA-dependent release of iron occurred at a rate comparable to that for the wild type enzyme.

When reconstitution of the iron center was achieved by method B, it was observed that the C26A, C30A, and C33A mutants could bind as much as 3 iron and sulfur atoms, whereas the wild type enzyme could bind almost 4 iron and 4 sulfur atoms (Table II). However, in the sample used for Mössbauer spectroscopy, the mutant was found to contain around 4 iron atoms/monomer, was analyzed by

Mössbauer Spectroscopic Properties of the Mutated Enzymes—One of the mutants, C30A, has been reconstituted under strict anaerobiosis (method B) with $^{57}$FeCl$_3$. This preparation, containing 4 iron atoms/monomer, was analyzed by Mössbauer spectroscopy at 77 and 4.2 K in an external field of 20 mT perpendicular to the $\gamma$-ray and at 4.2 K with an applied field of 7 T parallel to the $\gamma$-ray (Fig. 5). The major species in Fig. 5a (82%) is characterized by a doublet typical for mixed valence delocalized Fe$^{2.5+}$ sites (parameters $\delta = 0.47$ mm/s$^{-1}$; $\Delta E_p = 1.06$ mm/s$^{-1}$ and $\Gamma = 0.45$ mm/s$^{-1}$) as in a (4Fe-4S)$^{2+}$ or (3Fe-4S)$^{2+}$ cluster. The measurements in high field (Fig. 5, c and d) reveal that 54% of the total area belong to a diamagnetic species that is attributed to (4Fe-4S)$^{2+}$. The remaining 28% of the major doublet in Fig. 5a corresponds to Fe$^{3+}$ sites within a paramagnetic species, which is difficult to interpret. However, the best simulations of the different spectra (Fig. 5) were obtained assuming an $S = 2$ (3Fe-4S)$^{2+}$ cluster, with spin-Hamiltonian parameters (Table III) different from those found for the wild type protein $\beta$ (20). The Fe$^{2+}$ sites of this cluster (14%) exhibit a quadrupole doublet with the parameters $\delta = 0.42$ mm/s$^{-1}$, $\Delta E_q = 0.55$ mm/s$^{-1}$, and $\Gamma = 0.24$ mm/s$^{-1}$. The sample also contains a small amount of high spin ferrous iron (4%) with $\delta = 1.2$ mm/s$^{-1}$ and $\Delta E_p = 3.4$ mm/s$^{-1}$. Ferrous iron was also present in reconstituted wild type protein $\beta$.

The relatively large line width of the Fe$^{2.5+}$ doublet in the spectra of Fig. 5a and b, and the fact that only two cysteines are available for cluster coordination led us to fit the diamagnetic species in the spectra taken at 4.2 K with two distinct subspectra in the ratio 1:1. The resulting values are: $\delta_1 = 0.43$ mm/s$^{-1}$, $\Delta E_{p1} = 1$ mm/s$^{-1}$, $\Gamma_1 = 0.38$ mm/s$^{-1}$, and $\delta_2 = 0.5$ mm/s$^{-1}$, $\Delta E_{p2} = 1.18$ mm/s$^{-1}$, $\Gamma_2 = 0.38$ mm/s$^{-1}$. The parameters for subspectrum 1 are typical for conventional (4Fe-4S)$^{2+}$ clusters, whereas $\delta_2$ is significantly higher, in the range of the highest reported isomer shifts (21). This corroborates the idea that two Fe$^{2.5+}$ sites are not cysteine ligated, and the higher quadrupole splitting reflects a lower symmetry of these sites compared with those with sulfur-only coordination. The spectrum at 77 K was then reanalyzed successfully with the new parameters. The final parameter set of the various iron sites is summarized in Table III.

The observation that two different iron sites could be distinguished by Mössbauer spectroscopy in the C30A mutant led us to reanalyze the previously reported Mössbauer data of the wild type enzyme. The wild type (4Fe-4S)$^{2+}$ center was characterized previously by a single doublet with $\delta = 0.44$ mm/s$^{-1}$, $\Delta E_p = 1.0$ mm/s$^{-1}$, $\Gamma = 0.38$ mm/s$^{-1}$ (6). Using two quadrupole doublets with area ratio 1:3 and with the parameters obtained from the mutant slightly improved the fit (Fig. 6) and hence is in agreement with the idea that one Fe$^{2.5+}$ site is not cysteine-ligated.

In conclusion, the Mössbauer data show that a large portion
also be monitored by light absorption spectroscopy because the solution is bleaching during reduction. Reduction of the mutants also resulted in bleaching of the solutions in all cases, as shown from the decrease of the absorption between 360 and 700 nm (Fig. 3). However, the EPR signal characteristic of the (4Fe-4S)⁺ center, which could be observed in large amounts with the wild type enzyme, could not be detected. These results further confirm the conclusion that Cys-26, Cys-30, and Cys-33 are the cysteine ligands of the iron center. That the affinity of the mutated proteins for iron was not decreased drastically during reduction was checked by desalting the reduced mutants on Sephadex G-25 within the glove box and assaying the protein fractions for iron (data not shown).

Binding to Protein α—The mutations and the resulting changes at the iron cluster could affect the binding of protein β to protein α. To investigate this, an excess of reconstituted wild type or mutated protein β was incubated with protein α. The mixture was then loaded onto an affinity dATP-Sepharose column on which the αββ complex, and not protein β, binds. Elution of the complex was achieved with a buffer containing ATP, and the amount of protein β bound to protein α was quantitated by SDS-polyacrylamide gel electrophoresis of the ATP fraction and gel densitometry. This experiment showed that affinity of the mutated proteins for protein α was only slightly diminished with respect to wild type protein β (data not shown) but to an extent that could not account for the total loss of activity of these mutants.

DISCUSSION

The iron-sulfur center of the anaerobic ribonucleotide reductase, and also of the activase of the pyruvate formate-lyase, has a number of original properties. It can exist under different forms, (2Fe-2S), (3Fe-4S), and (4Fe-4S), depending on the redox conditions (6, 10, 20). Under strongly reducing conditions the (4Fe-4S)⁺ redox state accumulates, and the iron center becomes active during reductive activation of AdoMet, a process absolutely required for generation of a glycyl radical in the large component of the enzyme, protein α (9). Despite these rather unique properties nothing was known on the coordination environment of the iron-sulfur core of the ribonucleotide reductase-activating enzyme. This knowledge was important to understand whether the unique chemical properties of this class of iron-sulfur center were reflecting a novel coordination environment.

In the case of the activase of pyruvate formate-lyase, the mutation of the three cysteines of the conserved CXXXCCXC sequence led to inactive enzymes, suggesting that these cysteines were involved in iron binding (11). The nature of the fourth ligand was not investigated. The protein β of the anaerobic ribonucleotide reductase also contains such a sequence that is conserved among all class III ribonucleotide reductases (22). Also by site-directed mutagenesis of these cysteines to alanines we have generated totally inactive enzymes and thus conclude that Cys-26, Cys-30, and Cys-33 are ligands to the iron as in the activase of pyruvate formate-lyase. Because of the limited number of cysteines in protein β we were able to mutate all cysteines, and mutations at positions 19 and 96 gave active enzymes excluding a cysteine as the fourth ligand. We thus end up with an iron-sulfur center with only three cysteine ligands and an unknown fourth ligand. A reanalysis of the Mössbauer spectra of the (4Fe-4S)⁺ center of wild type protein β is in full agreement with a cluster containing two types of iron sites in a 3:1 ratio, with slightly different Mössbauer parameters: the iron site lacking a cysteine ligand has higher isomer shift and quadrupole splitting.

Identification of the fourth ligand is of course required for complete characterization of the cluster. In general, the iron...
atoms of (4Fe-4S) clusters are coordinated by the sulfur atom of four cysteinyl residues. However, there are notable exceptions and reported precursors of iron-sulfur centers with only three cysteine ligands. Aconitase is probably the most extensively studied example of such clusters (23). In this case, the fourth ligand of the (4Fe-4S) center is a solvent hydroxyl in the absence of substrate, whereas substrate binding results in a six-coordinate iron site with 2 oxygen atoms from the substrate. It is interesting to note that the iron center is rather unstable and can be degraded during oxidation to a (3Fe-4S) cluster that can in some cases decompose further to the apoprotein form (23). In one of the (4Fe-4S) centers in a Ni-Fe hydrogenase, one iron has a coordinating histidine residue replacing the cysteine (24). Finally, in the (4Fe-4S) center of a ferredoxin from Pyrococcus furiosus, one of the iron sites has an aspartate ligand in place of the cysteine (25). This cluster is stable and does not lose iron during purification, even though it can be converted to a (3Fe-2S) cluster. In this case, the fourth ligand is a coordinating histidine residue replacing the cysteine (26). This in some cases is caused by an improper folding of the protein, as a consequence of the absence or the incorrect insertion of the cluster, and increased susceptibility to degradation by cellular proteases. Here we show that the level of expression of all of the prepared protein β mutants were as for the wild type enzyme, indicating that the cluster had little effect on the protein folding.

Furthermore, mutations had only a minor effect on the capacity of protein β to assemble either a (4Fe-4S) or a (2Fe-2S) center, as in the wild type enzyme, and on the stability of these centers. In particular, detailed analysis of one of the mutants by Mössbauer spectroscopy shows unambiguously that a large proportion of the protein-bound iron is still in the form of a (4Fe-4S) center. It is not unusual that iron-sulfur proteins can assemble a cluster, with the correct nuclearity, when a cysteine ligand is mutated. In most cases this was observed with classical iron-sulfur clusters when the residue replacing one of the four cysteines was a serine. There are examples where replacement of a single Cys to Ser replacement appears to lead to intact (4Fe-4S) clusters, with only very small perturbations of their spectroscopic properties. These are cluster Fx of PsAB of photosystem I (27), cluster II in nitrate reductase (28), and the 4Fe cluster in subunit FrdB of fumarate reductase (29). In mutated ferredoxins, (2Fe-2S) clusters with only three cysteine ligands form spontaneously in vitro (30). In the case reported here, the (4Fe-4S) and (2Fe-2S) clusters have only 2 cysteines, one unknown ligand, and a non-coordinating residue (alanine) in place of a cysteine ligand. Although we expected such new clusters to be highly unstable, we were surprised to observe that they bind only slightly less iron, have light absorption spectra and stability similar to those of the wild type enzyme, and that they still can form a (4Fe-4S) cluster that degrades into stable (2Fe-2S) clusters during exposure to air, again as in the case of the wild type enzyme.

It should be noted that there is so far no example of a 4Fe-4S center with only two cysteine ligands. It is possible that the Cys to Ala mutants of the small component of the anaerobic ribonucleotide reductase reported here have such a cluster. Support for two distinct iron sites, in equal amounts, comes, in the case of the C30A mutant, from Mössbauer spectroscopy. However, at this stage, one cannot exclude that in the mutants, either Cys-19 or Cys-96 is recruited for stabilizing the cluster. Furthermore, considering the importance of DTT in this system, it is also possible that this exogenous thiol could provide additional sulfur coordination. Such a cluster would thus represent a novel structure in the growing list of iron-sulfur clusters, and further investigation is required.

Finally, even though the mutated clusters have retained most of their properties, they clearly lost activity. Whereas iron could be reduced by dithionite or DAF, as shown by light absorption spectroscopy (Fig. 3), a S = 1/2 (4Fe-4S) center could not be detected by EPR spectroscopy. This suggests that the mutations have greatly affected the cluster in its reduced form and provides an explanation for the loss of activity because ribonucleotide reductase activation strictly depends on the injection of one electron into a stable (4Fe-4S) cluster. We thus conclude that the CXXCXXXC sequence, present in the activating component of the anaerobic ribonucleotide reductase and in other enzymes, plays a crucial role in stabilizing a

![Mössbauer spectrum of the wild type enzyme](image)

**FIG. 6.** Mössbauer spectrum of the wild type enzyme recorded at 77 K in an applied field of 20 mT perpendicular to the γ-ray. Dashed lines are attributed to the different sites of the (4Fe4S)2+ cluster (~84%). Dashed-dotted lines refer to an Fe2+ contamination (~16%).

The Iron-Sulfur Center of Anaerobic Ribonucleotide Reductase

### Table III

Parameters used for a spin-Hamiltonian simulation of the spectra in Fig. 5, b–d

| δ | ΔE | Γ | η | β | D | E/D | A9 | A1 | A5 | Area |
|---|---|---|---|---|---|---|---|---|---|---|
| mm s⁻¹ | mm s⁻¹ | mm s⁻¹ | * | cm⁻¹ | | | | | | |
| [4FeS]²⁺ |
| Cys-ligated | 0.43 | 1.0 | 0.38 | 0.5 | 90 | 0.76 | 0.0 | −12 | −8 | −12 | 28 |
| Non-Cys-ligated | 0.5 | 1.18 | 0.38 | 1.0 | 90 | 0.76 | 0.0 | 12 | 5 | 9 | 14 |
| [2Fe4S]⁰ |
| Fe²⁺ sites | 0.43 | 1.0 | 0.38 | 0.5 | 90 | 0.76 | 0.0 | −12 | −8 | −12 | 28 |
| Fe³⁺ sites | 0.42 | 0.55 | 0.24 | 0.8 | 90 | 0.76 | 0.0 | 12 | 5 | 9 | 14 |
| Fe⁰ sites | 1.2 | −3.4 | 0.5 | 0.7 | 0 | 8 | 0.28 | −21 | −9 | −31 | 4 |

*δ, isomer shift; ΔE, quadrupole splitting; Γ, line width; η, asymmetry parameter; β, Euler angle relating electric field gradient tensor and zero-field splitting tensor; D, zero-field splitting; E/D, rhombicity; A5, 9, 15, magnetic hyperfine coupling tensor.
specific iron-sulfur cluster designed for AdoMet reduction and radical generation.

Note Added in Proof—The sequence of lysine 2,3-aminomutase from Clostridium subterminale SB4 has just been published (Ruzicka, F. J., Lieder, K. W., and Frey, P. A. (2000) *J. Bacteriol.* 182, 469–476).

**REFERENCES**

1. Jordan, A., and Reichard, P. (1998) *Annu. Rev. Biochem.* 67, 71–98
2. Mulliez, E., Ollagnier, S., and Fontecave, M. (1999) in *Iron Metabolism* (Ferreira, G. C., and Moura, J. J. G., eds) pp. 161–176, John Wiley and Sons, Weinheim
3. Eliasson, R., Pontis, E., Fontecave, M., Gerez, C., Harder, J., Jornvall, H., Krook, M., and Reichard, P. (1992) *J. Biol. Chem.* 267, 25541–25547
4. Bianchi, V., Eliasson, R., Fontecave, M., Mulliez, E., Hoover, D. M., Matthews, R. G., and Reichard, P. (1995) *Biochem. Biophys. Res. Commun.* 197, 792–797
5. Sun, X., Eliasson, R., Pontis, E., Anderson, J., Buist, G., Sjöberg, B.-M., and Reichard, P. (1995) *J. Biol. Chem.* 270, 2443–2446
6. Tamarit, J., Mulliez, E., Meier, C., Trautwein, A., and Fontecave, M. (1999) *J. Biol. Chem.* 274, 31291–31296
7. Sun, X., Ollagnier, S., Schmidt, P. P., Atta, M., Mulliez, E., Lepape, L., Eliasson, R., Graslund, A., Fontecave, M., Reichard, P., and Sjöberg, B.-M. (1996) *J. Biol. Chem.* 271, 6827–6831
8. Ollagnier, S., Mulliez, E., Gaillard, J., Eliasson, R., Fontecave, M., and Reichard, P. (1996) *J. Biol. Chem.* 271, 9419–9416
9. Ollagnier, S., Mulliez, E., Schmidt, P. P., Eliasson, R., Gaillard, J., Deronzier, C., Bergman, T., Graslund, A., Reichard, P., and Fontecave, M. (1997) *J. Biol. Chem.* 272, 24216–24223
10. Ollagnier, S., Meier, C., Mulliez, E., Gaillard, J., Schuennemann, V., Trautwein, A. X., Mattioli, T., Lütz, M., and Fontecave, M. (1999) *J. Am. Chem. Soc.* 121, 6344–6350
11. Kulzer, R., Pils, T., Kappl, R., Hüttermann, J., and Knappe, J. (1998) *J. Biol. Chem.* 273, 4897–4903
12. Guinaudeau, D., Florentin, D., Tse Sum Bui, B., Nuni, F., and Marquet, A. (1997) *Biochem. Biophys. Res. Commun.* 236, 492–496
13. Reed, K. E., and Cronan, J. E., Jr. (1993) *J. Bacteriol.* 175, 1325–1336
14. Ollagnier-de Choudens, S., and Fontecave, M. (1999) *FEBS Lett.* 453, 25–28
15. Mass, M. L., and Frey, P. A. (1990) *J. Biol. Chem.* 265, 18112–18115
16. Ho, S. N., Hunt, H. D., Horton, B. M., Pullen, J. K., and Pease, L. R. (1989) *Gene (Amst.)* 77, 51–59
17. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254
18. Fish, W. W. (1988) *Methods Enzymol.* 158, 357–364
19. Beinert, H. (1983) *Anal. Biochem.* 131, 373–378
20. Mulliez, E., Ollagnier-de Choudens, S., Meier, C., Cremonini, M., Luchinat, C., Trautwein, A. X., and Fontecave, M. (1999) *J. Biol. Inorg. Chem.* 4, 614–620
21. Bertini, I., Ciurl, S., Luchinat, C. (1995) *Struct. Bond.* 83, 1–53
22. Siedow, A., Cramm, R., Siddiqui, R. A., and Friedrich, B. (1999) *J. Bacteriol.* 181, 4919–4928
23. Beinert, H., Kennedy, M. C., and Stout, C. D. (1996) *Chem. Rev.* 96, 2335–2373
24. Volbeda, A., Charon, M.-H., Piras, P., Hatchikian, E. C., Frey, M., and Fontecilla-Camps, J. C. (1995) *Nature* 373, 580–587
25. Calzolai, L., Gorst, C. M., Zhao, Z.-H., Teng, Q., Adams, M. W. W., and La Mar, G. N. (1995) *Biochemistry* 34, 11373–11384
26. Moula, J. M., Davasse, V., Golizelli, M.-P., Meyer, J., and Quinkal, I. (1996) *J. Biol. Inorg. Chem.* 1, 1–4
27. Warren, P. V., Smart, L. B., McIntosh, L., and Golbeck, J. H., (1993) *Biochemistry* 32, 4411–4419
28. Augier, V., Guigliarelli, B., Asso, M., Bertrand, P., Frixon, C., Giordano, G., Chippaux, M., and Blasco, F., (1993) *Biochemistry* 32, 2013–2023
29. Kowal, A. T., Werth, M. T., Manodori, A., Cecchini, G., Schroder, I., Gunsalus, R., and Johnson, M. K. (1995) *Biochemistry* 34, 12284–12293
30. Xia, B., Cheng, H., Reed, G. H., and Markley, J. L. (1996) *Biochemistry* 35, 9488–9495