THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 21, Issue of May 26, pp. 15669–15675, 2000

Printed in U.S.A.

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

AN IRON-SULFUR CENTER WITH ONLY THREE CYSTEINES*

Received for publication, December 13, 1999, and in revised form, February 11, 2000

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Class III anaerobic ribonucleotide reductase small component, named protein β, contains a (4Fe-4S) center. Its function is to mediate electron transfer from reduced flavodoxin to *S*-adenosylmethionine, required for the introduction of a glycyl radical in the large component, named protein α, which then becomes active for the reduction of ribonucleotides. By site-directed mutagenesis we demonstrate that the three cysteines of the conserved C*XXX*C*XX*C sequence are involved in iron chelation. Such a sequence is also present in the activase 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 deoxyribonucleotides, required for DNA synthesis (1). The enzymatic system catalyzes the reduction of ribonucleoside triphosphates into the corresponding deoxyribonucleotides by formate (2). It consists of several proteins and low molecular weight components. The reductase, named protein α, in the form of a dimer α_2, contains the active site where substrates and formate react and allosteric effectors bind. However, as isolated it is not active. We have shown that it is activated during anaerobic incubation with a reducing system (NADPH + flavodoxin reductase + flavodoxin, which can *in vitro* be replaced by dithionite or photoreduced deazaflavin), dithiothreitol (DTT),[1] *S*-adenosylmethionine (AdoMet), and a specific activating component, named protein β, which functions catalytically with regard to activation of protein α (3–6). It is remarkable that the only modification of protein α, during the process that makes it catalytically competent, is the introduction of an air-sensitive glycyl radical at position 681 (7). As a consequence, the activated enzyme is active only under strict anaerobic conditions.

Protein β 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 $(4Fe\text{-}4S)^{2+}$ and $(4Fe\text{-}4S)^{+}$ redox states (6, 8). Protein β is essential during anaerobic activation of protein α because it catalyzes the one-electron transfer from reduced flavodoxin to AdoMet required for the formation of the glycyl radical. As a matter of fact it has been shown that the reduced $(4Fe\text{-}4S)^{+}$ cluster is able to reduce AdoMet, and it is postulated that this reaction results in the homolytic cleavage of its *S*-C(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 β stabilizes $(2Fe\text{-}2S)^{2+}$ 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 C*XXX*C*XX*C motif common to biotin synthase and the activating components of pyruvate formate-lyase and ribonucleotide reductase (Fig. 1*A*) 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 β. In this work we changed the five cysteines of protein β 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 C*XXX*C*XX*C motif conserved in all known ribonucleotide reductase sequences, a few of which are displayed in Fig. 1*B*, are important for activity.

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¶ Supported by a postdoctoral fellowship from the Spanish government (Secretaría de Estado de Universidades e Investigación).

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[1] The abbreviations used are: DTT, dithiothreitol; AdoMet, *S*-adenosylmethionine; DAF, 5-deaza-7,8-demethyl-10-methylisoalloxazine; T, tesla.

A

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NRDG ECOLI   CVHECPGC
PFLA ECOLI   CLMRCLYC
BIOB ECOLI   CPEDCKYC
LIPA ECOLI   CTRRCPFC
Consensus    C---C--C
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B

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                    10         20         30         40         50         60
NRDG ECOLI   MNYHQYYPVDIVNGPGTRCTLFVSGCVHECPGCYNKSTWRVNSGQPFTKAMEDQIINDLN   60
NRDG HAEIN   MNYLQYYPTDVINGEGTRCTLFVSGCTHACKGCYNQKSWSFSAGVLFDDVMEQQIINDLK   60
NRDG BPT4    MNYDRIYPCDFVNGPGCRVVLFVTGCLHKCEGCYNRSTWNARNGQLFTMNTVKELASHLS   60
Consensus    MNY.++YP.D.+NG+G+R++LFV+GC.H.C.GCYN.++W....G++F+..++.+++++L.

                    70         80         90        100        110        120
NRDG ECOLI   DTRIKRQGISLSGGDPLHPQNVPDILKLVQRIRAECPGKDIWVWTGYKLDELNAAQMQVV  120
NRDG HAEIN   DTRIKRQGLTLSGGDPLHPLNVETLLPFVQRVKRECPDKDIWVWTGYKLDELDKQQRAML  120
NRDG BPT4    KSYI--QGLTLTGGDPLYPQNREEISNLVSWVKARFPEKDIWLWTGYKFEDIK--QLEML  116
Consensus    +++I++QG++L+GGDPL+P+N++.++.+V+++++++P.KDIW+WTGYK++++...Q..++

                   130        140        150        160
NRDG ECOLI   DLINVLVDGKFVQDLKDPSLIWRGSSNQ-VVHHLR------                    154
NRDG HAEIN   PYIDVLIDGKFIQEQADPSLVWRGSANQ-IIHRFKL-----                    155
NRDG BPT4    KYVDVIIDGKYEKNLP-TKKLWRGSDNQRLWSNTDGVWKHD                    156
Consensus    .+++V++DGK+.+.+.++++.WRGS.NQ...+.........
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FIG. 1. *Panel A*, the cysteines motif common to the activating components of the *E. coli* anaerobic ribonucleotide reductase (*NRDG*) and pyruvate formate lyase (*PFLA*), *E. coli* biotin synthase (*BIOB*), and *E. coli* lipoate synthase (*LIPA*). *Panel B*, comparison of the sequences of the activating components of class III ribonucleotide reductase of *E. coli*, *Haemeophilus influenzae* (*HAEIN*), and bacteriophage T4 (*BPT4*). The three conserved cysteines are *underlined*. In the *E. coli* sequence, the mutated cysteines are in *bold* characters.

EXPERIMENTAL PROCEDURES

Materials—Enzymes and other components of the anaerobic ribonucleotide reductase system have been obtained as described previously (6). $^{57}Fe_2O_3$ 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. $Fe(NH_4)_2(SO_4)_2$ was from Aldrich. AdoMet was from Roche Molecular Biochemicals. 5-Deaza-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 isolated by agarose gel electrophoresis, were purified using the QIAEX II gel extraction kit from Qiagen, digested with *Sph*I and *Bam*HI, and subcloned into *Sph*I/*Bam*HI-digested pN9. The entire *nrdG* gene was sequenced. Besides the desired mutations, we found two silent mutations in all mutated genes, one at position +21 (TAC instead of TAT) and the second at position +27 (GTT instead of GTC).

Protein Purification and Reconstitution—Wild type and mutated β proteins were overproduced and purified as described previously (6, 8). Two methods for the reconstitution of enzymes with iron and sulfide have been used. In method A, the protein (10 mg/ml) was incubated at 4 °C under anaerobic conditions within tubes connected to a manifold under a constant flux of moist N_2 with a 6-fold molar excess of Na_2S and either $Fe(NH_4)_2(SO_4)_2$ or $^{57}FeCl_3$ in 0.1 M Tris-HCl, pH 8.0, in the presence of 5 mM DTT. After 2 h, EDTA (2 mM final concentration) was added, and the reaction was stopped after 30 min. After chromatography through a Sephadex G-25 column equilibrated with a DTT-free deaerated buffer, the colored fractions were collected under air and concentrated over a YM-10 Diaflo membrane (Amicon). In the second procedure (method B) all steps of the reconstitution procedure were made anaerobically at 18 °C inside a glove box (Jacomex BS531 NMT) in a N_2 atmosphere containing less than 2 ppm O_2. As reported previously, methods A and B are used to generate preparations with one (2Fe-2S) and one (4Fe-4S) cluster per polypeptide chain, respectively. For Mössbauer experiments samples were prepared with method B, and the final concentration was 0.5–0.7 mM.

Protein concentrations were determined by the Bradford method (17). Protein-bound iron was determined colorimetrically with bathophenantroline after acid denaturation of protein (18). Sulfide content was determined using the Beinert method (19).

Preparation of Reduced Samples—Reduction of reconstituted wild type and mutated β proteins was performed inside the anaerobic glove box. DAF was dissolved in dimethyl sulfoxide, diluted with water to 0.5 mM, and stored inside the box in the dark. Protein (100 μM) was prepared in 0.1 M Tris-HCl, pH 8.0, and irradiated in the presence of 20–50 μM DAF for 60 min. Reduction could be monitored by light absorption directly inside the box. To avoid exposure to oxygen, EPR tubes were frozen directly inside the box in a well filled with isopentane cooled from outside the box by liquid nitrogen.

TABLE I
Generation of mutated genes

Mutagenic oligonucleotides were complementary to the coding strand (Cs) or to the noncoding strand (Ncs). Two mutagenic oligonucleotides, complementary to each other, are used for each mutation, in two rounds of polymerase chain reaction as described.

Mutation[a]	Mutagenic oligonucleotides[b]	
C19A	5′-CAGGGTTGCACGAGTGCC-3′	Cs
	5′-GGCACTCGTGCAACCCTG-3′	Ncs
C26A	5′-ATGAACAGCCCCGGAGAC-3′	Cs
	5′-GTCTCCGGGGCTGTTCAT-3′	Ncs
C30A	5′-TAGCAACCGGGCGCTTCA-3′	Cs
	5′-TGAAGCGCCCGGTTGCTA-5′	Ncs
C33A	5′-TTTGTTATAGGCACCGGG-3′	Cs
	5′-CCCGGTGCCTATAACAAA-3′	Ncs
C96A	5′-GTCTTTACCCGGAGCCTC-3′	Cs
	5′-GAGGCTCCGGGTAAAGAC-3′	Ncs

[a] 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.

[b] 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 dCTP/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 consecutive 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 samples 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.

EPR Spectroscopy—EPR first derivative spectra were recorded on a Bruker EMX (9.5 GHz) EPR spectrometer equipped with an ESR 900 helium flow cryostat (Oxford Instruments).

Mössbauer Spectroscopy—^{57}Fe-Mössbauer spectra were recorded on 200-μl cups containing the protein (0.5–0.7 mM) with a conventional constant acceleration spectrometer using a ^{57}Co source in a Rh matrix (254 MBq). Measurements at 4.2 and 77 K were performed using a bath cryostat (Oxford Instruments) with an electromagnet mounted outside the cryostat producing a field of 20 mT perpendicular to the γ-ray. High field measurements were performed with a cryostat equipped with a superconducting magnet (Oxford Instruments). The spectra were analyzed assuming Lorentzian line shape, the isomer shift is quoted relative to α-Fe at room temperature.

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.

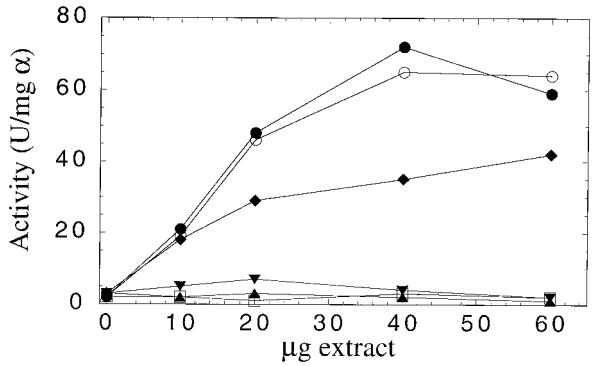

FIG. 2. **Ribonucleotide reductase activating capacity of wild type and mutant crude extracts.** Protein α (4 μg) was activated for 45 min with increasing amounts of crude extracts obtained from cells overexpressing wild type (●) and mutants C19A (♦), C26A (▼), C30A (▲), C33A (□), and C96A (○) and assayed for CTP reduction. Specific activity is given in units/mg of α protein.

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[a]	S[a]	[4Fe-4S][b]	Activity[c]	Fe[a]	S[a]	[4Fe-4S][b]	Activity[c]	Fe[a]	S[a]
				%				%		
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	<5	2.5	2.5	0	<5	1.1	1.0
C30A	1.5	1.5	0	<1	2.9	2.8	0	<5	1.7	1.6
C33A	2.1	1.9	0	<1	3.2	3.2	0	<1	1.6	1.5

[a] Iron and sulfur content are expressed in atoms/β protein (17.5 kDa).
[b] Amount of [4Fe-4S] S = ½ per β protein in samples reduced by dithionite or deazaflavin determined by EPR spectroscopy.
[c] Maximal ribonucleotide reductase activity of α protein activated with increasing amounts of each β preparation (wild type and mutants); 100% = 300 units/mg of α.

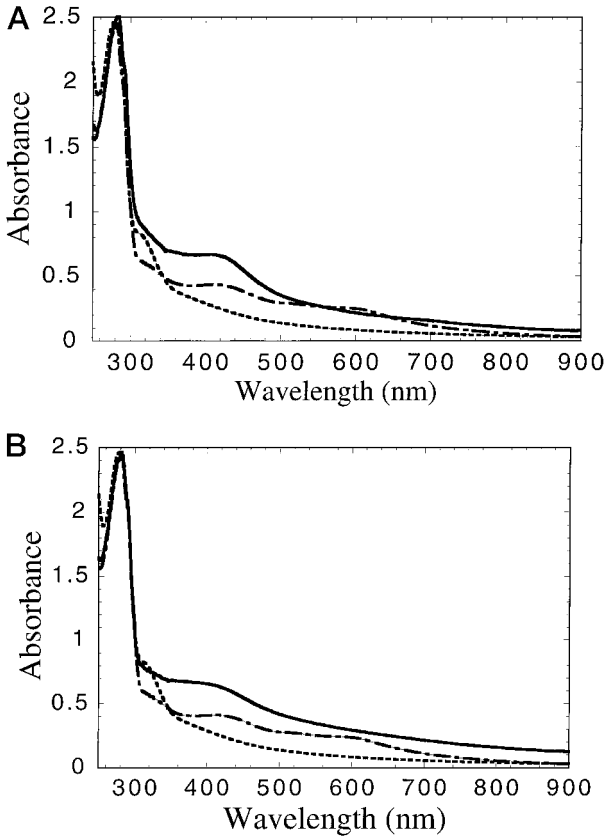

FIG. 3. **UV-visible light absorption spectra of the wild type (*panel A*) and C30A (*panel B*) β protein (60 μM) in 50 mM Tris-HCl, pH 8, after reconstitution by method A (*dotted-dashed line*), method B (*solid line*), and after reduction by photoreduced deazaflavin (*dotted line*).**

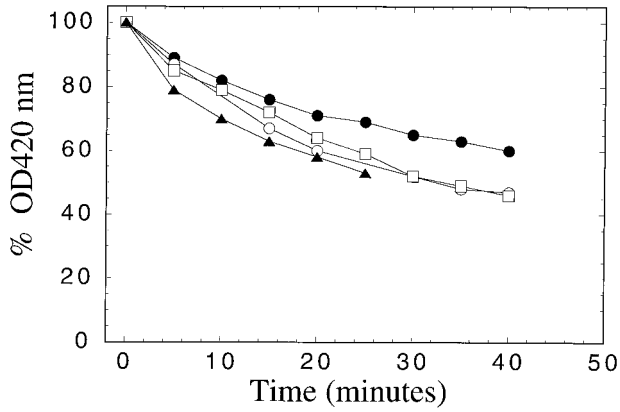

FIG. 4. **Stability of the iron-sulfur center of β proteins to EDTA treatment.** 100 μM wild type (●), C26A (▲), C30A (○), and C33A (□) β variants, reconstituted with method A, were incubated with 100 μM EDTA in 50 mM Tris-HCl, pH 8.0, at 18 °C. Destruction of the cluster was monitored by the decrease of optical density at 420 nm.

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/chain. The iron center of the mutant proteins had much in common with that of the wild type enzyme. First, they all displayed a similar UV-visible spectrum, recorded inside the glove box (Fig. 3). Second, the intensity of the 420 nm band decreased as a consequence of exposure to air (data not shown). The resulting species, after gel filtration, contained less iron (Table I) and displayed a light absorption spectrum similar to that of the proteins reconstituted by method A.

It thus seems that deletion of a cysteine does not have a dramatic effect on the iron and sulfide binding capacity of protein β.

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 γ-ray and at 4.2 K with an applied field of 7 T parallel to the γ-ray (Fig. 5). The major species in Fig. 5*a* (82%) is characterized by a doublet typical for mixed valence delocalized $Fe^{2.5+}$ sites (parameters $\delta = 0.47$ mm·s^{-1}; $\Delta E_q = 1.06$ mm·s^{-1} and $\Gamma = 0.45$ mm·s^{-1}) as in a $(4Fe\text{-}4S)^{2+}$ or $(3Fe\text{-}4S)^{0}$ 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 $(4Fe\text{-}4S)^{2+}$. The remaining 28% of the major doublet in Fig. 5*a* corresponds to $Fe^{2.5+}$ sites within a paramagnetic species, which is difficult to interpret. However, the best simulations of the different spectra (Fig. 5) were obtained assuming an $S = 2$ $(3Fe\text{-}4S)^{0}$ cluster, with spin-Hamiltonian parameters (Table III) different from those found for the wild type protein β (20). The Fe^{3+} 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 $\Delta E_q = 3.4$ mm·s^{-1}. Ferrous iron was also present in reconstituted wild type protein β.

The relatively large line width of the $Fe^{2.5+}$ doublet in the spectra of Fig. 5, *a* 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}, $\Delta E_q 1 = 1$ mm·s^{-1}, $\Gamma_1 = 0.38$ mm·s^{-1}, and $\delta 2 = 0.5$ mm·s^{-1}, $\Delta E_q 2 = 1.18$ mm·s^{-1}, $\Gamma_2 = 0.38$ mm·s^{-1}. The parameters for subspectrum 1 are typical for conventional $(4Fe\text{-}4S)^{2+}$ clusters, whereas δ2 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 $(4Fe\text{-}4S)^{2+}$ center was characterized previously by a single doublet with $\delta = 0.44$ mm·s^{-1}, $\Delta E_q = 1.0$ 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

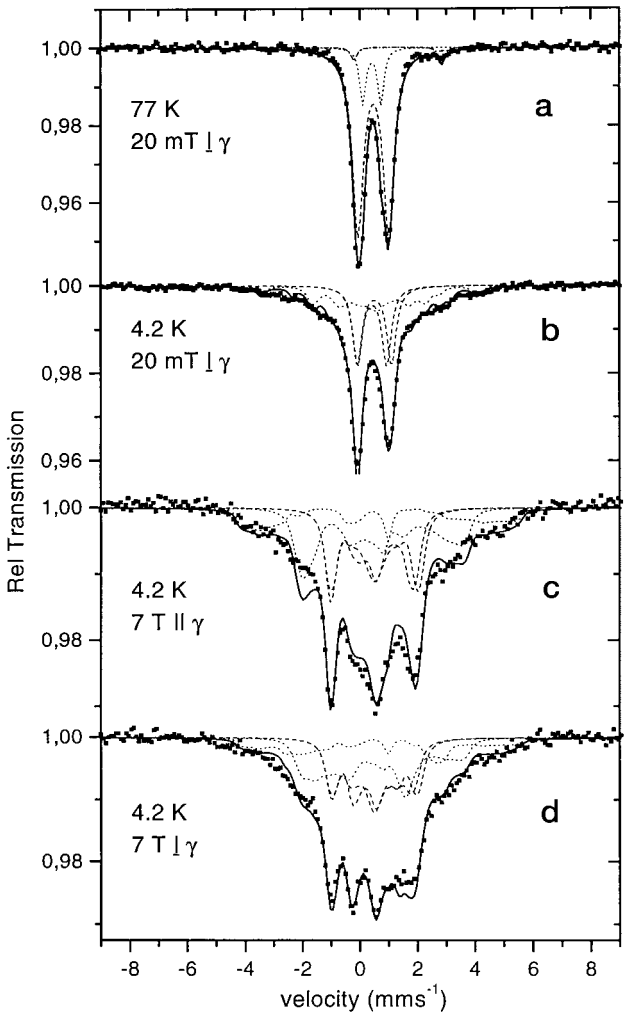

FIG. 5. **Mössbauer spectra of the mutant C30A.** *Panel a*, recorded at 77 K in an applied field of 20 mT perpendicular to the γ-ray; *dashed line*, $Fe^{2.5+}$ sites of $(4Fe4S)^{2+}$ and $(3Fe4S)^{0}$ (~82%); *dotted line*, Fe^{3+} site of $(3Fe4S)^{0}$ (~14%); *dashed-dotted line*, Fe^{2+} contamination (~4%). *Panel b*, recorded at 4.2 K in an applied field of 20 mT perpendicular to the γ-ray. *Panels c* and *d*, recorded at 4.2 K in applied fields of 7 T perpendicular and parallel to the γ-ray, respectively. In *panels b*, *c*, and *d*, the *dashed lines* are attributed to $(4Fe4S)^{2+}$ clusters (~54%), the *dotted lines* to $(3Fe4S)^{0}$ clusters (~42%). The subspectrum of the Fe^{2+} contamination is not shown, but it is included in the envelope spectrum (*solid line*).

of the iron is still assembled as $(4Fe\text{-}4S)^{2+}$ clusters in the C30A mutant. To our knowledge this is the first report of such a biological cluster with only two cysteine ligands.

Enzyme Activity—Mutants were assayed for CTP reduction, in combination with protein α, under strict anaerobic conditions, according to previously published procedures (3, 6). As shown in Table II, C26A, C30A, and C33A mutants reconstituted by either method A or B were catalytically incompetent. This suggests that Cys-26, Cys-30, and Cys-33 constitute cysteine sulfur ligands of the Fe-S core in protein β. However, as shown above, the loss of activity due to the mutations did not seem to reflect an impaired capacity of the proteins to chelate a (4Fe-4S) center.

Reduction of the Iron-Sulfur Center in the Mutated Enzymes—Anaerobic reduction by dithionite or DAF of the wild type enzyme reconstituted by either method A or B generates a S = 1/2 $(4Fe\text{-}4S)^{+}$ center, as shown by EPR spectroscopy. This reaction is essential for activity because only the $(4Fe\text{-}4S)^{+}$ center has the potential to reduce AdoMet and generate the essential glycyl radical (9). Formation of the reduced form can 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 $(4Fe\text{-}4S)^{+}$ 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 $\alpha_2\beta_2$ 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 $(4Fe\text{-}4S)^{+}$ 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 C*XXX*C*XX*C 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

TABLE III

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

δ, isomer shift; Δ*E*, 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; $A_{x,y,z}$, magnetic hyperfine coupling tensor.

	δ	Δ*E*	Γ	η	β	*D*	*E/D*	A_x	A_y	A_z	Area
	$mm \cdot s^{-1}$	$mm \cdot s^{-1}$	$mm \cdot s^{-1}$		°	cm^{-1}		*T*	*T*	*T*	%
$[4FeS]^{2+}$											
Cys-ligated	0.43	1.0	0.38	0.5							27
Non-Cys-ligated	0.5	1.18	0.38	1.0							27
$[3Fe4S]^0$											
$Fe^{2.5+}$ sites	0.43	1.0	0.38	0.5	90	0.76	0.0	−12	−8	−12	28
Fe^{3+} sites	0.42	0.55	0.24	0.8	90	0.76	0.0	12	5	9	14
Fe^{2+}	1.2	−3.4	0.5	0.7	0	8	0.28	−21	−9	−31	4

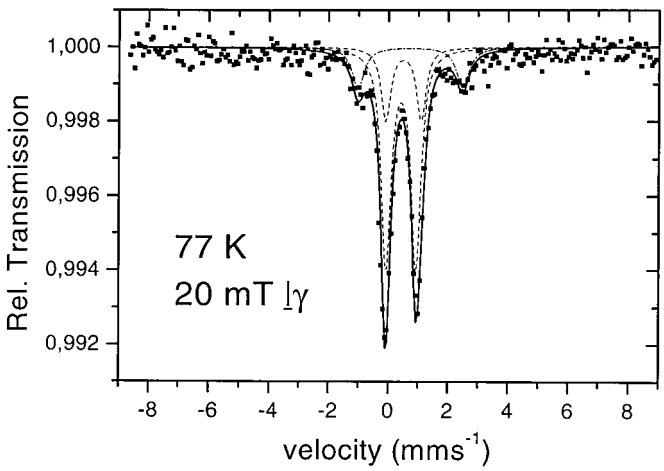

FIG. 6. **Mössbauer spectrum of the wild type enzyme recorded at 77 K in an applied field of 20 mT perpendicular to the γ-ray.** *Dashed lines* are attributed to the different sites of the $(4Fe4S)^{2+}$ cluster (~84%). *Dashed-dotted lines* refer to an Fe^{2+} contamination (~16%).

atoms of (4Fe-4S) clusters are coordinated by the sulfur atom of four cysteinyl residues. However, there are notable exceptions and reported precedents 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 residue (24). Finally, in the (4Fe-4S) center of a ferredoxin from the hyperthermophilic archaeon *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 $(3Fe-4S)^+$ cluster during oxidation. Histidine, aspartate, or glutamate and water are thus possible candidates for playing the role of the fourth ligand in the anaerobic ribonucleotide reductase.

In a number of examples, replacement of cysteine ligands to an iron-sulfur cluster using site-directed mutagenesis has resulted in a significant reduction of the amount of protein produced (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 C*XXX*C*XX*C sequence, present in the activating component of the anaerobic ribonucleotide reductase and in other enzymes, plays a crucial role in stabilizing a

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).

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The Activating Component of the Anaerobic Ribonucleotide Reductase from *Escherichia coli* : AN IRON-SULFUR CENTER WITH ONLY THREE CYSTEINES

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J. Biol. Chem. 2000, 275:15669-15675.
doi: 10.1074/jbc.275.21.15669