Assembly of 2Fe-2S and 4Fe-4S Clusters in the Anaerobic Ribonucleotide Reductase from *Escherichia coli*

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Abstract: *Escherichia coli* contains a specific ribonucleotide reductase for deoxyribonucleotide synthesis and growth under anaerobiosis. The $\alpha_2\beta_2$ enzyme contains an iron−sulfur center on its small β_2 subunit that is involved in the one-electron reduction of *S*-adenosylmethionine and in the generation of a glycyl radical on the large α_2 subunit. By a variety of spectroscopic methods (light absorption, resonance Raman, and Mössbauer spectroscopy) and metal and sulfide analysis, it is shown that the metal center is a $(2Fe\text{-}2S)^{2+}$ cluster. Reduction by photoreduced deazaflavin or dithionite converts these centers mostly into (4Fe-4S) clusters, in both the $(4Fe\text{-}4S)^{1+}$ and $(4Fe\text{-}4S)^{2+}$ states, as is unambiguously demonstrated by Mössbauer spectroscopy at 4.2 and 77 K, in the presence of small (10 mT) or high (5.3 or 7 T) fields. The structure and the function of the iron−sulfur center of the anaerobic ribonucleotide reductase are discussed in relation with other members of a class of enzymes with similar metal centers and functions (reduction of *S*-adenosylmethionine).

Escherichia coli uses different enzymes for the synthesis of deoxyribonucleotides during aerobic and anaerobic growth.[1] While the aerobic ribonucleotide reductase (RNR) has been extensively studied and characterized, less is known on the anaerobic enzyme. It is the prototype for a group of class III anaerobic RNRs also found in other anaerobically growing microorganisms both among archaea and bacteria. It consists of an $\alpha_2\beta_2$ complex, with α_2 being the large homodimeric component encoded by the *nrd*D gene and β_2 the small component encoded by the *nrd*G gene.[2]

Both components can be expressed separately and combined to give an active enzyme.[2] While the small component binds to α_2 as a β_2 dimer, as unambiguously shown from sucrose gradient centrifugation experiments, it consists, in the absence of α_2, of a mixture of monomeric β and dimeric β_2 forms, with a minor amount of polymeric forms, in equilibrium, as shown by gel-exclusion chromatography analysis of β solutions.[2,3] It thus seems that binding to α_2 shifts the equilibrium to the dimeric form.

In the active form protein α_2 contains an oxygen-sensitive glycyl radical supposed to be required for radical activation of the sugar moiety of the ribonucleotide substrate.[4] The presence of an iron−sulfur center in protein β_2 was early concluded from the light absorption and electron paramagnetic resonance properties of the purified active holoenzyme and from iron and sulfide analysis.[5] Previous studies have shown that the pure recombinant protein β_2, purified from overproducing *E. coli* cells, could be obtained only as an apoprotein devoid of enzymatic activity, as a consequence of iron lability.[2] So far all attempts to obtain a protein with significant amounts of iron failed. Under anaerobic conditions, it is however possible to reconstitute an iron center, with each polypeptide chain incorporating a maximal amount of 2.0 ± 0.2 Fe and 2.2 ± 0.2 S.[2] The reconstituted protein is EPR silent and fully enzymatically active. The only spectroscopic evidence so far for the presence of a (4Fe-4S) center in the reconstituted protein β_2 was the observation, under reducing conditions (dithionite or photoreduced deazaflavin), of an EPR signal at $g = 2.02$ and 1.93, with temperature and microwave power dependences consistent with a $S = 1/2$ $(4Fe\text{-}4S)^{+}$ reduced form.[2,6] This preliminary analysis led us to conclude that protein β_2 contained a (4Fe-4S) center that links its two polypeptide chains.[2,6]

The glycyl radical on α_2 is generated, under anaerobic conditions, during incubation of $\alpha_2\beta_2$ with *S*-adenosylmethionine (AdoMet) together with NADPH, flavodoxin, and flavodoxin reductase and dithiothreitol (DTT).[7−11] Photochemically reduced deazaflavin[11] or dithionite[6] can substitute for NADPH and the flavodoxin system. During the reaction AdoMet is reduced and

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(1) Reichard, P. *Science* **1993**, *260*, 1773−1777.
(2) Ollagnier, S.; Mulliez, E.; Gaillard, J.; Eliasson, R.; Fontecave, M.; Reichard, P. *J. Biol. Chem.* **1996**, *271*, 9410−9416.
(3) Sun, X.; Eliasson, R.; Pontis, E.; Anderson, J.; Buist, G.; Sjöberg, B.-M.; Reichard, P. *J. Biol. Chem.* **1995**, *270*, 2443−2446.
(4) Sun, X.; Ollagnier, S.; Schmidt, P. P.; Atta, M.; Mulliez, E.; Lepape, L.; Eliasson, R.; Graslünd, A.; Fontecave, M.; Reichard, P.; Sjöberg, B.-M. *J. Biol. Chem.* **1996**, *271*, 6827−6831.
(5) Mulliez, E.; Fontecave, M.; Gaillard, J.; Reichard, P. *J. Biol. Chem.* **1993**, *268*, 2296−2299.
(6) Ollagnier, S.; Mulliez, E.; Schmidt, P. P.; Eliasson, R.; Gaillard, J.; Deronzier, C.; Bergman.; Gräslund, A.; Reichard, P.; Fontecave, M. *J. Biol. Chem.* **1997**, *272*, 24216−24223.
(7) Eliasson, R.; Pontis, E.; Fontecave, M.; Gerez, C.; Harder, J.; Jörnvall, H.; Krook, M.; Reichard, P. *J. Biol. Chem.* **1992**, *267*, 25541−25547.
(8) Mulliez, E.; Ollagnier, S.; Fontecave, M.; Eliasson, R.; Fontecave, M. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 8759−8762.
(9) Harder, J.; Eliasson, R.; Pontis, E.; Ballinger, M. D.; Reichard, P. *J. Biol. Chem.* **1992**, *267*, 25548−25552.
(10) Bianchi, V.; Reichard, P.; Eliasson, R.; Pontis, E.; Krook, M.; Jörnvall, H.; Haggärd-Ljungquist, E. *J. Bacteriol.* **1995**, *175*, 1590−1595.

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cleaved to 5′-deoxyadenosine and methionine.[9] We have recently shown that the iron−sulfur center plays a crucial role in that reaction since it catalyzes the electron transfer to AdoMet.[6] Accordingly, enzyme activity is well correlated to the iron content in protein β_2[5] and AdoMet efficiently oxidizes the EPR active reduced form of the cluster.[6] This reaction results in the formation of methionine and 5′-deoxyadenosine and in the generation of the glycyl radical on protein α_2. It is remarkable that the activation process begins in the metal site of β_2 and ends up in α_2.

Given the importance of the iron−sulfur center of protein β_2 in the activation of the anaerobic RNR and the emergence of the notion, still too little appreciated, that iron−sulfur clusters, in general, might play catalytic functions during reduction reactions, such as the reductive activation of AdoMet, we found it worth better characterizing the active site of protein β_2. Here we report an extensive spectroscopic characterization of the iron−sulfur center of the anaerobic ribonucleotide reductase from *E. coli* in both oxidized and reduced forms. We show that, in fact, reconstituted active protein β_2 contains two $(2Fe\text{-}2S)^{2+}$ clusters, one per chain, which, under reduction by dithionite or photoreduced deazaflavin, are converted into one active $(4Fe\text{-}4S)^{+}$ center.

Experimental Procedures

Materials. Enzymes and other components of the anaerobic ribonucleotide reductase system have been obtained as previously described.[2,3,9−11] $^{57}Fe_2O_3$ was converted into its chloride by dissolving it in a hot concentrated (35%) hydrochloric acid of analytical grade (Carlo Erba) and repetitively concentrated in water. $^{56}Fe(NH_4)_2(SO_4)_2$ was from Aldrich. AdoMet was from Boehringer Mannheim. Sodium dithionite, titrated under anaerobic conditions with $K_3Fe(CN)_6$, was from Fluka. 5-Deaza-7,8-demethyl-10-methylisoalloxazine (5-DAF) was available in our laboratory. Ferredoxin IV from *Rhodobacter capsulatus* was provided by Dr. Y. Jouanneau (CEA, Grenoble, France).

Analysis. Protein concentration was determined by the method of Bradford,[12] standardized by amino acid analyses of the small and large subunits. Protein-bound iron was determined under reducing conditions with bathophenantroline disulfonate after acid denaturation of the protein[13] and labile sulfide by Beinert's method.[14]

Methods. (a) Reconstitution of the Small Protein. The iron−sulfur center of the β_2 protein was anaerobically reconstituted in the presence of 5 mM DTT during incubation with a 6-fold molar excess of Na_2S and either $^{56}Fe(NH_4)_2(SO_4)_2$ or $^{57}FeCl_3$ for 3 h at 4 °C, followed by incubation with 2 mM EDTA for 30 min. After chromatography over an anaerobic G-25 Sephadex column, the colored fractions were collected and concentrated under aerobic conditions over a YM10 Diaflo membrane (Amicon). Each preparation contained between 1.8 and 2.1 Fe and 2−2.4 S^{2-} per β polypeptide chain, as determined by colorimetric analysis. For all spectroscopic experiments, β_2 reconstituted protein contained DTT at 2−10 mM final concentration except for resonance Raman spectroscopy (30 mM).

(b) Enzymatic Activity. In the first activating step, the holoenzyme $\alpha_2\beta_2$ (2.0 μg), in a total volume of 20 μL, was deaerated with moist argon on a manifold during 30 min at room temperature, with different concentrations of DTT (0−50 mM). In parallel, the activation mixture was deaerated (in the dark when deazaflavin was used as reducing agent) and 15 μL were added to the protein solution so that the final concentrations were 10 mM sodium formate, 30 mM potassium chloride, 500 μM AdoMet, in 30 mM Tris−HCl buffer, pH 8. When deazaflavin was used as the source of electrons, the activating mixture additionally contained 5 μM 5-DAF and was irradiated for 60 min with a slide projector, 20 cm away from the reaction mixture. When the physiological system was used, the activating mixture additionally contained flavodoxin, flavodoxin reductase, and NADPH (final concentrations of 20 μg/mL, 40 μg/mL, and 1.25 mM, respectively) and was likewise incubated for 60 min on the manifold.

In the second step, 15 μL of the substrate mixture (giving a final concentration of 1.4 mM [^{3}H]CTP (20−30 cpm/pmol), 1 mM ATP, 10 mM $MgCl_2$, and 10 mM HCOONa) was added to initiate the reduction of the substrate. The reaction was stopped after 20 min by opening to air and addition of 0.5 mL of 1 M $HClO_4$. The solution was then worked up as described earlier.[7] One unit of enzyme activity is defined as the formation of 1 nmol of dCTP per min.

(c) UV−Visible Absorption. UV−visible spectra of the β_2 protein, in 30 mM Tris−HCl, pH 7.5, 5 mM DTT, were recorded under anaerobic conditions directly inside the glovebox with an HP 8453 diode array spectrophotometer coupled to the measurement cell by optical fibers (Photonetics system).

(d) EPR Spectroscopy. EPR first derivative spectra were recorded on a Varian E109 (9.5 GHz) EPR spectrometer equipped with an ESR 900 Helium flow cryostat (Oxford Instruments). Double integrals of the EPR signals were evaluated by using a computer on-line with the spectrometer. Spin concentrations in the protein samples were determined by calibrating double integrations of the EPR spectra with a standard sample of a 2Fe-2S protein (62 μM ferredoxin IV of *R. capsulatus*).

All the experiments were performed inside a glovebox (Jacomex BS531 NMT) in a N_2 atmosphere containing less than 2 ppm O_2. Stock solutions of dithionite were prepared in 30 mM Tris−HCl, pH 8. 5-Deazaflavin was dissolved in DMSO, diluted with water to 500 μM, and stored inside the box in the dark. Protein β_2 (40−250 μM) was prepared in 30 mM Tris−HCl, pH 8, 5 mM DTT. Then, either dithionite (2−10 molar excess) or 5-DAF (catalytic amount) was added to the protein solution. After 60 min of incubation with dithionite or irradiation with 5-DAF the EPR tube was frozen in liquid nitrogen inside the box.

(e) Mössbauer Spectroscopy. ^{57}Fe-Mössbauer spectra were recorded on 400 μL cups containing the protein (0.25−1 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 by using a bath cryostat (Oxford Instruments) with a permanent magnet mounted outside the cryostat producing a field of 10 mT perpendicular to γ. High-field measurements were performed with a cryostat equipped with a superconducting magnet (Oxford Instruments). The spectra were analyzed assuming Lorentzian line shape, and the isomer shift is quoted relative to α-Fe at room temperature.

(f) Resonance Raman Spectroscopy. The Resonance Raman spectrum of the reconstituted β_2 protein (5 μL, 1.5 mM) was recorded at 15 K with 413. nm excitation from a Kr^+ laser (Coherent, Innova 90), with a spectral resolution of 6 cm^{-1}. The spectra were averaged by the summation of 40−50 independent scans and the ice spectrum was subtracted. No additional smoothing was performed.

Results

The Requirement for Dithiothreitol. In the following, dithiothreitol (DTT) was included in all protein preparations (2−10 mM). As a matter of fact, previous studies had shown that DTT had a number of positive contributions to the enzyme system. First, for still unidentified reasons, it greatly stabilizes the protein that otherwise spontaneously precipitates. Second, it also stabilizes the iron center since higher amounts of iron were recovered in the protein when chromatography of the reconstituted protein β_2 was carried out in the presence of DTT.[2] Third, as shown in Figure 1, DTT is absolutely required for activity, with both the enzymatic NADPH/flavodoxin/flavodoxin reductase system and photoreduced deazaflavin as the reducing agent. The molecular basis of this effect is still unknown and requires further investigation.

A $(2Fe\text{-}2S)^{2+}$ Center in the Reconstituted Small Subunit of the Anaerobic Ribonucleotide Reductase. (a) UV−Visible and Resonance Raman Spectroscopy. The light absorption spectrum of the reconstituted β_2 protein (2.1 Fe/β, 30 μM) is

(11) Bianchi, V.; Eliasson, R.; Fontecave, M.; Mulliez, E.; Hoover, D. M.; Matthews, R. G.; Reichard, P. *Biochem. Biophys. Res. Commun.* **1995**, *197*, 792−797.
(12) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248−254.
(13) Fish, W. W. *Methods Enzymol.* **1988**, *158*, 357−364.
(14) Beinert, H. *Anal. Biochem.* **1983**, *131*, 373−378.

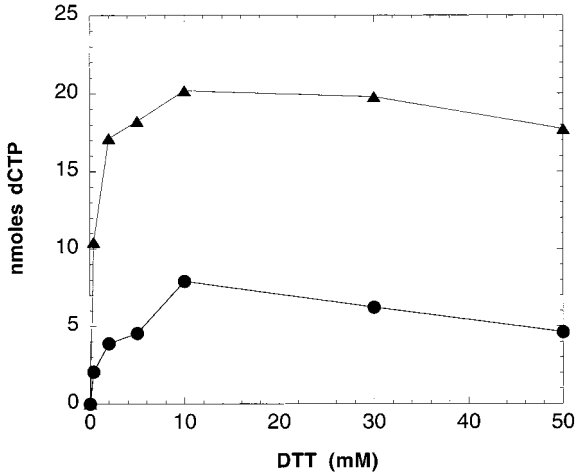

Figure 1. DTT is required for enzymatic activity. The holoenzyme (2 μg) was activated in the presence of an increasing amount of DTT (0−50 mM) with AdoMet (0.5 mM), potassium chloride (30 mM), sodium formate (10 mM), and either photoreduced 5-DAF (5 μM) (▲) or the physiological system (flavodoxin 20 μg/mL, flavodoxin reductase 40 μg/mL, NADPH 1.25 mM) (●) as reducing agent. After 60 min the mixture was assayed for CTP reduction, as described under Experimental Procedures.

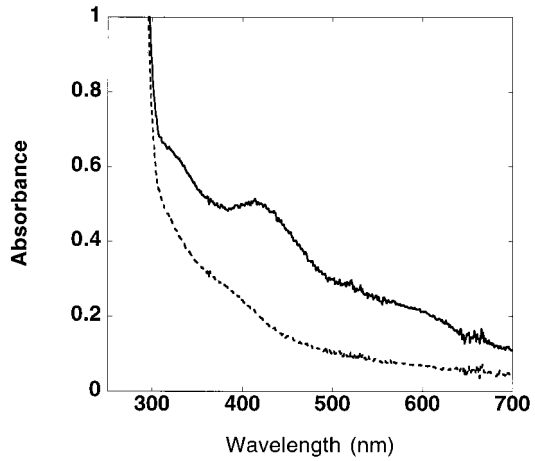

Figure 2. UV−visible light absorption spectra of the β_2 protein (30 μM) in 30 mM Tris−HCl, pH 8, 5 mM DTT, in the oxidized form (solid line) and after anaerobic reduction (dashed line) with 10 μM 5-DAF irradiated for 60 min at 18 °C.

shown in Figure 2. This spectrum is more similar to that of a protein containing a $(2Fe\text{-}2S)^{2+}$ center, with transitions at 325 (21.1 mM^{-1} cm^{-1}), 418 (16.3 mM^{-1} cm^{-1}), 520 (9.0 mM^{-1} cm^{-1}), and 600 nm (6.6 mM^{-1} cm^{-1}), even though they are less well resolved than in most $(2Fe\text{-}2S)^{2+}$ proteins.

In Figure 3 is shown the 413.1 nm excited resonance Raman (RR) spectrum of the β_2 protein at 1.5 mM. The bands at 287, 336, and 397 cm^{-1} together with a broad shoulder at 350 cm^{-1} are well in the range of those observed for an oxidized $[2Fe\text{-}2S]^{2+}$ center, such as found in adrenodoxin or spinach ferredoxin (281−295 and 326−350 cm^{-1} for the two lowest energy Fe−S stretching modes).[15−17] A comparable spectrum was recently reported for the related pyruvate formate lyase activating enzyme and interpreted as arising from a $[2Fe\text{-}2S]^{2+}$ center.[18]

(b) EPR and Mössbauer Spectroscopy. The X-band EPR spectrum of the reconstituted β_2 protein at 4−20 K (0.05−100 mW) displays a small nearly isotropic signal around $g = 2.02$ accounting for less than 2% of the total iron.[2] This signal is characteristic for an oxidized $S = 1/2$ $(3Fe\text{-}4S)^{+}$ center.

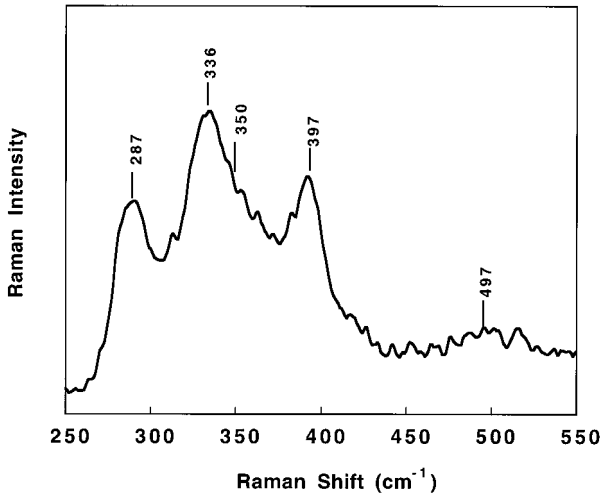

Figure 3. Resonance Raman spectrum of the β_2 protein (1.5 mM) in 0.1 M Tris−HCl, pH 8, 30 mM DTT, taken at 15 K with 413.1 nm excitation from a Kr^{+} laser, spectral resolution 6 cm^{-1}.

In Figure 4A the 4.2 K Mössbauer spectrum of 1 mM ^{57}Fe-reconstituted protein (2.05 ± 0.05 Fe and 2.00 ± 0.05 S per β) in a small external field of 10 mT is shown. The simulation is consistent with the presence of two components. The major component I (65% of the total area) consists of a quadrupole doublet with parameters ($\delta = 0.28$ mm s^{-1}, $\Delta Eq = 0.59$ mm s^{-1}) in the range for high-spin Fe(III) with a tetrahedral sulfur coordination. When recorded at 4.2 K in a 5.3 T external field, component I shows magnetic splitting that can be simulated by using the nuclear Hamiltonian only (Figure 4B), i.e., component I is diamagnetic ($S = 0$). This rules out mononuclear iron sites as in rubredoxin and oxidized $(3Fe\text{-}4S)^{+}$ clusters since they are paramagnetic and the spectra would exhibit substantial magnetic hyperfine interactions under the conditions of Figure 4B. Instead this demonstrates that component I represents a diamagnetic $(2Fe\text{-}2S)^{2+}$ cluster, with both irons being in the ferric high-spin state coupled antiferromagnetically, resulting in a spin $S = 0$.

The minor component II (35% of total area) exhibits at 4.2 K in a small field (10 mT) a broad unresolved magnetic pattern (Figure 4A) and at 4.2 K in a 5.3 T external field a magnetically split pattern with broad lines (Figure 4B), while at 77 K the hyperfine pattern has collapsed to a quadrupole doublet with the parameters $\delta = 0.35$ mm s^{-1} and $\Delta Eq = 0.88$ mm s^{-1} (Figure 4C). This behavior indicates small superparamagnetic iron clusters. A similar unidentified broad component was also observed in the Mössbauer spectrum of the oxidized form of the fumarate nitrate reduction (FNR) regulatory protein.[19] The latter was previously suggested to contain an iron−sulfur center comparable to that of the anaerobic ribonucleotide reductase.

We found that the proportion of component I increased as the concentration decreased. At 250 μM concentration, for example, it accounted for more than 75% (data not shown). It is likely that under enzyme assay conditions, where the protein

(15) Han, S.; Czernuszewicz, R. S.; Kimura, T.; Adams, M. W. W.; Spiro, T. G. *J. Am. Chem. Soc.* **1989**, *111*, 3505−3511.
(16) Fu, W.; Drozdzewski, P. M.; Davies, M. D.; Sligar, S. G.; Johnson, M. K. *J. Biol. Chem.* **1992**, *267*, 15502−15510.
(17) Crouse, B. R.; Sellers, V. M.; Finnegan, M. G.; Dailey, H. A.; Johnson, M. K. *Biochemistry* **1996**, *35*, 16222−16229.
(18) Broderick, J. B.; Duderstadt, R. E.; Fernandez, D. C.; Wojtuszewski, K.; Henshaw, T.; Johnson, M. K. *J. Am. Chem. Soc.* **1997**, *119*, 7396−7397.
(19) Khoroshilova, N.; Popescu, C.; Münck, E.; Beinert, H.; Kiley, P. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 6087−6092.

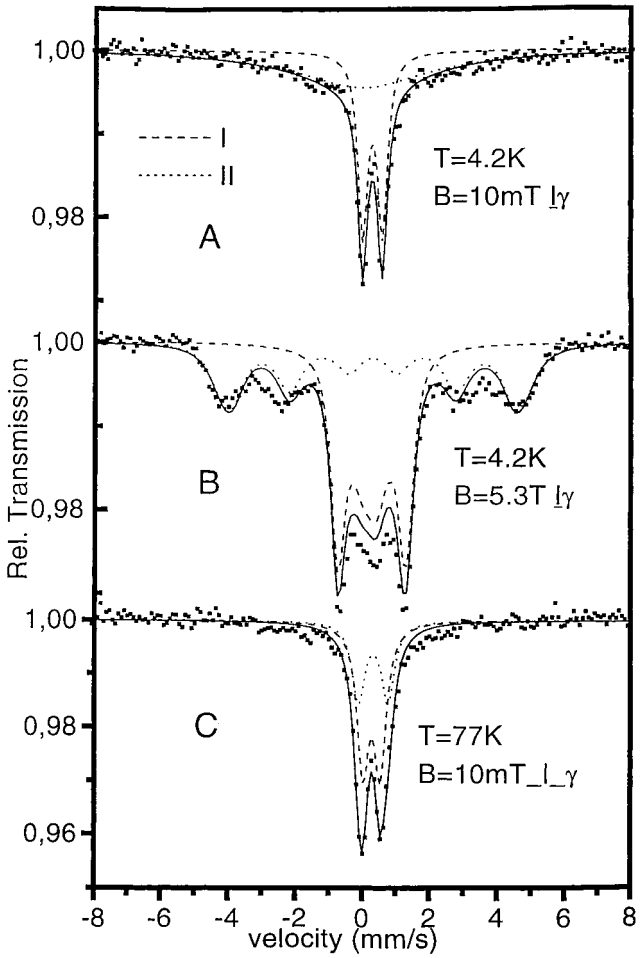

Figure 4. Mössbauer spectra of the oxidized β_2 protein (1 mM) in 30 mM Tris−HCl, pH 8, 2 mM DTT, recorded at 4.2 K in an applied field of 10 mT perpendicular (A) and in 5.3 T parallel (B) to the γ-beam. Spectrum C was taken at 77 K in an applied field of 10 mT perpendicular to the γ-beam. Subspectrum I corresponds to $(2Fe\text{-}2S)^{2+}$ clusters and subspectrum II to contaminant superparamagnetic iron clusters (see text). Solid lines are simulated spectra.

concentration is in the micromolar range, component II is insignificant.

A (4Fe-4S) Center in the Reduced Small Subunit of Ribonucleotide Reductase. (a) UV−Visible Spectroscopy. In Figure 2 is shown the light absorption spectrum of the β_2 protein anaerobically treated by a 10-fold excess of dithionite or by a catalytic amount of 5-deazaflavin in the presence of light. Most striking is the almost complete bleaching of the solution and the absence of transitions at around 540 nm, which are commonly observed in reduced $(2Fe\text{-}2S)^{+}$ clusters.

(b) EPR Spectroscopy. In Figure 5A is shown the X-band EPR spectrum, recorded at 10 K and 100 μW microwave power, of the ^{57}Fe-reconstituted β_2 protein (0.25 mM, 1.8 Fe/β) after reduction by dithionite. A very similar spectrum was obtained with photoreduced deazaflavin as the reducing agent. The EPR signal of the reduced cluster has apparent g values at 2.02 and 1.92, most similar to what we have already reported.[6] This signal is characteristic for $S = 1/2$ species integrating, under nonsaturating conditions, to about 0.6 (dithionite) up to 0.9 (deazaflavin) spin per β_2 protein. In fact, measurements at various microwave powers and higher temperatures demonstrated the presence of two components: one with fast relaxing properties, which broadened above 20 K, and assigned to a $(4Fe\text{-}4S)^{+}$ cluster, and a second one, with slower relaxation characteristics, detectable up to 70 K and assigned to a $(2Fe\text{-}2S)^{+}$ cluster. At temperatures above 35 K, when the contribution of the $(4Fe\text{-}4S)^{+}$ vanishes due to line broadening, there is still a rhombic EPR signal with g values at 2.04, 1.93, and 1.88 characteristic for $(2Fe\text{-}2S)^{+}$ centers (Figure 5B). The relative proportion of

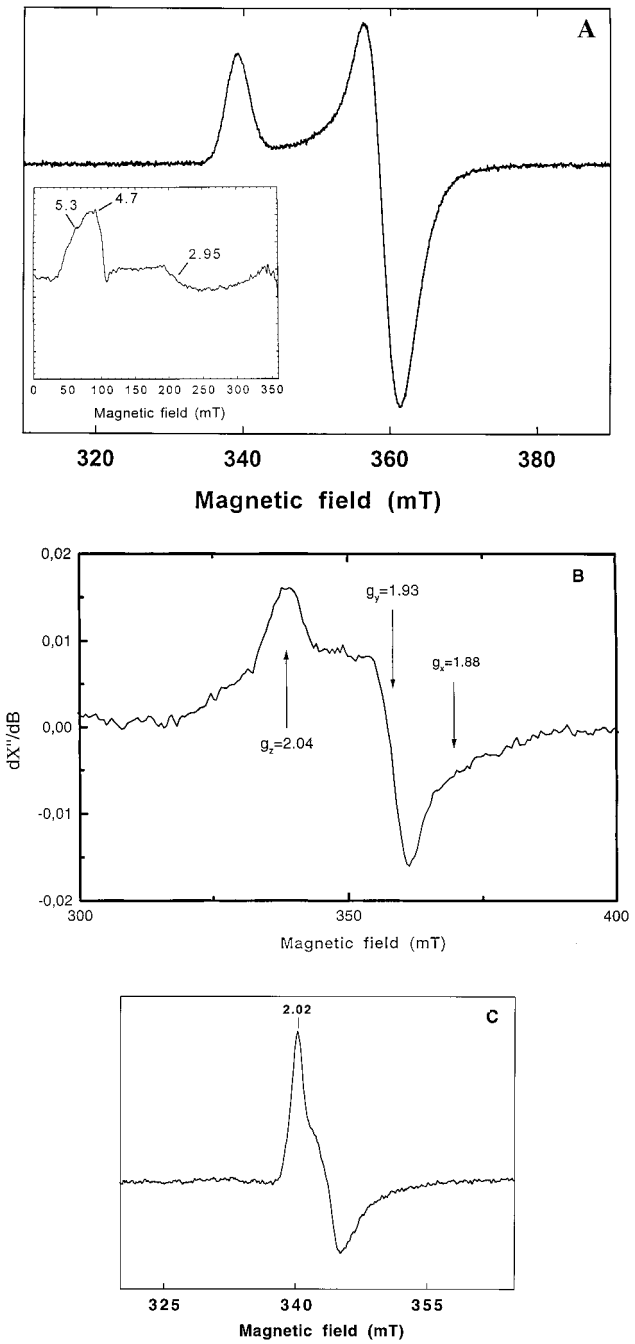

Figure 5. X-band EPR spectra of the reduced β_2 protein in 30 mM Tris−HCl, pH 8, 2 mM DTT (Sample A). (A) EPR signal obtained during reduction of the ^{57}Fe-β_2 protein (250 μM) with dithionite (500 μM) upon 60 min of incubation at 18 °C. Temperature 10 K; microwave power 100 μW; modulation amplitude 1 mT. (Inset) Low-field signals obtained during reduction of the ^{56}Fe-β_2 protein at high concentration (1 mM). Temperature 4 K; microwave power 5 mW; modulation amplitude 1.25 mT. (B) Same sample as in part A recorded at 43 K. Microwave power 200 mW; modulation amplitude 0.5 mT. (C) ^{56}Fe-reconstituted protein (40 μM) reduced with photoreduced 5-DAF (40 μM) and incubated in the dark for 3 min at room temperature with thionin (3 molar excess). Temperature 10 K; microwave power 1 mW; modulation amplitude 1 mT.

the second component greatly decreased with decreased protein concentration. In the case of dithionite, the EPR-active species is stable for hours under strict anaerobic conditions. If photoreduced deazaflavin is used instead as a reducing agent, it is rather unstable in the absence of irradiation and spontaneously decays, even under anaerobic conditions, to an EPR-silent species. The EPR-active and the EPR-silent reduced samples will be named A and B, respectively.

At much higher protein concentrations (1 mM, 0.7 spin per dimer), additional resonances at g values of 5.3, 4.7, and 2.95, that can be assigned to a $S = 3/2$ spin multiplet, were observed (Figure 5A, inset). Because of the multiplicity and the broadness of the resonances it is difficult to quantitate this spin state. At this concentration the signal is estimated to account for less than 10% of the iron present in the sample. Such a $S = 3/2$ state has only been observed with $(4Fe\text{-}4S)^{1+}$ clusters so far.[20] At 250 μM, this signal is hardly detectable.

Finally, treatment of sample A under anaerobic conditions with strong oxidants such as hydrogen peroxide or thionin instantaneously generated a new EPR-active species with high yield (70%), observable only during the first minutes of the reaction (Figure 5C). This EPR signal (shape, temperature dependence, and microwave power saturation properties) was characteristic of an oxidized $(3Fe\text{-}4S)^{+}$ center (data not shown). This provides further support to the hypothesis that A contains a $(4Fe\text{-}4S)^{+}$ center since $(3Fe\text{-}4S)^{+}$ centers are usually obtained from $(4Fe\text{-}4S)^{+}$ centers, under oxidative conditions.

(c) Mössbauer Spectroscopy. Samples A and B obtained as described above with a 250 μM ^{57}Fe-reconstituted protein preparation (1.8 Fe/β) were characterized by Mössbauer spectroscopy. The EPR-silent sample B exhibits, at 4.2 K in a small external field of 10 mT, two quadrupole doublets (component I with $\delta = 0.45$ mm s^{-1} and $\Delta Eq = 1.11$ mm s^{-1}, 95%, and component II with $\delta = 0.31$ mm s^{-1} and $\Delta Eq = 0.70$ mm s^{-1}, 5%; Figure 6A). This shows the presence of two populations of iron. The parameters of component I are indicative of tetrahedrally sulfur-coordinated, delocalized mixed-valent $Fe^{2.5+}$, while the parameters of component II are representative for tetrahedrally sulfur-coordinated Fe^{3+}. Applying a 5.3 T external field yields a magnetic pattern (Figure 6B), the simulations of which show that 85% of the iron belongs to a diamagnetic and 15% to a paramagnetic species. The diamagnetic ground state and the δ and ΔEq values strongly indicate that the major part of the iron is assembled in $(4Fe\text{-}4S)^{2+}$ clusters ($S = 0$), each consisting of two antiferromagnetically coupled $Fe^{2.5+}$-$Fe^{2.5+}$ dimers.[21] To characterize the remaining paramagnetic part (15%) we make use of the following observations: (i) the sample is EPR silent, (ii) it contains 5% Fe^{3+}, and (iii) 95% of the iron correspond to $Fe^{2.5+}$ but only 85% are assembled in diamagnetic $(4Fe\text{-}4S)^{2+}$ clusters, i.e., 10% must be assembled in paramagnetic clusters. With these observations it is tempting to assume that the remaining part of the iron (15%) is assembled in $(3Fe\text{-}4S)^{0}$ clusters ($S = 2$, EPR silent), each consisting of one $Fe^{2.5+}$ dimer and one Fe^{3+} monomer. Accordingly we have used published spin-Hamiltonian parameters from $(3Fe\text{-}4S)^{0}$ to simulate the paramagnetic background (15%) in Figure 6B.[22]

The EPR-active sample A has been studied by Mössbauer spectroscopy in a low field (10 mT) at both 4.2 (data not shown) and 77 K (Figure 7A) and also at 4.2 K in external fields of 1 (Figure 7B) and 7 T (Figure 7C).

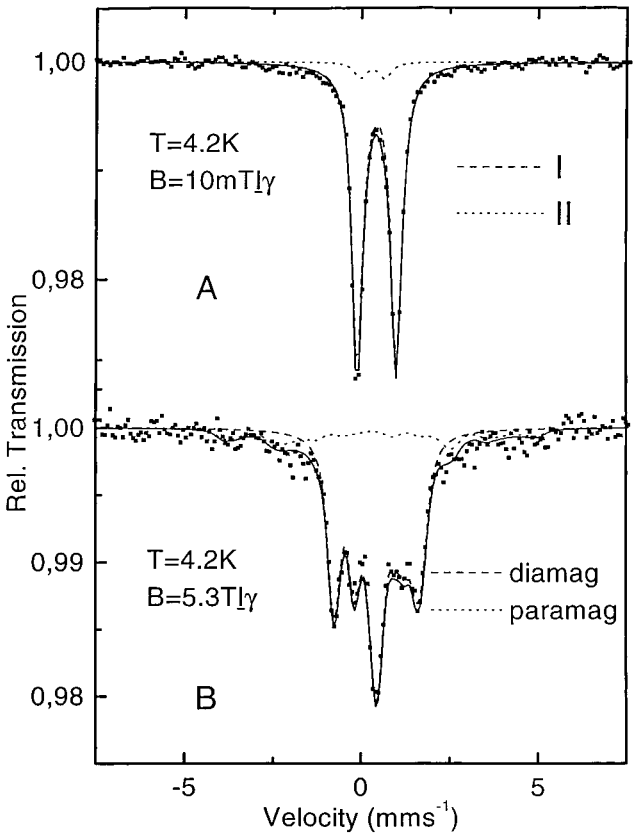

Figure 6. Mössbauer spectra of the reduced β_2 protein (250 μM) (Sample B), recorded at 4.2 K in an applied field of 10 mT (A) and 5.3 T (B), each perpendicular to the γ-beam. The two quadrupole doublets in part A correspond to delocalized mixed-valence $Fe^{2.5+}$ (I, 95%) and Fe^{3+} (II, 5%) sites. The diamagnetic subspectrum in part B (dashed line, 85%) corresponds to $(4Fe\text{-}4S)^{2+}$ and the paramagnetic subspectrum (dotted line, 15%) to $(3Fe\text{-}4S)^{0}$ clusters. Solid lines are simulated spectra.

The two magnetically split spectra (Figure 7, parts B and C) consist of a diamagnetic and paramagnetic part. The diamagnetic part has parameters ($\delta = 0.45$ mm s^{-1}, $\Delta Eq = 1.04$ mm s^{-1}) similar to those of component I, $(4Fe\text{-}4S)^{2+}$, of the EPR-silent sample B (Figure 6). The paramagnetic part is expected to reflect the two major EPR-active components of sample A, assigned to $(4Fe\text{-}4S)^{1+}$ and $(2Fe\text{-}2S)^{1+}$ clusters. Therefore the measured spectra (Figure 7A−C) were fitted by superposition of subspectra of $(2Fe\text{-}2S)^{1+}$, $(4Fe\text{-}4S)^{2+}$, and $(4Fe\text{-}4S)^{1+}$ clusters. The individual subspectra of these clusters were simulated with parameters which were either taken from our own EPR measurements (g values) or were basically known from the litterature.[21,23,24] In the present case we had to adjust slightly some of the parameters (Table 1) as, for example, the $A_{x,y,z}$ values for the Fe^{3+} site of $(2Fe\text{-}2S)^{1+}$ clusters from −41.2, −36.6, −31.6 T[24] to −48, −48, −48 T, to account for the outermost lines in Figure 7B. Applying this procedure, the spectra in Figure 7A−C consistently indicate that the reduced β_2 subunits can accommodate 2Fe as well as 4Fe clusters, with $(2Fe\text{-}2S)^{1+}$ accounting for 20%, $(4Fe\text{-}4S)^{2+}$ for 30%, and $(4Fe\text{-}4S)^{1+}$ for 50% of the total amount of iron. No $S = 3/2$ species was considered in the fitting procedure, since at the concentration used (250 μM) the corresponding signals were hardly detectable.

(20) Hagen, W. R. *Adv. Inorg. Chem.* **1992**, *38*, 165−216.
(21) Middleton, P.; Dickson, P. D. E.; Johnson, C. E.; Rush, J. D. *Eur. J. Biochem.* **1978**, *88*, 135−141.
(22) Papaefthymiou, V.; Girerd, J.-J.; Moura, J. J. G.; Moura, E.; Münck, E. *J. Am. Chem. Soc.* **1987**, *109*, 4703−4710.
(23) Fee, J. A.; Yoshida, T.; Hille, R.; Tarr, G. E.; Hearshen, D. O.; Dunham, W. R.; Day, E. P.; Kent, T. A.; Münck, E. *J. Biol. Chem.* **1984**, *259*, 124−133.
(24) Lloyd, S. G.; Franco, R.; Moura, J. J. G.; Moura, I.; Ferreira, G. C.; Huynh, B. H. *J. Am. Chem. Soc.* **1996**, *118*, 9892−9900.

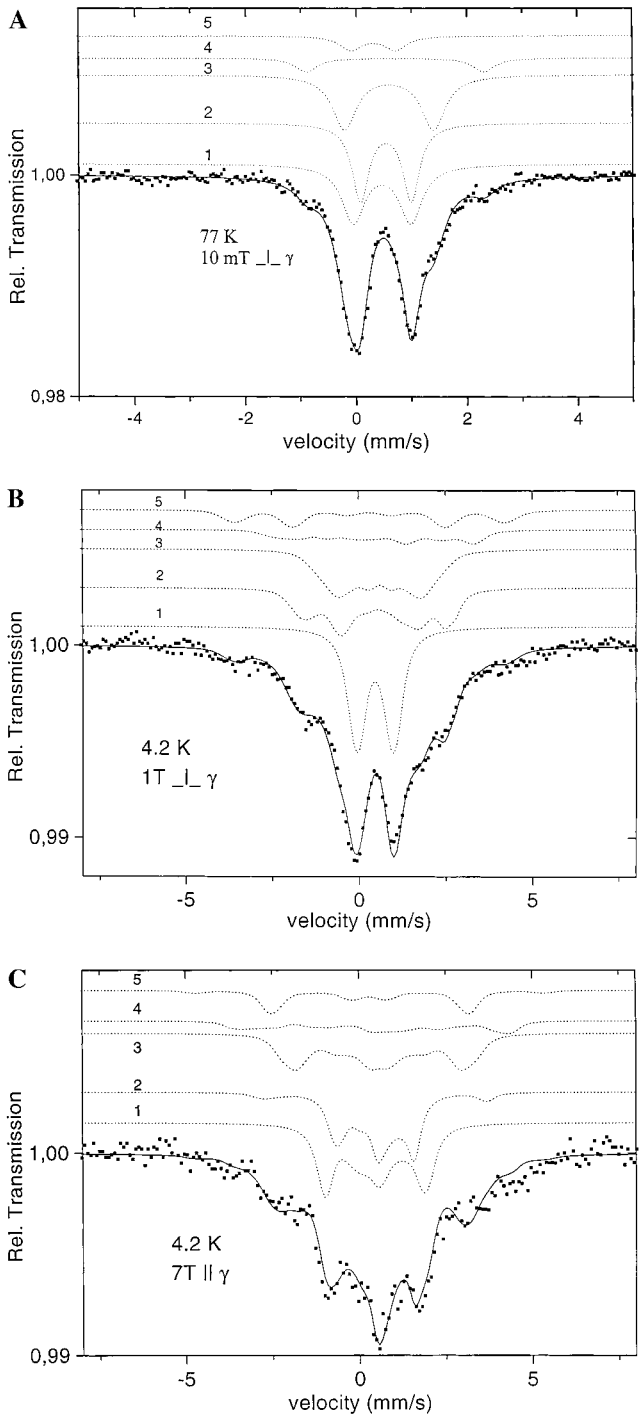

Figure 7. Mössbauer spectra of the reduced β_2 protein (250 μM) (Sample A), recorded at 77 K, 10 mT perpendicular to γ (A); 4.2 K, 1 T perpendicular to γ (B); and 4.2 K, 7 T parallel to γ (C). All spectra were simulated (solid lines) with three species with hyperfine parameters summarized in Table 1. Subspectrum 1 (30%) is attributed to diamagnetic $S = 0$ $(4Fe\text{-}4S)^{2+}$ clusters. Subspectra 2 and 3 (together 50%) are assigned to paramagnetic $S = 1/2$ $(4Fe\text{-}4S)^{+1}$ clusters, and subspectra 4 and 5 (together 20%) correspond to paramagnetic $S = 1/2$ $(2Fe\text{-}2S)^{1+}$ clusters.

To check whether the formation of the (4Fe-4S) centers occurs through disassembly of (2Fe-2S) precursors, we decided to perform the reduction of ^{57}Fe-reconstituted β_2 in the presence of ^{56}Fe. Release of ^{57}Fe and incorporation of ^{56}Fe in the cluster might be an indication of disassembly. Unfortunately, addition of a small excess of $FeCl_3$ in the presence of DTT under anaerobic conditions resulted in precipitation of the protein.

Discussion

The anaerobic ribonucleotide reductase contains, in its small subunit, an iron−sulfur center that was previously shown to catalyze electron transfer from flavodoxin to *S*-adenosylmethionine.[6] Chemical reducing agents such as dithionite or photoreduced deazaflavin can substitute for flavodoxin. By doing so the metal center participates in the oxidation of glycine 681 of the large subunit and the generation of the catalytically essential glycyl radical. Until now our working hypothesis was that the iron−sulfur center was a (4Fe-4S) center linking two polypeptide chains.[2,6] This figure has now to be corrected slightly on the basis of the full spectroscopic characterization of the iron center reported here.

It is for the first time unambiguously demonstrated that a large proportion of reduced clusters is actually of the cubane type, thus confirming our first hypothesis. Mössbauer spectroscopy showed that at least 80% of the iron in the reduced sample was either in the EPR-active $S = 1/2$ $(4Fe\text{-}4S)^{+}$ form, containing formally one ferric and three ferrous irons, or the EPR-silent $S = 0$ $(4Fe\text{-}4S)^{2+}$ form, containing formally two ferric and two ferrous iron. The former state is expected to be highly reactive. This is seen during oxidation with oxidants such as hydrogen peroxide and thionin which convert it into the oxidized $S = 1/2$ $(3Fe\text{-}4S)^{+}$ state. This is also reflected in the fact that in the absence of a reductive pressure (for example, in the absence of irradiation after reduction by deazaflavin), it is spontaneously, by an unknown mechanism, converted to the $(4Fe\text{-}4S)^{2+}$ state. The instability of this reduced state may also explain the formation of minor amounts of $(3Fe\text{-}4S)^{0}$ in some preparations. It is tempting to suggest that such a high reactivity is required for an efficient reduction of AdoMet. Indeed the $(4Fe\text{-}4S)^{+}$ cluster was previously shown to be very reactive with regard to AdoMet, resulting in cluster oxidation, reductive cleavage of the sulfonium functionality, and generation of the glycyl radical.[6]

The unexpected observation reported here is that the (4Fe-4S) cluster was produced by reduction of two $(2Fe\text{-}2S)^{2+}$ clusters. As a matter of fact, the spectroscopic data (resonance Raman and Mössbauer spectroscopies) and iron and sulfur quantitation were in agreement with the presence of ferric iron exclusively, mostly within $(2Fe\text{-}2S)^{2+}$ centers in the reconstituted small subunit. A minor population of iron has been shown to reside within unidentified superparamagnetic clusters, whose proportion was minimal in the presence of DTT (data not shown). The reductive conversion from (2Fe-2S) to (4Fe-4S) centers is rather unusual in the iron−sulfur chemistry and has been encountered only very recently, in the case of the iron-sulfur centers of FNR and biotin synthase.[19,25]

During reduction, we obtained evidence, in some experiments, for the presence of reduced $(2Fe\text{-}2S)^{+}$ centers, suggesting that such a one-electron reduced form was an intermediate of the reaction. Furthermore, the Mössbauer parameters of both the oxidized and reduced (2Fe-2S) centers were significantly different from those of exclusively sulfur-coordinated iron sites. The isomer shift of the oxidized center lies between the isomer shifts of the two Fe^{3+} sites of the oxidized Rieske protein (compare 0.28 mm s^{-1} to $\delta(S_2N_2Fe^{3+}) = 0.32$ mm s^{-1} and to $\delta(Fe^{3+}S_4) = 0.24$ mm s^{-1}).[23] The isomer shifts of the ferrous and the ferric site of the reduced cluster are higher than expected for exclusively sulfur-coordinated iron sites.[24] It is thus likely that iron coordination in the dimer is mixed, with sulfur and non-sulfur ligands, in agreement with the finding that only three

(25) Duin, E. C.; Lafferty, M. E.; Crouse, B. R.; Allen, R. M.; Sanyal. I.; Flint, D. H.; Johnson, M. K. *Biochemistry* **1997**, *36*, 11811−11820.

Table 1. Parameters Used for a Spin-Hamiltonian Simulation[33] of Subspectra 1−5 in Figure 7A−C[a]

subspectra	Γ, mm s^{-1}	δ, mm s^{-1}	ΔEq, mm s^{-1}	η	β, deg	g_x[b]	g_y[b]	g_z[b]	A_x,[c] T	A_y,[c] T	A_z,[c] T	rel area, %
1. $(4Fe)^{2+}$ $Fe^{2.5+}$ sites	0.5	0.46	1.04	0.6	0							30
2. $(4Fe)^{1+}$ $Fe^{2.5+}$ sites	0.44	0.53	0.92	1	0	1.92	1.92	2.02	−25 (−23.1)	−26 (−23.8)	−21 (−20.4)	25
3. $(4Fe)^{1+}$ Fe^{2+} sites	0.52	0.59	1.61	1	90	1.92	1.92	2.02	5.0 (6.3)	6.0 (9.9)	20 (19.3)	25
4. $(2Fe)^{1+}$ Fe^{2+} sites	0.48	0.70	3.2	1	0	1.88	1.93	2.04	10 (10.3)	15 (15.5)	24 (25.8)	10
5. $(2Fe)^{1+}$ Fe^{3+} sites	0.48	0.31	0.8	1	0	1.88	1.93	2.04	−48 (−41.2)	−48 (−36.8)	−48 (−31.6)	10

[a] Γ: line width. δ: isomer shift. ΔEq: quadrupole splitting. η: asymmetry parameter. β: Euler angle relating electric field gradient tensor and magnetic hyperfine coupling tensor. $g_{g,y,z}$: **g** tensor. $A_{x,y,z}$: magnetic hyperfine coupling tensor. [b] From our own EPR measurements. [c] Values in brackets refer to published parameters for $(4Fe\text{-}4S)^{1+}$ (ref 21) and $(2Fe\text{-}2S)^{1+}$ (ref 24).

cysteines in the N-terminal part of the protein are fully conserved among homologous proteins, as shown by sequence comparisons.[3]

Concerning the mechanism of the (2Fe-2S) to (4Fe-4S) conversion, it is still impossible to discriminate between a pure dimerization of two closely associated (2Fe-2S) centers, with no transient decomposition of the clusters and formation of a (4Fe-4S) center linking the two β chains, and a decomposition of the original clusters followed by reassembly of the (4Fe-4S) cluster. The latter possibility is supported by the observation that some iron (the "superparamagnetic particles" observed by Mössbauer spectroscopy) present in the oxidized sample, but not in the $(2Fe\text{-}2S)^{2+}$ centers, is found after reduction within defined 3Fe and 4Fe clusters.

In the case of ribonucleotide reductase the finding that reduction results in the formation of (4Fe-4S) centers raises the following questions:

The first obvious question is whether the reduced (4Fe-4S) center is at the interface of two polypeptide chains, using ligands of both, as previously suggested, or is present in only half of the polypeptides, due to the limited amount of iron available. There is still no strong experimental evidence for any of these two possibilities and further studies are required to answer the question. However, it is important to note that the activase of the pyruvate-formate lyase (PFL), which shows strong homologies to the β protein[3] and also combines a reduced iron−sulfur center and AdoMet for generation of a glycyl radical on PFL,[26,27] is a monomer carrying one (4Fe-4S) center per protein. A (4Fe-4S) center at the interface of a dimer thus does not appear to be absolutely required for carrying out AdoMet activation and reduction. Furthermore, on the basis of the dimeric nature of the $\alpha_2\beta_2$ holoenzyme and the availability of two identical glycine residues, one on each α chain, it seems more likely that each β chain has the potential to carry an active (4Fe-4S) center involved in the radical formation of the corresponding α chain.

The second question is related to the first one. As discussed above, the presence of one (4Fe-4S) center per β chain would be a likely hypothesis. On the contrary, the pure protein used in this study could not chelate more than 2 irons and 2 sulfides which generated $(2Fe\text{-}2S)^{2+}$ centers. This was shown during reconstitution of the iron center which was an obligatory preparation step since iron was lost during expression and/or purification, even when this was achieved anaerobically. Even though the oxidized protein might actually be a (2Fe-2S) protein, especially if one considers that the reconstituted $(2Fe\text{-}2S)^{2+}$ enzyme was fully active during catalysis of AdoMet reduction and formation of the glycyl radical, we cannot rule out that the limited incorporation of iron was an artifact of reconstitution or that the recombinant protein did not have the proper folding to incorporate a (4Fe-4S) center per polypeptide chain. The native protein instead might contain a (4Fe-4S) center per β chain which, under the anaerobic highly reducing conditions within the cell, would be continuously retained with no formation of a fully oxidized center. As it was recently used to study the FNR iron−sulfur protein in vivo,[28] analysis of whole *E. coli* cells by Mössbauer spectroscopy might give some insight into the structure of the cluster within the cell.

Ribonucleotide reductase and lysine aminomutase, LAM,[29] were for some time the only enzymes known to carry an iron−sulfur center for reduction of *S*-adenosylmethionine and for radical generation. It appears now that the iron−sulfur/AdoMet combination is used more extensively by enzymes than previously expected. The present study provides one of the most complete characterizations of this class of iron−sulfur centers. There is good evidence for such a chemistry in (i) anaerobic DNA synthesis (ribonucleotide reductase),[6] (ii) lysine metabolism (lysine aminomutase),[29] (iii) anaerobic pyruvate metabolism (pyruvate-formate lyase),[26,27] (iv) formation of C−S bonds (biotin synthase),[25,30] (v) DNA repair (spore photoproduct lyase)[31] and (vi) toluene metabolism (benzylsuccinate synthase).[32] In all cases it is very likely that the enzyme reaction involves controlled free radicals. It is also remarkable that in all these systems (except for LAM for which no sequence is known so far) the iron−sulfur protein contains the unusual CXXXCXXC sequence which probably provides the cysteines involved in iron chelation. This sequence is likely to give the metal center specific spectroscopic and chemical properties. The FNR protein, which contains such a sequence and is not involved in AdoMet reduction, also carries an iron−sulfur center very similar to that of ribonucleotide reductase.[19]

Acknowledgment. We are grateful to the European Union for financial support.

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(26) Külzer, R.; Pils, T.; Kappl, R.; Hüttermann, J.; Knappe, J. *J. Biol. Chem.* **1998**, *273*, 4897−4903.

(27) Wagner, A. F.; Frey, M.; Neugebauer, F. A.; Schäfer, W.; Knappe, J. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 996−1000.

(28) Popescu, C. V.; Bates, D. M.; Beinert, H.; Münck, E.; Kiley, P. J. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13431−13435.

(29) Lieder, K. W.; Booker, S.; Ruzicka, F. J.; Beinert, H.; Reed, G. H.; Frey, P. A. *Biochemistry* **1998**, *37*, 2578−2585.

(30) Shaw, N. M.; Birch, O. M.; Tinschert, A.; Venetz, V.; Dietrich, R.; Savoy, L.-A. *Biochem. J.* **1998**, *330*, 1079−1085.

(31) Rebeil, R.; Sun, Y.; Chooback, L.; Pedraza-Reyes, M.; Kinsland, C.; Begley, T. P.; Nicholson, W. L. *J. Bacteriol.* **1998**, *180*, 4879−4885.

(32) Leuthner, B.; Leutwein, C.; Hörth, H.; Haehnel, W.; Schiltz, E.; Schägger, H.; Heider, J. *Mol. Microbiol.* **1998**, *28*, 615−628.

(33) Trautwein, A. X.; Bill, E.; Bominaar, E. L.; Winkler, H. *Structure and Bonding*; Springer: New York, 1991; Vol. 78, pp 1−21.