Activation of Class III Ribonucleotide Reductase from *E. coli*. The Electron Transfer from the Iron−Sulfur Center to *S*-Adenosylmethionine†

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ABSTRACT: The anaerobic ribonucleotide reductase (ARR) from *E. coli* is the prototype for enzymes that use the combination of *S*-adenosylmethionine (AdoMet) and an iron−sulfur center for generating catalytically essential free radicals. ARR is a homodimeric α2 protein which acquires a glycyl radical during anaerobic incubation with a [4Fe-4S]-containing activating enzyme (β) and AdoMet under reducing conditions. Here we show that the EPR-active $S = 1/2$ reduced $[4Fe\text{-}4S]^+$ cluster is competent for AdoMet reductive cleavage, yielding 1 equiv of methionine and almost 1 equiv of glycyl radical. These data support the proposal that the glycyl radical results from a one-electron oxidation of the reduced cluster by AdoMet. Reduced protein β alone is also able to reduce AdoMet but only in the presence of DTT. However, in that case, 2 equiv of methionine per reduced cluster was formed. This unusual stoichiometry and combined EPR and Mössbauer spectroscopic analysis are used to tentatively propose that AdoMet reductive cleavage proceeds by an alternative mechanism involving catalytically active [3Fe-4S] intermediate clusters.

Ribonucleotide reductases (RNRs) catalyze the reduction of ribonucleotides to their corresponding 2′-deoxyribonucleotides (*1*). These enzymes are of utmost importance because there are no other biosynthetic pathways for the de novo formation of DNA building blocks. The general chemical strategy of these enzymes is to generate and use a protein radical to activate the substrate by abstraction of the 3′-hydrogen atom of its ribose moiety (*2*). This then allows the 2′-C−OH bond to be reduced to a C−H bond by reducing equivalents provided either by active site redox cysteines or by an exogenous reductant. Three classes of RNRs are presently recognized which differ widely in their cofactors, reflecting very different ways to generate the key protein radical (*3*, *4*).

Whereas introduction of this radical has been studied in great detail in the case of class I and II ribonucleotide reductases, much less is known for the last member of the family. Class III is found in strict or facultative anaerobic microorganisms (*1*). The enzyme from *E. coli* is considered as the prototype for this class. It is a homodimer α2 of 2 × 80 kDa which, in its active form, carries a catalytically essential and oxygen-sensitive glycyl radical on the C-terminal part of the polypeptide (*5*, *6*). The maximum amount of radical that can be generated so far is one per dimer, thus suggesting that, within the dimer, only half of the subunits can acquire the radical (*7*).

The generation of the glycyl radical in vitro depends on the concerted action of four components: (1) an enzymatic source of electrons, the NADPH/flavodoxin oxidoreductase/flavodoxin system, which can be substituted for by strong chemical reductants such as sodium dithionite or photoreduced deazaflavin (*8*−*10*); (2) *S*-adenosylmethionine (AdoMet),[1] which is reductively cleaved into methionine and presumably to the 5′-deoxyadenosyl radical proposed to be the H-atom-abstracting species during conversion of the glycine residue of protein α to the glycyl radical (*10*−*12*); (3) dithiothreitol (DTT), which maintains a pair of essential cysteines on protein α in the dithiol form (*13*, *14*); (4) an iron−sulfur protein, named protein β and containing a peculiar (4Fe-4S) cluster, which mediates the electron transfer from flavodoxin to AdoMet. In the presence of the α2 homodimer, protein β forms a tight α2β2 complex (*15*). However, under the reducing conditions of enzyme activation, a single molecule of protein β is able to introduce the glycyl radical into several α polypeptides. This fact has led to the notion that protein β could be defined as an activase (*16*).

According to Scheme 1, which shows our working hypothesis for the generation of the radical that is commented under Discussion, one predicts the formation of one methionine per radical. In experiments using flavodoxin as the

Scheme 1: Hypothetical Mechanism for the Activation of Anaerobic Ribonucleotide Reductase

$$[4Fe\text{-}4S]^{2+} + e^- \longrightarrow [4Fe\text{-}4S]^+ \quad (1)$$

$$[4Fe\text{-}4S]^+ + \text{AdoMet} \rightleftharpoons [4Fe\text{-}4S]^{2+} + \text{Ado}^\circ + \text{methionine} \quad (2)$$

$$\text{Ado}^\circ + \text{Gly-H} \longrightarrow \text{Gly}^\circ + \text{5′-deoxyAdenosine} \quad (3)$$

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[1] Abbreviations: AdoMet, *S*-adenosylmethionine; DTT, dithiothreitol; ARR, anaerobic ribonucleotide reductase.

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reducing system, such a ratio was indeed observed during formation of the radical into one subunit of protein α2 (*7*). However, once this step was completed, methionine formation further proceeded and leveled off when a methionine:radical ratio of approximately 2 was reached. This decoupled methionine formation reaction is still not understood.

The anaerobic ribonucleotide reductase system belongs to an emerging class of enzymes which utilize the combination of AdoMet, a reducing sytem, and an Fe-S cluster to generate catalytically essential free radical species. The latter can be a protein radical (pyruvate-formate lyase, benzylsuccinate synthase) or a substrate radical (lysine amino-mutase, biotin synthase) (*2, 17*). The mechanism shown in Scheme 1 is thus, in its main lines, also proposed for a number of such related systems even if the chemistry involved in the reductive cleavage of AdoMet remains largely obscure.

In the case of pyruvate-formate lyase, another glycyl radical enzyme, and lysine amino-mutase, preliminary results have established that radical formation was associated with the oxidation of the reduced $(4Fe\text{-}4S)^{+}$ cluster, with a 1 to 1 stoichiometry between radical formation and cluster oxidation (*18, 19*).

The iron−sulfur center of protein β has been the subject of recent investigations in our laboratory. It is an intriguing species for the following reasons: (i) only three cysteines, belonging to the conserved characteristic CXXXCXXC sequence, are used for iron chelation by the polypeptide, thus suggesting a specific function for the fourth unidentified coordination site (*20*); (ii) it can exist in different stable forms of different nuclearity, (4Fe-4S), (3Fe-4S), and (2Fe-2S) clusters, that are interconvertible, depending on the redox conditions (*13, 21*); (iii) the redox potential for the $(4Fe\text{-}4S)^{2+/1+}$ couple is low, consistent with the suggestion that the reduced $(4Fe\text{-}4S)^{+}$ cluster is the active reducing species during the reduction and the cleavage of AdoMet (*7*); (iv) protein β displays its own AdoMet reductase activity with no absolute requirement for protein α but only in the presence of DTT (*10*). However, little is known both of the mechanistic details of that reaction and of the role of DTT.

In the present paper, we confirm that the $(4Fe\text{-}4S)^{+}$ but not the $(4Fe\text{-}4S)^{2+}$ cluster is competent for AdoMet reduction and glycyl radical formation, and we present a detailed investigation of this reaction, using EPR and Mössbauer spectroscopy. This study essentially supports the mechanism proposed in Scheme 1 for the formation of the glycyl radical in protein α. However, results obtained in the absence of protein α show that the latter strongly influences the reaction between the iron cluster and AdoMet both in terms of its kinetics and in terms of its outcome and suggest alternative mechanisms.

EXPERIMENTAL PROCEDURES

Materials. Enzymes and other components of the anaerobic ribonucleotide reductase system have been obtained as previously described (*5, 7, 16*). $^{57}Fe_2O_3$ 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. $Fe(NH_4)_2(SO_4)_2$ was from Aldrich. AdoMet was from Roche Diagnostics. Sodium dithionite [87.3% pure as determined by titration with $K_3Fe(CN)_6$] was from Fluka.

Analysis. Protein concentration was determined by the method of Bradford, standardized by amino acid analyses of each different protein (*22*). Protein-bound iron was determined under reducing conditions with bathophenantroline disulfonate after acid denaturation (*23*) and labile sulfide by Beinert's method (*24*).

Methods. (a) Reconstitution of the Iron−Sulfur Center of the β Protein. All the steps of the reconstitution were done anaerobically inside a glovebox (Jacomex BS531 NMT) in an N_2 atmosphere containing less than 2 ppm of O_2. The iron−sulfur center of the β protein was reconstituted in 0.1 M Tris-HCl, pH 8.0, 50 mM KCl, 5 mM DTT (buffer A) during incubation with a 6-fold molar excess of Na_2S and either $Fe(NH_4)_2(SO_4)_2$ or $^{57}FeCl_3$ for 3 h at 18 °C, followed by incubation with a 2-fold molar excess of EDTA for 30 min. After chromatography over an anaerobic G-25 Sephadex column equilibrated with a DTT-free buffer, the brown-colored fractions were collected and concentrated over a YM 10 Diaflo membrane (Amicon). Each preparation contained between 3.5 and 4.0 Fe and 3.5 and 4.5 S^{2-} per β polypeptide chain, as determined by colorimetric analysis (see above).

(b) AdoMet Reduction by Reduced Protein β or αβ. All the manipulations were done inside the glovebox. Reduction of the iron−sulfur center (β or $\alpha\beta$) was performed in buffer A with a 10-fold molar excess of sodium dithionite at 18 °C. Reduction could be monitored by light absorption spectroscopy directly inside the glovebox. After 60 min, the solution was carefully desalted over an anaerobic G-25 Sephadex column equilibrated with in 0.1 M Tris-HCl, pH 8.0, 50 mM KCl. Then, the reduced protein (β or $\alpha\beta$) was reacted with AdoMet, and the reaction was initiated with addition of 5 mM DTT. At time intervals (from 0 to 50 min for β and from 0 to 15 min for $\alpha\beta$), an aliquot (2−5 nmol of protein) was withdrawn, precipitated with a solution of 1 M TCA (10% v/v), and assayed for methionine formation. In parallel, 200 μL solutions were transferred into an EPR tube immediately frozen in a well filled with isopentane cooled from outside the box by liquid nitrogen. When the experiments were performed with ^{57}Fe-protein, 200 μL solutions were also transferred in a Mössbauer cup and frozen as above.

(c) UV−Visible Absorption Spectroscopy. UV−visible spectra were recorded inside the glovebox with a Hewlett-Packard 8453 diode array spectrophotometer coupled to the measurement cell by optical fibers (Photonetics Systems).

(d) Methionine Determination. Aliquots precipitated with TCA were centrifuged, and the supernatant was collected, dried, and diluted in sodium citrate, pH 2.0. Then, AdoMet reductase activity was measured at 570 nm by the formation of ninhydrine-derivatized methionine determined by HPLC (System 7300, Beckman) calibrated with pure amino acid standards.

(e) 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). 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 recorded under nonsaturating conditions (i) with a standard sample of 200 μM $CuCl_2$ and 5 mM EDTA in Tris buffer, pH 8.0, for the reduced iron−sulfur center, or (ii) with a

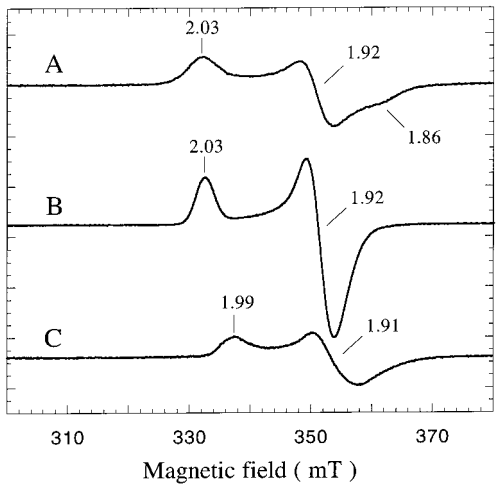


FIGURE 1: X-band EPR spectra of reduced protein β (420 μM) in 0.1 M Tris-HCl, pH 8.0, 50 mM KCl (A); after addition of 5 mM DTT (B); and after 1 min incubation of (B) with 1.5 mM AdoMet (C). For each spectrum, g values are indicated. Recording conditions: temperature, 10 K; microwave power, 0.1 mW; modulation amplitude, 1 mT; gain, 10^5.

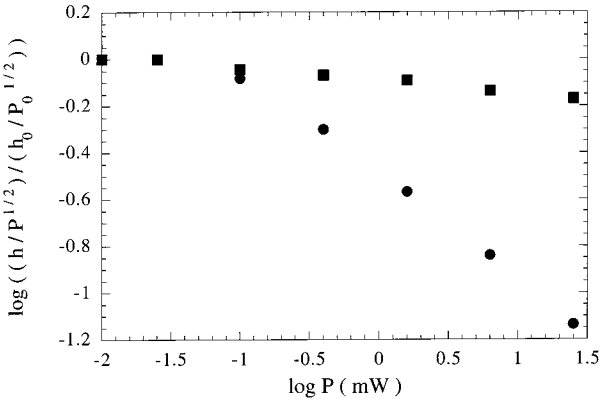


FIGURE 2: Microwave power (P) saturation of the X-band EPR signal of the anaerobically reduced protein β, before (●) and after 1 min incubation with 1.5 mM AdoMet (■). Both samples, 420 μM, in buffer A. The parameter h is the normalized EPR derivative signal amplitude obtained with microwave power P; h_0 and P_0 are the corresponding quantities under nonsaturating conditions. Temperature, 10 K; modulation amplitude, 1 mT.

standard sample of a 175 μM flavodoxin (half-reduced form), calibrated by UV−visible spectroscopy (ϵ_{350} = 4900 M^{-1}·cm^{-1}), for the glycyl radical (*7*). Microwave power (P) saturation experiments were carried out at 10 K by measuring the EPR signal amplitude (h) and normalizing it to the maximum value (h_0).

(f) Mössbauer Spectroscopy. ^{57}Fe Mössbauer spectra were recorded on 200 μL cups containing the protein (0.6 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 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) producing a field of 7 T perpendicular to the γ-ray. The spectra were analyzed assuming Lorentzian line shape, and the isomer shift is quoted relative to α-Fe at room temperature.

RESULTS

The Reduced (4Fe-4S)$^+$ Cluster, but Not the Oxidized (4Fe-4S)$^{2+}$Cluster, Is Active during AdoMet Reductive Cleavage. Anaerobic reaction of reduced protein β with AdoMet was assayed from the formation of methionine, one of the products of the reductive cleavage of AdoMet, and from the amount of reduced cluster by EPR spectroscopy, as the (4Fe-4S)$^+$ cluster displays a characteristic S = 1/2 EPR signal (Figure 1) (*16*). In the presence of protein α, the reaction can also be monitored from the formation of the glycyl radical by EPR spectroscopy since the latter has a characteristic EPR signature, with a doublet (a_H= 14 G) signal at g = 2.002, easily differentiated from that of the reduced cluster (*6*).

In a standard experiment, protein β was first reduced with a 10-fold molar excess of dithionite and carefully desalted over a Sephadex G-25 column, under strict anaerobiosis using a 0.1 M Tris buffer, pH 8.0, containing 50 mM KCl. This procedure afforded only incomplete reduction of protein β with, from one preparation to an other, a maximal amount of 25−60% of β polypeptides containing a (4Fe-4S)$^+$ cluster as determined from the intensity of the rhombic signal (g = 2.03, 1.92, 1.86) shown in Figure 1A. In the following, these partially reduced preparations will be referred as "reduced protein β". In the presence of 5 mM DTT, the rhombic EPR signal was changed into an axial one with g values at 2.03 and 1.92 (Figure 1B). Since no oxidation of the cluster by AdoMet occurred in the absence of DTT (*10*), the buffer was supplemented with 5 mM DTT (buffer A) in all the following experiments. When reduced protein β was reacted with an excess of AdoMet in buffer A, the axial signal of the cluster changed, during the mixing time, into a different one with g values at 1.99 and 1.91 (Figure 1C). The new signal, which is assigned to a cluster−AdoMet complex, displayed very different microwave power saturation properties (Figure 2). As shown in Figure 3A, it then decayed with a rate constant of 0.15 ± 0.05 min^{-1}, and the reaction was completed within 20 min. In parallel, methionine was formed with the same rate constant within error, showing that methionine formation was associated with cluster oxidation. However, Figure 3A shows that, all along the reaction, 1.8−2.0 mol of methionine was formed per mole of cluster converted to an EPR-silent form. This experiment has been repeated many times, and the same approximately 2:1 ratio was obtained with different preparations having widely different amounts of reduced clusters (Figure 3B). It is concluded that the reduced cluster is competent for AdoMet cleavage, even in the absence of protein α, and the reaction can be described with the equation:

$$(4Fe\text{-}4S)^+ + 2AdoMet \rightarrow 2\ methionine + EPR\text{-}silent\ cluster$$

However, this stoichiometry was unexpected considering that (4Fe-4S)$^+$ clusters are one-electron reductants. This raised the question whether the (4Fe-4S)$^{2+}$ cluster could provide the additional electron.

To address this point, protein β [containing one EPR-silent (4Fe-4S)$^{2+}$ cluster per polypeptide] was incubated with an excess of AdoMet (1.5 mM) in buffer A, inside an anaerobic glovebox at 18 °C. No significant production of methionine

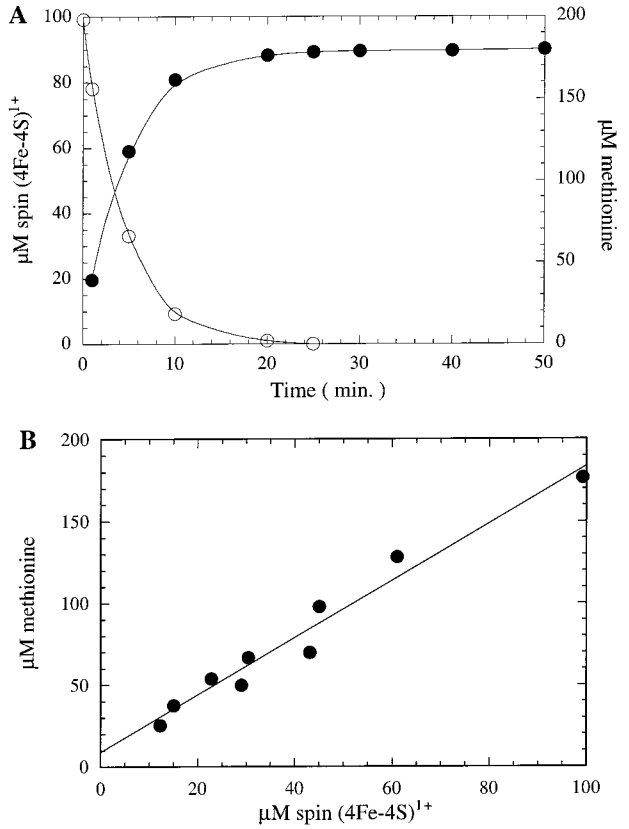


FIGURE 3: (A) Time course for the reaction of reduced protein β with AdoMet. The β protein (440 μM) was first reduced for 60 min by a 10-fold molar excess of dithionite in buffer A and carefully desalted over a Sephadex G-25 column using DTT-free buffer A under strict anaerobiosis. Then, reduced protein β was reacted with 1.5 mM AdoMet, and the reaction was initiated with addition of 5 mM DTT. At the indicated times, the reduced iron−sulfur center (○) and methionine (●) were assayed as described under Experimental Procedures. (B) Correlation between the amount of originally present reduced cluster and the amount of methionine, the latter measured after 30 min reaction with AdoMet (1.5 mM) in buffer A. Curve fit: *R* (correlation factor), 0.98; slope, 1.8.

was observed, whether protein α was present or not, in stoichiometric amounts, in the reaction mixture (data not shown). This demonstrated that the $(4Fe\text{-}4S)^{2+}$ center was not competent for AdoMet cleavage to methionine.

Oxidation of the Reduced Cluster by AdoMet Essentially Results in the Formation of a $(4Fe\text{-}4S)^{2+}$ Cluster with Minor Formation of a $(3Fe\text{-}4S)^{0}$ Cluster. To characterize the final EPR-silent iron center, similar experiments were carried out with a preparation of protein β reconstituted with ^{57}Fe, reduced with dithionite, desalted, and incubated with AdoMet, as described above. The solution was transferred to cups for Mössbauer analysis before (sample 1) and at two different times (5 min, sample 2; and 30 min, sample 3) after initiating the reaction with DTT. In parallel, samples were analyzed by EPR spectroscopy and for methionine formation. All experiments described below were performed in the anaerobic box. Mössbauer spectra recorded at 77 and 4.2 K in a 20 mT external field are presented in Supporting Information (Figure 7A−F).

The 77 K Mössbauer spectrum of sample 1, containing protein β and AdoMet, exhibits four quadrupole doublets ($\delta = 0.53$ mm·s^{-1} and $\Delta E_Q = 0.75$ mm·s^{-1} accounting for 20% of the total iron, $\delta = 0.59$ mm·s^{-1} and $\Delta E_Q = 1.40$ mm·s^{-1} accounting for 20% of the total iron, $\delta = 0.47$ mm·s^{-1} and $\Delta E_Q = 1.00$ mm·s^{-1} accounting for 57% of the total iron, and $\delta = 0.42$ mm·s^{-1} and $\Delta E_Q = 0.50$ mm·s^{-1} accounting for 3% of the total iron). Decreasing the temperature to 4.2 K (Table 1, sample 1, Figure 4A) results in a magnetic splitting of the two first doublets, indicating a paramagnetic ground state for these components; the equal ratio between each iron suggests that they are assembled in the same paramagnetic cluster; the Spin Hamiltonian simulation of the 20 mT and 7 T spectra clearly assigns these signals (together 40% of the total iron) to the $Fe^{2.5+}$ and Fe^{2+} sites of the same $S = 1/2$ EPR-active $(4Fe\text{-}4S)^{+}$ cluster. A magnetic splitting at low temperature (20 mT or 7 T applied external field) has also been observed for the $\delta = 0.42$ mm·s^{-1} signal (3% of total iron), as well as for part of the

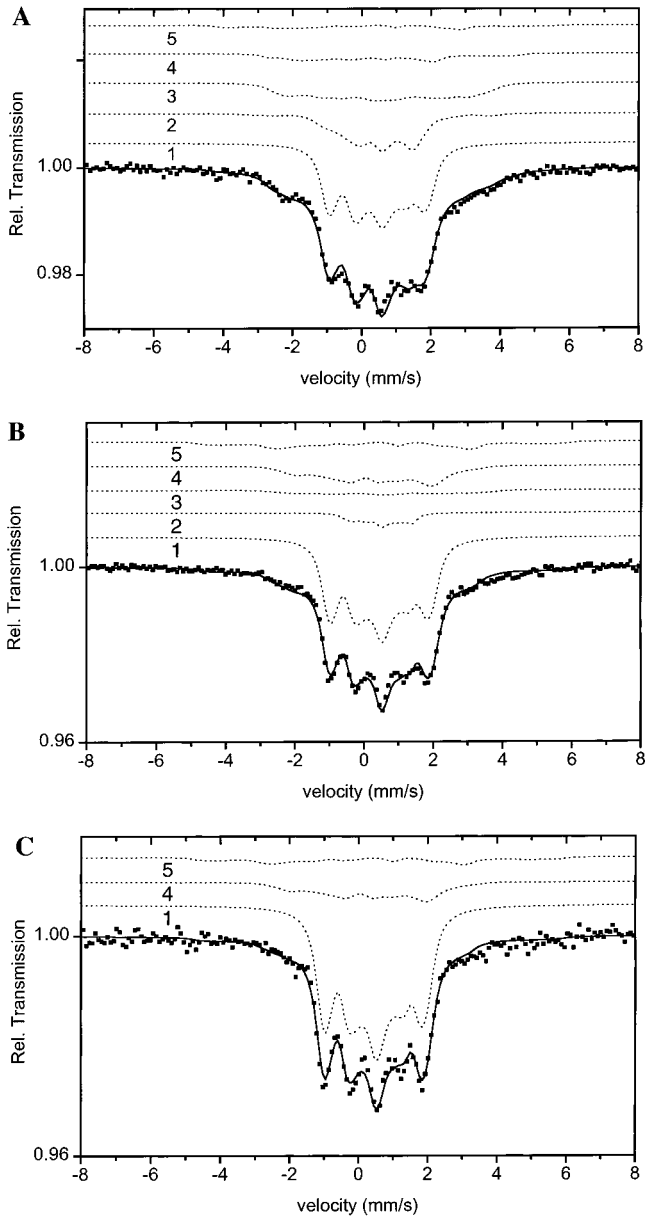


FIGURE 4: Mössbauer spectra of reduced protein β (0.6 mM) in the presence of AdoMet (panel A, AdoMet + β; panel B, AdoMet + β + DTT after 5 min; panel C, AdoMet + β + DTT after 30 min) recorded at 4.2 K in a 7 T external field perpendicular to the γ-ray. The spectra were simulated (solid lines) with hyperfine parameters summarized in Table 1. Subspectra (dotted lines): (1) $Fe^{2.5+}$ sites of the $S = 0$ $(4Fe\text{-}4S)^{2+}$ cluster; (2 and 3) respectively Fe^{2+} and $Fe^{2.5+}$ sites of the $S = 1/2$ $(4Fe\text{-}4S)^{+}$ cluster; (4 and 5) respectively $Fe^{2.5+}$ and Fe^{3+} sites of the $S = 2$ $(3Fe\text{-}4S)^{0}$ cluster.

Table 1: Parameters Used for the Spin Hamiltonian Simulation (*32*) of Subspectra 1−5 of Figure 4A−C[a]

sample	subspectra	Γ, mm·s^{-1}	δ, mm·s^{-1}	ΔE_Q, mm·s^{-1}	η	β, deg	$\mathbf{A}_x$, T[b]	$\mathbf{A}_y$, T[b]	$\mathbf{A}_z$, T[b]	rel area, %[c]
1	1. $(4Fe\text{-}4S)^{2+}$ $Fe^{2.5+}$ sites	0.45	0.47	1.00	0.6					49
	2. $(4Fe\text{-}4S)^{+}$ Fe^{2+} sites	0.56	0.62	1.70	1	90	6.4 (5.0)	10.0 (6.0)	24.0 (20.0)	20
	3. $(4Fe\text{-}4S)^{+}$ $Fe^{2.5+}$ sites	0.40	0.53	0.73	0	0	−22.3 (−25.0)	−32.8 (−26.0)	−21.3 (−21.0)	20
	4. $(3Fe\text{-}4S)^{0}$ $Fe^{2.5+}$ sites	0.45	0.47	1.00	1	90	−15.0 (−12/−16)	−6.0 (−8/−16)	−13.0 (−12/−11)	8
	5. $(3Fe\text{-}4S)^{0}$ Fe^{3+} sites	0.30	0.42	0.50	0	90	12 (12/11.6)	5 (5/11.6)	9 (9/11.6)	3
2, 3	1. $(4Fe\text{-}4S)^{2+}$ $Fe^{2.5+}$ sites	0.44	0.45	1.08	0.7					65; *80*
	2. $(4Fe\text{-}4S)^{+}$ Fe^{2+} sites	0.56	0.62	1.70	1	90	6.4	7.5	23.8	5; *0*
	3. $(4Fe\text{-}4S)^{+}$ $Fe^{2.5+}$ sites	0.35	0.53	0.73	1	0	−28.4	−30.2	−25.0	5; *0*
	4. $(3Fe\text{-}4S)^{0}$ $Fe^{2.5+}$ sites	0.44	0.45	1.08	1	90	−5.0	−9.7	−12.9	17; *13*
	5. $(3Fe\text{-}4S)^{0}$ Fe^{3+} sites	0.33	0.39	0.45	1	90	13.3	4.5	10.3	8; *7*

[a] Abbreviations: Γ, line width; δ, isomer shift; ΔE_Q, quadrupole splitting; η, asymmetry parameter; β, Euler angle relating electric field gradient tensor and magnetic hyperfine coupling tensor; $\mathbf{A}_{x,y,z}$, magnetic hyperfine coupling tensor. [b] **A** represents the term $\mathbf{A}/(g_N \cdot \mu_N)$; values in parentheses refer to published parameters for $(4Fe\text{-}4S)^{+}$ (*13*) and $(3Fe\text{-}4S)^{0}$ (*20, 21*). [c] First ratio related to sample 2, second ratio (italic) to sample 3.

$\delta = 0.45$ mm·s^{-1} signal (8% of total iron). This is interpreted by the presence of 11% of the total amount of iron belonging to a $S = 2$ EPR-silent $(3Fe\text{-}4S)^{0}$ cluster (the signal at $\delta = 0.42$ mm·s^{-1} being the Fe^{3+} site and $\delta = 0.45$ mm·s^{-1} the mixed-valent $Fe^{2.5+}$ dimer); due to its low ratio, the Spin Hamiltonian simulation of the 20 mT and 7 T spectra was performed with published **D**-, **E**/**D**-, and **A**-tensors (*20*) for the minor Fe^{3+} site of this cluster. We had to slightly adjust the **A**-tensor of the $Fe^{2.5+}$ site to account for the outermost broadening of the spectrum (Table 1). The resulting **A**-tensor (−15, −6, −13 T) is close to the published values for class III RNR $(3Fe\text{-}4S)^{0}$ clusters (*20, 21*) but lower than those for FdII $(3Fe\text{-}4S)^{0}$ clusters (*25*). The remaining iron (49%) is assembled in a diamagnetic cluster as shown by the 7 T measurements: the parameters of this component ($\delta = 0.47$ mm·s^{-1} and $\Delta E_Q = 1.00$ mm·s^{-1}) are typical for the tetrahedrally sulfur-coordinated mixed-valent dimers $Fe^{2.5+}$ (*13, 20, 21*), clearly indicating that this iron is assembled in an oxidized $(4Fe\text{-}4S)^{2+}$ cluster. After 5 min incubation with DTT, the same complete analysis was carried out (Table 1, sample 2, Figure 4B). In agreement with the EPR spectrum, the Mossbauer data showed that the reduced cluster has partly disappeared (now accounting for only 10% of the total Fe compared to 40% in sample 1). The same δ and ΔE_Q values as for sample 1 were used to simulate the 77 K subspectra while the **A**-tensors were slightly adjusted to fit the low-temperature spectra. Both the $(3Fe\text{-}4S)^{0}$ and $(4Fe\text{-}4S)^{2+}$ clusters were observed in higher amounts compared to sample 1. The parameters of the $Fe^{2.5+}$ sites assembled in $(4Fe\text{-}4S)^{2+}$ had not changed much ($\delta = 0.45$ mm·s^{-1} and $\Delta E_Q = 1.08$ mm·s^{-1}); however, this cluster accounts now for 65% of the total iron compared to only 40% before the oxidation occurred. Simulation of the low-temperature subspectra of $(3Fe\text{-}4S)^{0}$ (25% of total iron compared to only 11% before addition of DTT) showed that the Mossbauer parameters are basically comparable to those of sample 1; the lower isomer shift for the Fe^{3+} site (0.39 mm·s^{-1}) and the lower $\mathbf{A}_x$-component for the $Fe^{2.5+}$ site (−5.0 T) reflect a slightly different electronic charge and spin density repartition in this cluster in comparison with unreacted sample 1. Finally, at 30 min, when the reaction was completed, no reduced $(4Fe\text{-}4S)^{+}$ cluster was present anymore; while $(3Fe\text{-}4S)^{0}$ has decreased from 25% to 20% of total iron, the $(4Fe\text{-}4S)^{2+}$ cluster has increased from 65% to 80% (sample 3, Figure 4C). Each cluster type of sample 3 has been simulated with the same hyperfine parameters as for sample 2; the only free parameters were the respective relative areas. The reaction with that preparation also generated 2 methionine equiv per reactive cluster (data not shown). From this study, it appears that, during the reaction of sample 1 with AdoMet, the reduced $(4Fe\text{-}4S)^{+}$ cluster was converted to the corresponding $(4Fe\text{-}4S)^{2+}$ cluster, accompanied by the formation of a significant amount of $(3Fe\text{-}4S)^{0}$ clusters.

Reaction of the Reduced Cluster with AdoMet in the Presence of Protein α: Formation of the Glycyl Radical. The reaction of reduced protein β with AdoMet was carried out in the presence of a stoichiometric amount of protein α, i.e., with the $\alpha 2\beta 2$ complex. In that experiment, about 50% of the β polypeptides initially contained a reduced cluster, as determined by EPR spectroscopy. Oxidation of the reduced cluster by AdoMet occurred at a much faster rate than in the absence of protein α, since the iron center became EPR-silent after less than 2 min. A rate constant was thus difficult to determine accurately, but was at least 10 times larger than that determined in the absence of protein α. At the same rate, methionine was formed together with the glycyl radical, as shown from the appearance of the characteristic EPR signal. At completion, exactly 1 equiv of methionine per reacted cluster was formed (Figure 5). This ratio was highly reproducible with enzyme preparations containing various proportions of reduced cluster. The radical yield was significantly smaller and varied from 0.5 to 0.9 from one preparation to the other. The great sensitivity of the radical, in particular with regard to traces of oxygen, is likely to be responsible for these variations. These one-

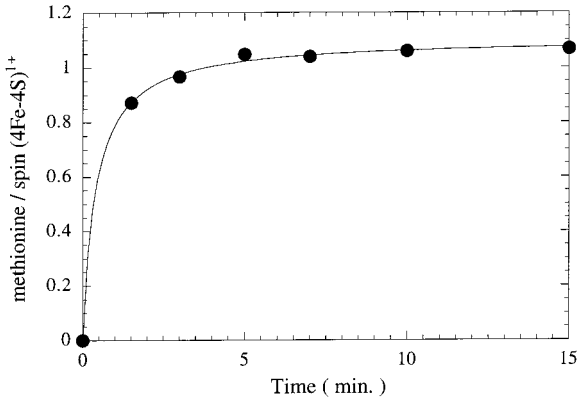


FIGURE 5: Time course formation for the reaction of reduced α2β2 complex with AdoMet. The α2β2 protein (110 μM) was reduced, desalted as in Figure 3, and then reacted with AdoMet (1.5 mM) in buffer A. At the indicated times, methionine was assayed as described under Experimental Procedures.

turnover experiments confirmed that the reduced (4Fe-4S)$^{+}$ form of the cluster is competent for AdoMet reductive cleavage to methionine and for glycyl radical formation. They also showed that protein α binding to protein β makes the reaction kinetically more efficient and affects the stoichiometry of the reaction.

DISCUSSION

Understanding the mechanism of the activation of anaerobic ribonucleotide reductase, which is addressed in this study, may be of general interest. Actually, it is now established that a large number of enzymatic systems use the combination of an iron−sulfur center, AdoMet, and a reducing agent to generate essential free radicals (*2*, *17*). Scheme 1 describes our working hypothesis, already proposed in 1993 (*26*). The reducing agent first tranfers one electron to the iron cluster of protein β. In the reduced state, protein β binds AdoMet. The existence of a protein β/AdoMet complex has been established by a filter binding assay and by EPR spectroscopy (*14*, *15*). The fact that the EPR properties of the reduced cluster are greatly affected by the presence of AdoMet, as shown previously and confirmed here (Figures 1 and 2), suggests that AdoMet lies in close proximity to the iron center. Then, one electron is transferred from the cluster to AdoMet, leading to 1 equiv of methionine and 1 equiv of the 5′-deoxyadenosyl radical which finally reacts with the glycine residue of protein α to generate the key glycyl radical. From that hypothetical scheme, it is expected that one reduced cluster generates one methionine and one glycyl radical. In previous papers, DTT was shown to be absolutely required for activation of ribonucleotide reductase. However, we recently demonstrated that its function resided in the reduction of a critical disulfide in protein α. With reduced preparations of protein α, DTT is not required anymore for the generation of the radical (*14*).

The first step of the reaction (Scheme 1), i.e., the reduction of the cluster by flavodoxin has been addressed in a previous paper (*7*). Here we investigate the subsequent steps and in particular the reaction of the cluster of protein β with AdoMet.

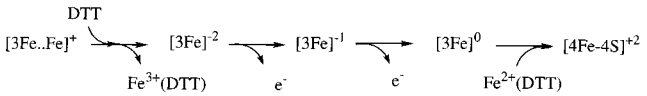
During the reaction between the α2β2 reduced protein with AdoMet, both methionine and glycyl radical are formed in association with the oxidation of the cluster. One methionine is formed per reacted reduced cluster, in full agreement with the proposed mechanism (Scheme 1). Slightly less radical was generated (0.5−0.9) probably due to ineffective radical introduction and/or high sensitivity of the radical to traces of oxygen that might occur under the in vitro conditions used in this study. On the whole, the results reported here support the notion that radical generation depends on the transfer of a single electron from the reduced cluster to AdoMet. The same conclusion was reached in the case of the pyruvate-formate lyase activating system and lysine amino-mutase (*18*, *19*).

When AdoMet was incubated with reduced protein β alone, no reductive cleavage could be detected, showing that this reaction is thermodynamically unfavorable as in the case of lysine amino-mutase (*27*). Only in the presence of DTT could the coupled formation of methionine and oxidation of the cluster be observed. In that case, the electron transfer from the cluster to AdoMet proceeded at a much lower rate than in the presence of protein α. The fact that the reaction becomes thermodynamically favorable in the presence of DTT (*7*) may be a consequence of DTT binding to the cluster. In one case, it has been shown that exogenous thiolate binding to a (4Fe-4S) cluster results in a 200 mV negative shift of the redox potential (*28*). Thus, it appears that DTT is required both in the presence and in the absence of protein α but for very different reasons.

In contradiction with our working hypothetical mechanism and with the notion that (4Fe-4S)$^{+}$ clusters are one-electron reductants, two methionines were formed per reacted cluster in the reaction with protein β alone.We previously reported a ratio of 3, but we believe that this discrepancy may be explained by the care taken here to avoid the presence of a strong reducing agent in the reaction mixture with the introduction of a gel filtration step, in the anaerobic box, after preparation of the reduced cluster (*10*). On the other hand, during the same reaction with AdoMet, the cluster has experienced a one-electron oxidation since it was found to end up essentially as a (4Fe-4S)$^{2+}$ cluster, as shown by Mössbauer spectroscopy, and, furthermore, we show that the (4Fe-4S)$^{2+}$ cluster has no competence for cleavage of AdoMet. This thus raises the question of the origin of the second electron.

Our present explanation to these conflicting observations is the following and introduces DTT as an important player. We propose that, in the absence of protein α, binding of AdoMet to protein β, adjacent to the reduced cluster, destabilizes it and allows DTT to chelate out its ferric iron, generating a "hyper-reduced" intermediate (3Fe-4S)$^{2-}$ cluster. It is indeed possible that, as the cluster of protein β has only three iron atoms bound to cysteine ligands, the fourth iron is more accessible and prone to chelation (*20*). As a matter of fact, there are strong indications that DTT binds to the cluster of protein β in the absence of protein α (Figure 1 and unpublished results). The (3Fe-4S)$^{2-}$ intermediate is proposed to provide two successive electrons for the one-electron reduction of two molecules of AdoMet, yielding two methionines and a (3Fe-4S)^{0} cluster, through a reactive (3Fe-4S)$^{1-}$ intermediate (Scheme 2). Previous results from this laboratory have indeed pointed to the unusual stability of (3Fe-4S)^{0} clusters in this protein (*21*). Finally, the expelled iron is reincorporated as a ferrous iron−DTT complex into the cluster during reaction with (3Fe-4S)^{0} to generate the

Scheme 2: Proposed Mechanism for the AdoMet Reductase Activity of Protein β



final $(4Fe\text{-}4S)^{2+}$ cluster. In this scheme, one ferric iron is chelated off from the cluster by DTT, reduced, and reincorporated as a ferrous iron, and thus the lacking electron is proposed to be provided by DTT. In addition to the observed stoichiometry, we take as a support to that mechanism the observation by Mössbauer spectroscopy that $(3Fe\text{-}4S)^{0}$ clusters are accumulating during the reaction and then disappear, partly. Hyper-reduced $(3Fe\text{-}4S)^{2-}$ clusters are known as low redox potential reductants and have been characterized, mainly by electrochemistry, in a number of (3Fe-4S) and (7Fe-4S) ferredoxins (*29*). These studies have shown that $(3Fe\text{-}4S)^{2-}$ clusters release two electrons in a cooperative reaction (*30*). In the present case, a $(3Fe\text{-}4S)^{1-}$ cluster intermediate is proposed to be capable of reducing a second molecule of AdoMet, in agreement with its presumed strong reducing power (*30*). This is speculative since no $(3Fe\text{-}4S)^{1-}$ cluster has ever been observed so far, and thus further experiments are required to substantiate this proposal.

Finally in the case discussed here, whether the reaction proceeds through direct electron transfer within the AdoMet−cluster complex remains to be determined. Alternative mechanisms might be considered, in particular those implying an initial nucleophilic attack of the cluster on the electrophilic 5′-deoxyadenosyl carbon of AdoMet and the release of 1 equiv of methionine, as suggested by P. Frey (*31*). Sulfur bridges of the $(3Fe\text{-}4S)^{2-}$ clusters are proposed to be nucleophilic enough for such a reaction.

In light of the present results, a mechanism involving (3Fe-4S) clusters during glycyl radical formation within $\alpha 2\beta 2$ might be considered.

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SUPPORTING INFORMATION AVAILABLE

Mössbauer spectra of the reduced protein β (0.6 mM) in the presence of AdoMet recorded at 4.2 and 77 K in a 20 mT external field (Figure 7A−F) (6 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

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