Activation of Class III Ribonucleotide Reductase by Flavodoxin: A Protein Radical-Driven Electron Transfer to the Iron−Sulfur Center

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Received July 26, 2000; Revised Manuscript Received January 19, 2001

ABSTRACT: In its active form, *Escherichia coli* class III ribonucleotide reductase homodimer α_2 relies on a protein free radical located on the Gly^{681} residue of the α polypeptide. The formation of the glycyl radical, namely, the activation of the enzyme, involves the concerted action of four components: *S*-adenosylmethionine (AdoMet), dithiothreitol (DTT), an Fe−S protein called *β* or "activase", and a reducing system consisting of NADPH, NADPH:flavodoxin oxidoreductase, and flavodoxin (fldx). It has been proposed that a reductant serves to generate a reduced $[4Fe\text{-}4S]^{+}$ cluster absolutely required for the reductive cleavage of AdoMet and the generation of the radical. Here, we suggest that the one-electron reduced form of flavodoxin (SQ), the only detectable product of the in vitro enzymatic reduction of flavodoxin, can support the formation of the glycyl radical. However, the redox potential of the Fe−S center of the enzyme is shown to be ∼300 mV more negative than that of the SQ/fldx couple and not shifted to a more positive value by AdoMet binding. It is also more negative than that of the HQ/SQ couple, HQ being the fully reduced form of flavodoxin. Our interpretation is that activation of ribonucleotide reductase occurs through coupling of the reduction of the Fe−S center by flavodoxin to two thermodynamically favorable reactions, the oxidation of the cluster by AdoMet, yielding methionine and the 5′-deoxyadenosyl radical, and the oxidation of the glycine residue to the corresponding glycyl radical by the 5′-deoxyadenosyl radical. The second reaction plays the major role on the basis that a Gly-to-Ala mutation results in a greatly decreased production of methionine.

For its anaerobic growth, *Escherichia coli* depends on an anaerobic ribonucleotide reductase (class III) which provides the cell with the four deoxyribonucleotides used for DNA chain elongation and repair (*1*). The enzyme is a homodimer α_2 (2×80 kDa) whose activity and specificity toward each of the four substrates is allosterically controlled by nucleoside triphosphates (*2*). In its active form, the protein carries an oxygen-sensitive free radical located on the Gly^{681} residue on the C-terminus of the polypeptide (*3*, *4*). This radical is essential for activity as it triggers the first committed step of the reduction process, namely, the abstraction of the sugar $H_{3'}$ of the ribonucleotide (*5*). The substrate radical is then reduced to the deoxy form by three reducing equivalents provided by formate and the glycine residue which is converted back to its radical form for another cycle (*6*).

The activation of protein α, which consists of the introduction of the glycyl radical, is a complex reaction mediated by the concerted action of four components: (i) an electron source, whose physiological version is flavodoxin (fldx)[1] reduced by NADPH:flavodoxin oxidoreductase (fpr) which in vitro can be substituted by strong chemical reductants such as sodium dithionite or photoreduced 5-deazaflavin (*7*−*9*), (ii) *S*-adenosylmethionine (AdoMet) which in the process is reductively cleaved to methionine and presumably to the elusive 5′-deoxyadenosyl radical (Ado°) which is supposed to be the precursor of the glycyl radical on protein α (*9*, *10*), (iii) dithiothreitol (DTT), a nonphysiological reductant (*11*), and (iv) a 17.5 kDa protein called *β* or "activase" which contains a $[4Fe\text{-}4S]^{2+}$ center (*12*−*15*). This center is highly oxygen sensitive, and oxidized preparations of protein *β* contain $[2Fe\text{-}2S]^{2+}$ centers exclusively (*11*). When protein *β* binds to protein α, a stable α2*β*2 holoenzyme is formed (*12*).

The anaerobic ribonucleotide reductase system belongs to an emerging class of enzymes which utilizes the combination of AdoMet and an Fe−S cluster to generate catalytically essential free radical species (*16*−*20*). The general concept (Scheme 1) is that the rather unreactive AdoMet serves as a source of 5′-deoxyadenosyl radicals which are powerful oxidants for H atom abstraction reactions and that activation of AdoMet requires a reduction step in which an iron−sulfur center plays a catalytic function (*21*). In its reduced $[4Fe\text{-}4S]^{+}$ form, the cluster of protein *β* has been shown to be competent for reduction and cleavage of AdoMet, thus promoting the introduction of the essential glycyl radical in protein α (*9*).

The combination of AdoMet and an Fe−S cluster is reminiscent of the well-known chemistry of the coenzyme B_{12} and may have been a primitive version of the latter (*22*).

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[1] Abbreviations: fldx, flavodoxin; fpr, NADPH: flavodoxin oxidoreductase; Gly°, glycyl radical; AdoMet, *S*-adenosylmethionine; Ado°, 5′-deoxyadenosyl radical; DTT, dithiothreitol; SQ, flavodoxin semiquinone; HQ, flavodoxin hydroquinone; WT, wild type.

10.1021/bi001746c CCC: $20.00
Published on Web 02/23/2001

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

$$[4Fe\text{-}4S]^{2+} + fldx_{red} \rightleftharpoons [4Fe\text{-}4S]^{+} + fldx_{ox} \quad (1)$$

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

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

Because of the wider than anticipated use of radical chemistry in biological systems, the understanding of the chemistry at work with the AdoMet/Fe−S combination represents an exciting challenge.

In the study reported here, we demonstrate that the reduction of the iron−sulfur cluster by the flavodoxin system is a thermodynamically unfavorable reaction. It is proposed that electron transfer occurs and activation of ribonucleotide reductase proceeds due to the coupling of that reaction to two thermodynamically favorable reactions, the oxidation of the reduced cluster of protein β by AdoMet and the homolytic cleavage of the C−H bond of the Gly^{681} of protein α, with the latter playing the major role.

EXPERIMENTAL PROCEDURES

All common reagents were obtained from commercial sources and used without further purification. 5-Deaza-7,8-demethyl-10-methylisoalloxazine (5-DAF) was kindly supplied by J. L. Décout (UJF, Grenoble, France) and utilized as a 1 mM stock solution in a 20/80 (v/v) CH_3CN/H_2O mixture.

Every protein used in this study was at least 90% pure as judged from highly loaded (5−10 μg) Coomassie blue-stained SDS−PAGE gels.

Protein α was obtained from anaerobic cultures of recombinant *E. coli* cells [JM109(DE3)] transformed with plasmid pRSS containing the *nrd*D gene as described previously (*23*). An additional ion exchange step (DE52) was inserted in the described protocol before the dATP affinity chromatography was carried out.

Mutant protein α G681A was obtained by the same procedure that was used for the wild type. The mutated plasmid was a kind gift from X. Sun (Stockholm, Sweden). The pure protein was analyzed for its N-terminal amino acid sequence.

Protein β (apo form) was obtained as previously described (*9*, *11*). The Fe−S center was inserted by chemical reconstitution using a 6.0-fold molar excess of ferrous ammonium sulfate and sodium sulfide in the presence of 5 mM DTT within an anaerobic glovebox (Jacomex, BS531 NMT) operating in a N_2 atmosphere containing <2 ppm O_2. The $[4Fe\text{-}4S]^{2+}$ form was obtained by desalting the reconstitution mixture over a long G25 Sephadex column with a DTT-free Tris buffer (pH 8.0) at a rate of 0.2 mL/min inside the anaerobic box (*14*). The $[2Fe\text{-}2S]^{2+}$ form was obtained by running the desalting step aerobically outside the box (*11*).

Plasmid pEE1010 (a generous gift from E. Haggard-Ljungquist, Stockholm, Sweden) was used for *E. coli* K12 transformation. The recombinant cells were used for overexpression of the NADPH:flavodoxin reductase (fpr). The purification was based on a previously described procedure (*24*).

Recombinant *E. coli* XL1-Blue DH01 overexpressing *E. coli* flavodoxin was a kind gift from D. Hoover (Ann Arbor, MI). The purification was based on a previously described procedure (*25*). However, the pure protein was isolated as a mixture of holo and apo forms (65/35), and an additional reconstitution−desalting step with purified FMN (C18, SepPak, Waters) was therefore required.

Analysis. Protein concentrations were determined by the Bradford assay with bovine serum albumin as a standard (*26*). Protein-bound iron was determined under reducing conditions with bathophenanthroline disulfonate after acid denaturation of the protein (*27*), and labile sulfide was assayed according to Beinert's method (*28*).

UV−Visible Absorption Spectroscopy. UV−visible spectra were recorded with a Cary1 Bio (Varian) spectrophotometer. Spectra of anaerobic samples could also have been recorded inside the glovebox using a Hewlett-Packard 8453 diode array spectrophotometer equipped with optical fibers connected to a cell holder inside the box.

EPR Spectroscopy. EPR first-derivative spectra under nonsaturating conditions 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 and spin concentrations were obtained through the Win-EPR software using 1 mM Cu(EDTA) and 200 μM fldx semiquinone [$\epsilon_{585} = 4900\ M^{-1}\ cm^{-1}$ (*24*)] standards for Fe−S centers and organic radicals, respectively.

Mössbauer Spectroscopy. ^{57}Fe Mössbauer spectra were recorded on 200 μL cups containing the protein (250−400 μM) as previously described (*11*).

Electrochemistry. Electrochemical experiments were performed under an N_2 atmosphere in a closed three-electrode cell as described in ref *29*. The three-electrode design included a platinum wire counter electrode (KO266) from EG&G Instruments, a glassy carbon working electrode (B30M540-XM540), and a saturated Ag/AgCl reference (MI-401F) micro electrode both from Radiometer Analytical (Copenhagen, Denmark). The three electrodes were connected to a EG&G Instruments model 273A potentiostat/galvanostat controlled with model software. The tip of the working electrode was prepared by careful polishing with ultrafine alumina and then heated for 1 h at 60 °C in 65% HNO_3, rinsed with water, and equilibrated in 1 M Tris buffer (pH 8.0). The acid treatment was repeated at room temperature before each subsequent measurement. The deaerated sample (10 μL) was placed on the working electrode, and the other electrodes were positioned to contact the drop. Other experimental conditions are described in the legend of Figure 5.

Preparation of the Two Reduced Forms of Flavodoxin. The one-electron (semiquinone, SQ) reduced form of flavodoxin [800 μM in Tris (pH 8.0)] was prepared inside the anaerobic box in the presence of catalytic (20 μM) 5-deazaflavin by exposing it to the light from a slide projector operating outside the box. The reaction was stopped when 60% of the SQ was formed as assayed from the absorbance

at 585 nm (~30 min). The SQ form was found to be stable for at least 5 h inside the box.

The two-electron (hydroquinone, HQ) reduced form of flavodoxin [800 μM in Tris (pH 8.0)] was prepared as described for SQ except that substoichiometric 5-deazaflavin (500 μM) and prolonged irradiation were found necessary for completion of the reaction. After 5−6 h, the HQ form (λ_{max} = 383 nm, ϵ = 6200 M^{-1} cm^{-1}) was found to contain less than 3−5% SQ as determined by EPR analysis. The HQ form was stable in the dark for at least 5 h inside the box.

Enzyme Assays. Ribonucleotide reductase activity was measured by the published dCTP assay performed on a manifold flushed with moist oxygen-free N_2 (*11*). One unit of enzyme activity corresponds to 1 nmol of dCTP produced per minute.

AdoMet reductase activity was measured at 570 nm by the level of formation of nihydrine-derivatized methionine analyzed by HPLC (System 7300, Beckman) calibrated with pure amino acid standards.

Activation of protein α was assessed by EPR spectroscopy from the amplitude of the glycyl radical doublet signal (a_H = 15 G) at g = 2.002. A typical experiment carried out at 10 °C within the anaerobic box was as follows. Protein α (120 μM) was mixed with fldx (12 μM), fpr (6 μM), NADPH (2.5 mM), DTT (5 mM), and AdoMet (1.5 mM) in a final volume of 2.0 mL in 50 mM Tris and 100 mM KCl (pH 8.0). At time zero, a 100 μL aliquot was mixed with 15 μL of 1 M TCA and used for the initial determination of the amount of methionine. At the same time, a 200 μL aliquot was transferred to a calibrated EPR tube and directly frozen inside the box. The g = 2.0 semiquinone signal at time zero was subtracted from each subsequent measurement. Then the reaction was initiated by adding the β protein (120 μM). At time intervals, 200 μL was withdrawn for EPR analysis and 100 μL mixed with TCA for the determination of the amount of methionine. Recording conditions were as follows: temperature of 20 and 40 K, microwave power of 0.5 and 2 μW, modulation of 1 mT, and gain of 10^5.

RESULTS

Activation of Ribonucleotide Reductase by the NADPH: Flavodoxin Reductase−Flavodoxin System. Enzyme activation was carried out during anaerobic incubation of protein α and a stoichiometric amount of protein β (one $[4Fe\text{-}4S]^{2+}$ center per polypeptide) with an excess of AdoMet, in the presence of DTT and a reducing agent. It is usually assayed from the ability of the activated enzyme to catalyze CTP reduction by formate. In the following experiments, the electrons are provided by flavodoxin enzymatically reduced by the NADPH:flavodoxin reductase system (*8*). Activation can also be monitored by X-band EPR spectroscopy, from the amount of glycyl radical generated during the reaction. This radical is characterized by a doublet EPR signal at g = 2.002 (*3*, *4*). Finally, during activation, AdoMet is reductively cleaved and the reaction can also be followed from the amount of produced methionine (*9*, *10*).

In Figure 1 is shown the time course for the flavodoxin-dependent formation of both the radical and methionine. Because preliminary experiments had shown that the stability of the radical was improved at lower temperatures, the reaction was carried out at 10 °C inside the anaerobic box. Under these conditions, the data showed a linear formation of the glycyl radical as a function of time, up to a maximum reached after 40−60 min and corresponding to ~0.4 radical per protein α. It should be noted that from one experiment to another, the radical yield varied from 0.35 to 0.5 Gly° per protein α but never exceeded 0.5. Then, further incubation resulted in a slight loss in the level of the radical. Figure 1 also shows that during the enzyme activation step, 1 methionine per radical was formed, but whereas activation was complete within 40 min, the cleavage of AdoMet to methionine further proceeded yielding a maximal amount of about 2 methionines per glycyl radical. No glycyl radical and no methionine could be detected when the experiment was carried out in the absence of protein β.

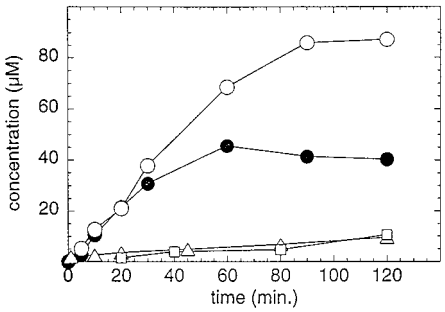

FIGURE 1: Activation of the anaerobic ribonucleotide reductase in the presence of the flavodoxin system. The β protein (120 μM) was incubated in 0.1 M Tris and 50 mM KCl (pH 8.0) at 10 °C inside an anaerobic glovebox with NADPH (2 mM), DTT (5 mM), fldx (12 μM), and fpr (6 μM) either alone (squares) or in the presence of the α protein (circles) (120 μM) or the G681A α mutant (triangles) (120 μM). The reaction was initiated with AdoMet (1.5 mM). At the indicated times, 100 μL was mixed with 15 μL of 1 M TCA for methionine assessment (white symbols) and 200 μL transferred to a calibrated EPR tube for glycyl radical assessment (black circles) as described in Experimental Procedures.

The role of protein α in driving the reductive cleavage of AdoMet to methionine was investigated with the following experiments. In the first one, protein α was omitted, and as shown in Figure 1, only a very small amount of methionine was generated. Furthermore, when the same experiment was carried out with an inactive protein α mutant in which the critical glycine residue, Gly^{681}, was changed into alanine, no glycyl radical could be observed by EPR spectroscopy, as expected, and the amount of methionine that was formed was comparable to that obtained with protein β alone (Figure 1).

The fact that the mutant α protein could bind protein β, as tightly as did the WT α protein, was qualitatively shown during experiments in which stoichiometric amounts of α and β were loaded onto a dATP−Sepharose affinity column. Such a column selectively binds protein α, but protein β is also retained as a consequence of tight binding to protein α (*12*). We observed that also with mutant protein α, protein β was fully retained on the column (data not shown).

In addition, AdoMet was shown to bind to the α2β2 mutant holoenzyme with an affinity comparable to that of the WT enzyme. In this experiment, AdoMet binding was assayed during titration of the enzyme monitored by EPR spectroscopy since the reduced cluster−AdoMet complex is characterized by EPR features (spectrum d, Figure 2) significantly different from those of the reduced cluster in

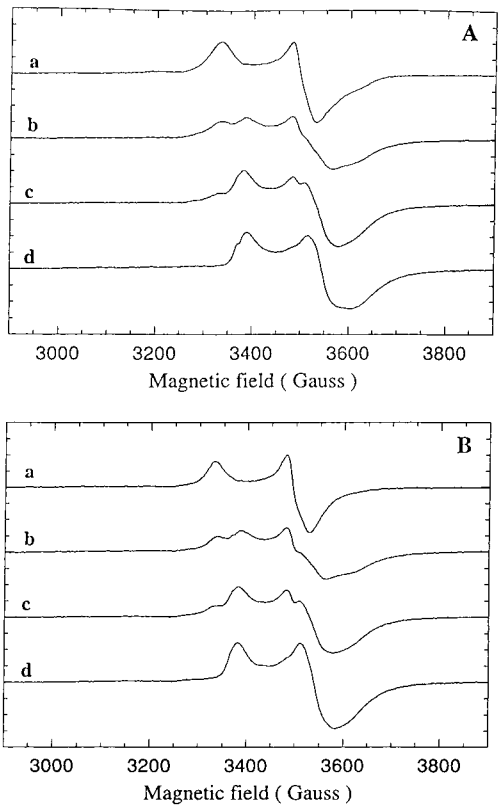

FIGURE 2: Binding of AdoMet to the WT (A) or the G681A (B) holoenzymes monitored by X-band EPR spectroscopy. The proteins (50 μM) were reduced anaerobically for 45 min by a 10-fold excess of sodium dithionite, yielding 25 μM spin for both preparations (spectrum a in panels A and B). Spectra b−d were obtained by addition of a 0.5-, 1-, and 2-fold molar excess of AdoMet with respect to protein. Recording conditions: temperature of 10 K, microwave power of 20 mW, gain of 5×10^4, and modulation of 1 mT.

the absence of AdoMet (spectrum a, Figure 2) (*9*). As shown in Figure 2A, an increased level of addition of AdoMet to the WT enzyme resulted in the conversion of spectrum a to spectrum d with intermediate spectra consisting of mixtures of both components. It is clear from the comparison of panels A and B of Figure 2, which shows the results of the titration experiment with the Gly-to-Ala mutant enzyme, that the same concentration of AdoMet gave the same proportion of the complex in WT and mutant enzymes, demonstrating that these two forms of the enzyme had similar affinities for AdoMet.

Activation of Ribonucleotide Reductase by the Half-Reduced and Fully Reduced Forms of Flavodoxin. In agreement with a recent study (*24*), we have observed by light absorption spectroscopy that reduction of flavodoxin by NADPH in the presence of flavodoxin reductase only generates the half-reduced form (SQ). Accordingly, throughout the reaction, a clean isobestic point at 520 nm was observed. Therefore, under these conditions, no evidence for the presence of HQ, the fully reduced form, could be obtained. If present, as a consequence of SQ disproportionation, HQ is not detectable.

SQ was independently prepared by chemical reduction inside the anaerobic box during irradiation of flavodoxin in the presence of catalytic amounts of deazaflavin. Irradiation was stopped at ~60% conversion to minimize the level of over-reduction to the HQ form. Under these conditions, a maximal amount of 0.1% HQ, at equilibrium, would be present in the reaction mixture, as calculated from the redox potential of the SQ/fldx and HQ/SQ couples. SQ was found to be stable for at least 5 h inside the anaerobic box. Addition of a 5-fold molar excess of SQ to an equimolar mixture of protein α and protein β in the presence of AdoMet, at 10 °C in the dark, was shown to result in the formation of the glycyl radical (Figure 3). An accurate quantitation of that radical was not easy since SQ is also an $S = {}^1/_2$ organic radical, in excess, and subtraction of its contribution is required to observe the EPR signal of the glycyl radical. However, one can estimate that glycyl radical formation occurred at a rate of 3 μM/min. When the same reaction was achieved with 600 μM HQ, prepared as described in Experimental Procedures, the rate of radical formation was about the same (data not shown).

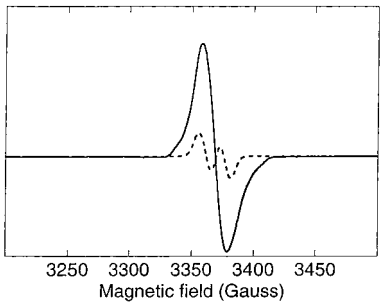

FIGURE 3: SQ supports glycyl radical formation. The holoenzyme (120 μM $\alpha\beta$) was reacted in 0.1 M Tris and 50 mM KCl (pH 8.0) with a 5-fold molar excess of SQ in the presence of AdoMet (1.5 mM) and DTT (5 mM). X-band EPR difference spectrum (---) obtained by subtracting an optimized amount of a standard SQ signal from the EPR signal of the mixture (—) after reaction for 10 min at 10 °C. Optimization was achieved by superimposition of the difference spectrum with that of a standard glycyl radical independently prepared. Recording conditions: temperature of 20 K, microwave power of 2 μW, and modulation of 1 mT.

This result supported the notion that during activation of ribonucleotide reductase by SQ solutions (chemically or enzymatically prepared), electrons were essentially provided by SQ and only marginally by the small amount of HQ at equilibrium with disproportionating SQ.

Reduced Flavodoxin Is Unable To Reduce the [4Fe-4S]$^{2+}$ Center of Protein β. During incubation of the holoenzyme $\alpha 2\beta 2$ with the enzymatic reducing system under anaerobic conditions, the only detectable EPR signal between 4 and 50 K, over a large range of microwave power, was that of the flavin radical from the SQ form of flavodoxin (data not shown). In particular, the EPR signal characteristic for the $S = {}^1/_2$ [4Fe-4S]$^+$ cluster could not be detected. The two signals can be easily differentiated since they have very different temperature dependence and microwave power saturation properties. To better mimic the activation reaction, the same experiment was carried out in the presence of a large excess of *S*-adenosylhomocysteine, an efficient inhibitor of the reaction, suggested to compete with AdoMet for binding to the enzyme (*10*). Again, under these conditions, the signal of the reduced cluster was not detected. No reduced cluster could be detected when either reduced form of flavodoxin, SQ or HQ, in excess, was used as the reducing agent or when protein α was omitted from the reaction mixture. Conversely, a near-quantitative yield of flavodoxin SQ (29 μM) was obtained when reduced protein β (50 μM),

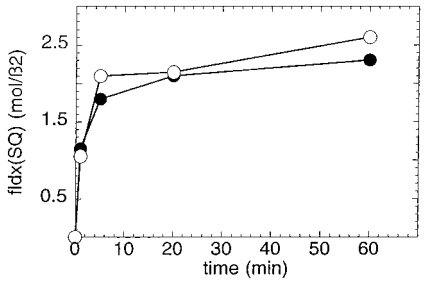

FIGURE 4: Flavodoxin hydroquinone (HQ)-mediated reductive conversion of the [2Fe-2S] to [4Fe-4S] centers. The β (●) or $\alpha\beta$ (○) proteins were incubated in 0.1 M Tris and 50 mM KCl (pH 8.0) at 18 °C inside an anaerobic glovebox with DTT (5 mM) and a 15-fold molar excess of flavodoxin HQ. At the indicated times, 200 μL aliquots were withdrawn and transferred to an EPR tube for SQ quantitation.

with a $[4Fe\text{-}4S]^{+}$ center (30 μM), generated during reduction by photoactivated deazaflavin, was reacted with flavodoxin (62 μM) (data not shown).

These experiments demonstrate that, in contrast to strong reducing agents such as photoreduced deazaflavin and dithionite in excess (*8*, *9*), reduced flavodoxin, in either the SQ or HQ form, is unable to convert the $[4Fe\text{-}4S]^{2+}$ center of protein β into the reduced $[4Fe\text{-}4S]^{+}$ center, to a detectable extent, even in the presence of protein α. The thermodynamically favorable electron flow is from the reduced protein β to flavodoxin and not the reverse.

It is interesting to note that the reaction of HQ (15-fold molar excess) with air-oxidized preparations of protein β, containing one $[2Fe\text{-}2S]^{2+}$ center per polypeptide (*11*), either in the presence or in the absence of protein α, resulted in the fast formation of SQ up to a plateau corresponding to 2 SQ molecules per protein β, after reaction for 5 min (Figure 4). Analysis of the resulting protein by Mössbauer spectroscopy in an experiment with ^{57}Fe-reconstituted protein β showed that the $[2Fe\text{-}2S]^{2+}$ clusters were quantitatively converted to $[4Fe\text{-}4S]^{2+}$ clusters (data not shown). Mössbauer signatures for both types of clusters have been obtained previously (*11*). No further reduction to the $[4Fe\text{-}4S]^{+}$ form could be observed by either EPR or Mössbauer spectroscopy. These observations are in agreement with the equation

$$2[2Fe\text{-}2S]^{2+} + 2HQ \rightarrow [4Fe\text{-}4S]^{2+} + 2SQ$$

Previously, such a reductive conversion of [2Fe-2S] to [4Fe-4S] clusters had been achieved with dithionite or photoreduced deazaflavin (*11*).

With SQ as the reducing agent, almost no reduction and no $[2Fe\text{-}2S]^{2+}$ to $[4Fe\text{-}4S]^{2+}$ conversion could be observed after reaction for 20 min (data not shown).

A Very Low Redox Potential of the FeS Cluster. In Figure 5 is shown the square-wave voltammograms for solutions of the $\alpha 2\beta 2$ holoenzyme [200 μM in 0.1 M Tris, 50 mM KCl (pH 8.0), and 5 mM DTT] at 23 °C, recorded directly at a carbon electrode in the absence of mediators. A redox active couple was observed at $E'^{\circ} = -550$ mV (vs NHE) that was assigned to the $[4Fe\text{-}4S]^{2+/1+}$ couple. When AdoMet (10 mM) was included in the protein solution in the absence of DTT, the measured redox potential was shifted to a more negative value [$E'^{\circ} = -620$ mV (vs NHE)].

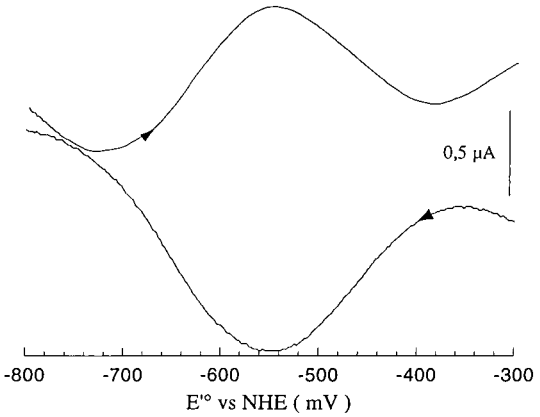

FIGURE 5: Forward and reverse square-wave voltammograms of 10 μL of $\alpha\beta$ protein, 19 mg/mL, in 100 mM Tris, 50 mM KCl (pH 8.0), and 5 mM DTT at a bare glassy carbon electrode. Conditions: temperature of 23 °C, forward sweep potential of 0 to −1.0 V, reverse sweep potential of −1.0 V to 0, frequency of 10 Hz, pulse height of 25 mV, current range of 10 μA, and filter of 5.3 Hz. Background buffer was subtracted.

DISCUSSION

The anaerobic ribonucleotide reductase from *E. coli* is the prototype of a whole class of enzymes which employs the combination of *S*-adenosylmethionine, AdoMet, and an iron−sulfur cluster to generate free radicals. The resulting radical serves for the initiation of C−OH bond reduction, during ribonucleotide reduction, and also for C−C bond cleavage, in pyruvate formate lyase and photoproduct lyase (*16*, *19*), for C−C bond formation, in benzylsuccinate synthase (*20*), and for insertion of a sulfur atom into nonactivated C−H bonds, in biotin and lipoate synthases (*18*, *30*). From sequence comparisons, it appears that all these enzymes contain a unique [4Fe-4S] cluster coordinated by three cysteines present in a conserved CXXXCXXC motif and by a fourth unidentified ligand. It is very likely that these systems employ a common mechanism for generating free radicals from AdoMet, and understanding of that mechanism in the case of one of the most extensively characterized members of this class of enzymes, the anaerobic ribonucleotide reductase, might provide general insight into the chemistry of the different enzymes.

A key aspect of this reaction resides in the fact that generation of free radicals depends on the one-electron reduction of AdoMet by the reduced cluster, as a prerequisite for homolysis of the S−C(5′-deoxyadenosyl) bond. In previous studies on ribonucleotide reductase (*9*) and more recently on pyruvate formate lyase and lysine 2,3-aminomutase (*31*, *32*), the $[4Fe\text{-}4S]^{+}$ form of the cluster has been actually shown to be competent for AdoMet reduction. There is thus a requirement for a source of electrons which allows reduction of the iron cluster. In the case of ribonucleotide reductase, this source is the NADPH:flavodoxin reductase−flavodoxin system (*7*, *8*). It is remarkable that some of the other AdoMet-dependent systems mentioned above also depend on flavodoxin for activity. This is the case for the PFL activating enzyme (*33*) and biotin synthase (*34*) and further supports the notion of a common class of enzymes, with similar electron transfer mechanisms. These are the subject of this study.

First, it is confirmed that the enzymatic reducing system (NADPH, flavodoxin reductase, and flavodoxin), in which

flavodoxin is absolutely required, is able to make the holoenzyme fully active. During turnover, under the in vitro anaerobic conditions used in our experiments, the actual electron donor seems to be SQ, the half-reduced form of flavodoxin. A similar conclusion was reached for other enzyme systems requiring NADPH, flavodoxin reductase, and flavodoxin for activity, such as biotin synthase, cytochrome P-450c17, and methionine synthase (*24*, *35*, *36*). Indeed, SQ was the only detected reduced species during enzymatic reduction of Fldx and has proved to be capable of supporting the formation of the glycyl radical (Figure 3). During the anaerobic growth of *E. coli*, the major reduced form of flavodoxin is instead HQ, the fully reduced one, which is produced by the action of a thiamin diphosphate-dependent pyruvate-flavodoxin oxidoreductase (*37*). Thus, in vivo it is likely that HQ donates the electron to the anaerobic ribonucleotide reductase.

However, we show here that the one-electron reduction of the $[4Fe\text{-}4S]^{2+}$ center requires a highly negative redox potential ($E^\circ = -550$ mV vs NHE at pH 8.0), and thus, reduction by the half-reduced SQ [$E^\circ = -256$ mV vs NHE at pH 7.0 (*24*)] flavodoxin is thermodynamically unfavorable. Accordingly, no reduced $[4Fe\text{-}4S]^{+}$ center could be detected by EPR spectroscopy when the $\alpha 2\beta 2$ holoenzyme was treated with enzymatically reduced flavodoxin or with chemically prepared SQ, in the absence of AdoMet. No reduced cluster could be detected either when HQ was used as the reducing agent again in agreement with the fact that the redox potential (E'°) of the HQ/SQ couple is only -440 mV vs NHE at pH 7.0 (*24*). The very negative potential of the cluster also explains why it is so difficult to reduce the cluster even with strong reducing agents such as dithionite in excess or photoreduced deazaflavin (*8*, *9*). On the other hand, HQ has a redox potential low enough to effect the redox conversion of the two $[2Fe\text{-}2S]^{2+}$ centers of the oxidized form of the β protein to the $[4Fe\text{-}4S]^{2+}$ cluster (Figure 4). This reaction also confirms that the inability of HQ to generate a reduced cluster has a thermodynamic origin and is not due to some sort of ineffective binding between flavodoxin and protein β in the absence of AdoMet.

This reaction and the observation, consistent with the above redox potentials, that the reduced cluster can convert fldx to the SQ form demonstrate that fldx and protein β interact with each other and that electron transfer between them can occur.

It is very important to note that, even though these experiments are preliminary, AdoMet binding to the holoenzyme did not result in a shift of the redox potential of the cluster toward more positive values that would make electron transfer from flavodoxin thermodynamically favorable. This is in line with the observation that reduction of the holoenzyme by flavodoxin in the presence of *S*-adenosylhomocysteine did not generate EPR-detectable amounts of reduced cluster. This result seems to differentiate ribonucleotide reductase from lysine aminomutase. As a matter of fact, even though, in the latter case, no measurements of redox potentials were reported to support the notion that AdoMet binding to this system was key to the reduction of the clusters, it was shown that the EPR signals of reduced clusters could be observed only in the presence of AdoMet or *S*-adenosylhomocysteine (*17*). In the case of pyruvate formate lyase, there are contradictory reports in the literature as far as the requirement of AdoMet for observation of reduced clusters is concerned (*16*, *38*). Further experiments are required to understand these differences.

It is thus proposed that the solution to the above thermodynamic paradox of the activation reaction resides in the coupling of the endergonic reduction of the cluster by reduced flavodoxin (either SQ or HQ) to two thermodynamically favorable reactions which drive the whole process toward products, methionine, 5′-deoxyadenosine, and the glycyl radical (Scheme 1). The first one (reaction 2 of Scheme 1) is the oxidation of the reduced cluster by AdoMet which, in the case of ribonucleotide reductase, has been previously shown to occur quantitatively, in the presence of DTT, to generate methionine (*9*). This does not seem to be the case for all related enzymes (*17*, *32*). The second one (reaction 3 of Scheme 1) is the reaction of the putative resulting 5′-deoxyadenosyl radical with the glycine residue generating the glycyl radical. The thermodynamic driving force of the latter originates from the difference between the C−H bond dissociation energies of 5′-deoxyadenosine (100 kcal/mol) and glycine (79 kcal/mol) and the improved stability of the glycyl radical with regard to that of the 5′-deoxyadenosyl radical (*39*). Consistent with this interpretation is the dramatic consequence of mutating the critical glycine residue of protein α on the amount of methionine that is formed during the reduction of AdoMet (Figure 1). We have clearly shown that this effect is not due to an impaired capacity of either the mutant to bind the iron−sulfur protein β or the resulting complex to bind AdoMet efficiently (Figure 2). It is thus clear that the role of protein α in shifting the equilibrium toward products depends on the presence of a glycine residue that can be converted to a radical. The fact that only a very small amount of methionine is formed in the absence of WT protein α indicates that reaction 2 is not exergonic enough to overcome the unfavorable reduction by flavodoxin.

There are other examples of reactions whose enthalpic costs are reduced by coupling to the formation of a stable species. The latter can be a radical as in the present case, and this is exemplified by class II ribonucleotide reductases whose activity depends on adenosylcobalamin (AdoCbl) and for which it was shown that the endergonic homolysis of the cobalt−carbon bond of AdoCbl (dissociation energy = 30 kcal/mol) is coupled to the formation of a cysteinyl radical (*40*). In the reactivation of oxidized methionine synthase, the formation of the stable methyl cobalamin cofactor forces the flavodoxin system to generate a Co(I) intermediate with an electrochemical gap of more than 200 mV, in a reaction driven by its subsequent facile reaction with AdoMet (*41*, *42*).

Two observations are more intriguing and require further experiments. The first one is that the glycyl radical could not be incorporated into more than one-half of the molecules of protein α. Incomplete radical introduction has been reported also in the case of pyruvate formate lyase which was shown to contain 1 ± 0.04 spin per protein dimer (*43*). Whether this reflects an ineffective introduction of the radical under the present in vitro conditions for enzyme activation or the fact that it is the result of an intrinsic property preventing one polypeptide chain of the α_2 dimer from being radicalized, once the radical has been introduced in the other, is unknown at that stage. One cannot exclude as a third the

possibility that the radical is indeed generated in each subunit but that, for unknown reasons, is unstable in half of the polypeptides and then disappears.

Such a scenario could explain the second intriguing observation, namely, that methionine formation proceeded even after maximal glycyl radical formation was achieved (Figure 1). As a matter of fact, we have above discussed the importance of generating the glycyl radical for driving the reductive cleavage of AdoMet by flavodoxin. It should be noted that these results corroborate earlier data from Harder et al. (*10*), who found, with a much more crude preparation, a "decoupling" between CTP reductase activity and methionine formation, the latter still being formed when the maximal activity (and hence, the maximum of glycyl radical) was reached. At this stage, it is difficult to understand these stoichiometries, and further experiments are required to fit in our working mechanistic hypothesis.

In conclusion, we had previously demonstrated that the activation of AdoMet was dependent on the reduction of the Fe−S cluster (*9*). The investigation presented here on how the cluster is reduced by flavodoxin adds new insights into the understanding of the fascinating chemistry of the radical-generating Fe−S/AdoMet combination. The next step is to understand at a molecular level the mechanism of the reduction of AdoMet by the reduced cluster. This is currently under study.

ACKNOWLEDGMENT

We thank C. Meier and A. X. Trautwein (Institut für Physik, Medizinische Universität, Lübeck, Germany) for Mössbauer spectroscopy analysis and J. P. Andrieu and J. Gagnon (IBS, LEM, Grenoble, France) for assaying the AdoMet reductase activity by HPLC.

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BI001746C