For deoxyribonucleotide synthesis during anaerobic growth, *Escherichia coli* cells depend on an oxygen-sensitive class III ribonucleotide reductase. The enzyme system consists of two proteins: protein α, on which ribonucleotides bind and are reduced, and protein β, of which the function is to introduce a catalytically essential glycyl radical on protein α. Protein β can assemble one [4Fe-4S] center per polypeptide enjoying both the [2Fe-2S] and [4Fe-4S] redox state, as shown by iron and sulfide analysis, Mössbauer spectroscopy (\( \delta = 0.43 \) mm/s, \( \Delta E_Q = 1.0 \) mm/s, [4Fe-4S]\(^{2+}\)), and EPR spectroscopy (\( g = 2.03 \) and \( 1.93 \), [4Fe-4S]\(^{1+}\)). This iron center is sensitive to oxygen and can decompose into stable [2Fe-2S]\(^{2+}\) centers during exposure to air. This degraded form is nevertheless active, albeit to a lesser extent because of the conversion of the cluster into [4Fe-4S] forms during the strongly reductive conditions of the assay. Furthermore, protein β has the potential to activate several molecules of protein α, suggesting that protein β is an activating enzyme rather than a component of an \( \alpha_2\beta_2 \) complex as previously claimed.

Ribonucleotide reductases (RNRs)\(^1\) catalyze the reduction of ribonucleotides into deoxyribonucleotides and thus provide the cell with a balanced supply of the DNA precursors (1–3). *Escherichia coli* uses different ribonucleotide reductases during aerobic and anaerobic growth. The active form of the anaerobic enzyme (class III RNR) is characterized by the presence of a catalytically essential glycyl radical and an iron-sulfur center as well as the requirement for formate as the hydrogen donor (4–6). It is found in other anaerobically growing microorganisms among bacteria, phages, and methanogens (7).

The anaerobic RNR was originally isolated as a dimeric α\(_2\) (160 kDa) inactive form that could be activated by anaerobic incubation with a complex activating system consisting of S-adenosylmethionine (AdoMet), a reducing system (NADPH, flavodoxin reductase, and flavodoxin), and an additional 17-kDa β protein, provisionally called activase (8, 9). During the reaction, a radical is introduced at a specific glycine residue (Gly-681) of protein α. The activated protein α, encoded by the *nrdD* gene, thus contains the glycyl radical, the substrate site, and two additional sites where allosteric effectors (deoxyribonucleotides) bind and regulate the activity (4, 10–12). The recently determined three-dimensional structure of a mutant form of the enzyme from bacteriophage T4, in which the essential glycine has been changed to an alanine, suggests that the function of the radical is to abstract a hydrogen atom from an adjacent cysteine close to the substrate (13). The resulting thyl radical is then supposed to initiate the reaction by removing the 3'-hydrogen atom of the ribose (14). How reduction by formate and formation of the deoxyribonucleotide proceed from the sugar radical remains to be established.

The small β protein, encoded by the *nrdG* gene, proved to be an unusual enzyme. First, in solution in the absence of the large protein, it occurred in a monomer-polymer equilibrium, with \( \beta_1 \) and \( \beta_2 \) being the major species. The addition of protein \( \alpha_2 \) shifts the equilibrium to the \( \beta_2 \) form and results in a very tight 1:1 complex between dimers of the two proteins, as shown from sucrose gradient centrifugation (5) and from the impossibility of separating them during gel filtration or by affinity chromatography on dATP-Sepharose gel, on which only protein \( \alpha \) can bind because of its affinity for dATP (5, 10). It was thus concluded that \( \beta_2 \) was not an activating enzyme but rather a component of the system and that the anaerobic ribonucleotide reductase had an \( \alpha_2\beta_2 \) structure (5).

Second, whereas the presence of an iron-sulfur center was suggested early from the light absorption properties of the enzyme and from iron and sulfide analysis (15), very little iron could be retained during purification of the protein. However, treatment of the β protein with ferrous iron and sulfide generated a well defined [2Fe-2S]\(^{2+}\) cluster, as shown from Mössbauer and Raman resonance spectroscopy (16).

Third, EPR and Mössbauer spectroscopies of the protein after reduction with photoreduced deazaflavin or dithionite showed that the reduced centers were almost exclusively [4Fe-4S] cubane clusters (5, 16). The reductive [2Fe-2S] to [4Fe-4S] conversion is a remarkable reaction, even though it has been recently also observed with other iron-sulfur proteins, such as the transcription factor FNR, the activating enzyme of the pyruvate formate-lyase and biotin synthase (17–19). Whether the [4Fe-4S] center, in the reduced anaerobic ribonucleotide reductase, was at the interface of two β polypeptide chains was first suggested as a likely possibility but not firmly established experimentally (5).

Fourth, formation of the glycyl radical was shown to depend on the one-electron reduction of S-adenosylmethionine by the reduced [4Fe-4S]\(^{2+}\) center (20). It was proposed that reduced AdoMet can undergo homolysis of the S—C(5'-deoxyadenosyl) bond to generate methionine and the 5'-deoxyadenosyl radical, presumably responsible for abstraction of the hydrogen atom of the glycine residue.

Here we report evidence that previous models for the iron center of RNR need to be revised. As a matter of fact, we show,
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] 1− protein (62 μm ferredoxin IV of R. capsulatus).

Reduction of Iron-Sulfur Centers—Reduction of iron-sulfur centers were performed inside the anaerobic glove box. 5-DAF was dissolved in Me2SO, diluted with water to 500 μM, and stored inside the box in the dark. Protein (100 μM) was prepared in 100 mM Tris-HCl, pH 8.0, and irradiated in the presence of 5-DAF (20–50 μM) for 60 min. Reduction could be monitored by light absorption directly inside the box. To avoid exposure to oxygen, EPR tubes were frozen directly inside the box in a water filled with isopentane cooled from outside the box by liquid nitrogen.

Mössbauer Spectroscopy—257Fe Mössbauer spectra were recorded on 200-μl samples containing the protein (0.25 mM) with a conventional constant acceleration spectrometer using a 57Co source in a Rh matrix (254 Mβq). Measurements at 4.2 and 77 K were performed using a bath cryostat (Oxford Instruments) with an electromagnet mounted outside the cryostat, producing a field of 20 mT perpendicular to the γ-ray. High-field measurements were performed with a cryostat equipped with a superconducting magnet (Oxford Instruments). The spectra were analyzed assuming Lorentzian line shape, and the isomer shift is quoted relative to α-Fe at room temperature.

RESULTS

Anaerobic Reconstitution of the Apoprotein β—The basis of this work was the suspicion that previous preparations of the small component of the anaerobic ribonucleotide reductase suffered from insufficient anaerobiosis. This led us to use a high quality anaerobic glove box and to carry out all experiments within the box. To avoid any contamination of protein samples with oxygen, also during light absorption spectroscopic analysis, the glove box was equipped with optical fibers, which allowed samples to be monitored spectrophotometrically directly inside the box.

The apoprotein form of protein β was obtained in large quantities by purification from overexpressing E. coli cells (5). It was incubated in the box in the presence of a 6-fold excess of sodium sulfide and ferrous sulfate, with respect to β, and dithiothreitol. After a 2-h reaction at 18 °C, the sample was desalted by chromatography on a Sephadex G-25 column inside the box. This preparation was called sample A. A portion of this preparation was opened to air outside the box for 1 h at 4 °C and then desalted, to give sample B. A third sample, C, prepared under the previously reported conditions and characterized as a [2Fe-2S] 1− protein, with one [2Fe-2S] center per polypeptide chain, was used for comparison (16). All three samples were assayed for iron and sulfur content, light absorption, EPR and Mössbauer spectroscopic properties, and ribonucleotide reductase activity. Spectra of sample C were found to be identical to earlier published preparations of the same type (16), showing the reproducibility of the reconstitution procedure and thus are not shown here.

Iron and Sulfide Content—Sample A contained 3–4 iron and 3–4 sulfur atoms/protein β, with slight variations from one preparation to another. Under these conditions, we never obtained 1.8–2 iron and sulfur atoms/chain, as previously reported (16) and as for sample C. Sample B contained only 1.7–2 iron and 1.8–2 sulfur atoms/chain, showing that about half of the iron and sulfide content was lost during the exposure of sample A to air.

Light Absorption Spectroscopy—Samples A and B displayed significantly different UV-visible spectra (Fig. 1). The time course for conversion of sample A to B, under exposure to air, could be monitored by light absorption spectroscopy (data not shown). The reaction is a rather slow process (t1/2 = 25 min at 11 °C) for which sample B is the true final product, not further transformed in a time scale of hours. Spectrum B was like that of sample C, with a band at 420 nm together with a broad band at 590 nm. Spectrum A was twice as intense at 420 nm and had
no band at 590 nm. In the anaerobic box, sample A was stable for days.

**EPR Spectroscopy**—Sample C was EPR-silent as were previously published comparable preparations. Even though most of the B preparations were also EPR-silent, in some cases a small EPR signal could be detected, which was characteristic of a [3Fe-4S]1+ center and accounted for less than 10% of total iron (data not shown). Sample A was also most often EPR-silent, but in some cases a small EPR signal could be observed, accounting for about 0.1 spin/β polypeptide and similar to that obtained during reduction of samples A, B, and C (see below).

Anaerobic reduction of samples A and B with photoreduced deazaflavin or dithionite generated an EPR signal (shown in Fig. 2 for sample A) characteristic for a $S = \frac{1}{2}$ species, with g values at 2.03 and 1.93. The temperature dependence and the microwave power saturation properties are consistent with a [4Fe-4S]1+ center (Fig. 2, inset). A and B spectra were very similar in shape and properties to that of the anaerobically reduced sample C, as prepared for this work or previously reported (5, 20). However, quantitation of the EPR signal of sample A demonstrated that the amount of spins accounted for reported (5, 20). However, quantitation of the EPR signal of reduced sample C, as prepared for this work or previously similar in shape and properties to that of the anaerobically accounts only for 24%. From one experiment to another, this amount varied from 15 to 25%. The second doublet (76%) in sample B shows parameters ($\delta = 0.3$ mm/s$^{-1}$, $\Delta E_Q = 0.56$ mm/s$^{-1}$) typical for tetrahedrally sulfur-coordinated high spin Fe$^{3+}$ as in [2Fe-2S]$^{2+}$ centers. These parameters are comparable with those previously reported for a C-type sample (16). Again, that these two components were diamagnetic, thus excluding the presence of [3Fe-4S] centers, was demonstrated from the Mössbauer spectrum taken at 4.2 K with an applied field of 7 T parallel to the γ-ray (Fig. 4B). Note that this B preparation was EPR-silent.

**Enzyme Activity**—The enzyme reaction consists of two steps. In the first one, the enzyme is activated by introducing the glycycl radical into protein $\alpha$, during anaerobic incubation of a mixture of protein $\alpha$ and protein $\beta$, with enzymatically reduced flavodoxin and AdoMet. In the second step, CTP is added in the same anaerobic tube, and the active enzyme catalyzes the reduction of CTP to dCTP by formate. The assay measures the amount of dCTP formed in the second step.

As shown in Fig. 5, sample A, with [4Fe-4S] centers, and sample B, derived from sample A by oxidation, were assayed for CTP reduction and compared. The addition of increasing amounts of protein $\beta$ to a fixed amount of protein $\alpha$ (4 µg), with an activation period fixed at 45 min, generated the results shown in Fig. 5A. The system became saturated with respect to protein $\beta$, in both cases with the same maximal specific activity. When the same experiment was done with sample C, containing [2Fe-2S] centers, the results obtained were superimposable on those obtained with sample B (data not shown).

However, saturation in the case of samples B and C occurred at significantly larger amounts of protein than in the case of sample A (Fig. 5). For example, whereas saturation was obtained for a $\beta/\alpha$ ratio of 0.1 for sample A, a $\beta/\alpha$ ratio of 0.4 was required for samples B and C. These data now suggest that $\beta$ is an activating enzyme capable of generating a glycycl radical in several molecules of protein $\alpha$ rather than a component of an $\alpha_5\beta_2$ holoenzyme.

This model was further supported by the data shown in Fig. 5B, in which the specific activity of protein $\alpha$ is reported as a function of the activation time for three different amounts of protein $\beta$ (sample A), substoichiometric with regard to protein $\alpha$. In fact, with a $\beta/\alpha$ ratio of 0.2, full activity was obtained for an activation period of less than 5 min. When this ratio was decreased to 0.06, full activity could also be obtained but only because of an extended activation time (about 40 min). With a $\beta/\alpha$ ratio of 0.03, as much as 75% of the activity was obtained after a 50-min activation time.

**DISCUSSION**

The ribonucleotide reductase from anaerobically grown *E. coli* is an enzyme that is extremely sensitive to oxygen, which makes it particularly difficult to handle. We previously showed that the glycycl radical reacted instantaneously during exposure to air, resulting in fragmentation of protein $\alpha$ and irreversible inactivation of the enzyme (8, 27). More recently we showed that the fully reduced iron cluster, identified as a [4Fe-4S]1+ center and responsible for AdoMet reduction and glycycl radical formation, was also sensitive to oxygen and degraded into 3Fe and 2Fe clusters during exposure to air (28).

The reactivity of the iron center explains why various forms can be obtained during reconstitution of the apoprotein, depending on the quality of the anaerobiosis. Previous preparations of reconstituted protein $\beta$ contained [2Fe-2S] centers exclusively, as shown by a combination of different spectroscopies (16). Now, by improving the anaerobic conditions and working exclusively within an anaerobic glove box, we show that the reconstituted protein can assemble a [4Fe-4S]$^{2+}$ center with two ferric and two ferrous ions per polypeptide chain. Iron and
sulfur analysis and UV-visible and Mössbauer spectroscopies unambiguously support such a conclusion. In particular, the Mössbauer parameters ($\delta = 0.43$ mm$\cdot$s$^{-1}$, $\Delta E_Q = 1.04$ mm$\cdot$s$^{-1}$) of the major iron species (80–90% of the total iron) are typical for Fe$_{2.5}^+$ centers, present in [4Fe-4S]$^{2-}$ clusters. A significant amount of mononuclear sulfur-coordinated Fe$^+$ was also observed. In some cases, probably because of optimal anaerobic conditions, a small amount of reduced [4Fe-4S]$^{2-}$ center can be retained during reconstitution, as shown by EPR spectroscopy of the reconstituted protein $\beta$. In any case, reduction with photoreduced deazaflavin afforded large amount of $S = 1/2$ [4Fe-4S]$^{2+}$ cluster.

During exposure to air, the cluster is oxidized and loses two iron atoms, which can be removed during filtration of the protein. That it is converted mainly into a [2Fe-2S]$^{2+}$ cluster is shown by a decreased intensity of the light absorption band at 420 nm and by characteristic Mössbauer parameters. The spectroscopic properties and the enzyme activity of this oxidized form are identical to those of the previously reported reconstituted forms, containing [2Fe-2S] centers (16).

It is thus now clear that the protein $\beta$ of the anaerobic ribonucleotide reductase is a [4Fe-4S] protein and that imperfect anaerobiosis was responsible for iron oxidation and labilization during reconstitution of the iron center and the prepa-
ration of a [2Fe-2S] protein. However, that previous preparations contained degraded forms of the cluster could not be easily concluded from enzyme activity because, as previously shown, the [2Fe-2S] centers of the protein have the potential, during the reductive conditions of the enzyme assay, to generate an active [4Fe-4S] center (15).

This [4Fe-4S] to [2Fe-2S] conversion is unlikely to have functional relevance. As a matter of fact, inside anaerobically growing cells, the reducing conditions are strong enough to maintain a [4Fe-4S] center, which probably shuttles between the two redox states, [4Fe-4S]²⁺ and [4Fe-4S]⁺. However, we cannot exclude the possibility that during transient exposure to air, the enzyme experiences a 4Fe to 2Fe conversion. This conversion could be beneficial for the cell because it would switch off the formation of the glycyl radical and prevent oxygen-dependent cleavage of the polypeptide at the glycine site (27). Furthermore, by limiting the loss of protein-bound iron, it would allow faster reconstitution of the cluster after restoring anaerobic conditions.

The present data now also rule out the previous suggestion that the [4Fe-4S] clusters, generated during reduction of the [2Fe-2S] forms of the protein β, are localized at the interface of two β polypeptides (5). Instead, it is likely that during reduction iron is mobilized to generate (4Fe-4S) centers in half of the polypeptides (16).

The improved anaerobic conditions and the higher amount of iron is oxidized forms of the enzyme.

The anaerobic ribonucleotide reductase presents a number of similarities with another enzymatic system of the anaerobic metabolism, the pyruvate formate-lyase (PFL). As a matter of fact, the PFL activating enzyme contains an iron center that is involved in the reduction of AdoMet by reduced flavodoxin and the generation of a glycyl radical on PFL (29, 30). Recently it was shown that this iron center was a [4Fe-4S] center that can be interconverted to a [2Fe-2S] center depending on the redox conditions (18, 31). The results reported here thus further extend the similarity between the PFL and the anaerobic RNR systems. Protein β and the PFL activase also share some sequence homology, with a common CXXXCXC motif, which presumably provides the cysteines for binding iron (18). This sequence is also found in the case of biotin synthase, which catalyzes the conversion of dethiobiotin to biotin. Biotin synthase is a homodimer containing one [2Fe-2S] center per monomer, which under reduction generate a [4Fe-4S] center (19). It is tempting to suggest that also in that case the enzyme is designed to assemble [4Fe-4S] centers and that the reported preparations are oxidized forms of the enzyme.
iron present in the preparations of protein \( \beta \) of the anaerobic RNR now available also explain another unexpected observation reported here. The holoenzyme was previously characterized as a tight \( \alpha_2\beta_2 \) complex, in agreement with sucrose gradient centrifugation studies and behavior on the affinity dATP-Sepharose column (5). In contrast, we now report that full activity of protein \( \alpha \) is obtained with a catalytic amount of protein \( \beta \), showing that one molecule of protein \( \beta \) is able to create a glycol radical in several molecules of protein \( \alpha \). Therefore protein \( \beta \) cannot be considered any longer as a component of an \( \alpha_2\beta_2 \) holoenzyme, as previously stated, but rather as an activating enzyme (“activase”) associated with protein \( \alpha \), the proper ribonucleotide reductase. This property again strengthens the similarity between the PFL and the anaerobic RNR systems.

Thus, whereas a tight association between radical-free protein \( \alpha \) and oxidized protein \( \beta \) undoubtedly occurs (5), this does not appear to be the case during enzyme activation. Several parameters, by themselves or in combination, might therefore affect this interaction: (i) the binding of flavodoxin to protein \( \beta \); (ii) the reduction of the iron-sulfur cluster of protein \( \beta \); (iii) the introduction of the glycol radical in protein \( \alpha \). This important aspect of the activation reaction deserves further investigation.

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The Anaerobic Ribonucleotide Reductase from *Escherichia coli* : THE SMALL PROTEIN IS AN ACTIVATING ENZYME CONTAINING A [4Fe-4S]2+ CENTER

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