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 ribonucleotide 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 chain, in which the function is to introduce a hydrogen atom from an adjacent cysteine close to the substrate (13). The resulting thiolate 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 β and β2 being the major species. The addition of protein α2 shifts the equilibrium to the β2 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 α can bind because of its affinity for dATP (5, 10). It was thus concluded that β2 was not an activating enzyme but rather a component of the system in which the anaerobic ribonucleotide reductase had an α2β2 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]1+ 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]1+ 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,
The [4Fe-4S] Center of the Anaerobic Ribonucleotide Reductase Activating Enzyme

By strict adherance to high quality anaerobic conditions probably not achieved in previous studies, that each β polypeptide has the ability to chelate 4 iron and 4 sulfur atoms per polypeptide chain, and we conclude that β is a [4Fe-4S]2+ and not, as previously reported, a [2Fe-2S]2+ enzyme. The [2Fe-2S]2+ center is in fact an air-degraded form of the cluster, which is convertible back to the [4Fe-4S] form under strong reducing conditions. The previous hypothesis that such a reduced cluster sits at the interface of the dimer has to be rejected. Furthermore, experiments are shown that demonstrate that β, with its [4Fe-4S] center, appears to function more like an activase for protein α than as a component of an αβ2 complex.

EXPERIMENTAL PROCEDURES

Materials

Enzymes and other components of the anaerobic ribonucleotide reductase system have been obtained as described previously (5, 8–10, 20, 21). FeO was converted into its chloride form by dissolving it in hot, concentrated (35%) hydrochloric acid of analytical grade (Carlo Erba) and repetitively concentrated in water. Fe(NH4)2(SO4)2 was from Aldrich. AdoMet was from Roche Molecular Biochemicals. 5-Deaza-7,8-demethyl-10-methyl-isoalloxazine (5-DADF) was available in our laboratory. Ferredoxin IV from Rhodobacter capsulatus and pyruvate:ferredoxin oxidoreductase from Clostridium pasteurianum were provided by Dr. Y. Jouanneau and Dr. J. M. Moulis, respectively (CEA, Grenoble, France).

Analysis

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

Methods

Reconstitution of the Iron-Sulfur Center of the β Protein—All of the steps of the reconstitution procedure were done anaerobically inside a glove box (Jacomet BS531 NMT) in an N2 atmosphere containing less than 2 ppm O2. The apoenzyme was incubated with a 6-fold molar excess of Na2S and either Fe(NH4)2(SO4)2 or 57FeCl3 in 0.1 M Tris-HCl, pH 8.0, and repetitively concentrated in water. Fe(NH4)2(SO4)2 was from Aldrich. AdoMet was from Roche Molecular Biochemicals. 5-Deaza-7,8-demethyl-10-methyl-isoalloxazine (5-DADF) was available in our laboratory. Ferredoxin IV from Rhodobacter capsulatus and pyruvate:ferredoxin oxidoreductase from Clostridium pasteurianum were provided by Dr. Y. Jouanneau and Dr. J. M. Moulis, respectively (CEA, Grenoble, France).

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 inside 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 equilibrated with the buffer, the colored fractions (sample A) were collected and concentrated over a YM10 Diaflo membrane (Amicon). Alternatively, protein β was reconstituted outside the box as described previously (16), within tubes connected to a manifold under a constant flux of moist N2, and then gel-filtrated on a Sephadex G-25 column equilibrated with Na2S-bubbled buffer. In this case, the protein was concentrated under anaerobic conditions (sample C).

RNR Activity—Activity assays were performed under anaerobic conditions inside a glove box. The assay comprised two steps, the activation step, which leads to the formation of the glycy radical, and the reduction step, in which CTP is reduced to dCTP. In the first step, 4 µg of the large α protein in a final volume of 25 µl were incubated for 45 min with different amounts of the β protein in the presence of 10 µM sodium formate, 30 mM KCl, 500 µM AdoMet, 20 µg/ml flavodoxin, 40 µg/ml flavodoxin reductase, 1.25 mM NADPH, and 10 mM dithiothreitol in 30 mM Tris-HCl buffer, pH 8.0.

In the second step, 15 µl of a substrate mixture (giving a final concentration of 1.4 mM [3H]CTP (20–30 cpm/pmol), 1 mM ATP, 10 mM MgCl2) were added to initiate the reduction of the substrate. The reaction was stopped after 20 min by moving the reaction solution outside of the glove box (exposing it to air) and adding to it 0.5 ml of 1 M HClO4. The solution was then worked up as described earlier (8). One unit of enzyme activity is defined as the formation of 1 nmol of dCTP/min.

UV-visible Absorption Spectroscopy—UV-visible spectra of aerobic samples were recorded with a Cary 1 Bio (Varian) spectrophotometer. Spectra could be also recorded inside the glove box using a Hewlett-Packard 8453 diode array spectrophotometer equipped with optical fibers connected to a cell holder inside the box. EPR Spectroscopy—EPR first derivative spectra were recorded on a Bruker EMX (9.5 GHz) EPR spectrometer equipped with an ESR 900 helium flow cryostat (Oxford Instruments). Double integrals of the EPR signals were evaluated by using a computer on-line with the spectrometer. Spin concentrations in the protein samples were determined by calibrating double integrations of the EPR spectra with a standard sample of a [2Fe-2S]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-DADF 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-DADF (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 wrap filled with isopentane cooled from outside the box by liquid nitrogen.

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

Discussion

In the second step, 15 µl of a substrate mixture (giving a final concentration of 1.4 mM [3H]CTP (20–30 cpm/pmol), 1 mM ATP, 10 mM MgCl2) were added to initiate the reduction of the substrate. The reaction was stopped after 20 min by moving the reaction solution outside of the glove box (exposing it to air) and adding to it 0.5 ml of 1 M HClO4. The solution was then worked up as described earlier (8). One unit of enzyme activity is defined as the formation of 1 nmol of dCTP/min.

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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]$^{2-}$ 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/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 $S = ½$ 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]$^{2+}$ 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 approximately 0.7–0.8 $S = ½$ species per polypeptide. This was the first time that such a small amount of signal could be obtained, because previous work could not show more than 0.3–0.45 $S = ½$ species per polypeptide (5, 16), amount also found here for samples B and C.

Mössbauer Spectroscopy—In Fig. 3A, the Mössbauer spectrum of an EPR-silent sample A is shown, measured at 77 K in an external field of 20 mT perpendicular to the γ-ray. The spectrum consists of two doublets. One doublet (δ = 0.43 mm/s$^{-1}$, $\Delta E_Q = 1.0$ mm/s$^{-1}$, 82%) is typical for tetrahedrally sulfur-coordinated Fe$^{2+}$ as in [4Fe-4S]$^{2+}$ centers. The diamagnetic nature of the major component is confirmed from the Mössbauer spectrum taken at 4.2 K with an applied field of 7 T parallel to the γ-ray (Fig. 3B). Actually, this component could be simulated using the nuclear Hamiltonian only. The other doublet shows parameters (δ = 0.86 mm/s$^{-1}$, $\Delta E_Q = 2.07$ mm/s$^{-1}$, 18%) for partially sulfur-coordinated Fe$^{2+}$ and most probably corresponds to a monomeric species (simulated with parameters for reduced Rubredoxin (26) with slightly changed hyperfine coupling tensor, i.e. $A_{xx} = -13$ T, $A_{yy} = -6$ T, $A_{zz} = -32$ T). The presence of small amounts of ferrous iron is not surprising because sample A is obtained by incubating ferrous sulfide with apoprotein β under anaerobiosis.

From Fig. 4A, it is clear that the signal corresponding to the [4Fe-4S]$^{2+}$ center has greatly decreased in sample B and now 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 (δ = 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 glycyl radical into protein α, during anaerobic incubation of a mixture of protein α and protein β, 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 β to a fixed amount of protein α (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 β, 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 β:α ratio of 0.1 for sample A, a β:α ratio of 0.4 was required for samples B and C. These data now suggest that β is an activating enzyme capable of generating a glycyl radical in several molecules of protein α rather than a component of an $\alpha_2\beta_2$ holoenzyme.

This model was further supported by the data shown in Fig. 5B, in which the specific activity of protein α is reported as a function of the activation time for three different amounts of protein β (sample A), substoichiometric with regard to protein α. In fact, with a β:α 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 β:α 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 glycyl radical reacted instantaneously during exposure to air, resulting in fragmentation of protein α and irreversible inactivation of the enzyme (8, 27). More recently we showed that the fully reduced iron cluster, identified as a [4Fe-4S]$^{+}$ center and responsible for AdoMet reduction and glycyl 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 β 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/s, $\Delta E_Q = 1.04$ mm/s) 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$^{2+}$ was also observed. In some cases, probably because of optimal anaerobic conditions, a small amount of reduced [4Fe-4S]$^{3-}$ 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$^{5/2}$  [4Fe-4S]$^{1-}$ 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 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 CXXXXXC 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.

The improved anaerobic conditions and the higher amount of
iron present in the preparations of protein β of the anaerobic RNR now available also explain another unexpected observation reported here. The holoenzyme was previously characterized as a tight αβ2 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 α is obtained with a catalytic amount of protein β, showing that one molecule of protein β is able to create a glycyl radical in several molecules of protein α. Therefore protein β cannot be considered any longer as a component of an αβ2 holoenzyme, as previously stated, but rather as an activating enzyme ("activase") associated with protein α, 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 α and oxidized protein β 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 β; (ii) the reduction of the iron-sulfur cluster of protein β; (iii) the introduction of the glycyl radical in protein α. 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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doi: 10.1074/jbc.274.44.31291

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