A Simplified Functional Version of the *Escherichia coli* Sulfite Reductase*

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*Escherichia coli* sulfite reductase (SiR) is a large and soluble enzyme with an αβ₄ quaternary structure. Protein α (or sulfite reductase flavoprotein) contains both FAD and FMN, whereas protein β (or sulfite reductase hemoprotein (SiR-HP)) contains an iron-sulfur cluster coupled to a siroheme. The enzyme is set up to arrange the redox cofactors in a FAD-FMN-Fe₄S₄-Heme sequence to make an electron pathway between NADPH and sulfite. Whereas α spontaneously polymerizes, we have been able to produce SiR-FP60, a monomeric but fully active truncated version of it, lacking the N-terminal part (Zeghouf, M., Fontecave, M., Macherel, D., and Covès, J. (1998) *Biochemistry* 37, 6114–6123). Here we report the cloning, overproduction, and characterization of the β subunit. Pure recombinant SiR-HP behaves as a monomer in solution and is identical to the native protein in all its characteristics. Moreover, we demonstrate that the combination of SiR-FP60 and SiR-HP produces a functional 1:1 complex with tight interactions retaining about 20% of the activity of the native SiR. In addition, fully active SiR can be reconstituted by incubation of the octameric sulfite reductase flavoprotein with recombinant SiR-HP. Titration experiments and spectroscopic properties strongly suggest that the holoenzyme should be described as an αβ₄ with equal amounts of α and β subunits and that the αβ₄ structure is probably not correct.

In *Escherichia coli*, sulfite reductase (SiR) catalyzes the transfer of six electrons from NADPH to sulfite to produce sulfide, which then is used during the synthesis of L-cysteine. Whereas α spontaneously polymerizes, we have been able to produce SiR-FP60, a monomeric but fully active truncated version of it, lacking the N-terminal part (Zeghouf, M., Fontecave, M., Macherel, D., and Covès, J. (1998) *Biochemistry* 37, 6114–6123). Here we report the cloning, overproduction, and characterization of the β subunit. Pure recombinant SiR-HP behaves as a monomer in solution and is identical to the native protein in all its characteristics. Moreover, we demonstrate that the combination of SiR-FP60 and SiR-HP produces a functional 1:1 complex with tight interactions retaining about 20% of the activity of the native SiR. In addition, fully active SiR can be reconstituted by incubation of the octameric sulfite reductase flavoprotein with recombinant SiR-HP. Titration experiments and spectroscopic properties strongly suggest that the holoenzyme should be described as an αβ₄ with equal amounts of α and β subunits and that the αβ₄ structure is probably not correct.

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1 The abbreviations used are: SiR, NADPH-sulfite reductase; SiR-FP, sulfite reductase flavoprotein; SiR-HP, sulfite reductase hemoprotein; SiR-FP60, the monomeric form of sulfite reductase flavoprotein; PCR, polymerase chain reaction.

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EXPERIMENTAL PROCEDURES

Materials—NADPH and sodium bisulfate were purchased from Sigma. TEMPO (2,2,6,6-tetramethyl-1-piperidinoyl, free radical) was isolated from Lancaster, and deazaflavin (5-deaza-10-methylisalloxazine) was synthesized by Dr. J.-L. Decout (Laboratoire de Chimie Bio-organique, Faculté de Pharmacie, Grenoble, France) according to a previously described method (16). All other chemicals were of the purest grade. Plasmid pJYW613 carrying the cysJIH operon region, including the cysJIII promoter, plus cysG from Salmonella typhimurium was purified from the NM522(pJYW613) strain kindly provided by Dr. N. M. Kredich (Duke University Medical Center, Durham, NC) and used as a template for the cloning experiments.

Cloning of SIR-HP—Standard recombinant DNA techniques were used to generate the construct as described previously (17). The plasmid pJYW613 carrying SIR, was used as a template for generating PCR products corresponding to the cys gene that encodes for the hemoprotein (β chain). The sense primer, 5’-ACGATGCATATGCAATGGC-3’ was designed to be complementary to the 5’ end of cysJ and to incorporate an EcoRI site (italic letters) followed by a NdeI site (underlined) that provides the start codon for the recombinant protein expression. The antisense primer, 5’-CCGATCTTTATGC-3’ was designed to complement the 3’ end of the cys gene and to incorporate a BamHI site (italic letters) downstream of the stop codon. The amplification was performed by PCR using the following scheme: 60 s at 94 °C, 90 s at 64 °C and 150 s at 68 °C for 12 cycles. This PCR product was first cloned into vector pET-3a (Novagen) between the NdeI and the BamHI sites, leading to the construct pET-apoHP. Another PCR product corresponding to the cys gene and the antisense primer, 5’-CCGATCTTTATGC-3’ that provides the siroheme sequence, was generated by PCR using pJYW613 as a template. The sense primer, 5’-CGGATCCCTGGCCGTCGCTGGCAATAATGTAAGGGGCC, was designed to include the Shine-Dalgarno motif of the cysG gene and the antisense primer, 5’-CGGATCTCTTAATCAGCATCCAGGGCC, was designed to anneal to the low NADPH oxidase activity of SiR-FP).

The results were confirmed by sequencing on both strands (Genome Express, Grenoble, France).

Overexpression and Purification of Proteins—SIR (the holoenzyme), SIR-FP (the flavoprotein), and SIR-FP60 (the monomeric form of the flavoprotein) were expressed and purified as described previously (4, 14). Overexpression of SIR-FP was achieved by using a B834(dEl3)lpSlyS E. coli strain (Novagen) transformed with pET-SIR-HP. The culture was grown for 2 h at 37 °C to an A600 of 0.6 and chilled on ice for 10 min before induction with 0.5 mM isopropyl-1-thio-β-D-galactoside. After 5.5 h of incubation at 20 °C, cells were harvested by centrifugation, and soluble extracts were prepared as described previously for SIR-FP (4). All subsequent steps were performed at 4 °C.

The pellet obtained after ammonium sulfate precipitation (60% final saturation) was dissolved in a minimal volume of 20 mM potassium phosphate (pH 7.4, buffer A) and dialyzed extensively against the same buffer throughout. Photochemical reductions in the presence of deazaflavin—EDTA were achieved in a 50 mM potassium phosphate buffer, pH 7.4, containing 10 mM EDTA, 5% glycerol, and 0.3 deazaflavin:protein ratio. Photoreduction of the protein sample was achieved with a slide projector and monitored spectrophotometrically as described previously (14). For EPR analysis, 200 μl of protein solution were transferred in 4-mm calibrated EPR tubes. All of them were capped and flash-frozen within the glove box before storage in liquid nitrogen until analysis.

RESULTS

Expression and Purification of Recombinant SIR-HP—From the pJYW613 plasmid containing the cysJIII operon, the cysJ gene was cloned as a NdeI-BamHI fragment under the control of the T7 promoter into vector pET-3a. The cysG gene was cloned downstream of the cys gene as a BamHI-BamHI fragment containing its own Shine-Dalgarno motif. cysG, encoding for the siroheme synthase, was introduced to achieve optimal incorporation of siroheme into the polypeptide (28). The resulting...
plasmid, named pET-SiR-HP, was then used to transform the E. coli B834(DE3)pLysS strain.

Maximal expression of soluble SiR-HP was obtained when cells were grown at 25 °C for 2 h after the addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside to the culture medium. Under these conditions, an intense band at 63 kDa is clearly visible after SDS-polyacrylamide gel electrophoresis performed on soluble extracts. However, because a large amount of protein was retained as insoluble material, the yield of recombinant SiR-HP protein was relatively low.

After anion-exchange chromatography on Q-Sepharose and filtration on Superdex-75, SiR-HP was obtained in a nearly pure form (more than 95% purity as deduced from SDS-polyacrylamide gel electrophoresis analysis; data not shown). Typically, 14 mg of that preparation could be obtained from 5 liters of culture. Furthermore, the behavior of the protein on Superdex-75 confirmed that SiR-HP was, in the absence of SiR-FP, a monomer in solution (data not shown).

**Spectroscopic Properties of Recombinant SiR-HP**—Solutions of SiR-HP are brownish, and their light absorption spectrum displays in the visible region the bands at 389, 590, and 712 nm characteristic for a ferriheme. The extinction coefficient at 590 nm was thus estimated at 18.5 mM⁻¹ cm⁻¹ for 0.9 mol siroheme/mol protein. The extinction coefficient at 590 nm was thus estimated at 18.5 mM⁻¹ cm⁻¹ for 0.9 mol siroheme/mol protein.

The spectrum shown in Fig. 1 is in perfect agreement with previously reported spectra of native SiR-HP (13, 29, 30). The siroheme content was quantitated spectrophotometrically after extraction with acetone-HCl, as described previously (19). On the basis of a careful composition analysis, recombinant SiR-HP was found to contain an average of 4.1 mol of iron and 2.7 mol of acid-labile sulfur per mole of protein. After subtraction of the contribution of the siroheme iron, SiR-HP appeared to contain a stoichiometric amount of Fe₄S₄ clusters (0.7 cluster/protein) and probably contained a small amount of adventitiously bound iron.

Fig. 2A shows the EPR spectrum of the SiR-HP. It exhibits a signal characteristic of a high spin $S = 5/2$ ferriheme with features at $g = 6.65, 5.23, and 1.98$, accounting for 0.9 iron/protein. Then, evidence for a cluster came from the EPR spectrum of reduced SiR-HP. After anaerobic reduction with photoreduced deazaflavin in the presence of KCN (to generate a low spin $S = 0$ ferriheme), the features due to ferriheme were no longer present in the EPR spectrum, and a new signal was observed with $g$ values at 2.04, 1.94, and 1.91, characteristic for a $S = 1/2$ (Fe₄S₄)⁺⁺ cluster (Fig. 2B). The temperature dependence and microwave power saturation properties of this signal are consistent with a (Fe₄S₄)⁺⁺ center. Again spin quantitation confirmed the presence of 0.7 cluster/protein. Both spectra in Fig. 2 are similar to those reported previously for native oxidized and reduced SiR-HP (29, 30).

All these results show that recombinant SiR-HP, although slightly depleted in iron sulfur cluster, contains both metal centers (one cluster and one siroheme) and closely resembles the native protein.

**SiR-HP and SiR-FP60 Form a Tight 1:1 Complex**—After incubation of a fixed amount of SiR-HP ($\beta$) with increasing amounts of SiR-FP60 ($\alpha'$), the solution was analyzed by native gel electrophoresis. As shown in Fig. 3, a discrete band with an apparent molecular mass of 127 kDa appeared, along with a band corresponding to SiR-FP60 when this protein was in excess in the incubation mixture (lane 5). This is in agreement with the formation of a complex resulting from the association of 1 mol of SiR-HP (theoretical mass, 65,843 Da) with 1 mol of SiR-FP60 (theoretical mass, 60,706 Da). During filtration on Superdex-75, the complex, eluted in the dead volume of the column, could be separated from free SiR-FP60 (data not shown) and analyzed for its spectroscopic properties.

Fig. 4 shows the light absorption spectrum of the 1:1 SiR-
was observed. From both experiments, one can calculate that a stoichiometric amount of the complementing protein was required for saturation. Furthermore, considering that the rate-limiting step is the electron transfer between SiR-FP and SiR-HP (29) and thus assuming that the activity is a function of the concentration of the complex, one can estimate from Fig. 5B an apparent $K_d$ value of about 10 nM. All these results can thus be explained by a very tight interaction between the two proteins that generates a 1:1 SiR-HP/SiR-FP60 complex.

When the same titration experiments were carried out with SiR-FP ($\alpha_2$) instead of SiR-FP60, saturation with respect to SiR-HP was also observed (data not shown). It appeared that saturation was obtained for a $\alpha_2\beta_2$ ratio close to 1 rather than 2, suggesting that the holoenzyme SiR is more likely described as an $\alpha_2\beta_2$ complex. Under these conditions, an apparent $K_d$ value of about 28 nM has been estimated.

**DISCUSSION**

One of the confusing features of the complex enzyme system sulfite reductase is the stoichiometry of its quaternary structure. Since 1970, on the basis of analytical ultracentrifugation experiments carried out by L. Siegel and collaborators, it has been accepted without further discussion that SiR has an $\alpha_4\beta_4$ structure, with protein $\alpha$ (SiR-FP) carrying flavin prosthetic groups (one FAD and one FMN, as more recently demonstrated), and protein $\beta$ (SiR-HP) carrying the iron-sulfur/siroheme-coupled center, where reduction of sulfite takes place. Considering the well-established fact that the electrons are shuttled, through FAD, from NADPH to FMN in protein $\alpha$, it is thus difficult to understand how the electron transfer between FMN in protein $\alpha$ and the metal center in protein $\beta$ proceeds. Indeed, there is an inconsistency between the proposed linear scheme for the electron transfer pathway (NADPH-FAD-FMN-FeS-siroheme-sulfite) and the above stoichiometry, which suggested that SiR was set up to make two FMNs converge on the same iron-sulfur/siroheme center within an $\alpha_2\beta_2$ functional unit. Looking at 20 years of reported studies on SiR, we observe that this inconsistency has never been raised.

What we now show is that a 1:1 stoichiometric combination of FAD + FMN and of the iron-sulfur/siroheme center is functional, revealing that there is no need for a 2-fold excess of the SiR-FP with regard to SiR-HP. This could be nicely demonstrated by using two original proteins. The first one, named SiR-FP60, is a monomorphic form of SiR-FP (protein $\alpha'$) that can be obtained by deleting the first 51 N-terminal amino acids, as shown previously. SiR-FP60 contains both FAD and FMN and retains both FAD- and FMN-dependent diaphorase activities of SiR-FP. The second one is a recombinant form of SiR-HP, which contains both the Fe$_4$S$_4$ center and the siroheme with spectroscopic properties (light absorption and EPR spectroscopy) similar to those of the corresponding centers in the native SiR. It consists of only one $\beta$ polypeptide chain. This confirms that, in the absence of protein $\alpha$, protein $\beta$ does not polymerize, and the presence of several $\beta$ polypeptides in SiR is a consequence of protein $\alpha$ polymerization. This had already been shown, but at that time, the experiments were carried out with a slightly denatured SiR-HP preparation obtained from a urea-treated SiR holoenzyme solution.

The results reported here demonstrate that SiR-FP60 ($\alpha'$) and SiR-HP ($\beta$) bind tightly to each other to form a 1:1 $\alpha'$-$\beta$ complex, which is able to catalyze the reduction of sulfite by NADPH. Although the activity is only 20% of the activity of the SiR holoenzyme, this strongly suggests that $\alpha\beta$, and not $\alpha_2\beta_2$, is the actual functional unit in SiR, consistent with the proposed linear electron transfer pathway discussed above. It thus follows that the currently proposed $\alpha_2\beta_4$ structure is probably incorrect, and in all probability, SiR is an $\alpha_2\beta_2$ complex, with
equal amounts of the α and β components. This is in agreement with our titration experiments carried out with SiR-FP and recombinant SiR-HP, which best fitted with such a stoichiometry. More direct evidence came from the observation that a 1:1 mixture of SiR-FP60 with SiR-HP gave a light absorption spectrum just superimposable to that of the native enzyme. Because this spectrum contains signatures for the cofactors of both α and β, this result makes totally inconsistent the idea that the native enzyme contains a 2:1 α:β mixture, as is still currently accepted. More experiments are required to further support this new scheme. Nevertheless, it should be noticed that direct determination of the size of SiR might be a difficult project because the native molecular weight of this protein is in the million-Da range.

As demonstrated previously in the case of the cytochrome P450 reductase activity of SiR-FP (31), the N-terminal part of this protein seems to play an important role. As indicated above, deletion of the first 51 amino acid residues results in a different quaternary structure of the holoenzyme and in decreased activity. Additional experiments, particularly structural investigations, are required to understand the molecular basis of these effects.

Finally, because the SiR-FP60/SiR-HP complex behaves as a simplified version of the SiR system with a much smaller size and retaining both cofactors and enzyme activity, it is an excellent candidate for crystallization and structural studies of the complex electron transfer pathway resulting in the 6-electron reduction of sulfite. SiR and the octameric SiR-FP have resisted crystallization thus far, and the only structural information available is the three-dimensional structure of SiR-HP, on one hand, and, on the other hand, that of SiR-FP60. It should be noted that, unfortunately, the SiR-FP60 crystal structure does not show any electron density related to the FMN binding domain. This disorder was interpreted as a consequence of a functional flexibility of that domain which probably serves to catch SiR-HP to locate the FMN cofactor close to the metal center. Thus, binding of SiR-HP to SiR-FP60 might stabilize the FMN domain, and the structure of the SiR-FP60/SiR-HP complex is expected to both allow determination of the structure of the FMN domain and reveal the molecular details of the NADPH to sulfite electron transfer pathway. This is our present goal.

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