Flavin Mononucleotide-Binding Domain of the Flavoprotein Component of the Sulfite Reductase from *Escherichia coli*

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Received September 19, 1996; Revised Manuscript Received February 12, 1997[⊗]

ABSTRACT: The flavoprotein component (SiR-FP) of the sulfite reductase from *Escherichia coli* is an octamer containing one FAD and one FMN as cofactors per polypeptide chain. We have constructed an expression vector containing the DNA fragment encoding for the FMN-binding domain of SiR-FP. The overexpressed protein (SiR-FP23) was purified as a partially flavin-depleted polymer. It could incorporate FMN exclusively upon flavin reconstitution to reach a maximum flavin content of 1.2 per polypeptide chain. Moreover, the protein could stabilize a neutral air-stable semiquinone radical over a wide range of pHs. During photoreduction, the flavin radical accumulated first, followed by the fully reduced state. The redox potentials, determined at room temperature [E_1' ($FMNH^{\bullet}/FMN$) = −130 ± 10 mV and E_2' ($FMNH_2/FMNH^{\bullet}$) = −335 ± 10 mV], were very close to those previously reported for *Salmonella typhimurium* SiR-FP [Ostrowski, J., Barber, M. J., Rueger, D. C., Miller, B. E., Siegel, L. M., & Kredich, N. M. (1989) *J. Biol. Chem. 264*, 15796−15808]. Both the radical and fully reduced forms of SiR-FP23 were able to transfer their electrons to cytochrome *c* quantitatively. Altogether, the results presented herein demonstrate that the N-terminal end of *E. coli* SiR-FP forms the FMN-binding domain. It folds independently, thus retaining the chemical properties of the bound FMN, and provides a good model of the FAD-depleted form of native SiR-FP. Moreover, the FMN prosthetic group in SiR-FP23 and native SiR-FP is compared to that of cytochrome P450 reductase and bacterial cytochrome P450, which also contain one FAD and one FMN per polypeptide chain.

NADPH:sulfite reductase (SiR)[1] participates in the assimilation of sulfate which leads to the biosynthesis of L-cysteine (Kredich, 1971). It catalyzes the six-electron reduction of sulfite to sulfide. In *Escherichia coli* and *Salmonella thyphimurium*, SiR is a multimeric and soluble hemoflavoprotein of 780 kDa with an $\alpha_8\beta_4$ subunit structure (Siegel et al., 1971). Each α chain encoded for by the *cysJ* gene binds two flavin prosthetic groups, namely one FAD and one FMN (Eschenbrenner et al., 1995a,b). The β polypeptide chain is a hemoprotein containing one Fe_4S_4 cluster and one siroheme. The crystal structure of β has been recently solved at 1.6 Å resolution (Crane et al., 1995).

The unique electron transfer pathway from NADPH to sulfite has been elucidated. In the flavoprotein component (SiR-FP) of the enzyme, FAD receives the electrons directly from NADPH, while FMN mediates their rapid transfer from FAD to the hemoprotein component (SiR-HP) where sulfite reduction takes place (Siegel et al., 1971, 1974). However, electronic leakages can occur from the FMN site to artificial acceptors such as cytochrome *c* or ferricyanide, while $AcPyADP^+$ or free exogenous flavins may receive electrons directly from FAD (Siegel et al., 1974; Ostrowski et al., 1989; Eschenbrenner et al., 1995a). Even though the physiological importance of such a flavin reductase activity is still unclear, there is indirect evidence for its participation in prokaryotes in iron mobilization (Covès et al., 1993a), activation of oxygen (Gaudu et al., 1994), or regulation of ribonucleotide reductase (Covès et al., 1993b), the enzyme responsible for the biosynthesis of deoxyribonucleotides. Moreover, it was also shown that SiR can act as a paraquat reductase in *E. coli* (Gaudu & Fontecave, 1994).

The flavoprotein component of the sulfite reductase from *E. coli* or *S. typhimurium* belongs to a family of enzymes which includes microsomal NADPH−cytochrome P450 reductases (CPR), *Bacillus megaterium* cytochrome P450 (P450BM-3), and nitric oxide synthases with high sequence similarities among them (Porter & Kasper, 1986; Porter, 1991; Bredt et al., 1991; Karplus & Burns, 1994). The common feature of these enzymes is the presence on one polypeptide chain of two flavinic prosthetic groups, one FAD and one FMN, which serve to shuttle electrons from NADPH to metal centers. In the case of P450BM-3 and nitric oxide synthases, the metal center (heme) belongs to the same chain, whereas it belongs to a different protein in the case of SiR (the iron−sulfur cluster plus siroheme) and CPR (the heme

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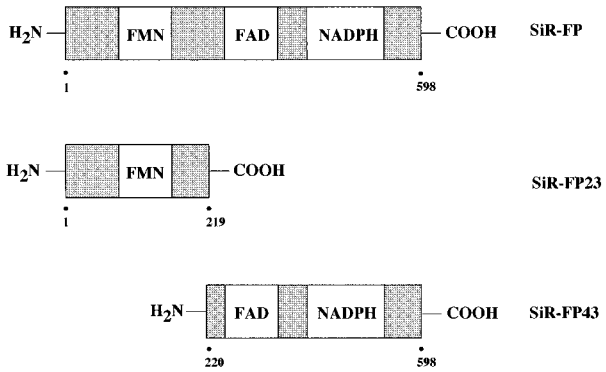
⊗ Abstract published in *Advance ACS Abstracts,* April 15, 1997.

[1] Abbreviations: SiR, NADPH:sulfite reductase; SiR-FP, sulfite reductase flavoprotein; SiR-HP, sulfite reductase hemoprotein; SiR-FP23, FMN-binding domain of sulfite reductase flavoprotein; SiR-FP43, FAD-binding domain of sulfite reductase flavoprotein; $AcPyADP^+$, 3-acetylpyridine adenine dinucleotide phosphate; CPR, NADPH−cytochrome P450 reductase; P450BM-3, *Bacillus megaterium* cytochrome P450; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy free radical.

S0006-2960(96)02374-4 CCC: $14.00

Scheme 1: Schematic Representation of the Flavin-Binding Domains of SiR-FP[a]



[a] Only the monomeric form of SiR-FP and SiR-FP23 is shown. SiR-FP43 is really a monomer.

of cytochrome P450). Another striking property of these enzymes is the fact that they all share significant sequence homology with two distinct classes of flavoproteins. Their C-terminal region shows homology with the FAD-containing ferredoxin−$NADP^+$ reductases (FNRs) and their N-terminal part with the FMN-containing bacterial flavodoxins (Ostrowski et al., 1989; Karplus & Burns, 1994). This strongly suggested that those enzymes evolved as a fusion of two ancestral flavin-containing electron transport proteins and that they are organized in functional domains, one FAD and one FMN, folding independently.

As a matter of fact, we have shown previously that SiR-FP can be dissected by limited proteolysis in its two flavin domains (Eschenbrenner et al., 1995b). The FMN-binding domain (flavodoxin-like module, called SiR-FP23 in this paper in reference to its molecular mass when denaturated) starts at the N-terminal end of the native SiR-FP and has been shown to be responsible for the polymerization of the α chains (Eschenbrenner et al., 1995b). The FAD-binding domain (FNR-like module, called SiR-FP43) is monomeric and corresponds to the C-terminal end of native SiR-FP, starting either with valine 218 or threonine 220 (Eschenbrenner et al., 1995b). The NADPH-binding domain belongs to this fragment. Thus, SiR-FP43 remains able to catalyze FAD-dependent reductions in the presence of NADPH, *i.e.* reduction of free flavins or $AcPyADP^+$ (Eschenbrenner et al., 1995b). Both domains retain their specific prosthetic group even in the absence of the other domain. A schematic representation of the flavin domains of SiR-FP is given in Scheme 1.

FMN is loosely bound to SiR-FP and can be removed selectively. Thus, large amounts of FMN-depleted SiR-FP could be prepared and used to delineate specific properties of FAD. In contrast, FAD is tightly bound to the protein, and all attempts to remove it have been unsuccessful (Faeder et al., 1974; Ostrowski et al., 1989). In order to study the physicochemical properties of FMN in SiR-FP specifically, we have constructed a vector that could be used to express a peptide corresponding to SiR-FP23 in *E. coli*. For the first time, large amounts of the FMN-binding domain of SiR-FP could be purified and characterized. It can accept FMN as a cofactor exclusively and is able to stabilize an air-stable neutral semiquinone radical. Both the radical and the fully reduced forms of SiR-FP23 can transfer electrons to cytochrome *c* very efficiently. Redox properties of SiR-FP23 are comparable to those of the FMN moiety of native SiR-FP.

MATERIALS AND METHODS

Materials. Riboflavin, FAD, and cytochrome *c* were purchased from Sigma. TEMPO was from Lancaster, and dye mediators were from Fluka. FMN, Pefabloc, and Taq polymerase were supplied by Boehringer Mannheim. Sephacryl S-400 HR was from Pharmacia LKB Biotechnology Inc., and hydroxylapatite Bio-Gel HTP was from Bio-Rad. All other chemicals were of the purest grade.

pCysJ and deazaflavin were available in the laboratory.

Construction of the Expression Plasmid. The plasmid pCysJ (Eschenbrenner et al., 1995a) encoding for SiR-FP was used as a template for PCR amplification of a synthetic DNA fragment encoding for the FMN-binding domain previously identified (Eschenbrenner et al., 1995b), with suitable restriction sites for subcloning into the expression vector pET-3a (Novagene). The sense primer 5′-GGG-GAATTCATATG**ACGACACCGGCTCCACTG** includes an *Eco*RI site followed by a *Nde*I site (underlined). The nucleotides shown in bold correspond to the DNA region coding for the N-terminal amino acid sequence of the native protein (TTPAPL). The recombinant protein is expected to start with a threonine since the *Nde*I site encodes the initiator methionine. The antisense primer 5′-GGCGGATCCT-CA**AGCGACGGATTGCGAAGGTGC** was designed to incorporate a *Bam*HI site (underlined) and a stop codon in the coding sequence of SiR-FP to construct a protein truncated between alanine 219 and threonine 220. Nucleotides shown in bold are complementary to the corresponding region in pCysJ. The amplification of the target sequence was carried out by PCR upon 1 μg of linearized plasmid pCysJ with 2.5 units of Taq polymerase and 100 pmol of each primer in the recommended buffer. The amplification was performed according to the following scheme with a Robocycler gradient 40 instrument (Stratagene): 60 s at 94 °C, 90 s at 53 °C, and 120 s at 72 °C for 12 cycles. After ethanol precipitation and agarose gel electrophoresis, the single PCR product was excised from the gel and recovered using Jetsorb (Genomed Inc., Research Triangle Park, NC). After digestion with *Eco*RI and *Bam*HI, it was purified again by agarose gel electrophoresis and ligated into plasmid pUC18 cut with *Eco*RI and *Bam*HI. After transformation in *E. coli* DH5α, a positive clone was selected and the recombinant plasmid pUC-SiR-FP23 was extracted using the Flexiprep kit (Pharmacia). The DNA insert was sequenced on both strands with Taq polymerase using the Applied Biosystem protocol carried out by GENOME EXPRESS (Grenoble, France). The plasmid pUC-SiR-FP23 was shown to encode the expected construct with a single amino acid mutation at position 156 resulting in a serine (AGC) instead of a threonine (ACG). Sequencing of the plasmid pCysJ which has served as a template gave the definitive demonstration of the occurrence of a serine at position 156, suggesting an error in the initial sequencing (Ostrowski et al., 1989) and proving that this study was not carried out on a mutant form of the FMN-binding domain. Finally, the *Nde*I-*Bam*HI fragment obtained by digestion of pUC-SiR-FP23 was cloned into plasmid pET-3a cut with the same enzymes to yield the expression plasmid pET-SiR-FP23.

Expression of the Recombinant SiR-FP23 Protein. The expression plasmid pET-SiR-FP23 was introduced into the T7 RNA polymerase-containing host *E. coli* B834(DE3)-pLysS, and a preculture was grown until an absorbance of

0.8 was reached at 600 nm in 20 mL of LB medium supplemented with carbenicilline (200 μg mL^{-1}) and chloramphenicol (34 μg mL^{-1}). Cells were then pelleted by centrifugation, resuspended in the same volume of fresh culture medium, and stored overnight at 4 °C. Then, 2 L of the same medium was inoculated at 5% with the overnight stock. Protein expression was induced at an absorbance of 0.5 at 600 nm by addition of 0.4 mM isopropyl thio-β-galactopyranoside (IPTG). To monitor the induction of SiR-FP23, 1 mL of the culture was sampled at various times. Cells were pelleted by centrifugation (14000*g*, 1 min) and suspended in 100 μL of SDS/PAGE sample buffer. After being heated at 100 °C for 3 min, 10 μL of the sample (corresponding to 100 μL of culture) was subjected to SDS/PAGE, and proteins were revealed by Coomassie blue staining. For large scale protein purification, cells were harvested 2.5 h after induction by centrifugation and frozen at −80 °C.

Purification of SiR-FP23. The frozen cells (12 g) were thawed in 24 mL of 100 mM Tris/HCl (pH 9) containing 80 mM KCl and 2 mM Pefabloc and lysed by freeze−thawing rupture. The total protein extract was recovered by centrifugation during 90 min at 45 000 rpm in a 60 TI rotor (Beckman) and used for further purification.

The total protein extract was treated as previously described for the purification of SiR-FP (Eschenbrenner et al., 1995a). The pellet obtained after ammonium sulfate precipitation (60% final saturation) was dissolved in 50 mM Tris/HCl (pH 7.5) containing 50 mM NaCl, 5% glycerol, and 2 mM Pefabloc (buffer A) and loaded on a Sephacryl S-400 HR column (2.6 × 93 cm, 494 mL) previously equilibrated with buffer A without Pefabloc. Elution was performed at 1 mL min^{-1}, and 5 mL fractions were collected. Fractions were assayed for protein (absorbance at 280 nm) and for flavin content (absorbance at 450 nm). The flavin-containing fractions (60 mL, 108 mg of protein) were pooled. Seventy-two milligrams of protein was fractionated on a hydroxylapatite column (2.6 × 6.6 cm, 35 mL) previously equilibrated with 20 mM potassium phosphate buffer at pH 7 (buffer B). Proteins were loaded at 0.5 mL min^{-1}. The column was washed at 1 mL.min^{-1} with buffer B until the base line was recovered. Then, elution was made at 1.5 mL min^{-1} with a linear gradient from 20 to 500 mM KPO_4 during 150 min followed by a plateau for 70 min at 500 mM KPO_4. FMN-containing proteins eluted during this plateau. They were pooled and desalted by cycles of concentration−dilution in 50 mM Tris/HCl (pH 7.5) and 5% glycerol using a Diaflo cell equiped with a YM-30 membrane (Amicon Co.). The protein solution obtained (7.4 mL, 66 mg of protein) was aliquoted and stored at −80 °C for further use. At this stage, SiR-FP23 was electrophoretically pure.

Native SiR-FP and SiR-FP43 were obtained as previously described (Eschenbrenner et al., 1995a,b).

Cofactor Analysis. A sample of pure SiR-FP23 was boiled for 10 min in the dark, chilled on ice, and microcentrifuged for 10 min to pellet the denaturated protein. An aliquot of the supernatant was analyzed by thin-layer chromatography on silica gel 60 F_{254} (Merck) with butan-1-ol/acetic acid/water (12:3:5 by volume) development system. Pure FMN and FAD were run separately or as a mixture under the same conditions as a control.

Reconstitution of SiR-FP23 and Binding Measurements. For reconstitution experiments, SiR-FP23 was incubated with a 2-fold excess of either FMN, FAD, or riboflavin during 60 min at ice temperature in the dark. Excess flavin was removed either by filtration on Sephadex G-25, extensive dialysis, ammonium sulfate precipitation, or a combination of the three. Optical spectra of SiR-FP23 were recorded before and after reconstitution. In each case, concentration of protein-bound FMN was calculated after heat denaturation of the protein from the absorbance of free FMN using an extinction coefficient of 12.2 mM^{-1} cm^{-1} at 450 nm.

Determination of the dissociation constant for the SiR-FP23−FMN complex was performed by fluorimetric analysis as described for the native sulfite reductase (Faeder et al., 1974). Briefly, to solutions of 200 nM FMN were added increasing amounts of SiR-FP23, and we monitored the resulting flavin fluorescence quenching. All solutions were in 50 mM Tris/HCl (pH 7.5) and 5% glycerol and the final volume was 2.5 mL. The protein−FMN mixtures were incubated at 25 °C in the dark for 1 h, and then the fluorescence of the solution, due to the free flavin, was measured using a Perkin-Elmer LS 450 fluorimeter. The excitation wavelength was 450 nm, and the emission spectrum was recorded between 480 and 700 nm. A single emission maximum was observed at 530 nm.

Spectroscopic Characterization of SiR-FP23. Absorption spectra of SiR-FP23 were recorded at room temperature in a quartz cell (10 mm light path) of 1 mL. Anaerobic experiments were run in the same cell capped with a rubber septum.

Photochemical reduction of SiR-FP23 was carried out as follows. A solution of 2.8 μM deazaflavin in 50 mM Tris/HCl (pH 7.5), 10 mM EDTA, and 20% glycerol, in a capped cuvette, was made anaerobic by extensive argon bubbling in the dark. The experiment was also run with 50 mM glycine/NaOH (pH 9.2) as the buffer. In that case, protein solutions were exchanged with the same buffer by filtration on Sephadex G-25 equilibrated at pH 9.2. Deazaflavin was then photoreduced by illumination for 2 min with a commercial slide projector placed at a distance of 30 cm. Complete photoreduction was checked by recording the spectrum of the solution using a Kontron Uvikon 930 spectrophotometer. Then, a previously deaerated sample of SiR-FP23 in the working buffer was introduced anaerobically in the cuvette. The final volume was 1 mL, and the absorbance at 455 nm was approximately 0.4. A first spectrum was recorded. The protein solution was then subjected to 2 min periods of illumination, and a spectrum was recorded after each period until complete reduction of the protein. Other spectra were recorded immediately after air admission and later for monitoring the reoxidation of the sample.

In a separate experiment, a 200 μL aliquot of the reaction mixture was sampled after air admission when the absorbance at 589 nm reached a maximum, placed into an EPR tube, and frozen in liquid nitrogen for further EPR analysis. EPR first-derivative spectra were recorded at 100 K using a Bruker ESP 300E spectrometer operating at 9.41 GHz. The microwave power was set at 20 μW, and the modulation amplitude was 0.495 mT. Quantification of the semiquinone radical was done by integration of the EPR signal and comparison with the integrated signal of a standard solution of TEMPO of known concentration recorded under the same conditions.

Potentiometric Titrations. The redox titrations were carried out anaerobically at 22 °C in 50 mM Tris/HCl at pH

7.5 under an argon atmosphere. Reduction was conducted by stepwise additions of small quantities of 10−20 mM sodium dithionite in the same buffer. The redox potentials were measured with a combined Pt−Ag/AgCl/KCl (3 M) microelectrode and given in the text with respect to a standard hydrogen electrode.

Optical measurements were performed with a Kontron 932 spectrophotometer equipped with a specially designed anaerobic sample cuvette containing 1.5 mL of protein solution corresponding to 62 μM FMN, in the presence of the following mediators at 3 μM each: resorufine (−51 mV), 2-hydroxy-1,4-naphthoquinone (−145 mV), phenosafranine (−255 mV), and neutral red (−375 mV). After each addition of sodium dithionite, the solution was left for 5 min so the thermodynamic equilibrium could be reached and the absorbance was recorded at 640 nm.

The redox titrations monitored by EPR spectroscopy were carried out similarly in an anaerobic Dutton-type glass cell containing 4 mL of a SiR-FR23 solution corresponding to 46.5 μM FMN. The mixture of the above-mentioned mediators was complemented with methylene blue (+11 mV), benzyl viologen (−350 mV), and methyl viologen (−440 mV). In that case, the final concentration of each mediator was 5 μM. For EPR titration at room temperature, a 30 μL sample of the protein solution was removed at each desired potential and transferred anaerobically into an argon-flushed EPR capillary tube (1 mm inner diameter). The tube was then stoppered, and the EPR spectrum was immediately recorded. For each tube, the signal stability was checked for at least 30 min. The titration monitored by EPR at low temperature (105 K) was carried out in the same way, except that 160 μL aliquots were transferred in 3 mm inner diameter tubes and frozen in liquid nitrogen for subsequent examination.

Protein Determination. The protein concentration was determined using bovine serum albumin as a standard (Bradford, 1976) and the commercial Bio-Rad protein assay solution. The denaturated molecular mass of SiR-FP23 was estimated by 0.1% SDS/15% polyacrylamide gel electrophoresis (Laemmli, 1970).

Cytochrome c Reduction. NADPH−cytochrome *c* reductase activity was carried out in a final volume of 0.5 mL containing 50 mM Tris/HCl (pH 7.5), 0.25 mM NADPH, 0.1 mM cytochrome *c*, and an appropriate amount of protein. Rates were measured spectrophotometrically at room temperature. The absorbance change was followed at 550 nm using an extinction coefficient of 22 mM^{-1} cm^{-1}.

In order to assay the reactivity of the reduced forms of the FMN cofactor, a solution of SiR-FP23 in 50 mM Tris/HCl (pH 7.5) and 20% glycerol corresponding to 26.6 μM FMN was fully photoreduced by illumination in the presence of deazaflavin as described above. Then a solution of cytochrome *c* previously deaerated by argon flushing was introduced anaerobically (final concentration of 50 μM), and the change in absorbance at 550 nm corresponding to the appearance of reduced cytochrome *c* was recorded. The half-reduced form of SiR-FP23 was generated by oxidation of the fully reduced form upon admission of air. Then its ability to reduce cytochrome *c* was assayed as described above. In all experiments, the initial absorbance at 550 nm of a mixture of oxidized SiR-FP23 and cytochrome *c* was subtracted from the experimental values.

RESULTS

Overexpression of SiR-FP23. E. coli B834(DE3)pLysS was transformed with the expression plasmid pET-SiR-FP23 encoding SiR-FP23, the presumed N-terminal FMN-binding domain of the flavoprotein component of sulfite reductase. SiR-FP23 is a peptide of 23 kDa, with alanine 219 chosen as the last residue. Maximal expression was observed 2.5 h after addition of IPTG to the growth medium (not shown), and SiR-FP23 was recovered in soluble extracts (about 15−20% of the total soluble proteins). These extracts were indigo-blue and slowly turned green to reach a yellow color after about 24 h at 0 °C. These color changes indicated that the overexpressed polypeptide was a flavoprotein, and the blue species is typical of the neutral semiquinone forms of flavins (Massey & Palmer, 1966) which, in the presence of oxygen, is converted to the yellow oxidized form.

Purification and Characterization of SiR-FP23. Soluble extracts were concentrated by ammonium sulfate precipitation and fractionated by filtration on a Sephacryl S-400 HR column. A flavin-containing fraction was eluted after the dead volume of the column, indicating that SiR-FP23 was, under nondenaturing conditions, a polymer of 8−10 subunits. The flavoprotein was then further purified to apparent homogeneity by chromatography on hydroxylapatite. Typically, 66 mg of pure SiR-FP23 was obtained from 2 L of culture. Analysis of the flavin cofactor in pure SiR-FP23 was done by thin-layer chromatography of the supernatant of a heat-denatured protein sample. This unambiguously showed that SiR-FP23 contained FMN exclusively (not shown). The same supernatant was also analyzed spectrophotometrically. Using an extinction coefficient of 12.2 mM^{-1} cm^{-1} for free FMN, we calculated a ratio of about 0.47 mol of FMN per mole of monomer of SiR-FP23. This could suggest that the affinity of SiR-FP23 for FMN was weaker than that of the intact protein. This point was ruled out by measuring the dissociation constant for the SiR-FP23−FMN complex. Fluorimetric analysis led us to estimate the dissociation constant in the nanomolar range, in perfect agreement with the value of 9 nM determined for the native sulfite reductase−FMN complex (Faeder et al., 1974). We can conclude that SiR-FP23 had retained binding capacities similar to that of the native enzyme. The purification of a partially FMN-depleted protein could indicate that the flavin cofactor either had been lost during purification, in agreement with the known lability of FMN in SiR-FP (Siegel et al., 1974), or was a result of the large overexpression of SiR-FP23. However, SiR-FP23 could be reconstituted by incubation with an excess of flavin. Reconstitution with FMN led to a maximum ratio of 1.2 mol of FMN per mole of protein monomer, while reconstitution with riboflavin or FAD was ineffective since the flavin content was unchanged after treatment of the protein. This shows that the first 219 residues of SiR-FP form the specific FMN-binding domain and that the recombinant SiR-FP23 protein folds correctly to bind FMN exclusively, as native SiR-FP does.

A pure preparation of SiR-FP23 gave a light absorption spectrum (Figure 1, spectrum 1) with absorption maxima at 455 and 370 nm, characteristic of an oxidized flavoprotein. An extinction coefficient of 10.5 mM^{-1} cm^{-1} at 455 nm was obtained, in very good agreement with the estimated extinction coefficient of other related flavoproteins (Massey & Palmer, 1966; Iyanagi et al., 1981; Ostrowski et al., 1989;

Table 1: Extinction Coefficients of the Different Redox Forms of SiR-FP23

	extinction coefficient (mM^{-1} cm^{-1})		
	455 nm	589 nm	640 nm
oxidized	10.5	1.01	
semiquinone	4.22	4.88	3.95
hydroquinone	1.5	0.33	

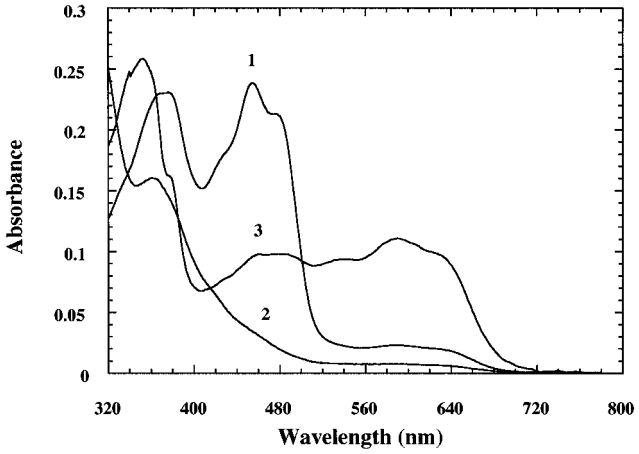


FIGURE 1: Absorption spectra of the three redox states of SiR-FP23: spectrum 1, oxidized SiR-FP23 (22.8 μM in FMN) obtained directly after purification; spectrum 2, fully reduced state obtained by anaerobic photoreduction in the presence of deazaflavin−EDTA as described in Materials and Methods; and spectrum 3, semiquinone state obtained from a short incubation of fully reduced SiR-FP23 with air.

Black, 1994). The presence of small amounts of FMN semiquinone species is shown from the residual characteristic absorption between 560 and 640 nm. An additional feature of the optical spectrum of SiR-FP23 was a shoulder at 490 nm.

Generation of the Blue Neutral FMN Radical and Determination of the Extinction Coefficients of the Three Redox Forms of SiR-FP23. Reduction of SiR-FP23 at pH 7.5 and 9.2 was achieved by illumination under anaerobic conditions in the presence of deazaflavin−EDTA (Massey & Hemmerich, 1978) and monitored spectrophotometrically (Figures 2 and 3). At pH 7.5 (Figure 2A,B), reduction of SiR-FP23 proceeded in two distinct steps. The first one was characterized by the progressive appearance of the absorption band characteristic of the neutral flavin semiquinone species with a maximum at 589 nm and a shoulder at 640 nm (Figure 2A). This band appeared with the concomitant decrease of the absorbance at 455 nm. These spectral modifications gave two tight isosbestic points at 504 and 362 nm. The 589 nm band reached a maximum within 12 min. Upon further photoreduction, both bands at 455 and 589 nm decreased until the protein was reduced to the hydroquinone state (Figure 2B). This second reduction step was characterized by three isosbestic points at 424, 384, and 328 nm. At pH 9.2, in contrast, reduction of SiR-FP23 was much faster and the intermediate flavin semiquinone started to be reduced before it was quantitatively formed (Figure 3). This experiment also showed that the semiquinone species is predominantly in the neutral form even at pH 9.2, suggesting that the pK_a associated with the formation of the red anionic flavin semiquinone is significantly increased with respect to the pK_a of 8.3 observed for the free flavin (Müller, 1987).

During exposure to air, at pH 7.5, the fully reduced form was converted to the radical form almost instantaneously.

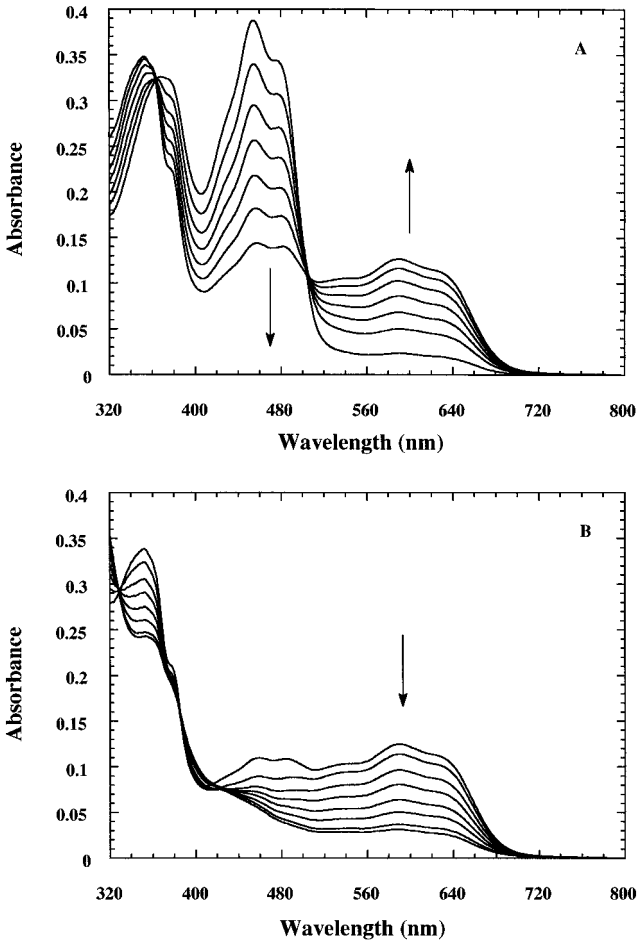


FIGURE 2: Photoreduction of SiR-FP23 at pH 7.5. A solution containing SiR-FP23 (38 μM in FMN) in Tris/HCl buffer at pH 7.5 was photoreduced in the presence of deazaflavin−EDTA as indicated in Materials and Methods. A spectrum was recorded after each 2 min period of illumination. The arrows point in the direction of spectral changes: (A) spectra at 0, 2, 4, 6, 8, 10, and 12 min of illumination and (B) spectra at 14, 16, 18, 20, 22, 24, 26, and 28 min of illumination.

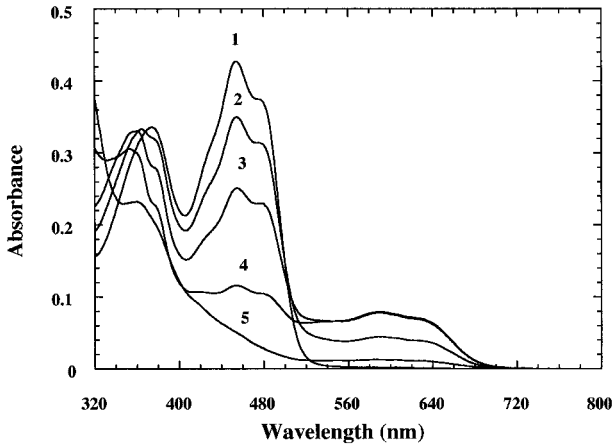


FIGURE 3: Photoreduction of SiR-FP23 at pH 9.2. A solution containing SiR-FP23 (40 μM in FMN) in glycine/NaOH buffer at pH 9.2 was photoreduced in the presence of deazaflavin−EDTA. Spectra 1−5 were obtained respectively after 0, 2, 4, 6, and 10 min of illumination.

The absorbance at 589 nm was substantially higher (0.185) than the maximal value observed during photoreduction of oxidized SiR-FP23 (0.13). Accurate quantification of the radical was achieved by comparing the intensity of its characteristic X-band EPR spectrum, recorded at 100 K, to that of a standard solution of TEMPO, a stable nitroxide.

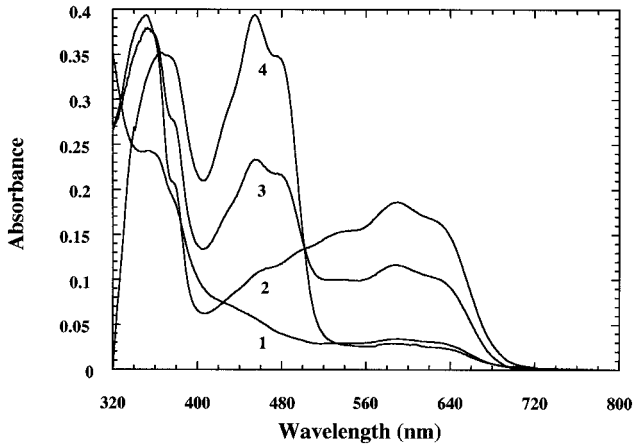


FIGURE 4: Oxidation of the hydroquinone state of SiR-FP23. Spectrum 1 corresponds to the last spectrum of Figure 2B. The solution was then shaken with air, and spectrum 2 was recorded immediately. Spectra 3 and 4 were recorded respectively after 5 and 15 h of incubation at room temperature in the presence of oxygen.

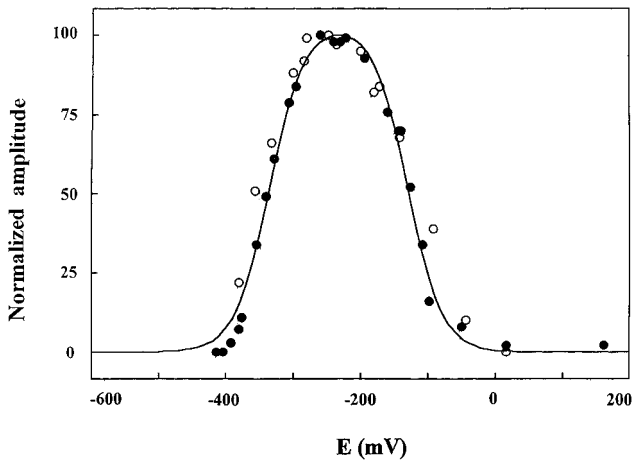


FIGURE 5: Potentiometric titrations of SiR-FP23 FMN cofactor at room temperature. Experiments were conducted as described in Materials and Methods. (●) Normalized variations of the semiquinone absorbance at 640 nm. (○) Normalized amplitude of the semiquinone EPR signal recorded with the following conditions: temperature, 22 °C; microwave power, 1 mw; and modulation amplitude, 1 mT. The solid line is the titration curve calculated with the redox potentials given in Table 2.

Double integration of the signals gave a radical concentration of about 36.5 μM. Since the initial oxidized flavin concentration was 37.6 μM, as deduced from the absorbance at 455 nm, we can conclude that about 97% of $FMNH_2$ has been converted to FMNH$^\bullet$ upon reoxidation while about 70% of FMN was converted to semiquinone upon one-electron reduction. From these results, we can calculate an extinction coefficient at 589 nm of 4.88 mM^{-1} cm^{-1}. The light absorption spectra of the redox forms of SiR-FP23 are depicted in Figure 1, and the associated Table 1 gives the extinction coefficients calculated from these spectra at different wavelengths.

The fact that less radical was obtained during reduction of FMN than during oxidation of $FMNH_2$ has also been observed with the FAD-depleted form of CPR (Kurzban et al., 1990). It may reflect that, during photoreduction of FMN, the thermodynamic equilibrium between the different redox species is slow to be reached. The neutral semiquinone species was found to be greatly stable in the presence of oxygen at pH 7.5 since complete reoxidation took several hours under aerobic conditions (Figure 4). The reaction was much faster at pH 9.2 since complete reoxidation of $FMNH_2$ took 30 min with the transient formation of FMNH$^\bullet$ (not shown).

Our data demonstrate that SiR-FP23 can yield the neutral radical species over a wide range of pHs and that the semiquinone radical is particularly air-stable at pH 7.5.

Redox Properties of the FMN Cofactor of SiR-FP23. Potentiometric titration of SiR-FP23 was first monitored by optical spectroscopy. Dye mediators were carefully chosen to achieve redox equilibrium between the protein and the electrode in the studied potential range and to give a minor spectral contribution in the 550−660 nm region corresponding to the absorption band of the flavin semiquinone. The absorbance variations were then recorded at 640 nm, a wavelength where the contribution of the mediator mixture is negligible while the absorbance of the semiquinone species is still important. Its extinction coefficient at this wavelength was estimated to be 3.95 mM^{-1} cm^{-1} from spectrum 2 of Figure 1 (Table 1). Upon reduction with dithionite, the A_{640} values showed a bell-shaped variation, consistent with formation and then reduction of the semiquinone species (Figure 5). The reversibility of the redox process was checked by reoxidizing the fully reduced protein with small addition of air. The variations of the semiquinone concentration were well fitted with a titration curve calculated by considering two successive $n = 1$ redox processes with an E'_1 (FMNH$^\bullet$/FMN) of -130 ± 10 mV and an E'_2 ($FMNH_2$/FMNH$^\bullet$) of -335 ± 10 mV (Table 2, Figure 5). The maximal amount of semiquinone was obtained at about −235 mV, with an A_{640} value corresponding to 92% of the FMN content of SiR-FP23. Within the experimental error, this value is in agreement with the theoretical maximum of 96% deduced from the redox potentials using the following equations where K_{sq} is the stability constant of the semiquinone species:

Table 2: Redox Potentials of the FMN Cofactor of Different Related Proteins

	redox potential (mV)			
	SiR-FP23[a]		*S. typhimurium* SiR-FP[b]	microsomaol cytochrome P450 reductase[c]
	RT[d]	105 K		
FMNH$^\bullet$/FMN	−130	−50	−152	−110
$FMNH_2$/FMNH$^\bullet$	−335	−350	−327	−270

[a] This work. Values are given at ±10 mV. Potentials were determined at pH 7.5. [b] From Ostrowski et al. (1989). Potentials were determined at pH 7.7. [c] From Iyanagi et al. (1974). Potentials were determined at pH 7.0. [d] RT means room temperature.

$$K_{sq} = \exp[(E'_1 - E'_2)F/RT]$$

with

$$K_{sq} = [FMNH^\bullet]^2/[FMN][FMNH_2]$$

In order to compare these redox properties with those previously determined for the FMN moiety of *S. typhimurium* SiR-FP (Ostrowski et al., 1989), the titration of SiR-FP23 was also followed by EPR spectroscopy at low temperature (105 K). Under these conditions, the low-potential part of the titration curve was similar to that obtained by optical spectroscopy. In contrast, the high-potential part corresponding to the titration of the FMNH$^\bullet$/FMN couple showed a +80 mV shift (Table 2). The same results were obtained

in both the reductive and oxidative experiments, and the spin quantitation of the maximal amount of the FMNH• radical gave about 85% of the FMN content.

The differences observed between the optical and the low-temperature EPR analysis prompted us to monitor the titration by EPR at room temperature (22 °C). Great cares were taken to avoid reoxidation of the protein in the EPR tube during the spectral recording. The relative amplitude of the FMNH• radical signal was plotted as a function of the redox potential (Figure 5) and showed variations remarkably similar to those obtained during the optical titration. Thus, freezing of the protein solution appears to have significantly affected the redox analysis.

Cytochrome c Reductase Activity of Reduced SiR-FP23. SiR-FP23 is a recombinant form of the flavoprotein component of sulfite reductase, lacking the NADPH binding site and the FAD fragment serving as an entry for the electrons from NADPH. Obviously, NADPH was unable to reduce the FMN cofactor of the protein (not shown). However, SiR-FP23 can be artificially reduced to the hydroquinone state by photoreduced deazaflavin−EDTA, and the semiquinone state is easily generated from the hydroquinone by admission of air. We have assayed the ability of the two reduced forms of SiR-FP23 to transfer electrons to cytochrome *c*. $FMNH_2$ (26.5 μM) was able to reduce 91% of a 50 μM solution of cytochrome *c*, while 26.1 μM FMNH• could reduce 56% of the same solution of cytochrome *c*. The reaction was instantaneous. We can conclude that all the electrons stored in the reduced forms of SiR-FP23 are easily transferred to cytochrome *c*. This suggests that, as in SiR-FP, the flavin cofactor in SiR-FP23 has retained its chemical reducing properties.

However, SiR-FP23 alone or in combination with SiR-FP43, the fragment containing FAD and the NADPH-binding site, was unable to catalyze the reduction of cytochrome *c* by NADPH. SiR-FP and, to a much lesser extent, the FAD-containing fragment were previously demonstrated to be able to catalyze such a reduction (Eschenbrenner et al., 1995a,b). Upon truncation of native SiR-FP, the transfer of electrons from FAD to FMN is hindered.

DISCUSSION

We have utilized recombinant DNA techniques to construct the expression vector pET-SiR-FP23 encoding a peptide of 23 kDa corresponding to the first 219 amino acids of the flavoprotein component of sulfite reductase. As a matter of fact, preliminary studies (Ostrowski et al., 1989; Eschenbrenner et al., 1995b) and sequence homologies with CPR strongly suggested that the FMN domain resided in the N-terminal end of SiR-FP. The overexpressed protein, named SiR-FP23, that was purified in two steps (gel filtration and hydroxylapatite) was a large polymer of the 23 kDa polypeptide. The number of monomers within the protein has not been determined but is likely to be around 8−10 as shown by gel filtration. This tendency to aggregate is consistent with our previous suggestion that the N-terminal end of the flavoprotein component is responsible for its polymerization which gives an α_8 protein within the sulfite reductase holoenzyme (Eschenbrenner et al., 1995b). Whether the polymerization of the flavoprotein subunit and the large size of sulfite reductase are of functional importance remains to be determined.

Several structural and functionnal features of SiR-FP23 indicate that the N-terminal fragment of SiR-FP is the actual FMN-binding domain, which folds independently to retain FMN as a cofactor, exclusively, and to display chemical properties similar to those of FMN in the native SiR-FP protein.

First, the purified protein only contained FMN as a flavin. Second, as it was partly flavin-depleted, we could show that full reconstitution of SiR-FP23 could only be achieved with FMN and not with FAD and riboflavin. Third, photoreduction in the presence of deazaflavin−EDTA proceeded as a stepwise reaction at pH 7.5 with accumulation of the one-electron-reduced radical form (FMNH•), in the first step, followed by conversion of FMNH• to the fully reduced hydroquinone form ($FMNH_2$) in the second step. This reflects the fact that the midpoint potential of the FMNH•/FMN couple ($E_1' = -130 \pm 10$ mV), determined by potentiometric titration of SiR-FP23 at room temperature, is much more positive than that of the $FMNH_2$/FMNH• couple ($E_2' = -335 \pm 10$ mV). Also in the case of native SiR-FP from *S. typhimurium* (Ostrowski et al., 1989), reduction proceeded in two distinct stages and the corresponding redox potentials as well as those of the FMN cofactor in CPR were in the same range as those determined in this study (Table 2). The fact that almost no radical could be observed during reduction of a FMN-depleted SiR-FP form showed that the semiquinone species formed during reduction of SiR-FP was FMNH• almost exclusively (Ostrowski et al., 1989; Eschenbrenner et al., 1995a), and not FADH•. SiR-FP23 has thus retained the ability of the FMN-binding domain to stabilize a radical species. This radical absorbs at 589 nm with an extinction coefficient of 4880 M^{-1} cm^{-1}, similar to those recently obtained in the case of flavoproteins also stabilizing a semiquinone form (Gassner & Ballou, 1995; Poole, 1996; Macheroux et al., 1996). It has be noted that CPR also stabilizes FMNH• specifically (Iyanagi et al., 1974) while the bacterial cytochrome P450, P450BM-3, rather stabilizes FADH• (Sevrioukova et al., 1996), even though in all cases FAD is the entry for the electrons. The protein thus can modulate the redox potentials of the flavins as shown from the various stabilities of semiquinones in different enzymes of the same class. It is interesting to point out that the redox titration of SiR-FP23 monitored by EPR spectroscopy gave a much higher redox potential for the FMNH•/FMN couple at cryogenic than at room temperature. The potential of the $FMNH_2$/FMNH• couple was not greatly affected (Table 2). Such a temperature dependence is well-documented. It could be due to a shift in pH occurring during freezing of the protein solution in the EPR tubes (Williams-Smith et al., 1977) or to electron re-equilibration related to changes of midpoint potentials with temperature (Palmer & Olson, 1980). In our case, it seems unlikely that the positive shift of E_1' (FMNH•/FMN), observed upon freezing, arises from changes in pH only since Tris buffer is known to induce alkaline pH shifts (Williams-Smith et al., 1977) which would lead to a negative shift of E_1'. These observations reveal marked differences of the temperature dependence of the flavin redox properties between SiR-FP23 and the whole SiR-FP protein. This likely arises from variations of the factors determining the entropic contribution to the redox free energy of the flavin group, like the polarity of its environment, its electrostatic interactions with charged groups, or, more generally, its interactions with the solvent (Bertrand et al., 1994).

Fourth, both the semiquinone and the hydroquinone forms of SiR-FP23 are able in the absence of external reductant to

transfer electrons to cytochrome *c*, used as a standard electron acceptor. In both cases, the reaction is fast and quantitative. In particular, $FMNH_2$ gives its two electrons to two molecules of cytochrome *c* immediately with no observable transient $FMNH^{\bullet}$. This is the first time that such a reaction can be directly studied. Our results are in marked contrast with previous suggestions, based on stopped-flow studies, that the FMN cofactor of SiR-FP cycled between the hydroquinone and the semiquinone state during catalytic reduction of ferricyanide or menadione by an excess of NADPH (Siegel et al., 1971), as a consequence of the relatively low reactivity of the semiquinone species. Cytochrome *c* was not included in this study. Such a mechanism could be actually explained if the rate of $FMNH^{\bullet}$ reduction to $FMNH_2$ by NADPH is much faster than the rate of electron transfer from $FMNH^{\bullet}$ to an electron acceptor during catalysis in the presence of NADPH.

It was also reported that reaction of SiR with one NADPH per flavin (*i.e.* under conditions more comparable to our conditions) and an excess of cytochrome *c* resulted in a stoichiometry of 1.5 cytochrome *c* reduced per NADPH added (Siegel et al., 1972), suggesting that one of the flavins had a rather unreactive semiquinone state. That flavin was not identified but was suggested to be FMN and not FAD. Certainly, this point has to be reinvestigated in light of our present observation that $FMNH^{\bullet}$ may be not as unreactive as it has been previously thought. It has to be mentioned that, in the case of CPR, FMN operates between the hydroquinone and the semiquinone states with only $FMNH_2$ being the electron donor to cytochrome *c* or cytochrome P450 (Vermilion et al., 1981), whereas in the case of P450BM-3, only $FMNH^{\bullet}$, and not $FMNH_2$, is capable of reducing the heme iron (Sevrioukova et al., 1996). This again shows that, in spite of considerable sequence homologies and probable similar foldings, the reactivity of the FMN cofactor can be modulated within the class of FAD- and FMN-binding enzymes.

Finally, the FMN cofactor of SiR-FP23 was unable to accept electrons from SiR-FP43, the purified FAD-binding fragment containing the NADPH binding site. As a matter of fact, SiR-FP23 was unable to catalyze cytochrome *c* reduction by NADPH in the presence of SiR-FP43. Truncation of SiR-FP has thus totally blocked the electron transfer between the two flavin prosthetic groups. This was suggested earlier by an analysis of the activities of SiR-FP during proteolytic cleavage (Eschenbrenner et al., 1995b). It seems likely that the communication between the two prosthetic groups in SiR-FP depends on the relative orientation of the flavin subdomains. This orientation is probably governed by the inter-flavin domain connecting SiR-FP23 and SiR-FP43. In the case of CPR (Smith et al., 1994) and P450BM-3 (Sevrioukova et al., 1996), on the contrary, combination of the domains provided a functional enzyme active in the reduction of cytochrome *c*. A major difference between SiR-FP23 and these systems is that the former is an extensively aggregated form of the FMN-binding polypeptide. This large polymerization may greatly disturb the binding to the FAD domain.

Our data show convincing evidence that the N-terminal end of SiR-FP is the polymerization site and the FMN-binding domain. It folds independently, and the FAD-binding domain is not required for the effective and selective recognition of FMN. SiR-FP23 can be considered as a model of a FAD-depleted form of SiR-FP since the reactivity and redox potential of FMN in SiR-FP are conserved in the recombinant protein. Moreover, they show strong similarities with those of the FMN moiety in CPR and P450BM-3. This further supports the flavodoxin origin for the FMN-binding domain within this class of structurally related enzymes.

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BI9623744