The Flavoprotein Component of the *Escherichia coli* Sulfite Reductase: Expression, Purification, and Spectral and Catalytic Properties of a Monomeric Form Containing both the Flavin Adenine Dinucleotide and the Flavin Mononucleotide Cofactors

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ABSTRACT: The flavoprotein component (SiR-FP) of the sulfite reductase from *Escherichia coli* is an octamer containing one FAD and one FMN per polypeptide chain. SiR-FP60, a SiR-FP fragment starting with alanine-52, was overexpressed in *E. coli* and purified as a monomer. The N-terminal part of the native protein contains thus all the determinants required for the polymerization. SiR-FP60 retains both FAD and FMN with comparable contributions of the two flavins and the catalytic properties of SiR-FP. Thus, SiR-FP60 can be considered as a reliable simplified model of the sulfite reductase flavoprotein component. The formation and the stabilization of the neutral FMN semiquinone is thermodynamically favorable in SiR-FP60 upon reduction with photoreduced deazaflavin, dithionite, or NADPH. Generation of $FMNH^{\bullet}$ is explained from a disproportionation of electrons between the reduced and oxidized FMN moieties during an intermolecular reaction, as shown with SiR-FP23, the FMN-binding domain of SiR-FP. The neutral FAD semiquinone can be observed only within SiR-FP43, the isolated FAD-binding domain. NADPH was used as a titrant or in excess to demonstrate that electron transfer is possible only because the FMN cofactor is coupled to FAD as an electron acceptor in the protein. The electron distribution within the various reduced forms of SiR-FP60 has been compared with that of the reduced forms of cytochrome P450 reductase, bacterial cytochrome P450, and nitric-oxide synthase. Despite the conservation of the bi-flavin-domain structure between these proteins over evolutionary time, each of them provides significantly different flavin reactivities.

In *Escherichia coli* and *Salmonella thyphimurium*, sulfite reductase (SiR)[1] mediates the transfer of electrons from NADPH to sulfite to produce sulfide which is, with *O*-acetylserine, the substrate of *O*-acetylserine (thiol)-lyase, the enzyme catalyzing the synthesis of L-cysteine (*1*).

SiR is a complex and soluble hemoflavoprotein of 780-kDa with an $\alpha_8\beta_4$ subunit structure. The electron transport cofactors are clearly distributed within the protein, and the sequence of electron flow is known (*2*, *3*). The flavoprotein component (SiR-FP) of the enzyme is composed of the 8 α chains. Each of them binds one FAD and one FMN as prosthetic groups and contains a NADPH-binding domain (*4*, *5*). Each β polypeptide chain contains one Fe_4S_4 cluster and one siroheme, and is thus defined as the hemoprotein component (SiR-HP) of the native sulfite reductase (*6*). In SiR-FP, two electrons are transferred from NADPH first to FAD and subsequently to FMN before being transferred one by one to SiR-HP where the six-electron reduction of sulfite takes place (*3*). SiR-FP can be characterized independently from its diaphorase catalytic properties. As a matter of fact, SiR-FP is able to transfer electrons either from the FMN site to artificial acceptors such as ferricyanide or cytochrome *c*, while FAD can reduce directly $AcPyADP^+$ or free exogenous flavins (*3*, *4*, *7*).

SiR-FP belongs to a family of proteins which includes NADPH-cytochrome P450 oxidoreductase (CPR), nitric-oxide synthase (NOS), and P450 BM-3 (*8*−*11*). These enzymes, in common, contain both FAD and FMN and shuttle electrons from NADPH to metal centers via the flavin prosthetic groups. These proteins were suggested to evolve from a common ancestor arising through the fusion of genes encoding single-flavin-containing electron transport protein (*8*). As a consequence, the enzymes mentioned above have discrete regions with an N-terminal domain that binds FMN and a C-terminal domain that binds FAD and recognizes NADPH specifically. In all cases, the FMN-binding domain shows significant homology with bacterial flavodoxins (*12*),

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[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; SiR-FP60, the monomeric form of sulfite reductase flavoprotein; $AcPyADP^+$, 3-acetylpyridine adenine dinucleotide phosphate; CPR, NADPH-cytochrome P450 reductase; P450 BM-3, *Bacillus megaterium* cytochrome P450; BMR, flavoprotein domain (reductase) of P450 μ_B-3; NOS, nitric-oxide synthase; TEMPO, [2,2,6,6-tetramethyl-1-piperidinyloxy, free radical]. Enzyme: NADPH-sulfite reductase (EC 1.8.1.2.).

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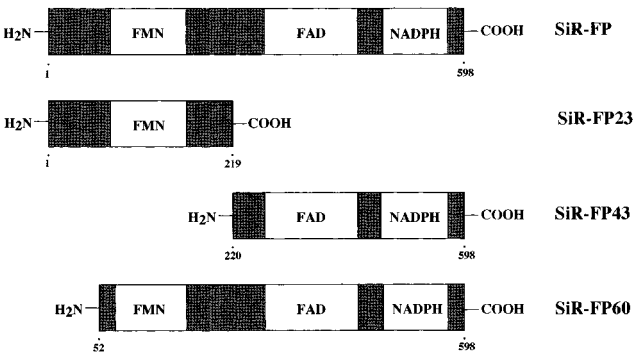
while the FAD-binding domain is related to the ferredoxin-$NADP^+$ reductases (*13*). Despite their similarities in sequences and operating cycles, the proteins of the SiR-FP family share differences in physiological roles and quaternary structure, with sulfite reductase being the more complex in terms of number of subunits and of redox cofactors.

Efforts have been made to obtain simplified versions of the SiR molecule in order to investigate the electron transport mechanism within the protein. Mutant strains of *S. typhimurium* or *E. coli* lacking an active *cysI* gene encoding for the β-subunit were used to purify SiR-FP as an octamer (*2*). The flavoprotein component can also be obtained from native SiR upon dissociation by urea-treatment followed by dilution and dialysis (*14*). In this case, the octameric structure of SiR-FP is also maintained, while SiR-HP behaves as a monomer. More recently, we have constructed an *E. coli* strain especially designed for overexpressing SiR-FP under the control of its own promoter (*4*). The purified protein is an octamer. SiR-HP is thus not required for the oligomerization of SiR-FP. In all cases, the endogenous flavin catalytic properties of SiR are retained by isolated SiR-FP, even if it is clear that a significant denaturation of the protein occurs during urea-dependent dissociation of the holoenzyme (*4*).

FMN can be removed specifically from SiR-FP. FMN-depleted SiR-FP was used to delineate the chemical and catalytic properties of endogenous FAD (*3*, *4*, *7*). Further simplification of SiR-FP progressed when we obtained the FMN- and the FAD-binding domains separately by limited proteolysis (*5*). We have suggested a quaternary structure in which the FMN-binding domains cooperate for polymerization in a head-to-head subunit interaction while the FAD-binding domain may exist as monomeric extensions in the native protein (*5*). The polymeric feature of the FMN-binding domain was demonstrated further by the specific expression of the protein encoded for by the plasmid pET-SiR-FP23 (*15*). This protein corresponding to the first 219 amino acids of SiR-FP was purified as a large polymer of 8−10 subunits 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 (*15*). These results show unambiguously that the N-terminal domain of SiR-FP contains all the determinants required for the polymerization of the α-chains.

In this paper, we describe a more precise identification of the polymerization site of SiR-FP and the construction of a vector that could be used to express a protein with alanine-52 as the starting amino acid. This protein, corresponding to a monomer, has been purified and characterized. It was called SiR-FP60 (Scheme 1) in reference to its native molecular mass. It represents a new simplified version of SiR-FP since it can bind FMN and FAD as cofactors and retains most of the catalytic and chemical properties of native SiR-FP. This tool, together with the isolated FMN- and FAD-binding domains, was used to reevaluate the electron-accepting properties of SiR-FP. Even if reduction experiments have been presented already (*2*, *16*), such a detailed study, in light of the recent reevaluation of the flavin content of the protein, has never been described. The results presented in this paper can be directly compared with those obtained with CPR, BMR (the flavoprotein domain of P450 BM-3), and NOS.

Scheme 1: Schematic Representation of the Proteins Used in This Paper[a]



[a] Only the monomeric forms of SiR-FP and SiR-FP23 are shown. SiR-FP43 and SiR-FP60 are really monomers.

EXPERIMENTAL PROCEDURES

Construction of the Expression Plasmid and Expression of the Recombinant SiR-FP60 Protein. The expression plasmid pET-SiR-FP60, expected to encode for the monomeric form of SiR-FP containing both FAD and FMN, was constructed as described for pET-SiR-FP23 (*15*), the plasmid encoding for the FMN-binding domain, except for the primers used. The sense primer 5′-ACGGAATTCATATG-**GCAACGCCAGCGCCAGCTGCAGAAATGC** contains the nucleotides (in bold) coding for a N-terminal amino acid sequence starting with alanine-52 (ATPAPAAEM). The overhanging sequence includes an *Eco*RI site followed by a *Nde*I site (underlined) carrying the initiator codon ATG. The antisense primer 5′-GGCGGATCC**TTAGTAGACATCTC-GCTGATAACGGCG** was designed to incorporate a *Bam*HI site (underlined) downstream of the stop codon in the coding sequence of SiR-FP. The nucleotides (in bold) are complementary of the C-terminal coding sequence of SiR-FP. The authenticity of the DNA insert generated by PCR was confirmed by sequencing. The expression plasmid pET-SiR-FP60 was used to transform the T7 RNA polymerase-containing host *E. coli* B834(DE3)pLysS. Conditions of culture and expression were as described for the expression of SiR-FP23 (*15*).

Purification of SiR-FP60. The pellet from 4 L of culture (about 20 g) was suspended in 100 mM Tris/HCl (pH 9) containing 80 mM KCl and 2 mM Pefabloc (Boehringer Mannheim) and lysed by freeze−thawing rupture. The total protein extract was recovered by centrifugation for 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 (*4*). The pellet obtained after ammonium sulfate precipitation (60% final saturation) was dissolved in 150 mL of 50 mM Tris/HCl (pH 7.5) (buffer A) and loaded at 1 mL min^{-1} on a Q-Sepharose fast-flow (Pharmacia LKB Biotechnology Inc.) column (2.6 cm × 9.5 cm, 50 mL) previously equilibrated with buffer A. The column was washed at 1 mL min^{-1} with the same buffer until the baseline was recovered. Then, elution was performed at the same flow rate with a linear gradient from 0 to 500 mM NaCl during 500 min. Fractions (8 mL) were collected and assayed for protein (absorbance at 280 nm), flavin content (absorbance at 450 nm), and flavin reductase activity. The active fractions, eluting at about 300

mM NaCl, were pooled and fractionated on a hydroxylapatite (Bio-Gel HTP from Bio-Rad) column (2.6 cm × 7.5 cm, 40 mL) as described for SiR-FP23 (*15*), except that SiR-FP60 was eluted at about 200 mM potassium phosphate buffer, pH 7.0. Active fractions were concentrated using a Diaflo cell equipped with a YM-30 membrane (Amicon Co.) before being filtered on a Superdex-75 column (Pharmacia LKB Biotechnology Inc.) previously equilibrated with buffer A. Elution was run at 0.8 mL min^{-1}, and the active fractions were pooled and concentrated again by ultrafiltration (3.5 mL, 55 mg of protein) before being aliquoted and stored at −80 °C for further use. At this stage, SiR-FP60 was electrophoretically pure.

Native SiR-FP, SiR-FP43, and SiR-FP23 were obtained as previously described (*5*, *15*, *17*). Nomenclature of the purified peptides reflects their molecular masses when denaturated, as judged from SDS−PAGE analysis (Scheme 1).

Spectroscopic Methods. Absorption spectra were recorded at room temperature in a quartz cell (10 mm light path) of 1 mL using a Kontron Uvikon 930 or a Hewlett-Packard 8452A diode array spectrophotometer. Anaerobic experiments were run in the same cell capped with a rubber septum or in a glovebox under overpressure of nitrogen. X-band EPR first-derivative spectra and quantification of the semiquinone radical were performed as previously described (*15*).

Reduction of the Different Proteins and Disproportionation Experiment. For the titration experiments, the protein samples were made oxygen-free by incubation for at least 1 h in a glovebox under overpressure of nitrogen. Sodium dithionite solutions were prepared in anaerobic buffers. Dithionite and NADPH concentrations were calculated using extinction coefficients of 8 mM^{-1} cm^{-1} at 315 nm and 6.22 mM^{-1} cm^{-1} at 340 nm, respectively. At the end of the experiments, the stability of the titrants was checked using the same method. After each addition of titrant, the solution was left for 10 min at room temperature so the thermodynamic equilibrium was reached, and a spectrum was recorded. Protein conditions are detailed in Results or in the legend of the concerned figures.

For the anaerobic reductions with an excess of NADPH, the protein samples and the NADPH solution were deaerated separately by argon flushing. Spectra were recorded before and after anaerobic addition of a few microliters of a concentrated solution of NADPH in order to minimize the variation of volume. Alternatively, the spectrophotometric cuvette contained also a mixture of glucose 6-phosphate and glucose-6-phosphate dehydrogenase at respectively 2 mM and 4 U mL^{-1} final concentration as a NADPH-regenerating system.

Photochemical reductions in the presence of deazaflavin−EDTA were carried out as previously described (*15*). The one-electron-reduced form of SiR-FP60 (FAD/$FMNH^{\bullet}$) was generated by oxidation of the fully reduced form upon air admission.

Disproportionation experiments were conducted with SiR-FP23, the FMN-binding domain of SiR-FP. A sample of SiR-FP23 corresponding to 11.9 nmol of FMN was fully reduced by illumination in the presence of deazaflavin−EDTA in the glovebox. Then, a sample of oxidized protein corresponding to 8 nmol of FMN was added, and the resulting spectra were recorded immediately and after the equilibrium was reached.

Enzymic Assays and Chemical Reactivity of Reduced SiR-FP60. NADPH-dependent reactions were carried out as previously described (*5*). The reactions were initiated by the addition of the protein solution. One unit of activity corresponds to the amount of protein catalyzing the oxidation of 1 nmol of NADPH per min or the reduction of 1 nmol of acceptor ($AcPyADP^+$ or cytochrome *c*) per minute. Specific activity is defined as units per milligram of protein.

The ability of the four-electron-reduced form ($FADH_2$/$FMNH_2$) or the one-electron-reduced form (FAD/$FMNH^{\bullet}$) of SiR-FP60 to reduce cytochrome *c* stoichiometrically was assayed as outlined previously for SiR-FP23 (*15*).

Cofactor Analysis and Protein Determination. Flavins were extracted from pure samples of protein denaturated by boiling for 10 min in the dark. Flavin content was analyzed by thin-layer chromatography as previously described (*15*) and quantitated by fluorometric analysis (*18*) using a Perkin-Elmer LS 450 fluorimeter. Concentration of standard flavin solutions was determined spectrophotometrically by means of their absorbances at 450 nm, using an extinction coefficient of 12.2 mM^{-1} cm^{-1} for FMN and of 11.3 mM^{-1} cm^{-1} for FAD (*19*).

Protein concentration was determined using bovine serum albumin as a standard (*20*) and the commercial Bio-Rad protein assay solution. Denaturated molecular mass of SiR-FP60 was estimated by 0.1% SDS−15% polyacrylamide gel electrophoresis (*21*), and its native molecular mass was ascertained by using a Bio-Rad precast 4−20% gradient polyacrylamide gel run under nondenaturating conditions or after elution of the calibrated Superdex-75 filtration column. N-terminal amino acid sequence determinations were performed by Dr. J. Gagnon (Institut de Biologie Structurale, Grenoble, France) using an Applied Biosystems gas-phase sequenator model 477A with on-line analysis of the phenylthiohydantoin derivatives.

RESULTS

Purification and Characterization of the 18-kDa Proteolytic Fragment. As previously shown, incubation of pure concentrated SiR-FP led to the progressive cleavage of the protein between the FAD- and the FMN-binding domains (*5*). As a matter of fact, during the first 17 h incubation, two main fragments accumulated as controlled by SDS−PAGE with apparent molecular masses of 43- and 23-kDa. After 24 h incubation, a third fragment appeared with an apparent molecular mass of 18-kDa under denaturating conditions along with the 43- and 23-kDa fragments (see Figure 1, ref *5*). In a previous study, when a completely cleaved preparation of SiR-FP was subjected to filtration on Superdex-75, only the major peaks were separated and analyzed while the third minor one was neglected.

We have now purified the protein responsible for this third peak. It corresponds to a dimeric form of the 18-kDa proteolytic fragment. The spectral analysis of this peptide reveals the presence of the visible absorbance due to flavins. Characterization of the flavin cofactor was done by thin-layer chromatography of a heat-denaturated protein sample and identified it as FMN exclusively (not shown). The N-terminal amino acid sequencing of the 18-kDa fragment was performed by gas-phase sequence analysis. Three sequences were detected in the following proportions:

Table 1: Purification of SiR-FP60 from *E. Coli* B834(DE3)pLysS(pET-SiR-FP60) Extracts Issued from 1 L of Culture

	protein (mg)	flavin reductase activity	
		units (nmol min^{-1})	specific activity (nmol min^{-1} mg^{-1})
extracts	161	328 000	3 200
Q-Sepharose	11.8	168 400	14 300
hydroxylapatite	5.9	99 400	16 900
Superdex-75	4.4	88 100	20 000

AATPA (38%), ATPAP (28%), and TPAPA (34%). This corresponds to three peptides, starting with alanine-51, alanine-52, or threonine-53, respectively.

Taken together, these results strongly suggest that the 18-kDa fragment was derived from the 23-kDa fragment during prolonged incubation of native SiR-FP under proteolytic conditions. The loss of the 50 first amino acids thus led to a peptide still able to bind the FMN cofactor but with a simplified quaternary structure, since SiR-FP23, the FMN-binding domain of SiR-FP, is at least octameric while the 18-kDa fragment behaves in fact as a dimer. This observation prompted us to construct a vector that could be used to express a new form of SiR-FP retaining the two flavin-binding domains but simplified with respect to the number of α-subunits.

Overexpression and Purification of SiR-FP60. *E. coli* B834(DE3)pLysS was tranformed with the expression plasmid pET-SiR-FP60 encoding SiR-FP60, a protein with alanine-52 chosen as the first residue and thus presumably devoid of the majority of the determinants required for the oligomerization in native SiR-FP. Maximal expression was observed about 2 h after addition of IPTG to the growth medium. SiR-FP60 was recovered in the soluble extracts and assayed for its flavin reductase activity in the presence of riboflavin and NADPH (*17*). A specific activity of 3200 was obtained, to be compared to about 50 for the extracts from the parental strain B834(DE3)pLysS. This shows an approximately 65-fold overexpression of SiR-FP60.

Soluble extracts stored as ammonium sulfate pellets were extensively diluted, and SiR-FP60 was purified to homogeneity by a three-step procedure. Flavin reductase activity was chosen for following the purification (Table 1). Anion-exchange chromatography on Q-sepharose fast-flow was a very efficient step, while hydroxylapatite chromatography and filtration on Superdex-75 led to the elimination of minor contaminants. In addition, the last filtration step enabled us to obtain SiR-FP60 in the typical working buffer (50 mM Tris/HCl pH 7.5) and to estimate the native molecular mass of the purified protein. SiR-FP60 eluted from the filtration as a monomer of about 57 000 Da. The monomeric feature of SiR-FP60 was ascertained by native gel electrophoresis. This second technique gave a single band with a molecular mass comparable with that determined by filtration (not shown). This value fits very well with the theoretical molecular mass of 60 706 Da deduced from the amino acid sequence.

Flavin Content and Catalytic Properties of SiR-FP60. A pure preparation of SiR-FP60 gave a light absorption spectrum characteristic of an oxidized flavoprotein with absorption maxima at 276, 386, and 458 nm (Figure 1A). The supernatant of a heat-denaturated sample of SiR-FP60 of known absorbance and concentration was analyzed by thin-layer chromatography and spectrophotometry. TLC analysis showed the presence of both FAD and FMN. Accurate quantification of the flavin content of several independent preparations of SiR-FP60 by fluorimetric analysis demonstrated that SiR-FP60 contained 1.7 ± 0.2 mol of flavin/mol of protein with comparable contribution of FAD and FMN (0.7−0.9 mol/mol of protein). The extinction coefficient of oxidized SiR-FP60 was thus found to be 10.4 ± 0.1 mM^{-1} cm^{-1} at 458 nm, a value in perfect agreement with those of SiR-FP [10.9 mM^{-1} cm^{-1} at 456 nm (*7*)] and SiR-FP23 [10.5 mM^{-1} cm^{-1} at 455 nm (*15*)].

The ability of SiR-FP60 to catalyze NADPH-dependent reductions was monitored spectrophotometrically with the typical electron acceptors used to characterize SiR-FP. Kinetic parameters, K_m and V_m, for NADPH and for the different acceptors were obtained from linear Lineweaver−Burk plots (Table 2). The parameters for NADPH were obtained with riboflavin as the second substrate.

In agreement with the presence in comparable proportions of the two flavinic cofactors, SiR-FP60 retained the capacity to catalyze both FAD- and FMN-dependent reductions, i.e., reduction of free flavins and transhydrogenation of AcPy-ADP$^+$ from the FAD site and reduction of cytochrome *c* or ferricyanide from the FMN site. K_m values for NADPH and riboflavin for SiR-FP60 were comparable to those previously reported in the case of SiR-FP (*4*). Specific activities obtained with the monomeric protein were significantly higher. As previously shown for SiR-FP (*4*), cytochrome *c* is the electron acceptor which is reduced at the higher rate and recognized with the better affinity. The most noticeable change is the K_m for ferricyanide which is 8-fold weaker for SiR-FP60 (82 μM) when compared with native SiR (11 μM) (*3*). However, the two proteins reduced this substrate at comparable rates.

In terms of its catalytic properties, we can conclude that SiR-FP60 is an excellent model of the octameric flavoprotein component of SiR.

Photoreduction of SiR-FP60 and Determination of the Extinction Coefficient of Its One-Electron-Reduced Form. Photoreduction of fully oxidized SiR-FP60 (21.7 μM total flavin concentration) was achieved by illumination at pH 7.5 and under anaerobic conditions in the presence of 2.8 μM deazaflavin and 10 mM EDTA. Three distinct steps were observed during this process (Figure 1). During the first step, the absorbance at 458 nm decreased while a long-wavelength absorption band at 589 nm with a shoulder at 630 nm characteristic of a neutral blue semiquinone increased in parallel. As previously demonstrated for SiR-FP (*7*) and for SiR-FP23 (*15*), the FMN-binding domain of SiR-FP, these spectral changes occurred with isosbestic points at 362 and 504 nm (Figure 1A,D) and are thus assigned to the transition from the oxidized form of the protein (FAD/FMN) to the one-electron-reduced form (FAD/FMNH$^\bullet$). In the following, we will take as a general criterion the absence of variation of the absorbance at 504 nm as the indication for such a transition.

During the second step, we observed a further decrease of the absorbance at 458 nm, while the absorbance at 589 nm remained quite stable (Figure 1B,D). The two isosbestic points were progressively lost. A likely hypothesis is that FAD started to be reduced, while FMNH$^\bullet$ remained stable.

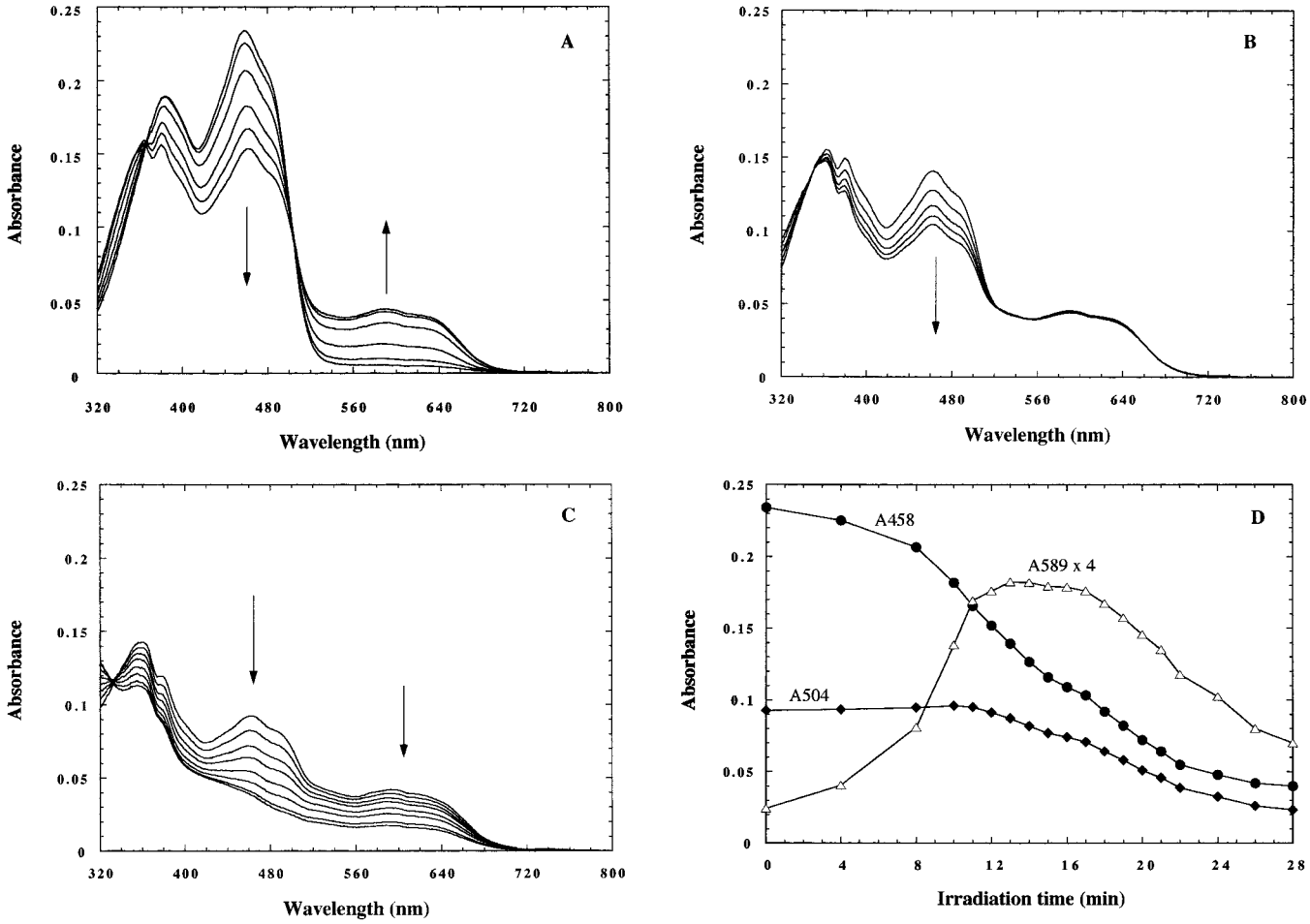


FIGURE 1: Photoreduction of oxidized SiR-FP60. A solution containing SiR-FP60 (14.5 μM) in 50 mM Tris/HCl (pH 7.5) was photoreduced in the presence of deazaflavin−EDTA as previously described (*9*). The arrows point in the direction of spectral changes. (A) Spectra were recorded after 0, 4, 8, 10, 11, and 12 min of irradiation. (B) Spectra at 13, 14, 15, 16, and 17 min of irradiation. (C) Spectra at 18, 19, 20, 21, 22, 24, 26, and 28 min of irradiation. (D) Plot of absorbance changes at 458 nm (●), 589 nm × 4 (△), and 504 nm (◆) as a function of irradiation time.

Table 2: Kinetic Parameters for the Substrates of the Diaphorase Activities of SiR-FP60

substrate	second substrate	K_m (μM)	V_m nmol min^{-1} mg^{-1}
riboflavin	NADPH	69	40 900
AcPyADP$^+$	NADPH	65	22 700
cytochrome *c*	NADPH	2.2	98 000
$K_3Fe(CN)_6$	NADPH	82	76 000
NADPH	riboflavin	75	70 200

The last spectrum of this phase should thus be predominated by the three-electron-reduced form $FADH_2$/FMNH$^\bullet$.

Then in the third step, both bands at 458 and 589 nm decreased with an isosbestic point at 328 nm until complete bleaching of the protein, indicating that the four-electron-reduced state of SiR-FP60 was reached (Figure 1C,D).

In another independent experiment, during exposure to air, the fully reduced form was converted to the radical form almost instantaneouly (not shown) as previously shown with SiR-FP23 (*15*). This one-electron-reduced form is stable enough to allow accurate spin quantification. This was done by recording the X-band EPR spectrum of the radical at 100 K and comparing it to that of a standard solution of TEMPO, a stable nitroxide. Starting with a pure preparation of SiR-FP60 corresponding to 52.6 μM total flavin concentration, as deduced from the absorbance at 458 nm, and containing 0.8 FAD and 0.7 FMN, 22.6 μM radical was obtained upon reoxidation. Assuming that the radical was exclusively formed on FMN, we concluded that 92% of FMN was under the one-electron-reduced form. From these results, we could calculate an extinction coefficient at 589 nm of 5.4 mM^{-1} cm^{-1}, a value slightly higher that the value of 4.88 mM^{-1} cm^{-1} obtained with SiR-FP23 (*15*) or of 4 mM^{-1} cm^{-1} obtained with SiR-FP (*7*).

Titration of SiR-FP60 with Dithionite. Spectral changes associated with dithionite reduction of SiR-FP60 were recorded spectrophotometrically under anaerobic conditions using the protein solution described above.

Addition of 0.5 mol of dithionite/mol of SiR-FP60 (one electron-equivalent added) resulted in spectral changes (Figure 2) characteristic of the formation of FMNH$^\bullet$, as described above. We could calculate that more than 85% of FMNH$^\bullet$ was generated under these conditions.

When a second electron-equivalent was added (1 mol of dithionite/mol of SiR-FP60), we observed the continuous decrease of A_{458}, the loss of the isosbestic point at 504 nm, and the relative stability of the radical content as judged by the value of A_{589}. It is thus tempting to suggest that a pseudo two-electron-reduced form was obtained. This form should be a 1:1 mixture of two different reduced forms (FAD/FMNH$^\bullet$ and $FADH_2$/FMNH$^\bullet$).

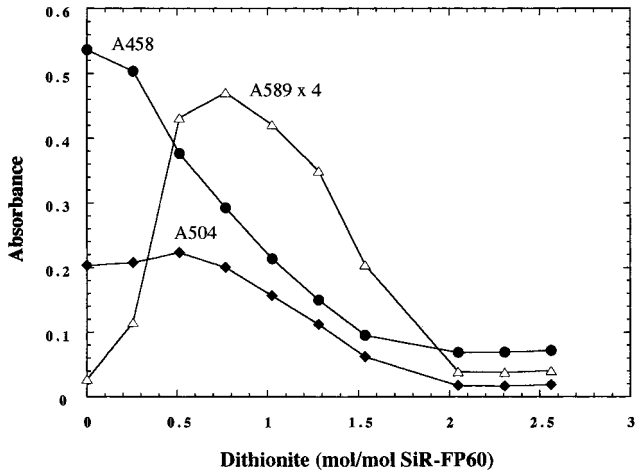


FIGURE 2: Anaerobic titration of oxidized SiR-FP60 with sodium dithionite. SiR-FP60 (34.4 μM) in 50 mM Tris/HCl, pH 7.5, was titrated in a glovebox under overpressure of nitrogen, at room temperature, with sodium dithionite (1.3 mM). Spectra were recorded at least 10 min after each addition of titrant. The plot represents the absorbance changes at 458 nm (●), 589 nm × 4 (△), and 504 nm (◆) as a function of sodium dithionite added. The values have been corrected for dilution.

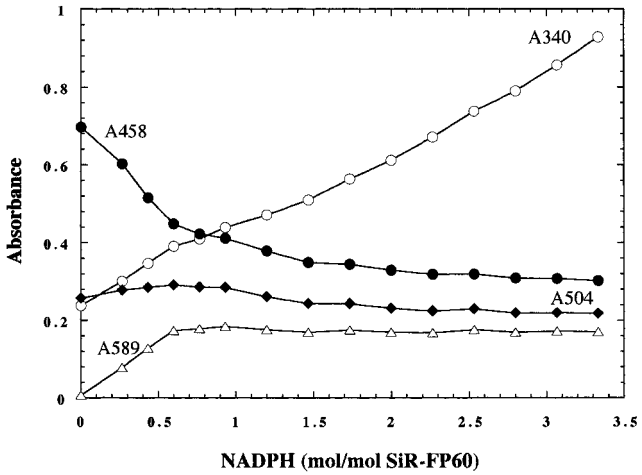


FIGURE 3: Anaerobic titration of oxidized SiR-FP60 with NADPH. SiR-FP60 (40.6 μM) in 50 mM Tris/HCl (pH 7.5) was titrated in a glovebox under overpressure of nitrogen, at room temperature, with NADPH (0.8 mM). Spectra were recorded at least 10 min after each addition of titrant. The plot represents the absorbance changes at 458 nm (●), 589 nm (△), 504 nm (◆), and 340 (○) as a function of NADPH added. The values have been corrected for dilution.

The addition of a third and then a fourth electron (1.5 and 2 mol of dithionite/mol of SiR-FP60) was characterized by the continuous decrease of all three absorbances. Complete bleaching of the protein ($FADH_2$/$FMNH_2$) was obtained with the addition of the fourth electron.

Reduction of SiR-FP60 with NADPH. A solution of SiR-FP60 containing 0.75 mol of FAD and 0.9 mol of FMN/mol of protein and corresponding to 67.3 μM total flavin concentration was titrated with NADPH anaerobically (Figure 3). Addition of up to 0.5 mol of NADPH/mol of enzyme reduced it to the FAD/$FMNH^\bullet$ form as deduced from the characteristic spectral changes, with about 88% of FMN present as a semiquinone under these conditions. Further addition of NADPH produced minimal changes in the flavin spectra. The absorbance at 589 nm remained stable, while the absorbance at 458 nm decreased continuously but slowly. The isosbestic point at 504 nm remained tight only until the addition of 1 equiv of NADPH. The regular increase of A_{340}, due to evolution of the flavin spectrum in the early step of the reaction, reflected then the accumulation of NADPH. These spectral evolutions indicated that $FMNH^\bullet$ could not be further reduced with NADPH, while FAD was only poorly reduced, probably to the $FADH_2$ form. This interpretation is supported by the absorbance increase in the very long-wavelength region (>700 nm) which is due to the formation of a charge-transfer complex between reduced FAD and $NADP^+$ (not shown).

On the contrary, a single addition of an 8-fold molar excess of NADPH resulted in an instantaneous reduction of SiR-FP60, with a large decrease of the absorbances at 458 and 504 nm and the formation of only 53% of $FMNH^\bullet$, as deduced from the absorbance at 589 nm (not shown). These spectral changes indicated that under these conditions FAD was fully reduced and FMN was reduced to a mixture of $FMNH^\bullet$ and $FMNH_2$. The proportion of $FMNH^\bullet$ decreased as the reductive pressure increased, as demonstrated from experiments with a 34-fold molar excess of NADPH (30% of $FMNH^\bullet$) or with a 6-fold molar excess of NADPH in the presence of a NADPH-regenerating system (not shown). In this latter case, the protein was fully reduced, and an excess of NADPH was not required.

The final state of reduction of SiR-FP60, when NADPH is used as the reductant, is different depending on whether the protein was fully oxidized or one-electron-reduced at the beginning of the experiment. This was shown by an experiment in which we have generated the stable FAD/$FMNH^\bullet$ form of the protein (by exposure to air after anaerobic photoreduction in the presence of deazaflaflavin−EDTA) and reacted it with an excess of NADPH. As monitored by light absorption spectroscopy, the semiquinone remained stable, while only a small decrease of the absorbances at 458 and 504 nm was observed (not shown). These spectral changes were interpreted as a poor conversion of FAD to $FADH_2$ and no reduction of $FMNH^\bullet$.

Photoreduction of SiR-FP43. SiR-FP43, the FAD-binding domain of native SiR-FP, has been used to observe the blue FAD semiquinone for the first time. This fragment was obtained from SiR-FP or SiR-FP60 by limited proteolysis occurring during the prolonged incubation of the concentrated protein at 30 °C (*5*). After purification by filtration on Superdex-75, SiR-FP43 was shown to contain FAD exclusively as a cofactor and to catalyze NADPH-dependent reactions (*5*).

SiR-FP43 was reduced under anaerobic conditions by illumination in the presence of deazaflavin−EDTA. As shown in Figure 4A, FAD in oxidized SiR-FP43 has absorbance maxima at 392 and 461 nm. Quantification of the FAD content allowed the determination of an extinction coefficient of 9.4 mM^{-1} cm^{-1} at this latter wavelength. Upon illumination, A_{461} decreased continuously while a long-wavelength absorbance, with a maximum around 610 nm and a shoulder at 640 nm, appeared first and then disappeared when SiR-FP43 was fully reduced (Figure 4B). This intermediate spectrum, characteristic of the neutral blue FAD semiquinone (inset of Figure 4A), had never been reported previously in any fragment of SiR-FP. Differences with the FMN semiquinone spectrum are clearly evident from the position of maxima and shoulder absorbances. Upon admis-

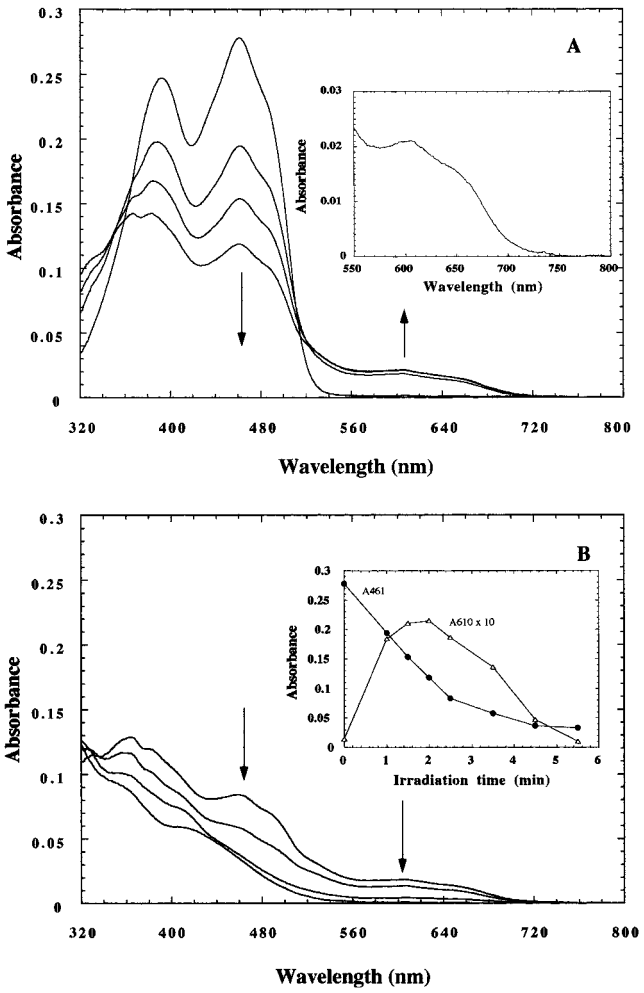


FIGURE 4: Photoreduction of oxidized SiR-FP43. A solution containing SiR-FP43 (29.6 μM) in 50 mM Tris/HCl (pH 7.5) was photoreduced in the presence of deazaflavin−EDTA as previously described (*9*). The arrows point in the direction of spectral changes. (A) Spectra were recorded after 0, 1, 1.5, and 2 min of irradiation. The inset shows the characteristic spectrum of the blue neutral FAD semiquinone. (B) Spectra at 2.5, 3.5, 4.5, and 5.5 min of irradiation. The inset is a plot of absorbance changes at 461 nm (●) and 610 nm × 10 (△) as a function of irradiation time.

sion of air and within less than 5 min, fully reduced SiR-FP43 oxidized completely with the transient appearance of FADH• (not shown).

Electron Transfer between Fully Reduced and Fully Oxidized FMN. A preparation of pure SiR-FP23 containing 0.47 mol of FMN/mol of monomer was used for the disproportionation experiment. Oxidized SiR-FP23 corresponding to 11.9 nmol of FMN was completely reduced under strict anaerobic conditions by illumination in the presence of deazaflavin−EDTA. Then, a volume of oxidized SiR-FP23 corresponding to 8 nmol of FMN was introduced in the cuvette, and the resulting spectra were recorded immediately and after the equilibrium was reached. Figure 5 shows that the spectrum characteristic of the neutral semiquinone (FMNH•) appeared instantaneously and that its absorbance was maximum after reaction for 5 min. At this stage, we can calculate, using the extinction coefficient of 4.88 mM^{-1} cm^{-1} at 589 nm previously determined (*15*), that 16.6 nmol of FMNH• was produced. In other words, within 5 min, the reaction of disproportionation was complete. This result shows unambiguously that interprotein electron transfers are possible with SiR-FP23. In all probability, it is also the case with native SiR-FP and SiR-FP60.

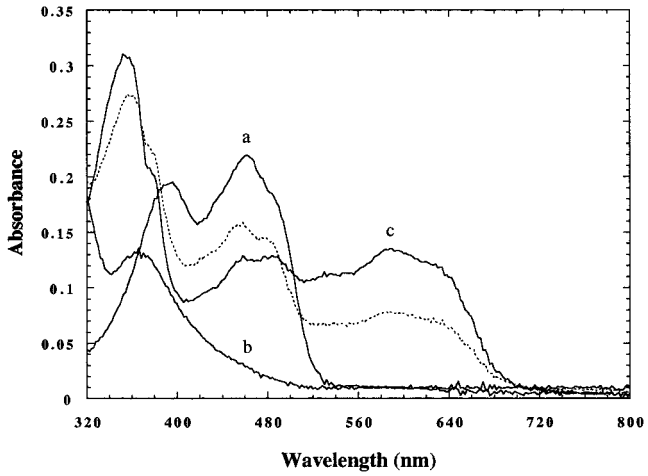


FIGURE 5: Disproportionation between fully reduced and fully oxidized SiR-FP23. The experiment was carried out in a glovebox under overpressure of nitrogen, at room temperature. A solution containing SiR-FP23 (560 μL, 21.2 μM of FMN) in 50 mM Tris/HCl (pH 7.5) was fully reduced in the presence of deazaflavin−EDTA as previously described (*9*). Absorbance spectra were recorded before (a) and after the photoreduction (b). An amount of 40 μL of oxidized SiR-FP23 (197.9 μM of FMN) was added. Spectra were recorded immediately (dotted line) and 5 min later when the equilibrium was reached.

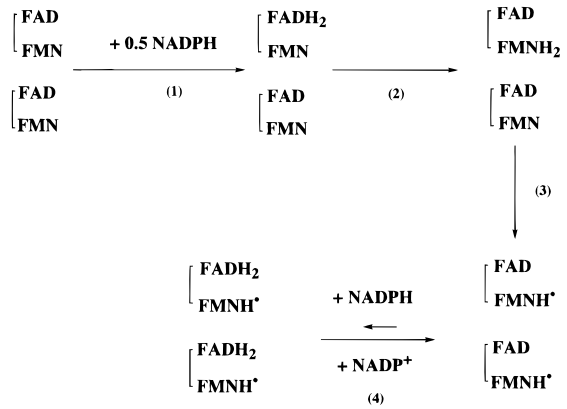
Reactivity of Reduced SiR-FP60. We have assayed the ability of reduced SiR-FP60 to transfer electrons from the FMN site to cytochrome *c*. In a first experiment, SiR-FP60 was reduced by illumination in the presence of deazaflavin−EDTA. As seen above, the fully reduced state of the enzyme (four electron-equivalents) can be easily obtained by photoreduction, while the stable one-electron-reduced state is generated from the hydroquinones by admission of air. A solution of fully reduced SiR-FP60 (22.2 μM in total flavin) was able to reduce 80% of a 50 μM solution of cytochrome *c*, while the one-electron-reduced form, corresponding to 10.7 μM FMNH•, reduced 24% of the same solution of cytochrome *c*. The two reactions were instantaneous.

It is thus easy to conclude that, in both cases, the totality of the electrons stored in the reduced protein were almost quantitatively transferred to cytochrome *c*. The same results were previously obtained with the FMN-binding domain of SiR-FP (*15*). This shows that the deletion of the 51 first amino acids does not disturb the chemical reactivity of the FMN-binding domain and confirms that the communication between the two flavin-binding domains is not altered in SiR-FP60.

DISCUSSION

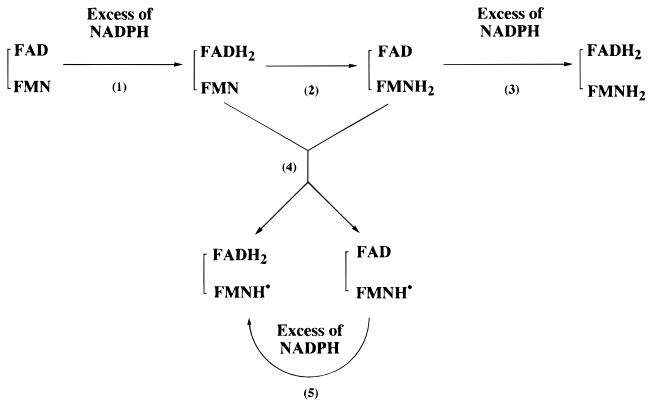
The interpretation of the results obtained from preliminary reduction experiments conducted with native SiR-FP suffered from the complexity of its quaternary structure and of the lack of precision in the determination of the flavin content (*2*, *16*). To study the exact electron distribution among the various reduced forms of SiR-FP and to compare it directly with the results obtained with CPR, BMR, and NOS, we have developed a new simplified model of the protein regarding its quaternary structure. A detailed study of the peptidic fragments issued from limited proteolysis of the

Scheme 2: Proposed Mechanism for the Reduction of SiR-FP60 with NADPH during Titration Experiments[a]



[a] The SiR-FP60 is purposely divided in two half-populations. The first event (steps 1 to 3) leads to the formation of the one-electron-reduced state of the protein FAD/FMNH•. The key disproportionation reaction is depicted in step 3. The final reduced state of SiR-FP60 depends on the equilibrium depicted in step 4.

Scheme 3: Proposed Mechanism for the Reduction of SiR-FP60 with an Excess of NADPH[a]



[a] After full reduction of FAD (step 1) and then transfer of the electrons to FMN (step 2), two reactions compete for leading either to the four-electron-reduced state of the protein (step 3) or its three-electron-reduced state (steps 4 and 5). This latter reaction involves a disproportionation of the electrons between the reduced and the oxidized form of the FMN cofactor.

flavoprotein component of sulfite reductase led us to conclude that the polymerization site of the α-chains occurs mainly between threonine-1 and alanine-51. We have used DNA techniques to construct the expression vector pET-SiR-FP60 encoding for a protein of 60-kDa starting with alanine-52. The overexpressed protein, named SiR-FP60, was purified as a monomer containing both FAD and FMN as cofactors with comparable contribution of the two flavins. Since SiR-FP60 displays catalytic properties very similar to those of native or recombinant SiR-FP (*3*, *4*), it can be considered as an excellent simplified model of the flavoprotein component of SiR.

The redox potentials of the different flavin couples have been previously determined (*7*) for native SiR-FP (E'_1 (FMNH•/FMN) = −152 mV, E'_2 ($FMNH_2$/FMNH•) = −327 mV and E'_3 (FADH•/FAD) = −382 mV, E'_4 ($FADH_2$/FADH•) = −322 mV). We have also demonstrated that the redox potentials of the FMN couples are conserved in isolated recombinant SiR-FP23, the FMN-binding domain of SiR-FP (E'_1 (FMNH•/FMN) = −130 mV, E'_2 ($FMNH_2$/FMNH•) = −335 mV), indicating that the protein is organized in two discrete flavin-binding domains (*15*). We can thus assume that these values are also conserved in SiR-FP60.

Scheme 2 gives a representation of the different phases occurring during the NADPH titration experiment. Addition of 0.5 mol of NADPH per mol of protein generates first the FAD/FMNH• form (90% yield). This is explained if one assumes that NADPH reacts with half of FAD by a two-electron reduction to generate $FADH_2$ (step 1). This reaction is thermodynamically unfavored since the average potential of the FAD-$FADH_2$ reaction (−352 mV) should be more negative than that of the NADPH/$NADP^+$ couple (−340 mV). Then, electron transfer is possible only because the protein has coupled a FMN cofactor as an electron acceptor to FAD. Thus the reaction is driven by the intramolecular oxidation of $FADH_2$ by FMN (step 2), since the average redox potential of the $FMNH_2$/FMN couple is −240 mV. We now explain the formation of about 100% FMNH• from the existence of a disproportionation reaction (step 3) between the reduced molecules (FAD/$FMNH_2$) and the oxidized ones (FAD/FMN) as a consequence of the following intermolecular reaction:

$$FMN + FMNH_2 \rightarrow 2\ FMNH^{\bullet}$$

We have shown that this is likely to occur on the basis of careful experiments with SiR-FP23, the FMN-binding domain of native SiR-FP. Further additions of NADPH aliquots (step 4) lead only to partial reduction of the FAD. The final reduced state of the protein is thus the result of an equilibrium in which FAD is poorly reduced and FMNH• remained stable, the redox potential of the $FMNH_2$/FMNH• couple being too low for efficiently driving the electrons to FAD. When an excess of NADPH is used, instead of small aliquots, to reduce a preparation of SiR-FP60 containing previously more than 90% FMNH•, the same results are obtained.

On the contrary, when an excess of NADPH is used under anaerobic conditions for reducing a fully oxidized sample of SiR-FP60, the final state of the protein is a mixture of the three- and four-electron-reduced states whose proportions depend on the NADPH concentration. The reaction can be decomposed as depicted in Scheme 3. All the FAD is two-electron-reduced immediately (step 1), and the two electrons are transferred to FMN (step 2). During this transfer, two reactions (steps 3 and 4) compete for leading to two different reduced states. In step 3 (rate constant = k_3), the FAD/$FMNH_2$ form can gain two more electrons from NADPH, while in step 4 (rate constant = k_4), a disproportionation can occur between the products of step 1 and step 2. The remaining oxidized FAD is then fully reduced with NADPH (step 5). The ratio k_3/k_4 should be NADPH concentration dependent and govern the exact final reduced state of the protein. We have also demonstrated that SiR-FP60 can be fully reduced, even in the presence of a substoichiometric amount of NADPH if $NADP^+$ is regenerated continuously to NADPH. Under these latter conditions, $NADP^+$ could not accumulate and the thermodynamic equilibrium of the reaction was shifted, thus allowing the instantaneous full reduction of SiR-FP60. This result is in agreement with a midpoint potential of enzyme-bound FAD more negative than that of the NADPH/$NADP^+$ couple. The fully reduced state

of SiR-FP60 is also easily obtained with low redox potential reductants such as photoreduced deazaflavin or sodium dithionite, this latter compound being thus very convenient for the titration of the totality of the redox centers.

A common intermediate observed during the titration experiments conducted with SiR-FP60 is the one-electron-reduced form FAD/FMNH•. This form should be defined as the air-stable semiquinone form of the protein, since anaerobically fully reduced SiR-FP60 is instantaneously converted to this radical form upon admission of air and then FMNH• is slowly oxidized. This behavior is in agreement with the redox potentials of the different FMN couples (see above). The 130−140 mV difference between these two values explains the high degree of thermodynamic stabilization of the FMNH• radical. In marked contrast, the two FAD redox couples are separated by only 60 mV. As a consequence of the thermodynamic instability and of the presence of FMN as an internal oxidant, the other possible one-electron-reduced state of SiR-FP60, FADH•/FMN, has never been observed. However, using SiR-FP43, the isolated FAD-binding domain of SiR-FP, we were able to generate the blue neutral FAD semiquinone by photoreduction of the protein in the presence of deazaflavin−EDTA. Under these conditions, the FAD radical was stable enough to be observed by spectrophotometry. The spectral features of FADH• are similar to those of FMNH•, except that a bathochromic shift leads to an absorbance maximum at 610 nm and a shoulder at 640 nm. As expected, air reoxidation of the fully reduced protein is very fast with the transient apparition of FADH•.

Even if the proteins of the SiR-FP family are genetically and functionally related, the intimate reactivity of each protein can be modulated within this class of enzymes. For instance, SiR-FP and CPR have been postulated to share a common mechanism of electron transfer whereby the physiological acceptor (the β-subunit of SiR and cytochrome P450, respectively) can obtain single electrons at constant potentials (*2*, *16*, *22*). Reducing equivalents enter the enzyme always in a reaction of NADPH with fully oxidized FAD, and only fully reduced FMN is able to reduce the acceptor. These two proteins share other common features. For instance, when fully reduced CPR is exposed to air, $FADH_2$ and $FMNH_2$ are rapidly oxidized with $FADH_2$ giving FAD and $FMNH_2$ giving FMNH• (*23*−*27*). Addition of one electron-equivalent of CPR causes also the conversion of the oxidized enzyme into the air-stable semiquinone FMNH• (*24*). As with SiR-FP60, full reduction with NADPH is reached in the presence of the mixture of glucose 6-phosphate and glucose-6-phosphate dehydrogenase (*28*). However, in marked contrast with SiR-FP60, the one-electron-reduced form of CPR (FAD/FMNH•) was converted to the semiquinone of the other flavin (FADH•/$FMNH_2$) during anaerobic reduction by NADPH (*28*, *29*).

The situation is different with BMR. This protein does not form significant amounts of an air-stable semiquinone during the air reoxidation of the reduced enzyme (*30*), suggesting that the environment of its FMN moiety must be somewhat different. No flavin semiquinone formation was observed after the addition of a first mol of NADPH (two electron-equivalent) per mol of enzyme. Then, 1.5 additional mol of NADPH per mol of BMR produces an absorbance increase at 585 nm characteristic of a neutral blue semiquinone species (*30*, *31*). Moreover, $FMNH_2$ was suggested to be unreactive during electron transfer to the heme iron (*30*). The proposed mechanism for the reduction of P450 BM-3 involves the participation of the anionic form of the FMN semiquinone as the reductant of the heme iron (*30*, *31*).

Concerning NOS, titration experiments of the flavin cofactors led to the demonstration of an air-stable semiquinone on this protein (*32*, *33*). The flavin responsible for the binding of an unpaired electron has not been identified so far but should be FMN in all probability.

In the absence of a NADPH-regenerating system, native SiR-FP, CPR, BMR, and NOS resist full reduction with NADPH, as SiR-FP60 does, whereas complete bleaching of these proteins requires the use of a stronger reductant. This behavior suggests that the redox potentials of their flavinic cofactors should be comparable.

In summary, the monomeric form of SiR-FP is an excellent model of the octameric native protein and a very good tool for studying its electron-accepting capacities. While the bi-domain structures of the protein of the SiR-FP family has been conserved over evolutionary time, they provide different flavin environments and thus different flavin reactivities. In this respect, SiR-FP seems to be more similar to CPR than to BMR, albeit that FADH• was not detected during reduction of the protein. Among these proteins, the crystal structure of CPR has been solved very recently at 2.6 Å resolution (*34*). The overall structural organization of these related enzymes should be very similar according to their considerable sequence homologies. CPR is composed of four structural domains, and the two flavin isoalloxazine rings are juxtaposed for communicating through the methyl groups of their xylene rings located at about 4 Å from each other. A precise study of the flavin environments in each protein of the SiR-FP family requires the comparison of the crystal structure of these proteins.

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