Overexpression of the FAD-binding domain of the sulphite reductase flavoprotein component from *Escherichia coli* and its inhibition by iodonium diphenyl chloride

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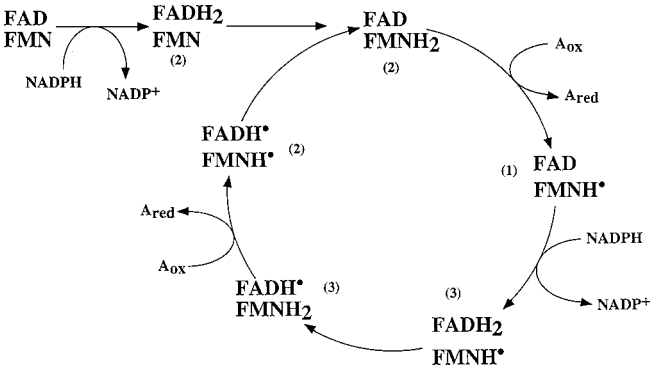
SiR-FP43, the NADPH- and FAD-binding domain of the *Escherichia coli* sulphite reductase flavoprotein component (SiR-FP), has been overexpressed and characterized. It folds independently, retaining FAD as a cofactor and the catalytic properties associated with the presence of this cofactor. Iodonium diphenyl chloride (IDP) was shown to be a very efficient inhibitor of SiR-FP43 and SiR-FP60, the monomeric form of SiR-FP, containing both FMN and FAD as cofactors ($K_i = 18.5 \pm 5\ \mu M$, maximal inactivation rate $= 0.053 \pm 0.005\ s^{-1}$). In both cases, inactivation was shown to result from covalent binding of a phenyl group to FAD exclusively, in marked contrast with previous results obtained with cytochrome P450 reductase (CPR), where FMN and a tryptophan were phenylated, but not FAD. However, our kinetic analyses are in agreement with the inhibition mechanism demonstrated with CPR [Tew (1993) Biochemistry **32**, 10209–10215]. Nine different FAD phenylated adducts were isolated and, for the first time, two FAD phenylated adducts were identified directly after extraction from a protein. Taken together, our results have shown that flavoprotein inactivation by IDP is not a reliable indicator for a flavin radical intermediate in catalysis.

Key words: flavin semiquinone, irreversible inactivation, phenylated adducts.

INTRODUCTION

In *Escherichia coli*, NAPH-sulphite reductase (SiR) is involved in the sulphate assimilation pathway leading to the biosynthesis of L-cysteine and organic sulphur compounds [1]. The flavoprotein component of the enzyme is composed of eight α-chains (SiR-FP) encoded for by the *CysJ* gene [2]. Each of the α-chains contains two distinct flavinic domains: an N-terminal flavodoxin-like domain that binds one FMN and a C-terminal ferredoxin-$NADP^+$ reductase-like (FNR-like), FAD-containing and NADPH-binding domain [3,4]. This type of organization is a characteristic of the family of proteins which includes NADPH-cytochrome P450 reductase (CPR), nitric oxide synthase (NOS) and *Bacillus megaterium* cytochrome P450. These proteins were suggested to have evolved from a common ancestor arising through the fusion of genes encoding a single-flavin-containing electron-transport protein [5,6]. Amino acid sequence analysis of the SiR-FP family members and structural alignments with flavodoxins and FNRs predict a very similar overall structure for these proteins [6,7]. However, among this family of enzymes, only the X-ray crystallographic structure of CPR has been obtained [7].

The flavin cofactors of SiR-FP serve to shuttle electrons from NADPH to the haemoprotein (SiR-HP) component of SiR, where the six-electron reduction of sulphite to sulphide takes place [8]. During catalytic turnover, SiR-FP and CPR have been postulated to share a common mechanism of electron transfer whereby the physiological acceptor (SiR-HP or cytochrome P450 respectively) can obtain single electrons at constant potential [9,10]. 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 (Scheme 1). In this mechanism, FAD cycles between fully reduced and fully oxidized



Scheme 1 Schematic representation of the SiR-FP catalytic cycle (see also [9])

Numbers in parentheses refer to the number of extra electrons contained in the enzyme when compared with the oxidized form.

Abbreviations used: SiR, NADPH-sulphite reductase; SiR-FP, sulphite reductase flavoprotein; SiR-HP, sulphite reductase haemoprotein; SiR-FP23, FMN-binding domain of sulphite reductase flavoprotein; SiR-FP43, FAD-binding domain of sulphite reductase flavoprotein; SiR-FP60, the monomeric form of sulphite reductase flavoprotein; FNR, ferredoxin-$NADP^+$ reductase; ESI–MS, electrospray ionization MS; CPR, NADPH-cytochrome P450 reductase; NOS, nitric oxide synthase; $AcPyADP^+$, 3-acetylpyridine adenine dinucleotide phosphate; $AcPyADP^+$-THase, $AcPyADP^+$-transhydrogenase; IDP, iodonium diphenyl chloride.

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states (probably with the transient appearance of a semiquinone), whereas FMN cycles only between fully reduced and semiquinone states [9]. Furthermore, in SiR-FP and CPR, the formation and the stabilization of a neutral FMN semiquinone is thermodynamically favourable [2,11,12]. SiR-FP can also transfer electrons either from the FMN site to artificial acceptors such as ferricyanide or cytochrome *c*, or from FAD to 3-acetylpyridine adenine dinucleotide phosphate ($AcPyADP^+$) or free exogenous flavins [2,13].

CPR and NOS were demonstrated to be irreversibily inactivated by iodonium compounds such as iodonium diphenyl chloride (IDP) or diphenylene iodonium chloride [14–16]. Moreover, iodonium salts inhibit the activity of a variety of other flavoproteins, such as mitochondrial NADH-dehydrogenase, neutrophil and macrophage NADPH oxidases, xanthine oxidase, nicotine oxidase or protoporphyrinogen oxidase [17–22]. On the basis of the reactivity of CPR or neutrophil NADPH oxidase with IDP, inhibition is proposed to occur through a reductive mechanism [14,15,19]. A reduced flavin tranfers one electron to IDP first. This results in the formation of a flavin semiquinone and a neutral diphenyliodonyl radical which disproportionates to iodobenzene and an extremely reactive phenyl radical. Then, a radical recombination between the phenyl radical and the flavin semiquinone is proposed to give a phenylated adduct [14,15,23]. ^{14}C-labelled IDP was used to identify the targets of the inhibitor in CPR. Radioactive labelling was recovered both on the polypeptide chain on or around Trp-419 and on FMN [14].

In order to investigate the possible inhibitory effect of IDP on SiR-FP, we have used a recently described SiR-FP fragment named SiR-FP60 [4]. SiR-FP60 retains both FAD and FMN with comparable contributions of the two flavins and the catalytic properties of the native protein, and it has the advantage of being monomeric [4]. In this paper, we demonstrate that SiR-FP60 is inhibited upon incubation with IDP and NADPH, as were CPR and NOS. However, in marked contrast with CPR, only FAD was modified. These results prompted us to construct a vector that could be used to express specifically and in large quantities a polypeptide corresponding to the FAD-binding domain of SiR-FP. This polypeptide was named SiR-FP43 with respect to its native molecular mass. Overexpressed SiR-FP43 was first enzymically characterized and then used to analyse the kinetics of the reaction of FAD with IDP. At least nine different phenylated FAD adducts were isolated. Among the five major products, two were identified. The difference of reactivity between SiR-FP and CPR towards IDP further illustrates the subtle differences between these two homologous proteins.

EXPERIMENTAL

Construction of the expression plasmid and expression of the recombinant SiR-FP43 protein

The construction of the expression plasmid pET-SiR-FP43, expected to encode for the FAD-containing and NADPH-binding domain of SiR-FP, was as described for the construction of pET-SiR-FP60 [4], the plasmid encoding for the monomeric FMN- and FAD-binding form of SiR-FP, except for the sense primer used. The N-terminal amino acid sequence was chosen to start with Ala-218, so the sense primer was designed as follows: 5′- ACGGAATTCATATG**GCTACTGGCGCGGTAAATGAA.** The coding sequence in bold is preceeded by an overhanging sequence containing an *Eco*RI site followed by an *Nde*I site (underlined) carrying the initiator codon ATG. The antisense primer was complementary to the C-terminal coding sequence of SiR-FP, as described for the construction of pET-SiR-FP60 [4]. The authenticity of the DNA insert generated by PCR was confirmed by sequencing and by MS analysis of the overexpressed protein. *E. coli* B834(DE3)pLysS was used as the host strain for the expression plasmid pET-SiR-FP43. Conditions of culture and expression were as previously outlined [11].

Purification of SiR-FP43

Total protein extracts were routinely obtained as previously described [13]. The pellet obtained after ammonium sulphate precipitation (60 % final saturation) was dissolved in a minimum volume of 50 mM Tris/HCl (pH 7.5) and loaded at 1 ml·min^{-1} on an Ultrogel AcA54 (Sepracor) filtration column (2.6 cm × 62 cm, 330 ml) previously equilibrated with the same buffer. Elution was run at the same flow rate and 3 ml fractions were collected. Flavin-containing fractions, as judged from the absorbance at 450 nm, were assayed for their flavin reductase activity. Active fractions were pooled and the two further purification steps on hydroxyapatite (Bio-Gel HTP from Bio-Rad) and Superdex-75 filtration column (Pharmacia LKB Biotechnology Inc.) were as described for SiR-FP60 [4]. At this stage, SiR-FP43 was electrophoretically pure.

SiR-FP60 and SiR-FP23 (the FMN-binding domain of SiR-FP) were obtained as previously described [4,11].

Protein analysis

Protein concentration was estimated using BSA as a standard [24] and the commercial Bio-Rad protein assay solution. The denaturated molecular mass of SiR-FP43 was estimated by 0.1 % SDS/12 %-PAGE [25], and its native molecular mass was ascertained by using a Bio-Rad precast 4–20 % gradient polyacrylamide gel run under non-denaturating conditions, or after elution of the calibrated Superdex-75 filtration column.

LC coupled with electrospray ionization MS (LC–ESI–MS) analyses were performed with a single quadrupole API 100 mass spectrometer (Applied Biosystems), 140 B pumps and 785 A detector (Perkin-Elmer). Reverse-phase LC was carried out on a 1 × 150 mm C_4 column (Vydac 214TP5115, 5 μm particle size). The mobile phase consisted of A: water/0.1 % formic acid and B: water/acetonitrile/formic acid (20/80/0.1 %). Protein (1 μg) was injected and a 20 min gradient from 10 % to 80 % of B was used.

Photochemical reduction of SiR-FP60 in the presence of deazaflavin and EDTA was run essentially as described in [11], except that the reaction was done under a nitrogen atmosphere in a glove-box. Complete photoreduction was checked by recording the spectrum of the solution with a Hewlett-Packard 8453 diode-array spectrophotometer. The one-electron reduced form of SiR-FP60 was obtained by air exposure of anaerobically fully reduced protein [4].

Cofactor analysis

Absorption spectra were recorded at room temperature in a quartz cell (10 mm light path) of 1 ml in volume using a Kontron Uvikon 930 spectrophotometer. Flavins were extracted from pure samples of protein, denatured by boiling for 3 min in the dark. The concentration of FAD was determined spectrophotometrically by means of its absorbance at 450 nm, using a molar absorption coefficient of 11.3 mM^{-1}·cm^{-1} [26]. Flavin adducts were separated on a C_{18} HPLC column (Macherey-Nagel Nucleosil 100-5, 4 mm × 25 cm) using an ammonium acetate (20 mM, pH 5.7) to methanol gradient (0–100 %) over 20 min at 0.8 ml·min^{-1}. The column was connected to a Hewlett-Packard Series 1100 system equipped with a diode-array spectrophoto-

meter. FMN and/or FAD extracted from pure preparations of SiR-FP43, SiR-FP23 or SiR-FP60 were injected separately for standardization of the retention times and of the separation conditions. Ion-trap electrospray MS analyses were run with an LCQ Thermoquest Finnigan apparatus.

Assay procedures

NADPH-dependent reactions were carried out as previously described, with either riboflavin, AcPyADP$^+$ or ferricyanide as electron acceptor [3,4]. The reactions were initiated by the addition of an appropriate amount of the protein solution. One unit of activity corresponds to the amount of protein catalysing the oxidation of 1 nmol of NADPH per min or the reduction of 1 nmol of AcPyADP$^+$ per min. Specific activity is defined as units per milligram of protein.

IDP (Fluka) was prepared as 10 mM stock solution in DMSO. Time-dependent inactivation of SiR-FP60 or SiR-FP43 was performed by adding various amounts of IDP to the total assay mixture. The effect of NADPH on the inhibition of SiR-FP60 or SiR-FP43 by IDP was studied by preincubating an appropriate amount of each enzyme for 5 min in the presence of an excess of NADPH (270 μM). Then, 0–7 μM IDP was added to give a final volume of 100 μl. The mixture was incubated at room temperature for 10 min, a time sufficient for inactivation to be complete. Then, the NADPH-dependent reactions of a 10 μl aliquot were assayed in the presence of the required complete assay mixture. Under these conditions, the amount of IDP present in the assay mixture was negligible. Assays were run at least in triplicate.

The irreversibility of the inactivation of SiR-FP43 by IDP was checked by measuring the reductase activity of an inactivated sample of protein before and after passage through an NAP-10 column (Pharmacia LKB Biotechnology Inc.). A control experiment with DMSO in place of IDP was run in parallel.

The involvement of phenyl radical in IDP inhibition of SiR-FP43 was checked by EPR spectrometry (Bruker EMX EPR spectrometer) as described in [15] with *N*-t-butyl-α-phenylnitrone (Sigma) as a spin trap.

RESULTS

FAD cofactor is the target of inhibition by IDP

SiR-FP60 can be assayed by its AcPyADP$^+$-transhydrogenase activity (AcPyADP$^+$-THase) or its ferricyanide reductase activity [3,4]. By using FMN-depleted SiR-FP, it was demonstrated that FAD was directly involved in the transhydrogenation of AcPyADP$^+$ via a Ping Pong Bi Bi mechanism [13]. FMN is the site of reduction of ferricyanide and this reaction requires the presence of both FAD and FMN [13]. Figure 1 shows that both SiR-FP60 activities are inhibited by IDP. A possible explanation of these observations is that FAD is the target of IDP in SiR-FP60 and that inhibition of the ferricyanide activity is a consequence of the modification of the FAD cofactor, making it unable to transfer electrons from NADPH to FMN.

Moreover, it is clearly evident that in the two cases, there are both time- and concentration-dependences on the inhibition by IDP. Progress curves closely resemble those obtained with CPR or neutrophil NADPH oxidase [14,19]. This suggests that SiR-FP60 responds to the inhibition by IDP in a similar manner, i.e. inactivation should be a two-step process requiring binding of IDP to the reduced protein before inhibition proceeds. As SiR-FP60 was shown to be inhibited by the reaction of its FAD cofactor with IDP, we have overexpressed the 43 kDa peptide corresponding to the FNR-like domain of the native protein.

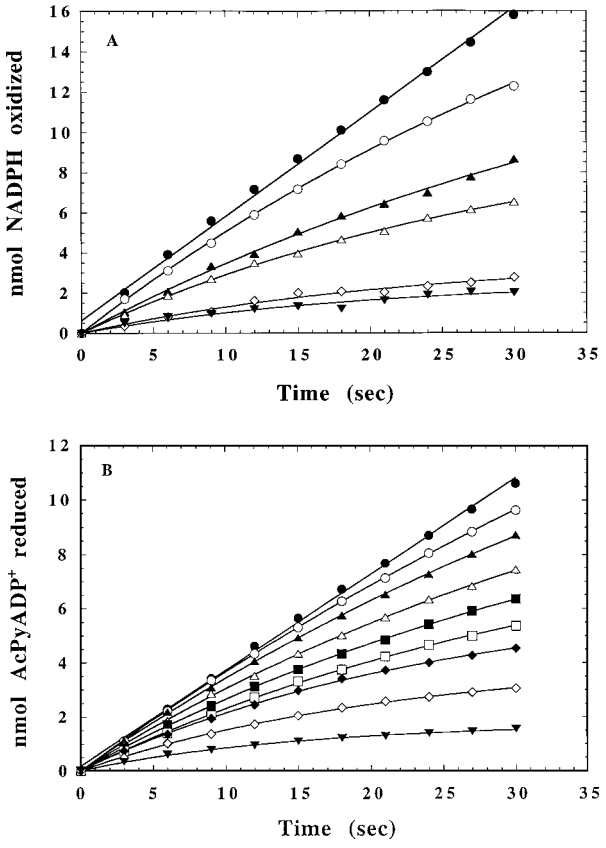

Figure 1 Inhibition of SiR-FP60 by IDP

(**A**) Ferricyanide reductase activity and (**B**) AcPyADP$^+$-THase activity of SiR-FP60 measured as a function of time in the absence (●) or in the presence of IDP: (○) 5 μM; (▲) 10 μM; (△) 15 μM; (□) 20 μM; (■) 30 μM; (◆) 40 μM; (◇) 60 μM; (▼) 100 μM.

Overexpression, purification and characterization of SiR-FP43

Previous preparations of SiR-FP43 obtained by limited proteolysis were not strictly homogenous, since cleavage occurred at different positions [3]. On the basis of N-terminal sequence analysis, Ala-218 was chosen as the starting amino acid and *E. coli* B834(DE3)pLysS was transformed with the expression vector pET-SiR-FP43.

Maximal expression was observed about 2 h after addition of isopropyl β-D-thiogalactoside to the growth medium. SiR-FP43 was recovered in the soluble extracts and assayed for its flavin reductase activity in the presence of riboflavin and NADPH [27]. A specific activity of 3500 was obtained, compared with about 50 for the extracts from the parental strain B834(DE3)pLysS. This shows an approx. 70-fold overexpression of SiR-FP43.

Soluble extracts stored as ammonium sulphate pellets were diluted in a minimal volume of 50 mM Tris/HCl, pH 7.5 (buffer A), and SiR-FP43 was purified to homogeneity by a three-step procedure. Flavin reductase activity was chosen for monitoring the purification (Table 1). After filtration on AcA 54 and hydroxyapatite chromatography in phosphate buffer, filtration on Superdex-75 improved the purification slightly and allowed SiR-FP43 to be obtained in the typical working buffer A. SiR-FP43 was eluted from the calibrated Superdex-75 column as a monomer of about 42000 Da (results not shown). This value fits very well with the theoretical molecular mass of 42925 Da

Table 1 Purification of SiR-FP43 from *E. coli* B834(DE3)pLysS(pET-SiR-FP43) extracts from 1 litre of culture

	Protein (mg)	Flavin reductase activity		Specific activity ($nmol \cdot min^{-1} \cdot mg^{-1}$)	Fold increase of specific activity
		Units ($nmol \cdot min^{-1}$)	Yield (%)		
Extracts	124	43 9660	100	3500	1
AcA 54	37	36 6120	83	9900	2.8
Hydroxyapatite	11.8	12 3900	28	10 500	3.0
Superdex-75	8	92000	21	11 500	3.3

Table 2 Kinetic parameters for the substrates of the diaphorase activities of SiR-FP43

The reported values correspond to the average for experiments performed in duplicate.

Substrate	Second substrate	K_m (μM)	V_m ($nmol \cdot min^{-1} \cdot mg^{-1}$)
NADPH	Riboflavin	88	30 000
$AcPyADP^+$	NADPH	190	72 860
Riboflavin	NADPH	66	23 260

deduced from the amino acid sequence. ESI–MS gave a molecular mass of 42931 ± 5 for SiR-FP43 (see below).

A pure preparation of SiR-FP43 gave a UV/visible light absorption spectrum characteristic of an oxidized flavoprotein with absorption maxima at 394 and 462 nm. The supernatant of a heat-denaturated sample of SiR-FP43 of known absorbance and concentration was analysed spectrophotometrically and by reverse-phase HPLC. These techniques unambiguously showed that SiR-FP43 contained 1 mol of FAD exclusively per mol of protein. The molar absorption coefficient of oxidized SiR-FP43 was thus found to be $9.33\ mM^{-1} \cdot cm^{-1}$ at 462 nm, a value in perfect agreement with that of $9.4\ mM^{-1} \cdot cm^{-1}$ reported for SiR-FP43 obtained by limited proteolysis [4].

The ability of SiR-FP43 to catalyse FAD-dependent reactions, i.e. transhydrogenation of $AcPyADP^+$ or reduction of free flavins, was monitored spectrophotometrically [3,4,27]. Kinetic parameters, K_m and V_m, were obtained from linear Lineweaver–Burk plots (Table 2). The parameters for NADPH were obtained with riboflavin as the second substrate. K_m and V_m values obtained with $AcPyADP^+$ are somewhat different from those reported for SiR-FP60. In spite of the ability of SiR-FP43 to fold independently, this suggests an influence of the FMN-binding domain on the kinetic parameters of the FAD-binding domain.

Inhibition of SiR-FP43 by IDP

Reaction of transhydrogenation of $AcPyADP^+$ in the presence of NADPH was chosen for studying the inhibition of SiR-FP43 by IDP, since such a reaction is absolutely characteristic of the presence of the FAD cofactor [13]. Figure 2(A) shows the time-dependent inactivation of SiR-FP43 by increasing concentrations of IDP at fixed concentrations of NADPH and $AcPyADP^+$. We calculated that more than 100 μM IDP were required to inhibit 31 nM SiR-FP43. As observed above with SiR-FP60, the inhibition is clearly time- and concentration-dependent. The rate of enzyme inhibition at each inhibitor concentration can be obtained by fitting each progress curve to the equation:

$$V = A(1 - e^{-kt})/k$$

where V is the turnover of substrate and A the initial rate. Under these conditions, k can be derived, and plotting k against IDP concentration (Figure 2B) shows a saturation behaviour with respect to the concentration of inhibitor. This indicates that binding of IDP to the enzyme is a prerequisite for inactivation. A maximal value of $0.053 \pm 0.005\ s^{-1}$ was estimated for the inhibition rate. Moreover, this representation allows the calculation of an apparent K_i for IDP [28]. A value of 18.5 ± 5 μM was obtained.

The rate of SiR-FP43 inhibition was also examined at fixed concentrations of IDP (20 μM) and $AcPyADP^+$ (472 μM) and increasing concentrations of NADPH (Figure 2C). Under these conditions, k increased to a maximal value, showing that IDP can be considered as an uncompetitive inhibitor with respect to NADPH. This strongly suggests that reduced SiR-FP43 was the target of IDP. Finally, increasing $AcPyADP^+$ concentration, at a fixed amount of both IDP and NADPH (Figure 2D), caused a decrease in the inhibition rate.

The effect of preincubation of SiR-FP43 in the presence of an excess of NADPH before the introduction of IDP was also studied. Under these conditions, it was possible to determine the minimal IDP/protein ratio required for complete inhibition of the protein (Figure 3). The same experiment was also carried out with SiR-FP60 (Figure 3), except that either $AcPyADP^+$ or ferricyanide was used as the electron acceptor during the enzymic assay. In both cases, 4 mol of IDP are required for the inhibition of 1 mol of protein. This result should be compared with the ratio of more than 3000 calculated in the presence of the substrate. This difference reflects the protective effect of the substrate competing with IDP for the reduced enzyme. Moreover, obtaining the same IDP/protein ratio for SiR-FP43 and SiR-FP60 provides additional evidence that the FAD site was the target

The fully inactivated SiR-FP43 sample, described above, was filtered on an NAP-10 column to remove low-molecular-mass compounds, such as NADPH and IDP, and was found to be devoid of $AcPyADP^+$-THase activity, even after 24 h of incubation (results not shown). In a control experiment in which DMSO replaced IDP, the activity remained constant. Inhibition was therefore resistant to removal of the inhibitor and NADPH, dilution in the assay mixture and 24 h of incubation. We can thus conclude that under these conditions, SiR-FP43 was irreversibly inactivated by IDP.

In the following, SiR-FP43 inhibited by addition of an excess of IDP to the NADPH-reduced enzyme will be called inactivated SiR-FP43.

FAD is covalently modified in inactivated SiR-FP43

Reduction of SiR-FP43 with NADPH caused the bleaching of the yellow colour of FAD, but the typical FAD UV/visible spectrum was recovered after thermal denaturation of the protein, due to reoxidation of the cofactor (Figure 4). In inactivated SiR-

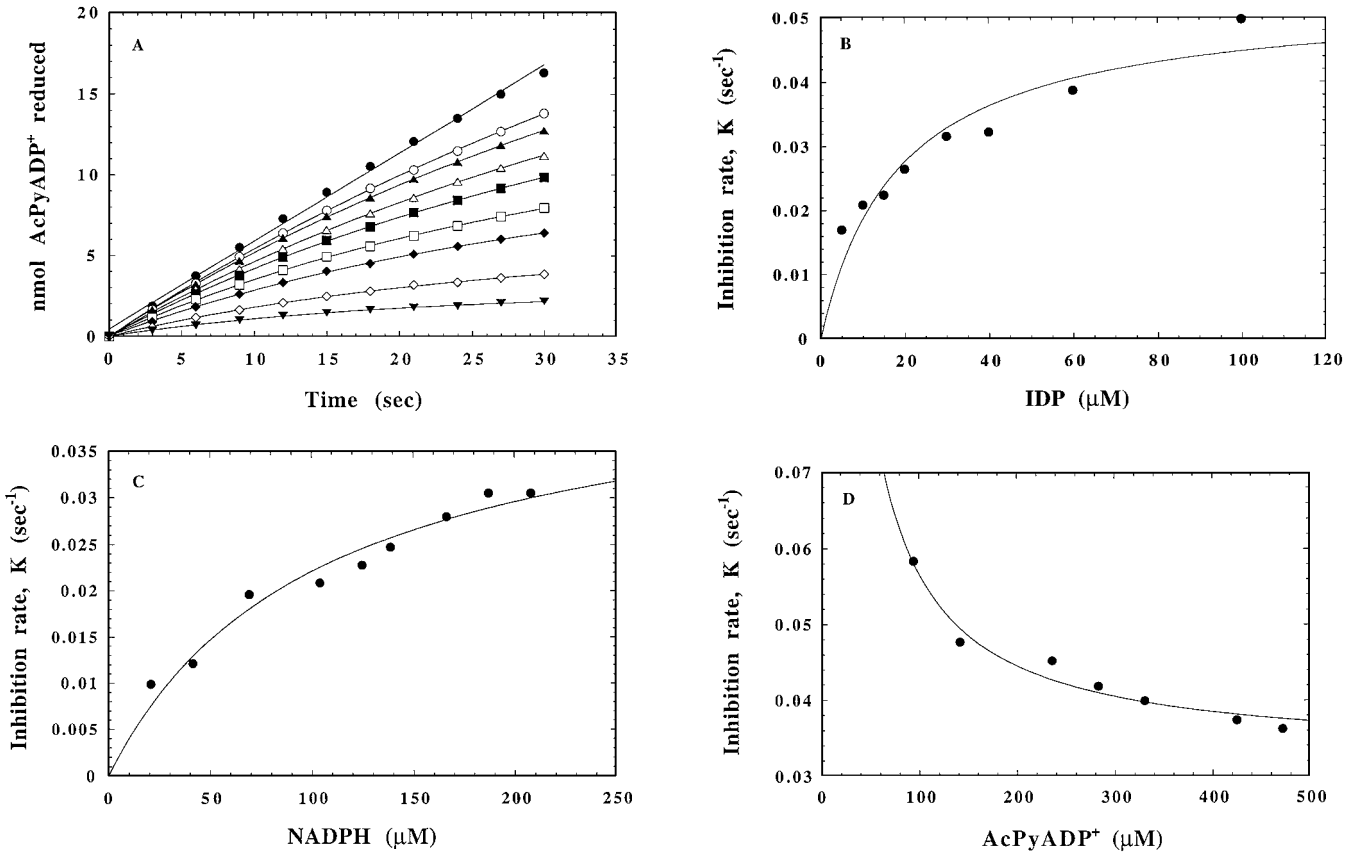

Figure 2 Inhibition of SiR-FP43 by IDP

(**A**) AcPyADP$^+$-Thase activity of SiR-FP43 measured as a function of time in the absence (●) or in the presence IDP: (○) 5 μM; (▲) 10 μM; (△) 15 μM; (■) 20 μM; (□) 30 μM; (◆) 40 μM; (◇) 60 μM; (▼) 100 μM. (**B**) Plot of the inhibition rat (k) as a function of IDP concentration. k values were calculated from the results in (**A**). (**C**) Plot of k as a function of NADPH concentration in the presence of 20 μm IDP and 472 μM AcPyADP$^+$. (**D**) Plot of k as a function of AcPyADP$^+$ concentration in the presence of 20 μM IDP and 250 μM NADPH.

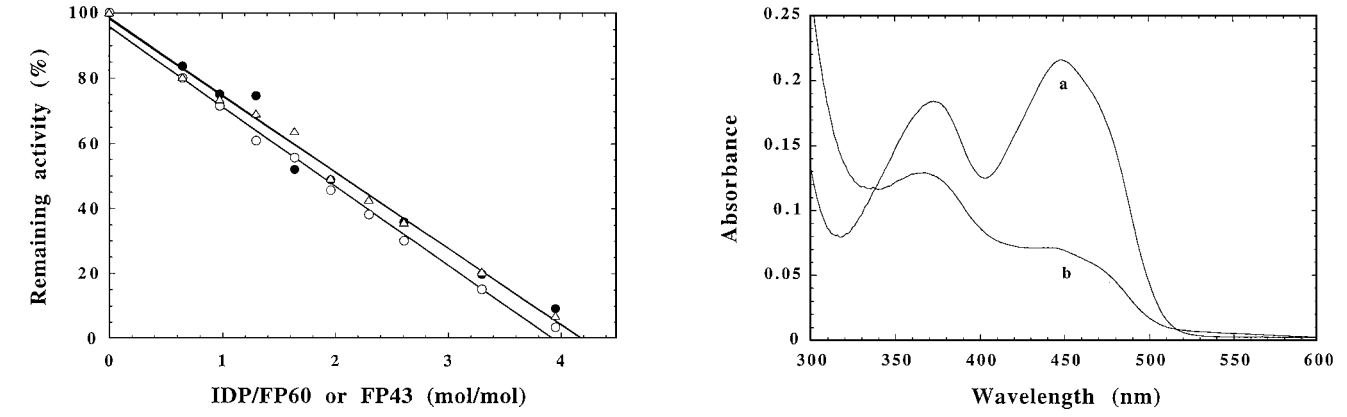

Figure 3 Determination of the minimal IDP/protein ratio required for complete inhibition

SiR-FP60 (9.1 μg) or SiR-FP43 (6.6 μg) was incubated in 100 μl final volume of Tris/HCl (50 mM, pH 7.5) in the presence of 270 μM NADPH for 5 min. Then, an increasing amount of IDP in a constant minimal volume was added to each tube and the mixture was incubated again for 5 min. Then, 10 μl of sample was assayed for AcPyADP$^+$-THase activity with SiR-FP43 (●) or for AcPyADP$^+$-THase activity (○) and ferricyancide reductase activity (△) with SiR-FP60. Remaining activity was expressed as the percentage of total activity assayed under the same conditions but in the absence of inhibitor.

Figure 4 Modification of FAD during inhibiton of SiR-FP43 by IDP

SiR-FP43 (375 μg) was incubated in 500 μl of Tris/HCl (50 mM, pH 7.5) in the presence of 250 μM NADPH for 5 min. Then, 2 μl of 10 mM IDP (40 μM final) were added. In the control experiment, 2 μl of DMSO was added. After 5 min of incubation, the two samples were heat-denaturated by boiling in the dark for 3 min. Denaturated protein was removed by centrifugation and the spectrum of the supernatants was recorded. Spectrum a, control experiment; spectrum b, IDP-inhibited SiR-FP43.

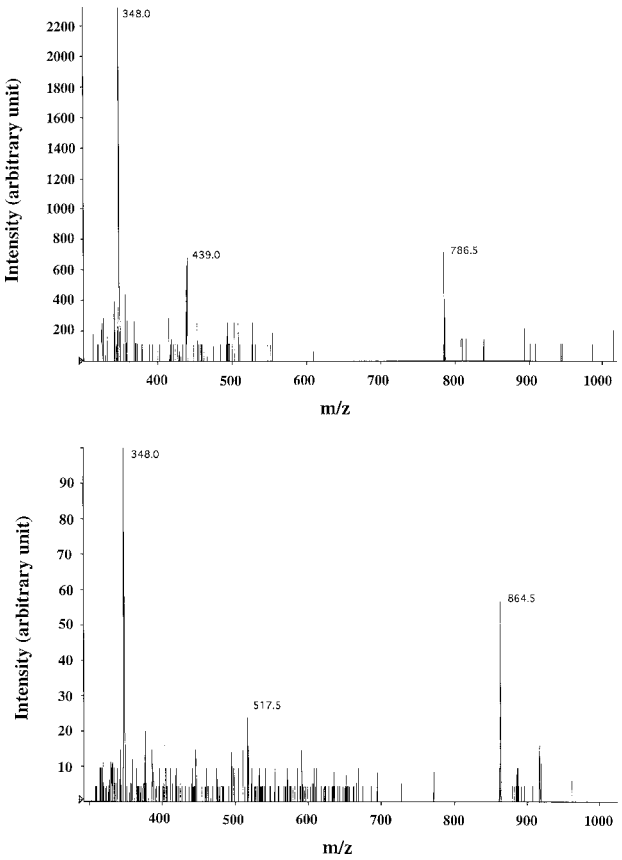

Figure 5 Electrospay analysis (positive-ion mode) of FAD extracted before (top) or after (bottom) inhibition of SiR-FP43 by IDP

Operating conditions are described in the Experimental section.

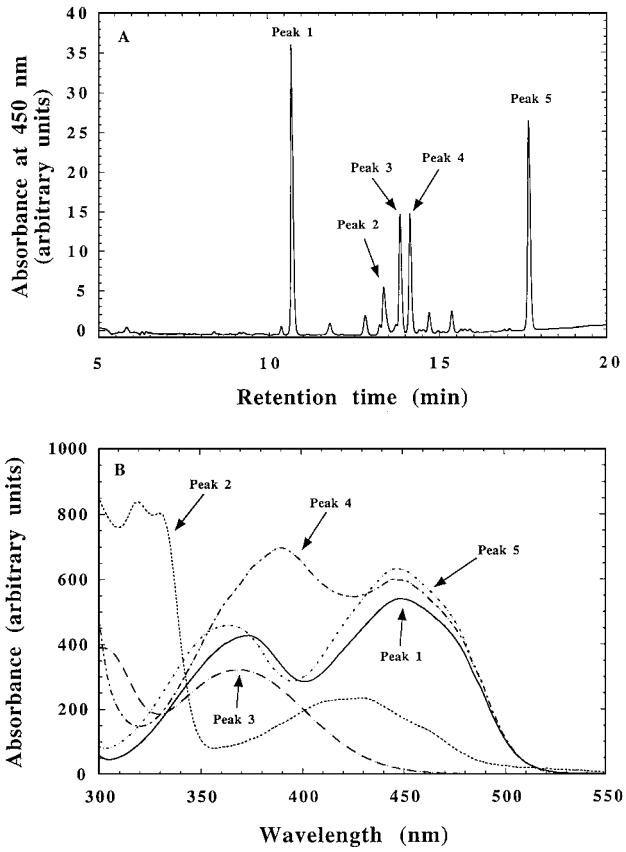

Figure 6 Separation and identification of the FAD phenylated adducts

(**A**) Typical chromatogram recorded at 450 nm obtained after HPLC separation of FAD adducts on a C_{18} column; operating conditions are described in the Experimental section. (**B**) UV/visible spectra of the compounds responsible for peaks 1–5.

FP43, the chromophore was definitively lost and the UV/visible spectrum of the FAD cofactor extracted by thermal denaturation of the protein was altered (Figure 4), suggesting that FAD was modified by reaction with IDP.

During MS analysis, the FAD cofactor was extracted and separated from the protein. In a control experiment run with an active protein (Figure 5, top), a molecular ion (MH^+) with $m/z = 786.5$, corresponding to native FAD, is clearly visible along with two fragments at $m/z = 348$ and 439, resulting from a fragmentation of FAD between the β-phosphorus and the oxygen of the pyrophosphate moiety of the flavin, giving the nucleotidic part of the molecule with the α-phosphorus at 348 and the flavinic part with one associated phosphorus at 439. With inactivated SiR-FP43 (Figure 5, bottom), the molecular mass obtained for the flavin was 864.5 and corresponded to FAD with one attached phenyl ring ($m/z = 78$). Along with the modified cofactor, two fragments were present with molecular masses of 348 and 517.5. This shows that only the flavinic part of the molecule was modified by the covalent attachment of one phenyl, very likely on the isoalloxazine ring as previously described [14,23].

Whether the polypeptide chain was also altered was checked by electrospray MS analysis of inactivated SiR-FP43 and comparison with the active control protein. After extraction of the cofactor, the two proteins had a comparable molecular mass of 42931 ± 5 (results not shown), corresponding to the theoretical mass of the protein, and proving that the polypeptide chain was thus not modified by addition of one or more phenyl groups.

Analysis of the flavin adducts by HPLC and MS

The spectrum depicted in Figure 4 closely resembles that of the mixture of three different phenylated FMN adducts obtained by reaction of pure photoreduced FMN with IDP [23]. Each of these compounds had a characteristic UV/visible spectrum resulting from the addition of one phenyl group either on the N-5, C-8 or C-4a position. In order to elucidate whether the modified FAD, extracted from inactivated SiR-FP43, consisted of several phenylated adducts, a sample of the reaction product was analysed by HPLC on a C_{18} column. Although the separation was followed at several wavelengths, only the typical chromatogram obtained at 450 nm is shown in Figure 6(A). This chromatogram shows at least nine peaks eluting between 10 and 18 min. The UV/visible spectrum of each of the separated compounds allowed the identification of three of them. Peak 1 had the retention time and the spectral characteristics of FAD (Figure 6B). The spectra of peaks 2 and 3 probably correspond to the C-8 and C-4a adducts respectively. The identification of the C-4a adduct was based on the fact that the loss of the 450 nm peak indicated the stabilization of a reduced flavin chromophore.

Moreover, the spectrum of this compound was very similar to those described by Ghisla et al. [29] and by Kemal and Bruice [30] for dihydroflavins with C-4a substitution and to the C-4a-phenyl adduct of FMN characterized in [23]. The description of the spectrum of the C-8 adduct [23], with maxima at 320, 330 (shoulder) and around 430 nm, fits perfectly with those of the compound giving peak 2. To our knowledge, phenyl addition giving the compound responsible for peak 4 has never been reported. Peak 4 was characterized by light absorption spectroscopy (Figure 6B).

Peak 5, eluting at 17.7 min, was easily isolated and was relatively intense. We have thus tried to characterize it by electrospray ion trap MS. This compound was eluted 7 min after authentic FAD and was thus much more hydrophobic. The spectrum was that of an oxidized flavin and was very similar to that of FAD, except for the marked hypsochromic shift in the 360 nm region. Unfortunately, this compound was not stable enough to resist freeze-drying concentration and it was thus impossible to accumulate it. MS analysis directly after HPLC purification showed a product with a mass corresponding to FAD with one phenyl adduct (results not shown). However, this product was destroyed during the experiment and ion trapping was impossible. FAD could be modified in a position which would not eliminate the typical flavin yellow colour; however, for reasons that are as yet unclear, this modified compound is unstable.

The involvement of a phenyl radical in the reaction of IDP with FAD was ascertained by trapping this radical with *N*-t-butyl-α-phenylnitrone [15] and identifying the product of the reaction by its characteristic six-line EPR signal (results not shown).

Finally, it should be noted that when flavins extracted from IDP-inhibited SiR-FP60 were separated on a C_{18} column and compared with those extracted from a control protein, the peaks corresponding to FMN had the same intensity and only FAD adducts were observed (results not shown). The same result was obtained when IDP was added to photoreduced SiR-FP60 (results not shown) under anaerobiosis or to the one-electron reduced form of SiR-FP60 ($FAD/FMNH^{\circ}$). These results confirm that none of the reduced forms of FMN in SiR-FP60 reacts with IDP.

DISCUSSION

SiR-FP60, the monomeric model of the SiR-FP component, was shown to be inactivated by the reaction of its reduced FAD cofactor with IDP. This was confirmed with the recombinant 43 kDa SiR-FP43 protein, which corresponds to the FNR-like domain of SiR-FP containing the NADPH-binding site and the FAD cofactor only. In addition, SiR-FP43 was found to fold independently, exhibiting the NADPH-dependent reductase activities associated with the presence of FAD in SiR-FP. SiR-FP43 was thus chosen for delineating the reaction of IDP with FAD.

Inhibition of flavoproteins by IDP is supposed to result from electron transfer from reduced flavins to IDP, the one-electron reduced form of IDP generating a phenyl radical which couples with the flavin semiquinone radical [14,15]. An amino acid in the flavin site can also be alkylated. With SiR-FP43, IDP was shown to be an uncompetitive inhibitor with respect to NADPH, and $AcPyADP^+$ a competitive inhibitor for the inactivation. The apparent K_i calculated for IDP ($18.5 \pm 5\ \mu M$) demonstrates a very good affinity of the inhibitor for the enzyme when this value is compared with the apparent K_m values of the different substrates (Table 2). The maximal rate of inactivation ($0.053 \pm 0.005\ s^{-1}$) reflects a very fast inhibition process. If we consider that inactivation by IDP is irreversible and that a phenyl radical intermediate is generated upon reaction of IDP with SiR-FP43 and NADPH, these results show that IDP is a very efficient inhibitor of SiR-FP43, probably reacting with the reduced protein according to the mechanism described by Tew [14].

This reductive mechanism was suggested from work with rat NADPH-CPR [14], an enzyme sharing a common genetic origin and a common operating cycle with SiR-FP [5,9,10]. However, some subtle differences have been noticed already between the two enzymes [4,11] and reaction with IDP illustrates an additional point of divergence. Firstly, the phenyl radical from IDP reacted in CPR with both FMN and a tryptophan residue. Second, kinetic analysis of the inhibition of CPR by IDP gave an apparent K_m value of 2.8 mM and an inactivation rate value of $5.2\ min^{-1}$. Third, 21 mol of IDP are required for complete inhibition of reduced CPR [14]. We can conclude that IDP was not a very efficient inactivator of CPR, but the most intriguing result was the difference for homologous proteins as far as the target site is concerned. In the case of SiR-FP60 and SiR-FP43, it is perfectly clear that FAD reacted exclusively with IDP. The mass of the SiR-FP43 polypeptide remained unchanged after inhibition by IDP, and FMN extracted from inactivated SiR-FP60 was not modified.

It was proposed that the flavoenzymes which function as one-electron donors, accepting two electrons but giving them one-by-one to an acceptor, were sensitive to and inhibited by iodonium compounds [15], whereas those which transfer two electrons during catalysis were insensitive. With SiR-FP, we demonstrate that such a hypothesis is not correct. Despite the fact that the FMN moiety in SiR-FP possesses the redox potential required for reduction of IDP (the potential of the $FMNH_2/FMNH^{\bullet}$ couple is -335 mV [2,11], compared with a midpoint potential of -332 mV for IDP [31]), and is well known to stabilize a semiquinone radical [2,4,11], no reaction between reduced FMN and IDP could be observed. We can thus conclude that, without calling the reductive mechanism of Tew [14] into question, inactivation by IDP is not a reliable indicator for flavin radical intermediates in catalysis. What is more likely, we suggest, is that IDP did not gain access to the FMN site in SiR-FP60, whereas it can be recognized as a pseudo-substrate at the NADPH- and $AcPyADP^+$-binding site.

Among the nine products separated by HPLC on a reverse-phase column, three were identified. The less hydrophobic compound was identified as the authentic FAD. Moreover, two compounds that were eluted later because of the chemical modification were identified as FAD phenylated adducts in the C-4a or C-8 position, on the basis of their spectral similarities with FMN derivatives previously characterized by NMR and MS [23]. Surprisingly, the phenylated adduct in the N-5 position could not be observed. Moreover, recovery of authentic FAD after cofactor extraction does not seem to be compatible with complete inhibition of the protein. Since the FMN N-5 adduct was found to be highly unstable [23], we suggest that formation of the FAD N-5 adduct actually occurs during full inhibition of SiR-FP43, but this adduct is converted into FAD during thermal denaturation of the protein. Finally, the UV/visible spectrum of the more hydrophobic compound showed similarity with that of FAD, but the difference in elution time demonstrated the presence of an additional hydrophobic group. This compound was found to be also unstable and hence we could not obtain good mass spectral data. It has previously been reported that FAD adducts were transformed after isolation and thus resisted identification [19].

In conclusion, through the study of the reaction of its model proteins with IDP, we have shown that SiR-FP is highly sensitive to inhibition by iodonium compounds. This is an additional difference which separates SiR-FP and CPR. For the first time, some FAD phenylated adducts extracted from a protein were identified. These results demonstrate that protein-bound flavins can react as free flavins. Further work and a scale-up of our flavin purification procedures are needed for the identification of all the isolated adducts.

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