NADPH-sulfite reductase flavoprotein from *Escherichia coli*: contribution to the flavin content and subunit interaction

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Abstract The flavoprotein component (SiR-FP) of the sulfite reductase of *E. coli* is an octamer of the 66 kDa α subunit. It was shown to be cleaved in two peptide fragments. The 23 kDa fragment has been purified as a polymer of 8–10 subunits. It corresponds to the N-terminal part of the native protein and was shown to contain essentially FMN as cofactor. The 43 kDa fragment is monomeric. It contains exclusively FAD and remains able to catalyze efficiently NADPH-dependent reductions. One can conclude that each α-chain of SiR-FP is composed of two distinct domains, one binding FAD and the other FMN and that the FMN-binding domains cooperate for a head-to-head subunit interaction.

Key words: Sulfite reductase; Flavoprotein; Flavin; Quaternary structure; *Escherichia coli*

1. Introduction

NADPH-sulfite reductase of *Escherichia coli* or *Salmonella typhimurium* is a multimeric and soluble hemoflavoprotein which catalyses the six-electron reduction of sulfite to sulfide [1,2]. It provides the cell with reduced sulfur for the biosynthesis of L-cysteine. The native enzyme is composed of two different peptides with a subunit structure $\alpha_8\beta_4$ for the holoenzyme. The octamer constituent α is a flavoprotein (SiR-FP; M_r = 528,000) and the tetramer β is a hemoprotein (SiR-HP; M_r = 252,000). The holoenzyme has been proposed to contain 4 FMN, 4 FAD, 4 Fe_4S_4 clusters and 4 sirohemes. Thus each α chain, all coded for by the *cysJ* gene, might bind only one flavin prosthetic group, FAD or FMN. In addition, SiR-FP contains the sites for specific recognition of NADPH and thus provides the entry port for electrons [2–4].

The electron transfer pathway from NADPH to sulfite has been clearly demonstrated [3,5]. FAD serves as an input center for receiving electrons from NADPH, while FMN serves as a mediator for the rapid transfer of these electrons to SiR-HP where sulfite is reduced. However, electrons can also be transferred from FMN to other acceptors such as cytochrome *c* or ferricyanide or from FAD directly (i.e. without the mediation of endogenous FMN) to AcPyADP$^+$ or free flavins (riboflavin, FMN, FAD) [3–6].

We have recently constructed an α overproducing strain and completely characterized the NADPH:flavin oxidoreductase activity of SiR-FP [7]. In contradiction with previous reports, we have demonstrated that each α subunit contains two flavin prosthetic groups, very likely one FAD and one FMN. Moreover, sequence homology of SiR-FP to NADPH:cytochrome P450 reductase and NO synthase, in which a single polypeptide chain binds a FAD and 1 FMN suggests that each α chain possess one binding site for FMN in the N-terminal part of the polypeptide and one binding site for FAD in its C-terminal part. A binding domain for NADPH can also be found in the C-terminal sequence [4,8–10].

To solve definitively this question we have undertaken cristallogenesis of pure recombinant SiR-FP. During preparation of concentrated samples of SiR-FP, we observed a sensible loss in activity due to the spontaneous cleavage of the protein when glycerol was omitted.

Here, we report the purification and the characterization of the two fragments derived from cleaved SiR-FP. This provides a strong new support to the notion that α binds FAD and FMN at distinct sites.

2. Materials and methods

2.1. Materials

Riboflavin, 3-acetylpyridine adenine dinucleotide phosphate (AcPyADP$^+$), cytochrome *c* and ferricyanide were purchased from Sigma. Superdex-75 was from Pharmacia LKB Biotechnology Inc. All other chemicals were of the purest grade.

SiR-FP was purified from the α overproducing strain as previously described [7]. Protein concentration was estimated by the method of Bradford using bovine serum albumin as a standard [11].

2.2. Enzymic assays

NADPH-dependent reactions were carried out as previously described [4,7] with 0.25 mM NADPH, an electron acceptor and an appropriate amount of protein. Electron acceptors were present at the following concentrations: 0.1 mM riboflavin, 0.3 mM ferricyanide, 0.2 mM AcPyADP$^+$ and 0.1 mM cytochrome *c*. Absorbance changes were followed using a Kontron Uvikon 930 spectrophotometer at 340 nm for riboflavin and ferricyanide; at 363 nm for AcPyADP$^+$ and at 550 nm for cytochrome *c*. The following extinction coefficients were used: NADPH, ε_{340} = 6.22 $mM^{-1} \cdot cm^{-1}$; AcPyADP$^+$, ε_{363} = 5.6 $mM^{-1} \cdot cm^{-1}$; reduced cytochrome *c*, ε_{550} = 22 $mM^{-1} \cdot cm^{-1}$. The reaction was initiated by the addition of the protein fraction. Specific activities are defined in the legend of Table 1.

2.3. Other assays

FAD and FMN extracted from the purified fragments were measured fluorimetrically according to [12] using a Perkin Elmer LS 450 fluorimeter.

NH_2-terminal amino acid sequence determination was performed by Dr. J. Garin and M. Vinçon (Centre d'Etudes Nucléaires, Grenoble, France) using an Applied Biosystems gas-phase sequencer model 477 A with on-line analysis of the phenylthiohydantoin derivatives.

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3. Results

3.1. Spontaneous cleavage of SiR-FP and identification of the cut site

From the overproducing strain LS 1312(pCYS J) containing the gene coding for the α subunit of the sulfite reductase, large amounts of pure and fully active SiR-FP were obtained in two purification steps [7]. When the protein is stored in liquid nitrogen in the presence of 5% glycerol at a concentration below 1 mg/ml, it is not degraded for months, as judged from the SDS-PAGE pattern.

Concentration of the protein at more than 1 mg/ml in the absence of glycerol led to an almost complete loss of the cytochrome *c* reductase activity within 26 h at 25° C, while the flavin reductase activity decreased in the same time to 80% of the original value (Fig. 1A). A control of the integrity of the protein by SDS-PAGE revealed that after 30 min incubation, two peptides with apparent molecular masses of 43 and 26 kDa are present along with the remaining intact SiR-FP α subunit (M_r = 66,000). After 36 h, all the α subunits have apparently be cleaved into the 43 and 26 kDa peptide fragments (Fig. 1B).

The NH_2-terminal amino acid sequencing of these two fragments was performed by gas-phase sequence analysis. The 10-first amino acids of the 26 kDa polypeptide were unambigously identified as the N-terminal sequence of the native protein. Analysis of the 43 kDa fragment gave two sequences equally represented (VATGAVNE and TGAVNE). This corresponds to a cut site located between serine-217 and valine-218 or between alanine-219 and threonine-220. According to these results, the calculated molecular masses of the two fragments are 42,820 or 43,024 Da and 23,230 or 23,430 Da. The sum of these values fits very well with a molecular mass of 66,237 Da for the intact α subunit.

This kind of cleavage could be attributed to a thermolysine-type protease contaminating our SiR-FP preparations. Interestingly, addition of 1 mM EDTA in the storage buffer prevents the cleavage and SiR-FP keeps stable for several days at 4°C under these conditions.

3.2. Purification and characterization of the fragments

A completely cleaved preparation of SiR-FP was subjected to filtration on Superdex-75. The elution profile showed two main peaks (data not shown). Peak I eluted in the dead volume indicating a native molecular mass higher than 75 kDa, while peak II corresponded to a protein with a molecular mass of about 45 kDa. SDS-PAGE analysis showed that peak I is a polymer of the 23 kDa fragment, while peak II contains the 43 kDa fragment which is thus a monomer. Native gel electrophoresis confirmed these results since peak I gave a broad band centered at about 200–230 kDa indicating that the 23 kDa fragment is a polymer with 8–10 subunits. Peak II gave a discrete band with the expected molecular mass of 43 kDa, confirming again the monomeric feature of this polypeptide.

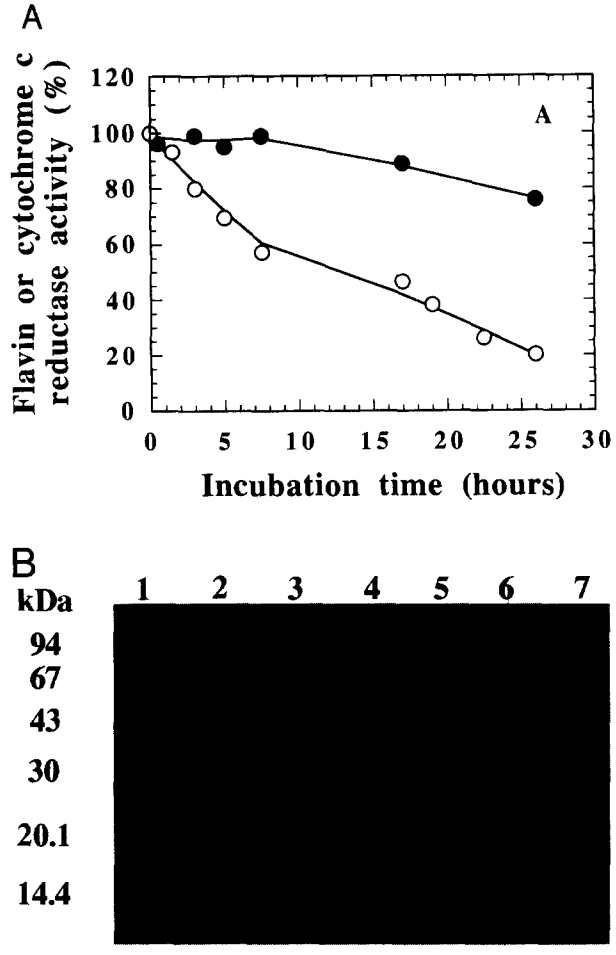

Fig. 1. Spontaneous cleavage of the α subunit of SiR-FP. The cleavage was followed by measuring the flavin reductase (●) and the cytochrome *c* reductase (○) activities (panel A) and by SDS-PAGE analysis (panel B). Enzymatic activities were measured as described in section 2 on aliquots removed at the indicated time intervals from a 8 mg/ml solution of SiR-FP incubated at 25° C in the absence of glycerol. For gel analysis, each line contains 1.5 μg of protein. Lane 1 shows protein molecular weight markers, while lanes 2–7 represent samples removed at 0, 0.5, 7.5, 17, 27 and 36 h, respectively. Gel was stained with Coomassie blue.

Table 1
Flavin content and catalytic activities of SiR-FP and derived peptide fragments

Sample	Flavin prosthetic groups[a]		Acceptors for NADPH-dependent reduction[b]		
	FAD	FMN	riboflavin	$AcPyADP^+$	cytochrome *c*
Native SiR-FP	0.94	0.71	14,000	20,000	90,000
23 kDa fragment	0.01	0.70	1,760	1,540	8,920
43 kDa fragment	0.98	0	13,130	16,830	2,090

[a]Flavins were measured fluorimetrically as described in section 2. Values are expressed as mol flavin per mol polypeptide and are the mean of at least two determinations.
[b]NADPH : acceptor oxidoreductase activities were assayed as described in section 2. Values are expressed as nmol acceptor reduced per min and per mg protein. They are the mean of three determinations.

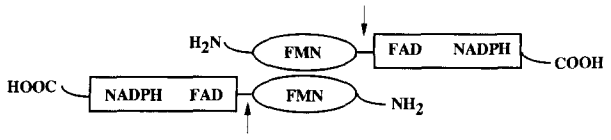

Fig. 2. Schematic representation of the building blocks for the polymerization of the α subunits of SiR-FP. The N-terminal regions of α bind FMN and are the site of subunits interactions which proceed until completion of the polymeric structure (8–10 subunits). The C-terminal regions bind FAD and contain the NADPH-binding sites. They may exist as independent monomeric extensions. Arrows indicate the site of cleavage.

The spectral analysis of each purified fragments revealed the presence of the visible absorbance due to flavins. By fluorimetry, it was possible to determine the flavin content of the two polypeptides. As shown in Table 1, the 43 kDa polypeptide was found to contain exclusively FAD (0.98 mol per mol fragment), while the 23 kDa fragment contained 0.01 mol FAD and 0.7 mol FMN per mol fragment. These values show unambiguously that each α chain of SiR-FP contains both FMN and FAD probably equally represented. The clearly established lability of FMN [2,5] could explain the lower FMN content in the 23 kDa fragment. The presence of FAD in trace amount in this fragment could be due to contamination of peak I with very small amount of remaining intact SiR-FP polymerized in its octameric form.

Catalytic properties of each fragment are depicted in Table 1. The 43 kDa polypeptide was able to catalyze the NADPH-dependent reduction of free flavins and $AcPyADP^+$ with specific activities corresponding to those previously reported for the FMN-depleted SiR-FP preparation [7]. This indicates clearly that the NADPH-binding site belongs to the 43 kDa fragment. Moreover, because of the loss of the FMN-binding peptide, the 43 kDa fragment was unable to catalyze efficiently the NADPH-dependent reduction of ferricyanide or cytochrome *c*.

The 23 kDa fragment displayed only slight activities, corresponding in all probability to contamination of peak I with remaining intact SiR-FP, confirming that it did not contain the NADPH-binding site.

4. Discussion

SiR-FP, the flavoprotein subunit of the sulfite reductase, is an octamer in its active form and was previously found to contain 8 flavin prosthetic groups: 4 FAD and 4 FMN [2,5].

However, the recent reevaluation of the flavin content [7] and sequence homologies of α to NADPH:cytochrome P450 reductases [5,8] and to NO-synthases [9,10], two families of enzymes containing both FAD and FMN as prosthetic groups and shuttling electrons from NADPH to external acceptors via the flavin cofactors, suggest that the ribitylphosphate moiety of FMN could bind amino acids in the region 69–79, while the region 149–180 is a putative site for its pyrimidine part. The phosphate moiety of FAD might be bound to the region 236–288 and the isoalloxazine ring is suggested to bind the 386–392 part. A NADPH-binding domain could also exist in the C-terminal region with the pyrimidine moiety of NADPH binding to the 455–598 region which contains a GPGTG motif [5,6]. Moreover, the comparison of the amino acid sequence of SiR- FP with those of flavodoxin from *D. vulgaris*, a FMN-binding protein, and of the spinach ferredoxin:$NADP^+$ oxidoreductase, a FAD-binding protein, has also revealed a FMN-binding domain and a FAD-binding domain respectively in the N-terminal region and in the C-terminal region of the SiR-FP α-subunit [5].

In this paper, we have taken advantage of the spontaneous cleavage of SiR-FP in two fragments for confirming our model with two flavin cofactors per α chain [7] and for characterizing the interaction between the subunits in SiR-FP. The 23 kDa fragment is polymeric with a native molecular mass around 200–230 kDa. Its NH_2-terminal amino acid sequence is that of the native SiR-FP and we found essentially FMN in this fragment. The catalytic activities of this fragment in NADPH-dependent reactions are very low, probably due to contamination of the preparation with a small amount of intact SiR-FP. In contrast, the 43 kDa fragment is monomeric and contains exclusively one mol FAD per mol fragment. This is in perfect agreement with the model we have recently proposed for the structure of SiR-FP, in which each α-chain contains 2 flavin cofactors [7]. The NH_2-terminal sequence analysis of the 43 kDa fragment gave two starting amino acids, valine-218 or threonine-220. Moreover, the 43 kDa fragment remains able to catalyze the NADPH-dependent reduction of free flavins or $AcPyADP^+$. Thus, the NADPH-binding domain belongs to this fragment.

We thus suggest a quaternary structure (Fig. 2) in which the FMN-binding domains cooperate for polymerization in a head-to-head subunit interaction. In this putative model, the site of cleavage remains always accessible and the FAD and NADPH domains may exist as independent monomeric extensions in the native polymer. It is very likely that the β hemoprotein subunit, required for sulfite reduction, is in interaction with the FMN-binding domain of α. Crystallographic studies of native SiR-FP and of the 43 kDa fragment are in progress and should give more informations about the complex structural organization of the fascinating enzyme SiR-FP.

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