Incorporation of iron–sulphur clusters in membrane-bound proteins
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Abstract
The completely sequenced genome of the cyanobacterium Synechocystis PCC 6803 contains several open reading frames, of which the deduced amino acid sequences show similarities to proteins known to be involved in FeS cluster synthesis of nitrogenase (Nif proteins) and other FeS proteins (Isc proteins). In this article, the results of our studies on these proteins are summarized and discussed with respect to their relevance in FeS cluster incorporation in chloroplasts. In cyanobacteria, there appears to exist several pathways for FeS cluster synthesis.

Introduction
Iron–sulphur (FeS) proteins are present in all organisms studied so far. The nature of their redox-active centre has attracted significant interest, and was clarified in the 1960s by the chemical synthesis of FeS clusters in apoproteins [1,2]. However, the chemical synthesis of FeS clusters required millimolar concentrations of iron salts and sulphide, both known to be highly toxic for living cells. Therefore the sources of iron and sulphur in vivo had to be different from those used in the in vitro reconstitution experiments. The first light on the biological mechanism of FeS cluster synthesis was shed in 1993 when Dean and colleagues discovered the enzymic activity of a protein, NifS [3], an enzyme essential for the synthesis of active nitrogenase Fe protein in Azotobacter vinelandii [4]. NifS was shown to be a cysteine desulphurase, which converts free cysteine into alanine. The sulphur from free cysteine becomes bound to a cysteine side chain in the active centre of NifS. In the presence of a reductant this sulphur is released as sulphide. Another protein essential for the synthesis of active nitrogenase Fe protein, NifU, was shown to be able to bind one iron ion at its N-terminal part [5]. When the Fe-binding NifU was then incubated with NifS in the presence of cysteine and a reductant a (relatively instable) FeS cluster is assembled at NifU [6]. This preassembled cluster is thought to be transferred then to the apoprotein of nitrogenase.

Proteins with similarity to NifS and NifU are encoded in the genome of almost all organisms, including the nitrogen-fixing organisms themselves [7]. Biochemical and genetical studies demonstrated that at least A. vinelandii, E. coli, yeast and the cyanobacterium Synechocystis PCC 6803 contain proteins with a function similar to NifS responsible for FeS cluster synthesis in proteins other than nitrogenase. With NifU, the situation is more complicated, since the protein from A. vinelandii consists of three domains, and some
organisms contain "NifU-like" proteins with similarity to only one of these domains. In *Synechocystis* there exists an open reading frame (ORF) (ss12667) that encodes a protein with similarity to the C-terminal domain of NifU from *A. vinelandii*, but the N-terminal Fe/FeS-binding domain is missing. The same situation exists in a number of Archaea, of which the genome has been completely sequenced. Therefore, although essentially similar proteins are involved in FeS cluster synthesis in various organisms, the mechanism appears to differ significantly. In addition, in many, but by no means all, prokaryotic organisms, the NifS and NifU homologues are encoded in the so-called *isc* gene clusters/operons, which contain up to seven additional genes [7] that are important for FeS cluster assembly [8]. In cyanobacteria such as *Synechocystis*, the situation is even more complex, because there exists an additional FeS assembly mechanism involving C-DES, a cyst(e)ine desulphurase that can be used *in vitro* to assemble the FeS cluster of ferredoxin [9]. In the following article, our current knowledge concerning FeS cluster assembly in cyanobacteria is summarized, and its significance in terms of chloroplasts will be discussed.

**Synechocystis NifS-like proteins Slr0387, Slr0704 and Slr0077**

Among the three *Synechocystis* NifS-like proteins, Slr0387 is the most similar protein to NifS from *A. vinelandii*, whereas Slr0077 shows relatively low sequence similarity. In order to obtain sufficient material to study the enzymic activities, the three proteins were overexpressed in *E. coli*. After overexpression of the ORFs slr0387 and slr0704, a significant increase of cysteine desulphurase activity was detected in extracts from *E. coli* cells [10] (K. Jaschkowitz and A. Seidler, unpublished results). The gene product of slr0387 was purified and its enzymic activity was studied in detail. The enzyme converted cysteine into alanine and sulphur or, in the presence of a reductant, sulphide. In the presence of Fe$^{3+}$ and a reductant FeS clusters were assembled into the apo-Rieske FeS protein [11] and apo-ferredoxin. Similar data were recently obtained also by Kato et al. [12]. Using site-directed mutagenesis, several residues important for the enzymic activity were identified. Cys$^{358}$ was essential for the production of alanine and sulphide, but not for substrate binding [10]. We concluded that, after binding of free cysteine to Slr0387, the sulphur is transferred to Cys$^{358}$ forming a cysteine persulphide. The data obtained are consistent with Slr0387 being a cysteine desulphurase involved in FeS cluster synthesis.

In a second set of experiments, we studied the function of the three ORFs by deletion mutagenesis. Antibiotic-resistance cassettes were introduced into all three ORFs, replacing a significant part of the gene. We were able to inactivate the ORFs slr0387 and slr0704, but slr0077 appeared to be essential for the growth of *Synechocystis*. In preliminary studies, the slr0704 mutant did not show any significant phenotype. However, cells in which slr0387 was partially replaced by a chloram-
phenicol (Cm) resistance gene contain severely less pigments compared with wild-type cells (Figure 1). The absorption maxima of chlorophyll (445 and 685 nm) as well as for phycobilisomes (630 nm) were significantly reduced. In order to investigate whether only the FeS cluster-containing photosystem I was reduced, fluorescence spectra at 77 K were recorded from wild-type and mutant cells. Upon excitation at 435 nm, fluorescence of chlorophyll bound to photosystem I emitted light at 685 and 695 nm, whereas photosystem I chlorophyll emitted light at 725 nm. Both photosystems seem to be equally affected, since the spectra from wild-type and mutant cells are almost superimposable (Figure 1). Although photosystem II is not a FeS cluster-containing complex, FeS proteins might be involved in the synthesis of assembly factors and/or cofactors of the photosystem.

The function of Slr0077 is not clear, but it cannot be excluded that this protein is also involved in FeS cluster synthesis; perhaps it is involved in the maturation of a specific subset of FeS proteins. For A. vinelandii it has been already shown that NifS is not able to replace IscS. The iscS gene could not be deleted, not even when the protein was isolated with bound iron. This opens the possibility that this protein provides the iron for the assembly of FeS clusters. Further studies are required to clarify the exact role of this protein.

### Synechocystis NifU-like protein Ssl2667

The deduced amino acid sequence of ssl2667 corresponds to the C-terminal portion of A. vinelandii NifU and to the NifU protein of some other nitrogen-fixing species, such as *Rhodobacter capsulatus*. Insertion of a kanamycin-resistance cassette into ORF ssl2667 led to kanamycin-resistant *Synechocystis* cells. However, complete segregation was not obtained, indicating that this ORF is indispensable for cell growth.

In the deduced amino acid sequence of the protein there is a Cys-Xaa-Xaa-Cys motif. Such sequence motifs have been found in thioredoxins and FeS proteins. These cysteines are indeed located on the surface of the protein, because after overexpression in *E. coli* the protein was isolated as a monomer with an intramolecular disulphide bridge, or as a dimer with two intermolecular disulphide bridges (M. Wollenberg and A. Seidler, unpublished work). In vitro studies indicated that this protein releases sulphur from Slr0387 for the formation of FeS clusters (M. Wollenberg and A. Seidler, unpublished work). Data concerning the assembly of an FeS cluster at Ssl2667 have been obtained recently [13].

### Synechocystis IscA proteins

In the nif gene cluster from *A. vinelandii* there is an ORF upstream of *nifS* that has not been assigned, because its inactivation did not result in any detectable change in phenotype [14,15]. However, in the isc gene clusters an ORF (*iscA*) is found that encodes a protein with high similarity to the ORF upstream of *nifS*, thereby indicating that this ORF represents a 'true' gene. In the *Synechocystis* genome, two proteins with similarity to IscA are encoded. Inactivation of both ORFs (slr1417 and slr1565) revealed that they are dispensable for growth of *Synechocystis*, and no obvious change in phenotype was observed. This is similar to the situation observed in other bacteria. However, the two proteins might replace each other in function. A change in phenotype might be observable only after inactivation of both genes. The inactivation of the equivalent genes in yeast led to a severe change in phenotype in relation to the integrity of mitochondria and FeS cluster synthesis [16,17].

The product of ORF slr1417 was over-expressed in *E. coli* and purified. Interestingly, the protein was isolated with bound iron. This opens the possibility that this protein provides the iron ions for the assembly of FeS clusters. Further studies are required to clarify the exact role of this protein.

### Comparison to FeS cluster assembly in the chloroplast

The assembly of the FeS cluster of plant ferredoxin was shown to occur in isolated chloroplasts [18]. This assembly depends on the presence of cysteine, NADPH (or light) and ATP [19,20]. However, no enzymes involved in this assembly process have been identified so far. In the completely sequenced genome of *Arabidopsis thaliana*, there are numerous genes with homology with NifS, the C- and N-terminal portion of *A. vinelandii* NifU and IscA. Almost all of the deduced proteins carry N-terminal transit peptides indicating a location of the proteins either in mitochondria or chloroplasts. Interestingly, there are only two genes coding for proteins with similarity to NifS. One is nearly 70% identical with *Synechocystis* Slr0387, and was shown recently to be located in mitochondria [21]. Using the program 'targetP' the other protein was predicted to be located in the chloroplast. This protein is less
similar to NifS than is Slr0387, but shows strong similarity to Slr0077. It will be interesting to see whether this protein is involved in the maturation of FeS proteins.

We found that under some conditions the *Synechocystis* NifU-like protein can release sulphur from Slr0387 after this enzyme has reacted with free cysteine, with concomitant oxidation of the cysteine side chains at NifU. The same reaction might have occurred in lysed chloroplasts in the work of Takahashi et al. [19]. This would explain the need for light or NADPH for sulphide production, because the oxidized NifU-like protein would have to be reduced, and the most likely reductase is ferredoxin–thioredoxin oxidoreductase. This enzyme itself becomes reduced in the light via ferredoxin, which, in turn, is reduced by photosystem I.

**Concluding remarks**

Significant progress has been made in recent years in terms of understanding FeS cluster assembly in various organisms. The mechanism of this process seems to be similar, but by no means identical, in various organisms. Cyanobacteria are apparently different from most other organisms in which FeS assembly is currently under investigation, particularly concerning the role of the NifU/IscU-like protein and the presence of a cyst(e)ine desulphurase other than the NifS-like proteins.

**References**


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**Assembly-controlled regulation of chloroplast gene translation**

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**Abstract**

Studies of the biogenesis of the photosynthetic protein complexes in the unicellular green alga *Chlamydomonas reinhardtii* have pointed to the importance of the concerted expression of nuclear and chloroplast genomes. The accumulation of chloroplast- and nuclear-encoded subunits is concerted, most often as a result of the rapid proteolytic disposal of unassembled subunits, but the rate of synthesis of some chloroplast-encoded subunits from photosynthetic protein complexes, designed as CES proteins (Controlled by Epistasy...