Role of NifS in Maturation of Glutamine Phosphoribosylpyrophosphate Amidotransferase†

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Glutamine phosphoribosylpyrophosphate amidotransferase from *Bacillus subtilis* is synthesized as an inactive precursor that requires two maturation steps: incorporation of a [4Fe-4S] center and cleavage of an 11-residue NH₂-terminal propeptide. Overproduction from a multicopy plasmid in *Escherichia coli* leads to the formation of soluble proenzyme and mature enzyme forms as well as a small fraction of insoluble proenzyme. Heterologous expression of *Azotobacter vinelandii* *nifS* from a compatible plasmid increased the maturation of the soluble proenzyme three- to fourfold without influencing the content of the insoluble fraction. These results support a role for NifS in heterologous Fe-S cluster assembly and enzyme maturation.

Glutamine phosphoribosylpyrophosphate (PRPP) amidotransferase catalyzes the initial reaction in de novo purine nucleotide synthesis and is the key regulatory enzyme in the pathway. Genes encoding glutamine PRPP amidotransferase have been cloned from a number of members of the domains *Bacteria*, *Eucarya*, and *Archaea* (21). However, only the enzymes from *Escherichia coli* and *Bacillus subtilis* have been purified to homogeneity and are well characterized. The *B. subtilis* enzyme is representative of a subclass of glutamine PRPP amidotransferases found in all higher eukaryotes. The *B. subtilis* *purF*-encoded enzyme is synthesized as a proenzyme that requires two maturation steps for activity (11). The maturation steps are incorporation of a [4Fe-4S] cluster and cleavage of an 11-amino-acid NH₂-terminal propeptide. Although the Fe-S center does not have a role in catalysis (19), an intact cluster is necessary for optimal propeptide processing (10). Lability of the Fe-S cluster to molecular oxygen results in a loss of native structure, leading to enzyme proteolysis and turnover (6, 18). This proteolysis is part of the developmental regulation that shuts down biosynthesis prior to *B. subtilis* sporulation.

There is no information about how the [4Fe-4S] cluster is synthesized and incorporated into the enzyme. Cluster assembly has not been reconstituted in vitro because the apoenzyme is subject to aggregation and is insoluble (20). NifS is one component of an *Azotobacter vinelandii* enzyme system that is required for incorporation of Fe-S clusters into nitrogenase in vivo (8) and in vitro (22). NifS is a pyridoxal phosphate-dependent cysteine desulfurase that converts the sulfur of cysteine to the inorganic sulfide required for nitrogenase metallocluster formation (23). NifS homologs have been detected in other bacteria (17), including *E. coli* (5), and Zheng and Dean (22) have proposed that NifS-like proteins might have a general role in mobilizing the sulfur required for metallocluster formation. In support of this idea, NifS from *A. vinelandii* or *E. coli* has been shown to function for in vitro reconstitution of Fe-S clusters in apo forms of *E. coli* FNR (9), SoxR (7), and dihydroxy-acid dehydratase (5). However, there has been no evidence to date for a general role of a NifS-like activity in

metallocluster synthesis in vivo. In this report we show that *A. vinelandii* *nifS* promotes the maturation of *B. subtilis* glutamine PRPP amidotransferase hyperproduced in *E. coli*. These results suggest that a NifS-like protein is involved in Fe-S cluster synthesis in this enzyme and that coexpression of *nifS* may increase heterologous metallocluster synthesis in other proteins during overexpression.

NifS and enzyme production. In order to determine whether NifS can contribute to glutamine PRPP amidotransferase proenzyme maturation, *nifS* and *purF* were coexpressed from compatible plasmids in *E. coli* B834 (DE3) and their expression was compared with *purF* expression in the absence of *nifS*. For this purpose, two plasmids were constructed for overexpression of *purF*-encoded glutamine PRPP amidotransferase. Plasmid pET-BsF contains a 1.6-kb *NdeI/HindIII* *B. subtilis* *purF*⁺ fragment inserted into the corresponding sites of pET24a (Novagen). Transcription is driven by a T7 promoter. Translation starts at Met⁻¹¹ (numbering from Cys¹ in the mature enzyme [11] at the *NdeI* site). Plasmid pTrc-BsF-NifS is a bicistronic *purF*-*nifS* expression vector. It was constructed by first inserting the *NdeI/HindIII* *purF*⁺ fragment into the corresponding sites of pET14b (Novagen). *purF* was then excised with *NcoI* and *BamHI* and ligated into pTrc99A (4) to yield pTrc-BsF. This results in the addition of a 20-codon extension containing a His₆ tag from pET14b to the 11 codons corresponding to the *purF* propeptide. A 1.2-kb *XbaI/HindIII* *A. vinelandii* *nifS* fragment (23) containing a T7 ribosome binding site was then inserted into pTrc-BsF to give pTrc-BsF-NifS. Transcription is driven by the strong Trc promoter in this plasmid. Two monocistronic *nifS* expression plasmids were constructed in pMCL210 (14), which contains p15A *ori*. In pLNifS, transcription is driven by P_{lac}, and in pT7NifS, transcription is driven by a T7 promoter. For overproduction of *B. subtilis* glutamine PRPP amidotransferase, *E. coli* B834 (DE3) (3) bearing pET-BsF was grown in 1 liter of Luria-Bertani (LB) medium containing 25 µg of kanamycin per ml at 37°C to mid-log phase. At an optical density at 620 nm of 0.5, cells were induced with 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 h at room temperature. The cells were collected by centrifugation and stored in liquid nitrogen prior to analysis. To examine the effect of NifS on maturation of glutamine PRPP amidotransferase, *E. coli* B84 (DE3) bearing pET-BsF and either pLNifS or pT7NifS was grown as described above except that the LB

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FIG. 1. Overexpression of *nifS* and *purF* shown by SDS-polyacrylamide gel electrophoresis of soluble and pellet fractions from *E. coli* extracts. Cells were induced by IPTG at room temperature for 16 h. Soluble extract for lane 9 was from uninduced cells. The *nifS* gene was expressed from a T7 (T) or *lac* (L) promoter. Lanes contain 40 μ g of soluble (S) protein or the quantity of pellet (P) protein that was obtained from the corresponding soluble fraction. Electrophoresis was carried out for 100 min. GPAT, glutamine PRPP amidotransferase.

medium contained 25 μ g of chloramphenicol per ml in addition to kanamycin. For overexpression of bicistronic *purF-nifS*, strain B834 (DE3)/pTrc-BsF-NifS was grown as described above in LB medium containing 100 μ g of ampicillin per ml. The cells were induced with IPTG for 5 h at 37°C. Cell extracts were prepared as previously described (2). After centrifugation of the disrupted cells, the cell pellet was taken up in a volume of sodium dodecyl sulfate (SDS) gel buffer corresponding to that of the supernatant fraction and was heated in boiling water for 15 min. Equal aliquots of the supernatant and pellet fractions were loaded on an SDS-8% polyacrylamide gel that was run at 100 V for 100 min or 3 h (see Fig. 1 and 2).

The effect of NifS on glutamine PRPP amidotransferase hyperproduction is shown in Fig. 1. NifS production driven from either the T7 or *lac* promoter in the absence of *purF* is shown in lanes 1 and 2, respectively. Overproduction of glutamine PRPP amidotransferase in the absence of NifS is shown in lanes 7 and 8. Glutamine PRPP amidotransferase was the major protein and was mostly soluble, as seen by comparing lane 7 (insoluble fraction) with lane 8 (soluble enzyme). Comparison of lanes 8 and 9 shows the induction of *purF* by IPTG, and lane 10 shows the pattern of proteins in *E. coli* B834 (DE3). Coexpression of *nifS* and *purF* is shown in lanes 3 to 6. The data show that *nifS* expression had no significant effect on the relative accumulations of the soluble and insoluble forms of glutamine PRPP amidotransferase directed by the two-plasmid system. It should be noted that NifS accumulation in the two-plasmid system was reduced from that obtained from hyperexpression of *nifS* alone. Due to the comigration of NifS with a protein from the host cells, the level of NifS production in the two-plasmid system was obscured.

NifS and enzyme maturation. As a consequence of the conditions for gel electrophoresis, mature and unprocessed forms of glutamine PRPP amidotransferase were not resolved in the experiment whose results are shown in Fig. 1. To separate mature and unprocessed enzyme, the fractions were diluted 10-fold and the time of electrophoresis was extended from 100 min to 3 h. Lanes 1 and 2 in Fig. 2 show the soluble and insoluble fractions from expression of *purF* alone. The soluble enzyme in lane 1 was resolved into a larger unprocessed form and a minor fraction of mature enzyme. Lane 2 contains the insoluble unprocessed enzyme. The effect of *nifS* expression from the *lac* promoter was to increase the fraction of mature soluble enzyme (lane 3); there was no visible effect on the amount of insoluble proenzyme (lane 4). A further increase in

the proportion of mature enzyme was obtained when *nifS* was expressed from the T7 promoter (lane 5), perhaps due to an increased level of NifS protein compared to that from the weaker *lac* promoter. In this case also, the approximate content of insoluble proenzyme was not visibly changed (lane 6). These results suggest that NifS can function in the maturation of *B. subtilis* glutamine PRPP amidotransferase.

Enzyme assays were carried out to quantitate the maturation seen in Fig. 2. Enzyme activity in cell extracts (2) was assayed by measuring the formation of the product glutamate. The standard assay contained 2.5 mM PRPP, 20 mM glutamine, 10 mM MgCl₂, 1 mM EGTA, 50 mM Tris-HCl (pH 8.0), 1 mg of bovine serum albumin per ml, and approximately 0.1 to 1 μ g of protein in a volume of 100 μ l. Incubation was carried out for 6 min at 37°C. Reactions were stopped in a boiling water bath for 2 min, and the concentration of glutamate was determined by the glutamate dehydrogenase method (13). The control reaction mixture contained all the components listed above except PRPP. The protein concentration was determined by the Bradford procedure (1). An enzyme unit corresponds to the production of 1.0 nmol of product per min. Specific activity is in units per milligram of protein.

There is a direct relationship between glutamine PRPP amidotransferase maturation and enzyme activity since propeptide cleavage is required to generate the N-terminal nucleophile (11, 12) needed for catalysis (16). In a two-plasmid system, NifS derived from either pLNifS or pT7NifS increased the glutamine PRPP amidotransferase specific activity in cell extracts from strain B834 (DE3) bearing pET-BsF fourfold, from a specific activity of 1.1 U/mg of protein without NifS to 4.8 to 5.0 U/mg of protein with NifS. A control experiment with cells not carrying a *purF* plasmid showed that essentially all of the enzyme activity was plasmid encoded. Although these data support the hypothesis that NifS can contribute to glutamine PRPP amidotransferase maturation and activation, assays with cell extracts do not distinguish between (i) increased maturation resulting in more active enzyme and (ii) increased specific activity of the existing mature enzyme. For this reason, we purified the enzyme from cells in which *purF* was overexpressed in the presence or absence of *Azotobacter nifS*.

For enzyme purification from strain B834 (DE3) bearing pET-BsF or pET-BsF and pLNifS, we used 10 g of cells from 5 liters of medium or 6 g of cells from 3 liters of medium, respectively. Glutamine PRPP amidotransferase was purified by a recently described procedure (2). Enzyme purity was estimated to be greater than 95% by SDS-polyacrylamide gel electrophoresis.

The data shown in Table 1 have been normalized to an equivalent amount of extract protein to facilitate the comparison. In this experiment, in which cells bearing plasmid pET-BsF or both pET-BsF and pLNifS were obtained from 10 or 6 liters, respectively, of growth medium, the *nifS* plasmid in-

FIG. 2. Resolution of proenzyme and mature enzyme by SDS-polyacrylamide gel electrophoresis. Abbreviations are the same as in Fig. 1. All lanes contain either 4 μ g of soluble fraction or the quantity of pellet fraction obtained from each soluble fraction. Electrophoresis was carried out for 3 h.

TABLE 1. Summary of enzyme purifications

Purification step	Protein		Activity		Yield (%)
	Concn (mg/ml)	Total amt (mg)	Sp act (U/mg)	Total (U)	
pET-BsF (PurF)					
Extract	17.2	300 ^a	1.4	421	100
Protamine	18.5	315	1.2	378	90
DEAE	1.00	22.5	10.9	245	58
(NH ₄) ₂ SO ₄	19.1	9.7	27.1	262	62
pET-BsF/pLNifS (PurF + NifS)					
Extract	20.7	300 ^b	4.5	1,311	100
Protamine	18.5	244	4.9	1,196	91
DEAE	0.88	32.0	17.2	550	42
(NH ₄) ₂ SO ₄	16.8	22.9, 30.2 ^c	27.0	619, 816 ^c	47

^a Extract from 10 g of cells gave 654 mg of protein. Data were normalized to 300 mg of protein.

^b Extract from 6 g of cells gave 331 mg of protein. Data were normalized to 300 mg of protein.

^c Value obtained for a calculated recovery of 62%.

creased enzyme activity threefold in the starting cell extract. After the three-step purification to more than 95% homogeneity, enzyme specific activities were identical for the two preparations. Because of a somewhat lower recovery from the ion-exchange step, the final yield of enzyme from pLNifS-bearing cells was lower than from cells without pLNifS. When corrected for this difference in yield there was threefold more purified protein and total activity from the *nifS*-bearing cells. The effect of NifS was thus to increase the production of fully active enzyme and not to increase the specific activity of partially active enzyme.

It is necessary to describe the results of two experiments in which no effect of NifS was detected. First, coexpression of pET-BsF and pT7NifS in strain B834 (DE3) at 37°C rather than room temperature resulted in mostly insoluble glutamine PRPP amidotransferase and no increased enzyme maturation over that obtained in the absence of NifS (data not shown). In view of the possibility that there was insufficient NifS to handle the massive glutamine PRPP amidotransferase overproduction at 37°C, a second series of experiments was carried out with a *purF-nifS* bicistronic operon driven by a *tac* promoter in plasmid pTrc-BsF-NifS. Induction by IPTG at 37°C resulted in a large overproduction of both glutamine PRPP amidotransferase and NifS as determined by gel electrophoresis (data not shown). However, most of the glutamine PRPP amidotransferase was insoluble, and NifS had no effect on maturation. With plasmid pTrc-BsF-NifS we were unable to obtain overexpression at room temperature. Overall, these results indicate that NifS enhanced the maturation of soluble proenzyme but had no effect on preventing or decreasing insolubility.

Model for maturation. Glutamine PRPP amidotransferase from *B. subtilis* is synthesized as an inactive proenzyme, as are the homologous enzymes from all higher eukaryotes. The enzyme from *B. subtilis* is the only one of these that has been purified and carefully characterized, and it therefore is useful as a model for studies on glutamine PRPP amidotransferase maturation. In heterologous expression, soluble and insoluble proenzymes as well as soluble mature enzyme have been detected. The mature enzyme contains one [4Fe-4S] cluster per subunit (6, 15). The insoluble proenzyme lacks [4Fe-4S] clusters, and the propeptide is uncleaved (6). The soluble proenzyme is also uncleaved (16), but this enzyme form has not been isolated and its Fe-S cluster content is unknown. The accumu-

lated evidence suggests that an intact [4Fe-4S] cluster is required for propeptide processing to take place and propeptide processing is required for enzyme activity with glutamine as a substrate. Based on the NifS-mediated conversion shown in Fig. 2, a working model for maturation can be proposed. We assume that Fe-S cluster assembly occurs cotranslationally or immediately after synthesis since the mature apoenzyme aggregates (20). Therefore, when overexpression in a heterologous system exceeds the capacity for Fe-S cluster incorporation, insoluble apo proenzyme accumulates. We suggest that recombinant NifS in concert with other proteins required for mobilization of Fe may synthesize and incorporate [4Fe-4S] clusters into each subunit of the tetramer at the appropriate time during or immediately after translation. Considering that NifS has been shown to increase the proportion of mature, active enzyme at the expense of the soluble proenzyme, the latter species may have an incomplete complement of [4Fe-4S] clusters, either fewer than four clusters per tetramer or incompletely assembled clusters. Increased *nifS* dosage may accelerate cluster completion and rescue soluble proenzyme, but it has no effect on the aggregated insoluble enzyme. This function of *Azotobacter* NifS in the heterologous system presumably mimics the role of host cell NifS-like protein(s) in glutamine PRPP amidotransferase maturation.

A. vinelandii nifS mutants are defective in nitrogenase metalcluster assembly (8), whereas other Fe-S-containing proteins appear to be functional, implying the existence of multiple species of NifS-like proteins. It is therefore significant that *A. vinelandii* NifS, although designed for nitrogenase metalcluster synthesis, can function with heterologous proteins in vivo as shown here and in vitro for Fe-S cluster assembly into *E. coli* FNR (9), SoxR (7), and dihydroxy-acid dehydratase (5). These results support the suggestion of Zheng and Dean (22) that NifS activity provides a general mechanism for incorporation of sulfur into metalclusters. A NifS two-plasmid system may also be useful for identifying *nifS* homologs and genes for Fe mobilization from *A. vinelandii* and other organisms having roles in metalcluster synthesis for housekeeping enzymes and proteins.

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