Glutamine phosphoribosylpyrophosphate amidotransferase (PRPP) 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] 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.

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. 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 -3 (numbering from Cys 1 in the mature enzyme) and is necessary for proper 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 dihydroxyacyl 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.
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 as 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 μg of protein in a volume of 100 μl. 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-
at 37°C, a second series of experiments was carried out with a massive glutamine PRPP amidotransferase overproduction over that obtained in the absence of NifS (data not shown). In PRPP amidotransferase and no increased enzyme maturation than room temperature resulted in mostly insoluble glutamine pET-BsF and pT7NifS in strain B834 (DE3) at 37°C rather which no effect of NifS was detected. First, coexpression of the bicistronic operon driven by a tac promoter in plasmid pT7NifS was insoluble, and NifS had no effect on maturation. Overall, these results indicate that NifS activity provides a general mechanism for incorporation of sulfur into metalloclusters. 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 metallocluster synthesis for housekeeping enzymes and proteins.

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 pT7NifS. 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 pT7NifS 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 accumulated 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 metallocluster 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 metallocluster synthesis, can function with heterologous proteins in vivo as shown here and in vitro for Fe-S cluster assembly into E. coli ENR (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 metalloclusters. 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 metallocluster synthesis for housekeeping enzymes and proteins.

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