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Mutagenesis of Ligands to the [4Fe-4S] Center of *Bacillus subtilis* Glutamine Phosphoribosylpyrophosphate Amidotransferase*

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Several mutations were constructed in residues thought to provide ligands for a [4Fe-4S] cluster in *Bacillus subtilis* amidophosphoribosyltransferase using site-directed mutagenesis of cloned *purF*. These replacements confirm the identification of cysteinyl ligands to the Fe-S center. Of five mutant enzymes, two had no activity, two less than 25% of the wild type activity, and one was lethal and could not be studied. The Fe content of the two mutant enzymes with partial activity was similar to that of the wild type. Results of partial characterization suggest that the [4Fe-4S] cluster is not involved in allosteric regulation and does not play a specific role in the ammonia- or glutamine-dependent reactions of the enzyme. At least partial enzymatic activity is required for NH_2-terminal processing. Pulse labeling experiments suggest that processing is a slow post-translational process which is dependent upon cellular factors. A relationship between Fe-S centers and NH_2-terminal processing of an undecapeptide leader suggests a functional connection between these two structural elements in amidophosphoribosyltransferase.

Amidophosphoribosyltransferase (glutamine phosphoribosylpyrophosphate amidotransferase (EC 2.4.2.14)) catalyzes the first reaction of *de novo* purine nucleotide synthesis (Equation 1).

$$\text{Glutamine} + \text{5-phosphoribosyl-1-pyrophosphate} \rightarrow \text{phosphoribosylamine} + \text{glutamate} + PP_i \quad (1)$$

Similar to other glutamine amidotransferases, NH_3 can substitute for glutamine *in vitro* and *in vivo* (1–3), in which case the products are phosphoribosylamine and PP_i. In contrast to the *Escherichia coli* enzyme (4, 5), *Bacillus subtilis* amidophosphoribosyltransferase contains one [4Fe-4S] cluster per subunit (6, 7). Destruction of the [4Fe-4S] with O_2 or by chelation with *o*-phenanthroline inactivates the enzyme (8, 9). The [4Fe-4S] clusters in amidophosphoribosyltransferase are in the diamagnetic (+2) state (6) and do not function in electron transfer during catalysis (10), consistent with the absence of any overall oxidation-reduction reaction in conversion of substrates to products. Several studies suggest a regulatory role for the Fe-S center. Amidophosphoribosyltransferase is one of a number of enzymes of nucleotide and amino acid biosynthesis that are inactivated during sporulation of *B. subtilis* (11). Inactivation of amidophosphoribosyltransferase *in vivo* requires O_2 (12) which appears to react with the [4Fe-4S] cluster (6).

The *B. subtilis* gene, *purF*, encoding amidophosphoribosyltransferase was cloned and sequenced by Makaroff *et al.* (13). Cloned *B. subtilis purF* was expressed in *E. coli*, and amidophosphoribosyltransferase was active *in vitro* and functional *in vivo*. Fe-S centers were assembled into the *B. subtilis* enzyme synthesized in *E. coli*. By comparison of the derived amino acid sequence of amidophosphoribosyltransferase with ferredoxins, 4 cysteinyl residues were identified as candidates for ligands to the Fe-S center.

Comparison of the NH_2-terminal amino acid sequence derived from *B. subtilis purF* with that determined from the purified mature enzyme indicated that 11 amino acids are removed from the primary translation product (13) to expose an NH_2-terminal active site cysteine (14). Processing was abolished in a mutant in which Cys-12 was replaced by phenylalanine (3). In addition, a tentative link was noted between NH_2-terminal undecapeptide processing and Fe-S content. Amidophosphoribosyltransferase synthesized in an *in vitro* coupled transcription-translation system in the absence of Fe-S was not processed.

In this report we describe the construction and characterization of five mutations in amino acids implicated in binding the amidophosphoribosyltransferase Fe-S cluster. Two of the mutations, serine replacements of Cys-448 and Cys-451, inactivate the enzyme and thereby confirm the function of these residues in binding Fe-S. NH_2-terminal undecapeptide processing was abolished in Fe-S mutants exhibiting no amidophosphoribosyltransferase activity, but processing was complete in mutants having partial enzyme activity.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Phage—*E. coli* strain AB352 (*thr*, *leuB6*, *purF*, *thi*, *lacZ4*, *rpsL8*, *supE44*) was used as the host for *purF* plasmids. Minimal media used for enzyme purification and growth analysis included salts (15), 0.5% glucose, 0.2% acid-hydrolyzed casein, 2 μg/ml thiamine, and trace minerals (16). Adenine (20 μg/ml) was added to allow growth of purine auxotrophs. Ampicillin was used as an antibiotic at 50 μg/ml. *E. coli* strains JM103, JM105 and GM119 used for growth of M13 phage have been described (3). Plasmid pPZ2 (13) is a pBR322 recombinant that contains *purF* on a 1.6-kilobase pair fragment of *B. subtilis* DNA. Plasmids pRK9 (17), pUC8, and pUC13 (18) have been described. The M13 phage used were M13mp11 and M13mp11w (w designates wild type). M13 growth, cloning, and DNA isolation were essentially as described by Messing (19).

Plasmid and Phage Construction—A 1.2-kilobase pair *Hin*cII fragment containing the 3′ end of *purF* was transferred from pPZ2 into the *Sma*I site of pUC13 to yield pPF1.2. The cloned insert was then transferred to M13mp11 as an *Eco*RI/*Bam*HI fragment. The resulting phage, M13mp11PF1.2, contains a segment of *purF* noncoding strand

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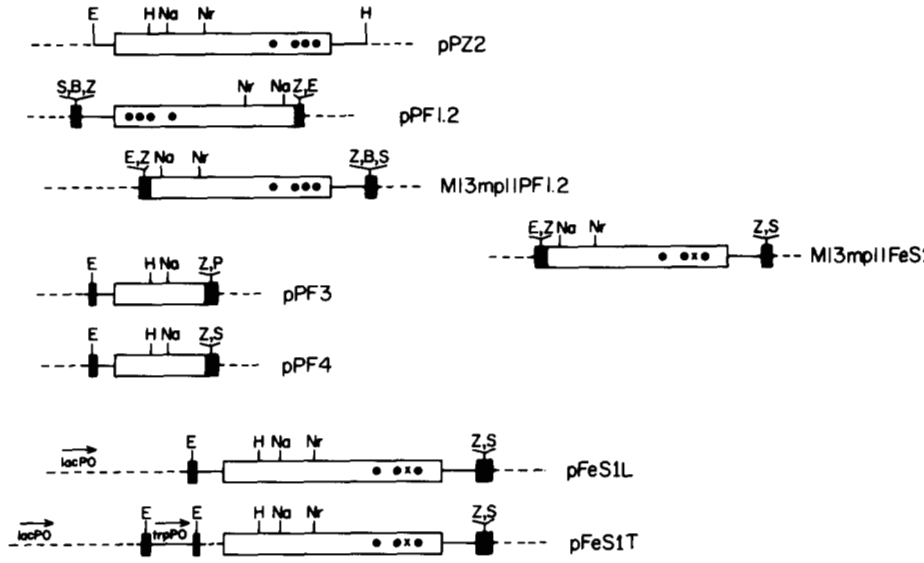

FIG. 1. **Schematic representation of *purF*-derived plasmids and phage employed for mutagenesis.** Figures are not drawn to scale. Only relevant regions are shown. Symbols used: *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hin*cII; *Na*, *Nar*I; *Nr*, *Nru*I; *P*, *Pst*I; *S*, *Sst*I; *Z*, destroyed *Hin*cII site; *open bar*, *purF* coding region; *solid bar*, polylinker DNA; ●, cysteine residues thought to be involved in the [4Fe-4S] binding sites; ×, mutations in the Fe-S site; *solid line*, *B. subtilis* DNA; *dashed line*, vector DNA.

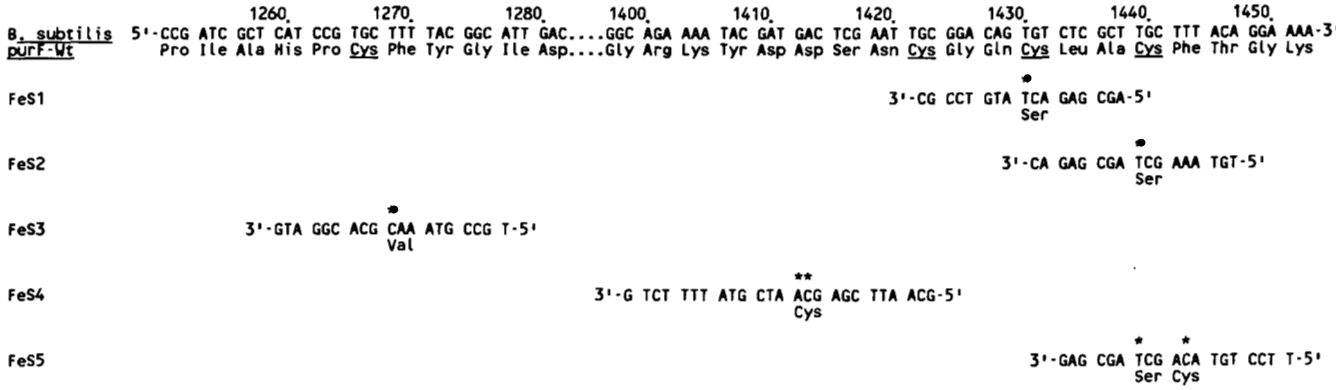

FIG. 2. **Nucleotide and corresponding amino acid sequence of a portion of *B. subtilis purF*, and the nucleotide sequences of the FeS oligodeoxyribonucleotides.** The nucleotide sequence is numbered as in Ref. 13, * indicates nucleotides different from the wild type sequence. Cysteine residues implicated as ligands to the [4Fe-4S] center are *underlined*. Amino acid replacements are shown under the mutagenic oligodeoxyribonucleotides. The oligodeoxyribonucleotide sequences are in the coding stand.

DNA from nucleotides 437 to 1648 (Fig. 1).

Synthetic Oligodeoxyribonucleotides—The mutagenic oligodeoxyribonucleotides synthesized for this study are shown in Fig. 2. The FeS1 and FeS2 17-mers were synthesized by the triester method on a 1% cross-linked polystyrene support (20). After deprotection the oligonucleotides were purified by high performance liquid chromatography as described (3). The FeS3, FeS4, and FeS5 mutagenic oligonucleotides as well as the *purF*-specific sequencing primer were synthesized on an Applied Biosystems Model 380A DNA synthesizer. Purification on a 20% polyacrylamide/7 M urea gel was followed by crush soak (21) and isolation on a Sep-Pak (Anspec) Column.

Oligonucleotide-directed Mutagenesis—Mutagenesis was conducted using the procedure of Bauer *et al.* (22). Mutagenic oligonucleotides, Fig. 2, were annealed to a gapped heteroduplex constructed from M13mp11w and M13mp11PF1.2 at a ratio of approximately 100:1. Annealing and primed synthesis conditions have been described (3). Single-lane dideoxy sequencing was used to screen for the FeS mutants.

DNA Sequence Analysis—DNA sequences were determined by the dideoxy procedure of Sanger *et al.* (23) using either the universal primer (Pharmacia) or a *purF*-specific sequencing primer (CGTGAGGAAGCACTGTA) extending from nucleotides 1493 to 1477. The polyacrylamide/urea gel electrophoresis system of Biggen *et al.* (24) was used for resolving DNA fragments.

Reconstruction and Expression of purF—To allow the rapid reconstruction of several *purF* FeS mutants, a series of plasmids was constructed. The relevant plasmids and phage used for reconstruction are shown in Fig. 1. An *Eco*RI/*Nru*I fragment (nucleotides 1–739) containing the 5′ end of *purF* was transferred from pPZ2 to pUC8 to yield pPF3. The polylinker *Pst*I site of pPF3 was replaced with an *Sst*I linker after it had been made blunt-ended with DNA polymerase I Klenow fragment. The plasmid is designated pPF4 and was the recipient of the five different *purF* FeS mutations. The mutations in M13 are designated M13mp11FeS1-M13mp11FeS5. During the final reconstruction step, the five FeS mutations were transferred by replacing the *Nar*I/*Sst*I fragment of pPF4 with the *Nar*I/*Sst*I fragment from the M13mp11FeS1-M13mp11FeS5 mutant phage (Fig. 1). These five mutant *purF* genes in plasmids are designated pFeS1L-pFeS5L and pFeS1T-pFeS5T. Representative examples, pFeS1L and pFeS1T, are shown in Fig. 1.

The gene orientation in the pFeSL plasmids is such that *purF* is expressed from the *lac* promoter (The L in pFeSL designates the *lac* promoter.) For higher level expression of *purF* an *Eco*RI fragment containing the *Serratia marcescens trp* promoter (17) was inserted into the *Eco*RI site of a pFeSL plasmid. This construction is designated pFeST with T indicating a *trp* promoter. Indole acrylic acid (40 mg/liter) was used to derepress the *trp* promoter. Plasmids carrying the wild type *purF* gene are designated pFeSwtL or pFeSwtT.

Enzyme Purification—Amidophosphoribosyltransferase was purified from strains AB352/pFeSwtL, AB352/pFeS3L, and AB352/pFeS4L. Four liters of cells were grown to late log phase, harvested, and frozen at −20 °C prior to enzyme purification (3, 7). Enzyme activity was monitored by the glutamate dehydrogenase method (4) during purification. The purification properties of the two mutants were essentially the same as the wild type enzyme. The enzymes were stored at −80 °C in buffer A (8) containing 2 mM AMP and 5 mM dithiothreitol and prepared for use by dialysis against 50 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 2 mM $MgCl_2$, 2 mM dithiothreitol.

Enzyme Assay—NH_3-dependent amidophosphoribosyltransferase activity was assayed by the [^{35}S]cysteine method (4, 25). Glutamine-dependent activity was assayed by the same [^{35}S]cysteine method and by the glutamate dehydrogenase method (4). Glutamine-dependent activity determined by the two methods was similar (±10%). A unit of activity is defined as the amount of enzyme forming 1 μmol of product/min at 37 °C. Protein concentration was determined by the method of Lowry (26) during enzyme purification. The concentration of purified amidophosphoribosyltransferase was determined spectrophotometrically at 278 nm using an extinction coefficient $A^{1\%}_{278} = 9.6$ (6) or by amino acid analysis.

Analyses—NH_2-terminal amino acid sequences of purified enzymes and *in vivo* labeled immune-precipitated enzymes were determined by Mark Hermodson, Purdue University, using the procedures of Mahoney *et al.* (27). Amino acid derivatives were identified by high performance liquid chromatography. Samples from radiosequencing were dried under nitrogen, taken up in 50 μl of methanol, and counted for radioactivity. Samples for amino acid analysis were hydrolyzed in 6 N HCl for 22 h (110 °C) *in vacuo*. Amino acid analyses were performed by Jim Cook, Purdue University, with a Durrum D500 amino acid analyzer.

The iron content of the wild type, FeS3, and F3S4 enzymes was determined as described (5). Enzyme samples (approximately 2.5 mg) were dialyzed against deoxygenated, Chelex-treated 10 mM NH_4HCO_3 and lyophilized prior to hydrolysis in 6 N HCl for 22 h (110 °C) *in vacuo*. Approximately 150 μg were removed for amino acid analysis to quantitate the amount of protein present. A carefully measured aliquot of the remaining sample was dried under nitrogen and sent to William Seifert, The University of Texas Health Science Center at Houston, for iron analysis by x-ray fluorescence spectrography. An equal volume of dialysis buffer was used as the blank.

In Vivo Labeling and Immune Precipitation—Cells were grown, labeled, and immune-precipitated essentially as described (28, 29). Plasmid-bearing cells were grown in minimal media to mid log phase, harvested, and resuspended in MOPS[1] medium (28) containing 0.05% glucose, 2 μg/ml thiamine, and trace minerals. For SDS-polyacrylamide gel electrophoresis, cells were labeled with 100 μCi of [^{35}S] methionine (1000 Ci/mmol). For radiosequencing, cells were labeled with 100 μCi of [2,3,4,5-^{3}H]leucine (120 Ci/mmol) and 50 μCi of [U-^{14}C]isoleucine (310 mCi/mmol). Labeling and chase conditions varied with individual experiments and are described in the appropriate figure legends. Cells were lysed in the presence of 30 mg/ml SDS for 10 min at 100 °C. Buffer solution (10 mg/ml Triton X-100, 50 mM Tris-hydrochloride (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.2 mg/ml NaN_3) was added and amidophosphoribosyltransferase immune-precipitated (29). Anti-amidophosphoribosyltransferase was a gift from R. L. Switzer, University of Illinois, Urbana, IL.

SDS-Polyacrylamide Gel Electrophoresis—Samples of purified enzyme or *in vivo* labeled, immune-precipitated enzyme were analyzed using a 15% polyacrylamide discontinuous gel system (30). Proteins were visualized by staining with Coomassie Blue (31) or by exposure to Kodak X-Omat AR film at −20 °C after treatment with EN^{3}HANCE®.

RESULTS

Mutant Isolation—Four cysteine residues thought to provide ligands to the [4Fe-4S] cluster in *B. subtilis* amidophosphoribosyltransferase were previously identified by comparison with bacterial ferredoxins (13). To confirm this identification of the binding site and to investigate the role of the [4Fe-4S] cluster, several mutations were generated using site-directed mutagenesis. A summary of the amino acid replacements is shown in Fig. 3.

Mutations FeS1 and FeS2 were designed to replace Cys-448 and Cys-451, residues thought to be ligands in the 4Fe-4S cluster. The mutations were generated by annealing mutagenic 17-mers, shown in Fig. 2, to the complementary region of *purF* generating T:T mismatches at nucleotides 1430 and 1439, for FeS1 and FeS2, respectively. Mismatches are identified by asterisks in Fig. 2. Both codon changes, TGT to AGT and TGC to AGC, result in the replacement of cysteine

[1] The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; SDS, sodium dodecyl sulfate.

	393	445	448	451
WILD TYPE	C F ----------	D S N C G Q C L A C F T G		
FeS-1	C F	D S N C G Q S L A C F T G		
FeS-2	C F	D S N C G Q C L A S F T G		
FeS-3	C V	D S N C G Q C L A C F T G		
FeS-4	C F	C S N C G Q C L A S F T G		
FeS-5	C F	D S N C G Q C L A S C T G		

FIG. 3. **Amino acid sequence of wild type and FeS mutant amidophosphoribosyltransferase.** Amino acids are numbered as in Ref. 13.

with serine. The frequency of incorporation of the oligonucleotides for these mutations were FeS1, 1 mutant in 24 screened, and FeS2, 3/24.

The FeS3 mutation is a Phe to Val change at amino acid 394. The corresponding Phe in the *Azotobacter vinelandii* ferredoxin [4Fe-4S] cluster is involved in aligning the fourth cysteinyl ligand in the binding site (32). A 19-mer (Fig. 2) was used to generate the T→G (nucleotide 1268) transversion which was obtained at a frequency of 1/24.

In an attempt to generate a subtle change in the position of the binding site in the protein, an Asp to Cys substitution at amino acid 442 was generated in mutant FeS2 to produce the double mutant FeS4 (Fig. 3). A mutagenic 24-mer was used to generate a GA→TG change at nucleotides 1412 and 1413 (Fig. 2). The 2-base pair change was obtained at a frequency of 1/12 and should have the effect of shifting the -Cys-X-X-Cys-X-X-Cys- binding site toward the NH_2 terminus of the protein by three amino acids.

Mutation FeS5 was constructed to examine the effect of changing the spatial relationship of the 3 core cysteine residues with respect to each other. For mutant FeS5, the 19-mer shown in Fig. 2 was annealed to the complementary FeS2 single-stranded DNA. A T→G transversion at nucleotide 1443 was generated at a frequency of 1/12 in the FeS2 mutant to yield FeS5 (Fig. 2). This mutation resulted in a Phe to Cys replacement at amino acid 452 as shown in Fig. 3. In all cases the desired mutations were first identified by single lane sequencing. DNA sequence analysis using all four reactions confirmed the mutations and established that there were no other changes for at least 250 nucleotides surrounding the planned mutation.

Expression of the FeS Mutant Genes in E. coli—The five *purF* FeS mutants as well as a wild type *purF* gene were reconstructed as described under "Experimental Procedures." *purF* is expressed in the pFeSL plasmid derivatives from the *lac* promoter. Higher level expression was obtained in the pFeST derivatives in which an *Eco*RI fragment containing the *S. marcescens trp* promoter (17) is cloned upstream of *purF*. The two constructions used for *purF* expression in these studies are diagramed in Fig. 1.

When *B. subtilis purF*$^+$ is expressed in *E. coli*, amidophosphoribosyltransferase is functional and can complement a *purF* mutation (13). The function of the FeS mutants was first tested *in vivo* using the *E. coli purF* strain AB352. Plasmids pFeS3L and pFeS4L conferred nearly wild type growth to strain AB352 (Table I). AB352/pFeS3L and AB352/pFeS4L had doubling times of 56 and 60 min, respectively. This compares to a doubling time of 50 min for AB352/

TABLE I

Properties of wild type and FeS mutant amidophosphoribosyltransferase

E. coli strain AB352 (*purF*) bearing plasmid pFeSwtL (*purF*$^+$) or pFeS1L-pFeS4L was analyzed for amidophosphoribosyltransferase function. Growth rate, enzyme purification and assay, NH_2-terminal enzyme processing, Fe content, and inhibition of glutamine-dependent amidophosphoribosyltransferase were determined as described under "Experimental Procedures." Growth rate is expressed as doubling time. Unless specified, amino acid analysis was used to quantitate protein concentration.

purF	Enzyme activity					
	Growth	Processed	Gln	NH_3	Fe content	AMP inhibition
	min		*units/mg protein*		*g atom/subunit*	
Wild type	50	Yes	28.6	41.5	6.0 ± 1.5 (3.0)[a]	Yes
FeS1	NG[b]	No	<0.01[c]	ND[d]	ND	ND
FeS2	NG	No	<0.01[c]	ND	ND	ND
FeS3	56	Yes	4.4	6.5	4.8 ± 1.1 (2.4)	Yes
FeS4	60	Yes	6.9	5.5	4.0 ± 0.79 (2.0)	Yes

[a] Mean values ± standard deviation were calculated from three independent determinations made on single enzyme preparations. Values in parentheses were calculated using an extinction coefficient of $A_{278}^{1\%} = 9.6$ to calculate protein content (5).

[b] NG, no growth.

[c] Assayed in crude cellular extracts.

[d] ND, not determined.

pFeSwtL, and indicates that the mutant enzymes are active. However, the plasmids pFeS1L and pFeS2L, failed to confer purine-independent growth to strain AB352. As shown below, *purF* genes in pFeS1L and pFeS3 are transcribed and translated to yield inactive amidophosphoribosyltransferase.

Unexpectedly, *purF* containing the FeS5 mutation could not be reconstructed and expressed in *E. coli*. Several attempts to reconstruct the gene in alternative plasmid orientations were unsuccessful. All pFeS5 plasmids isolated contained Phe at amino acid 452. This reversion was the only problem encountered in expressing and maintaining FeS mutations, indicating the FeS5 mutation is most likely lethal to the cell.

Several possibilities were considered for the inability of pFeS1L and pFeS2L to restore *purF* function to *E. coli* AB352. (i) The mutant genes may not be expressed due to incorrect gene reconstruction. (ii) The mutant enzymes could be unstable and rapidly degraded. (iii) The enzymes could be stable but inactive. To determine whether or not the FeS1 and FeS2 mutant enzymes were actually synthesized from the reconstructed genes, amidophosphoribosyltransferase was immune-precipitated from plasmid-bearing strain AB352 radiolabeled with [^{35}S]methionine. Fig. 4*A* shows the results of SDS-polyacrylamide gel electrophoresis of enzyme that was immune-precipitated from cells harboring pFeSwtT (*lane 1*), pFeS1T (*lane 2*), and pFeS2T (*lane 3*) after a 30-min pulse with [^{35}S]methionine. *Lane 1* contains two species of amidophosphoribosyltransferase with slightly different mobilities. *Lanes 2* and *3* each contain one species that migrates with the larger form of the wild type enzyme in *lane 1*. The total amount of immune-precipitable radioactivity was approximately the same for all three enzymes. Thus the FeS1 and FeS2 mutant enzymes are synthesized and exhibit a steady state level comparable to the wild type, yet are unable to complement an *E. coli purF* mutant strain. This result suggests that the FeS1 and FeS2 mutations inactivate the enzyme but do not perturb protein folding enough to result in rapid degradation of the enzyme. The data also suggest that when wild-type amidophosphoribosyltransferase is overproduced at sufficiently high levels, two species of the enzyme are present.

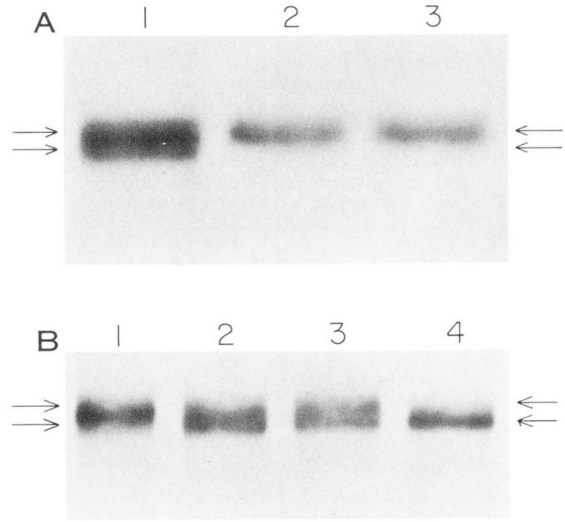

FIG. 4. In *A*, SDS-polyacrylamide gel electrophoresis of *B. subtilis* wild-type, FeS1, and FeS2 amidophosphoribosyltransferase is shown. *E. coli* strain AB352 containing the plasmids pFeSwtT (*lane 1*), pFeS1T (*lane 2*), and pFeS2T (*lane 3*) was grown in the presence of indole acrylic acid and pulsed with ^{35}[S]methionine for 30 min. Amidophosphoribosyltransferase was immune precipitated as described under "Experimental Procedures." Equal quantities of radioactively labeled immune-precipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The *arrows* point to two enzyme species (unprocessed and processed). In *B*, effect of high level expression on processing of wild type amidophosphoribosyltransferase is shown. AB352/pFeSwtT (*lanes 1* and *2*) and AB352/pFeSwtL (*lanes 3* and *4*) were labeled for 15 min in the presence of [^{35}S]methionine. Unlabeled methionine (0.2 mg/ml) and chloramphenicol (0.25 mg/ml) were added and cells grown for an additional 40 min (*lanes 2* and *4*). Samples were analyzed as described in the text. Equal quantities of radioactivity were applied to each lane.

NH_2-terminal Processing—*B. subtilis* amidophosphoribosyltransferase undergoes a processing event in which 11 amino acids are clipped from the NH_2 terminus of the primary translation product to yield the mature enzyme (13). The factors involved in this processing event are not known. To investigate the nature of the two species observed in Fig. 4*A* and test the possibility that a rate-limiting step may be involved in NH_2-terminal processing, a pulse-chase experiment was conducted. Fig. 4*B* shows the results obtained from cells containing PFeSwtT and pFeSwtL after a 15-min pulse with [^{35}S]methionine (*lanes 1* and *3*) and a 40-min chase with unlabeled methionine in the absence of further protein synthesis (*lanes 2* and *4*). Two species of amidophosphoribosyltransferase were initially present in cells containing either pFeSwtT (*lane 1*) or pFeSwtL (*lane 3*). However, after the 40-min chase, cells bearing pFeSwtT still contained both species of amidophosphoribosyltransferase (*lane 2*), whereas only the smaller enzyme form was present in cells containing pFeSwtL (*lane 4*). Immunoprecipitation indicated that cells harboring the pFeSwtT plasmid contained 6-fold more radiolabeled amidophosphoribosyltransferase than cells which contained pFeSwtL. This result indicates that high level expression of *purF* results in the overproduction and accumulation of unprocessed amidophosphoribosyltransferase enzyme. Only processed enzyme was detected when *purF* was expressed from a weaker plasmid promoter (3).

To confirm that the two species represent processed and unprocessed forms of amidophosphoribosyltransferase, immune precipitates from cells harboring pFeSwtT and pFeSwtL were radiosequenced. Amidophosphoribosyltransferase was labeled by growing AB352/pFeSwtT and AB352/pFe-

SwtL in the presence of [^{3}H]leucine and [^{14}C]isoleucine. Immune precipitates were subjected to automated Edman degradation. Muture amidophosphoribosyltransferase has leucine at positions 20 and 23 and isoleucine at positions 6 and 15 (13). Amidophosphoribosyltransferase in which the NH_2-terminal processing event has not occurred should yield a pattern of leucine at positions 2 and 8 and isoleucine at positions 5, 17, and 26. Radiosequencing results are shown in Figs. 5 and 6. *Arrows* indicate positions predicted to contain radioactivity. The labeling pattern obtained from AB352/pFeSwtL is that expected for a fully processed mature form of amidophosphoribosyltransferase (Fig. 5). A more complex labeling pattern is observed from amidophosphoribosyltransferase immune precipitated from AB352/pFeSwtT (Fig. 6). Leucine at cycles 2 and 8 and isoleucine at cycles 5, 17, and 26 are expected for the non-processed primary translation product. The release of leucine at cycles 20 and 23 and isoleucine at cycle 6 and 15 is evidence for mature enzyme in this mixture. The labeling pattern for enzyme immunoprecipitated from cells bearing plasmid pFeSwtT is therefore consistent with a mixture of both processed and unprocessed forms of amidophosphoribosyltransferase. These results thus confirm the assignment of processed and unprocessed forms of the enzyme to the two species detected by SDS-polyacrylamide gel electrophoresis shown in Fig. 4, *A* and *B*.

Accumulation of unprocessed primary translation product suggests that NH_2-terminal clipping may be a relatively slow process. To examine the rate of processing, amidophosphoribosyltransferase was labeled for 5 min and immune-precipitated at various times after protein synthesis had been terminated. Seven minutes after termination of protein synthesis, approximately half of the amidophosphoribosyltransferase synthesized during the 5-min labeling period was processed (Fig. 7). Intermediate forms of processed enzyme were not readily detectable, suggesting that processing is not the result of an aminopeptidase. Thus, pulse chase experiments on wild type amidophosphoribosyltransferase indicate that NH_2-terminal processing is a slow event that occurs post-translationally.

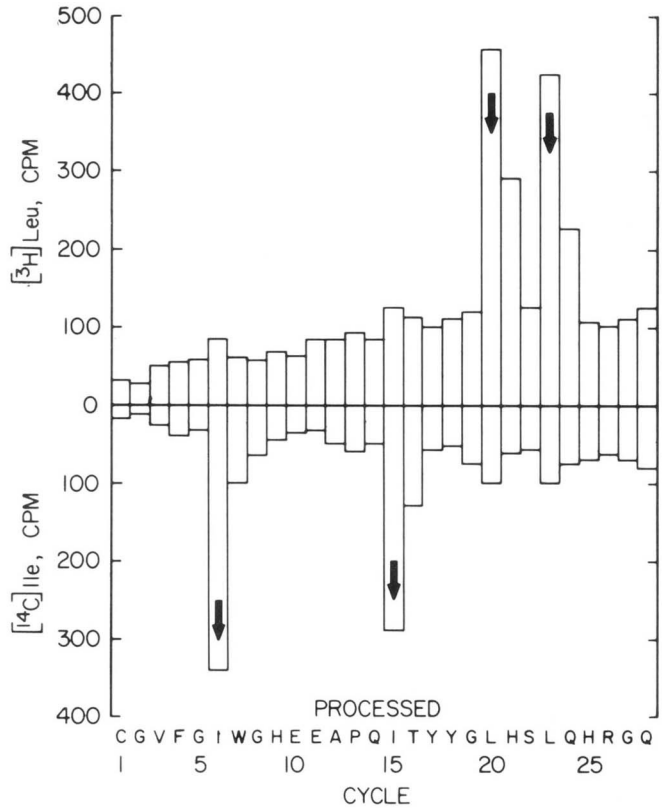

FIG. 5. **Radiosequencing of amidophosphoribosyltransferase produced from pFeSwtL.** NH_2-terminal Edman degradation was carried out on [^{3}H]leucine- and [^{14}C]isoleucine-labeled amidophosphoribosyltransferase as described in the text. Cells were labeled for 40 min prior to a 40-min chase in the presence of excess unlabeled amino acids. *Arrows* point to positions predicted to contain radioactivity. The amino acid sequence of the processed form of the enzyme is shown at the bottom of the figure.

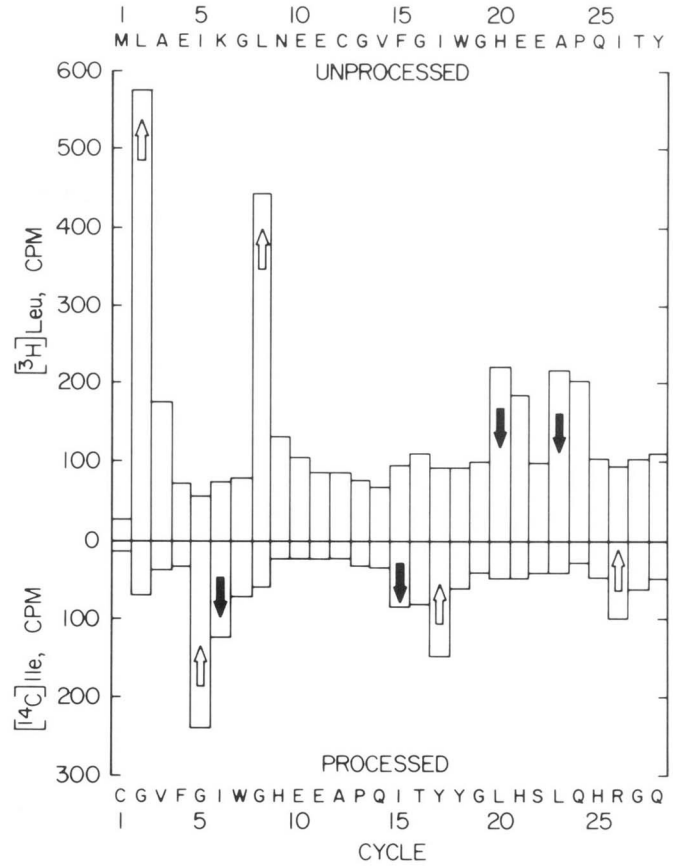

FIG. 6. **Radiosequencing of amidophosphoribosyltransferase produced from pFeSwtT.** Experimental details are described in the legend to Fig. 5 and the text. *Open arrows* indicate positions in the unprocessed form of the enzyme sequence shown at the *top* of the figure, predicted to contain radioactivity. *Solid arrows* indicate positions in the processed form of the enzyme predicted to contain radioactivity. The amino acid sequence of the processed form of the enzyme is shown at the *bottom* of the figure.

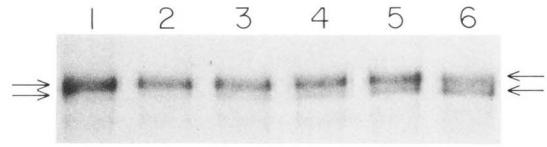

FIG. 7. **Time course for processing of amidophosphoribosyltransferase in AB352/FeSwtL.** Cells were pulse-labeled for 5 min in the presence of [^{35}S]methionine. At time zero, unlabeled methionine (0.20 mg/ml) and chloramphenicol (0.25 mg/ml) were added and samples removed at 0 (*lane 1*), 0.5 (*lane 2*), 1.0 (*lane 3*), 3.0 (*lane 4*), 5.0 (*lane 5*), and 7.0 (*lane 6*) min. Samples were analyzed as described in the text. *Arrows* point to unprocessed and processed forms of the enzyme.

Characterization of the FeS1 and FeS2 Mutant Amidophosphoribosyltransferase—Strains AB352/pFeS1T and AB352/pFeS2T were grown for enzyme purification. As shown in Fig. 8, amidophosphoribosyltransferase is readily visualized by

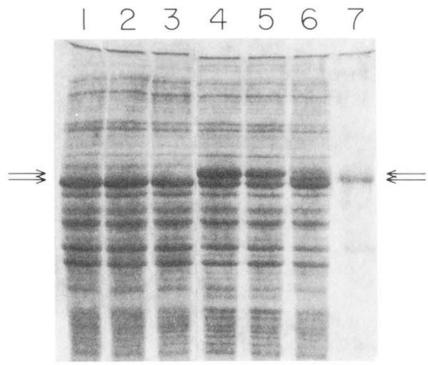

FIG. 8. **Amidophosphoribosyltransferase in cell extracts under induced and non-induced conditions.** 0.2 ml of exponentially growing cells was collected, lysed in SDS (30 mg/ml) at 100 °C, and subjected to SDS-polyacrylamide gel electrophoresis. *Lanes 1–3* contain cell extracts from strain AB352 harboring pFeS1, pFeS2, and pFeSwt plasmids in which *trp* promoter expression is directed opposite to *purF*. *Lanes 4–6* contain cell extracts from AB352/pFeS1T, AB352/pFeS2T and AB352/pFeSwtT, respectively. *Lane 7*, purified amidophosphoribosyltransferase.

Coomassie staining of SDS-polyacrylamide gels of crude cellular extracts. Amidophosphoribosyltransferase in extracts from AB352/pFeS1T (*lane 4*) and pFeS2T (*lane 5*) migrates slower than purified wild type amidophosphoribosyltransferase (*lane 7*). Protein bands can be visualized in extract from AB352/pFeSwtT (*lane 6*) which migrate with purified amidophosphoribosyltransferase (*lane 7*) and the mutant enzymes (*lanes 4* and *5*). Thus under steady state conditions both processed and unprocessed forms of amidophosphoribosyltransferase are present in AB352/pFeSwtT. *Lanes 1–3* contain extracts of cells containing plasmids with the *trp* promoter directed in the opposite orientation. These strains do not produce amidophosphoribosyltransferase. It is estimated by visual inspection that up to 15% of the total cellular protein is amidophosphoribosyltransferase. Despite enzyme overproduction of the FeS1 and FeS2 mutants, amidophosphoribosyltransferase activity was undetectable. Although stable in growing cells, the mutant enzymes appeared to be unstable during purification. In addition, the FeS1 and FeS2 mutant enzymes were insoluble and had an affinity for the membrane fraction. As a result, the FeS1 and FeS2 mutant enzymes were refractory to purification.

The results of electrophoresis shown in Figs. 4*A* and 8 suggest that the mutant enzymes may be unprocessed. To investigate this possibility further, radiosequencing of these enzymes was conducted. The data in Fig. 9 show that the labeling pattern of the FeS2 enzyme was indicative of unprocessed enzyme. A virtually identical labeling pattern was obtained for the FeS1 mutant (not shown). Thus, the FeS1 and FeS2 mutations inactivate the enzyme, and NH_2-terminal processing does not occur. These results are recorded in Table I.

Purification and Characterization of FeS3 and FeS4 Mutant Amidophosphoribosyltransferase—The FeS3 and FeS4 mutant enzymes as well as wild type amidophosphoribosyltransferase were purified from *E. coli* strain AB352 containing the plasmids pFeS3L, pFeS4L, and pFeSwtL, respectively. Based on SDS-polyacrylamide gel electrophoresis and protein sequence analysis the enzyme preparations were greater than 95% pure. Table I summarizes several properties of the enzymes. Automated Edman degradation for 12 cycles indicated that both mutant enzymes and the wild type were fully processed.

The glutamine- and NH_3-dependent activities of the mutant enzymes were decreased to approximately 13–24% of the wild type. The specific activity of the wild type enzyme was comparable to that obtained previously from *B. subtilis purF* expressed in *E. coli* (13).

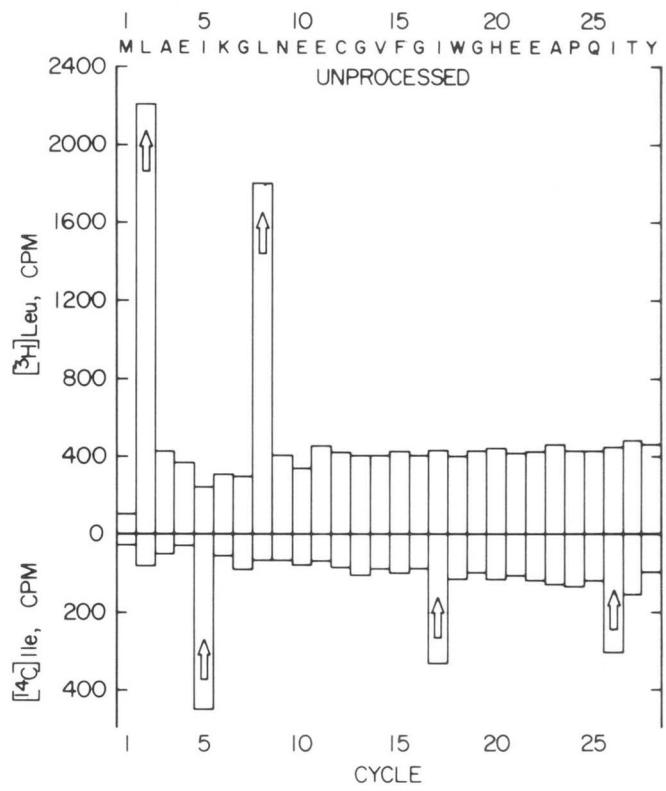

FIG. 9. **Radiosequencing of amidophosphoribosyltransferase produced from pFeS2T.** NH_2-terminal Edman degradation was carried out on [^{3}H]leucine- and [^{14}C]isoleucine-labeled amidophosphoribosyltransferase as described in the text. Cells were labeled for 40 min. *Arrows* indicate positions predicted to contain radioactivity. The amino acid sequence of the unprocessed form of the enzyme is shown at the *top* of the figure.

The iron content of the purified enzymes was determined and recorded in Table I. The wild type enzyme contained 6.0 ± 1.5 g atoms of Fe/subunit. This value is about 2-fold higher than previously reported (13) and results from a difference in methods used for measurement of protein. The Fe content of the mutant enzymes was somewhat lower than the wild type. However, based on the variations in replicate determinations, we conclude that the two mutants and the wild type enzymes likely have similar Fe contents.

B. subtilis amidophosphoribosyltransferase is subject to end product inhibition by purine ribonucleotides (33). AMP is the most effective inhibitor. Since Vollmer *et al.* (10) have suggested that the Fe-S cluster may serve a structural or regulatory role, we investigated the capacity of the FeS3 and FeS4 mutant enzymes for allosteric regulation by AMP. The data in Fig. 10 show that inhibition by AMP was indistinguishable for the two mutant enzymes and the wild type.

DISCUSSION

The primary structures of amidophosphoribosyltransferase from *E. coli* and *B. subtilis* are approximately 40% conserved and are likely homologous (13). It is therefore not unexpected that these enzymes exhibit similar catalytic and allosteric regulatory properties (4–7) and employ an identical mechanism for glutamine amide transfer (5, 14). However, amidophosphoribosyltransferase enzymes from these two bacteria exhibit two key structural differences. (i) The *B. subtilis* enzyme is synthesized with an NH_2-terminal undecapeptide

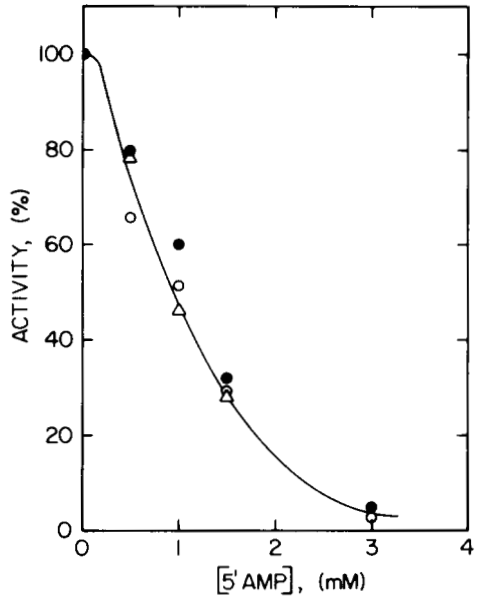

FIG. 10. **Inhibition of amidophosphoribosyltransferase by AMP.** Glutamine-dependent activity was measured by the glutamate dehydrogenase method. ●, wild type amidophosphoribosyltransferase; ○, FeS3 mutant amidophosphoribosyltransferase; △, FeS4 mutant amidophosphoribosyltransferase.

leader sequence which is processed to expose an active site cysteine residue at the NH_2 terminus. In the *E. coli* enzyme, only the initiator Met is removed to yield a mature enzyme having an NH_2-terminal active site cysteinyl residue. (ii) *B. subtilis* but not *E. coli* amidophosphoribosyltransferase contains one [4Fe-4S] cluster per subunit that is essential for activity but appears to have no direct role in catalysis (10). The present series of experiments confirms the previously ascribed cysteinyl [4Fe-4S] ligands (13), provides additional information about undecapeptide processing, and supports a relationship between Fe-S function and NH_2-terminal processing.

The Fe-S binding site was identified by mutations that partially or completely inactivate amidophosphoribosyltransferase. Enzyme activity was abolished by replacement of either Cys-448 or Cys-451 with serine. Activity was partially restored to the Ser-451 mutant (FeS2) by introduction of a cysteine at position 442 (FeS4). The most direct interpretation of these replacements is that the triad C^{445}-X-X-C^{448}-X-X-C^{451} provides three ligands to the [4Fe-4S] cluster and the closely related triad C^{442}-X-X-C^{445}-X-X-C^{448} can also provide three ligands and is partially functional. We consider unlikely the alternative possibility that Ser-448 (FeS1) and Ser-451 (FeS2) mutations inactivate the enzyme due to an altered conformation which can be partially corrected by a second site Cys-442 mutation (FeS4). Evidence for Cys-393 as the fourth cysteinyl ligand to Fe was provided by replacement of Phe-394 to Val in FeS3. This mutation, which likely promotes a subtle change in the orientation of Cys-394, partially inactivates the enzyme. The alternative explanation that the Val-394 mutation perturbs the structure of the catalytic domain previously assigned (34) to amino acids 354–450 is considered unlikely. In the *E. coli* enzyme, the residue corresponding to Val-394 is also valine. The *E. coli* enzyme and the FeS4 mutant enzyme both have the conserved sequence V-Y-G-I-D at this position.

Although analytical measurements indicate somewhat lower values for Fe content in the FeS3 and FeS4 mutant enzymes compared to the wild type, the data are insufficient to establish a significant difference between the enzymes. We conclude that the enzyme preparations from the two mutants and wild type contained similar amounts of Fe. A stoichiometry of 6 g atoms of Fe/subunit for the *B. subtilis* enzyme made in *E. coli* (Table I) compares with a previous estimate of 3.2 g atoms/subunit (13). This discrepancy results from the methods used to determine protein. Previously, the stoichiometry for Fe content was based on an extinction coefficient of 9.6 at 278 nm for a 1% solution of enzyme subunit. This extinction coefficient was determined for enzyme purified from *B. subtilis*. Based on amino acid analysis and supported by NH_2-terminal amino acid sequencing, an extinction coefficient of 19.2 at 278 nm for a 10 mg/ml solution of enzyme subunit purified from *E. coli* was calculated. Further work is required to resolve this discrepancy and to characterize the Fe-S cluster in the *B. subtilis* enzyme made in *E. coli*.

Based on measurements of specific activity, it appears unlikely that the [4Fe-4S] cluster has a specific role in either the NH_3-dependent reaction or in glutamine amide transfer since both catalytic reactions were lost in parallel in FeS mutants (Table I). A role of the Fe-S center in allosteric regulation is excluded based on normal feedback inhibition by AMP for the residual enzyme activity of FeS mutants. We conclude from these experiments that the Fe-S cluster has no specific role in catalysis or allosteric regulation. This conclusion is in agreement with the work of Vollmer *et al.* (10), which showed that the [4Fe-4S] cluster does not function to transfer electrons during catalysis. If the Fe-S center has a role in O_2-dependent inactivation (9) that precedes amidophosphoribosyltransferase degradation during sporulation, mutations FeS1 and FeS2 might promote increased degradation during log phase growth. *purF* containing mutations FeS1 to FeS4 has been integrated into the *B. subtilis* chromosome in preparation for such experiments.[2]

Expression of $purF^+$ genes from plasmids pFeSwtL and pFeSwtT results in overproduction of *B. subtilis* amidophosphoribosyltransferase in *E. coli*, and indicates that NH_2-terminal undecapeptide processing is a post-translational rate limiting step. The most direct explanation for slow processing in this heterologous system is that an essential enzyme or factor is required which becomes limiting when amidophosphoribosyltransferase is overproduced. It was previously reported (3) that replacement of Cys-12 abolished glutamine-dependent activity as well as undecapeptide processing. Furthermore, a link was noted between NH_2-terminal processing and Fe-S content. A relationship between catalytic activity and processing was maintained in the FeS mutants. Mutant enzymes FeS1 and FeS2, having no activity, were not processed, whereas mutant enzymes FeS3 and FeS4, having partial activity, were processed. We suggest two alternative possibilities for the connection between functional Fe-S centers and NH_2-terminal processing of amidophosphoribosyltransferase. (i) A [4Fe-4S] center is required to maintain a native conformation which is required for activity and for processing. (ii) Processing requires glutamine-dependent catalytic activity which is dependent upon an Fe-S center. At present we cannot choose between these possibilities nor evaluate whether processing is factor-dependent, autocatalytic (3), or a combination of both.

It is intriguing that amidophosphoribosyltransferase from *E. coli* (5) and *Saccharomyces cerevisiae* (35) lacks both an Fe-S cluster and an NH_2-terminal undecapeptide leader. The basis for the connection between Fe-S cluster and NH_2-terminal leader could be that (i) the leader is required for

[2] C. Makaroff, D. E. Ebbole, and H. Zalkin, unpublished experiments.

assembly of the Fe-S cluster and/or (ii) the Fe-S cluster is required to remove the leader. Based on this connection, we predict that human amidophosphoribosyltransferase (36), an Fe-S protein, is synthesized with an undecapeptide leader.

Amidophosphoribosyltransferase purified from cells bearing plasmids pFeSwtL, pFeS3L, and pFeS4L was fully processed. Unprocessed enzyme is most likely removed during purification. If NH_2-terminal undecapeptide processing is required for glutamine-dependent activity, the unprocessed enzyme would not be detected during purification. It is known that the unprocessed Phe-12 mutant and the wild type enzyme chromatograph differently during purification (3). If unprocessed forms of the wild type, FeS3, and FeS4 enzymes also chromatograph differently from mature enzyme, they were likely discarded during purification.

SDS-polyacrylamide gel electrophoresis of the FeS1 and FeS2 mutant enzymes indicate that the enzymes are stable in growing cells. Based on their insolubility in extracts, we suggest that the stability of inactive FeS1 and FeS2 enzymes may result from formation of insoluble aggregates within the cell which are resistant to degradation by proteases. A second possibility that the mutant enzymes assume the native conformation is unlikely but can not be ruled out. Previous attempts to remove the Fe-S cluster from the wild type enzyme have yielded insoluble aggregates of denatured enzyme (9).

In contrast to the FeS1 and FeS2 mutant enzymes which are stable in the cell even under high level expression, the FeS5 mutation was extremely unstable. Several attempts to reconstruct the FeS5 mutant *purF* failed. In all cases a C → A reversion was observed which restored the wild type Phe residue at position 452. This result suggests that the FeS5 mutation is lethal to the cell. The apparent lethality of FeS5 is a likely result of the mutant enzyme interfering with normal cellular functions.

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