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Evidence That the Iron-Sulfur Cluster of *Bacillus subtilis* Glutamine Phosphoribosylpyrophosphate Amidotransferase Determines Stability of the Enzyme to Degradation *in Vivo**

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Bacillus subtilis **glutamine P-Rib-PP amidotransferase contains a [4Fe-4S] cluster which is essential for activity. The enzyme also undergoes removal of 11 NH_2-terminal residues from the primary translation product *in vivo* to form the active enzyme. It has been proposed that oxidative inactivation of the FeS cluster *in vivo* is the first step in degradation of the enzyme in starving cells. Four mutants of amidotransferases that alter cysteinyl ligands to the FeS cluster or residues adjacent to them have been prepared by site-directed mutagenesis, expressed in *Escherichia coli*, and characterized (Makaroff, C. A., Paluh, J. L., and Zalkin, H. (1986) *J. Biol. Chem.* 261, 11416–11423). These mutations were integrated into the *B. subtilis* chromosome in place of the normal *purF* gene. Inactivation and degradation *in vivo* of wild type and mutant amidotransferases were characterized in these integrants. Mutants FeS1 (C448S) and FeS2 (C451S) failed to form active enzyme, assemble FeS clusters, or undergo NH_2-terminal processing. The immunochemically cross-reactive protein produced by both mutants was degraded rapidly ($t_{1/2}$ = 16 min) in exponentially growing cells. In contrast the wild type enzyme was stable in growing cells, and activity and cross-reactive protein were lost from glucose-starved cells with a $t_{1/2}$ of 57 min. Mutant FeS3 (F394V) contained an FeS cluster and was processed normally, but had only about 40% of normal specific activity. The FeS3 enzyme was also inactivated by reaction with O_2 *in vitro* about twice as fast as the wild type. The amidotransferase produced by the FeS3 integrant was stable in growing cells but was inactivated and degraded in glucose-starved cells more rapidly ($t_{1/2}$ = 35 min) than the wild type enzyme. Mutant FeS4 (C451S, D442C) also contained an FeS cluster and was processed; the enzyme had about 50% of wild type-specific activity and reacted with O_2 *in vitro* at the same rate as the wild type. Inactivation and degradation of the FeS4 mutant *in vivo* in glucose-starved cells proceeded at a rate ($t_{1/2}$ = 45 min) that was somewhat faster than normal. The correlation between absence of an FeS cluster or enhanced lability of the cluster to O_2 and increased degradation rates *in vivo* supports the conclusions that stability of the enzyme *in vivo* requires an intact FeS cluster and that O_2-dependent inactivation is the rate-determining step in degradation of the enzyme. The fact that mutant FeS3 was processed normally but degraded rapidly argues against a role for NH_2-terminal processing in controlling degradation rates.**

Glutamine phosphoribosylpyrophosphate amidotransferase (EC 2.4.2.14) catalyzes the first step in *de novo* purine nucleotide biosynthesis:

Glutamine + P-Rib-PP → 5-phosphoribosyl-1-amine + glutamate + PPi.

The *Bacillus subtilis* enzyme has been purified and extensively characterized (1–3) and its corresponding gene, *purF*, has been cloned and sequenced (4). The *B. subtilis* enzyme is homologous to *Escherichia coli* glutamine P-Rib-PP amidotransferase, but in contrast to the *E. coli* enzyme (5) contains a [4Fe4S] center (6, 7) and an 11-amino acid NH_2-terminal sequence that is processed to yield the mature enzyme (4). The FeS center has been extensively characterized but as yet its role is unknown (7, 8).

B. subtilis glutamine P-Rib-PP amidotransferase is known to undergo a complex inactivation and degradation process. The enzyme is specifically inactivated and degraded in cells under carbon, amino acid, or nitrogen starvation (9–11). *In vitro* inactivation of amidotransferase has been shown to require molecular oxygen and to accompany oxidation of the FeS center. It has been proposed that the FeS center is involved in *in vivo* inactivation process (9–11). Makaroff and co-workers (12) have employed site-directed mutagenesis to investigate the role of the FeS center. Four mutations were constructed in and around the proposed [4Fe4S] cluster-binding site. The mutations are shown in Fig. 1 along with the wild type sequence.

While it appears as though the FeS cluster is necessary for NH_2-terminal processing and to maintain the proper tertiary structure of the enzyme (12), it is not known if the FeS cluster is directly involved in the inactivation and degradation of the enzyme. To investigate this possibility we have integrated the mutant *purF* genes into the *B. subtilis* chromosome and examined their effect on processing, inactivation, and degradation *in vivo*. We have also compared the rates of *in vitro* inactivation of amidotransferase in these mutants with rates of inactivation measured *in vivo*. The results show that mutant enzymes which fail to form FeS clusters *in vivo* or in

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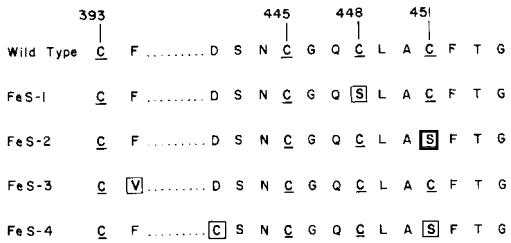

FIG. 1. **Amino acid replacements in the 4Fe-4S center.** Numbering of amino acids is from the NH_2 terminus of the primary translation product.

TABLE I

Plasmid and phage used for integration of the purF FeS mutations

The abbreviation used is: kb, kilobase.

Plasmid	Description
pPZ1	*B. subtilis purF*$^+$ *purM*$^+$ cloned on 3.3-kb *Eco*RI fragment in pBR322 (4)
pPZ3	1.7-kb *Hin*cII/*Eco*RI fragment containing *purM*$^+$ cloned in pUC13
pPZ4	1.7-kb *purM*$^+$ fragment cloned into *Pst*I/modified *Hin*dIII site of pUC18
pPZ5	Chloramphenicol acetyltransferase gene from pDE194 cloned between *Bam*HI/*Pst*I sites of pPZ4
pFeS1L	Contains Cys (448) → Ser mutation, designated C448S, in *purF* (12)
pFeS2L	C451S mutation in *purF* (12)
pFeS3L	F394V mutation in *purF* (12)
pFeS4L	C451S and D442C mutations (12)
pFeS1-I	*Eco*RI/*Sst*I fragment from pFeS1L containing *purF* cloned into pPZ5
pFeS2-I	*Eco*RI/*Sst*I fragment from pFeS2L cloned into pPZ5
pFeS3-I	*Eco*RI/*Sst*I fragment from pFeS3L cloned into pPZ5
pFeS4-I	*Eco*RI/*Sst*I fragment from pFeS4L cloned into pPZ5
pFeSwt-I	*Eco*RI/*Sst*I fragment from pFeSwtL cloned into pPZ5
pFeSwtT	Wild type *purF* (12)
pUC18	Cloning vector (29)
pUC13	Cloning vector (30)
pDE194	Contains the chloramphenicol acetyltransferase gene from pC194 cloned into pUC8 (16)

which the clusters are more readily destroyed by oxidation are also degraded more rapidly *in vivo*.

EXPERIMENTAL PROCEDURES

Strains and Media—*E. coli* strains AB352 (*thr, leuB6, purF, thi, lac24, rpsL8, supE44*) and JM83 (13) were used for plasmid construction. Both *E. coli* strains AB352 and LE392 (*hsdR514, metB1, trpR55*) were used as hosts for the expression of plasmid-encoded *purF* genes. Mutant *purF* genes were integrated into the chromosome of *B. subtilis* strain 168 (*trpC2*). L-broth was employed as a rich growth medium. Minimal growth medium contained M9 salts (14) supplemented with 0.5% glucose, 0.2% acid-hydrolyzed casein, 2 μg/ml thiamin, and trace minerals (15). Antibiotic concentrations were ampicillin 50 μg/ml and chloramphenicol 5 μg/ml. Indole acrylic acid (40 mg/l) was used to induce expression from the *trp* promoter.

Construction of Integration Plasmids—Table I lists plasmids used in this study and their relevant properties. Plasmids having *purF* mutations in FeS-liganding residues or their neighbors (12) are designated pFeS1L to pFeS4L (L designates *lac* promoter). In these plasmids *purF* is transcribed from the *lac* promoter (Fig. 2). pPZ3 was constructed by cloning a 1.5-kilobase *Hin*cII/*Eco*RI fragment containing *purM* DNA from pPZ1 into pUC13 (Fig. 2). For construction of pPZ4, the *Eco*RI site of pPZ3 and the *Hin*dIII site of pUC18 were blunt-ended and the plasmids were recut with *Pst*I. The blunt-ended *purM* fragment from pPZ3 having *Pst*I and modified *Eco*RI ends was ligated into the digested vector to yield pPZ4. The chloramphenicol acetyltransferase gene (16) from pDE194 was then cloned between the *Bam*HI and *Pst*I sites of pPZ4 to yield pPZ5. The final integration plasmids, designated pFeS1-I to pFeS4-I and pFeSWt-I, were then constructed by transferring an *Eco*RI/*Sst*I fragment from pFeSL plasmids containing a mutant or wild type *purF* gene into the *Eco*RI and *Sst*I polylinker sites in pPZ5. A representative integration plasmid (pFeS1-I) is shown at the bottom of Fig. 2.

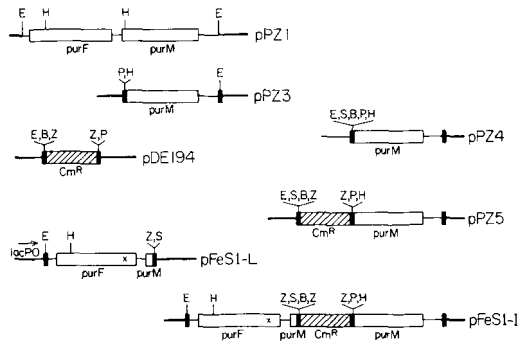

FIG. 2. **Schematic representation of vehicles used in the construction of *purF* integration plasmids.** For details of plasmid construction see "Experimental Procedures." Symbols used: *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hin*cII; *P*, *Pst*I; *S*, *Sst*I; *Z*, destroyed *Hin*cII site; ×, mutated FeS center; *open bars*, coding region; *closed bars*, polylinker DNA; *light line*, *B. subtilis* noncoding DNA; *heavy line*, vector DNA. Figures are not drawn to scale and only relevant regions are shown.

Integration of Mutants—Integration plasmids linearized with *Eco*RI were used to transform *B. subtilis* strain 168 to chloramphenicol resistance using the procedure of Anagnostopoulos and Spizizen (17).

Southern Analysis—*B. subtilis* chromosomal DNA was purified (18) from a chloramphenicol-resistant colony from each of the putative integrant strains, digested with *Eco*RI, and subjected to Southern analysis (14). Southern blots were probed with a 450-base pair *Eco*RI/*Hin*cII fragment from the 5′ end of *purF*, labeled with [α-^{32}P-dCTP] by primer extension. Stringent conditions were used for hybridization and washing (19).

Enzyme Assays—Cells were grown in minimal medium supplemented with adenine (7.5 μg/ml) and tryptophan (10 μg/ml) and harvested 30 min after adenine depletion, as evidenced by a sharp decrease in growth rate. Cells were resuspended (3 ml/g) in buffer A (1) containing 2 mM AMP, 5 mM dithiothreitol, and 1.0 mM phenylmethylsulfonyl fluoride and broken using a French press. Glutamine P-Rib-PP amidotransferase was assayed in crude cellular extracts by the glutamine dehydrogenase method (20). Protein concentrations were determined by the method of Lowry (21). Activities are expressed as μmol of glutamate produced min^{-1} mg^{-1} protein.

Inactivation and Degradation Experiments—Cell cultures were grown in buffered minimal medium supplemented with 50 μg/ml of 20 amino acids, 0.1% glucose, 5 μg/ml adenosine, and salts (11). A 5-ml culture grown in 0.8% supplemented nutrient broth for 5 h was used to inoculate 50 ml of buffered minimal medium containing 0.3% glucose and 50 μg/ml adenosine. This culture was grown overnight at 37 °C and used as an inoculum. Growth was monitored using a Klett-Summerson colorimeter with a No. 66 red filter. Integration of the *purF* genes resulted in a Pur$^-$ phenotype due to disruption of the *purM* gene. In order to express the *purF* gene in the cells containing this integration, the cell cultures were grown in buffered minimal medium containing 5 μg/ml of adenosine and allowed to starve for purines for 1.5 h. After derepression of the *purF* gene, 100 μg/ml of adenosine and guanosine were added to the medium. After resumption of logarithmic growth, samples were removed for activity and immunochemically cross-reactive material (CRM)[1] measurements. Cells were harvested by centrifugation at 4 °C for 2 min at 17,000 × *g*. Cell pellets were washed in ice-cold 50 mM Tris-HCl, pH 7.9, containing 1 M KCl and 1 mM phenylmethylsulfonyl fluoride. In experiments with BsFeS1 and BsFeS2, *N*-tosyl-L-lysine chloromethyl ketone, iodoacetamide, and *p*-chloromercuriphenylsulfonic acid were added at 1 mM each to prevent proteolysis.

[1] The abbreviations used are: CRM, immunochemically cross-reactive material; SDS, sodium dodecyl sulfate.

Iron and Inorganic Sulfide Analysis—Iron analysis was performed as described (7), except that tripyridyl-*S*-triazene was replaced by 3-(4-phenyl-2-pyridyl)-5-phenyl-1,2,4-triazine disulfonic acid (G. Fredric Smith Chemicals) as the chromogenic reagent. Inorganic sulfide was assayed by the method of Beinert (7, 22).

Production of Anti-amidotransferase IgG—Rabbit anti-amidotransferase serum was obtained by injecting female New Zealand rabbits with 1 mg of purified amidotransferase emulsified in Freund's complete adjuvant. Half was injected into the footpads and half under the skin on the back. Secondary immunization was administered by injecting 50 mM Tris-HCl, pH 7.9, containing 1 mg of antigen into the marginal ear vein. Secondary immunization was given on 3 successive days and was followed by bleeding 3 days thereafter. Immunoglobulin G was purified by protein A-agarose affinity chromatography and titered by precipitation of amidotransferase that was labeled by reductive methylation with [^{14}C]formaldehyde (23).

A strain of *B. subtilis*, KK1, was prepared in which the gene for chloramphenicol acetyltransferase replaced the promotor for the purine gene cluster (24). Extract of KK1 cells was incubated for 0.5 h at 37 °C with anti-amidotransferase IgG prior to each experiment. The antibody was then centrifuged for 2 min in a Microfuge to remove immunoprecipitate that formed. This preparation was used as primary antibody in immunoblotting experiments. The preabsorption with KK1 extracts was essential to obtain antibodies that were monospecific toward amidotransferase CRM.

Measurement of Immunologically Cross-reacting Material by Quantitative Immunoblotting—Crude cell extracts were separated on a SDS-polyacrylamide gel and the proteins transferred to a nitrocellulose sheet (Gelman Biotrace NT) using an American Bionetics Polyblot transfer apparatus. The nitrocellulose was blocked using 2% nonfat milk in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20 (TBST) for 10 min with shaking. Primary antibody was diluted 1:10,000 with TBST and incubated with the nitrocellulose blot for 30 min at room temperature. Detection was performed using ^{125}I-labeled protein A as secondary probe. Bands of cross-reacting material were located using the Protoblot system (Promega, Inc.). The bands corresponding to amidotransferase native molecular weight were excised from the nitrocellulose and counted in a gamma counter. Background counts were determined by running a lane of extract of strain KK1 and determining the radioactivity in the area corresponding to amidotransferase.

Quantitation of Amidotransferase Protein in Immunoprecipitates and Insoluble Fractions—E. coli AB352 (Leu$^-$) or LE392 (Met$^-$) cells were grown in culture media that contained either [^{3}H]leucine or [^{35}S]methionine, respectively, of known specific radioactivity. When a sample of amidotransferase was isolated from crude extracts of these cells by immunoprecipitation or sedimentation (see below), the nmol of amidotransferase subunit in the precipitates was calculated from their radioactivity and the known amino acid composition of the enzyme (4).

Iron Analysis of Immunoprecipitates—E. coli strain AB352 containing plasmids was grown in Vogel-Bonner (25) glucose medium containing Miller-Stadtman (15) metal salts except that $FeCl_3 \cdot 6H_2O$ was 14 μg/liter, and [^{3}H]leucine of known specific radioactivity was added. Cell extracts were prepared in argon-sparged 50 mM Tris-HCl pH 7.9 (buffer B) + 10 mM β-mercaptoethanol by sonication under an argon stream, and extracts were centrifuged at 115,000 × *g* for 30 min. In order to obtain a uniform background of nonspecifically absorbed radioactivity and Fe, it was necessary to obtain immunoprecipitates of approximately equal size. To do this the concentration of wild type and mutant enzymes was estimated by rocket electrophoresis (26). This permitted the concentration of wild type enzyme to be adjusted by dilution with AB352/pUC8 extract to equal that of the mutant enzymes. Rabbit anti-amidotransferase was added to the extracts, and immune complexes were precipitated by addition of fixed *Staphylococcus aureus* cells (Sigma). To correct for nonspecific radioactivity and iron which might contaminate the immunoprecipitate, a mock immunoprecipitation was done with nonradioactive apoamidotransferase added to labeled crude extract of AB352/pUC8. The apoamidotransferase used in this procedure was prepared by incubation of purified amidotransferase with 1,10-phenanthroline to remove iron (1).

Fe and S^{2-} Analysis of Amidotransferase Protein in Insoluble Precipitates—Plasmid-bearing *E. coli* LE392 cells were grown in Vogel-Bonner (25) medium containing [^{35}S]methionine. Extracts were centrifuged at 11,000 × *g* for 30 s, and the pellets were assayed for Fe and S^{2-}. The amidotransferase protein in the pellets was solubilized in SDS sample buffer containing 4 M urea and was quantitated following electrophoresis on an SDS-polyacrylamide gel. The gel was dried and exposed to x-ray film using Lightning Plus enhancing screens. The exposed film was used to locate the bands in the original gel, and these bands were excised, soaked in 200 μl of H_2O, and digested overnight with 20 ml of scintillation fluid containing TS-2 tissue solubilizer (RPI, Inc.). The radioactivity in the vials was used to calculate total amidotransferase protein in the pellet.

Immunochromatography—A 1-ml protein A-Sepharose column (Repligen) was derivatized with 10 mg of anti-amidotransferase IgG by cross-linking with dimethyl suberimidate (27). Extract containing amidotransferase was prepared in buffer A containing 1% Triton X-100 and 1 M NaCl. After loading the extract, the column was washed extensively with 50 mM Tris-HCl, pH 7.9, 1% Triton X-100, and 2 M NaCl. Antigen was eluted with either 4 M guanidine HCl or 0.1 M glycine, pH 1.5.

Protein Sequencing—Automated Edman sequencing was performed by the Protein Sequencing Center at the University of Illinois, Urbana, using an Applied Biosystems sequenator.

Measurement of Inactivation of Amidotransferase in Vitro—Extracts of *E. coli* strain AB352-containing plasmids pFeSwtT, pFeS3L, and pFeS4L were mixed with oxygen-sparged buffer and incubated at 37 °C as described by Bernlohr and Switzer (28). Aliquots were removed at various times and assayed for amidotransferase. Control samples received argon sparging in place of oxygen.

RESULTS

Further Characterization of FeS1 and FeS2 Mutant Enzymes—Four mutations in amino acid ligands to the [4Fe-4S] cluster of *B. subtilis purF* were previously constructed using site-directed mutagenesis (12). These four mutations and the wild type sequence are shown in Fig. 1. Wild type and mutant proteins FeS1 and FeS2 were produced from plasmids in *E. coli* as previously described (12). These proteins were expressed to approximately 40% of the cell protein, as estimated by SDS-polyacrylamide gel electrophoresis. The cell extracts from strains bearing mutant plasmids contained no glutamine-dependent amidotransferase activity. Approximately 7% of the FeS1 and FeS2 mutant enzymes was present in crude extracts as soluble CRM; the remainder was insoluble material (Table II). In contrast, more than half of the overproduced, plasmid-encoded wild type enzyme was soluble in *E. coli* extracts. The insoluble material precipitated by centrifuging these extracts at 11,000 × *g* for 30 s was approximately 80% pure amidotransferase. The Fe and S^{2-} content of the insoluble forms of wild type and mutant enzymes was measured by assay of these precipitates without further purification. Iron content of the soluble forms of the wild type and mutant enzymes was analyzed after immunoprecipitation as described under "Experimental Procedures." The results of these analyses are shown in Table II. It can be seen that, when either wild type or the FeS1 and FeS2 mutant enzymes were overproduced in *E. coli*, insoluble forms accumulated

TABLE II

Iron and sulfide content of soluble and insoluble forms of B. subtilis wild type and mutant P-Rib-PP amidotransferases produced in an E. coli host

Plasmid	Amido-transferase fraction	Amount of amidotransferase nmol/mg protein[a]	FeS/subunit	S^{2-}/subunit
pFeSwt-I	Soluble	0.7	4.3 ± 0.5	ND[b]
pFeSwt-I	Insoluble	0.4	0.1 ± 0.06	1.7 ± 0.2
pFeS1-I	Soluble	0.2	1.0 ± 0.5	ND
pFeS1-I	Insoluble	2.2	0.4 ± 0.2	1.0 ± 0.1
pFeS2-I	Soluble	0.2	1.1 ± 0.6	ND
pFeS2-I	Insoluble	2.7	0.4 ± 0.1	1.0 ± 0.1

[a] mg protein refers to total soluble protein in the crude extract.
[b] ND, not determined because S^{2-} was shown to contaminate control immunoprecipitates.

that did not contain significant Fe and contained relatively little S^{2-}. The soluble form of wild type *B. subtilis* amidotransferase produced in *E. coli* contained, as shown previously (4), a normal FeS cluster (Table II). However, the soluble forms of the FeS1 and FeS2 mutant enzymes did not contain Fe. Either these mutant proteins cannot accept an FeS cluster, or it is extremely labile and destroyed by gentle immunoprecipitation. The insoluble forms of amidotransferase may represent protein which was produced faster than FeS cluster insertion can take place in *E. coli* since even the wild type gene led to formation of amidotransferase protein without cluster. The soluble form of the enzymes, however, are likely to be the same as is produced in *B. subtilis* when the corresponding genes are integrated into the chromosome.

Mutant enzymes FeS3 and FeS4 were previously purified by conventional means (12). Iron analysis showed that these proteins contained a nearly normal content of iron (12). Properties of the four mutant enzymes purified from the *E. coli* host are summarized in Table III.

Integration of FeS Mutants—To characterize the effect of the mutations in *B. subtilis in vivo*, we have replaced the wild type copy of *purF* with each of the four FeS mutants. A series of integration plasmids was constructed that contained a mutant copy of *purF*, a chloramphenicol acetyltransferase gene, and *purM* DNA. The integration plasmids were constructed such that a double recombination event, once in *purF* and once in *purM*, would transfer the chloramphenicol resistance gene and, most likely, the FeS mutation into the chromosome. A representative integration plasmid as well as a diagram of the *purF* region of the *B. subtilis* strain 168 chromosome before and after integration is shown in Fig. 3.

Chloramphenicol-resistant colonies originally selected on rich medium were screened for adenine-dependent growth. Integrants of each of the mutants and the wild type *purF* gene failed to grow in the absence of adenine. Ebbole and Zalkin (24) have shown that *purF* is part of a *pur* gene cluster in *B. subtilis* and that *purM* lies downstream of *purF*. During the construction of the intergation plasmids it was necessary to join vector polylinker DNA and the chloramphenicol resistance gene to codon 10 of *purM*. When the plasmids are integrated into the chromosome, disruption of *purM* occurs and results in purine auxotrophy.

A chloramphenicol-resistant colony representing each of the FeS mutants, FeS1 to FeS4, and wild type *purF* was picked and characterized further. For simplicity the strains will be designated BsFeS1 through BsFeS4 and BsFeSwt to indicate their relationship to the pFeS plasmids from which they were generated.

TABLE III

Properties of mutant P-Rib-PP amidotransferases and B. subtilis integrants studied in this work

Enzyme	Gln-dependent activity[a]	FeS cluster[b]	NH_2 terminally processed? in *E. coli*[c]	Amount produced in *B. subtilis* integrants	
				Activity[d]	CRM[d]
	% of wild type			*units/mg*	*% of BsFeSwt*
Wild type	100	Yes	Yes	0.1	100
FeS1	0	No	No	<0.002	8
FeS2	0	No	No	<0.002	5
FeS3	15	Yes	Yes	0.03	79
FeS4	24	Yes	Yes	0.04	80

[a] Enzymes were isolated from plasmid-bearing *E. coli*. The wild type, FeS3 and FeS4 enzymes were purified to homogeneity (12). Isolation of FeS1 and FeS2 enzymes is described in the text.

[b] Data for wild type, FeS3, and FeS4 from Ref. 12. Data for FeS1 and FeS2 are from Table II.

[c] From Ref. 12.

[d] Results from this work.

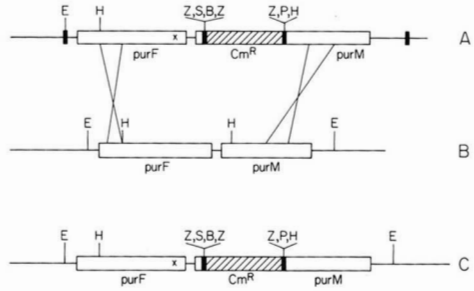

FIG. 3. **Schematic representation of the integration of mutant *purF* plasmids into the *B. subtilis* chromosome.** *A*, a representative integration plasmid (pFeS1-I). *B*, structure of the *purF* region of the chromosome in strain 168. *C*, structure of the *purF* region of the chromosome in a BsFeS integrant strain. Symbols used are the same as those used in Fig. 2. Figures are not drawn to scale.

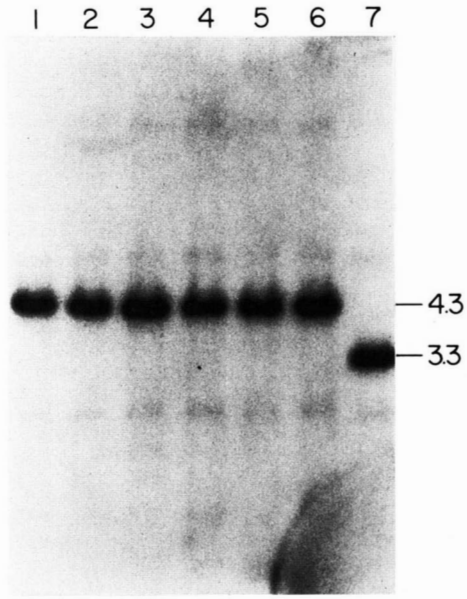

FIG. 4. **Southern blot analysis of *B. subtilis* strain 168 and BsFeS integrant DNA.** Chromosomal DNA was digested with *Eco*RI. *Lane 1*, BsFeS-Wt; *lanes 2–5*, BsFeS-1 through BsFeS-4, respectively; *lane 6*, BsFeS-5, an integrant of mutant FeS-5 (12) not studied in this work; *lane 7*, 168; and probed with a [α-^{32}P]dCTP-labeled fragment of *purF*.

Characterization of Integrants—To verify integration as diagrammed in Fig. 3 for the BsFeS wild type and mutant strains, chromosomal DNA from strain 168 and the integration strains was digested with *Eco*RI and subjected to Southern analysis. The data in Fig. 4 show that there is one copy of *purF* present in the chromosome of the integration strains and it is contained on a 4.3-kilobase *Eco*RI fragment (*lanes 1–6*). In the chromosome of strain 168, *purF* is present on a 3.3-kilobase *Eco*RI fragment (*lane 7*). The increase in size of the *Eco*RI fragment is due to insertion of the chloramphenicol acetyltransferase gene between *purF* and *purM*. Thus, analysis of the integrants at the DNA level indicates that the wild type chromosomal copy of *purF* has been successfully replaced by the plasmid borne FeS mutants.

For measurement of amidotransferase levels in BsFeS integrants (Table III), cells were grown in the presence of low levels of adenine (8 μg/ml) and harvested approximately 30 min after the adenine had been depleted from the medium. These conditions were previously used to obtain derepressed enzyme levels while avoiding the inactivation and degradation

of the enzyme which occurs late in the growth cycle (1). Crude extracts of BsFeSwt exhibited an amidotransferase level of 0.1 units/mg protein, which is comparable to previously reported values (1). Enzyme activity in strains BsFeS1 and BsFeS2 was undetectable. Likewise amidotransferase from pFeS1L and pFeS2L was undetectable in *E. coli in vitro* and *in vivo* (12). BsFeS3 and BsFeS4 contained activities of 0.03 and 0.04 units/mg protein, respectively. Thus, amidotransferase levels in crude extracts from the integration derivatives are consistent with the activities observed for the plasmid-encoded enzymes in *E. coli* and indicate that the mutations have been transferred to the chromosome. Immunochemical determinations of the levels of amidotransferase protein in BsFeSwt and BsFeS1 to BsFeS4 are also shown in Table III. BsFeS3 and BsFeS4 produced nearly normal amounts of amidotransferase, but BsFeS1 and BsFeS2 produced only 5–8% of normal levels. The low level of FeS1 and FeS2 CRM could result from either insolubility in crude extract or rapid degradation of these mutants *in vivo*. The first possibility was eliminated by analyzing pellets prepared from centrifugation of sonicated cell suspension as is done in the normal extraction procedure. The pellets were solubilized in SDS-polyacrylamide gel electrophoreis sample buffer plus 4 M urea and subjected to electrophoresis on a 10% SDS gel for immunoblotting. Less than 10% of the total FeSwt, FeS1, and FeS2 CRM was found in these pellets. It will be shown below that the low level of CRM in BsFeS1 and BsFeS2 is probably the result of rapid degradation of these mutant enzymes *in vivo*.

Wild type amidotransferase undergoes normal NH_2-terminal processing in both *B. subtilis* and *E. coli* (4). Mutants FeS3 and FeS4 have also been shown to be processed normally in *E. coli* (12). Thus, it may be assumed that the amidotransferase produced by the BsFeSwt, BsFeS3, and BsFeS4 integrants are all processed normally. The FeS1 and FeS2 mutants are not processed in *E. coli* (12), so we determined whether they are processed in the BsFeS1 and BsFeS2 integrants. This was examined in two ways. First, SDS-polyacrylamide gel electrophoresis on 12.5% slab gels followed by Western immunoblotting permitted the unprocessed form of amidotransferase to be resolved from processed enzyme (12). The migration of amidotransferase CRM from extracts of both BsFeS1 and BsFeS2 was slightly slower than amidotransferase CRM from BsFeSwt, which indicates that these mutant proteins are not processed normally. Second, a small amount of amidotransferase from BsFeS2 was isolated by immunoabsorption chromatography and subjected to automated Edman degradation. The identifies of the phenylthiohydantoin derivatives released confirmed that at least the majority of amidotransferase from BsFeS2 had not undergone NH_2-terminal processing. Since the electrophoretic behavior of CRM from BsFeS1 and BsFeS2 was identical, we concluded that the enzyme from BsFeS1 was also unprocessed.

Inactivation and Degradation of FeSwt in B. subtilis—Levels of CRM were measured by quantitative immunoblotting with ^{125}I-protein A as described under "Experimental Procedures." Over the range of CRM measured in these experiments, radioactivity in the amidotransferase band increased linearly with the amount of cell extract loaded on the polyacrylamide gel. In the experiments described in Figs. 5 and 6 and in Table IV, adenosine and guanosine were added to permit exponential growth until glucose was exhausted. The nucleosides also fully repressed amidotransferase synthesis so there was no contribution of newly synthesized enzyme to the CRM measured by immunoblotting. Thus, rates of CRM disappearance in these experiments represent rates of degradation only. Fig. 5 shows that amidotransferase activity and

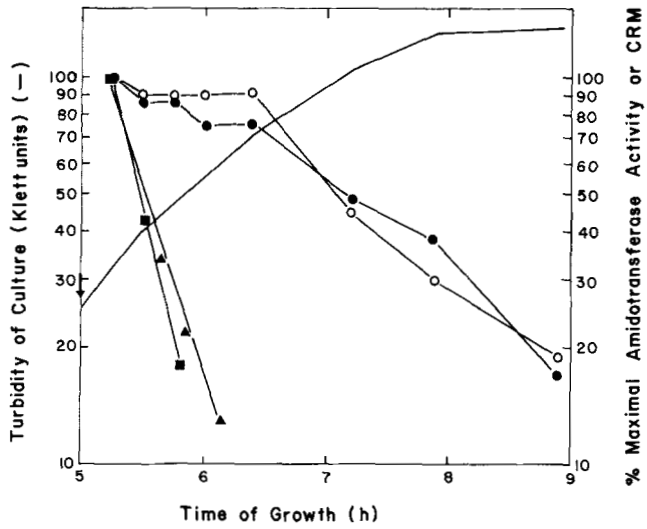

FIG. 5. **Disappearance of FeSwt amidotransferase activity and CRM of FeS1 and FeS2 CMR in logarithmically growing and glucose-starved *B. subtilis* cells.** BsFeSwt, BsFeS1, and BsFeS2 were cultured in buffered minimal medium plus 5 μg/ml adenosine and 0.1% glucose. After 1.5 h of adenosine starvation, 100 μg/ml each of adenosine and guanosine were added to the culture to repress further amidotransferase synthesis and to allow the cells to grow (↓). Samples were removed during logarithmic growth and glucose starvation. The cells were harvested, extracted, assayed for amidotransferase activity, and analyzed for amidotransferase CRM by quantitative immunoblotting with ^{125}I-protein A. Symbols used: ○, FeSwt activity; ●, FeSwt CRM; ■, FeS1 CRM; ▲, FeS2 CRM.

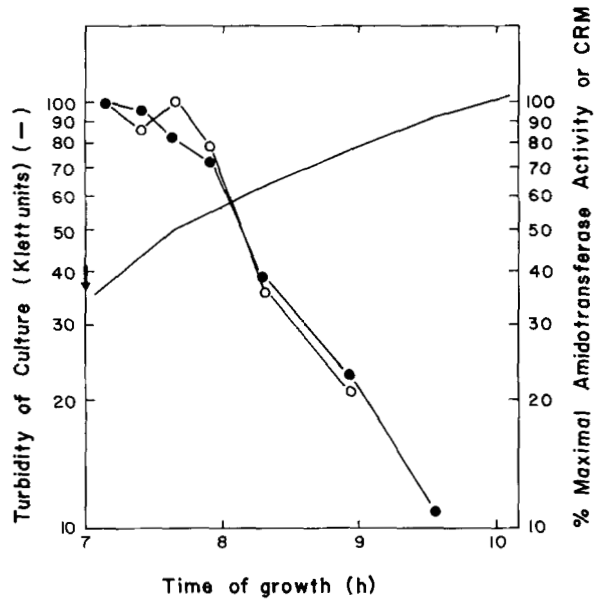

FIG. 6. **Amidotransferase activity and CRM of BsFeS3 in logarithmically growing and glucose-starved cells.** The experiment was performed as in legend to Fig. 5. Symbols used: ○, FeS3 activity; ■, FeS3 CRM.

CRM in BsFeSwt were stable during exponential growth and disappeared in parallel upon glucose starvation (Fig. 5). This result is in agreement with previous results of Ruppen and Switzer (11). The half-life of amidotransferase in stationary phase was 57 ± 3 min, which is faster than the 90 min half-life measured by Ruppen and Switzer in strain LC168. We do not know the reason for this difference.

Degradation of Mutant Amidotransferase Proteins in B. subtilis—CRM of BsFeS1 and BsFeS2, which produce amidotransferases lacking FeS clusters, was lost very rapidly

TABLE IV

Stability of native and mutant P-Rib-PP amidotransferase activity and protein in B. subtilis integrants

Strain studied	Stability of amidotransferase *in vivo*				Stability of amidotransferase *in vitro*[a]
	Half life of activity[b]		Half-life of cross-reactive protein[b]		
	Exponential phase	Stationary phase	Exponential phase	Stationary phase	Half life of activity[b]
	min				
BsFeSwt	Stable[c]	57 ± 3	Stable	65 ± 3	28 ± 1
BsFeS1	NA[d]	NA	16 ± 1	UD[e]	NA
BsFeS2	NA	NA	16 ± 4	UD	NA
BsFeS3	Stable	30 ± 5	Stable	38 ± 4	17 ± 0.3
BsFeS4	Stable	47 ± 3	Stable	43 ± 5	27 ± 1

[a] Stability of amidotransferase activity *in vitro* was measured in crude extracts on O_2-saturated buffer as described by Bernlohr and Switzer (28) in studies with the purified enzyme.

[b] All experiments were repeated at least three times. Uncertainties are given as average deviations from the mean values.

[c] Stable denotes a half-life of about 4 h or longer; longer half-lives could not be accurately measured under the experimental conditions used.

[d] NA, activity was <0.002 units/mg.

[e] UD, half-life was not determined because no (<5%) CRM was detectable in the stationary phase cells.

during exponential growth, during which time the wild type enzyme was stable (Fig. 5, Table IV). The rate of degradation of these mutant enzymes in exponential phase was four times faster than that of wild type enzyme in stationary phase (Table IV).

The mutant amidotransferases which contain an FeS cluster, BsFeS3, and BsFeS4, were stable during logarithmic growth, as was seen for the wild type enzyme. Upon glucose starvation, BsFeS3 activity and CRM were lost with half-lives of 30 ± 5 and 35 ± 4 min, respectively (Fig. 6, Table IV). This degradation was about twice as fast as the wild type enzyme. Activity and CRM for BsFeS4 during glucose starvation declined slightly faster than BsFeSwt (Table IV), but not as rapidly as BsFeS3. The half-lives of activity and CRM for this mutant were 47 ± 3 and 43 ± 5 min, respectively.

Oxygen-dependent Inactivation of Wild Type, FeS3, and FeS4 Mutant Amidotransferases in Vitro—Reaction of oxygen with the FeS cluster of purified amidotransferase has been shown to cause rapid inactivation of the enzyme, which was accompanied by destruction of the secondary and tertiary structure. Bernlohr and Switzer (28) have proposed that inactivation of amidotransferase *in vivo* also results from oxidation of the FeS cluster. Since the mutants BsFeS3 and BsFeS4 were inactivated faster than the wild type *in vivo*, we tested the sensitivity of these mutant proteins to oxidation in cell extracts *in vitro*. The rate of oxidation of plasmid-encoded wild type and mutants FeS3 and FeS4 in crude extracts of *E. coli* AB352 was measured by the method of Bernlohr and Switzer (28). The wild type enzyme was inactivated *in vitro* with a half-life of 28 ± 1 min in oxygen-sparged buffer, as seen previously (28). Mutant BsFeS3 had a half-life of 17 ± 0.3 min under these conditions, while mutant BsFeS4 was inactivated at a rate similar to the wild type enzyme ($t_{½}$ = 27 ± 1 min). When argon was substituted for oxygen in these experiments, all three enzymes were completely stable.

DISCUSSION

It was shown previously that *E. coli* cells were able to carry out normal NH_2-terminal processing and FeS cluster insertion reactions on the primary translation product of the plasmid-encoded *B. subtilis purF* gene (4). These modifications were carried out by the *E. coli* host despite the fact that the *E. coli* P-Rib-PP amidotransferase undergoes neither modification (4, 5). This result made it possible to study *B. subtilis* amidotransferase maturation in an *E. coli* overproduction system (12, 31). As with many cloned proteins, overproduction of wild type amidotransferase yielded a large amount of insoluble material. We have shown that this material is neither NH_2 terminally processed[2] nor does it contain an FeS cluster (Table II). We suggest that in this case synthesis of the primary translation product outruns the processing events and that failure to insert an FeS cluster results in precipitation of an incorrectly folded protein. Results of Souciet *et al.* (31) also suggest that NH_2-terminal processing requires FeS cluster insertion but that FeS cluster insertion can occur without NH_2-terminal processing. Both events are apparently necessary for glutamine-dependent enzyme activity.

Overproduction of FeS1 and FeS2 mutant proteins in *E. coli* also resulted in the formation of a large fraction of insoluble enzyme. Only 6–7% of the mutant protein was soluble, as compared with 62% for the wild type produced under the same conditions (Table II). As was shown for the insoluble wild type enzyme, the insoluble mutant enzymes contained no Fe or S^{-2} and were not processed at the NH_2 terminus. It was also important to analyze the soluble mutant enzymes produced in *E. coli* because the *B. subtilis* integrant strains produced mostly soluble mutant enzyme. Methods were developed to determine the Fe content of the soluble FeS1 and FeS2 enzymes, which had previously been refractory to purification. Our data indicate that the soluble FeS1 and FeS2 proteins contain no FeS clusters and are not processed at the NH_2 terminus. We conclude that the lack of cluster insertion and NH_2-terminal processing in these mutants is due to the amino acid replacements and is not an artifact of overproduction and precipitation *in vivo*, as appears to be the case for the insoluble wild type enzyme. We further conclude that the inability to incorporate an FeS cluster results in an improperly folded enzyme that is poorly soluble, cannot undergo normal NH_2-terminal processing, and does not have glutamine-dependent activity. Consistent with this idea, the FeS3 and FeS4 mutant proteins, which were capable of FeS cluster formation, were soluble, processed at the NH_2 terminus and had at least a fraction of normal activity.

As noted above, we have assumed that the posttranslational maturation of amidotransferase in *E. coli* is the same as occurs in *B. subtilis*. This has been exhaustively documented for the wild type enzyme (4). The issue has not been fully tested with the mutant integrants because they produce quite small amounts of enzymes, which in two cases were very rapidly degraded *in vivo*. However, in all cases where it was examined, the properties of the mutant enzymes produced by the integrants are exactly those predicted from the studies in *E. coli*. Mutants FeS1 and FeS2 were inactive, as expected, and were not processed at their NH_2 termini. Mutants FeS3 and FeS4 were active but had specific activities less than half the wild type enzyme, as expected (12). They also appear from electrophoresis and Western blotting to be processed normally.

The major purpose for studying the integrants of mutant *B. subtilis* amidotransferases was to examine the effects of the mutations on the inactivation and degradation of this enzyme in growing and nutrient-starved *B. subtilis*. Since both wild type and mutant enzymes are relatively stable in *E. coli*, a specific system governing the degradation of these enzymes exists in *B. subtilis*. It has been previously proposed that this system involves the following two steps (1, 28): rate-

[2] J. Grandoni, unpublished experiments.

limiting inactivation of the enzyme by reaction of the FeS cluster with O_2, followed by degradation of the inactive protein. The present observations cannot establish this model unequivocally, but they provide striking support for it. The BsFeSwt integrant behaved as expected from studies with wild type *B. subtilis* cells. Amidotransferase was stable during growth; upon glucose starvation activity and CRM disappeared simultaneously. In the BsFeS1 and BsFeS2 integrants, which produced mutant proteins that did not assemble FeS clusters, the amidotransferase CRM was very rapidly degraded during exponential growth and undetectable during starvation. This indicates that amidotransferase protein in which an FeS cluster is not inserted or in which it has been destroyed by oxidation is very labile *in vivo*, as previously proposed.

The BsFeS3 integrant produces a mutant enzyme that does form an FeS cluster. This cluster is somewhat abnormal, however, as shown by the lower specific activity of this enzyme and the enhanced reactivity of the enzyme with O_2 *in vitro*. The lower specific activity was demonstrated both for the purified FeS3 mutant enzyme (12) and in extracts of the integrant (Table III). Strikingly, the activity of the FeS3 enzyme in the integrant was essentially stable in growing *B. subtilis* cells, but both activity and CRM disappeared twice as fast during starvation as the wild type amidotransferase. These results accorded well with the suggestion that normal amidotransferase is protected against O_2 in growing cells but that O_2-dependent destruction of the FeS cluster leads to immediate degradation of the protein. The enhanced relative lability to O_2 of the FeS3 enzyme matched exactly the enhanced rate of degradation *in vivo*. Furthermore, FeS3 mutant enzyme was processed normally at its NH_2 terminus. Thus, enhanced degradation did not result from differences in NH_2-terminal structure. This leads us to deduce that the same was also true for the FeS1 and FeS2 mutant proteins, which were not processed.

The FeS4 mutant protein also had a slightly altered FeS cluster, as judged from the lower specific activity of the mutant enzyme. This alteration was not detectable in studies of the rate of inactivation by O_2 *in vitro*, however. The FeS4 mutant protein was relatively stable in growing cells, but was inactivated and degraded somewhat more rapidly than the wild type enzyme, but more slowly than the FeS3 mutant protein. This modest enhancement in degradation rate presumably resulted from a more labile FeS cluster, but we did not demonstrate this directly by our *in vitro* oxidation experiments.

It is striking that in all cases the rate of inactivation of amidotransferase *in vivo* was equal within experimental error to the rate of degradation. This supports the conclusion that inactivation is the rate-limiting step in degradation. The results in this paper reinforce the previous proposal that the inactivation step *in vivo* is the reaction of O_2 with the FeS cluster of the enzyme. The mechanisms used by *B. subtilis* cells to protect the FeS cluster in growing cells against O_2 have been extensively studied but are not yet fully understood (10, 28).

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REFERENCES

1. Wong, J. Y., Bernlohr, D. A., Turnbough, C. L., and Switzer, R. L. (1981) *Biochemistry* **20,** 5669–5674
2. Vollmer, S. J., Switzer, R. L., Hermodson, M. A., Bower, S. G., and Zalkin, H. (1983) *J. Biol. Chem.* **258,** 10582–10585
3. Meyer, E., and Switzer, R. L. (1979) *J. Biol. Chem.* **254,** 5397–5402
4. Makaroff, C. A., Zalkin, H., Switzer, R. L., and Vollmer, S. J. (1983) *J. Biol. Chem.* **258,** 10586–10593
5. Tso, J. Y., Hermodson, M. A., and Zalkin, H. (1982) *J. Biol. Chem.* **257,** 3532–3536
6. Wong, J. Y., Meyer, E., and Switzer, R. L. (1977) *J. Biol. Chem.* **252,** 7424–7426
7. Averill, B. A., Dwivedi, A., Debrunner, P., Vollmer, S. J., Wong, J. Y., and Switzer, R. L. (1980) *J. Biol. Chem.* **255,** 6007–6010
8. Vollmer, S. J., Switzer, R. L., and Debrunner, P. G. (1983) *J. Biol. Chem.* **258,** 14284–14293
9. Turnbough, C. L., and Switzer, R. L. (1975) *J. Bacteriol.* **121,** 108–114
10. Bernlohr, D. A., and Switzer, R. L. (1983) *J. Bacteriol.* **153,** 937–949
11. Ruppen, M. E., and Switzer, R. L. (1983) *J. Biol. Chem.* **258,** 2843–2851
12. Makaroff, C. A., Paluh, J. L., and Zalkin, H. (1986) *J. Biol. Chem.* **261,** 11416–11423
13. Vieria, J., and Messing, J. (1982) *Gene (Amst.)* **19,** 259–268
14. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15. Miller, R. E., and Stadtman, E. R. (1972) *J. Biol. Chem.* **247,** 7407–7419
16. Worinouchi, S., and Weisblum, B. (1982) *J. Bacteriol.* **150,** 815–825
17. Anagnostopoulos, C. A., and Spizizen, J. (1961) *J. Bacteriol.* **81,** 741–746
18. Marmur, J. (1961) *J. Mol. Biol.* **3,** 208–218
19. Davis, R. W., Botstein, D., Roth, J. R. (1980) *A Manual for Genetic Engineering: Advanced Bacterial Genetics*, pp. 159–177, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Messenger, L. J., and Zalkin, H. (1979) *J. Biol. Chem.* **254,** 3382–3392
21. Layne, E. (1957) *Methods Enzymol.* **3,** 447–454
22. Beinert, H. (1983) *Anal. Biochem.* **131,** 373–378
23. Jentoft, N., and Dearborn, D. G. (1983) *Methods Enzymol.* **91,** 570–579
24. Ebbole, D. J., and Zalkin, H. (1987) *J. Biol. Chem.* **262,** 8274–8287
25. Vogel, H. J., and Bonner, D. M. (1956) *J. Biol. Chem.* **218,** 97–106
26. Svendsen, J. P., Weeke, B., and Johansson, B.-G. (1983) *Scand. J. Immunol.* **17** (Suppl. 10), 3–20
27. Reeves, H. C., Heeren, R., and Malloy, P. (1981) *Anal. Biochem.* **115,** 194–196
28. Bernlohr, D. A., and Switzer, R. L. (1981) *Biochemistry* **20,** 5675–5681
29. Norrander, J., Kempe, T., and Messing, J. (1983) *Gene (Amst.)* **26,** 101–108
30. Vieria, J., and Messing, J. (1982) *Gene (Amst.)* **19,** 259–268
31. Souciet, J.-L., Hermodson, M. A., and Zalkin, H. (1988) *J. Biol. Chem.* **263,** 3323–3327