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Cloning of the *Bacillus subtilis* Glutamine Phosphoribosylpyrophosphate Amidotransferase Gene in *Escherichia coli*

NUCLEOTIDE SEQUENCE DETERMINATION AND PROPERTIES OF THE PLASMID-ENCODED ENZYME*

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The *Bacillus subtilis* gene encoding glutamine phosphoribosylpyrophosphate amidotransferase (amidophosphoribosyltransferase) was cloned in pBR322. This gene is designated *purF* by analogy with the corresponding gene in *Escherichia coli*. *B. subtilis purF* was expressed in *E. coli* from a plasmid promoter. The plasmid-encoded enzyme was functional *in vivo* and complemented an *E. coli purF* mutant strain. The nucleotide sequence of a 1651-base pair *B. subtilis* DNA fragment was determined, thus localizing the 1428-base pair structural gene. A primary translation product of 476 amino acid residues was deduced from the DNA sequence. Comparison with the previously determined NH_2-terminal amino acid sequence indicates that 11 residues are proteolytically removed from the NH_2 terminus, leaving a protein chain of 465 residues having an NH_2-terminal active site cysteine residue. Plasmid-encoded *B. subtilis* amidophosphoribosyltransferase was purified from *E. coli* cells and compared to the enzymes from *B. subtilis* and *E. coli*. The plasmid-encoded enzyme was similar in properties to amidophosphoribosyltransferase obtained from *B. subtilis*. Enzyme specific activity, immunological reactivity, *in vitro* lability to O_2, Fe-S content, and NH_2-terminal processing were virtually identical with amidophosphoribosyltransferase purified from *B. subtilis*. Thus *E. coli* correctly processed the NH_2 terminus and assembled [4Fe-4S] centers in *B. subtilis* amidophosphoribosyltransferase although it does not perform these maturation steps on its own enzyme. Amino acid sequence comparison indicates that the *B. subtilis* and *E. coli* enzymes are homologous. Catalytic and regulatory domains were tentatively identified based on comparison with *E. coli* amidophosphoribosyltransferase and other phosphoribosyltransferase (Argos, P., Hanei, M., Wilson, J., and Kelley, W. (1983) J. Biol. Chem. 258, 6450–6457).

Glutamine phosphoribosylpyrophosphate amidotransferase (EC 2.4.2.14) is a glutamine amidotransferase which catalyzes the initial reaction in the *de novo* pathway for purine nucleotide synthesis (Equation 1).

$$\text{P-Rib-PP}^1 + \text{glutamine} \rightarrow \text{phosphoribosylamine} + \text{glutamate} + \text{PP}_i \quad (1)$$

Amidophosphoribosyltransferases from *Bacillus subtilis* (1) and *Escherichia coli* (2) have been purified to homogeneity and characterized. Several catalytic and regulatory properties of the enzymes from the two organisms are similar. Chemical modification studies with glutamine affinity analogs have identified an NH_2-terminal active site cysteine residue in both enzymes that is essential for the glutamine amide transfer catalytic function (3, 4). Amidophosphoribosyltransferases from *B. subtilis* and *E. coli* are both subject to end product inhibition by purine nucleotides (2, 5). The two enzymes exhibit a major structural difference. *B. subtilis* amidophosphoribosyltransferase contains a [4Fe-4S] cluster which is essential for activity (6, 7), whereas an Fe-S center is not present in *E. coli* amidophosphoribosyltransferase (2, 3). The role of the Fe-S center in *B. subtilis* amidophosphoribosyltransferase is not understood. A possibility is that it may participate in a specific O_2-dependent inactivation (8, 9) that occurs late in the growth cycle. There is little information about gene regulation but important differences in the two organisms appear likely.

E. coli purF has recently been cloned and sequenced as a first step to gain further information on gene regulation and enzyme structure (10). We report here the cloning in *E. coli* of *B. subtilis purF*,[2] its nucleotide sequence, and purification of the plasmid-encoded amidophosphoribosyltransferase. These data provide significant new insights into structural features that contribute to amidophosphoribosyltransferase function.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP (>2000 Ci/mmol), 5′-deoxynucleoside [α-^{32}P]triphosphates (3000 Ci/mmol), and L-[^{35}S]methionine (>1000 Ci/mmol) were purchased from Amersham Corp. Restriction endonucleases were purchased from commercial suppliers. Phage T4 DNA

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[1] The abbrevations used are: P-Rib-PP, 5-phosphoribosyl-1-pyrophosphate; amidophosphoribosyltransferase, glutamine phosphoribosylpyrophosphate amidotransferase; kb, kilobase pair; bp, base pair.

[2] Five genes of purine nucleotide synthesis have been identified and designated *purA–purE* (11). Gene-enzyme correlations have not been made. We arbitrarily designate the *B. subtilis* gene encoding amidophosphoribosyltransferase *purF* by analogy with the corresponding *E. coli* gene.

ligase was from New England Biolabs. Phage T4 polynucleotide kinase and *E. coli* DNA polymerase Klenow fragment were obtained from Bethesda Research Laboratories. Calf intestinal phosphatase was a product of Boehringer Mannheim. *E. coli* strains TX158 (ara Δlac ϕ (*purF* 200-lac::λpl(209)) (12) and LE392 (13) have been described. DNA was isolated from *B. subtilis* strain 168 (*trpC*2) as described (14) and was purified by banding in CsCl/ethidium bromide. Plasmid pSB5 was the source of *E. coli purF* (10). Amidophosphoribosyltransferase was purified from *B. subtilis* (1) and *E. coli* (3).

Media—Medium E (15) supplemented with 0.5% glucose, 2 μg/ml of thiamin, and appropriate antibiotic was used as the minimal growth medium for *E. coli*. L broth or nutrient agar (Difco) was employed as rich media for *E. coli*. For enzyme production, *E. coli* strain TX158 bearing plasmid pPZ2 was grown as described (3). *B. subtilis* was grown in Penassay broth (Difco). Antibiotic concentrations were ampicillin, 25 μg/ml, and tetracycline, 10 or 20 μg/ml.

DNA Isolation—A rapid procedure was used to screen transformed strains for plasmids (16). This procedure was scaled up for large scale preparation of plasmid. Plasmid DNA was banded in CsCl/ethidium bromide. DNA fragments for sequencing were isolated by preparative electrophoresis on 5% polyacrylamide gels and extracted by the crush-soak procedure (17).

Preparation of B. subtilis Plasmid Pool—Chromosomal DNA (2 μg) from *B. subtilis* strain 168 was digested to completion with the restriction endonuclease *Eco*RI, and the resulting fragments were ligated into the *Eco*RI site of pBR322. The ligated mixture was used to transform *E. coli* strain LE392 to ampicillin resistance. All of the transformants (approximately 1.4×10^5) were collected and plasmid DNA was isolated.

Restriction Endonuclease Digestions and Ligation of DNA Fragments—Digestion of DNA with restriction endonucleases was carried out using conditions recommended by the supplier. Conditions for ligation of DNA fragments have been described (10).

DNA Sequence Determination—DNA sequences were determined by the method of Maxam and Gilbert (17). DNA fragments were end labeled either by using [γ-^{32}P]ATP and polynucleotide kinase or by filling in with [α-^{32}P]dNTP and *E. coli* DNA polymerase I Klenow fragment. Strand separation was carried out at either 22 or 5 °C. The polyacrylamide/urea gel system described by Sanger and Coulson (18) was used. DNA sequences were analyzed by computer (19, 20).

Hybridization Analysis of Cloned B. subtilis purF—B. subtilis chromosomal DNA and pPZ1 plasmid DNA were digested with *Eco*RI, electrophoresed on a 0.7% agarose gel, and then transferred to nitrocellulose (21). The probe was pPZ1 labeled by nick translation (21). Hybridization was in 65% formamide at 42 °C (21).

In Vitro Enzyme Synthesis—Coupled transcription/translation was carried out using plasmids pSB5 (*E. coli purF*) and pPZ1 (*B. subtilis purF*) in the standard S-30 system (22). Proteins were radioactively labeled with [^{35}S]methionine. The amidotransferases were immunoprecipitated with antibodies specific for each enzyme. Immune precipitates were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and radioactive proteins visualized by autoradiography.

Purification and Characterization of Amidophosphoribosyltransferase from Cloned purF—Amidophosphoribosyltransferase was purified from *E. coli* TX158/pPZ2 cells by the procedure previously developed for purifying the enzyme from *B. subtilis* cells (1). To obtain enzyme of acceptable purity it was necessary to repeat the final ammonium sulfate precipitation step an additional two times, precipitating the enzyme by adding ammonium sulfate to 15 to 20% of saturation in the absence of AMP and dithiothreitol. The enzyme was assayed, and its Fe and S^{2-} content were determined as previously described (1).

Analyses—Sequenator analysis was performed with a Beckman sequenator, model 890, using the procedures of Mahoney *et al.* (23). Carboxyl-terminal analyses were performed essentially as described by Oroszlan *et al.* (24) using carboxypeptidase B (diisopropylfluorophosphate treated, Sigma) and/or carboxypeptidase A (diisopropylfluorophosphate treated). Release of amino acids was quantitated by amino acid analysis.

The *E. coli* and *B. subtilis* amino acid sequences were aligned by computer using their nucleotide sequences (20). Searches for homologous nucleotide sequences were performed in the following manner. Every possible span of length L bases from *E. coli purF* was aligned with all possible stretches of length L in *B. subtilis purF*. The total base difference for each oligonucleotide match was determined. Only the first and second base positions were examined to accommodate genomes of differing GC content. Since the third base in codons is degenerate, it is strongly influenced by the overall GC content of the genome. The length L was initially chosen as 45 bases, of which only 30 were actually compared. This length made reasonable allowances for gaps while preserving statistical significance. For sequences with little or no homology, an additional comparison was made using a length L of 30 bases in which 20 were actually compared.

RESULTS

Cloning of B. subtilis purF—B. subtilis purF was isolated from a plasmid pool containing *Eco*RI fragments of *B. subtilis* chromosomal DNA ligated into the *Eco*RI site of pBR322. Selection for *purF* was in *E. coli purF* strain TX158. In a typical experiment strain TX158 was transformed with 1 μg of plasmid pool and of approximately 1×10^5 ampicillin-resistant transformants, 150 were purine independent. All Pur$^+$ transformants examined contained a 7.3-kb plasmid with a 3-kb insert in the *Eco*RI site of pBR322. A representative plasmid was saved and designated pPZ1. Plasmid pPZ1 was mapped with several restriction endonucleases. A map is shown in Fig. 1.

In an effort to localize the gene and reduce the size of the insert, the 3-kb *Eco*RI fragment was subcloned. Partial digestions with *Hinc*II followed by religation allowed isolation of a series of plasmids having deletions of internal *Hinc*II fragments. Of these deletions, plasmid pPZ2 in which the 1.7-kb *Hinc*II fragment *a* (Fig. 1) was removed retained *purF* gene function. Other *Hinc*II deletions of pPZ1 lost the capacity to transform strain TX158 to purine prototrophy. Plasmid pPZ2 contained a 1.65-kb *Hinc*II-*Eco*RI *B. subtilis* insert (Fig. 1, fragment *b*) in partially shortened pBR322. Plasmid pPZ2

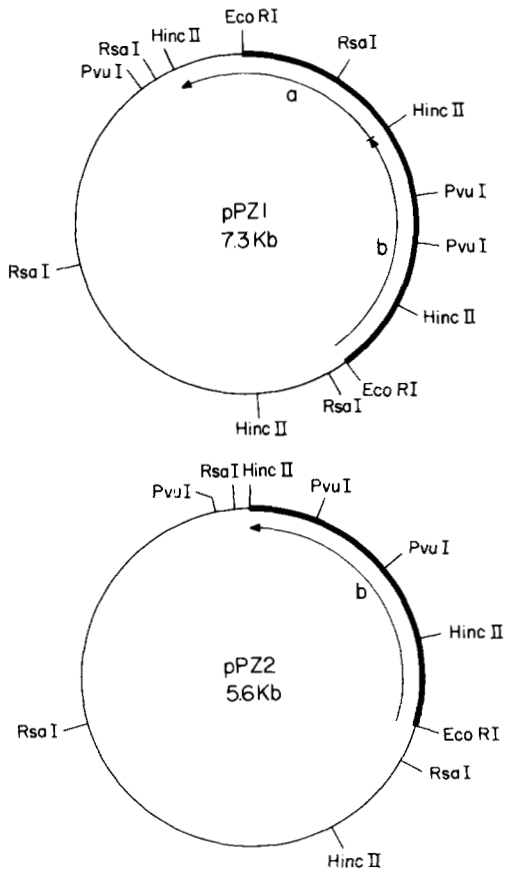

FIG. 1. **Restriction maps of plasmids and DNA inserts.** The *heavy line* represents *B. subtilis* chromosomal DNA, and the *light line* represents DNA from the pBR322 cloning vector. Segment *a* is a 1.7-kb *HincII* fragment that was deleted from plasmid pPZ1 in construction of pPZ2. Segment *b* is a 1.65-kb *Eco*RI-*Hinc*II fragment of *B. subtilis* DNA that contains *purF*.

conferred a Pur$^+$ tetracycline-resistant phenotype upon strain TX158.

A calculation indicated that based on a M_r of approximately 50,000 for *B. subtilis* amidophosphoribosyltransferase (1) a *purF* structural gene of approximately 1.4 kb is expected. The 1.65-kb insert in pPZ2 is thus of sufficient size to contain the *purF* structural gene.

The Cloned Insert Is Derived from B. subtilis—To confirm that pPZ1 does in fact carry *B. subtilis purF* which encodes amidophosphoribosyltransferase the following experiments were conducted. Southern blot analysis of plasmid and chromosomal DNA was carried out to establish that the cloned insert is derived from *B. subtilis* DNA. *B. subtilis* chromosomal DNA and pPZ1 plasmid DNA were digested with *Eco*RI. Samples were electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose, and probed with nick translated pPZ1. The data in Fig. 2 show hybridization of the probe to pBR322 and the cloned *Eco*RI insert (lane *1*) and to an identical 3-kb *Eco*RI fragment from *Eco*RI-digested chromosomal DNA (lane 2). Cross-hybridization of *E. coli purF* with *B. subtilis* DNA was not detected with the hybridization conditions that were employed.

In vitro coupled transcription/translation was used to confirm that *B. subtilis* amidophosphoribosyltransferase was encoded by the cloned gene. For *in vitro* enzyme synthesis the S-30 was prepared from *E. coli* strain TX158 to ensure the complete absence of endogenous *E. coli* amidophosphoribosyltransferase. [^{35}S]Methionine-labeled proteins synthesized from plasmids pPZ1 (*B. subtilis*) and pSB5 (*E. coli* (10)) were immune precipitated with antisera specific for each enzyme. The immune precipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis next to samples of the purified enzymes (Fig. 3). *B. subtilis* amidophosphoribosyltransferase synthesized *in vitro* (lane *1*) migrated in the same position as the enzyme purified from *B. subtilis* (lane *3*). The *B. subtilis* enzyme subunit made *in vitro* (lane *1*) or *in vivo* (lane *3*) is of slightly lower molecular weight than the M_r = 56,395 protein chain made by *E. coli in vitro* (lane 2) or *in vivo* lane *4*). We, therefore, conclude that plasmid pPZ1 encodes *B. subtilis* amidophosphoribosyltransferase and that *B. subtilis purF* is expressed in *E. coli*.

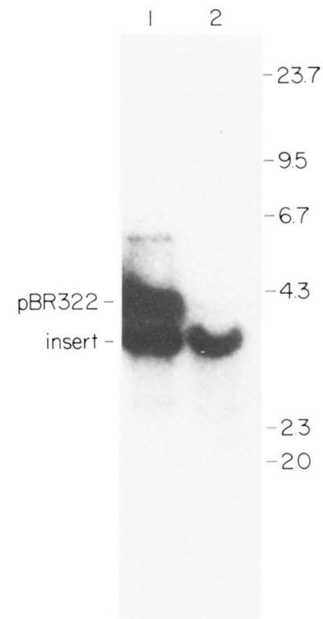

FIG. 2. **Southern blot analysis of pPZ1 plasmid and *B. subtilis* chromsomal DNA.** Lane *1*, pPZ1 plasmid DNA digested with *Eco*RI; lane *2*, *B. subtilis* chromosomal DNA digested with *Eco*RI. The hybridization probe was nick translated pPZ1.

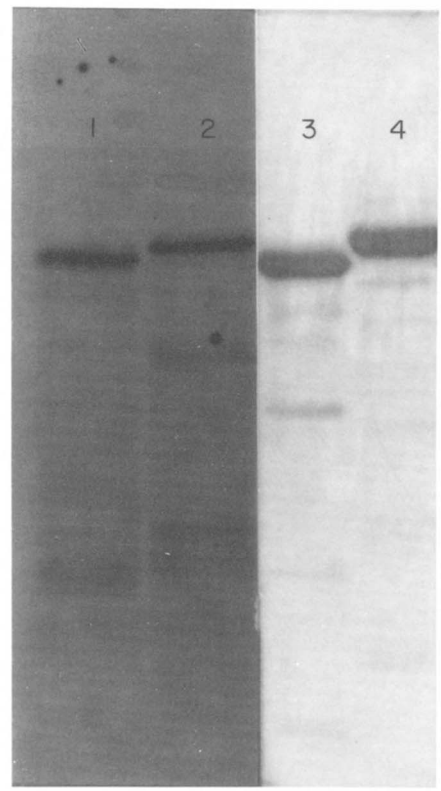

FIG. 3. **Comparison of *E. coli* and *B. subtilis* amidophosphoribosyltransferase made *in vitro* and *in vivo*.** Lanes *1* and *2*, immune precipitated *B. subtilis* and *E. coli* amidophosphoribosyltransferase synthesized *in vitro* from pPZ1 and pSB5, respectively. Lanes *3* and *4*, amidophosphoribosyltransferase purified from *B. subtilis* and *E. coli*, respectively.

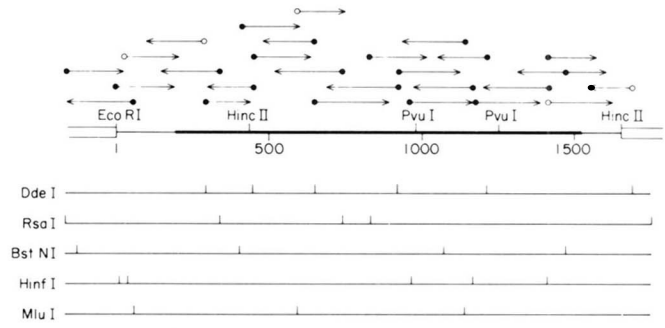

FIG. 4. **Restriction endonuclease sites and sequencing strategy used to establish the nucleotide sequence of *B. subtilis purF*.** Nucleotides are numbered from the 5′ proximal *Eco*RI site. *Arrows* indicate the extent of each sequence determination. *Arrows* originating from *closed circles* represent 5′ end labeled fragments. *Arrows* originating from *open circles* represent 3′ end labeled fragments. The *thick open line* represents pBR322. The *heavy line* represents the coding region. The *light line* represents flanking regions of *B. subtilis* DNA.

Nucleotide Sequence Determination—The nucleotide sequence of *purF* was determined using fragments isolated from plasmid pPZ2. A restriction map of the 1.65-kb insert as well as the sequencing strategy is shown in Fig. 4. Most fragments were 5′-labeled with [γ-^{32}P]ATP and polynucleotide kinase followed by strand separation. In several cases *Dde*I and *Hinf*I 3′ fragment ends were labeled by filling in with deoxynucleoside [α-^{32}P]triphosphate and DNA polymerase I Klenow fragment. Approximately 90% of the sequence was obtained from

both strands. The nucleotide sequences of all fragments were overlapped using different fragments to ensure that no small regions were missing. The nucleotide sequence of the 1651-bp *Eco*RI-*Hinc*II insert is shown in Fig. 5. The coding sequence is flanked by 88 bp at the 5′ end and 135 bp at the 3′ end.

Deduced Amino Acid Sequence—The amino acid sequence as deduced from the nucleotide sequence is shown in Fig. 5. The NH_2-terminal amino acid sequence of amidophosphoribosyltransferase purified from *B. subtilis* has been reported (4). The NH_2-terminal amino acid sequence of amidophosphoribosyltransferase corresponds exactly with residues 12 to 35 shown in Fig. 5. Thus residues 1 to 11 are post-translationally removed to yield the functional enzyme having an NH_2-terminal cysteine residue. As with *E. coli* amidophosphoribosyltransferase (3) the NH_2-terminal cysteinyl is an active site residue that is essential for the glutamine amide transfer function of the enzyme (4).

The CO_2H-terminal residue of amidophosphoribosyltransferase was determined and compared with that predicted by the nucleotide sequence. Digestion with carboxypeptidase B released approximately 1 mol of lysine per mol of enzyme. No other amino acids were released. Results of digestion with carboxypeptidases A plus B are shown in Table I. Release of lysine, threonine, leucine, valine, alanine, and glutamate, in the order named, corresponds with the sequence at the CO_2H terminus that was deduced from the DNA (Fig. 5). No other amino acids were released

A comparison of the reported (1) amino acid composition with that deduced from the DNA is given in Table II. All values are identical within the experimental errors of amino acid analysis. We conclude that the DNA sequence is free from frame shift errors that could alter the translational reading frame or lead to an incorrect translation stop codon. The DNA sequence encodes a primary translation product of

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          10        20        30        40        50        60        70        80
GAATTCTGGCGATTCAAAACCAAGACGGACAACAAATGATTCATGCGCAAACGAAAGAGCTTGAACGCGTATGGAAAGGAGCTATCCC

            100         110         120         130         140         150         160         170
ATG CTT GCT GAA ATC AAA GGC TTA AAT GAA GAA TGC GGC GTT TTT GGG ATT TGG GGA CAT GAA GAA GCC CCG CAA ATC ACG TAT TAC GGT
MET LEU ALA GLU ILE LYS GLY LEU ASN GLU GLU CYS GLY VAL PHE GLY ILE TRP GLY HIS GLU GLU ALA PRO GLN ILE THR TYR TYR GLY
                                     10                                  20                                      30

            190         200         210         220         230         240         250         260
CTC CAC AGC CTT CAG CAC CGA GGA CAG GAG GGT GCT GGC ATC GTA GCG ACT GAC GGT GAA AAG CTG ACG GCT CAC AAA GGC CAA GGT CTG
LEU HIS SER LEU GLN HIS ARG GLY GLN GLU GLY ALA GLY ILE VAL ALA THR ASP GLY GLU LYS LEU THR ALA HIS LYS GLY GLN GLY LEU
                                     40                                  50                                      60

            280         290         300         310         320         330         340         350
ATC ACT GAA GTA TTT CAA AAC GGC GAA CTC AGC AAA GTA AAG GGA AAA GGC GCT ATC GGG CAC GTT CGG TAC GCA ACG GCT GGA GGC GGC
ILE THR GLU VAL PHE GLN ASN GLY GLU LEU SER LYS VAL LYS GLY LYS GLY ALA ILE GLY HIS VAL ARG TYR ALA THR ALA GLY GLY GLY
                                     70                                  80                                      90

            370         380         390         400         410         420         430         440
GGA TAC GAA AAT GTT CAG CCG CTC CTC TTC CGT TCC CAA AAC AAC GGC AGC CTG GCG CTT GCT CAT AAC GGA AAT CTT GTC AAC GCC ACT
GLY TYR GLU ASN VAL GLN PRO LEU LEU PHE ARG SER GLN ASN ASN GLY SER LEU ALA LEU ALA HIS ASN GLY ASN LEU VAL ASN ALA THR
                                    100                                 110                                     120

            460         470         480         490         500         510         520         530
CAG CTG AAG CAG CAG CTC GAA AAT CAA GGG AGC ATC TTT CAA ACC TCT TCG GAT ACA GAG GTT TTG GCT CAC CTG ATC AAA AGA AGC GGA
GLN LEU LYS GLN GLN LEU GLU ASN GLN GLY SER ILE PHE GLN THR SER SER ASP THR GLU VAL LEU ALA HIS LEU ILE LYS ARG SER GLY
                                    130                                 140                                     150

            550         560         570         580         590         600         610         620
CAC TTC ACG CTG AAG GAT CAA ATT AAA AAC TCG CTT TCT ATG CTG AAA GGC GCC TAC GCG TTC CTG ATC ATG ACC GAA ACA GAA ATG ATT
HIS PHE THR LEU LYS ASP GLN ILE LYS ASN SER LEU SER MET LEU LYS GLY ALA TYR ALA PHE LEU ILE MET THR GLU THR GLU MET ILE
                                    160                                 170                                     180

            640         650         660         670         680         690         700         710
GTC GCA CTT GAT CCA AAC GGG CTG AGA CCG CTA TCC ATC GGC ATG ATG GGC GAC GCT TAT GTG GTC GCA TCA GAA ACA TGC GCA TTT GAC
VAL ALA LEU ASP PRO ASN GLY LEU ARG PRO LEU SER ILE GLY MET MET GLY ASP ALA TYR VAL VAL ALA SER GLU THR CYS ALA PHE ASP
                                    190                                 200                                     210

            730         740         750         760         770         780         790         800
GTC GTC GGC GCA ACG TAC CTT CGC GAG GTA GAG CCG GGA GAA ATG CTG ATC ATT AAT GAT GAA GGC ATG AAA TCA GAG CGT TTT TCC ATG
VAL VAL GLY ALA THR TYR LEU ARG GLU VAL GLU PRO GLY GLU MET LEU ILE ILE ASN ASP GLU GLY MET LYS SER GLU ARG PHE SER MET
                                    220                                 230                                     240

            820         830         840         850         860         870         880         890
AAT ATC AAT CGT TCC ATT TGC AGC ATG GAG TAC ATT TAT TTC TCC AGA CCA GAC AGC AAT ATT GAC GGT ATT AAT GTG CAC AGT GCC CGT
ASN ILE ASN ARG SER ILE CYS SER MET GLU TYR ILE TYR PHE SER ARG PRO ASP SER ASN ILE ASP GLY ILE ASN VAL HIS SER ALA ARG
                                    250                                 260                                     270

            910         920         930         940         950         960         970         980
AAA AAC CTT GGG AAA ATG CTG GCT CAG GAA TCC GCA GTT GAA GCT GAC GTC GTA ACC GGG GTT CCG GAT TCC AGT ATT TCA GCG GCG ATC
LYS ASN LEU GLY LYS MET LEU ALA GLN GLU SER ALA VAL GLU ALA ASP VAL VAL THR GLY VAL PRO ASP SER SER ILE SER ALA ALA ILE
                                    280                                 290                                     300

           1000        1010        1020        1030        1040        1050        1060        1070
GGC TAT GCA GAG GCA ACA GGC ATT CCG TAT GAG CTT GGC TTA ATC AAA AAC CGT TAT GTT GGC AGA ACG TTT ATT CAG CCG TCC CAG GCT
GLY TYR ALA GLU ALA THR GLY ILE PRO TYR GLU LEU GLY LEU ILE LYS ASN ARG TYR VAL GLY ARG THR PHE ILE GLN PRO SER GLN ALA
                                    310                                 320                                     330

           1090        1100        1110        1120        1130        1140        1150        1160
CTG CGT GAG CAA GGC GTC AGA ATG AAG CTG TCT GCG GTG CGC GGG GTT GTA GAA GGC AAA CGC GTC GTG ATG GTG GAT GAC TCT ATC GTG
LEU ARG GLU GLN GLY VAL ARG MET LYS LEU SER ALA VAL ARG GLY VAL VAL GLU GLY LYS ARG VAL VAL MET VAL ASP ASP SER ILE VAL
                                    340                                 350                                     360

           1180        1190        1200        1210        1220        1230        1240        1250
CGA GGA ACA ACT AGC CGC CGG ATT GTC ACG ATG CTA AGA GAG GCG GGT GCG ACA GAG GTG CAT GTG AAA ATC AGT TCA CCG CCG ATC GCT
ARG GLY THR THR SER ARG ARG ILE VAL THR MET LEU ARG GLU ALA GLY ALA THR GLU VAL HIS VAL LYS ILE SER SER PRO PRO ILE ALA
                                    370                                 380                                     390

           1270        1280        1290        1300        1310        1320        1330        1340
CAT CCG TGC TTT TAC GGC ATT GAC ACT TCC ACA CAT GAA GAA CTG ATC GCG TCT TCG CAT TCT GTC GGA GAA ATC CGT CAG GAA ATC GGA
HIS PRO CYS PHE TYR GLY ILE ASP THR SER THR HIS GLU GLU LEU ILE ALA SER SER HIS SER VAL GLY GLU ILE ARG GLN GLU ILE GLY
                                    400                                 410                                     420

           1360        1370        1380        1390        1400        1410        1420        1430
GCC GAT ACC CTC TCA TTT TTG AGT GTG GAA GGG CTG CTG AAA GGC ATC GGC AGA AAA TAC GAT GAC TCG AAT TGC GGA CAG TGT CTC GCT
ALA ASP THR LEU SER PHE LEU SER VAL GLU GLY LEU LEU LYS GLY ILE GLY ARG LYS TYR ASP ASP SER ASN CYS GLY GLN CYS LEU ALA
                                    430                                 440                                     450

           1450        1460        1470        1480        1490        1500        1510
TGC TTT ACA GGA AAA TAT CCG ACT GAA ATT TAC CAG GAT ACA GTG CTT CCT CAC GTA AAA GAA GCA GTA TTA ACC AAA
CYS PHE THR GLY LYS TYR PRO THR GLU ILE TYR GLN ASP THR VAL LEU PRO HIS VAL LYS GLU ALA VAL LEU THR LYS
                                    460                                 470

1520      1530      1540      1550      1560      1570      1580      1590      1600      1610      1620      1630
TAAAACTTGAAAAATGACATAAAGGCAGCGCAGTTCGGCTGCCTTTCTCTTTCTGCCCTCGTTCGGGGAGATATTTTGAAAAGCGCCTTAAAGGAGTGAATAGGATGTCTGAAGCATA

  1640      1650
TAAAAACGCAGGAGTTG
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FIG. 5. **Nucleotide and deduced amino acid sequence of *B. subtilis purF*.** The nucleotide sequence is numbered from the 5′ end of the fragment. Amino acids are numbered from the ATG codon. The first 11 residues are removed by post-translational processing. The two regions of dyad symetry possibly involved in transcription termination are *underlined*.

476 amino acids. After NH_2-terminal processing the mature protein chain contains 465 amino acids and has a calculated M_r of 50,397. This molecular weight is in close agreement with the value of approximately 50,000 previously determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1).

Purification and Properties of B. subtilis Amidophosphoribosyltransferase Synthesized in E. coli—E. coli strain TX158 bearing plasmid pPZ2 overproduced *B. subtilis* amidophosphoribosyltransferase. The specific activity in extracts of strain TX158/pPZ2 varied from 0.07 to 0.16 unit/mg. These values compare with a specific activity of 0.09 unit/mg obtained from derepressed *B. subtilis* (1). The plasmid-encoded enzyme was purified by a modification of the procedure of Wong *et al.* (1). The purified protein appeared to be greater than 95% pure by electrophoretic analysis on sodium dodecyl sulfate-containing polyacrylamide gels; a minor contaminant migrating at the dye front was detectable. NH_2-terminal sequence analysis of the purified protein indicated that this contaminant which stained poorly with Coomassie blue constituted 10 to 20 mol % of the sample. The purified enzyme had a specific activity of 27 units/mg. Specific activities in the range from 35 to 45 units/mg are generally obtained for the enzyme purified from *B. subtilis* (1, 7). Amidophosphoribosyltransferase from *E. coli* strain TX158/pPZ2 contained nearly normal amounts of the Fe-S center. The UV-visible absorption spectrum was identical with the *B. subtilis* enzyme; the A_{420}:A_{278} ratio was 0.24. The Fe and S^{2-} contents were 3.2 and 3.3 g atoms/mol of subunit, respectively. A range of 2.6 to 3.7 g atoms of Fe and 2.5 to 3.3 g atoms of S^{2-} per mol of subunit was found in various preparations of enzyme isolated from *B. subtilis* (7). The amidophosphoribosyltransferases from *B. subtilis* and *E. coli* TX158/pPZ2 were indistinguishable in activity neutralization assays using antibody raised against the enzyme isolated from *B. subtilis*. The lability of the enzyme from the two sources to O_2-saturated buffers was identical; both decayed with a half-life of 25 to 28 min at 37 °C and pH 7.9. Like the *B. subtilis* enzyme, amidophosphoribosyltransferase from *E. coli* TX158/pPZ2 possessed glutaminase activity equal to 0.5% of the amidotransferase activity.

TABLE I

Carboxyl-terminal analysis of amidophosphoribosyltransferase

Amidophosphoribosyltransferase (250 nmol) was treated with carboxypeptidases A plus B. Samples were removed at the indicated times and analyzed for amino acids released.

Time	Lys	Thr	Leu	Val	Ala	Glu
min	*mol amino acid/mol protein*					
15	0.95	0.11	0.13	ND[a]	ND	ND
60	0.86	0.38	0.38	0.24	0.03	ND
120	0.93	0.81	0.80	0.98	0.10	0.03

[a] ND, not detected.

NH_2-Terminal Amino Acid Sequence of B. subtilis Enzyme Obtained from E. coli—Highly purified amidophosphoribosyltransferase encoded by *B. subtilis purF* and obtained from *E. coli* strain TX158 was subjected to 24 cycles of automated Edman degradation. The amino acid sequence was identical

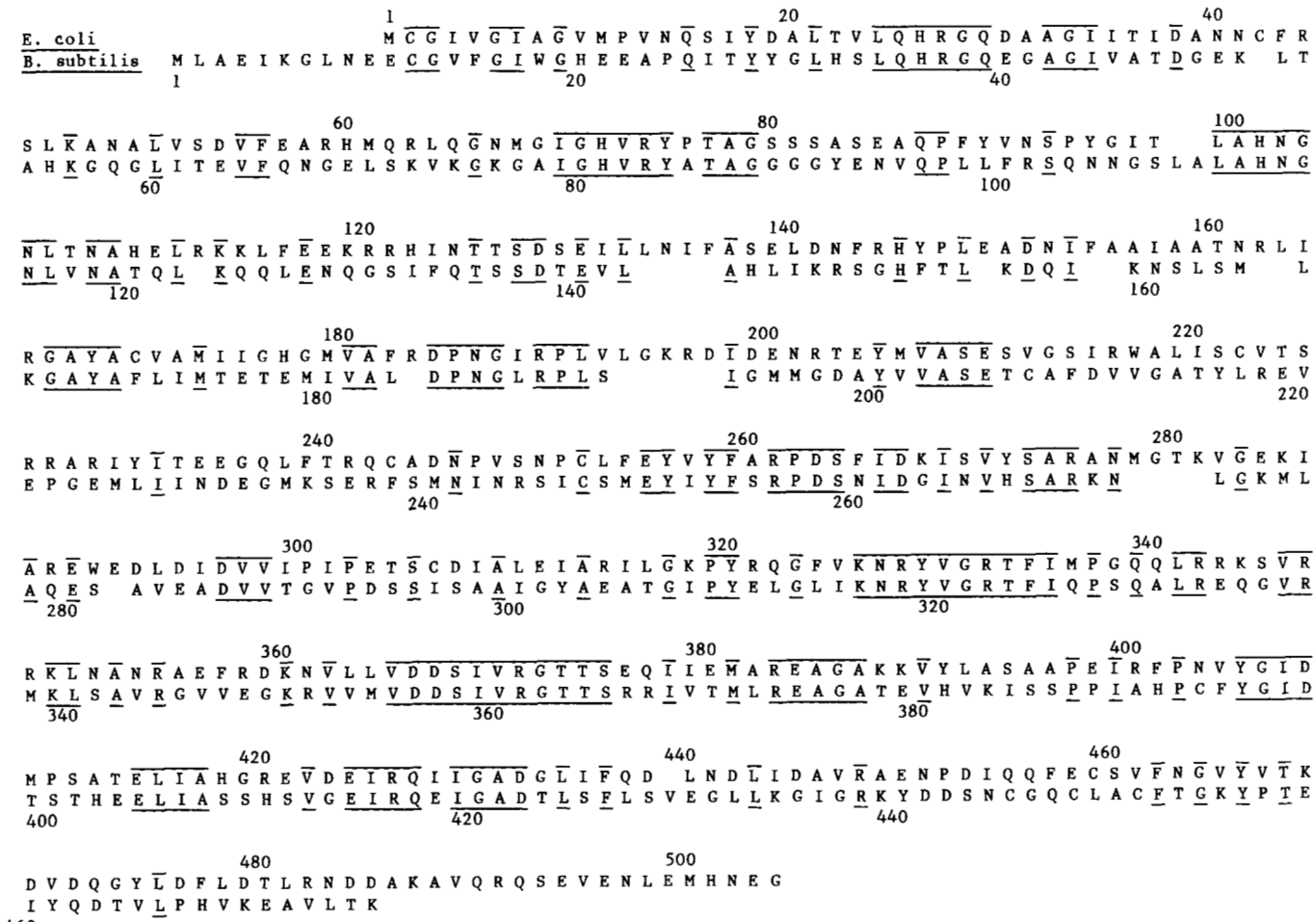

FIG. 6. ***E. coli* and *B. subtilis* amidophosphoribosyltransferase aligned to give maximum homology by computer (20).** Identical amino acids are *underlined* and *overlined*.

TABLE II

Comparison of amino acid composition of B. subtilis purF deduced from DNA sequence with that obtained by amino acid analysis

Amino acid	Residues per subunit	
	Deduced from DNA sequence[a]	Amino acid analysis[b]
Ala	35	34
Arg	22	20
Asn	18	
Asp	18	36
Cys	7	7
Gln	20	
Glu	33	54
Gly	49	47
His	14	13
Ile	33	30
Leu	38	37
Lys	22	22
Met	13	12
Phe	13	12
Pro	14	14
Ser	36	34
Thr	27	27
Trp	1	1
Tyr	16	13
Val	36	35

[a] The composition of the mature enzyme after NH_2-terminal processing is given.

[b] Wong *et al.* (1).

with that previously determined (4) for residues 1 to 24 for the enzyme purified from *B. subtilis*. The sequence corresponds to residues 12 to 35 shown in Fig. 5. We conclude that *B. subtilis purF* is expressed in *E. coli* and is correctly processed even though the processing is different from that used for the *E. coli* enzyme.

Computer Alignment of E. coli and B. Subtilis Amidophosphoribosyltransferase Sequences—The amino acid sequences of *E. coli* and *B. subtilis* amidophosphoribosyltransferases were aligned by computer using their nucleotide sequences. The alignment is shown in Fig. 6. A large portion of the *B. subtilis purF* gene, which encodes the first 426 amino acids of the *B. subtilis* amidophosphoribosyltransferase, was matched with the *E. coli* enzyme using a length L of 45 bases resulting in base difference counts for 30 compared positions. Visual inspection of the oligonucleotide matches was used to place the insertions and deletions such that the total base differences were kept to a minimum and contiguity in the sequence alignments was preserved. The 3′ end of the *B. subtilis purF* gene which encodes the last 50 amino acids does not exhibit sufficient homology with the *E. coli* enzyme to be aligned unequivocally using a length L of 45 nucleotides. To align this sequence a shorter length of 30 nucleotides was used to detect a possible greater frequency of insertions and deletions. Because the region of *B. subtilis* amidophosphoribosyltransferase containing the terminal 50 amino acids contains little homology with the *E. coli* enzyme, alternative alignments giving different matches are possible.

DISCUSSION

Expression and Cloning—Expression of *B. subtilis purF* in *E. coli* has facilitated the cloning and sequencing of this gene. The expression of *B. subtilis purF* in *E. coli* is surprising for several reasons. (*a*) Hybridization of *E. coli purF* to *B. subtilis* DNA was not obtained using standard stringency conditions. Even under hybridization conditions of reduced stringency little cross-hybridization was detected and served to indicate the limited nucleotide sequence homology between the two genes. (*b*) *B. subtilis* amidophosphoribosyltransferase contains Fe-S centers which are obligatory for function. Fe-S has not been detected in *E. coli* amidophosphoribosyltransferase. (*c*) Comparison of the deduced amino acid sequences following the initiator methionine in the two genes indicates a major difference in post-translational processing. Despite these differences *E. coli* purine auxotroph TX158 bearing plasmids pPZ1 or pPZ2 synthesizes amidophosphoribosyltransferase in amounts comparable to derepressed *B. subtilis* cells and grows at or near the wild type rate in minimal media. These results indicate that cloned *B. subtilis purF* is transcribed in *E. coli*, the mRNA is translated, and the protein chain is processed to yield active enzyme.

Analysis of the 5′ Flanking Region—The 1.65-kb cloned *Eco*RI-*Hinc*II fragment contains 88 bp of *B. subtilis* DNA that extends upstream from the *purF* coding region to the *Eco*RI site (Fig. 5). Although only a limited number of *B. subtilis* promoters have been sequenced to date (25, 26), it appears that the principal form of RNA polymerase holoenzyme containing σ^{55} recognizes promoters with −35 and −10 regions that are homologous to those in *E. coli* promoters (27, 28). The *B. subtilis* −35 and −10 promoter concensus sequences TTGACA and TATAAT, respectively, are not present in the 88-bp 5′ flanking region of *purF* suggesting the absence of a *B. subtilis* promoter in the cloned fragment. Reversal of the orientation of the *B. subtilis* insert in pPZ1 abolishes expression and supports the conclusion that transcription of *purF* is initiated from a plasmid promoter. A pBR322 promoter located between the *Eco*RI and *Hind*III sites which contributes to *bla* expression (29) likely serves to initiate *purF* transcription in pPZ1 and pPZ2.

The *purF* 5′ flanking region contains two sequences that exhibit perfect Shine-Dalgarno complementarity to the 3′ end of *B. subtilis* 16 S rRNA (30). The two sequences shown in Fig. 7 are separated by 17 bp. The downstream 8-nucleotide complementary sequence precedes the deduced ATG translation start by 7 nucleotides. These sequences could function in ribosome binding. *B. subtilis* translation appears to require more stringent mRNA-rRNA complementarity than is observed in *E. coli* ribosome binding sites (30). Multiple Shine-Dalgarno sequences were noted previously for the *B. subtilis* amylase gene (26) and *E. coli ompA* (31) and as discussed below also may be utilized by a gene downstream from *purF*. Multiple ribosome binding sites were suggested to contribute to efficient translation of *ompA* (31).

TGA triplets at nucleotides 37–39 and 62–64 are 49 and 24

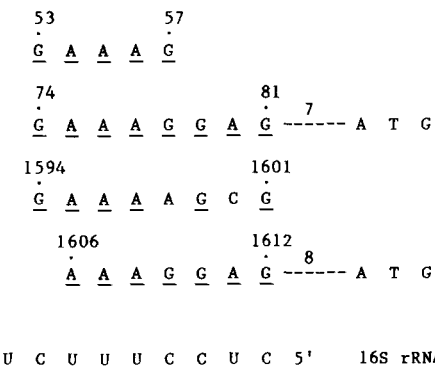

FIG. 7. **Putative ribosome binding sites and homology with the 3′ end of *B. subtilis* 16 S rRNA.** Numbered sequences are taken from Fig. 5. The *numbers over the dashed lines* indicate the number of nucleotides from the putative ribosome binding site to the ATG. *Underlined* nucleotides are complimentary to the 3′ end of 16 S rRNA. The upper two sequences are potential ribosome binding sites for purF. The lower two sequences are potential ribosome binding sites for a putative downstream coding region.

bp upstream, respectively, from the deduced *purF* translation start (Fig. 7). The latter TGA is situated between two putative ribosome binding sites. The proximity of a translation stop to the ribosome binding site in an intercistronic boundary is an important factor in determining the efficiency of translation initiation (32). If either of these TGA triplets were the translation stop for an upstream coding sequence, *purF* would be part of a *pur* operon. Dyad symmetries indicative of transcription terminators or operators were not detected in the 5′ flanking region by a computer search.

Analysis of the 3′ Flanking Region—The determined nucleotide sequence extends 134 bp downstream from the deduced TAA translation stop at nucleotides 1517–1519. A computer search of the region indicates there are two sequences capable of forming stem and loop structures characteristic of transcription termination sites (Fig. 5). The first region is centered 30 nucleotides from the TAA stop codon and is potentially capable of forming a stem and loop structure with a $\Delta G = -16$ kcal/mol (33). Located 6 nucleotides downstream from the first is a second region that might form a stem and loop with a $\Delta G = -9$ kcal/mol. Both regions are GC rich and are followed by short stretches of Ts in the DNA sequence. These regions are characteristic of transcription termination sites in *E. coli* (28) and *B. subtilis* (26, 34). A nucleotide sequence having the potential to form multiple stem and loop structures characteristic of terminators was identified at the 3′ end of the *B. subtilis* amylase gene (26). Whether either of the regions of dyad symmetry between nucleotides 1537–1587 functions as the terminator for the *purF* gene remains to be determined. It is interesting to note that two potential Shine-Dalgarno sequences complimentary to 16 S rRNA are also present in the region. They are located at nucleotides 1594–1601 and 1606–1612 (Fig. 7). The latter heptanucleotide sequence precedes by 8 bases an ATG. While the existence of a downstream gene has not been shown, this sequence has the potential for translation initiation of a downstream coding sequence.

Little is known about the gene-enzyme relationships for purine biosynthesis in *B. subtilis*. In *E. coli*, *purF* is unliked to other *pur* genes and may be regulated by the *purR* aporepressor (35). The present analysis of the *purF* flanking regions suggests the possibility that *purF* is part of an operon in *B. subtilis*. Further work is required to obtain a better understanding of the relationship of *purF* to other *B. subtilis pur* genes and to identify sequences involved in *purF* regulation.

Coding Region—Mature amidophosphoribosyltransferase has an NH_2-terminal cysteine which is residue 12 in the deduced sequence shown in Fig. 5. There are two possible translation start sites at ATG 71–73 and ATG 89–91. We favor the view that translation initiates at the latter site and yields a precursor of 476 amino acids which is then processed by proteolytic removal of 11 residues from the NH_2 terminus. Translation initiation at ATG 89–91 (Fig. 5) would allow utilization of a perfect octanucleotide Shine-Dalgarno sequence at an optimal distance of 7 bases from the initiation site (Fig. 7). The upstream ATG would have to use a ribosome binding site with 5-nucleotide complimentarity at a suboptimal distance of 13 bases from the initiation site.

Mature *B. subtilis* amidophosphoribosyltransferase is a protein chain of 465 amino acids having a calculated M_r of 50,397. The *B. subtilis* enzyme exhibits similar catalytic properties to the slightly larger *E. coli* amidophosphoribosyltransferase (503 amino acids), and both enzymes are subject to allosteric inhibition by adenine and guanine nucleotides (2, 5). The major structural feature that distinguishes the two enzymes is the essential Fe-S center in *B. subtilis* amidophosphoribosyltransferase. Common functional domains are expected to exhibit at least limited sequence homology. The two enzymes may, therefore, possess similar domains for catalysis and feedback inhibition but differ with respect to the unique structural features imposed by Fe-S centers.

In the amino acid sequence alignment shown in Fig. 6, *E. coli* and *B. subtilis* amidophosphoribosyltransferases exhibit 180 identities out of the 465 residues (39%). Residues 247–426 (*B. subtilis*) comprise a highly conserved region. Within this region there are 94/179 identities (52%). Based on calculations utilizing amino acid physical parameters thought to control protein folding and secondary structure prediction analysis of three phosphoribosyltransferases, Argos *et al.* (36) have identified two regions in *E. coli* amidophosphoribosyltransferase that exhibit predicted structural homology with two purine nucleotide phosphoribosyltransferases. A region including residues 234–353 in *E. coli* amidophosphoribosyltransferase was identified as a possible nucleotide binding domain and residues 354–450 were predicted to form a catalytic domain. The sequence comparison in Fig. 6 shows that conservation is greatest within these two putative domains. If the predictions of Argos *et al.* (36) are correct, it appears that the region between 247–426 of the *B. subtilis* enzyme contains domains for catalysis and allosteric regulation by purine nucleotides. It is interesting to note that the region of *E. coli* amidophosphoribosyltransferase from Lys-360 to Ser-375, predicted to contribute to the catalytic domain, is not only highly conserved (13/16 identities) in *B. subtilis* amidophosphoribosyltransferase (Fig. 6) but also in human hypoxanthine-guanine phosphoribosyltransferase (residues 127–142, 9/16 identities (36)). The high degree of conservation of primary sequence suggests that this region is important in the enzymatic activity of phosphoribosyltransferases.

The NH_2 termini of the two enzymes are a second region that show homology. Both enzymes employ an active site cysteine residue at the NH_2 terminus (3, 4). Homology at the NH_2 terminus supports the findings that the NH_2-terminal cysteine is located at the active site. We further suggest that conservation of residues in the NH_2-terminal portions of the two enzymes reflect structural requirements in folding required to bring the NH_2-terminal active site cysteine into proximity with other active site residues. Together the NH_2 terminus and residues within the region 247–426 may comprise the catalytic site of the protein.

From Leu-427 the CO_2H-terminal segment of *B. subtilis* amidophosphoribosyltransferase shows little homology to the *E. coli* enzyme. Using a length of 45 nucleotides no significant homologies above the 2 σ confidence level were found. Upon realigning using a comparison length of 30, several possible alignments were found but all exhibited a large number of mismatches. The final alignment shown in Fig. 6 was chosen to minimize the number of mismatches and yet keep continuity. In the CO_2H-terminal 50 amino acids there are only 7 conserved residues. However, in this region a Fe-S binding sequence has been identified, Cys(445)-Gly-Gln-Cys(448)-Ser-Ala-Cys(451). This arrangement of cysteinyl residues in the primary structure is characteristic for [4Fe-4S] clusters of the ferredoxin type (37). Of the 4 remaining cysteinyl residues in the protein chain, Cys-393 is the most likely fourth ligand to the FeS cluster because it lies in the sequence -Pro-Cys-Phe-Tyr- and the fourth ligands in 4Fe-4S proteins are always found near Pro residues, usually as -Cys-Pro (38). Interestingly, the fourth ligand in the Azotobacter ferredoxin 4Fe-4S cluster lies in the sequence -Pro-Val-Asp-Cys-Phe-Tyr- (39). The sequence around Cys-393 is -Pro-Cys-Phe-Tyr- (Figs. 5 and 6).

Post-translational Modification—Our data establish that *E. coli* can conduct two complex processing events on *B. subtilis* amidophosphoribosyltransferase that are not used for the *E. coli* enzyme. From the NH_2 terminus, 11 residues are removed to "expose" the NH_2-terminal active site cysteine. There is no information concerning the mechanism for this type of processing. The *E. coli* enzyme is processed by direct cleavage of the initiator methionine to yield a protein chain having an NH_2-terminal active site cysteine (10). It is tempting to speculate that NH_2-terminal trimming is essential for function of the cysteine in glutamine amide transfer.

The second post-translational modification involves assembly of the Fe-S center. *E. coli* has the capacity to synthesize Fe-S proteins (40) although *E. coli* amidophosphoribosyltransferase does not contain Fe-S. There appear to be several possibilities for incorporation of Fe-S into proteins. Fe-S centers may be assembled spontaneously *in vivo* from apoprotein, Fe^{2+}, Fe^{3+}, and S^{2-} or spontaneously prior to completion of translation. Alternatively, enzymes may be involved in the assembly process. If assembly is enzymatic, our results suggest that such enzymes have broad specificity for protein acceptor.

Codon Usage—The pattern of codon usage is relatively unbiased and is typical of moderately expressed bacterial genes such as the *E. coli trp* operon (41). Of the 61 sense codons only 3 are unused ATA (Ile), CCC (Pro), and AGA (Arg), while two others are used only once, CCT (Pro) and TGT (Cys). Strongly preferred codons are CCG (Pro) and AAA (Lys). Most other codons exhibit a fairly random distribution. Codon third position use is 51.9% GC which compares with a genome content of 43% GC (42).

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