Glutamine Phosphoribosylpyrophosphate Amidotransferase from Cloned Escherichia coli purF

NH$_2$-TERMINAL AMINO ACID SEQUENCE, IDENTIFICATION OF THE GLUTAMINE SITE, AND TRACE METAL ANALYSIS

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Glutamine 5-phosphoribosylamine pyrophosphate amidotransferase (amidophosphoribosyltransferase) was purified in large amounts from an Escherichia coli strain harboring a purF hybrid plasmid. Purified E. coli amidophosphoribosyltransferase lacks iron as well as other trace metals as determined by x-ray fluorescence spectrometry. The NH$_2$-terminal amino acid sequence of the enzyme was determined and is in agreement with that deduced from the DNA sequence. $[^{14}C]$-Diazo-5-oxo-norleucine (DON), an active site-directed affinity analog of glutamine, selectively inactivated the glutamine-dependent amidophosphoribosyltransferase. Inactivation was accompanied by incorporation of 1 eq of $[^{14}C]$DON per enzyme subunit. A 10-residue cyanogen bromide peptide labeled by $[^{14}C]$DON was isolated and sequenced. The NH$_2$-terminal cysteine of amidophosphoribosyltransferase was determined to be the residue alkylated by $[^{14}C]$DON. These results establish that the NH$_2$-terminal cysteine is the active site residue required for the glutamine amide transfer function of the enzyme. The experiments reported in this and the preceding article (Tso, J. Y., Zalkin, H., van Cleemput, M., Yanofsky, C., and Smith, J. M. (1982) 257, 3525-3531) demonstrate the application of affinity labeling, rapid peptide purification by high pressure liquid chromatography, and nucleotide sequence determination of a structural gene to localize an amino acid residue, peptide fragment, or functional domain in a long protein chain.

Glutamine phosphoribosylamine:pyrophosphate amidotransferase catalyzes the first specific reaction in the de novo pathway of purine biosynthesis. The enzyme can utilize either glutamine or NH$_3$ for the formation of phosphoribosylamine from 5-phosphoribosyl-1-pyrophosphate as shown in Equations 1 and 2.

Glutamine + PP-Ribose-P\(^{1}\) $\text{Mg}^{2+}$ $\rightarrow$ phosphoribosylamine + PP + glutamate (1)

The enzyme has been extensively purified from avian liver (1, 2), Bacillus subtilis (3), and E. coli (4). Amidophosphoribosyltransferases from avian liver and B. subtilis have molecular weights of approximately 200,000 and are composed of four identical subunits. The avian liver, mammalian (5) and B. subtilis enzymes have been reported to contain nonheme iron, although no role has been determined for the prosthetic group. E. coli amidophosphoribosyltransferase is either a trimer or tetramer of identical subunits and is devoid of iron (4). The amino acid sequence of E. coli amidophosphoribosyltransferase has been deduced from the DNA sequence of purF (6). The protein chain of 503 residues has a calculated $M_r$ of 56,263 which agrees well with the value of approximately 57,000 previously determined (4).

Amidophosphoribosyltransferase exhibits properties common to other glutamine amidotransferases. In addition to its capacity to utilize NH$_3$ in place of glutamine, the enzyme also has glutaminase activity which is stimulated by the substrate analog ribose 5-phosphate plus pyrophosphate (4). An essential cysteine residue is required for the glutamine-dependent activity. DON, a glutamine analog, selectively inactivates the glutamine-dependent amidophosphoribosyltransferase activity by an affinity labeling mechanism.

Further studies on the structure and function of amidophosphoribosyltransferase require the availability of larger amounts of enzyme than previously available. In this article, we describe a procedure for isolation of large quantities of E. coli amidophosphoribosyltransferase utilizing cloned purF. Homogeneous protein was used for NH$_2$-terminal amino acid sequence determination and isolation of the glutamine amide transfer active site of the protein. The active site cysteine required for glutamine amide transfer is the NH$_2$-terminal residue of amidophosphoribosyltransferase.

EXPERIMENTAL PROCEDURES

Materials—DON and $[^{14}C]$DON were synthesized according to the methods of Hartman (7) by Stanley G. Bower of this laboratory with precursor N-trifluoroacetyl-L-glutamic-y-oxo chloride-a-methyl ester provided by Dr. John S. Holcenberg, Medical College of Wisconsin, Milwaukee, WI. Idono[1-$^{14}$C]acetamide was from Amersham Corp., Arlington Heights, IL. 4-Vinylpyridine was from Sigma and was vacuum distilled before used. Chelex 100 was from Bio-Rad. Other chemicals and proteins were the highest grades commercially available and were used without further purification.

Enzyme Purification—E. coli amidophosphoribosyltransferase was purified from E. coli strain TX158 (8) harboring hybrid plasmid pSB2 (6). Plasmid pSB2 is a pBR322 derivative containing a purF insert. Cells were grown, for 14 h at 37 °C in salts (9), trace minerals (10), 0.5% glucose, 0.1% casein amino acids, 7.5 mM (NH$_4$)$_2$SO$_4$, 10 mM K$_2$HPO$_4$, and 20 µg of ampicillin/ml. Amidophosphoribosyltransferase was analyzed...
was purified from cell extracts as previously described (4) except that blue dextran-Sepharose chromatography and hydroxyapatite chromatography were omitted. Purity was estimated by sodium dodecyl sulfate gel electrophoresis (11). The enzyme retained full activity for long periods of time when stored in liquid nitrogen at 10 mM Tris-HCl, pH 7.4.

Enzyme Assay—The glutamine-dependent amidophosphoribosyltransferase activity was assayed by either the glutamate dehydrogenase method (assay I) or the DEAE filter-binding assay (method II) as previously described (4). An enzyme unit is defined as the formation of 1 μmol of product per min. Specific activity is units per mg of protein. Protein concentration was determined by the biuret or Lowry methods as described by Layne (12).

Trace Element Analysis—Dialysis buffer (10 mM NH₄HCO₃) was treated with Chelex 100 to remove trace metal ions. Amidophosphoribosyltransferase was extensively dialyzed against the deoxygenated buffer and lyophilized. No more than 25% of the enzyme activity was lost during dialysis. The small activity loss was attributed to oxidation of the enzyme (4).

Trace element analysis was performed using a Kevek model 0900 Ultra-Trace X-ray fluorescence spectrometer equipped with a model 7000 data acquisition/storage system and a 6100 data processor.

For sample preparation 3 μl of 10 N NaOH was added to 2 ml of a 5-ppm yttrium solution. Two hundred μl of this solution was added to 1.3 ml of protein. The solution was mixed by vortex, and 20 μl were pipetted onto a Mylar support slide. This slide was dried at 37°C for 30 min. The samples were then placed into the X-ray fluorescence analyzer. The X-ray beam produced was filtered with 7 mils of silver foil. The fluorescence emission spectrum of the sample was determined and compared with that of the internal standard, yttrium. A Mylar blank was subtracted from the sample spectrum, escape peaks were removed, and the spectrum was plotted.

Active Site Labeling—The glutamine-dependent amidophosphoribosyltransferase activity was selectively inactivated by DON. Enzyme (11 mg) was reacted with 130 μM [6-14C]DON (2900 cpm/nmol) in a reaction mixture containing 50 mM Tris-HCl buffer, pH 7.4, 30 μM PP-Ribose-P, and 50 μM MgCl₂ in a final volume of 2 ml. Inactivation proceeded at 22°C until more than 95% of the glutamine-dependent activity was lost. The reaction was then terminated by adding guanidine-HCl crystals to make a 6 M solution. After the solution was adjusted to pH 8.5 with Tris base, the enzyme was reduced with 20 mg of sodium borohydride for 1 h prior to alklylation with 40 μl of 4-vinylpyridine for 90 min. The protein was desalted by gel filtration on a Sephadex G-25 column (2.2 ml) in 50 mM Tris-HCl buffer and lyophilized. No more than 25% of the enzyme activity was lost during dialysis. The small activity loss was attributed to oxidation of the enzyme (4).

RESULTS

Purification of Amidophosphoribosyltransferase—The specific activity of amidophosphoribosyltransferase in the crude extract from strain TX168/pSB2 was approximately 90-fold higher than in extracts of the wild type strain lacking the plasmid. The enzyme was purified as previously described (4) except that the final three steps were omitted (Table I). Purity was greater than 96% based on sodium dodecyl sulfate gel electrophoresis. The final specific activity of 170 units/mg of protein is virtually identical with that previously obtained for the homogeneous enzyme. The results in Table I show that amidophosphoribosyltransferase constituted approximately 4.2% of the soluble protein in strain TX168/pSB2. The overall yield was approximately 0.2 g of enzyme per litre of cells, a value 160-fold higher than previously obtained (4).

NH₃-terminal Amino Acid Sequence—Automated Edman degradation of amidophosphoribosyltransferase through 23 cycles generated the NH₃-terminal amino acid sequence shown in Table II. Except for the first cycle, where the identification of the PTH amino acid was tentative, the identification and repetitive yield of PTH amino acids from cycle 2 to cycle 23 were satisfactory. The assignment of cysteine as the NH₃-terminal residue was confirmed by carboxamidomethylation (described below) and by the DNA sequence (6).

The observation of a single sequence with the quantity of each PTH amino acid consistent with the quantity of protein degraded supports the conclusion that the amidophosphoribosyltransferase preparation is homogeneous and that the enzyme contains identical subunits.

Active Site Labeling with [6-14C]DON—The glutamine analog DON is known to inactivate amidophosphoribosyltransferase by an affinity labeling mechanism (4). Amidophosphoribosyltransferase was reacted with [6-14C]DON to inactivate selectively the glutamine-dependent activity. The inactivated protein was reduced with sodium borohydride to stabilize the DON-enzyme adduct (19) and to cleave disulfide bonds.

| Step | Vol. mg | Protein mg | Activity μmol | Specific activity unit/mg | Yield % | Fold |
|------|---------|------------|---------------|--------------------------|---------|------|
| Crude homogenate | 688 | 22,650 | 16,310 | 0.72 | 100 | 1.0 |
| Ammonium sulfate | 185 | 8,730 | 17,290 | 2.0 | 100 | 2.7 |
| Heat step | 165 | 3,700 | 12,580 | 3.4 | 76 | 4.7 |
| DEAE-Sepharose | 32 | 530 | 9,010 | 17.0 | 55 | 23.6 |

Identification and quantitation of phenylthiohydantoin amino acids sequentially removed by automated Edman degradation of E. coli amidophosphoribosyltransferase.

The yield was estimated from the peak height of the high pressure liquid chromatogram. The data were obtained from the degradation of 3.5 mg (61 nmol) of 4-pyridylethylated amidophosphoribosyltransferase. Residue assignment for cycle 1 is tentative.

| Cycle | PTH amino acid | Yield nmol | Cycle | PTH amino acid | Yield nmol |
|-------|----------------|------------|-------|----------------|------------|
| 1 | Cys(PE) | ND | 13 | Asn | 12 |
| 2 | Gly | 50 | 14 | Gln | 14 |
| 3 | Ile | 64 | 15 | Ser | 9 |
| 4 | Val | 58 | 16 | Ile | 30 |
| 5 | Gly | 58 | 17 | Tyr | 22 |
| 6 | Ile | 54 | 18 | Asp | 18 |
| 7 | Ala | 43 | 19 | Ala | 29 |
| 8 | Gly | 54 | 20 | Leu | 20 |
| 9 | Val | 45 | 21 | Thr | 6 |
| 10 | Met | 29 | 22 | Val | 26 |
| 11 | Pro | 20 | 23 | Leu | 18 |

* S-(4-pyridylethyl)cysteine.

Not determined.
phoribosyltransferase were titrated in denatured enzyme and DON. In one experiment the sulfhydryl groups of amidophosphoribosyltransferase were alkylated by the NH₂-terminal cysteine is the active site residue alkylated by phosphoribosyltransferase. The first cycle indicates that the NH₂-terminal residue of amidophosphoribosyltransferase peptide was subjected to automated Edman degradation for the presence of 0.4% thioglycolic acid. The active site CNBr analysis even when the acid hydrolysis was performed in the presence of 6.2 eq of carboxamidomethyl groups per DON-treated enzyme.

Active Site Peptide Isolation and Sequence Determination—The [6-14C]DON-labeled S-pyridylethylated amidophosphoribosyltransferase was digested with cyanogen bromide, and the resulting peptides were fractionated by gel filtration (Fig. 1). Only one major radioactive fraction that eluted slightly ahead of the salt peak on gel filtration was obtained. Peptides in this radioactive fraction were resolved by high pressure liquid chromatography. Fig. 2 shows their elution profile on high pressure liquid chromatography. Only fraction j contained radioactivity. The amino acid analysis of the peptide in fraction j is shown in Table III. The amino acid composition of the DON-labeled CNBr peptide is very similar to that of the NH₂-terminal CNBr peptide except that it lacks the NH₂-terminal cysteine residue. No DON-amino acid adduct or its derivative was apparent from the composition analysis even when the acid hydrolysis was performed in the presence of 0.4% thioglycolic acid. The active site CNBr peptide was subjected to automated Edman degradation for 10 cycles. Sequence data in Table IV established that the DON-labeled peptide is the NH₂-terminal CNBr peptide. An unidentified PTH amino acid, which migrated in a position between PTH-glycine and PTH-serine in the high pressure liquid chromatogram, was released in the first cycle of Edman degradation. The concomitant release of radioactivity in the first cycle indicates that the NH₂-terminal residue of amidophosphoribosyltransferase is the residue alkylated by [6-14C] DON.

Identification of the Residue Alkylated by DON—Further experiments were conducted to unequivocally prove that the NH₂-terminal cysteine is the active site residue alkylated by DON. In one experiment the sulfhydryl groups of amidophosphoribosyltransferase were titrated in denatured enzyme and the peptide in fraction j is shown in Table III. The amino acid composition of the DON-labeled CNBr peptide is similar to that of the NH₂-terminal CNBr peptide except that it lacks the NH₂-terminal cysteine residue. No residue identification was made on entries with a dash because of the low yield.

Identification and quantitation of phenylthiohydantoin amino acids sequentially removed by automated Edman degradation of the 6-14C]DON-labeled CNBr peptide

The yield was estimated from the peak area of the high pressure liquid chromatogram. The data below were obtained from the degradation of 90,000 cpm of the 6-14C]DON-labeled CNBr peptide in the presence of 3 mg of Polybrene. X represents an unidentified residue. No residue identification was made on entries with a dash because of the low yield.

Fig. 1. Separation of a cyanogen bromide digest of amidophosphoribosyltransferase by gel filtration. Gel filtration was conducted using a column (2.5 × 100 cm) of Sephadex G-75 (superfine) in 0.1% trifluoroacetic acid. Fractions of 3.0 ml were collected. The yield was estimated from the peak area of the high pressure liquid chromatogram. The data below were obtained from the degradation of 90,000 cpm of the 6-14C]DON-labeled CNBr peptide in the presence of 3 mg of Polybrene. X represents an unidentified residue. No residue identification was made on entries with a dash because of the low yield.
enzyme subunit. In the untreated control there was incorporation of 6.9 eq of carboxamidomethyl groups per subunit. Thus DON had alkylated a cysteine residue which prevented subsequent labeling by iodo[1-14C]acetamide. The carboxamidomethylation of 6.9 cysteine residues per subunit compares with a content of 8 cysteine residues per subunit deduced from the DNA sequence (6). Somewhat incomplete alkylation by iodoacetamide under these conditions has been previously noted (22, 23).

In a second experiment the [1-14C]carboxamidomethylated, DON-treated, and untreated control samples were subjected to Edman degradation. The untreated control released approximately 1.0 eq of carboxamidomethylcysteine in the first cycle of Edman degradation. The DON-treated sample released only 0.1 eq of carboxamidomethylcysteine in the first cycle of Edman degradation. These results confirm that the NH2-terminal cysteine is the active site residue that is alkylated by DON.

**Trace Element Analysis—Extensively dialyzed E. coli Amidophosphoribosyltransferase was analyzed for trace elements using the very sensitive method of x-ray fluorescence spectrometry. Presence of trace elements in the enzyme sample as low as 1 ppm could be detected. As shown in the x-ray fluorescence spectrum of trace element analysis (Fig. 3) there were no trace elements detected in the sample of amidophosphoribosyltransferase other than a small amount of contaminating chloride. Elements which would have been detected between the chloride peak and the internal standard yttrium peak include K, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Ge, As, Se, Rb, and Sr. Mo, if present, would also have been detected after the yttrium peak. The sensitivity of the analysis was 1000-fold higher than would be required to detect iron at 1 g atom/mol of subunit of amidophosphoribosyltransferase. The trace element analysis confirms and extends the earlier conclusion that E. coli amidophosphoribosyltransferase lacks nonheme iron (4).**

**DISCUSSION**

Amidophosphoribosyltransferase, which catalyzes the first reaction in de novo purine biosynthesis, is an important site for control of purine nucleotide synthesis. Immediate problems encountered in previous studies of this enzyme from various sources including E. coli (4) were the laborious purification procedures and the low enzyme yield. Inactivation of the enzyme by air oxidation often further complicated the matter. Overproduction of E. coli amidophosphoribosyltransferase in a multicopy purF plasmid-bearing strain allowed preparation of fully active homogeneous enzyme in 160-fold larger amounts than previously available. It appears likely that the approximately 90-fold increased activity in extracts prepared from strain TX158/pSB2 results from the high copy number and uncertainties in assaying very low enzyme levels in crude extracts from wild type cells. The high yield of enzyme reflects the simple purification scheme that results from omitting the final 3 column chromatographic steps in the earlier procedure (4). For the first time there are sufficient quantities of homogeneous E. coli amidophosphoribosyltransferase available for protein chemistry studies. Large amounts of the B. subtilis enzyme have also been prepared (3).

In the present study we have utilized the purified enzyme to determine the NH2-terminal amino acid sequence, identify and localize the active site residue involved with glutamine utilization, and analyze for metal ions. The NH2-terminal amino acid sequence determined by automated Edman degradation agrees with that deduced from the DNA sequence (6) except for the absence of the initiator methionine residue. Thus Met-1 of the primary translation product must be processed to yield the functional enzyme having an NH2-terminal cysteine residue. Digestion with carboxypeptidases A, B, and Y was generally unsatisfactory due to the low yield of digestion. Glycine and glutamate were released in largest amounts (approximately 0.2 mol/mol of subunit) and a number of amino acids were present at levels of approximately 0.1 mol/mol of glutamate. Isoulation of the CO2H-terminal CNBr peptide has verified the CO2H-terminal sequence deduced from the DNA sequence (6).

Characterization of the glutamine amidotransfer active site of amidophosphoribosyltransferase should be viewed in light of structure-function studies on other glutamine amidotransferases. Amidophosphoribosyltransferase is one enzyme in a family of 13 glutamine amidotransferases (24). All glutamine amidotransferases thus far studied contain a cysteine residue essential for the glutamine-dependent activity. Alkylation of this cysteine residue leads to selective loss of the glutamine-dependent activity while the NH2-dependent activity is generally unaffected, suggesting distinct sites for glutamine binding and NH2 utilization. Previous studies with anthranilate synthase (25) have suggested a mechanism for glutamine utilization that appears applicable to amidophosphoribosyltransferase (4) and other glutamine amidotransferases. According to this proposed mechanism, glutamine binds and then forms a covalent intermediate with an active site cysteine. A glutamyl thioester is formed upon transfer of the amide, either directly to the second substrate or to the NH2 site. Hydrolysis of the thioester releases glutamate. Whereas in some glutamine amidotransferases, such as anthranilate synthase, the glutamine and NH2 sites are on distinct subunits, in other enzymes including amidophosphoribosyltransferase, both functions are on a single subunit. Of interest is the question whether gene fusion may account for the evolution of a single protein chain containing the two functional domains for glutamine binding-amide transfer and NH2-dependent synthesis.

Previous experiments have shown that the glutamine affinity analog DON alkylates a residue in amidophosphoribosyltransferase that is essential for glutamine-dependent activity but is not required for NH2-dependent activity (4). The present data establish that DON alkylates only 1 residue per subunit, the NH2-terminal cysteine. Thus the active site cysteine required for the glutamine amide transfer function is the NH2-terminal residue. Fusion of a glutamine amide transfer gene with the 5' end of an ancestral gene coding for an enzyme
that catalyzed NH₃-dependent P-ribosylamine synthesis remains a possibility for the evolution of E. coli amidophosphoribosyltransferase. However, it appears unlikely that the glutaminase binding-amide transfer domains of anthranilate synthase (26) and amidophosphoribosyltransferase evolved from a common ancestral gene since sequence homology adjacent to the active site cysteine is not apparent.

We are aware of only one other enzyme that directly utilizes a group at the NH₃ terminus for catalysis. A pyruvyl residue attached to an NH₃-terminal phenylalanine participates in catalysis in histidine decarboxylase from Lactobacillus 30a (27). The pyruvyl moiety in histidine decarboxylase arises from a complex post-translational processing event.

The experiments in this study demonstrate the feasibility of isolating an active site peptide from a [6-14C]DON-labeled amidotransferase. The key steps are to stabilize the DON-enzyme adduct by reduction with sodium borohydride and to avoid lengthy peptide purification procedures by the use of high pressure liquid chromatography. NaBH₄ reduction was previously used to stabilize a DON adduct of glutaminase-high pressure liquid chromatography. NaBH₄ reduction was shown to contain an essential iron-sulfur center (3, 5, 28).

Thus no obligatory role for transition metal ions in glutamine amidotransferase function.

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