Structure and Function of the Glutamine Phosphoribosylpyrophosphate Amidotransferase Glutamine Site and Communication with the Phosphoribosylpyrophosphate Site

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Glutamine phosphoribosylpyrophosphate (PRPP) amidotransferase from Escherichia coli exhibits a basal PRPP-independent glutaminase activity having a kcat/Km that is 0.3% of fully active enzyme. Binding of PRPP activates the enzyme by a structural change that lowers the Km for glutamine 100-fold and couples glutamine hydrolysis to synthesis of 5-phosphoribosylamine. By analysis of the x-ray structure of the glutamine site containing bound 6-diazo-5-oxonorleucine, a glutamine affinity analog, and by site-directed mutagenesis we have identified residues important for glutamine binding, catalysis, and coupling with PRPP. Tyr74 is a key residue in the coupling between the sites for glutamine and PRPP in the COOH-terminal domain. Arg23 and Asp127 have roles in glutamine binding. The x-ray structure indicates that there are no amino acid side chains sufficiently close to Cys1 to participate as a proton acceptor in formation of the thiolate needed for nucleophilic attack on the carboxamide of glutamine, nor as a general acid for amide thiolate needed for nucleophilic attack on the carboxylate group. Based on the x-ray model of the glutamine site and analysis of a mutant enzyme we propose that the free NH2-terminus of Cys1 functions as the proton acceptor and donor. The results indicate that the side chain of Asn101 and the backbone nitrogen of Gly102 function to stabilize a tetrahedral oxyanion resulting from attack of Cys1 on the glutamine carboxamide. Cys1, Arg23, Asn101, Gly102, and Asp127 are conserved in the NH2-terminal domain of a subfamily of amidotransferases that includes asparagine synthetase, glucosamine 6-phosphate synthase, and glutamate synthase, implying a common function in the four enzymes. Tyr74, on the other hand, is conserved only in glutamine PRPP amidotransferase sequences consistent with a specific role in interdomain coupling. The catalytic framework of key glutamine site residues supports the assignment of glutamine PRPP amidotransferase to a recently described Ntn (NH2-terminal nucleophile) hydrolase family of enzymes.

Glutamine PRPP1 amidotransferase catalyzes the initial reaction in de novo purine nucleotide biosynthesis and is the key regulatory enzyme in the pathway. Adenine and guanine nucleotides bind to an allosteric A-site and catalytic C-site and inhibit enzyme activity (Smith et al., 1994; Zhou et al., 1994). X-ray structures have been obtained for the inhibited Bacillus subtilis enzyme (Smith et al., 1994) and the homologous enzyme from Escherichia coli. Glutamine PRPP amidotransferase amino acid sequences are available from 18 prokaryotic and eukaryotic organisms. Although pairwise identity is 40% or more, indicative of homology, the sequences fall basically into two groups. Glutamine PRPP amidotransferases from human (Brayton et al., 1994), rat (Iwahana et al., 1993), chicken (Zhou et al., 1990), Drosophila (Clark, 1994), plants (Kim et al., 1995b; Ito et al., 1994), Mycobacterium leprae (accession number MLU15182), cyanobacteria (accession number U33211), and Lactobacillus (Gu et al., 1992) contain propeptide sequences preceding Cys1 and four conserved cysteine residues which are ligands to a [4Fe-4S] cluster in the B. subtilis enzyme. This group of enzymes thus most closely resembles the glutamine PRPP amidotransferase from B. subtilis. Enzyme sequences from yeast (Mänttäri and Zalkin, 1984b; Ledin et al., 1994), Neurospora, and Haemophilus (accession number U32800) are similar to E. coli glutamine PRPP amidotransferase in lacking the propeptide and cysteiny1 ligands to an Fe-S cluster. A sequence from Caenorhabditis elegans (accession number CET04A8) lacks a propeptide but contains the four cysteine residues that are Fe-S ligands in the B. subtilis enzyme. Whether the conserved cysteine residues are used as ligands to an Fe-S cluster is not known. Based on the x-ray structures of the enzymes from B. subtilis and E. coli the amino acids thought to be critical for catalysis and feedback regulation are conserved in all of the enzymes. It is therefore our working hypothesis that the mechanisms for catalysis and inhibition by nucleotide end products are highly similar in all of the enzymes.

Glutamine PRPP amidotransferase catalyzes the reaction: glutamine + PRPP → PRA + glutamate + PP. The enzyme also has the capacity to use NH3 in place of glutamine in vitro (Mänttäri and Zalkin, 1979) and in vivo (Mänttäri and Zalkin, 1984a). In addition, a glutaminase activity was detected that was less than 5% of the biosynthetic rate (Mänttäri and Zalkin, 1979; Kim et al., 1995a). From the x-ray structure of the nucleotide-inhibited enzyme there appear to be at least two barriers to catalysis (Smith et al., 1994). First, the

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1 The abbreviations used are: PRPP, 5-phosphoribosylpyrophosphate; PRA, 5-phospho-α-ribosylamine; DON, 6-diazo-5-oxonorleucine; PIPES, 1,4-piperazinediethanesulfonic acid; N-domain, NH2-terminal domain; C-domain, COOH-terminal domain.

2 C. A. Muchmore, J. Krahn, H. Zalkin, and J. L. Smith, manuscript in preparation.

3 D. Ebbole, personal communication.
inhibited enzyme is in a conformation described as "open" in which the glutamine and PRPP sites are too far apart for catalysis. Glutamine binds to a site in the NH₂-terminal domain (N-domain) and PRPP to a catalytic site (C-site) in the CO₂H-terminal domain (C-domain). Second, a structural change is required in the glutamine site that enables Cys⁵ to attack the carbamoyl of glutamine and initiate amide transfer. Given the requirement of PRPP for reaction of glutamine (hydrolysis and amide transfer) and for affinity labeling of Cys⁵ by glutamine analogs (Messenger and Zalkin, 1979), the binding of PRPP must in some way activate the enzyme permitting Cys⁵ to initiate glutamine amide transfer.

In the present work we have determined the x-ray structure of the E. coli glutamine PRPP amidotransferase glutamine site labeled by DON, a glutamine affinity analog. We have investigated the roles of pertinent amino acids in the glutamine site to determine how binding of PRPP to the C-site in the C-domain is communicated to the glutamine site in the N-domain. Residues important for the binding and reaction of glutamine and for amide transfer to PRPP were identified.

**EXPERIMENTAL PROCEDURES**

Plasmids and Strains—Plasmid pT7F1 containing the E. coli purF gene under the control of the T7α10 promoter and with the M13 replication origin was constructed for overexpression and mutagenesis. The plasmid was constructed in two steps. First, an Ndel-HindII fragment containing the gene was digested into the Ndel and HindII sites of pT7-SCA (U. S. Biochemical Corp.). Next, an Ndel site was inserted at the ATG initiation codon of purF by polymerase chain reaction. The DNA template was pGZ13 (Zhou et al., 1994) linearized by PstI and the primers were 5'-GGAAAAACGATATGGCGGTA-3' and 5'-GCCGTTTGGAAGCAACGTC-3'. The resulting polymerase chain reaction product, digested with Ndel, was ligated into the Ndel site of the intermediate described above for the Ndel of purF. The intact purF gene was confirmed by DNA sequencing.

E. coli strain BL21(DE3) (Studier et al., 1990) was the host for pT7F1 for enzyme overproduction. E. coli strain C 236 (mut., redA, pC105(Cm²)) and MV190 (Δ(lac-proAB), thi, supF, Δ(lac-recA) 306::tn10 (tet1) [F trdAb3, proAB I in 15 strains]) were used for site-directed mutagenesis (Kunkel et al., 1987). E. coli strain TX358 (purF, recA) (Tso et al., 1982) was used for in vivo assay of glutamine PRPP amidotransferase.

**Enzyme Purification—** Wild type E. coli glutamine PRPP amidotransferase and mutant enzymes were purified from plasmid pT7F1 and its derivatives in E. coli BL21(DE3) as described (Zhou et al., 1993). Plasmid pT7F1 was transformed into E. coli cells grown at 37 °C until mid-log phase (Miller, 1972) containing 140 µg/ml ampicillin. The purF gene was induced by 0.2 mM isopropyl-thi-β-d-galactopyranoside at midlog phase and growth was continued for 2–3 h. Enzyme was overproduced to approximately 30–40% of total soluble cell proteins. The enzyme was purified to approximately 95% homogeneity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Enzyme Assay—** Enzyme activity was assayed by measurement of product formation, either glutamate or PRA. The production of glutamate from glutamine reflects a glutaminase activity. Total glutaminase refers to the activity that is dependent upon PRPP plus the activity that is PRPP independent. The standard assay for total glutaminase activity contained 2.5 mTRPP (Sigma), 20–220 µM glutamine depending on the Kₘ, 10 mM MgCl₂, 1 mg/ml bovine serum albumin, 50 mM Tris-HCl (pH 8.0), and approximately 100 ng of enzyme in a total volume of 100 µl. Incubation was at 37 °C for 10 min. Reactions were quenched in a boiling water bath for 1 min and glutamate was determined by the glutamate dehydrogenase method (Messinger and Zalkin, 1979). A basal glutaminase activity was determined by subtracting the glutamate dehydrogenase activity (Rice and Meister, 1968; Stubbe, Massachusetts Institute of Technology, Cambridge, MA). The reactions contained in a volume of 40 µl, 6 mM PRPP, 20 mM glutamine, 2.5 mM ATP, 2 mM [¹⁵C]glycine (1800 cpm/nmol), 10 mM MgOAc, 50 mM Tris-HCl (pH 8.0), and approximately 100 ng of enzyme. Incubation was for 6 min at 37 °C. NH₃-dependent PRA synthesis was assayed by the same procedure except 150 mM NH₄Cl and Tris-HCl (pH 8.5) replaced glutamine and Tris-HCl (pH 8.0), respectively. Glycinamide ribonucleotide was isolated from the reaction mixture by ion exchange chromatography and was counted for radioactivity (Schedel et al., 1988).

To determine whether mutant enzymes retained glutamine- or NH₃-dependent activity in vivo, we measured the capacity of the enzyme to support growth of a purF auxotroph in medium containing either 50 mM NH₄Cl or 1 mM NH₄Cl as nitrogen source (Mei and Zalkin, 1989).

**Glutamine PRPP Amidotransferase Active Site**

Glutamine PRPP amidotransferase of wild type and all mutants, 0–20 µM for total glutaminase of enzymes in which the Kₘ was < 3 mM (Ala insertion, N101D, N101G, G102A), 0–220 µM for total glutaminase of mutants in which the Kₘ was > 15 mM (Arg⁹³, Tyr⁴⁸, and Asp¹¹¹ mutants). For assays with the low range of glutamine concentrations, two concentrations over 100 µM were also tested to verify saturation. For assays of enzymes having a Kₘ of > 100 µM for glutamine, the lowest standard errors were obtained using two glutamine concentrations <100 µM and about six concentrations between 100 and 220 µM. The maximum concentration of glutamine was limited to 220 µM by its solubility. The substrate concentrations used for other assays were 50 µM NH₄Cl, 150 mM NH₄Cl for NH₃-dependent PRA synthesis, and 0–540 µM PRPP. Glutamine was determined by the Michaelis-Menten equation using Ultrafit software (Bioisoft, Cambridge, UK).

**Nucleotide Inhibition—** Nucleotide inhibition of glutaminase activity by AMP and GMP was determined by the standard assay except that the MgCl₂ concentration was increased to 20 mM, PRPP was decreased to 1 mM (pH 8.0), and approximately 200 µM enzyme subunit in a total volume of 50 µl. The other concentration contained 10 mM Tris-HCl (pH 7.5) and approximately 200 µM enzyme subunit in a total volume of 50 µl. Dialysis was for 20 h at 4 °C in a rotating apparatus (Hoefs). Samples of 40 µl were retrieved from each chamber and were counted for radioactivity. Equilibrium binding data were fit to the Scatchard equation for nonlinear regression using Ultrafit. Under these conditions GMP is expected to bind to the A-site and AMP to the C-site (Zhou et al., 1994). Kinetic constants Kₘ and Vₘₐₓ were determined for the wild type enzyme, 20 mM glutamine was used for nucleotide inhibition of the PRPP-dependent glutaminase activity and 200 mM glutamine for the PRPP-independent or total glutaminase. In the case of Arg⁹³ and Tyr⁴⁸ mutants, 200 mM glutamine was used for nucleotide inhibition of all glutaminase assays.

**Equilibrium Dialysis—** Nucleotide binding was determined by equilibrium dialysis (Zhou et al., 1994) using chambers of 100 µl that were separated by a 12,000–14,000 molecular weight cut off dialysis membrane (Spectraphore). One chamber contained 200 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 35 mM [8-¹⁵N]GMP (0.1 µc), 0–2.0 mM unlabeled GMP, and 300 mM AMP in a total volume of 50 µl. The other chamber contained 10 mM Tris-HCl (pH 7.5) and approximately 200 µM enzyme subunit in a total volume of 50 µl. Dialysis was for 20 h at 4 °C in a rotating apparatus (Hoefl). Samples of 40 µl were retrieved from each chamber and were counted for radioactivity. Equilibrium binding data were fit to the Scatchard equation for nonlinear regression using Ultrafit. Under these conditions GMP is expected to bind to the A-site and AMP to the C-site (Zhou et al., 1994).

**Affinity Labeling by DON and Crystallization—** Approximately 20 ng of enzyme was incubated at room temperature in 2.0 ml of a mixture containing 50 mM potassium phosphate (pH 7.5), 3 mM PRPP, 5 mM MgCl₂, and 3 mM DON. After reaction for 30 min, enzyme was dialyzed against 10 mM Tris-HCl (pH 7.5). PRPP-dependent glutaminase activity was determined by measuring the increase in glutamine concentration where the synthesis of PRA was 38.5 units/mg, not significantly different from the activity of the untreated enzyme (43 units/mg). DON-inactivated glutamine PRPP amidotransferase was crystallized at 20 °C by sitting drop vapor diffusion. Drops containing 4 µl of enzyme (18.5 mg/ml) and 4 µl of well solution were equilibrated against a well solution of 13% polyethylene glycol 3550, 5% 2-propanol, 100 mM PIPES, 4 mM MgCl₂, and 4 mM MgSO₄ at pH 6.3. Crystals were transferred to an equivalent solution containing 12% polyethylene glycol, 10% 2-propanol, and 15% DL-meso-2,3-butandiol 10 min prior to freezing.
X-ray Structure Determination of DON-inactivated Enzyme—The structure of native E. coli glutamine PRPP amidotransferase has recently been determined with X-ray crystallography. DON-inactivated glutamine PRPP amidotransferase was crystallized under conditions identical to those used in structure determination of the native protein. The DON-enzyme crystals are isomorphous to those of the native enzyme (space group C2221, a = 316.9 Å, b = 157.5 Å, c = 106.3 Å, with two monomers of the tetrameric enzyme per asymmetric unit). X-ray diffraction data to 2.3 Å were collected from a single crystal, flash frozen to 120 K with an Oxford Cryostream, using an R-axis II imaging plate system mounted on a Rigaku RU-200 rotating anode (CuKα) operated at 100 mA and 50 kV. The data were processed and scaled using DENZO and Scalepack (Otwinowski, 1993). Statistics for the resulting data are shown in Table I. An initial difference electron density map was calculated using Fo(DON-enzyme) − Fo(native) and phases from the refined native structure. This indicated that differences were confined to the glutamine active site, with significant shifts of protein atoms being small, and limited to the Asp127 side chain and Cys1 sulfhydryl. These shifted side chain atoms and water molecules within 10 Å of Cys1 were removed from the refined native model. The truncated model was then refined against the DON-enzyme data in X-PLOR (Brünger, 1992) with 10 steps of isotropic temperature factor refinement and 20 steps of positional refinement. An IFo − IFc electron density map calculated from the resulting model showed clear density for the DON atoms. The initial model of the DON-Cys1 adduct fit this unbiased density remarkably well, demonstrating the accuracy of both the density and the model. This electron density is shown with the current refined model of the DON-enzyme active site in Fig. 3, which was drawn with Molscript (Kraulis, 1991) and Raster 3D (Bacon and Anderson, 1988; Merritt and Murphy, 1994). The completed model of two monomers was refined independently in X-PLOR, with positional and restrained individual temperature factor refinement. Analyses—NH2-terminal amino acid sequencing was carried out on an Applied Biosystems gas-phase sequenator model 470 using standard operating procedures. The mass spectrum of the glutamine PRPP amidotransferase wild type and Ala insertion mutant was determined by matrix-assisted laser desorption ionization mass spectrometry. By these methods wild type enzyme was not detected in the preparation of the Ala insertion. However, the limit of detection, although not precisely determined, was greater than 5%. RESULTS AND DISCUSSION Effect of PRPP on Glutamine Hydrolysis—Glutamine hydrolysis is markedly dependent upon PRPP and is tightly coupled to synthesis of PRA. Under the assay conditions used previously (10 mM glutamine), the basal rate of glutamine hydrolysis in the absence of PRPP was determined using conditions appropriate for the reaction with PRPP; the rate was between 0.8 and 4% of the rate with PRPP (Messenger and Zalkin, 1979; Kim et al., 1995a). To understand catalysis, it is necessary to determine how PRPP activates glutamine hydrolysis and how the amide of glutamine reacts with PRPP to form PRA. We have now found a higher rate of basal PRPP-independent glutaminase having a Vmax of approximately 10 units/mg and Kmax of 190 mM for glutamine (Fig. 1 and Table I). At high nonphysiological concentrations of glutamine the basal activity was 35% of the PRPP-dependent glutaminase. Values of Kmax/Km were 47 and 14,980 M−1 s−1 for basal and PRPP-dependent glutaminase, respectively. The data in Table I indicate that the effect of PRPP is to lower the Km for glutamine by over 100-fold and increase the Vmax approximately 3-fold. The basal glutaminase activity was inhibited by GMP and AMP (Fig. 2) indicating that the active site for this reaction is the same as that for PRPP-dependent hydrolysis of glutamine. The nucleotide concentration required for 50% inhibition was lower for reactions in which PRPP was omitted. These data support the idea that the competitive relationship between PRPP and nucleotide inhibitors (Messenger and Zalkin, 1979) results from alternative active and inactive enzyme conformations (Smith et al., 1994). The basal activity in the absence of PRPP thus reflects enzyme in a partially active conformation that can be further activated by PRPP or inhibited by nucleotides. In spite of the basal glutaminase activity, production of phosphoribosylamine was still tightly coupled to glutamine hydrolysis because PRPP-independent glutaminase activity was insignificant at glutamine concentrations of 20 mM or lower (Fig. 1). Thus, at concentrations of glutamine less than 20 mM the rates of total glutaminase determined by glutamate production
and glutamine-dependent synthesis of PRA were similar. The rate of PRA synthesis with NH₄ as nitrogen donor was 2.1 times the rate with glutamine as substrate (Table I). This is similar to a ratio of 2.8 determined previously by a different method (Messenger and Zalkin, 1979). The results indicate that the capacity for phosphoribosylamine synthesis exceeds that for glutamine hydrolysis.

Structure of the Glutamine Site—The current refined model contains 992 (98.4%) of the protein residues and 942 water molecules. Statistics for this model are in Table II. The glutamine active site is in a pocket in the N-domain. The arrangement of key residues in the glutamine site labeled with DON is shown in Fig. 3 and is illustrated schematically in Fig. 4. Distances for interacting atoms are given in Table III. DON is shown in Fig. 3 and is illustrated schematically in Fig. 4. The largest shifts are 0.5 Å for Cys 1 S, Asp127 carboxyl, and up to 0.7 Å for the Asp127 side chain and may be due primarily to the covalently attached DON which has an additional carbon atom compared to a glutamine thioester intermediate. Overall, the DON structure is quite similar to the predicted structure of glutamine modeled into the site (Smith, 1995).

**TABLE II**

| Data collection and refinement statistics for DON-inactivated glutamine PRPP amidotransferase |
|---------------------------------------------------------------|
| Resolution (Å) | 2.3 Å |
| Total No. observations | 192,620 |
| No. Unique reflections | 40,313 |
| R-merge | 5.4% (17.0%)* |
| Completeness | 91.8% (61.2%)a |
| Number of atoms | 8985 |
| Range of Data | 6.0–2.3 Å |
| Rwork | 15.2% |
| Rfree | 21.2% |
| R.m.s.d. bonds | 0.009 Å |
| R.m.s.d. angles | 1.79° |
| R.m.s.d. dihedrals | 23.0° |
| R.m.s.d. impropers | 1.31° |
| R.m.s.d. bonded B’s | 3.13 Å² |

*Values in parenthesis represent the highest resolution shell of data (2.38–2.30 Å). 

**Fig. 3.** Electron density map. Refined model of the DON-labeled active site overlaid with the original unbiased difference electron density map, contoured at 3σ. The map was calculated using the native structure with Cys 1 S, Asp127 carboxyl, and neighboring water molecules removed. Atoms are colored as follows: peptide C, yellow; DON C, white; N, blue; O, red; S, green.

The DON α- NH₂ group demonstrates good hydrogen bonding to the carboxyl of Asp 127. In addition, the DON α- NH₂ forms a strong hydrogen bond to the backbone carboxyl of Gly 102, demonstrating that this invariant residue also functions in substrate binding. A third hydrogen bond is formed to a water molecule, completing all possible hydrogen bond interactions to the α- NH₂ group.

The carboxyl oxygens of DON exhibit good hydrogen bond distances to Thr 76 and three serine side chains at positions 79, 126, and 128. Of these residues Thr 76 is invariant and at positions 126 and 128 there are either serine or threonine residues in the other glutamine PRPP amidotransferase sequences. Position 79 is totally nonconserved on the other hand. These interactions, by themselves, however, may be insufficient for effective binding of glutamine. This is suggested by temperature factors for the DON carboxyl atoms that are significantly higher than neighboring atoms, indicating that they are not tightly bound. It is possible to model Arg 73 in a favorable conformation that displaces only water molecules and results in hydrogen bonding to the carboxyl of DON or to the carboxyl of glutamine modeled in the site. In this configuration Arg 73 would displace two water molecules in contact with the substrate, creating an active site that more fully encloses the bound glutamine. The proximity of Arg 73 to DON is shown in Fig. 3.
subfamily.4 We have analyzed the effects of mutations of these amino acids as well as Tyr17 which is conserved in glutamine PRPP amidotransferase but not in the other enzymes.

Cys5—The NH2-terminal cysteine is an active site residue required for glutamine amide transfer. In earlier work (Mé and Zalkin, 1989), it was proposed that conserved His and Asp residues function in acid-base catalysis to generate the Cys5 thiolate for nucleophilic catalysis and in the steps of amide transfer and hydrolysis of a γ-glutamyl thioester intermediate. More recent evidence, however, indicates that the proposed His101 and Asp127 side chains are too far removed from Cys5 to participate in catalysis (Smith et al., 1994). Furthermore, these residues are not conserved using improved alignments with the larger group of more recently acquired sequences of Ntn amidotransferases. More importantly, there are no other amino acid side chains within hydrogen bonding distance to the γ-sulfhydryl of Cys5 in the x-ray structure of the E. coli enzyme that could function in acid-base catalysis.5 In the absence of an amino acid side chain to facilitate formation of the thiolate anion needed for nucleophilic catalysis and for the subsequent step of amide transfer and the proximity of the α-NH2 group to the γ-SH group of Cys5 (shown in Fig. 4), we have investigated whether a free Cys5 α-NH2 group is required for catalysis. An alanine residue was inserted between Met1 and Cys5 by site-directed mutagenesis to block the Cys5 amino group. The determined NH2-terminal amino acid sequence of the purified amidotransferase was Ala-Cys-Gly-Val-Phe, indicating cleavage of the glutamine binding/amide transfer domain required for catalysis. The main chain oxygen of Arg26 hydrogen bonds with the ε-amino group of Cys1, thus increasing its basicity. In addition, the α-NH2 group of Cys5 is close enough to the C-site that the alanine insertion may interfere sterically with the PRPP reaction. In penicillin acylase a similar role has been proposed for the α-NH2 group of the NH2-terminal serine to generate a seryl nucelophile for covalent catalysis (Duggleby et al., 1995).

Arg26—Arginines 26 makes a series of H bonds with residues involved in catalysis at the glutamine site (Fig. 4). The ε-N of Arg26 H-bonds to the main chain oxygen of Cys5, an interaction that should contribute to positioning Cys5 for catalysis. The main chain oxygen of Arg26 hydrogen bonds with the amino group of Cys5, thus increasing its basicity. In addition, the γ1-amino group of Arg26 is within H-bonding distance to the carboxamide oxygen of Asn101. To determine the functional consequence of these interactions two replacements of Arg26 were made, R26H and R26A. In both cases the mutant enzymes were extremely labile and could not be purified for analysis.

In vivo analysis of enzyme function was made by examining the capacity of the R26H and R26L enzymes to support growth of a purF mutant in medium containing either 50 mM NH4Cl or 1 mM NH4Cl as N-source. Cells having the R26L or R26H enzymes grew at the wild type rate (2-h doubling time) with 50 mM NH4Cl, but growth was severely limited (10-h doubling) in medium with 1 mM NH4Cl. This indicates that the glutamine-dependent activity was disabled in these mutants but there was sufficient NH3-dependent activity for wild type growth. An R26K mutant was reported previously to have little or no activity in the Ala-Cys enzyme is consistent with a role of the Cys5 α-NH2 group as a proton acceptor for thiolate formation and proton donor for amide nitrogen transfer. The basis for the reduced activity with NH4 as substrate is not certain, but the α-NH2 of Cys5 is close enough to the C-site that the alanine insertion may interfere sterically with the PRPP reaction. In penicillin acylase a similar role has been proposed for the α-NH2 group of the NH2-terminal serine to generate a seryl nucelophile for covalent catalysis (Duggleby et al., 1995).

4 Glutamine amidotransferase subfamilies were previously named G-type (Class I) and F-type (Class II) after prototype E. coli TrpG and PurF enzymes, respectively. With x-ray structures now available, the nomenclature has been revised to reflect the defining structural features of the glutamine binding/amide transfer domain required for catalysis. The key features are an NH2-terminal nucleophile (Ntn) (Brannigan et al., 1995) and a catalytic triad (Tesmer et al., 1996). The G-type (Class I) enzymes are in the Triad subfamily and F-type (Class II) enzymes in the Ntn subfamily.

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**Table III**

| Hydrogen bonds for interactions shown in Fig. 4 |
|-----------------------------------------------|
| A cutoff of 3.4 Å for N and O, and 3.7 Å for S was used. Values in this table pertain to the A subunit of the crystallographic asymmetric unit. Numbers are similar for the B subunit, which is less well ordered. |

| Protein | DON Protein | Distance (Å) |
|--------|-------------|-------------|
| Cys5   | S  | Cys5 | N  | 3.35 |
| Cys5   | Amino N | Arg26 | O  | 3.29 |
| Arg26  | Asp127 | Ala101 | O  | 3.26 |

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**Fig. 4. Interactions of DON in the glutamine site.** Schematic representation of the DON-labeled active site illustrating hydrogen bond interactions involving DON and key residues. DON and the Cys1 γS-DON C6-thioether bond are shown in red. Additional hydrogen bonds between DON and water molecules are not shown. Distances between atoms are given in Table III.
glutamine-dependent activity and to be subject to rapid proteolysis in vivo (Mei and Zalkin, 1989). Overall, the results indicate that Arg26 has roles maintaining a functional glutamine site and in structural integrity of the enzyme, in accord with the participation of Arg26 in an extensive network of salt bridges within the N-domain.

Asn101 and Gly102—Nucleophilic attack of Cys3 on the carbonyl amide of glutamine is postulated to lead to a transient tetrahedral adduct that would be stabilized by interactions with the amide of Asn101 and the backbone NH of Gly102. N101G, N101D, and G102A replacements were made for these residues of the putative oxyanion hole and the results are shown in Table IV. The N101G mutation had only a marginal 4-fold reduction in Vm, for glutamine-dependent activity while the Km for glutamine, Vm, and Km, for the NH3-dependent reaction, and nucleotide binding to regulatory sites were indistinguishable from the wild type. Inspection of the structure of the glutamine site (Fig. 3) suggests that Arg26 may occupy the space of Asn101 in the glycine replacement and thus provide surrogate oxyanion stabilization that could account for the residual 23% activity with glutamine. With a N101D mutation the Vm, for the glutamine reaction was reduced more than 2,500-fold, possibly as a result of repulsion between the carboxylate and the oxyanion species. The G102A replacement shows that Gly102 is critical for activity with glutamine but not for glutamine binding, NH3-dependent PRA synthesis, nor nucleotide binding. The structure of Gly102 indicates ϕ, ψ angles that are highly unfavorable for alanine. Therefore, the G102A mutation is likely to interfere with positioning of the backbone NH needed to stabilize an oxyanion intermediate.

Arg73 and Asp127—Binding of DON in the glutamine site involves hydrogen bonds between the carbonyl of Asp127 and the backbone oxygen of Gly102 with the ω amino group and by hydrogen bonds between the Oy of serines 79, 126, and 128 and Thr76 with the carboxyl group (Fig. 4). The side chain of Arg73 although not within hydrogen bonding distance to DON in the x-ray structure of the PRPP-free enzyme, nevertheless, is the only positively charged group capable of interacting with the DON carboxyl without backbone reorientations. Replacements were made for Gly102, Arg73, and Asp127. The Km for glutamine was similar to the wild type value for the G102A enzyme (Table IV). In the absence of an increase in glutamine Km for the G102A mutant there is no biochemical evidence to support a role of the Gly102 main chain oxygen in glutamine binding. Two effects were noted for Arg73 and Asp127 replacements and are seen in Table IV. Km values for the glutamine reaction were increased 137-fold in the Asp127 mutant and about 50-fold in the two Arg73 mutants relative to the wild type. These increased Km values are consistent with roles for Asp127 and Arg73 in glutamine binding. However, as shown in Table IV there were also significant reductions in Vm for the glutamine reaction, 46-fold in the D127A enzyme and 15-fold in the two arginine mutants. The decreased Vm, is restricted to glutamine site function since the NH3-dependent activity and nucleotide binding were similar to the wild type. The basis for the decreased rate of glutamine site catalysis for Arg73 mutants, as explained in the section on "Model for the Glutamine Site, Activation by PRPP, and Inhibition by Nucleotides" may be a structural perturbation that impacts on catalysis.

Tyr74—Binding of PRPP to the C-site is required to activate Cys for reaction with glutamine and amide transfer. Tyr74 is the closest N-domain residue to PRPP modeled into the C-site of the nucleotide inhibited enzyme (see Fig. 1 in Kim et al. (1995a)) and by virtue of its proximity to Cys3 could participate in coupling PRPP binding to glutamine hydrolysis and amide transfer. Tyr74 is conserved in all of the glutamine PRPP amidotransferase sequences, but not in the other Ntn amidotransferases. In order to investigate whether Tyr74 functions to couple PRPP binding to the reaction of glutamine we examined glutamine site function and PRA synthesis in three Tyr74 replacements, Y74S, Y74L, and Y74F. Data summarized in Table V, although complex, establish that Tyr74 exerts an important role in coupling glutamine site catalysis with synthesis of PRA. The glutamine site was characterized by two assays. First, a basal glutaminase activity was measured. Second, we determined the capacity of PRPP to activate the basal glutaminase by increase in Vm and reduction in glutamine Km. The extent of PRPP activation can be evaluated by comparing basal and total glutaminase in Table V. "Total glutaminase" is given in Table V, although complex, establish that Tyr74 exerts an important role in coupling glutamine site catalysis with synthesis of PRA. The glutamine site was characterized by two assays. First, a basal glutaminase activity was measured. Second, we determined the capacity of PRPP to activate the basal glutaminase by increase in Vm and reduction in glutamine Km. The extent of PRPP activation can be evaluated by comparing basal and total glutaminase in Table V. "Total glutaminase" is given in Table V. "Total glutaminase" is given in Table V, although complex, establish that Tyr74 exerts an important role in coupling glutamine site catalysis with synthesis of PRA. The glutamine site was characterized by two assays. First, a basal glutaminase activity was measured. Second, we determined the capacity of PRPP to activate the basal glutaminase by increase in Vm and reduction in glutamine Km. The extent of PRPP activation can be evaluated by comparing basal and total glutaminase in Table V. "Total glutaminase" is given in Table V, although complex, establish that Tyr74 exerts an important role in coupling glutamine site catalysis with synthesis of PRA.

There are four important results that were obtained from the Tyr74 replacements. (i) The basal glutaminase was perturbed. Activity was decreased 3-fold in Y74S and increased nearly 5-fold relative to wild type in the Y74L enzyme. As shown in Tables IV and V, Arg73 is critical for glutamine site function and the Tyr replacements may interact with Arg73 differently. The perturbation of basal glutaminase in Tyr74 mutants was confined to Vim; Km was not affected. (ii) There was little or no stimulation of basal glutaminase by PRPP in the Tyr74 mutants. This is shown by similar Vim values for basal and total glutaminase in the Y74S and Y74L enzymes. (iii) Little, if any, of the glutaminase was coupled to PRA synthesis. There was apparent 2-fold stimulation of glutaminase by PRPP in the Y74F enzyme. However, only one-third of this glutaminase activity was available for PRA synthesis. There is thus clear evidence for defective coupling of glutamine hydrolysis with PRA synthesis. The Km for PRPP, although technically difficult to determine due to the high basal glutaminase, appeared to be
similar to the wild type value. (iv) A decrease in $V_m$ for NH$_3$-dependent PRA synthesis was accompanied by small increases in $K_m$ for NH$_3$. Thus, these data show that Tyr$_{74}$ participates in communicating PRPP availability to the glutamine site, coupling of glutamine hydrolysis with amide transfer and the reaction of NH$_3$ with PRPP to make PRA.

The data in Table V also show the relationship between functions of Tyr$_{74}$ and Arg$_{73}$. In the two Arg$_{73}$ mutants the primary defect was impaired binding of glutamine as reflected by the unmeasurable $K_m$ for basal glutaminase. There was activation by PRPP which led to low but detectable total glutaminase having a high $K_m$. Most importantly, the resulting glutamine hydrolysis was coupled to PRA synthesis and NH$_3$-dependent PRA synthesis was comparable to wild type. When R73L and Y74L mutations were combined there was no stimulation of basal glutaminase by PRPP and no coupling of glutaminase with PRA synthesis. NH$_3$-dependent PRA synthesis was decreased marginally from the wild type level. Thus Arg$_{73}$ functions mainly in glutamine binding and catalysis and Tyr$_{74}$ in modulating glutamine site catalysis with the availability of PRPP and coupling synthesis of PRA to nitrogen from glutamine.

Nucleotide Inhibition—Since Tyr$_{74}$ has a role in communicating the binding of PRPP in the C-site to glutamine site catalysis in the N-domain, we investigated whether Tyr$_{74}$ was involved in communicating binding of nucleotides to the N-domain. Nucleotide inhibition of Arg$_{73}$ mutants was also examined because of the importance of this residue on glutamine site function and the possibility of coordinate effects of residues at positions 73 and 74. Inhibition of total glutaminase by nucleotides is shown in Fig. 5 for the wild type and three mutant enzymes. Y74L and R73L enzymes were each somewhat less sensitive to inhibition by AMP. The R73L but not the Y74L mutant was less sensitive than the wild type enzyme to inhibition by GMP. Similar results, not shown, were obtained for the Y74H, Y74F, and R73H enzymes. Most importantly, however, the R73L/Y74L double mutant was essentially insensitive to inhibition by concentrations of nucleotides that gave 95–100% inhibition of the wild type enzyme.

Binding of GMP to the double mutant was measured in order to determine whether loss of inhibition resulted from a binding defect or from loss of communication by bound nucleotide to the glutamine site. GMP binding in the presence of unlabeled AMP is shown in Fig. 6. The GMP binding stoichiometry extrapolated to $1.25 \pm 0.19$ equivalents with a $K_a$ of $221 \pm 76 \mu M$. This $K_a$ for GMP binding to the mutant is similar to that for binding to the wild type enzyme (Kim et al., 1995a) and indicates that nucleotide binding is not communicated to the glutaminase site in the feedback-insensitive R73L/Y74L enzyme.

Model for the Glutamine Site, Activation by PRPP, and Inhibition by Nucleotides—E. coli glutamine PRPP amidotransferase is a tetramer with each subunit organized in an $N$-domain, amino acids 1–251, and a $C$-domain, amino acids 252–504. Two half-sites constitute the active site. Glutamine binds to the $N$-domain and it is thought that Cys$_1$ attacks the carboxylic acid.
boxamide of glutamine to initiate amide transfer. The amide nitrogen of glutamine is transferred to PRPP bound to the second half-site (C-site) to yield PRA + PRP, and a putative γ-glutamyl thioester enzyme intermediate. Affinity labeling by DON results in a DON-cysteinyld adduct that is a model for the putative γ-glutamyl thioester intermediate. C-site residues 362–374 constitute a conserved PRPP fingerprint. Although a primary function of the C-site is binding PRPP, it has features in common with mononucleotide sites and is a binding site for AMP or GMP. Four intersubunit allosteric sites (A-sites) in the tetramer also bind nucleotides. Synergistic binding of AMP and GMP to the C- and A-sites, respectively, results in synergistic inhibition (Zhou et al., 1994).

We can distinguish three activity states of the enzyme, basal, active, and inhibited, that differ by the occupancy of the A- and C-sites. Nucleotide-free enzyme having no PRPP in the C-site can bind glutamine with low affinity and catalyze its hydrolysis at a rate up to about 35% of the fully activated rate. This form of the enzyme having unoccupied A- and C-sites thus defines a basal activity that can either be activated by PRPP or inhibited by nucleotides. The kinetically competitive relationship between PRPP and nucleotides (Messenger and Zalkin, 1979) thus results from alternative enzyme conformations mediated by the ligands. In the presence of PRPP the K_m for glutamine is decreased by 100-fold and the V_m for reaction increased by 3-fold. In this active state PRPP is the C-site ligand and the A-site is unoccupied.

How does PRPP activate the enzyme? At least two structural changes must take place. First, it is plausible to imagine that binding of PRPP to the C-site repositions Arg73 for high affinity binding of glutamine. Replacements of Arg73 and also Asp121, which participates in glutamine binding, led to significantly higher K_m values for glutamine. Once the substrate is precisely positioned by the Arg73 interaction, nucleophilic attack by Cys3 on the carboxamide can initiate amide transfer. Second, it is likely that the 16-Å space between the glutamine half-site and PRPP in the C-site must close to permit nucleotide transfer. Although it is not possible to explain how these changes take place without knowledge of the x-ray structure of the fully active enzyme, Tyr74 has a role in communicating PRPP binding to the glutamine site. Replacements of Tyr74 perturb the binding and reaction of glutamine and amide transfer.

Binding of nucleotides to the A- and C-sites converts the enzyme to the inhibited state. A “flag-loop” structure from the C-domain of one subunit is packed tightly against the N-domain of a neighboring subunit and through interactions with nucleotide in the A-site appears to hold apart the subunit’s N- and C-domains stabilizing the inactive open conformation (Smith et al., 1994). It also appears that Arg73 may have a role in inhibition in addition to its role in glutamine binding, since a replacement of Arg73 augmented the effect of a Tyr74 mutation and desensitized the enzyme to nucleotide inhibition. Tyr74 and Arg73 are involved in transmitting the inhibitory nucleotide binding signal to the glutamine site. An x-ray structure is needed for the active enzyme to precisely describe the changes in conformation.

Relationships to Asparagine Synthetase. Glucosamine-6-phosphate Synthase, and Glutamate Synthase—Glutamine PRPP amidotransferase, asparagine synthetase (Van Heek and Schuster, 1989), glucosamine 6-phosphate synthase (Badet et al., 1987), and glutamate synthase (Oliver et al., 1987) all use an N-terminal active site cysteine for glutamine amide transfer and contain N-domains having nine conserved amino acids. Boehrlein et al. (1994) have analyzed replacements of Arg30 and Asn74 in E. coli asparagine synthetase, residues that correspond to Arg26 and Asn101 in E. coli glutamine PRPP amidotransferase. A number of rules were suggested for this Arg residue including binding the glutamine substrate, mediating the glutaminyl half-reactions in asparagine formation, and controlling nitrogen release from glutamine. Suggested roles for Asn74 included stabilization of an oxynion intermediate and stabilization of a hypothetical hydroxyimine tautomeric species of glutamine substrate. It is likely that asparagine synthetase Arg30 and Asn74 as well as other conserved amino acids in the N-domains of asparagine synthetase, glucosamine 6-phosphate synthase, and glutamate synthase define the glutamine site in these enzymes and have similar functions in the four enzymes.

Ntn Hydrolases—A family of enzymes having an NH_2-terminal side chain, a common active site organization, and a common fold was identified recently and named the Ntn hydrolase family (Brannigan, 1995). Glutamine PRPP amidotransferase is an Ntn hydrolase and data reported here support the mechanisms proposed for the enzyme family. In addition to an NH_2-terminal nucleophile, Cys, Ser, or Thr, the catalytic framework includes a free NH_2 terminus and an oxianion hole. Analysis of the glutamine PRPP amidotransferase Ala insertion mutant supports the proposed role of a free NH_2 terminus as a general acid-base (Brannigan et al., 1995). The structure of glutamine PRPP amidotransferase-DON adduct and the properties of Asn101 and Gly102 mutants show that these residues likely constitute the oxianion hole for glutamine hydrolysis, as predicted. Interestingly, the binding of DON to glutamine PRPP amidotransferase (Fig. 3) is very similar to the binding of aspartate to the Ntn hydrolase, aspartylglucosaminidase (Oinonen et al., 1995). Interactions of Gly230, Arg111, and Asp214 with aspartylglucosaminidase correspond to Gly102, Arg73, and Asp127 interactions with DON in glutamine PRPP amidotransferase. The common ligand interactions in glutamine PRPP amidotransferase and other Ntn hydrolases support the proposed roles of specific glutamine PRPP amidotransferase active site residues and the idea that these are homologous enzymes.

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Structure and Function of the Glutamine Phosphoribosylpyrophosphate Amidotransferase Glutamine Site and Communication with the Phosphoribosylpyrophosphate Site

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