THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 26, Issue of June 28, pp. 15549–15557, 1996

Printed in U.S.A.

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

(Received for publication, January 11, 1996, and in revised form, March 25, 1996)

Jeong Hyun Kim‡, Joseph M. Krahn§, Diana R. Tomchick§, Janet L. Smith§, and Howard Zalkin‡

From the Departments of ‡Biochemistry and §Biological Sciences, Purdue University, West Lafayette, Indiana 47907-1153

Glutamine phosphoribosylpyrophosphate (PRPP) amidotransferase from *Escherichia coli* exhibits a basal PRPP-independent glutaminase activity having a k_{cat}/K_m that is 0.3% of fully active enzyme. Binding of PRPP activates the enzyme by a structural change that lowers the K_m 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. Tyr^{74} is a key residue in the coupling between the sites for glutamine in the NH_2-terminal domain and PRPP in the COOH-terminal domain. Arg^{73} and Asp^{127} have roles in glutamine binding. The x-ray structure indicates that there are no amino acid side chains sufficiently close to Cys^1 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 nitrogen transfer. Based on the x-ray model of the glutamine site and analysis of a mutant enzyme we propose that the free NH_2 terminus of Cys^1 functions as the proton acceptor and donor. The results indicate that the side chain of Asn^{101} and the backbone nitrogen of Gly^{102} function to stabilize a tetrahedral oxyanion resulting from attack of Cys^1 on the glutamine carboxamide. Cys^1, Arg^{73}, Asn^{101}, Gly^{102}, and Asp^{127} are conserved in the NH_2-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. Tyr^{74}, 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 (NH_2-terminal nucleophile) hydrolase family of enzymes.

Glutamine PRPP[1] 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*.[2] 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 Cys^1 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 (Mäntsälä and Zalkin, 1984b; Ludin *et al.*, 1994), *Neurospora*,[3] and *Haemophilus* (accession number U32800) are similar to *E. coli* glutamine PRPP amidotransferase in lacking the propeptide and cysteinyl 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_i. The enzyme also has the capacity to use NH_3 in place of glutamine *in vitro* (Messenger and Zalkin, 1979) and *in vivo* (Mäntsälä and Zalkin, 1984a). In addition, a glutaminase activity was detected that was less than 5% of the biosynthetic rate (Messenger 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

* This work was supported by United States Public Health Service Grants DK42303 (to J. L. S.) and GM24658 (to H. Z.). This is journal paper number 15023 from the Purdue University Agricultural Research Station. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Atomic models of native glutamine PRPP amidotransferase and the DON-inactivated enzyme described in this work have been deposited with the Protein Data Bank, Brookhaven National Laboratory, Upton, NY, under accession code 1ECF and 1ECG, respectively.

‡ To whom correspondence should be addressed. Tel.: 317-494-1618; Fax: 317-494-7897.

[1] The abbreviations used are: PRPP, 5-phosphoribosylpyrophosphate; PRA, 5-phospho-β-D-ribosylamine; DON, 6-diazo-5-oxonorleucine; PIPES, 1,4-piperazinediethanesulfonic acid; N-domain, NH_2-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 NH_2-terminal domain (N-domain) and PRPP to a catalytic site (C-site) in the CO_2H-terminal domain (C-domain). Second, a structural change is required in the glutamine site that enables Cys^1 to attack the carboxamide of glutamine and initiate amide transfer. Given the requirement of PRPP for reaction of glutamine (hydrolysis and amide transfer) and for affinity labeling of Cys^1 by glutamine analogs (Messenger and Zalkin, 1979), the binding of PRPP must in some way activate the enzyme permitting Cys^1 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 T7ϕ10 promoter and with the M13 replication origin was constructed for overexpression and mutagenesis. The plasmid was constructed in two steps. First, an *Nde*I-*Hin*dIII fragment containing a 3′ segment of the gene was ligated into the *Nde*I and *Hin*dIII sites of pT7-SCA (U. S. Biochemical Corp.). Next, an *Nde*I 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 *Pst*I and the primers were 5′-GGAAAAAGACATATGTGCGGTA-3′ and 5′-GCCGTTGTGGGCAAGCGT-3′. The resulting polymerase chain reaction product, digested with *Nde*I, was ligated into the *Nde*I site of the intermediate plasmid described above containing the 3′ segment 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 CJ236 (*dut, ung, rel*A, *pCJ105(Cm*^{R})) and MV1190 (Δ(*lac-pro*AB), *thi, supE,* Δ(*srl-rec*A) 306::*tn*10 (tet^R) [F′ *tra*D36, *pro*AB, *lac*I^q ZΔM15]) 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-bearing *E. coli* cells were grown at 37 °C in LB medium (Miller, 1972) containing 140 μg/ml ampicillin. The *purF* gene was induced by 0.2 mM isopropyl-1-thio-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 mM PRPP (Sigma), 20–220 mM glutamine depending on the K_m, 10 mM $MgCl_2$, 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 (Messenger and Zalkin, 1979). A basal PRPP-independent glutaminase activity was determined by the same procedure except for omission of PRPP in the reaction. PRPP-dependent glutaminase activity was calculated by subtracting the PRPP-independent activity from the total glutaminase activity. Control reactions contained all components except enzyme. Glutamine-dependent PRA synthesis was assayed by coupling the formation of PRA to the production of glycinamide ribonucleotide with glycinamide ribonucleotide synthetase (Schendel *et al*., 1988). Purified glycinamide ribonucleotide synthetase (Shen *et al*., 1990) was the generous gift of J. 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 [^{14}C]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. NH_3-dependent PRA synthesis was assayed by the same procedure except 150 mM NH_4Cl 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 (Schendel *et al*., 1988).

To determine whether mutant enzymes retained glutamine- or NH_3-dependent activity *in vivo*, we measured the capacity of the enzyme to support growth of a *purF* auxotroph in medium containing either 50 mM NH_4Cl or 1 mM NH_4Cl as nitrogen source (Mei and Zalkin, 1989). Growth with 1 mM NH_4Cl requires assimilation into glutamine and use of glutamine for PRA synthesis by glutamine PRPP amidotransferase, whereas 50 mM NH_4Cl will suffice for the NH_3-dependent activity of the enzyme. The wild type or mutant *purF* gene was cloned into the *Xba*I site of pTrc99A (Egon *et al*., 1988) and the recombinant plasmid transformed into *E. coli* strain TX358 (*purF recA*). The growth rate of plasmid-harboring cells was determined in M9 medium (Miller, 1972) supplemented with 0.5% lactose, 0.01% acid-hydrolyzed casein, 2 μg/ml thiamin, 100 μg/ml ampicillin, and 1 or 50 mM NH_4Cl.

Determination of K_m and V_m—The kinetic constants K_m and V_m were determined using the standard assays in which the concentration of one substrate was saturating and the concentration of the second substrate was varied. The concentration range for glutamine in the different glutaminase assays was adjusted for specific mutants: 0–220 mM for PRPP-independent glutaminase of wild type and all mutants, 0–20 mM for total glutaminase of enzymes in which the K_m was <3 mM (Ala insertion, N101D, N101G, G102A), 0–220 mM for total glutaminase of mutants in which the K_m was >15 mM (Arg^{73}, Tyr^{74}, and Asp^{127} mutants). For assays with the low range of glutamine concentrations, two concentrations over 100 mM were also tested to verify saturation. For assays of enzymes having a K_m of >100 mM for glutamine, the lowest standard errors were obtained using two glutamine concentrations <100 mM and about six concentrations between 100 and 220 mM. The maximum concentration of glutamine was limited to 220 mM by its solubility. The substrate concentrations used for other assays were: 0–130 mM glutamine for glutamine-dependent PRA synthesis, 0–150 mM NH_4Cl for NH_3-dependent PRA synthesis, and 0–540 μM PRPP for total glutaminase. Kinetic data were fit to the Michaelis-Menten equation using Ultrafit software (Biosoft, Cambridge, UK).

Nucleotide Inhibition—Inhibition of glutaminase activity by AMP and GMP was determined by the standard assay except that the $MgCl_2$ concentration was increased to 20 mM, PRPP was decreased to 1 mM and 0–15 mM AMP or 0–3 mM GMP were included. 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 Arg^{73} and Tyr^{74} 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 $MgCl_2$, 35 nM [8-^{3}H]GMP (0.1 μCi), 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 (Hoefer). 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; Kim *et al*., 1995a).

Affinity Labeling by DON and Crystallization—Approximately 20 mg 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 $MgCl_2$, 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 below the limit of detection whereas the NH_3-dependent 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 3350, 5% 2-propanol, 100 mM PIPES, 4 mM $MgCl_2$, and 4 mM $MgSO_4$ at pH 6.3. Crystals were transferred to an equivalent solution containing 12% polyethylene glycol, 10% 2-propanol, and 15% DL-*meso*-2,3-butanediol 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.[2] 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 $C222_1$, a = 116.9 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 II. 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 Asp^{127} side chain and Cys^1 sulfhydryl. These shifted side chain atoms and water molecules within 10 Å of Cys^1 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 |Fo| − |Fc| electron density map calculated from the resulting model showed clear density for the DON atoms. The initial model of the DON-Cys^1 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—NH_2-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 V_m of approximately 10 units/mg and K_m 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 k_{cat}/K_m 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 K_m for glutamine by over 100-fold and increase the V_{max} 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.

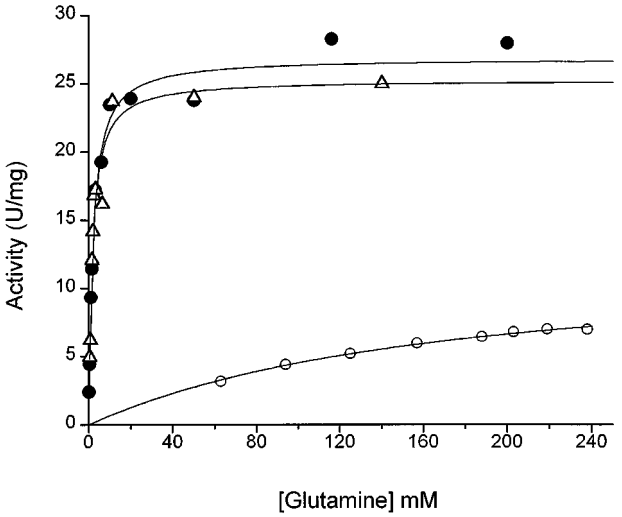

FIG. 1. **Effect of glutamine concentration on basal and PRPP-dependent glutaminase and PRA synthesis.** The symbols are: ●, PRPP-dependent glutaminase; ○, basal glutaminase; Δ, PRA synthesis.

TABLE I
Kinetic constants for wild type glutamine PRPP amidotransferase

Activity	V_m	K_m (glutamine or NH_3)
	units/mg	*mM*
Glutamine hydrolysis		
Total	35.8 ± 2.6	3.24 ± 0.91
PRPP-dependent	27.4 ± 2.0	1.72 ± 0.46
PRPP-independent	9.7 ± 2.5	193 ± 24
Glutamine-dependent PRA synthesis	24.5 ± 1.3	2.10 ± 0.31
NH_3-dependent PRA synthesis	52.3 ± 5.6	7.34 ± 3.95

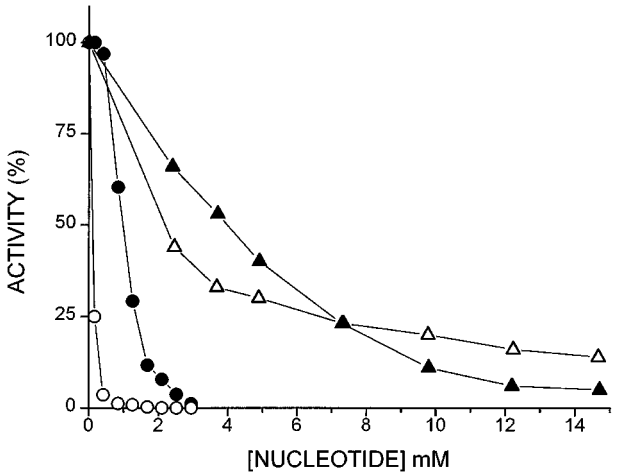

FIG. 2. **Inhibition of PRPP-dependent and basal glutaminase by AMP and GMP.** Standard assays were employed using 20 mM glutamine for PRPP-dependent glutaminase and 200 mM glutamine for the basal activity. The concentration of AMP or GMP was varied. The symbols are: ○, GMP and no PRPP; ●, GMP plus PRPP; Δ, AMP no PRPP; ▲, AMP plus PRPP.

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 NH_3 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 an affinity analog of glutamine, known to alkylate Cys^1 and to mimic the putative covalent glutamyl enzyme thioester intermediate resulting after amide transfer (Zalkin, 1992). The overall structure of the glutamine site of DON-inactivated enzyme is nearly identical to that of the native enzyme (not shown). The largest shifts are 0.5 Å for Cys^1 γS and up to 0.7 Å for the Asp^{127} 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%)[a]
Completeness	91.8% (61.2%)[a]
Number of atoms	8985
Range of Data	6.0–2.3 Å
R_{work}[b]	15.2%
R_{free}[b]	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 Å^2

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

[b] $R = (\Sigma||Fo|-|Fc||)/\Sigma|Fo|$ for all $F>1\sigma$. R_{free} represents 5% of unique reflections which were not used in refinement.

The DON α-NH_2 group demonstrates good hydrogen bonding to the carboxyl of Asp^{127}. In addition, the DON α-NH_2 forms a strong hydrogen bond to the backbone carbonyl 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-NH_2 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.

The carbonyl oxygen of DON hydrogen bonds to the backbone NH of Gly^{102} and to the amide of Asn^{101}. The orientation of these residues results in very favorable hydrogen bonds and supports the proposal that they constitute an oxyanion hole, stabilizing a tetrahedral glutaminyl oxyanion intermediate resulting from attack of Cys^1 thiolate on the carboxamide of glutamine (Brannigan *et al.*, 1995).

Mutagenesis of Residues Involved in Glutamine Binding and Stabilization of the Oxyanion Intermediate—Based on the x-ray structure of the DON-labeled enzyme and native enzyme into which glutamine has been modeled, a number of amino acid residues were identified that are in proximity to the glutamine site and are positioned to participate in binding and in reaction of the glutamine amide. These amino acids include Cys^1, Arg^{26}, Arg^{73}, Asn^{101}, Gly^{102}, and Asp^{127}, residues that are invariant in the 18 known glutamine PRPP amidotransferase sequences as well as in 20 asparagine synthetase, glucosamine 6-phosphate synthase, and glutamate synthase sequences. These four enzymes are members of an Ntn glutamine amidotransferase

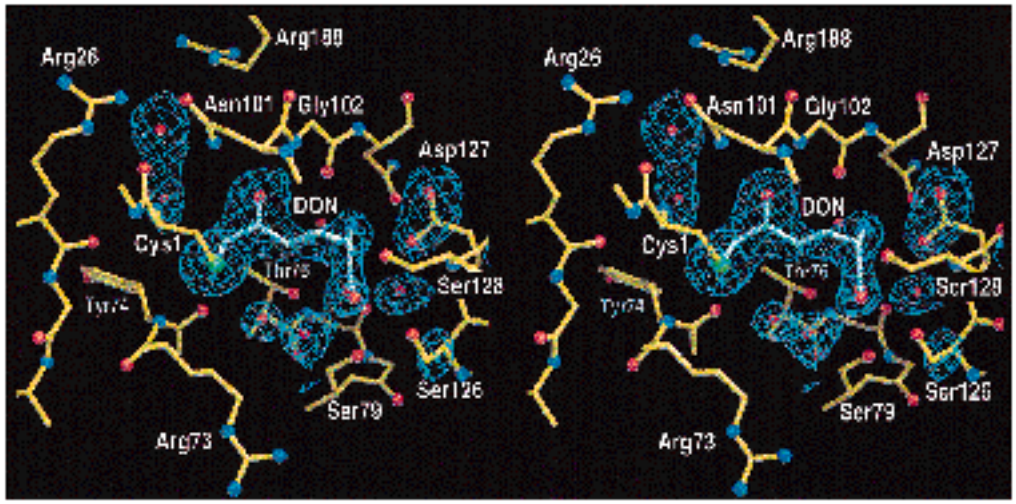

FIG. 3. **Electron density map.** Refined model of the DON-labeled active site overlaid with the original unbiased difference electron density map, contoured at 3σ. The map was calculated using the native structure with Cys^1 S, Asp^{127} carboxyl, and neighboring water molecules removed. Atoms are colored as follows: peptide C, yellow; DON C, white; N, blue; O, red; S, green.

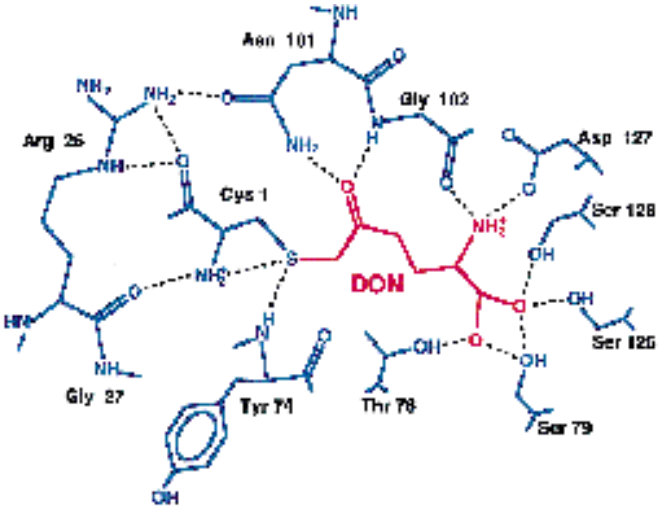

FIG. 4. **Interactions of DON in the glutamine site.** Schematic representation of the DON-labeled active site illustrating hydrogen bond interactions involving DON and key residues. DON and the Cys^1 γS-DON C_6 thioether bond are shown in red. Additional hydrogen bonds between DON and water molecules are not shown. Distances between atoms are given in Table III.

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 assymetric unit. Numbers are similar for the B subunit, which is less well ordered.

Atom				
DON		Protein		Distance
				(Å)
Carbonyl O		Gly^{102}	N	2.66
		Asn^{101}	Nδ	2.92
Amino N		Gly^{102}	O	2.64
		Asp^{127}	Oδ1	2.61
Carboxyl O_1		Ser^{79}	Oγ	3.06
		Ser^{126}	Oγ	2.88
		Ser^{128}	Oγ	2.88
		Gln^{86}	Nε	3.27
Carboxyl O_2		Thr^{76}	Oγ	3.00
		Ser^{79}	Oγ	2.80
		Asp^{127}	Oδ1	3.36
Protein		Protein		Distance
				(Å)
Cys^1	Sλ	Cys^1	N	3.35
		Tyr^{74}	N	3.43
Cys^1	Amino N	Arg^{26}	O	2.92
Cys^1	O	Arg^{26}	Nε	2.76
		Arg^{26}	Nε2	3.32
Arg^{26}	Nη2	Asn^{101}	O	2.76

subfamily.[4] We have analyzed the effects of mutations of these amino acids as well as Tyr^{74} which is conserved in glutamine PRPP amidotransferase but not in the other enzymes.

Cys^1—The NH_2-terminal cysteine is an active site residue required for glutamine amide transfer. In earlier work (Mei and Zalkin, 1989), it was proposed that conserved His and Asp residues function in acid-base catalysis to generate the Cys^1 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 His^{101} and Asp^{29} side chains are too far removed from Cys^1 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 Cys^1 in the x-ray structure of the *E. coli* enzyme that could function in acid-base catalysis.[2] 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-NH_2 to the γ-SH group of Cys^1 (shown in Fig. 4), we have investigated whether a free Cys^1 α-NH_2 group is required for catalysis. An alanine residue was inserted between Met^{-1} and Cys^1 by site-directed mutagenesis to block the Cys^1 amino group. The determined NH_2-terminal amino acid sequence of the purified amidotransferase was Ala-Cys-Gly-Val-Phe, indicating cleavage of the initiator Met in accord with the specificity of *E. coli* methionine aminopeptidase (Hirel *et al.*, 1989). Data in Table IV summarize the effects of the Ala insertion. Activity with glutamine was reduced about 20-fold compared to the wild type enzyme, whereas the K_m for glutamine was unchanged. Although wild type enzyme was not detected in the preparation of the Ala insertion we cannot distinguish whether all of the residual activity is due to the Ala insertion species or whether there was 5% or less contamination by enzme with Ala clipped off. C-domain catalysis was also perturbed as indicated by the 10-fold reduction in NH_3-dependent synthesis of PRA. Nucleotide sites in the C-domain were, however, functional as shown by the stoichiometry and K_d for GMP binding. Under these conditions GMP is bound to the nucleotide A-site and AMP to the C-site (Kim *et al.*, 1995a). Reduced glutamine-dependent activity in the Ala-Cys enzyme is consistent with a role of the Cys^1 α-NH_2 group as a proton acceptor for thiolate formation and proton donor for amide nitrogen transfer. The basis for the reduced activity with NH_3 as substrate is not certain, but the α-NH_2 of Cys^1 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-NH_2 group of the NH_2-terminal serine to generate a seryl nucleophile for covalent catalysis (Duggleby *et al.*, 1995).

Arg^{26}—Arginine 26 makes a series of H bonds with residues involved in catalysis at the glutamine site (Fig. 4). The ε-N and η-N of Arg^{26} H-bonds to the main chain oxygen of Cys^1, an interaction that should contribute to positioning Cys^1 for catalysis. The main chain oxygen of Arg^{26} hydrogen bonds with the amino group of Cys^1, thus increasing its basicity. In addition, the η^1-amino group of Arg^{26} is within H-bonding distance to the carboxamide oxygen of Asn^{101}. To determine the functional consequence of these interactions two replacements of Arg^{26} 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 NH_4Cl or 1 mM NH_4Cl as N-source. Cells having the R26L or R26H enzymes grew at the wild type rate (2-h doubling time) with 50 mM NH_4Cl but growth was severely limited (10-h doubling) in medium with 1 mM NH_4Cl. This indicates that the glutamine-dependent activity was disabled in these mutants but there was sufficient NH_3-dependent activity for wild type growth. An R26K mutant was reported previously to have little or no

[4] Glutamine amidotransferase subfamilies were previously named G-type (Class 1) 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 NH_2-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.

TABLE IV
Activities of glutamine site mutants

Enzyme	PRPP-dependent glutaminase		NH_3-dependent PRA synthesis		GMP binding[a]	
	V_m	K_m	V_m	K_m	Capacity	K_d
	units/mg	*mM*	*units/mg*	*mM*	*equiv*	*μM*
Wild type	27.4 ± 2.0	1.72 ± 0.46	52.3 ± 5.6	7.34 ± 3.95	0.91 ± 0.10	228 ± 56
Ala insert	1.27 ± 0.06	1.48 ± 0.21	4.68 ± 0.86	8.49 ± 4.4	1.09 ± 0.04	281 ± 24
N101G	6.42 ± 0.53	0.64 ± 0.08	58.7 ± 7.2	6.03 ± 3.3	0.78 ± 0.11	297 ± 160
N101D	0.01 ± 0.0002	1.42 ± 0.12	57.5 ± 5.3	9.17 ± 2.3	ND[b]	
G102A	0.02 ± 0.002	2.43 ± 0.82	52.3 ± 3.9	7.67 ± 2.9	0.79 ± 0.22	365 ± 120
D127A	0.59 ± 0.02	236 ± 17	51.7 ± 12	6.08 ± 3.3	ND	
R73H	1.92 ± 0.16	101 ± 18	54.3 ± 4.6	9.76 ± 1.9	0.72 ± 0.35	184 ± 87
R73L	1.82 ± 0.81	110 ± 6.4	53.1 ± 2.1	7.31 ± 2.5	0.70 ± 0.30	181 ± 101

[a] Binding in the presence of 300 μM AMP.
[b] Not determined.

glutamine-dependent activity and to be subject to rapid proteolysis *in vivo* (Mei and Zalkin, 1989). Overall, the results indicate that Arg^{26} has roles maintaining a functional glutamine site and in structural integrity of the enzyme, in accord with the participation of Arg^{26} in an extensive network of salt bridges within the N-domain.

Asn^{101} and Gly^{102}—Nucleophilic attack of Cys^1 on the carboxamide of glutamine is postulated to lead to a transient oxyanion tetrahedral adduct that would be stabilized by interactions with the amide of Asn^{101} and the backbone NH of Gly^{102}. 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 V_m for glutamine-dependent activity while the K_m for glutamine, V_m and K_m for the NH_3-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 Arg^{188} may occupy the space of Asn^{101} 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 V_m 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 Gly^{102} is critical for activity with glutamine but not for glutamine binding, NH_3-dependent PRA synthesis, nor nucleotide binding. The structure of Gly^{102} 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.

Arg^{73} and Asp^{127}—Binding of DON in the glutamine site involves hydrogen bonds between the carboxyl of Asp^{127} and the backbone oxygen of Gly^{102} with the α-amino group and by hydrogen bonds between the Oγ of serines 79, 126, and 128 and Thr^{76} with the carboxyl group (Fig. 4). The side chain of Arg^{73} 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 Gly^{102}, Arg^{73}, and Asp^{127}. The K_m for glutamine was similar to the wild type value for the G102A enzyme (Table IV). In the absence of an increase in glutamine K_m for the G102A mutant there is no biochemical evidence to support a role of the Gly^{102} main chain oxygen in glutamine binding. Two effects were noted for Arg^{73} and Asp^{127} replacements and are seen in Table IV. K_m values for the glutamine reaction were increased 137-fold in the Asp^{127} mutant and about 50-fold in the two Arg^{73} mutants relative to the wild type. These increased K_m values are consistent with roles for Asp^{127} and Arg^{73} in glutamine binding. However, as shown in Table IV there were also significant reductions in V_m for the glutamine reaction, 46-fold in the D127A enzyme and 15-fold in the two arginine mutants. The decreased V_m is restricted to glutamine site function since the NH_3-dependent activity and nucleotide binding were similar to the wild type. The basis for the decreased rate of glutamine site catalysis for Arg^{73} 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.

Tyr^{74}—Binding of PRPP to the C-site is required to activate Cys^1 for reaction with glutamine and amide transfer. Tyr^{74} 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 Cys^1 could participate in coupling PRPP binding to glutamine hydrolysis and amide transfer. Tyr^{74} is conserved in all of the glutamine PRPP amidotransferase sequences, but not in the other Ntn amidotransferases. In order to investigate whether Tyr^{74} functions to couple PRPP binding to the reaction of glutamine we examined glutamine site function and PRA synthesis in three Tyr^{74} replacements, Y74S, Y74L, and Y74F. Data summarized in Table V, although complex, establish that Tyr^{74} 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 V_m and reduction in glutamine K_m. The extent of PRPP activation can be evaluated by comparing basal and total glutaminase in Table V. "Total glutaminase" is given in Table V rather than PRPP-dependent glutaminase in order to avoid the technical difficulty of calculating a small PRPP-dependent activity in the presence of a high background of PRPP-independent basal activity, a particular problem in the Tyr^{74} mutants.

There are four important results that were obtained from the Tyr^{74} 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, Arg^{73} is critical for glutamine site function and the Tyr replacements may interact with Arg^{73} differently. The perturbation of basal glutaminase in Tyr^{74} mutants was confined to V_m; K_m was not affected. (ii) There was little or no stimulation of basal glutaminase by PRPP in the Tyr^{74} mutants. This is shown by similar V_m 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 K_m for PRPP, although technically difficult to determine due to the high basal glutaminase, appeared to be

TABLE V
Kinetic constants for wild type, Arg[73], and Tyr[74] enzymes

Enzyme	Total glutaminase			Basal glutaminase		PRA synthesis			
	Glutamine		PRPP	Glutamine		Glutamine		NH_3	
	V_m	K_m	K_m	V_m	K_m	V_m	K_m	V_m	K_m
	units/mg	*mM*	*μM*	*units/mg*	*mM*	*units/mg*	*mM*	*units/mg*	*mM*
Wild type	31.8 ± 2.6	2.64 ± .91	53 ± 12	9.70 ± 2.5	193 ± 24	24.5 ± 1.3	2.10 ± 0.31	52.3 ± 5.6	7.34 ± 3.95
Y74S	3.45 ± 0.29	228 ± 33	ND[a]	3.45 ± 0.29	228 ± 33	<0.035	ND[b]	20.6 ± 3.3	30.7 ± 7.7
Y74L	49.2 ± 6.5	46.0 ± 19	ND[a]	49.5 ± 16	179 ± 47	1.10 ± 0.13	13.5 ± 6.6	17.8 ± 2.5	21.5 ± 5.5
Y74F	32.3 ± 1.2	30.0 ± 5.6	40 ± 31	16.5 ± 7.3	240 ± 160	5.01 ± 0.23	32.8 ± 4.7	4.45 ± 0.84	12.0 ± 4.3
R73H	2.32 ± 0.19	111 ± 21	ND[b]	<0.5	>1000	1.08 ± 1.1	153 ± 110	53.1 ± 2.1	7.31 ± 2.5
R73L	2.22 ± 0.34	115 ± 12	ND[b]	<0.5	>1000	1.20 ± 0.91	161 ± 130	54.3 ± 4.6	9.76 ± 1.9
R73L/Y74L	2.37 ± 0.29	185 ± 44	ND[b]	2.37 ± 0.29	185 ± 44	<0.035	ND[b]	30.8 ± 4.3	34.1 ± 11

[a] Not determined due to little or no activation by PRPP.
[b] Not determined.

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 and from NH_3.

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 also 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 ± 0.19 equivalents with a K_d of 221 ± 76 μM. This K_d 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 glutamine 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 car-

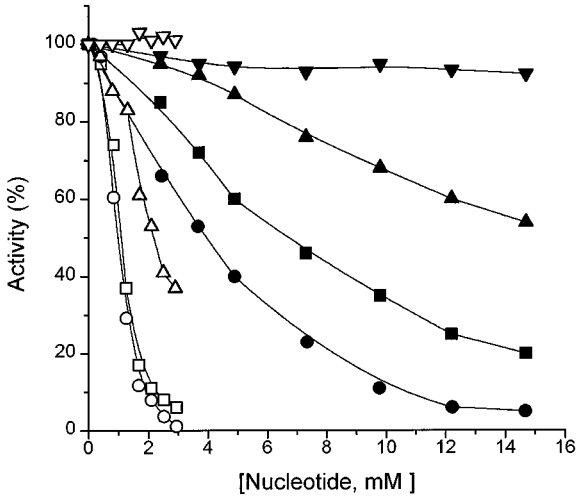

FIG. 5. **Desensitization of Arg^{73} and Tyr^{74} mutants to inhibition by AMP and GMP.** The total glutaminase activity was assayed using 200 mM glutamine. The specific activity was 1.56 units/mg for R73L and 1.46 units/mg for R73H and 1.46 units/mg for the double mutant. The symbols are: ●, wild type (AMP); ○, wild type (GMP); ▲, R73L (AMP); Δ, R73L (GMP); ■, Y74L (AMP); □, Y74L (GMP), ▼, R73L/Y74L (AMP); ▽, R73L/Y74L (GMP).

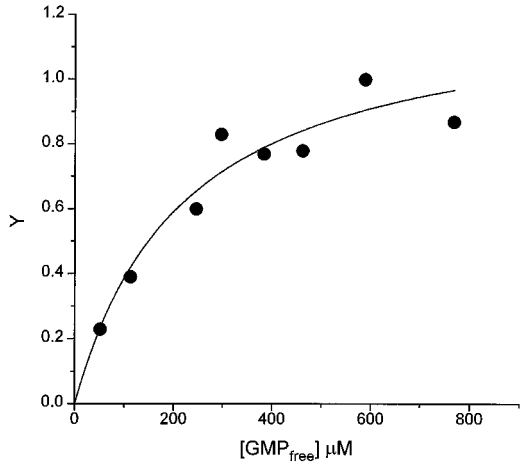

FIG. 6. **Binding of GMP to the R73L/Y74L mutant.** GMP binding was determined by equilibrium dialysis in the presence of 300 mM AMP. Equivalents (*Y*) of GMP are plotted against the free GMP concentration.

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 + PP_i and a putative γ-glutamyl thioester enzyme intermediate. Affinity labeling by DON results in a DON-cysteinyl 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 Arg^{73} for high affinity binding of glutamine. Replacements of Arg^{73} and also Asp^{127}, which participates in glutamine binding, led to significantly higher K_m values for glutamine. Once the substrate is precisely positioned by the Arg^{73} interaction, nucleophilic attack by Cys^1 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 nitrogen 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, Tyr^{74} has a role in communicating PRPP binding to the glutamine site. Replacements of Tyr^{74} 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 Arg^{73} may have a role in inhibition in addition to its role in glutamine binding, since a replacement of Arg^{73} augmented the effect of a Tyr^{74} mutation and desensitized the enzyme to nucleotide inhibition. Tyr^{74} and Arg^{73} 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. Boehlein *et al*. (1994) have analyzed replacements of Arg^{30} and Asn^{74} in *E. coli* asparagine synthetase, residues that correspond to Arg^{26} and Asn^{101} in *E. coli* glutamine PRPP amidotransferase. A number of roles were suggested for this Arg residue including binding the glutamine substrate, mediating communication between separate half-reactions involved in asparagine formation, and controlling nitrogen release from glutamine. Suggested roles for Asn^{74} included stabilization of an oxyanion intermediate and stabilization of a hypothetical hydroxyimine tatuomeric species of glutamine substrate. It is likely that asparagine synthetase Arg^{30} and Asn^{74} 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 nucleophilic 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 oxyanion 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 Asn^{101} and Gly^{102} mutants show that these residues likely constitute the oxyanion 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 Gly^{235}, Arg^{211}, and Asp^{214} with aspartate in aspartylglucosaminidase correspond to Gly^{102}, Arg^{73}, and Asp^{127} 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.

Acknowledgments—We thank Mary Bower and the Purdue Laboratory for Macromolecular Structure for synthesis of oligonucleotides and protein sequencing and Karl Wood and Connie Bonham for mass spectrometry.

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