Interdomain Signaling in Glutamine Phosphoribosylpyrophosphate Amidotransferase

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The glutamine phosphoribosylpyrophosphate (PRPP) amidotransferase-catalyzed synthesis of phosphoribosylamine from PRPP and glutamine is the sum of two half-reactions at separated catalytic sites in different domains. Binding of PRPP to a C-terminal phosphoribosyltransferase domain is required to activate the reaction at the N-terminal glutaminase domain. Interdomain signaling was monitored by intrinsic tryptophan fluorescence and by measurements of glutamine binding and glutamine site catalysis. Enzymes were engineered to contain a single tryptophan fluorescence reporter in key positions in the glutaminase domain. Trp63 in the glutamine loop (residues 73–84) and Trp482 in the C-terminal helix (residues 471–492) reported fluorescence changes in the glutaminase domain upon binding of PRPP and glutamine. The fluorescence changes were perturbed by Ile335 and Tyr74 mutations that disrupt interdomain signaling. Fluorescence titrations of PRPP and glutamine binding indicated that signaling defects increased the $K_d$ for glutamine but had little or no effect on PRPP binding. It was concluded that the contact between Ile335 in the phosphoribosyltransferase domain and Tyr74 in the glutamine site is a primary molecular interaction for interdomain signaling. Analysis of enzymes with mutations in the glutaminase domain C-terminal helix and a 404–420 peptide point to additional signaling interactions that activate the glutamine site when PRPP binds.

Glutamine PRPP amidotransferase, an N-terminal nucleophile type glutamine amidotransferase (1), catalyzes the first step in purine nucleotide synthesis, shown by Equation 1. This reaction takes place in two steps (Equations 2 and 3).

\[
\text{Gln} + \text{PRPP} + \text{H}_2\text{O} \rightarrow \text{PRA} + \text{PP} + \text{Glu} \quad \text{(Eq. 1)}
\]

\[
\text{Gln} + \text{H}_2\text{O} \rightarrow \text{Glu} + \text{NH}_3 \quad \text{(Eq. 2)}
\]

\[
\text{NH}_3 + \text{PRPP} \rightarrow \text{PRA} + \text{PP} \quad \text{(Eq. 3)}
\]

X-ray structures have defined the structure-function relationship (2, 3). An N-terminal glutaminase domain catalyzes the first half-reaction (Equation 2) and a C-terminal PRTase domain the second step (Equation 3). These catalytic sites are separated by 16 Å. Binding of PRPP to the PRTase catalytic site activates the glutaminase step (4, 5), a feature that prevents the wasteful hydrolysis of glutamine independent of PRA synthesis. We have investigated two key questions regarding the enzyme mechanism. First, how is the PRPP-binding signal communicated to the glutamine site over a distance of 16 Å? Second, how is NH$_3$ produced at the glutamine site sequestered from solvent and delivered to the PRTase domain for nucleophilic attack on PRPP to produce PRA and PP? The substrate for the second half-reaction, shown by Equation 3, is NH$_3$, not NH$_3^+$ (4). X-ray crystal structures of an *Escherichia coli* ligand-free enzyme (2) and an enzyme-substrate analog ternary complex (3) have provided the framework to investigate these questions. The ligand-free and ternary complex structures have been referred to as state I and state III conformers, respectively (6). The structure of the state I enzyme is incompatible with catalysis. The unfavorable properties include: (i) the PRPP site is exposed to solvent; (ii) an important arginine residue (Arg73) needed for glutamine binding is unfavorably positioned; and (iii) sites for glutamine hydrolysis and reaction of NH$_3$ with PRPP are separated by a 16 Å solvent-exposed space. These barriers to catalysis are corrected in the structure of the state III enzyme-substrate ternary complex. A PRTase “flexible loop” (residues 326–350) has closed over the bound PRPP, thus protecting it from hydrolysis. Arg73 is optimally positioned for binding glutamine and the glutamine, and PRTase sites are connected by a 20 Å NH$_3$ channel. However, the x-ray structures of the two enzyme conformers do not indicate how glutamine binds because in each conformer the glutamine site is closed thus restricting entry to the site. Thus, glutamine must initially bind to a different enzyme conformer. Recently, we have engineered enzymes containing a single tryptophan fluorescence reporter in positions that change conformation upon formation of the enzyme-substrate ternary complex (6). Measurements of steady state and pre-steady state intrinsic tryptophan fluorescence have identified an intermediate state II enzyme-PRPP conformer. This conformer can catalyze the reaction of external NH$_3$ with PRPP and can also bind glutamine with high affinity, in contrast to state I enzyme, which does not exhibit high affinity glutamine binding. Thus, PRPP binding transmits an interdomain signal to the glutamine site. From the differences seen in the x-ray structures of the inactive (state I) and fully active (state III) conformers, a structure-based model was developed to explain the interdomain signaling from the PRTase domain to the glutamine site (7, 8). Briefly, PRPP binding initiates several large and small conformational changes, which include closure of the 30-residue flexible loop over the bound substrate, a 4° change in the hinge between glutaminase and PRTase domains, and a 7° rotation and 4 Å translation of the neighboring subunit at a molecular 2-fold axis adjacent to the PRPP site. Together these changes lead to reorganization of two structures in the glutaminase domain: a “glutamine loop” (residues 73–84) that includes the...
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Enzyme activities and $K_m$ were determined for the wild type and enzymes having the single mutation indicated. $K_m$ values were determined by titrations using enzymes with C1S/W290F/S83W and C1S/W290F/R482W mutations. The C1S mutation prevented turnover of the enzyme. PRPP-glutamine complex, whereas the W290F mutation removed the only tryptophan helix in the glutaminase domain. A parental enzyme with C1S/W290F/S345W mutation was overproduced in E. coli strain B834 (10) and purified to homogeneity exactly as described previously (6). Glycerol (final concentration, 15%) was added to the enzyme in 50 mM Tris·HCl, pH 7.5, prior to storage at −20 °C. Two series of wild type and mutant enzymes were constructed for assay of ligand binding using intrinsic tryptophan fluorescence. These enzymes were engineered to contain a single tryptophan residue for assay of ligand binding using intrinsic tryptophan fluorescence. The first glutamine PRPP amidotransferase half-reaction, hydrolysis of glutamine (Equation 2), is dependent upon binding of PRPP to the PRTase domain (4, 5). PRPP binding lowers the $K_m$ for glutamine by 100-fold. Two factors limit glutamine binding to the ligand-free enzyme. First, a conformational change is needed to open the glutamine site to permit access of the substrate. Second, Arg73, which forms a salt bridge with the $\epsilon$-carboxyl group of glutamine (3) and is critical for glutamine binding and a C-terminal helix (residues 471–492) that appears to block access to the glutaminase site in both state I and state III enzymes. Contacts between the flexible loop and glutamine loop were proposed to trigger the changes in the glutaminase domain. Here we report the construction and analyses of mutant enzymes to evaluate the structure-based mechanism for interdomain signaling. New tryptophan reporters in the glutaminase domain detect the interdomain signaling upon PRPP binding that was not detected previously (6).

EXPERIMENTAL PROCEDURES

Enzyme Production and Purification—Wild type E. coli glutamine PRPP amidotransferase was produced from plasmid pETpurF (6). Mutations were constructed by the method of Kunel et al. (9) using pETpurF phagemid DNA. E. coli gltA-immunocytolysate, used for the Gln-PRA and NH3-PRA assays, was produced from plasmid pETEG (6) and purified as described. Glutamine PRPP amidotransferase was overproduced in E. coli strain B834 (10) and purified to homogeneity exactly as described previously (6). Glyceral (final concentration, 15%) was added to the enzyme in 50 mM Tris·HCl, pH 7.5, prior to storage at −20 °C. The specific activity of the wild type enzyme was approximately 60 units/mg protein determined by the glutaminase assay. Protein concentration was determined spectrophotometrically at 278 nm using an extinction coefficient of 8.12 for a 1% solution (4). The activity is dependent upon binding of PRPP to the PRTase domain (4, 5). PRPP binding lowers the $K_m$ for glutamine by 100-fold. Two factors limit glutamine binding to the ligand-free enzyme. First, a conformational change is needed to open the glutamine site to permit access of the substrate. Second, Arg73, which forms a salt bridge with the $\epsilon$-carboxyl group of glutamine (3) and is critical for glutamine binding and a C-terminal helix (residues 471–492) that appears to block access to the glutaminase site in both state I and state III enzymes. Contacts between the flexible loop and glutamine loop were proposed to trigger the changes in the glutaminase domain. Here we report the construction and analyses of mutant enzymes to evaluate the structure-based mechanism for interdomain signaling. New tryptophan reporters in the glutaminase domain detect the interdomain signaling upon PRPP binding that was not detected previously (6).

RESULTS AND DISCUSSION

Tryptophan Fluorescence Monitors Conformational Changes in the Glutaminase Domain—The first glutamine PRPP amidotransferase half-reaction, hydrolysis of glutamine (Equation 2), is dependent upon binding of PRPP to the PRTase domain (4, 5). PRPP binding lowers the $K_m$ for glutamine by 100-fold. Two factors limit glutamine binding to the ligand-free enzyme. First, a conformational change is needed to open the glutamine site to permit access of the substrate. Second, Arg73, which forms a salt bridge with the $\epsilon$-carboxyl group of glutamine (3) and is critical for glutamine binding and a C-terminal helix (residues 471–492) that appears to block access to the glutaminase site in both state I and state III enzymes. Contacts between the flexible loop and glutamine loop were proposed to trigger the changes in the glutaminase domain. Here we report the construction and analyses of mutant enzymes to evaluate the structure-based mechanism for interdomain signaling. New tryptophan reporters in the glutaminase domain detect the interdomain signaling upon PRPP binding that was not detected previously (6).

Enzyme Assays—Two assays were used to measure the overall reaction shown by Equation 1. Glutamate and PRA production were determined as described (6). The production of glutamate is referred to as a glutaminase half-reaction, hydrolysis of glutamine (Equation 2), and is dependent upon binding of PRPP to the PRTase domain (4, 5). PRPP binding lowers the $K_m$ for glutamine by 100-fold. Two factors limit glutamine binding to the ligand-free enzyme. First, a conformational change is needed to open the glutamine site to permit access of the substrate. Second, Arg73, which forms a salt bridge with the $\epsilon$-carboxyl group of glutamine (3) and is critical for glutamine binding and a C-terminal helix (residues 471–492) that appears to block access to the glutaminase site in both state I and state III enzymes. Contacts between the flexible loop and glutamine loop were proposed to trigger the changes in the glutaminase domain. Here we report the construction and analyses of mutant enzymes to evaluate the structure-based mechanism for interdomain signaling. New tryptophan reporters in the glutaminase domain detect the interdomain signaling upon PRPP binding that was not detected previously (6).

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## Table I

| Enzyme     | Position of mutation | Glutaminase $K_m$ | $K_N$ Glu | $K_G$ PRTase | PRPP Gln-PRA | NH3-PRA | Glutaminase units/mg | PRPP units/mg |
|------------|----------------------|------------------|------------|--------------|--------------|--------|----------------------|----------------|
| WT         |                      | 59.5 ± 7.2       | 1.57 ± 0.1 | ND           | ND           | 51.6 ± 6.2 | 152 ± 6.0            |                |
| S83W       | Gln loop             | 54.2 ± 1.8       | 1.15 ± 1.1 | 0.13 ± 0.1   | 11.9 ± 0.8   | 47.2 ± 6.3 | 125 ± 5.0            |                |
| R482W      | C-terminal helix     | 50.9 ± 0.8       | 6.04 ± 0.4 | 0.51 ± 0.05  | 5.68 ± 0.4   | 39.8 ± 2.2 | 174 ± 9.8            |                |

$^a$ WT, wild type.

$^b$ ND, not determined because Trp290 was in the wild type enzyme does not monitor changes in conformation (6). $K_N$ values for Glu and PRPP, determined with a C1S/W290F/S345W parental enzyme having no changes other than C1S in the glutaminase domain, are given in the first line of Table II.
Effect of mutations on enzyme activities and interdomain signaling

Enzyme activities and \( K_m \) were determined for the wild type and enzymes having the single mutation indicated. \( K_m \) was determined using the glutaminase assay. Each of the mutations was incorporated into a C1S/W290FS345W parental enzyme for determination of \( K_m \) values by fluorescence titration.

| Enzyme            | Position of mutation | Glutaminase \( K_m \) Gln | Glutaminase \( K_m \) Gln | \( K_m \) PRPP | Gln-PRA | NH\(_2\)PRA |
|-------------------|----------------------|---------------------------|---------------------------|--------------|--------|----------|
| Wild type/parental| Gln loop             | 59.5 ± 7.2                | 0.25 ± 0.01               | 2.72 ± 0.4   | 51.6 ± 6.2 | 13.2 ± 6.0 |
| R75A              | Gln loop             | 0.85 ± 0.5               | ND                        | ND           | ND     | ND       |
| Y74A              | Gln loop             | 1.66 ± 0.4               | ND                        | ND           | ND     | ND       |
| Y74F              | Gln loop             | 54.1 ± 6.1               | 19.3 ± 2.0                | 7.28 ± 0.99  | 2.90 ± 0.4 | 21.1 ± 0.7 |
| T76A              | Gln loop             | 15.0 ± 2.2               | 3.60 ± 1.1                | 2.19 ± 0.3   | 6.11 ± 1.4 | 21.0 ± 14 |
| I335A             | Flexible loop        | 1.22 ± 0.0               | ND                        | ND           | ND     | ND       |
| I335V             | Flexible loop        | 53.9 ± 8.3               | 38.5 ± 13                 | 15.2 ± 1.3   | 2.58 ± 0.4 | 42.4 ± 1.8 |
| D408A             | 404–420 peptide      | 54.2 ± 5.2               | 6.01 ± 1.2                | 2.82 ± 0.13  | 1.66 ± 0.3 | 23.9 ± 2.3 |
| R482A             | C-terminal helix     | 56.3 ± 4.8               | 5.23 ± 0.2                | 1.07 ± 0.14  | 3.98 ± 0.2 | 49.0 ± 5.2 |
| Δ476–505          | C-terminal helix     | ND                        | ND                        | ND           | 2.22 ± 0.2 | 53.2 ± 7.3 |

\( a \) ND, not determined because the \( K_m \) or \( K_i \) was too high or the activity was too low.

\( b \) Activity determined at 100 \( \mu \)M glutamine.

Fig. 1. Ribbon diagram of the glutamine PRPP amidotransferase active site region. Top panel, active state III ternary complex with 6-diazo-5-oxonorleucine (DON) bound to the glutamine site and carbocyclic PRPP (cPRPP) bound to the PRPP site. Backbone segments of the glutamine loop, C-terminal helix, and the PTase flexible loop that undergo major changes in conformation are highlighted and identified, as are key amino acids. Bottom panel, same view showing the three backbone regions and the positions of the key amino acids in the inactive ligand-free, state I conformer. In both views, amino acid side chains and ligands are shown by ball and stick models. Atoms are red for oxygen, blue for nitrogen, and green for phosphorus.

Incorporation of a bulky tryptophan side chain increased the \( K_m \) value for glutamine 7- and 4-fold in the S83W and R482W enzymes, respectively (Table I). However, given the nearly wild type activity, the intrinsic fluorescence of Trp\(^{383}\) and Trp\(^{382}\) was used to monitor changes in the glutaminase domain in response to substrate binding. Data in Fig. 2 (A and B) show that binding of PRPP quenched the fluorescence of the Trp\(^{383}\) and Trp\(^{382}\) reporters by about 25%. Because the site for PRPP binding is 27 Å from Trp\(^{383}\) and 19 Å from Trp\(^{382}\) (measured from C1 of PRPP to C atom), direct interaction with PRPP cannot account for this fluorescence quenching. Rather, interdomain signaling from the PRPP site likely changed the conformation and altered the microenvironment of the PTase reporters in the glutaminase domain. Binding of glutamine to the state II binary enzyme–PRPP complex to form the state III ternary enzyme-substrate complex further quenched the fluorescence of the tryptophan reporters by about 20%. In the absence of PRPP, glutamine binding was not detected. The fluorescence changes in the Trp\(^{383}\) glutamine loop and Trp\(^{482}\) C-terminal helix reporters were used to calculate \( K_f \) values for PRPP and glutamine, which are given in Table I. The \( K_f \) for PRPP binding was increased about 4-fold in the S83W enzyme and 2-fold in the R482W enzyme relative to a parental enzyme with a Trp\(^{385}\) reporter (compare data in Table I with line 1 in Table II). In addition, the \( K_f \) values for glutamine binding in the S83W and R482W enzymes varied only 2-fold from a parental Trp\(^{385}\) enzyme having only a C1S mutation in the glutaminase domain (compare Table I with line 1 in Table II). Thus, the S83W and R482W Trp reporters in the glutaminase domain detected the binding of PRPP and glutamine without major disruption of substrate binding or catalysis. The resulting fluorescence changes in the glutaminase domain therefore can be used to monitor interdomain signaling and the state II conformation.

Amino Acid Interactions Required for Signaling—From x-ray crystal structures of the state I and state III conformers, it was concluded that interdomain signaling results from closing of the PTase flexible loop, initiated by PRPP binding, and the ensuing contact of the flexible loop with the glutamine loop (7). An interaction of the methyl group of Ile\(^{335}\) in the flexible loop with the aromatic ring of Tyr\(^{74}\) in the glutamine loop seen in the active state III conformer (Fig. 1) may have signaled the repositioning of the glutamine loop and the C-terminal helix. However, from the state I and state III crystal structures it was not possible to assess the individual contribution that binding of each substrate made to the overall change in conformation. To assess the consequences of perturbing this interaction on interdomain signaling, we have constructed and analyzed a series of mutations of Ile\(^{335}\) and Tyr\(^{74}\) as well as other residues that contribute to signaling and to glutamine binding.

Ile\(^{335}\) and Tyr\(^{74}\)—Ile\(^{335}\) and Tyr\(^{74}\) are invariant residues in more than 20 glutamine PRPP amidotransferase sequences. I335A and I335V replacements were constructed to give hydro-
phobic side chains of differing size. As shown in Table II, the enzyme with an I335V replacement retained 80–90% of its capacity to hydrolyze glutamine and to transfer the resulting NH₃ to the PRTase domain for synthesis of PRA. Values for glutamine $K_m$ and $K_d$, given in Table II, provide direct evidence for a signaling defect. As shown in Table II, the $K_d$ for binding of PRPP to the valine mutant was unchanged from the wild type. However, values for $K_d$ and $K_m$ for glutamine were increased 60- and 25-fold, respectively, relative to the wild type. Thus, the binding of glutamine, but not PRPP, was perturbed by the I335V substitution, indicative of the signaling defect.

The Trp83 glutamine loop reporter and the Trp 482 C-terminal helix reporter also detected the glutaminase domain signaling defect because of the I335V mutation. As shown in Fig. 2 (E and F), the Val 335 replacement interfered with the quenching of Trp83 and Trp482 fluorescence by PRPP binding that normally occurred in the parental enzymes containing Ile 335 (Fig. 2, A and B). However, both of the mutant enzymes retained the capacity to bind glutamine and form a ternary complex. Thus, as reported by the microenvironment of the glutaminase domain Trp reporters, PRPP binding to the I335V enzyme led to an altered state II conformer, which, however, still retained the capacity to bind and hydrolyze glutamine.

The NH₃-PRA activity of the I335V enzyme was only approximately 20% that of the wild type activity, although the $K_m$ for NH₃ was unchanged (data not shown). The decreased activity might result from an altered structure of the glutamine loop because of interaction of Tyr74 with Val335 rather than with Ile335 in the wild type. According to the current model, external NH₃ enters the channel via the glutamine site (6). Amino acid replacements in the glutamine domain also perturb NH₃-PRA activity (6).

Replacement of Ile335 with Ala nearly disabled the enzyme. The three measured activities were reduced to 3% or less compared with the wild type enzyme. Although the $K_d$ for PRPP binding was similar to that for the wild type, transmission of the binding signal to the glutamine site was defective, as seen by the nearly 50-fold decrease in glutaminase activity (Table II) and by the fluorescence emission signal of the glutaminase domain Trp reporters (Fig. 2, C and D). In contrast to enzyme with Ile at position 335 there was no decrease in Trp83 or Trp482 fluorescence when glutamine was added to the I335A enzyme-PRPP complex. Similar results were obtained with enzyme containing a Trp345 flexible loop reporter (data not shown), indicating that the $K_d$ was too high to detect glutamine binding under these conditions. Thus, the glutamine site Trp reporters as well as the flexible loop Trp reporter sensed PRPP binding, but the normal state II conformation was not achieved, and glutamine could not bind to the defective binary complex.

The reduction in NH₃-PRA activity in the I335A enzyme (Table II) suggests a structural defect in the NH₃ channel resulting from replacement of the large hydrophobic side chain. Because the channel lacks a scaffold of hydrogen bonds, amino acid replacements with large change in volume, such as I335A, may result in a substantially different channel structure. Inhibition of NH₃-PRA activity in the I335A enzyme may even be due to a complete blockade of NH₃ entry to the channel.

The role of Tyr74 was probed by mutations to Phe and to Ala. The Y74F mutant was characterized by increases in $K_d$ and $K_m$ for glutamine of 29- and 12-fold, respectively, whereas the $K_d$ for PRPP binding was unchanged from the wild type enzyme (Table II). These data reflect a signaling defect. The comparable reduction in Gln-PRA and NH₃-PRA activity in the Y74F

FIG. 2. Fluorescence emission spectra of enzymes having a single tryptophan fluorescence probe in the glutaminase domain. All enzymes contained C1S and W290F mutations in addition to those listed. The C1S mutation prevented enzyme turnover.
enzyme suggests that in addition to a role in signaling, Tyr74 is needed for the structure of the NH₃ channel. The signaling defects in the Y74A mutant were exacerbated compared with the Phe replacement as shown by the low glutaminase activity, undetectable Gln-PRA activity, and the inability to detect glutamine binding by fluorescence titration. Binding of PRPP to the Y74A enzyme was unchanged from the wild type. Decreased NH₃-PRA activity could be due to either a problem of NH₃ entry at the glutamine site or a defect in the NH₃ channel. 

Arg73 and Thr76—According to the crystal structures of the state I and state III enzyme conformers, one role of interdomain signaling is to reposition residues, especially Arg73, to achieve high affinity glutamine binding. In contrast, residues required to catalyze glutamine hydrolysis (Cys1, Asn101, and Gly102) are positioned identically in state I and state III. According to this conclusion, mutations in residues required for glutamine binding should have a similar phenotype to signaling mutants. To test this conclusion Arg73 and Thr76 mutants were constructed. Arg73 and Thr76 are conserved residues in the glutamine loop that interact with the glutamine α-carboxylate (5, 7). Data in Table II show that although PRPP binding was not perturbed in an R73A enzyme, the $K_d$ for glutamine binding was too large to detect. As a consequence there was a large decrease in the glutaminase activity of the R73A enzyme. Although the Gln-PRA activity was too low to detect, synthesis of PRA from external NH₃ was 60% that of the wild type enzyme, indicative of a functional NH₃ channel. Similar perturbations in $K_m$ and $V_m$ values for glutaminase and Gln-PRA activities were obtained earlier for R73H and R73L enzymes (5).

Thr76 Oy also forms a hydrogen bond with the α-carboxyl of the glutamine substrate, although its position is not dependent upon the Ile335-Tyr74 interaction (5, 8). The T76A enzyme has increased $K_d$ (14-fold) and $K_m$ (9-fold) for glutamine, similar to the Y74F enzyme, supporting roles of both Thr76 and Tyr74 in glutamine binding. However, the lower glutaminase and Gln-PRA activities of T76A relative to the Y74F enzyme suggest that the direct interaction of Thr76 with glutamine is important to catalysis. Replacement of Thr76 with Ala (Table II) or Ser (data not shown) resulted in modestly higher NH₃-PRA activity relative to the wild type. There was no change in $K_m$ for NH₃ (not shown). The increase in NH₃-PRA activity, although small, was reproducible and supports the earlier suggestion (6) that small amino acid side chains in the glutamine loop facilitate access of external NH₃ to the NH₃ channel.

Asp408 and Arg482 and the C-terminal Helix—Amino acid residues in a 404–420 peptide sequence and in the C-terminal helix (positions 471–492) contribute to closing of the glutamine site in the state I ligand-free enzyme and in the state III enzyme-substrate ternary complex (2, 8). Structural changes resulting from PRPP and glutamine binding that were monitored by tryptophan fluorescence may have roles in opening and closing of the site to control access of glutamine and exit of glutamate. To assess functional interactions, we have mutated residues in the 404–420 peptide and the C-terminal helix that may participate in this aspect of the signaling between the PRPP and the glutamine sites. In the active state III but not in the inactive state I conformer, the backbone N of Asp408 forms a hydrogen bond with the ring OH of Tyr74 (Fig. 3). In addition, the Asp408 carboxyl group interacts with Ne and NH₃ of Arg482 and with the Lys487 side chain in the C-terminal helix. Arg482, in turn, hydrogen bonds with the backbone carbonyl groups of Gly78 and Pro75 in the glutamine loop, thus providing an additional link between the primary Ile335-Tyr74 interaction and the glutamine site (Fig. 3).

Although Asp408 does not interact directly with the glutamine substrate, the primary defect in the D408A enzyme was a decreased binding affinity for glutamine. The glutamine $K_d$ was increased 11-fold in the D408A enzyme. However, upon saturation with glutamine the rate for glutaminase was similar to that of the wild type enzyme. The phenotype of the D408A enzyme is most like that of Y74F, suggesting that the hydrogen bond between the backbone N of Asp408 and OH of Tyr74 is important for glutamine binding and that this interaction does not occur in the D408A enzyme. Interestingly, the glutamine binding affinity was only slightly perturbed in the R482A (Table II) and R482W (Table I) enzymes. Thus, the interactions of Arg482 with Asp408 and with the glutamine loop residues are
not essential for communicating the PRPP binding signal to the glutamine site. On the other hand, a deletion of the C-terminal helix (Δ476–505 enzyme) totally abolished glutamine binding and glutaminase and Gln-PRA activities, whereas about one-third of the NH₃-PRA activity was retained. This result points to an essential role for the C-terminal helix in the function of the glutamine site but not in NH₃ channel function. In the transition from state I to state III, two hydrogen bonds are lost and five are gained from side chains in the C-terminal helix to residues elsewhere in the structure. It is possible that some of the other residues forming these hydrogen bonds (Asp484, residues elsewhere in the structure. It is possible that some of the other residues forming these hydrogen bonds (Asp484, Asp485, Lys487, and Arg491) are important to the essential function of the C-terminal helix.

Ile335-Tyr74 Is the Key Interaction Required for Interdomain Signaling—Two glutamine PRPP amidotransferase half-reactions take place at physically separated sites in glutaminase and PRTase domains. In the state I ligand-free enzyme the two catalytic sites are disconnected by a 16 Å solvent exposed space, the flexible loop is open and poorly ordered, the PRPP site is solvent accessible, and the glutamine site is closed. PRPP binding triggers a series of conformational changes, which make the resulting state II enzyme competent to utilize external NH₃ for synthesis of PRA and to bind glutamine. In this work the state II conformer was detected by changes in fluorescence of tryptophan residues in two glutamine site positions and by activation of the glutamine site upon binding of PRPP to the PRTase domain. Activation was assessed by the $K_d$ for glutamine binding and by glutamine $K_m$, glutaminase, and Gln-PRA activities.

According to the current structure-based model (7, 8), the state I to state II transition requires several steps. First, binding of PRPP initiates closing of the PRTase flexible loop over the PRPP site and formation of the NH₃ channel to deliver external NH₃, entering via the glutamine site, for reaction with PRPP (6). These steps were monitored previously by changes in fluorescence emission of the Trp345 flexible loop reporter. Next, an interaction between the closed flexible loop and the glutamine loop must reposition Arg73 for high affinity glutamine binding and interactions between the glutamine loop, the C-terminal helix, and the 404–420 peptide must open the glutamine site. Although the precise order and structure of these changes has not been determined and is therefore speculative, they result in the state II conformer in which the PRPP binding signal has been communicated to the glutamine site. Additional changes of 4° in the interdomain hinge and 7° in the neighboring subunit orientation occur between state I and state III and may also influence state II. The analyses of mutant enzymes supports the structure-based insights into the molecular interactions for interdomain signaling.

A principal signaling interaction is between the side chains of Ile335 in the flexible loop and Tyr74 in the glutamine loop. Modest changes in either side chain, Ile335 to Val or Tyr74 to Phe, resulted in significant increases in $K_d$ and $K_m$ for glutamine, although the glutaminase $V_{max}$ was not significantly altered. However, replacement of either residue to alanine essentially abolished glutamine binding. It is noteworthy that each of the three tryptophans that were monitored, Trp345, Trp348, and Trp482, detected the same state II structure. That is to say, neither the Trp345 nor the Trp348 glutaminase domain reporters sensed PRPP binding in the Val335 signaling mutant. In the more disruptive Ala335 signaling mutant, fluorescence changes of the Trp345, Trp348, and Trp482 reporters each monitored a defective state II conformer that could not bind glutamine to form the state III ternary complex.

The results of enzyme characterization indicate that, in addition to the signaling contact, Ile335 and Tyr74 have at least one other function. Lower Gln-PRA activity relative to glutaminase activity in the Y74F and I335V enzymes suggests less efficient transfer of NH₃ through the NH₃ channel to the PRPP site. There were also large decreases in NH₃-PRA activity. These changes are consistent with defects in the NH₃ channel. Ile335 and Tyr74 form one wall of the NH₃ channel. Because the NH₃ channel structure is dependent upon hydrophobic amino acid side chains, replacements of these residues could alter or block the channel. Two factors, however, complicate the analysis of channel function. First, because NH₃-PRA activity likely depends on entry from the glutamine site as well as transfer through the channel, changes of NH₃-PRA activity should be viewed in parallel with changes in Gln-PRA activity. Changes in NH₃-PRA activity that exceed changes in Gln-PRA may reflect perturbation of NH₃ entry at the glutamine site. Second, the function of the NH₃ channel was difficult to evaluate in Y74A and I335A mutants because glutamine hydrolysis was so severely inhibited. In the absence of glutamine hydrolysis, the values for Gln-PRA are noninformative. It is therefore difficult to distinguish between defects because of NH₃ entry and those because of channel function to explain low NH₃-PRA activity in the Y74A and I335A mutant enzymes. Analyses of previous replacements of Tyr74 led to the similar conclusion that Tyr74 is a key residue in the coupling between sites for glutamine and PRPP in the two domains (5), although the molecular interactions were not recognized because the crystal structure for the active state III enzyme ternary complex was not yet available.

In addition to the enzymes with Tyr74 and Ile335 replacements, the 2-fold reduction in the ratio of glutaminase to GlnPRA activity in T76A and D408A enzymes suggests a perturbation in the channeling of NH₃ derived from glutamine hydrolysis to the PRPP site. However, the high NH₃-PRA activity in the T76A enzyme indicates normal channel function for delivery of external NH₃ to the PRPP site. A further analysis of channel function is in progress and will be reported elsewhere. In another class of mutants (R73A and the C-terminal helix deletion Δ476–505), a functioning NH₃ channel formed upon PRPP binding, but glutamine binding was abolished. These mutants and others discussed above show that channel formation, signaling, and glutamine binding are separable events, although several residues contribute to more than one event.

Signaling in Other Enzymes—Signaling from a second domain or subunit to a glutamine site to activate glutamine hydrolysis is typical in glutamine amidotransferases. For example, binding of ATP and bicarbonate to the large subunit of E. coli carbamoyl phosphate synthetase large subunit stimulates the $k_{cat}$ for glutamine hydrolysis at a site about 45 Å removed in the small enzyme subunit (11). Likewise, binding of chorismate to the anthranilate synthase component I subunit stimulates glutamine hydrolysis by the component II subunit by more than 30-fold (12). In neither of these cases nor in other glutamine amidotransferases has the signaling mechanism been determined. More generally, interdomain or intersubunit signaling may be an essential feature in enzymes with physically separated active sites connected by a tunnel. Intersubunit signaling between tryptophan synthase active sites in α and β subunits has been described in a series of elegant studies recently reviewed by Miles et al. (13). Tryptophan synthase signaling is accompanied by channeling of indole through a largely hydrophobic intersubunit tunnel, with similarities to the channeling of NH₃ through the glutamine PRPP amidotransferase tunnel.

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