

Mutation of the Adenylylated Tyrosine of Glutamine Synthetase Alters Its Catalytic Properties

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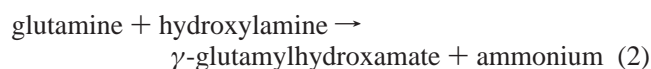
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ABSTRACT: Glutamine synthetase is central to nitrogen metabolism in the Gram-negative bacteria. The amount of glutamine synthetase in the cell and its catalytic activity are tightly regulated by multiple, sophisticated mechanisms. Reversible covalent modification of Tyr-397 is central to the regulation of glutamine synthetase activity, via esterification of the hydroxyl group to AMP in a process termed adenylylation. As expected, site-specific mutation of this surface-exposed Tyr-397 to Phe, Ala, or Ser was found to prevent adenylylation. Unexpectedly, these mutations had major effects on the catalytic characteristics of glutamine synthetase. The specific activities of each mutant were approximately doubled, the pH-activity profiles changed, and divalent-cation specificity was altered. Overall, Tyr397Phe behaved as if it were unadenylylated, while both Tyr397Ala and Tyr397Ser behaved as if they were adenylylated. Thus, subtle modifications in the environment of residue 397 are sufficient to induce changes previously thought to require adenylylation.

Examination of a metabolic map reveals glutamine located at the center of nitrogen metabolism. The key enzyme responsible for its synthesis is glutamine synthetase (EC 6.3.1.2), which catalyzes the ATP-dependent condensation of glutamate with ammonium



This activity is referred to as the biosynthetic reaction. Glutamine synthetase catalyzes a number of other reactions, including the transfer of the glutamyl moiety from glutamine to hydroxylamine (1). This is referred to as the transferase reaction



The bacterial glutamine synthetase molecule is an oligomer, composed of 12 identical subunits, oriented as two hexamers bound to each other. The structure of the dodecamer has been established by the crystallographic studies of Eisenberg and colleagues (2, 3). Each molecule has 12 active sites, but each is constructed by domains contributed by two adjacent subunits. The active site can be envisioned as a “bifunnel”, in which the two conical openings of the funnels are at the top and bottom of each active site (Figure 1A). The narrower stems of the two funnels are in the middle and contain the two divalent cation binding sites, presumably occupied by magnesium under physiological conditions. ATP enters the top funnel and binds to its substrate site, facilitated by interaction with one of the divalent cations. Glutamate enters the bottom funnel and binds to facilitate reaction with ATP

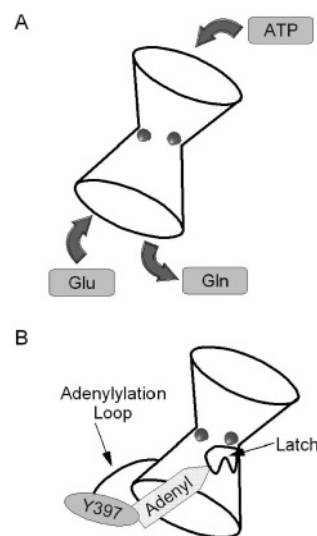


FIGURE 1: Bifunnel model of glutamine synthetase. (A) Substrate and product movements (3). ATP enters the upper funnel, while glutamate enters the lower funnel. After the binding of the third substrate, NH_4^+ , glutamine is synthesized and leaves through the lower funnel. (B) Possible mechanism of action of adenylylation (17). Adenylylation allows the loop containing Tyr-397 to bind to the latch, preventing it from closing during the catalytic cycle.

to produce γ -glutamyl phosphate and ADP. Ammonium attacks the δ -carbon of γ -glutamyl phosphate, releasing phosphate and forming glutamine. Glutamine then leaves the active site through the lower funnel, while ADP exits through the upper funnel.

Glutamine is the preferred nitrogen donor in the biosynthesis of histidine, tryptophan, carbamoyl phosphate, AMP, CTP, NAD, and glucosamine-6-phosphate. Thus, glutamine participates in the biosynthesis of all amino acids, protein, nucleic acids, and many polysaccharides. Glutamine synthetase is present at relatively high levels in organisms from

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Table 1: Comparison of Unadenylylated and Adenylylated Glutamine Synthetase (1)

characteristic	unadenylylated enzyme	adenylylated enzyme
Mg ²⁺ -dependent biosynthetic activity	yes	no
Mn ²⁺ -dependent biosynthetic activity	no	yes
Mg ²⁺ -dependent γ -glutamyltransferase activity	yes	no
Mn ²⁺ -dependent γ -glutamyltransferase activity	yes	yes
pH optimum for Mn ²⁺ -dependent γ -glutamyltransferase activity	8	6.9
sensitivity to feedback inhibition by His, Trp, CTP, AMP	absent	enhanced
K _m for ADP in the γ -glutamyltransferase assay	0.04 μ M	85 μ M
effect of monovalent cations (K ⁺ , Na ⁺ , Li ⁺ , Cs ⁺)	none or stimulated	inhibited
effect of 4.5 M urea	none	inactivated

bacteria to mammals. The enzyme from Gram-negative bacteria has been studied in great detail (1). In *Escherichia coli*, glutamine synthetase constitutes 1–2% of the total soluble protein and catalyzes the formation of glutamine at a rate of about 100 (μ mol/min)/mg protein. Not surprisingly, this high capacity to produce glutamine is highly regulated, being exquisitely matched to current cellular requirements. The level and activity of glutamine synthetase are controlled by multiple mechanisms including transcriptional control, cumulative feedback inhibition by at least 9 metabolites, regulation of susceptibility to proteolytic degradation, and reversible covalent modification of Tyr-397. The covalent modification of Tyr-397 involves formation of a phosphoester bond, although not via simple phosphorylation of the hydroxyl group. Rather, the tyrosine is modified by adenylation in which an AMP group is attached. Adenylation and deadenylation are controlled by a novel bicyclic cascade system whose details were elucidated by a number of investigators, especially Stadtman and collaborators (4).

The adenylylated and deadenylylated enzymes differ in many characteristics (Table 1). Notably, adenylation causes loss of biosynthetic activity with the physiological cation, magnesium. Consistent with this point, cells growing or maintained in media with glutamine have adenylylated glutamine synthetase. When glutamine is depleted, the glutamine synthetase is deadenylylated, allowing for production of glutamine to meet cellular requirements. Because the enzyme is a dodecamer, the number of adenylyl groups can range from 0 to 12, and this graded response allows the cell to closely match the rate of glutamine production with consumption. Glutamine synthetase with an average of five adenylyl groups per dodecamer is termed GS₅, unadenylylated enzyme is GS₀, and the fully adenylylated glutamine synthetase is GS₁₂.

When one wishes to study certain aspects of the glutamine synthetase molecule, this heterogeneity of adenylation status of subunits is a complicating factor. In the course of our investigation of the role of methionine residues in glutamine synthetase (5), we decided to obviate the problem of heterogeneity by constructing a site-specific mutant with Tyr-397 changed to Phe, which could not be adenylylated. Tyr-397 is surface-exposed in the available crystal structures, as expected from its required accessibility to the adenylyltransferase which mediates the attachment and removal of the adenylyl group. Given that this surface-exposed residue is located on a loop outside the bottom entrance to the bifunnel and that Tyr to Phe is a conservative alteration, no significant functional changes were expected. We were thus surprised to find that the catalytic characteristics of Tyr397Phe were quite different than those of the wild-type, unadenylylated

enzyme. We therefore undertook a more detailed investigation of the effect of amino acid substitutions at Tyr-397.

EXPERIMENTAL PROCEDURES

Expression and Purification of Wild-Type and Site-Specific Mutant Glutamine Synthetases. Unadenylylated, wild-type glutamine synthetase was obtained from *E. coli* YMC10 *pglu6*, which overproduces the enzyme (6). To prepare the adenylylated, wild-type glutamine synthetase and Tyr-397 mutants, we first obtained the glutamine synthetase coding region by PCR using 5' (5'-ggaattccatgatgccgctgaacacgtact-3') and 3' (5'-gttccgctcgagttgacgctgtagtacagctcaaacctac-3') primers. An *Nde*I and *Xho*I site was incorporated at the 5' and 3' ends, respectively. The gene was inserted into the *Nde*I and *Xho*I sites of a pET17b vector (Novagen, Madison, WI) to create the pETglnA plasmid for glutamine synthetase overexpression. The construct was sequenced to confirm that the desired sequence was obtained. Three site-specific Tyr-397 mutants (Tyr397Phe, Tyr397Ala, and Tyr397Ser) were constructed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Plasmids were transformed into BL21(DE3)pLysS competent cells (Stratagene) for overexpression of glutamine synthetase. The masses of the expressed proteins were confirmed to be within 1 mass unit of the calculated value by electrospray mass spectrometry (Agilent 1100 HPLC-MSD, Palo Alto, CA). Mass spectrometry also confirmed that the wild-type glutamine synthetase was highly adenylylated, while all three mutants were completely unadenylylated. The enzymes were purified by the zinc aggregation procedure as previously described (7). They were stored in 10 mM imidazole, 100 mM KCl, and 1 mM MnCl₂, pH 7.0, at 4 °C. Protein concentration was determined spectrophotometrically (1).

Activity Assays. For all activity assays, at least three replicates were run, and in the figures, activities are plotted as the mean with error bars showing 1 standard deviation. The pH 7.57 triethanolamine–dimethylglutarate buffer system was employed to determine γ -glutamyltransferase activity, the average state of adenylation, and pH profiles (8). The pH of the assay mixtures was determined at 23 °C, and enzyme assays were run at 37 °C. The effect of feedback inhibitors was measured with the 20 mM imidazole, pH 7.0, γ -glutamyltransferase assay (9) with hydroxylamine and glutamine at 20 mM (10). Cumulative inhibition was calculated as described (11).

The glutamine biosynthetic assay conditions were as described (12), although glutamine was determined directly by amino acid analysis after precolumn derivatization with *o*-phthalaldehyde (13). The assay solution was pH 7.5–7.6, 111 mM imidazole, 100 mM KCl, 56 mM MgCl₂, 56

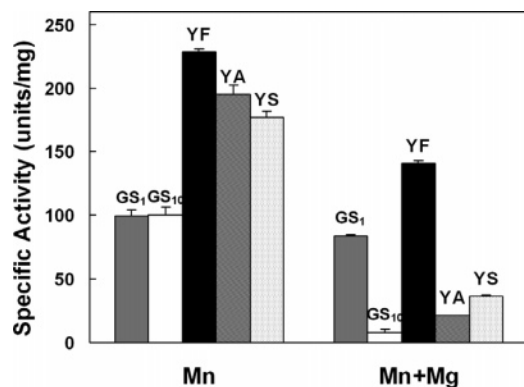


FIGURE 2: γ -glutamyltransferase activity of the glutamine synthetases. Activity in the presence of 400 μ M MnCl_2 alone or with 60 mM MgCl_2 was determined as described in Experimental Procedures. The wild-type enzymes GS_1 and GS_{10} had average states of adenylation of 1 and 10, respectively. The mutants are labeled YF for Tyr397Phe, YA for Tyr397Ala, and YS for Tyr397Ser.

mM NH_4Cl , 56 mM L-glutamate, and 100 mM ATP. For the assay, 180 μ L assay mix was placed in a 37 $^\circ\text{C}$ water bath for 5 min and the reaction then started by addition of 20 μ L glutamine synthetase (0.1–1.0 μ g). The reaction was stopped after 15 min by addition of 20 μ L 10% trifluoroacetic acid, which ensures that the pH is between 2 and 4. The solution was diluted 20-fold with water, and 5 μ L was used for amino acid analysis.

RESULTS

γ -Glutamyltransferase Activity. Both the unadenylylated and adenylylated enzymes have manganese-dependent γ -glutamyltransferase activity, although the pH optimum varies with the state of adenylation. The optimum shifts from about 8.0 to 6.9 as the state of adenylation increases from 0 to 12 (14). An isoactivity point occurs at pH 7.57, facilitating activity determination which is independent of the state of adenylation (8). As shown in Figure 2, wild-type GS_1 and GS_{10} have the same specific activity, as expected. Surprisingly, Tyr397Phe has a specific activity that is almost double that of the wild-type enzyme. While tyrosine and phenylalanine differ only in the hydroxyl group on tyrosine, this difference renders phenylalanine considerably more hydrophobic than tyrosine (15). We therefore constructed Tyr397Ala because the hydrophobicities of tyrosine and alanine are similar, and we made Tyr397Ser because serine is also a hydroxy-amino acid. All three mutants have increased specific activity compared to the wild-type.

Divalent Cation Specificity. The γ -glutamyltransferase activity of wild-type, unadenylylated enzyme is supported by either Mn^{2+} or Mg^{2+} , while the adenylylated enzyme is active only with Mn^{2+} in the absence of Mg^{2+} (1). This metal-dependence was observed in our wild-type preparations, as expected (Figure 2). The Tyr397Phe mutant behaves as the unadenylylated form, consistent with its inability to be adenylylated. However, the Tyr397Ala and Tyr397Ser behave as if they were adenylylated.

pH Optimum. As mentioned above, the pH optimum for the γ -glutamyltransferase activity shifts from about 8.0 to 6.9 as the state of adenylation increases from 0 to 12 (14). Given the increased specific activity of the Tyr-397 mutants

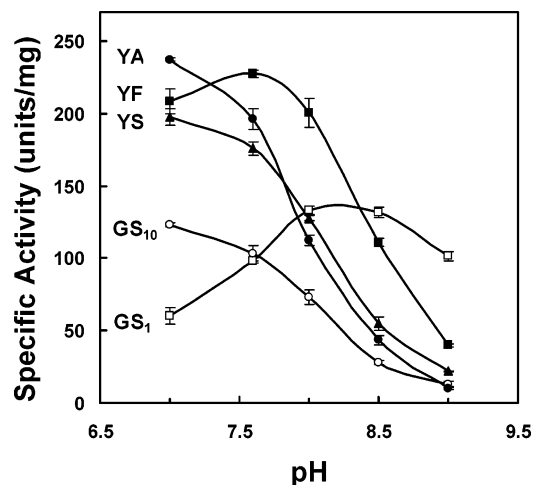


FIGURE 3: pH activity profiles. The abbreviations are GS_1 and GS_{10} , the wild-type enzymes, and YF, Tyr397Phe; YA, Tyr397Ala; and YS, Tyr397Ser.

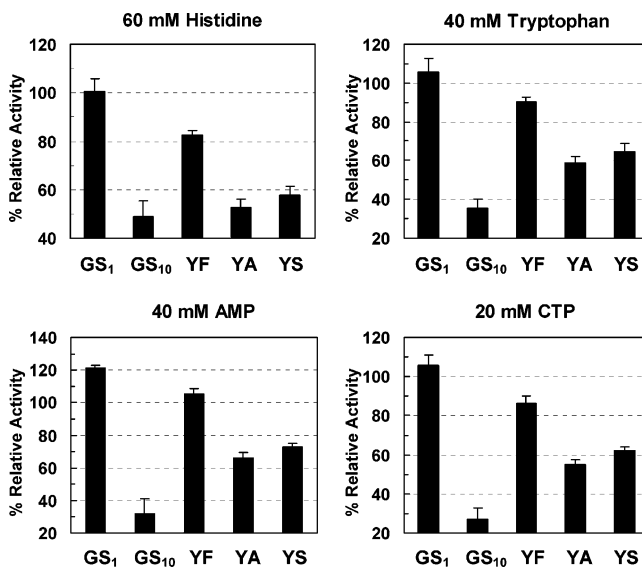


FIGURE 4: Effect of feedback inhibitors on γ -glutamyltransferase activity. The specific activity of the enzymes without additions was set to 100%. The abbreviations are GS_1 and GS_{10} , the wild-type enzymes, and YF, Tyr397Phe; YA, Tyr397Ala; and YS, Tyr397Ser.

and their varying metal specificities, we investigated the dependence of their activity on pH. As shown in Figure 3, the pH curves for wild-type GS_1 and GS_{10} match those in the literature, including an isoactivity point at pH 7.57 (8). The unadenylylated enzyme has a pH optimum at ~ 8.0 , while the activity of the adenylylated form increases with decreasing pH within the range studied. The shape of the pH-activity curves for Tyr397Ala and Tyr397Ser is similar to that of the adenylylated wild-type, while that of Tyr397Phe is similar to that of the unadenylylated enzyme but with the pH optimum shifted to a slightly lower pH. The specific activity of each mutant is ≥ 200 units/mg, a value much higher than that observed with either form of wild-type glutamine synthetase.

Feedback Inhibition. Adenylation renders glutamine synthetase sensitive to feedback inhibition by histidine, tryptophan, AMP, and CTP (1), as seen in Figure 4. The Tyr397Phe is modestly inhibited, while both Tyr397Ala and Tyr397Ser are more sensitive as observed for wild-type, adenylylated enzyme. Glutamine synthetase has been called

Table 2: Cumulative Feedback Inhibition^a

additions	GS ₁		GS ₁₀		Tyr397Phe		Tyr397Ala		Tyr397Ser	
	obs	calc	obs	calc	obs	calc	obs	calc	obs	calc
His	0		27		2		22		14	
Trp	-1		34		3		13		12	
AMP	-8		65		-5		13		10	
CTP	-6		26		5		5		3	
His, Trp	3	0	49	51	10	5	29	32	27	25
His, Trp, AMP	-4	-9	76	83	6	1	43	41	35	32
His, Trp, AMP, CTP	-16	-15	84	87	12	6	51	44	44	34

^a The table shows the percent inhibition observed for the individual metabolites and for mixtures. The concentrations added were His 15 mM, Trp 10 mM, AMP 10 mM, and CTP 5 mM. "Obs" is the observed inhibition, while "calc" is that calculated for the mixtures from the individual effects (11). Negative values indicate stimulation of activity.

a "molecular computer"¹ because of its ability to monitor and integrate the levels of many metabolites which require glutamine for their synthesis. This property is termed "cumulative feedback inhibition" and refers to the fact that the inhibition obtained by a combination of inhibitors is approximately the sum of their individual effects (16). Table 2 presents the results for cumulative feedback inhibition of the wild-type and mutant enzymes. The unadenylylated, wild-type enzyme was barely affected by the presence of all four metabolites, while the adenylylated (GS₁₀) form was strongly inhibited. Tyr397Phe behaved as if it were unadenylylated, being little affected. However, Tyr397Ala and Tyr397Ser exhibited a loss of about half their activity, a behavior closer to that of the adenylylated wild-type.

Glutamine Synthesis. The preceding studies described the behavior of the enzymes in the γ -glutamyltransferase reaction. The physiological role of glutamine synthetase is the synthesis of glutamine, and the unadenylylated, wild-type enzyme catalyzes this biosynthetic reaction with Mg²⁺ but not with Mn²⁺ (1). Thus, determination of the Mg²⁺-dependent biosynthetic activity specificity of the Tyr-397 mutants provides another method for characterizing the effect of side-chain alterations. Figure 5A shows that Tyr397Phe has substantial biosynthetic activity, similar to that of the unadenylylated, wild-type enzyme. In contrast, Tyr397Ala and Tyr397Ser are similar to the adenylylated enzyme, with little Mg²⁺-dependent biosynthetic activity. Because the biosynthetic activity decreases as a linear function of the fraction of adenylylated subunits, we can characterize Tyr397Phe as similar to GS₃, while Tyr397Ser and Tyr397Ala are similar to GS₉ and GS₁₀ (Figure 5B).

DISCUSSION

The studies of Stadtman and colleagues on regulation of glutamine synthetase led to the recognition that covalent, interconvertible, enzyme cascades provide the cell with a regulatory mechanism capable of integrating multiple metabolic inputs and also possessing a remarkable potential for amplification of signals (4). Reversible adenylylation of Tyr-397 is central to this regulatory mechanism. Adenylylation alters divalent cation specificity, pH optimum, susceptibility

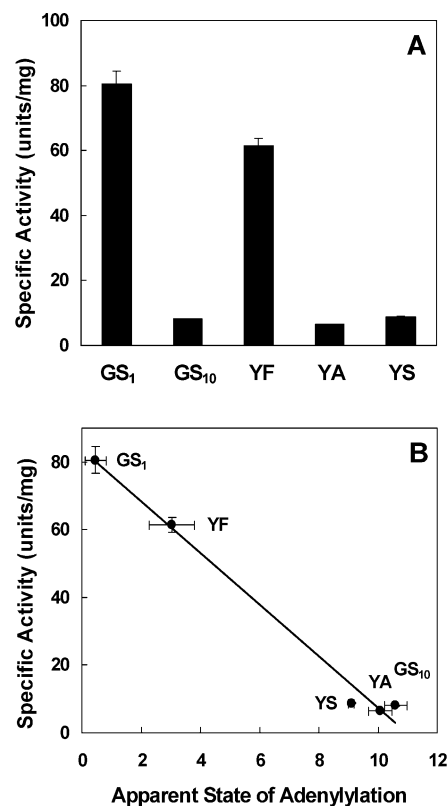


FIGURE 5: Biosynthetic activity (A) and correlation with the apparent state of adenylylation (B). The Mg²⁺-dependent biosynthetic activity was measured in the presence of 50 mM MgCl₂, while the apparent state of adenylylation was determined with the γ -glutamyltransferase assay, both as described in Experimental Procedures. The abbreviations are GS₁ and GS₁₀, the wild-type enzymes, and YF, Tyr397Phe; YA, Tyr397Ala; and YS, Tyr397Ser.

to feedback inhibitors, and catalytic activity. Esterification of the adenylyl group to the hydroxyl group of Tyr-397 substantially increases the molecular size of the residue and adds a negatively charged phosphate group. Site-specific mutation of Tyr-397 to a Phe, Ala, or Ser would prevent adenylylation but was not expected to alter the catalytic properties of glutamine synthetase.

However, mutation of Tyr-397 has impressive effects on the catalytic characteristics of the enzyme. Considering divalent cation specificity, pH dependence, feedback inhibition, and biosynthetic activity, conversion of tyrosine to either alanine or serine gave an enzyme with characteristics similar to that of adenylylated, wild-type glutamine synthetase. In contrast, conversion of the tyrosine to a phenylalanine gave an enzyme with characteristics of the unadenylylated wild-type. All three mutants had much higher γ -glutamyltransferase activities than the wild-type enzyme.

Several crystal structures of the Gram-negative glutamine synthetase, as well as that from *Mycobacterium tuberculosis*, have been elucidated in Eisenberg's laboratory (3). All are of the unadenylylated form because it has not yet been possible to obtain diffracting crystals of the adenylylated protein. Also, all of the available crystal structures were obtained with the Mn²⁺ form; none have yet been solved for the presumably physiological Mg²⁺ form. Given the documented differences in catalytic behavior with the two cations, there must be structural differences between the Mn²⁺ and Mg²⁺ forms (Table 1 and ref 1). However, consideration of the various unadenylylated, Mn²⁺ structures

¹ Goodsell D. S., "Molecule of the Month: Glutamine Synthetase", Research Collaboratory for Structural Bioinformatics Protein Databank, 2002. (http://www.rcsb.org/pdb/molecules/pdb30_1.html).



FIGURE 6: Tyr-397 moves near the Glu-327 flap in the presence of substrates. (A) In the absence of substrates,² the hydroxyl group of Tyr-397 is 13.5 Å from the α -carbon of Glu-327 and 25.1 Å from the n1 metal site (20). (B) With bound ADP and the glutamate analogue, phosphinothricin,³ the hydroxyl group of Tyr-397 is 3.4 Å from the α -carbon of Glu-327 and 12.5 Å from the n1 metal site (19). The figures were generated with the program ViewerLite (Accelrys, San Diego, CA). For clarity, only one of the active sites from the dodecamer is shown. The adenylation loop is shown in yellow, the Glu-327 flap in red, and the latch in orange. Tyr-397 and Glu-327 are drawn with balls and sticks.

allowed Eisenberg and colleagues to propose a model of the catalytic cycle which includes a number of loop motions (3, 17). One of these mobile loops is termed the latch (residues 50–64); following the binding of ATP, the latch moves closer to the active site where its position is stabilized by interactions established after movement. Tyr-397 is in the adenylation loop (residues 388–411), which is also capable of motion, moving it toward the active site. It is hypothesized that adenylation allows the hydrophobic lip of the latch to bind the adeny group, thus, preventing closure of the latch and blocking completion of the active site (17) (Figure 1B). It is somewhat difficult to reconcile this specific mechanism

with the behavior of our Tyr-397 mutants. Substitution of the tyrosine by alanine or serine produces a protein which cannot be adenylylated but whose catalytic characteristics were similar to those of the adenylylated, wild-type glutamine synthetase. These residues seem unlikely to be able to bind to the latch as hypothesized for the adenosine moiety of the adeny group. Nevertheless, they presumably induce a conformational change in the protein similar, in some ways, to that induced by adenylylation.

Biophysical studies of adenylylated glutamine synthetase, carried out before any crystal structures had been elucidated, established that the adeny group was close to the divalent cation binding site at the catalytic center of the enzyme (18). The phosphoryl group of the covalently bound adeny group was only 6 Å from the metal and was relatively immobilized. This led the authors to speculate that Tyr-397 was not only an anchoring point for attachment of the adeny group but was also important in the activity of the unadenylylated enzyme. Consistent with that proposal, we observed that all three mutants had increased γ -glutamyltransferase activity compared to the wild-type. Compared to the wild-type enzyme, the biosynthetic activity of Tyr397Ala and Tyr397Ser was reduced by 90%, while that of Tyr397Phe was only decreased by 25%. Thus, the hydroxyl group on the residue is not required for biosynthetic activity, but the phenyl moiety is important.

Tyr-397 and the adenylation loop can be visualized from the crystal structures determined without substrates (Figure 6A) and in the presence of the substrate ADP and the glutamate analogue, phosphinothricin (Figure 6B). The flexible Glu-327 flap closes the active site, a movement thought to be important in excluding water from the active site, thus, preventing hydrolysis of the enzyme-bound biosynthetic intermediate (19). In the absence of substrates, Tyr-397 is 13.5 Å from Glu-327 but, in the presence of substrates, is only 3.4 Å from Glu-327 (Figure 6). Thus, Tyr-397 may be important in ensuring the closure of the Glu-327 flap.

Determining the structure of the site-specific mutants should provide a rational basis for their observed characteristics and may give insight into the mechanism by which adenylation of Tyr-397 regulates glutamine synthetase. For now, we conclude that the immediate environment of the side chain of residue 397 modulates the activity of glutamine synthetase.

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³ PDB no. 1FPY (19). (<http://www.rcsb.org/pdb/cgi/explore.cgi?pdbId=1fpy>).

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