A Spectral Probe Near the Subunit Catalytic Site of Glutamine Synthetase from Escherichia coli

REVIEWED PYRIDOXAL 5'-PHOSPHATE-ENZYME COMPLEXES*

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In order to label phosphate binding sites, unadenylated glutamine synthetase from Escherichia coli has been pyridoxylated by reacting the enzyme with pyridoxal 5'-phosphate followed by reduction of the Schiff base with NaBH₄. A complete loss in Mg²⁺-supported activity is associated with the incorporation of 3 eq of pyridoxal-P/subunit of the dodecamer. At this extent of modification, however, the pyridoxylated enzyme exhibits substantial Mn²⁺-supported activity (with increased Kₘ values for ATP and ADP). The sites of pyridoxylation appear to have equal affinities for pyridoxal-P and to be at the enzyme surface, freely accessible to soluble. At least one of the three covalently bound pyridoxamine 5'-phosphate groups is near the subunit catalytic site and acts as a spectral probe for the interactions of the manganese-enzyme with substrates. A spectral perturbation of covalently attached pyridoxamine-P groups is caused also by specific divalent cations (Mn²⁺, Mg²⁺, or Ca²⁺) binding at the subunit catalytic site (but not while binding to the subunit high affinity, activating Me⁺⁺ site). In addition, the feedback inhibitors, AMP, CTP, L-tryptophan, L-alanine, and carbamyl phosphate, perturb protein-bound pyridoxamine-P groups. The spectral perturbations produced by substrate and inhibitor binding are pH-dependent and different in magnitude and maximum wavelength. Adenylation sites are not major sites of pyridoxylation.

Glutamine synthetase of Escherichia coli is a dodecamer of Mₑ = 600,000, composed of apparently identical subunits (1). Mechanisms of regulating glutamine synthetase activity have been reviewed (2, 3) and include feedback inhibition and enzymatically catalyzed covalent modification by adenylation-deadenylation cascade systems. Each subunit of glutamine synthetase has a catalytic site (4), an adenylylation site (5), two essential divalent cation sites (4), and separate binding sites for covalent attachment of 5'-adenylic acid residues per enzyme subunit. The glutamine synthetase adenylyltransferase was from a previous study (29). Pyridoxal 5'-phosphate (A grade) was obtained from Calbiochem Corp. Pyridoxamine 5'-phosphate/HCl, pyridoxal, 2-acetyl-L-lysine, L-methionine (SH)-sulfoximine, carbamyl phosphate, sodium borohydride, amino acids, and nucleotides were obtained from Sigma Chemical Co. Di- and trivalent metal ions were removed from amino acids prior to use (29). Solutions of carbamyl phosphate, nucleotides, and other effectors were prepared at the desired pH just before use. Standardized solutions (4) of 0.486 ± 0.004 mM MnCl₂, 1.00 ± 0.05 mM CaCl₂, and 0.60 ± 0.01 mM MgCl₂ kindly were supplied by Dr. John B. Hunt. Deionized, ultrafiltered water was obtained by passing distilled water through a Milli-Q reagent grade water system of Millipore Corp.

Pyridoxylated Glutamine Synthetase—The enzyme was dialyzed overnight at 4°C against 10 mM TEA/acetate, 10 mM MgCl₂ buffer at pH 8.0 ± 0.1. Solutions of pyridoxal 5'-phosphate in 0.15 M TEA/acetate (pH 8.0) and of 0.1 mM NaBH₄ in 20 mM TEA/acetate (pH 8.0) were prepared just prior to use. For Schiff base formation, the dialyzed enzyme (4 mg/ml) was incubated at room temperature with slow stirring in the presence of 50 mM MgCl₂, 50 mM TEA/acetate (pH 8.0), and 6 mM pyridoxal-P (unless otherwise specified). After 30 min, NaBH₄ was added in a 5-fold molar excess over the amount of pyridoxal-P present, and another 30 min was allowed for the reduction reaction. The modified protein then was precipitated by adding an equal volume of a saturated ammonium sulfate solution to the reducing mixture and the precipitate was collected by centrifugation and was dissolved in a buffer (pH 8.0) containing 20 mM imidazole/HCl, 100 mM KCl, and 20 mM MgCl₂; if necessary, a few drops of 1 M KOH were added to adjust the pH to 8.0. The precipitate then was centrifuged, resuspended in 20 mM imidazole/HCl, 200 mM KCl, and 10 mM MgCl₂, and stored as described previously (28). The enzyme preparation had an average of 1 eq of covalently bound 5'-adenylyl groups per mol (600,000g) of glutamine synthetase (GS). The glutamine synthetase used in this study to monitor interactions of E. coli glutamine synthetase with divalent cations, substrates, and feedback inhibitors.

EXPERIMENTAL PROCEDURES

Materials—Unadenylated glutamine synthetase was isolated and stored as described previously (28). The enzyme preparation had an average of 1 eq of covalently bound 5'-adenylyl groups per mol (600,000g) of glutamine synthetase (GS). The glutamine synthetase used in this study to monitor interactions of E. coli glutamine synthetase with divalent cations, substrates, and feedback inhibitors.

1 The abbreviations used were: GS₉, glutamine synthetase with an average state of adenylation n (where the number of adenylation sites for covalent attachment of 5'-adenyl acid residues per enzyme dodecamer equals 12); pyridoxal-P, pyridoxal 5'-phosphate; pyridoxamine-P, protein-bound pyridoxamine 5'-phosphate (N-ε-phosphopyridoxyl-lysyl derivative); TEA, triethanolamine.
Pyridoxylation of Glutamine Synthetase

7.2 to 7.4 containing 20 mM imidazole/HCl, 100 mM KCl, and 1 mM MnCl₂, or up to 50 mM MgCl₂. Control samples of glutamine synthetase that were treated as above without pyridoxal-P addition retained full enzymatic activity.

Gel filtration of the dialyzed pyridoxylated enzyme through a Sephadex G-25 column (0.9 x 55 cm) showed that more than 90% of the reduced pyridoxal-P was protein-bound. All procedures were performed in the absence of direct light. Covering glassware with aluminum foil was found to be adequate protection against light-stimulated cleavage of (pyridoxyl-P)-GS complexes. Storage was at 4°C.

Diluent cation free enzyme was prepared and checked for EDTA removal as described previously (31). These solutions were used soon after preparation.

Protein and Protein-bound Pyridoxamine-P Concentrations—Protein concentrations of unmodified glutamine synthetase were determined from spectrophotometric measurements at 290 or 280 nm (32). Blueret measurements (33) indicated that the pyridoxylated enzyme has an unchanged absorption at 280 nm. The amount of pyridoxyl-P covalently bound per mol of enzyme subunit was calculated from absorbption measurements (pH 7) at 280 and 326 nm on the basis of a subunit molecular weight of 50,000 for glutamine synthetase (1), assuming a value of 10,000 Å²/mg for the molar absorption coefficient of protein-bound pyridoxyl-P groups (34).

Characterization of Protein-bound Pyridoxamine-P Groups—A aliquots (3 mg) of the unadenylylated and pyridoxylated enzyme preparations were hydrolyzed overnight in 1 ml of 6 N HCl in evacuated tubes. N-e-Pyridoxyllysine in acid hydrolysates of the modified enzyme was identified as a fluorescent spot after ascending chromatography on Whatman No. 3MM paper using the following solvents (34): n-butyl alcohol:pyridine:acetic acid:water (30:20:6:24), Rₖ = 0.24; isopropanol:pyridine:acetic acid:water (30:40:6:24), Rₖ = 0.49. The chromatographic reference compound, N,N'-pyridoxyl-1-lysine, was synthesized from N-acetyl-1-lysine by the procedure of Forre et al. (34) except that the pyridoxylation reaction with pyridoxal was run in 90% methanol after dissolving the acetylated lysine in water. A measurement of the molar absorption coefficient for covalently bound pyridoxyl-P groups to glutamine synthetase was made from 280 and 326 nm absorptions at pH 7.2 and phosphate analyses (35) on acid hydrolysates, using the unhydroxylated, unmodified enzyme as a blank in the phosphate determination.

Enzyme Assays—Mg⁺⁺- or Mn⁺⁺-supported biosynthetic activities were measured at 30°C in a coupled assay system as described previously (32) except that assay solutions (with an initial absorbance at 340 nm of 2 and sufficient glutamine synthetase to give Δ4A₅₅₀/ΔT min = 0.06 to 0.30) were mixed just prior to sampling into a thermostated cell (0°C) attached to a Beckman DU equipped with a Gilford digital readout and an automatic data lister (Gilford, model 4008). Mg⁺⁺-supported (2) and Mn⁺⁺-supported γ-glutamyl transfer activities at pH 7.57 were measured as described before (4).

Kinetic parameters (Kₐ and Vₘₐₓ) were obtained from double reciprocal plots of velocity versus substrate concentration, using appropriate precautions in assays to ensure that initial velocities were measured at all substrate levels. All such plots were linear at moderate substrate concentrations with unmodified and pyridoxylated enzymes.

Difference Spectral Measurements—All spectra were recorded using a Cary model 15 spectrophotometer equipped with the 0 to 0.1 and 0 to 1 Å slide-wires. The temperature of the sample cell for most experiments was maintained at 25 ± 0.1°C by using a water-jacketed silica (200 ml) of 1-cm light path. After establishing a base-line on the 0.1 Å scale with the protein solution (2 to 2.5 mg/ml) in both the sample and reference cells, ligands were added to the enzyme solution in the sample compartment while the same dilutions (<5%) of the buffers, Ligand or buffer additions to enzyme solutions were made with appropriate precautions in assays to ensure that initial velocities were measured at all substrate levels. All such plots were linear at moderate substrate concentrations with unmodified and pyridoxylated enzymes.

Ultracentrifugation—A Beckman model E analytical ultracentrifuge, equipped with a rotor temperature and control unit, phase plate, schlieren, absorption optics, and photoelectric scanner with multiplexor was used. Sedimentation velocity experiments were at 20°C with a protein concentration of 4.4 mg/ml and a speed of 20,000 rpm using a two-place An-D rotor. Sedimentation coefficients were measured from schlieren photographs and appropriately corrected (37). A 12-mm cell with a Kel F double sector centerpiece and absorption optics at 326 or 280 nm were used to look for more slowly sedimenting species. For difference sedimentation measurements, two single sector cells (with one containing a 1° positive wedge window) were used. Before ultracentrifugal analyses, enzyme samples were dialyzed against several changes of 20 mM imidazole/HCl, 100 mM KCl, 1.0 mM MnCl₂ buffer at pH 7.25.

RESULTS AND DISCUSSION

Pyridoxylation of Unadenylylated Glutamine Synthetase.—The absorption spectrum of a protein incubated with pyridoxal 5'-phosphate provides a monitor of Schiff base formation (11–15, 38). When glutamine synthetase reacts with pyridoxal-P, a decrease in the maximum absorbance at 390 nm (free pyridoxal-P) and the appearance of a new absorption peak at 425 nm (Schiff base) are observed. In preliminary experiments (not shown), a variety of conditions (pH 6 to 9 with different buffers and either MgCl₂ or MnCl₂ present) were tested and optimal Schiff base formation was found to occur under the conditions given under "Experimental Procedures." Nucleotide substrates of glutamine synthetase inhibited Schiff base formation with pyridoxal-P (Table I, below). The Schiff base formed with pyridoxal-P is reversible; dialysis or gel filtration regenerates the unmodified enzyme.

All experiments reported in this paper involve the relatively stable reduced pyridoxyl 5'-phosphate-enzyme complexes. Lysyl residues of the enzyme are modified in the pyridoxylation reaction; N-e-pyridoxyllysine was identified in acid hydrolysates of the pyridoxylated protein. The amount of covalently attached pyridoxyl-P in pyridoxylated glutamine synthetase samples remained unchanged after storage in the dark for as long as 6 months. The enzyme derivative was completely stable also during spectrophotometric and activity studies. However, the protein-bound pyridoxyl-P groups were photolyzed within a few minutes during excitation at 330 nm in a Hitachi Perkin-Elmer MPF-2A spectrophotometer, which made fluorescent measurements with this derivative impossible.

In sedimentation velocity studies, the pyridoxylated enzyme had S₂₀,₀ₒ = 20.2 S, no more slowly sedimenting species could be detected with absorption optics. In difference sedimentation measurements, the pyridoxylated glutamine synthetase sedimented 4% faster than did the unmodified enzyme (ΔS = 0.8 S). Modification of glutamine synthetase by pyridoxylation therefore does not cause dissociation of the dodecamer or any significant change in shape.

Fig. 1 shows absorption spectra of unadenylated gluta-
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Fig. 2. Pyridoxylation of glutamine synthetase as a function of pyridoxal 5'-phosphate concentration. Reaction conditions are as described under "Experimental Procedures." The extent of modification (v) is given as the number of pyridoxal-5'-phosphate groups incorporated per enzyme subunit. Inset, Scatchard plot of these data.

Fig. 3. Effect of pyridoxylation on the Mg²⁺-supported biosynthetic activity of unadenylylated glutamine synthetase (GS). The enzyme was modified to different extents by variation of the pyridoxal 5'-phosphate concentration (Fig. 2). Enzyme activity is given as the percent of that of a control which was subjected to the same modification conditions (including reaction with NaBH₄) without pyridoxal 5'-phosphate present. Assay results with all substrates at saturating concentrations are given by the closed circles; open circles show results obtained in the same assays with subsaturating ATP concentrations as a function of pyridoxylation (Fig. 3).

Table I

| Ligand added | Pyridoxal-5'-incorporation | Mg²⁺-biosynthetic activity |
|--------------|---------------------------|---------------------------|
| ATP          | 34 ± 11⁰                   | 49 ± 9⁰                   |
| ADP          | 23                        | 37                        |
| AMP          | 20                        | 32                        |
| Atenesate    | 14                        | 35                        |
| Carbamyl-P   | 39                        | 60                        |
| CTP          | 25                        | 41                        |

* From four determinations with added ATP; all other values are from single experiments.
Random binding of substrates and rapid equilibria, \( K = \frac{2V'C}{(37x43)} \) were the same with pyridoxylated enzyme as they were with substrates (3). The kinetics of the phanyl-tyrosyl residue perturbation at 2S4 and 290 nm, attributed to the binding of a small amount of Me2+ near the covalently bound pyridoxamine-P groups at low concentrations of Me2+ (Curves 1 and 2, Fig. 4), presumably are due to the binding of a covalently bound pyridoxamine-P per subunit and were recorded after time-dependent changes were completed at 25°C. Metal ion additions were: Curve 1, 0.05 mM MnCl2; Curve 2, 0.4 mM MgCl2 or 0.8 mM CaCl2; Curve 3, 1.0 mM MnCl2; Curve 4, 4.0 mM MgCl2 or 5.0 mM CaCl2.

An explanation of the Mn2+-supported biosynthetic activity of GS1 measures only the small fraction (one-twelfth) of the adenylated subunits. The inactivity of unadenylated subunits in this assay has been attributed by Rhee et al. (42) to the very high affinity that this enzyme form has for MgADP in the presence of Pi. In contrast, the Mn2+-supported \( \gamma \)-glutamyl transfer reaction at pH 7.57, in which MgADP and arsenate are nonconsumable substrates, measures both adenylated and unadenylated subunits equally (4). Further, subunits within the dodecamer independently express Mn2+-supported \( \gamma \)-glutamyl transfer activity. The loss in the latter activity was linear with increasing equivalents of pyridoxal-P incorporated and coincided with the data shown in Fig. 3, taking into account the 42% residual Mn2+-supported transfer activity of the fully modified enzyme. A pH variation from pH 6.9 to 8.1 alters the absolute velocities but not the relative activities of the native and pyridoxylated enzymes in the Mn2+-supported \( \gamma \)-glutamyl transfer reaction. The same activity difference between unmodified and pyridoxylated enzymes was observed also in these assays when Pi (20 mM) was substituted for arsenate.

The activity results suggest that pyridoxylation of the dodecamer occurs near the nucleotide binding site of each subunit expressing activity. In obtaining the \( K_m \) data of Table II, double reciprocal plots of velocity versus nucleotide-manganese concentrations were linear in Mn2+-supported \( \gamma \)-glutamyl transfer assays. If some of the subunits within a dodecamer had not been modified, those presumably would have expressed activity with \( K_m = 10^{-8} M \) for the MnADP and double reciprocal plots of velocity versus MnADP concentration would have been biphasic reflecting \( K_m \) values of \( 10^{-9} \) and \( 10^{-10} M \) for MnADP. This was not the case.

The \( K_m \) data of Table II illustrate that pyridoxylase is like adenyllylation in that it causes a large decrease in the affinity of the enzyme for MnATP or for MnADP.2 Adenylylation of glutamine synthetase, however, produces an increase in the Mn2+-supported biosynthetic specific activity (3) whereas pyridoxylation of the unadenylated enzyme causes a partial inactivation (shown by the decrease in \( V_{max} \) observed in the Mn2+-supported transfer assay). We conclude that pyridoxylated, unadenylated subunits with a lower affinity for MnADP must express some Mn2+-supported biosynthetic activity and that it is therefore a coincidence that MnADP must express some Mn2+-supported biosynthetic activity. The loss in this latter activity was linear with increasing equivalents of pyridoxal-P incorporated and coincided with the data shown in Fig. 3, taking into account the 42% residual Mn2+-supported transfer activity of the fully modified enzyme. A pH variation from pH 6.9 to 8.1 alters the absolute velocities but not the relative activities of the native and pyridoxylated enzymes in the Mn2+-supported \( \gamma \)-glutamyl transfer reaction. The same activity difference between unmodified and pyridoxylated enzymes was observed also in these assays when Pi (20 mM) was substituted for arsenate.

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Fig. 5. Hill plots of spectrophotometric titrations at 25°C of divalent cation-free, pyridoxylated glutamine synthetase with Mg²⁺ (O, •) or Ca²⁺ (∆, △). The conditions were as described in the legend to Fig. 4 with 0.05 mM modified enzyme subunits. Changes in absorbance upon Me⁺ addition were monitored at 290 nm (○, ●; ΔA₃₉₀ = +0.060) and at 328 nm (●, △; ΔA₃₂₈ = −0.028) where ΔA₃₂₈ and ΔA₃₉₀ are the observed final change in absorbance at 290 or 328 nm at a given Me⁺ concentration and the maximum change in absorbance at each wavelength observed at saturating concentrations of Me⁺, respectively. The free millimolar concentration of Me⁺ was calculated assuming that [Me⁺]free = [Me⁺]total − 0.05(ΔA₃₂₈/ΔA₃₉₀)₃₂₈ + 0.05(ΔA₃₂₈/ΔA₃₉₀)₃₉₀ where absorbance changes at 290 and 328 nm measure Me⁺ binding to two sites of the enzyme subunit. The results are from titrations of three different pyridoxylated enzyme preparations with Mg²⁺ and Ca²⁺.

protein-bound pyridoxamine-P groups produced by the binding of Mn²⁺ or Mg²⁺ are different, with maxima at 338 or 328 nm, respectively. Since Mn⁺⁺ can competitively displace Mg²⁺ from the enzyme (4, 43), this wavelength difference could be verified by adding Mn²⁺ to the pyridoxylated magnesium enzyme in an experiment (not shown) and observing the perturbation maximum shift from 338 to 328 nm. The difference in absorbance between maxima produced by Mn²⁺ versus Mg²⁺ (or Ca²⁺) could be related to the electronic structures of these metal ions, in which case the aromatic ring of a covalently bound pyridoxamine-P group must be very near the bound metal ion at the subunit n₂ site. Titrations of Me⁺⁺-free, pyridoxylated glutamine synthetase with Co²⁺ (36) were unsuccessful due to a Co⁺⁺-induced aggregation of the modified enzyme.

The only groups in pyridoxamine 5'-phosphate capable of forming a stable metal ion chelate are the phenolic and adjacent aminooxytide groups (40, 41). The binding of Mn⁺⁺ to pyridoxamine 5'-phosphate at pH 7.2 is very weak (40); stability constants for Mg²⁺ (41) and Ca²⁺ at neutral pH would be even lower than that for Mn⁺⁺. In the absence of tertiary structure provided by the protein, these divalent cations are not expected to form stable chelates with the secondary amine derivative N₆-phosphopyridoxyl-l-lysyl-GLS at neutral pH.

The results from spectrophotometric titrations of Me⁺⁺-free, pyridoxylated glutamine synthetase with Mg²⁺ and Ca²⁺ are presented as Hill plots in Fig. 5. A stoichiometry of one Me⁺⁺ binding site per subunit for each wavelength perturbation was assumed for the calculations of free Me⁺⁺ in Fig. 5. Both sets of data with Mg²⁺ and Ca²⁺ at either 290 or 328 nm could be fit with slopes of unity, suggesting that each type of site in the dodecamer is equivalent or noninteracting (i.e. not cooperative). This has been found previously for Mn⁺⁺ binding to the unmodified enzyme (43). The most straightforward interpretation of the results shown in Fig. 5 is that the 290 nm data measure the titration of the high affinity n₁ sites (Kₐ = 8000 M⁻¹) whereas the 328 nm data measure the saturation of n₂ sites (Kₐ = 1000 M⁻¹). The n₁ sites of pyridoxylated glutamine synthetase appear to have about a 3-fold lower affinity for Mg²⁺ or Ca²⁺ than measured previously with the unmodified enzyme (4, 43). It has not been possible before to measure the binding of Mg²⁺ or Ca²⁺ to n₂ sites, but unadenylated glutamine synthetase has Kₐ = 1 mM for Mg²⁺ (4, 43), and this value reflects n₂ site saturation under assay conditions.

For the experiments of Figs. 4 and 5, EDTA had been removed from the modified enzyme prior to titrations with the divalent cations. EDTA had been used at an enzyme subunit concentration of 0.050 mM in a buffer of 20 to 50 mM imidazole/HCl (pH 7.3 or 8.3) and 100 mM KCl with 1 mM MnCl₂, 50 mM MgCl₂, or no divalent cation present. Pyridoxylated glutamine synthetase (P-Pxy-GS) preparations contained 2.6 or 3.2 eq of covalently bound pyridoxyl-P groups per subunit. Spectrophotometric titrations with different ligands were performed at the pH of maximum absorbance change; ΔA₃₉₀ (at λ₃₀₀) is for a saturating concentration of ligand; Kₐ = Cₜ − 0.025, where Cₜ is the total concentration (millimolar) of ligand required to produce 50% of the maximum spectral change. When known, Kₐ₃₉₀ values (with appropriate literature references) for the unmodified enzyme (GS) are given.

| Table III | Spectral perturbations of protein-bound pyridoxamine-P groups |
|-----------|---------------------------------------------------------------|
| **Mn⁺⁺ present and ligand added** | **ΔA₃₉₀ x 10⁷** | **Kₐ₃₉₀** |
| **pH 7.3** | **pH 8.3** | **P-Pxy-GS** | **GS** | **Ref.** |
| Mn⁺⁺ | ATP | +10 | +68 | 322⁺⁺ | 0.04 | 0.005 | 44 |
| | ADP | −38 | −8 | 328 | 0.02 | 0.003 | 4 |
| | AMP | −30 | −8 | 328 | 0.9 | 0.13 | 6 |
| | Pₜ | +12⁺⁺ | +12⁺⁺ | 328 | 1.4 | 42 |
| Glutamate | 0 | 0 | | | |
| Glutamine | 0 | 0 | 6.4 | 4 | 28 |
| Met-sulfoximine | −9 | −8 | 345 | 0.14 | 0.03 | 45 |
| Carbamyl-P, tight | +9 | +56 | 334 | 0.7 | <0.5 | 46 |
| Carbamyl-P, loose | −50 | 325 | 14 | | |
| Tryptophan | 0 | −58 | 333 | 1.1 | 0.91 | 46 |
| CTP | +5 | +63 | 334 | 0.15 | | |
| GlcN-6-P | +5 | 327 | | | |
| GlcN-12-P | 0 | 0 | | | |

For the experiments of Figs. 4 and 5, EDTA had been removed from the modified enzyme prior to titrations with the divalent cations. EDTA had been used at an enzyme subunit concentration of 0.050 mM in a buffer of 20 to 50 mM imidazole/HCl (pH 7.3 or 8.3) and 100 mM KCl with 1 mM MnCl₂, 50 mM MgCl₂, or no divalent cation present. Pyridoxylated glutamine synthetase (P-Pxy-GS) preparations contained 2.6 or 3.2 eq of covalently bound pyridoxyl-P groups per subunit. Spectrophotometric titrations with different ligands were performed at the pH of maximum absorbance change; ΔA₃₉₀ (at λ₃₀₀) is for a saturating concentration of ligand; Kₐ₃₉₀ = Cₜ − 0.025, where Cₜ is the total concentration (millimolar) of ligand required to produce 50% of the maximum spectral change. When known, Kₐ₃₉₀ values (with appropriate literature references) for the unmodified enzyme (GS) are given.

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| GlcN-12-P | 0 | 0 | | | |

*These are not AA max values because Pi at >4.5 mM produces strong perturbations.
Me⁺ ions. When EDTA was present in difference spectra measurements similar to those shown in Curves 3 and 4 of Fig. 4, additional perturbations of covalently bound pyridoxamine-P groups were observed. These studies were not pursued but the observations suggest that the metal-EDTA complex binds to the modified enzyme.

**Substrate- and Inhibitor-induced Spectral Changes—**
Spectral perturbations of protein-bound pyridoxamine-P groups produced by interactions of substrates and inhibitors with the Mn⁺⁺-, Mg⁺⁺-, or Me⁺⁺-free forms of pyridoxylated glutamine synthetase are summarized in Table III. Absorbance changes occurred within 15 s after the addition of each effector; no effect of time on the degree of perturbation was observed for as long as 3 h. Examples of difference spectra are shown in Fig. 6. Substrate or inhibitor concentrations required to give 50% of the maximum spectral changes were used to calculate $K_D$ values for pyridoxylated glutamine synthetase in Table III; comparable $K_D$ values (when known) for the unmodified enzyme are included. The effect of modification on this parameter varies from a quite pronounced decrease in the apparent affinity of the modified enzyme for ATP or ADP to a negligible affinity change for the inhibitor L-tryptophan. The spectral change observed with a particular ligand can be pH-dependent and this necessitated testing perturbations with each effector at several pH values (within the range of interest for glutamine synthetase). The results from titrations at pH 7.3 and 8.3 are given in Table III. Between these pH values, spectral changes were intermediate in amplitude to those given in Table III. None of the ligands of Table III affected the spectral properties of free pyridoxamine 5'-phosphate.

Spectral perturbations of protein-bound pyridoxamine-P groups produced by the nucleotide substrates (ATP and ADP) are Me⁺⁺ ion-dependent. These are maximum with the manganese enzyme complex, greatly diminished with the magnesium enzyme complex, and nonexistent with the Me⁺⁺-free, pyridoxylated enzyme. This is consistent with the activity results of Table II, in which the pyridoxylated enzyme was shown to have Mn⁺⁺-supported but little Mg⁺⁺-supported activity. When added to the manganese enzyme derivative, ATP and ADP induce spectral changes that differ both in direction and pH dependence (Table III, Fig. 6). ATP produces a relatively large increase in absorption at pH 8.3 but little absorbance change at pH 7.3, whereas ADP produces a large decrease in absorption at pH 7.3 but only a small perturbation at pH 8.3. The maximum wavelength of the ATP-induced difference spectrum varied with the manganese enzyme derivative used (2.6 or 3.2 eq of pyridoxamine-P/subunit) and was either 322 or 334 nm, respectively. This variation in maximum wavelength was observed only with ATP.

Spectral changes caused by inorganic phosphate, a substrate of the reverse biosynthetic reaction (3, 42), do not have the Me⁺⁺ ion- or pH-dependence observed with ATP and ADP (Table III). Titrations of the modified enzyme at low Pᵢ concentrations (<4.5 mM) gave similar absorbance changes in the presence of Mn⁺⁺ or Mg⁺⁺, but the complete spectral change produced by Pᵢ presumably can be observed only with the manganese enzyme complex (Fig. 6) because precipitation occurs in the Mn⁺⁺ system. The change in the absorption of the covalently bound pyridoxamine-P groups produced by Pᵢ in the presence of either metal ion is positive, which may relate to a binding of Pᵢ to the protein at the site for the γ-phosphate of ATP (42). Interestingly, Pᵢ produces a small negative absorbance change with the Me⁺⁺-free, modified enzyme in which case the protein is in an inactive conformation.

The AMP-induced spectral perturbations of the protein-bound pyridoxamine-P groups in the manganese enzyme complex are similar in direction, amplitude, and pH dependence to those of ADP (Fig. 6). However, the binding of AMP to glutamine synthetase, unlike that of ADP, is independent of metal ion (6, as reflected in the absorption changes observed with the Me⁺⁺ ion-free enzyme derivative (Table III). Although AMP is an allosteric inhibitor of this enzyme (6, 49, 50), AMP can substitute for ADP in a Mn⁺⁺-supported γ-glutamyl transfer reaction catalyzed by unadenylylated glutamine synthetase (2, 42). Antagonism between AMP and ATP was indicated by titrations of the MnATP enzyme derivative with AMP at pH 8.3.

The addition of L-glutamate or L-glutamine to the pyridoxylated manganese enzyme has no effect on the absorption spectrum although both substrates would be expected to bind to the protein (28, 47). A transition state analogue of the glutamine synthetase reaction (51), L-methionine (SR)-sulfoximine, produces only a small (red-shifted) spectral perturbation (Table III). However, these compounds have marked effects on the absorption of the protein-bound pyridoxamine-P groups of the ADP-manganese enzyme complex. Since the binding of ADP and L-glutamate to the enzyme are synergistic (7, 5, 28), the difference spectrum for ADP + Glx in Fig. 6 is representative of a different conformation than those stabilized by either substrate alone. Spectrophotometric titrations of the ADP-manganese enzyme pyridoxamine-P complex with L-glutamine or L-glutamate yielded $K_D$ values of 24 or 18 mM, respectively; these values are ~10-fold greater than those measured previously with the unmodified enzyme in the presence of Me⁺⁺ and ADP (7, 28). The ATP-induced difference spectrum of Fig. 6, in contrast to that of ADP, is unchanged by the presence of 24 mM L-glutamate.

**Fig. 6.** Substrate-induced difference spectra of pyridoxylated glutamine synthetase at 25°C. The modified enzyme at 0.05 mM subunit concentration with 2 eq of covalently bound pyridoxamine-P groups per subunit was in a buffer containing 20 mM imidazole/HCl, 100 mM KCl, and 1.0 mM MnCl₂ (or 50 mM MgCl₂ for Pᵢ addition, only) at pH 7.3 (or pH 8.3 for the ATP perturbation, Table III). The maximum absorbance change produced by each ligand or ligand combination is shown. Ligands added singly were: 0.6 mM ATP, 0.5 mM ADP, 6.3 mM AMP, 40 mM Pᵢ; Combinations of added ligands in the presence of 1.0 mM MnCl₂ were: 0.24 mM ADP + 0.72 mM L-methionine (SR)-sulfoximine Met(O)(NH); 0.30 mM ADP + 60 mM L-glutamate or L-glutamine (Glx); 0.30 mM ADP + 1.0 mM Pᵢ.
The spectral changes observed with carbamyl phosphate are more complex than those with the other effectors of Table III and these are illustrated in Fig. 7. Relatively low concentrations of carbamyl phosphate produce positive spectral changes when added to the modified manganese–enzyme at pH 8.3 (e.g. Curve 1 of Fig. 7). The estimated $K_D$ value for this spectral change is 0.7 mM and is designated as that obtained by a titration of a “tight” binding site in Table III. Increasing concentrations of carbamyl phosphate (Curves 2 to 6 in Fig. 7) produce negative spectral perturbations and alter the wavelength of maximum absorption change. The $K_D$ value estimated for this second binding site (designated “loose” in Table III) is 14 mM. The results from two types of experiments with the manganese–enzyme pyridoxamine–P complex at pH 8.3 indicated that the tight binding of carbamyl phosphate was to the subunit catalytic site. (a) With just carbamyl phosphate to saturate the tight binding site (Curve 1, Fig. 7), the addition of ADP at 80% of the enzyme subunit concentration produced the spectral change of ATP (Fig. 6). In fact, Meister and co-workers (46) have determined that carbamyl phosphate can bind to the catalytic site of glutamine synthetase and can phosphorylate ADP to synthesize ATP. (b) When the modified manganese–enzyme at pH 8.3 was titrated with ATP to produce the maximum spectral perturbation (Fig. 6, Table III), subsequent additions of carbamyl phosphate only caused the negative spectral change associated with the loose binding of carbamyl phosphate. Thus, the loose binding of carbamyl phosphate appears to be to allosteric inhibitor sites of the enzyme.

**Adenylylation and Pyridoxylation**—Since the unadenylated enzyme was modified in these studies, two types of experiments were performed to see whether lysyl residues near adenylylation sites (1, 5) are pyridoxylated. After preparing fully adenylylated glutamine synthetase (GS$_{ADP}$) as described previously (28, 32), the adenylylated and unadenylated enzymes were pyridoxylated in parallel reactions. Exactly 3.2 equiv of pyridoxamine–P groups per subunit were covalently attached to either enzyme form. This suggests that pyridoxylation is independent of the availability of adenylation sites. The pyridoxylated, unadenylated, and adenylylated enzymes also had about the same Mn$^{2+}$–supported activities at pH 7.57; 44 and 30 units/mg, respectively. If adenylylation caused an exposure of new pyridoxylation sites (while burying others), it must do so without much affecting the Mn$^{2+}$–supported transfer activity of the modified enzyme. In a separate experiment, we tried to adenylylate the pyridoxylated, unadenylated glutamine synthetase preparation enzymatically. Using large excesses of the glutamine synthetase adenylyltransferase (39, 32) in the adenylyltransferase assay system (53), the maximum incorporation of radioactively labeled [14C]AMP into the pyridoxylated protein was about 50% of that into unmodified GS$_{ADP}$. This could be due to the pyridoxylated enzyme being a poor substrate for adenylyltransferase (which is likely) or to some pyridoxylation having occurred near adenylylation sites. Although these results are somewhat equivocal, we can conclude that lysyl residues at adenylylation sites are not major sites of pyridoxylation in unadenylated glutamine synthetase.

**Topography of Pyridoxylation Sites in Glutamine Synthetase**—Whether or not pyridoxylation occurs at three unique sites of each subunit of the dodecamer must await peptide analysis. Amino acid sequence and three-dimensional structural information is not yet available for the assignment of pyridoxylation sites. Nevertheless, the results presented here indicate that at least one pyridoxylation site is near the catalytic site of each subunit of glutamine synthetase.

The uniformity of labeling near the nucleotide binding sites of the enzyme is indicated by the activity data of Fig. 3 and Table II. One pyridoxylation site appears to be near the γ-phosphate position of ATP. Carbamyl phosphate can bind to...
this site and can phosphorylate ADP to synthesize ATP. Since the maximum wavelength of the absorption change produced by ATP (Table III) was a function of the extent of pyridoxyl-
ination (2.6 or 3.2 eq of pyridoxamine-P groups/subunit), ATP binding appears to perturb two covalently bound pyridoxamine-P groups. At least one pyridoxamine-P group is very near the $n_2$ metal ion binding site of the enzyme subunit. 

However, the affinity of the pyridoxylated enzyme for Mg$^{2+}$ or Ca$^{2+}$ (Fig. 5) is too low to obtain the stoichiometry of pyridoxamine-P labeling at this site. Since the Me$^{2+}$ is known to chelate the nucleotide at the catalytic site, it can be assumed nevertheless that all metal ion binding at $n_2$ subunit sites is producing the observed spectral perturbation of protein-bound pyridoxamine-P groups (Fig. 4). Interestingly, the binding of specific divalent cations (Mn$^{2+}$, Mg$^{2+}$, or Ca$^{2+}$) to the $n_1$ activating site of each subunit does not perturb covalently bound pyridoxamine-P groups. Also, lysyl residues near adenylation sites of the enzyme do not appear to be sites of pyridoxylation. Thus, the labeling of the dodecamer by pyri-
doxylation appears to occur at specific sites.

The spectral perturbations of protein-bound pyridoxamine-P groups caused by substrate and inhibitor binding to the enzyme at a given pH can arise from shifts in the environment that influence solvent accessibility and the $pK$ of the pyridi-
rium ion of the aromatic ring, which may involve also changes in the ionization of protein groups near pyridoxylation sites. For example, the equivalent of a 0.1 pH unit increase near the $pK$ (at a pH of ~8) of the pyridinium ion will produce a molar absorption change of about 220 m$^{-1}$cm$^{-1}$ at 326 nm. The pH dependence and different wavelength maxima of absorbance changes produced by various ligands of glutamine synthetase are brought about by the different conformations stabilized. 

The positive absorption change produced by ATP binding to the enzyme corresponds to an increased protonation of the pyridine nitrogen which may be why the ATP perturbation is not observed at pH 7.3 but is observed at more alkaline pH values. In contrast, ADP binding to the pyridoxylated enzyme produces an absorption decrease which corresponds to a de-
crease in the degree of protonation of the pyridine nitrogen. This may be why the perturbation by ADP is observed at pH 7.3 and not at pH 8.3.

Metal ion binding at the subunit catalytic site appears to involve a direct electronic interaction between a very near covalently bound pyridoxamine-P group and the protein-bound metal ion. The absorption change produced by Mn$^{2+}$ and by Mg$^{2+}$ or Ca$^{2+}$ binding to pyridoxylated glutamine synthetase had different wavelength maxima which presumably relate to the electronic configurations of these divalent cations. Since the Me$^{2+}$ chelates the $\gamma,\beta$-phosphates of ATP or the $\alpha,\beta$-phosphates of ADP, there must be flexibility in the conformation at the subunit catalytic site for accommodating ATP or ADP binding. An indication that the catalytic site is able to assume different conformations in the presence of various combinations of substrates is shown by the spectral perturbations produced by ADP + Glx, ADP + methionine sulfoximine, or ADP + P (Fig. 6). These were not equal to the sum of the spectral perturbations produced by either ligand alone (Fig. 6, Table III).

Covalently attached pyridoxamine-P groups near catalytic sites must be able to move out of the way for activity to be expressed since the pyridoxylated enzyme has substantial Mn$^{2+}$-supported activity (with a lower $V_{max}$). An effect of pyridoxylation is to lower the affinity of the manganese-enzyme for nucleotide substrates (Tables II and III). The inability of Mg$^{2+}$ to support activity of the pyridoxylated enzyme may relate to the relatively low affinity that the unmodified magnesium enzyme has for nucleotide substrates. Spectral perturbations of protein-bound pyridoxamine-P groups are observed for ATP with the manganese-enzyme but not with the magnesium-enzyme (Table III). The effect of pyridoxylation in decreasing the affinity of the enzyme for nucleotide substrates has precedent in the observations of Greenwell et al. (15) who found that the introduction of one pyridoxamine-P group per catalytic chain in aspartate transcarbamylase produced apparent inactivation but only a decrease in the affinity of the enzyme for a tightly binding, bisubstrate analogue.

The labeling of phosphate binding sites at or near allosteric inhibitor sites is difficult to assess. By definition, an allosteric inhibitor can affect the conformation of the active site of a regulatory enzyme. Desensitization of pyridoxylated glutamine synthetase to various inhibitors has not been tested. However, carbamyl phosphate clearly is bound to two types of sites, one of which is identifiable as the subunit catalytic site. Also, L-tryptophan is an interesting example of an inhibitor producing a large negative spectral perturbation of covalently attached pyridoxamine-P groups while apparently binding too far away from pyridoxylation sites so that the enzyme has an unchanged affinity for this inhibitor. Further, the spectral change produced by tryptophan is maximum at pH 8.3 and is the same with Mn$^{2+}$ or Mg$^{2+}$ present (Table III). Thus, the tryptophan-induced spectral change of protein-bound pyridoxamine-P groups has a different origin than those produced by ATP or ADP binding.

The use of covalently bound pyridoxamine-P groups as spectral probes for ligand interactions with a complex regulatory protein is illustrated in the studies of this paper. The spectral and ionization characteristics of protein-bound pyri-
doxamine-P indicate that these groups are near the enzyme surface exposed to solvent, implying the same for the catalytic sites of glutamine synthetase.

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