Adenosine 5′-Triphosphate Analogues as Structural Probes for *Escherichia coli* Glutamine Synthetase†

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ABSTRACT: Introduction of specific structural probes into substrate binding sites of *Escherichia coli* glutamine synthetase is now possible. Various analogues of ATP substituted with an amino or sulfhydryl moiety at the 6- or 8-position of the purine ring have been found to substitute for ATP in the autoinactivation reaction of the manganese enzyme with L-Met-(*S*)-sulfoximine at pH 7. Dissociation of enzyme complexes containing an ADP analogue, L-Met-(*S*)-sulfoximine phosphate, and 2 equiv of Mn^{2+} is negligible at neutral pH. Prior to binding of the mercapto nucleotides to active sites, 6-mercaptopurine ribonucleoside triphosphate (6-S-ATP) and 8-mercaptoadenosine 5′-triphosphate (8-S-ATP) also have been further modified with fluorescent and chromogenic probes for energy-transfer measurements [Maurizi, M. R., Kasprzyk, P. G., & Ginsburg, A. (1986) *Biochemistry* (following paper in this issue)] or with electron-dense markers for electron microscopic and X-ray crystallographic structural analyses. Binding 6-S-ATP or 8-S-ATP to enzyme active sites at pH 7.1 produced red shifts of ~6 nm in nucleotide spectra characteristic for transfer of these nucleotide analogues into more acidic and hydrophobic environments. The spectrum of 6-S-ADP at active sites was more red-shifted than that of 6-S-AMP attached to adenylylation sites. The thiol group at the 6- or 8-position of the purine ring of the bound nucleotides was accessible for reactions with alkylating or mercurial reagents. Alkylation or mercaptide formation produced large blue shifts in the spectrum of enzyme-bound 6-S-ADP or 8-S-ADP at active sites or of 6-S-AMP covalently bound at adenylylation sites. At least one of two tryptophanyl residues in each subunit is very near the nucleotide binding site, as evidenced by changes in tryptophanyl residue fluorescence on binding ATP, mercaptonucleotides, or other ATP analogues.

Glutamine synthetase (GS)[1] from *Escherichia coli* is composed of 12 identical subunits of M_r 50 000 structurally arranged as two superimposed hexagonal rings (Valentine et al., 1968; Shapiro & Ginsburg, 1968). Each subunit of the enzyme has an active site containing binding sites for two divalent metal ions (n_1 and n_2), a nucleotide, and either L-glutamate or L-glutamine (Hunt et al., 1975; Shrake et al., 1977). Each subunit also has separate sites for feedback inhibitors and a regulatory site that undergoes enzyme-catalyzed reversible covalent modification by attachment of a 5′-adenylate group [reviewed by Stadtman & Ginsburg (1974)]. Intrasubunit distances between Mn^{2+} at n_1 and n_2 sites and carbon 2 of the adenine ring of the 5′-adenylate group at the adenylylation site are 10–12 Å (Villafranca et al., 1978). The binding of ligands at the active site has been observed to perturb the environment of covalently bound 5′-adenylate groups (Shrake et al., 1980; Rhee et al., 1981). In addition, lysyl residue 47 near the N-terminus of the subunit polypeptide chain appears to be in the vicinity of the nucleotide binding site (Pinkofsky et al., 1984).

Glutamine synthetase catalyzes the phosphorylation of the L-glutamate analogue L-Met-(*S*)-sulfoximine (Meister, 1974) by ATP in the presence of Mn^{2+} or Mg^{2+}, which results in enzyme inactivation due to the formation of a transition-state complex on each subunit (Weisbrod & Meister, 1973; Maurizi & Ginsburg, 1982a). This inactive complex consists of 2 equiv of Mn^{2+} and 1 equiv each of L-Met-(*S*)-sulfoximine phosphate and ADP per subunit (Maurizi & Ginsburg, 1982a). There is extreme synergism in binding the components of this complex at neutral pH: K_A' for Mn^{2+} binding to n_2 sites increases at least 4 orders of magnitude to $>10^9$ M^{-1} (Hunt & Ginsburg, 1980) as does the association constant for ADP ($K_A' \geq 10^{12}$ M^{-1}; Maurizi & Ginsburg, 1982a) which makes dissociation of these active site ligands negligible at pH 7. However, the inactive enzyme can be reactivated by dissociating the transition-state complex at pH ≃4–4.5 in the presence of high concentrations of neutral salt [KCl, LiCl, or $(NH_4)_2SO_4$] and then reinactivated by raising the pH to 7.2 in the presence of only the released ligands (Maurizi & Ginsburg, 1982a). The transition-state complex strengthens intersubunit bonding domains of the dodecameric enzyme (Maurizi & Ginsburg, 1982b). Substrates that increase the affinity of n_2 sites for Mn^{2+} (ADP, L-glutamate, or arsenate + ADP; Hunt et al., 1975; Hunt & Ginsburg, 1980) also stabilize the enzyme under denaturing conditions (Ciardi et al., 1973).

Our previous studies provided the mechanism used in this paper for specifically introducing structural probes into active

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[1] Abbreviations: GS, glutamine synthetase from *E. coli*; PMPS, *p*-mercuribenzenesulfonate; DABPM or Y adduct, [4-[[*p*-(dimethylamino)phenyl]azo]phenyl]mercuri; 6-S-ATP, 6-mercaptopurine riboside triphosphate; 8-S-ATP, 8-mercaptoadenosine triphosphate; AE-ATP, 8-[(2-aminoethyl)amino]-ATP; AH-ATP, 8-[(6-aminohexyl)amino]-ATP; aza-ε-ATP, 1,N^6-etheno-2-aza-ATP; 8-Br-ATP, 8-bromo-ATP; IAEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; AEDANS, adduct formed from IAEDANS by nucleophilic displacement of the iodide; neohydrin, 1-[3-(chloromercuri)-2-methoxypropyl]urea; (Gly-Met)PtCl, (glycyl-L-methioninato)platinum(II) chloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

sites of glutamine synthetase. Nucleotide analogues with large substituents at the 8- or 6-position of the purine ring of ATP have been found to substitute for ATP in the GS-catalyzed phosphorylation of L-Met-(*S*)-sulfoximine in the presence of Mn^{2+}. Some of the same probes could be introduced into adenylylation sites also, but this is not the emphasis in the present studies since others (Villafranca et al., 1978; Chock et al., 1979; Rhee et al., 1981) have described the attachment of fluorescent and spin-labeled probes at adenylylation sites of glutamine synthetase.

By use of the techniques discussed in this paper, the accompanying paper (Maurizi et al., 1986) describes fluorescent energy transfer measurements that locate active sites toward the outer edges of hexagonal rings near exterior surfaces of the dodecamer, away from the lateral plane between hexagonal rings.

Materials and Methods

Unadenylylated glutamine synthetase with an average of 0.8 equiv of covalently bound 5′-adenylate group per dodecamer as determined by assay (Stadtman et al., 1979) and UV spectral methods (Ginsburg et al., 1970) was isolated from an overproducing strain of *E. coli* (*pgln6*/YMC10) bearing multiple copies of the plasmid containing the *glnA* gene obtained from Dr. B. Magasanik, Massachusetts Institute of Technology, by the purification procedure of Woolfolk et al. (1966). Final specific activities in the γ-glutamyl-transfer assay at pH 7.57 and 37 °C (Stadtman et al., 1979) and in the Mg^{2+}-supported biosynthetic assay at pH 7.8 and 30 °C (Ginsburg et al., 1970) were 135 ± 5 and 77 units/mg, respectively. Glutamine synthetase preparations were stored at 4 °C as a suspension in 50% $(NH_4)_2SO_4$ containing 5 mM $MnCl_2$ and 5 mM imidazole; protein was collected as needed by centrifugation and dialyzed vs. three changes of 1000-fold volumes of buffer, pH 7.1, which usually contained 20 mM Hepes/KOH, 100 mM KCl, and 1 mM $MnCl_2$.

Adenylyl transferase, purified by the procedure of Caban & Ginsburg (1976), and aza-ε-ATP were gifts from Dr. S. G. Rhee of this laboratory. Pyruvate kinase, phosphoenolpyruvate, and dithiothreitol were from Boehringer-Mannheim. Myokinase (adenylate kinase), ATP, 2-mercaptoethanol, L-methionyl-(*RS*)-sulfoximine (containing ~53% of the *S* diastereoisomer; Shrake et al., 1982), 6-mercaptopurine riboside monophosphate (barium salt), Hepes, Tris, PMPS, iodoacetamide, [4-[[*p*-(dimethylamino)phenyl]azo]phenyl]-mercuric acetate, and trinitrobenzenesulfonic acid (picrylsulfonic acid) were from Sigma Chemical Co. Dansyl chloride was from Pierce and 5-[[[(iodoacetyl)amino]ethyl]amino]-naphthalene-1-sulfonic acid (IAEDANS) was from Molecular Probes. Neohydrin was from ICN Pharmaceutical, Inc. Dowex 1-X8 (100-200 mesh) and Chelex 100 (100–200 mesh) resins were from Bio-Rad. Hepes/KOH (20 mM) buffer at pH 7.1 containing 100 mM KCl was purified by passage through a column (5 × 33 cm) of Chelex 100 resin in the K^+ form (Hunt & Ginsburg, 1980) and was used for dialysis of inactive glutamine synthetase complexes for which the metal ion content was to be quantitated. Water was distilled and then deionized and filtered through a Millipore Q2 reagent-grade system prior to use.

Preparation of Derivatives of ATP. Derivatives of ATP and ADP were either obtained commercially or synthesized by procedures based on methods previously published. Most derivatives were separated from starting materials and breakdown products (mostly dephosphorylation) on small Dowex 1-X8 (acetate form) columns at 4 °C. Elution was usually with linear LiCl gradients in 1–2 mM HCl, as specified below. The solid products were obtained as lithium salts by crystallization with ethanol. Purity was checked by UV or visible spectrophotometry and by thin-layer ascending chromatography on poly(ethylenimine)–cellulose (PEI C600, Brinkman) with 1 or 1.5 M LiCl or on cellulose (without fluorescent indicator; no. 6064, Eastman) with isobutyric acid/H_2O/concentrated NH_4OH (66/33/1). The starting material for 8-substituted ATP or ADP analogues was the 8-bromo nucleotide either obtained commercially (P-L Biochemicals) or synthesized by the method of Ikehara & Uesugi (1969). Briefly, 2 g of ATP or ADP was dissolved in 50 mL of H_2O, and 650 μL of Br_2 was added dropwise with vigorous stirring. The mixture was stirred at room temperature in the dark for 2 h, and then 0.35 g of sodium metabisulfate was added. The entire mixture was taken to dryness by rotary evaporation and washed 3 times with EtOH, which was removed each time by rotary evaporation. The caked residue was dissolved in 100 mL of H_2O and pumped onto a 1.5 × 40 cm column of Dowex 1-X8 (acetate) and eluted with a linear gradient formed from 1 L of 0.10 M LiCl in 1 mM HCl and 1 L of 0.30 M LiCl in 1 mM HCl.

Conversion of 8-bromo-ATP to 8-S-ATP was accomplished by dissolving 100 mg of 8-Br-ATP in a solution (2.5 mL) of 1 M LiHS, which was made by bubbling H_2S into 1 M LiOH until the pH was lowered to 9. The reaction solution was left at room temperature overnight, diluted with ~10 volumes of water, and pumped onto a 10-mL column of Dowex 1-X8 (acetate). The column was then washed with water and eluted with a linear gradient formed from 250 mL of 0.1 M LiCl containing 1 mM HCl and 250 mL of 0.5 M LiCl containing 1 mM HCl; after ~350 mL of eluant had run through the column, the gradient solutions were replaced with 250 mL each of 0.4 M LiCl and 1.5 M LiCl in 1 mM HCl and elution was continued overnight. Fractions that contained no ATP were pooled (A_{300} > 8–9) and concentrated on a rotary evaporator, after which 9 volumes of absolute ethanol was added; this solution was left overnight in a freezer at −20 °C. The precipitate was dissolved in 2 mL of water and reprecipitated with 24 mL of absolute ethanol. After the second precipitate was washed with 10 mL of ethanol, it was dried under vacuum at room temperature and then stored in a desiccator at −20 °C as a white crystalline powder.

8-[(Aminoethyl)amino]-ATP and 8-[(6-aminohexyl)-amino]-ADP were made by allowing 100 mg of the respective 8-bromo nucleotides to react with a 10-fold molar excess of diaminoethane or diaminohexane in 3 mL of aqueous solution at 60 °C for 5 h (pH was between 11 and 11.5). The reaction mixture was diluted with water and passed through a Dowex 1-X8 (acetate) column. The nucleotides were eluted with a gradient of LiCl (250 mL each of 0 and 0.5 M LiCl in 1 mM HCl) and crystallized with ethanol.

8-(Allylamino)-ADP was made by incubating 200 mg of 8-Br-ADP dissolved in 2 mL of H_2O with 2 mL of allylamine and 0.40 mL of concentrated HCl at 103 °C overnight. The reaction mixture was diluted and run over a Dowex 1-X8 column (1 × 20 cm) and the 8-(allylamino)-ADP was eluted with a gradient formed from 250 mL each of 0–0.5 M LiCl in 1 M acetic acid.

Preparation of 8-S-AEDANS-ATP. 8-S-ATP (5 mg) was dissolved in 0.10 mL of 0.1 M dipotassium phosphate, and 8 mg of IAEDANS was added. After 5 min, another 0.10 mL of 0.1 M K_2HPO_4 was added, and the reaction was allowed to continue for 3 h at room temperature. The reaction mixture was diluted to 10 mL with water and passed over a 2-mL column of Dowex 1-X8. After being washed with 1.5 M LiCl,

the AEDANS-ATP was eluted with a linear gradient made with 25 mL each of 1.5 and 3.0 M LiCl.

6-Mercaptopurine riboside triphosphate (6-S-ATP) was prepared from commercially available 6-mercaptopurine riboside monophosphate (6-S-AMP) as follows: 100 mg of 6-S-AMP was incubated at room temperature overnight in a volume of 20 mL containing 100 mM Tris-HCl at pH 8.0, 10 mM $MgCl_2$, 20 mM potassium phosphoenolpyruvate, 100 mM KCl, 6 mg of pyruvate kinase, 1 mM ATP, 2 mg of myokinase, and 2 mM DTT. After this solution was diluted 2-fold with water, it was pumped onto a 10-mL column of Dowex 1-X8 (acetate), and the column was washed with 3 volumes of water. A LiCl linear gradient elution was used with 250 mL each of 0.1 and 0.5 M LiCl containing 1 mM HCl; if necessary, LiCl was increased to 1 M in the gradient elution (6-S-ATP elutes last). Fractions containing the 6-S-ATP (monitored by absorbance at 320 nm) were pooled and precipitated twice with ethanol and dried as described for 8-S-ATP. The isolated 6-S-ATP had a white crystalline appearance and was free of ATP.

Preparation of Aquo(glycyl-L-methioninato)platinum(II). Two milliliters of a 1 mM solution of (glycyl-L-methioninato)platinum(II) chloride (synthesized by J. J. Lipka, Brookhaven National Laboratory, using the procedure of Freeman and Golomb) was incubated with a 2-fold molar excess of $AgNO_3$ at room temperature for 1 h and then at 4 °C overnight. The precipitate of AgCl was removed by centrifugation, and the clear supernatant liquid was passed through a 4-mL column of Chelex 100 (Bio-Rad) to remove any remaining Ag^+. The colorless effluent contained the aquo(glycyl-L-methioninato)platinum(II), which was stable for months when stored at 4 °C in the dark. No evidence of light sensitivity of the Pt(II) complex was observed.

Reaction of 8-Mercapto-ATP with Aquo(glycyl-L-methioninato)platinum(II). Solid 8-S-ATP (lithium salt) was added to 1 mM aquoPt(II) complex, the pH was adjusted to 6.5 with diluted acetic acid, and the reaction mixture was left at room temperature for 3 h followed by overnight incubation at 4 °C. Nine volumes of ethanol was added to precipitate the 8-S-ATP–Pt(II) adduct; the precipitate was collected by centrifugation, dissolved in H_2O, and reprecipitated with ethanol. Unreacted aquo-Pt(II) remained soluble in 90% ethanol. Quantitative conversion of 8-S-ATP to the Pt(II) adduct was confirmed by thin-layer chromatography on poly(ethylenimine)–cellulose and by spectrophotometric analysis (see text).

Protein and Enzymatic Assays. Concentrations of glutamine synthetase were determined from published absorption coefficients (Ginsburg et al., 1970); a value for the subunit molecular weight of 50 000 was used in calculations. In the presence of ATP or its analogues, enzyme concentrations were obtained with the Coomassie blue protein reagent from Bio-Rad (filtered through a 0.45 μm Millipore filter before use) with purified GS as the standard and keeping the time of color development (>15 min) constant. Enzymatic activity was measured in the glutamyl transferase reaction at pH 7.57 (Stadtman et al., 1979).

Partially or fully inactive glutamine synthetase complexes were prepared by incubating the enzyme at pH 7.1 (25–37 °C) in the presence of 2 mM (*RS*)-L-Met-sulfoximine, 20 mM Hepes/KOH, 100 mM KCl, and 1 mM $MnCl_2$ with substoichiometric or excess amounts of either ATP or ATP analogue, respectively (Maurizi & Ginsburg, 1982a,b). Inactivation was monitored by enzymatic assay, and incubations were continued overnight at 4 °C to be certain that reactions were complete. With excess ATP or ATP analogues, >99% of the initial enzyme activity was lost, indicating that transition-state complexes were on all sites of the dodecamer. The enzyme complexes were stable at pH 7.1 during analysis and storage at 4 °C; after as long as 8 months storage at 4 °C, there was no detectable dissociation of the inactivating ligands.

Adenylylation of Glutamine Synthetase with 6-S-ATP. Glutamine synthetase (~8.5 mg) was incubated at 37 °C (pH 8) in 5 mL with 2 mM 6-S-ATP, 25 mM $MgCl_2$, 10 mM L-glutamine, and ~1000 units of adenylyl transferase (Ginsburg et al., 1970) for 2 h, after which time 5 mg more of 6-S-ATP was added, and incubation was continued at 4 °C overnight. The extent of adenylylation was assayed by the pH 7.57 γ-glutamyl transfer ± Mg^{2+} method of Stadtman et al. (1979). Glutamine synthetase was protected from light and repurified by twice precipitating in 30% (v/v) $(NH_4)_2SO_4$ at pH 4.45 (Woolfolk et al., 1966) and then dialyzing against 20 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.2.

Instrumentation. Absorbance measurements were made with a Perkin-Elmer Model 320 UV–visible spectrophotometer using black self-masking semimicrocuvettes or with a Hewlett-Packard Model 8450A spectrophotometer equipped with a 89100A temperature controller. Fluorescence measurements were made with a Perkin-Elmer Model 650-40 spectrofluorometer. Polarization measurements were made with a SLM-AMINCO Model 8000 spectrofluorometer. Solution temperatures in Perkin-Elmer instruments were maintained at ±0.2 °C by circulating constant temperature water through cell blocks. The Pt(II) and/or Mn^{2+} content of inactive glutamine synthetase complexes was quantitated on the Perkin-Elmer Model 603 atomic absorption spectrophotometer equipped with an HGA-2100 graphite furnace; sensitivities for Pt(II) $\simeq 10^{-6}$ M and for $Mn^{2+} \simeq 10^{-7}$ M.

Ultracentrifugation at 20 °C was performed as described by Shrake et al. (1980) with enzyme samples dialyzed against buffer of the same composition (20 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.1). The procedure of Howlett & Schachman (1977) was used for computing the difference in sedimentation coefficient (Δs) in a two-cell experiment with a precision of ±0.1%; $s_{20,w}$ = 21.0 S for unadenylylated glutamine synthetase (Shrake et al., 1980). Values for apparent specific volumes of 0.51 mL/g for L-Met-(*S*)-sulfoximine phosphate and 0.42 mL/g for ADP were used to correct for the change in buoyant weight due to ligand binding (Kirschner & Schachman, 1971).

Results and Discussion

Some Properties of the Inactive Glutamine Synthetase Complex.[2] A number of properties of the inactive complex of glutamine synthetase with L-Met-(*S*)-sulfoximine phosphate, ADP, and two Mn^{2+} indicate that the binding of the ligands to the active site perturbs the local conformation at the active site and has a large effect on the overall structure of the

[2] Definition of inactive complex: Mn–GS catalyzes a single turnover of phosphorylation of L-Met-(*S*)-sulfoximine by using ATP or analogues of ATP. The products L-Met-(*S*)-sulfoximine phosphate, ADP (or analogue), and two Mn^{2+} are tightly bound at the subunit active site ($K_A' > 10^{12}$), forming a catalytically inactive complex. From 1 to 12 subunits of the dodecameric GS can be inactivated. In this paper the inactive complexes will be named only with the nucleotide analogue; thus, "6-S-ADP inactive complex" refers to GS with 6-S-ADP, L-Met-(*S*)-sulfoximine phosphate, and two Mn^{2+} bound at each active site. GS with fewer than 12 subunits inactivated will be referred to as "*X*% inactive complex"; thus, an average of three inactivated subunits per dodecamer would be "25% inactive complex".

enzyme. Previous reports showed that inactive complexes of both the unadenylylated and the adenylylated enzymes are more resistant to denaturation at low pH and in 6 M guanidine hydrochloride (Maurizi & Ginsburg 1982a,b, 1985). In the present study, difference sedimentation velocity measurements at pH 7.1 of the fully inactive complex of unadenylylated enzyme vs. active, unadenylylated Mn·GS gave $\Delta s = 0.72$, corresponding to $(\Delta s/s)_{obsd} = 3.6\%$ and $(\Delta s/s)_{cor} = 1.1\%$ after correcting for increased buoyant weight due to ligand binding. Thus, the inactive enzyme complex has a substantially more compact structure than has the native Mn–enzyme. This value of $(\Delta s/s)_{cor}$ for the enzyme with the transition-state complex bound to all active sites is 2.4-fold greater than that previously observed for the binding of L-Met-(*RS*)-sulfoximine alone to the unadenylylated Mn enzyme (Shrake et al., 1980).

An altered conformation of the adenylylated enzyme upon active site ligand binding was also indicated by the finding that the 5′-AMP groups from the inactive, adenylylated enzyme complex were not removed either by incubation with the α-ketoglutarate-stimulated deadenylylation system of *E. coli* (Stadtman & Ginsburg, 1974) or by treatment with snake venom phosphodiesterase. Since either procedure normally removes covalently bound 5′-AMP groups from active glutamine synthetase, the presence of the transition-state complex at active sites must make the phosphodiester linkage of 5′-AMP groups at adenylylation sites inaccessible to enzymatic cleavage. Conversely, the adenylylation site of the unadenylylated enzyme in the presence of Mn^{2+}, L-Met-(*S*)-sulfoximine, and ATP is unavailable for enzyme-catalyzed adenylylation (Hunt & Ginsburg, 1980).

The inactive complex of glutamine synthetase also was found to be completely resistant to oxidation during incubation with Fe^{2+}, ascorbic acid, and O_2 (data not shown), under which conditions the unliganded enzyme is inactivated (Levine, 1983). The effect may be due to the inability of Fe^{2+} to bind to the inactive complex at one of the divalent metal ion sites occupied by Mn^{2+}, or it could reflect a shielding of the active site histidine, which is the target of the putative reaction radical in this oxidative reaction. In order to determine how active site ligand binding affects the structural stability of glutamine synthetase and how interactions between active sites or between active sites and other protein groups occur, we have attempted to introduce various structural probes into active sites of the enzyme.

Interactions with Analogues of ATP with Substitutions at the 8- or 6-Position of the Purine Ring. Table I gives apparent dissociation constants for the reversible complexes formed between unadenylylated, manganese glutamine synthetase, and various analogues of ATP and of ADP that are substituted at the 8- or 6-position of the purine ring. The K_D' values in Table IA were calculated from titration data obtained at pH 7.1 and 25 °C by either protein tryptophanyl fluorescence enhancement or nucleotide absorbance increases with the mercapto-ATP analogues (Figures 1 and 2 below) to monitor the extent of binding of each nucleotide to the enzyme. Table IB shows K_m values of the unadenylylated enzyme for ADP and for 8-[(6-aminohexyl)amino]-ADP and for 8-(allylamino)-ADP. The K_m values (pH 7.57 and 37 °C) are K_D' values under assay conditions since ADP is a nonconsumable substrate in the γ-glutamyl transfer reaction (Hunt et al., 1975).

Substitution at the 8-position of the purine ring of ATP produces a 7–60-fold decrease in the nucleotide binding affinity to the Mn–enzyme. However, all of the ATP analogues listed in Table IA, which includes 6-mercaptopurine ribonucleoside triphosphate (6-S-ATP), bind to glutamine synthetase with $K_A' \geq 2 \times 10^4\ M^{-1}$. Although the biosynthetic activity of the enzyme with each of the ATP analogues of Table IA was not tested, all of these ATP analogues serve as substrates for the phosphorylation of L-Met-(*S*)-sulfoximine in the presence of Mn^{2+}. Thus, each of these ATP analogues can form a tightly bound transition-state complex on each subunit of the enzyme (Maurizi & Ginsburg, 1982a). At neutral pH, there was no measurable dissociation of the various ADP analogues bound

Table I: Affinities of Unadenylylated, Manganese Glutamine Synthetase for ATP and ADP Analogues

Part A

ATP and ATP analogues	K_D' (μM)[a]
ATP	0.7
8-bromo-ATP (8-Br-ATP)	5.0
6-mercaptopurine ribonucleoside triphosphate (6-S-ATP)	17
8-mercapto-ATP (8-S-ATP)	28
8-[(aminoethyl)amino]-ATP (8-AE-ATP)	37
8-mercapto-ATP alkylated with *N*-(acetylamino)-5-naphthylamine-1-sulfonic acid (8-S-AEDANS-ATP)	44

Part B

ADP and ADP analogues	K_m (nM)[b]
ADP	40
8-[(6-aminohexyl)amino]-ADP	70
8-(allylamino)-ADP	70

[a] Titrations of the enzyme were performed in 20 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.1 at 25 °C. Monitoring either protein tryptophanyl residue fluorescence enhancement (excitation = 300 nm; emission = 350 nm) or absorbance changes with 6-S-ATP or 8-S-ATP (see Figures 1 and 2). A stoichiometry of binding was assumed to be 1 equiv per subunit in calculating K_D' values from Δ/Δ_{max} vs. nucleotide concentration curves. [b] K_m values were determined by the Mn^{2+}-supported γ-glutamyl transfer assay at pH 7.57 and 37 °C (Stadtman et al., 1979) with all substrates except ADP or the ADP analogue at saturating concentrations.

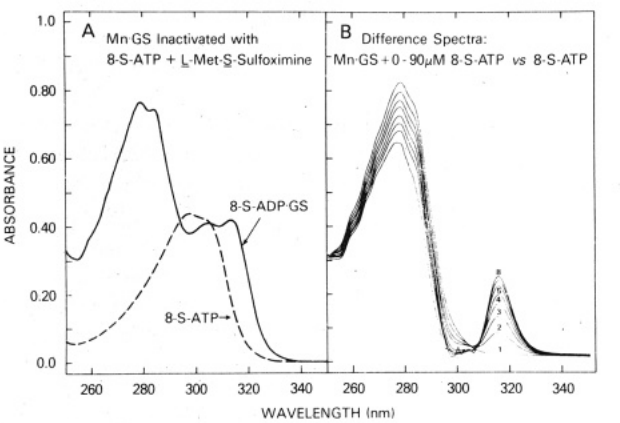

FIGURE 1: Spectral shift of 8-S-ATP on binding to unadenylylated glutamine synthetase. Spectra in (A) and (B) were recorded at 25 °C in a buffer of 20 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$ at pH 7.1. In (A), spectra of free 8-S-ATP and of glutamine synthetase (~0.85 mg/mL), which had been fully inactivated with 8-S-ATP, Mn^{2+}, and L-Met-(*S*)-sulfoximine, are shown by the dashed and solid lines, respectively. In (B), difference spectra were obtained by titrating glutamine synthetase (~15 μM subunit concentration) with 8-S-ATP in the sample cuvette while adding equivalent amounts of 8-S-ATP to the same volume of buffer in the reference cuvette. For difference spectra 1–8, 0, 10, 20, 30, 42, 56, 70, and 90 μM 8-S-ATP was added, respectively. The addition of 2 mM L-Met-(*RS*)-sulfoximine at the end of the titration of the enzyme produced no further spectral change.

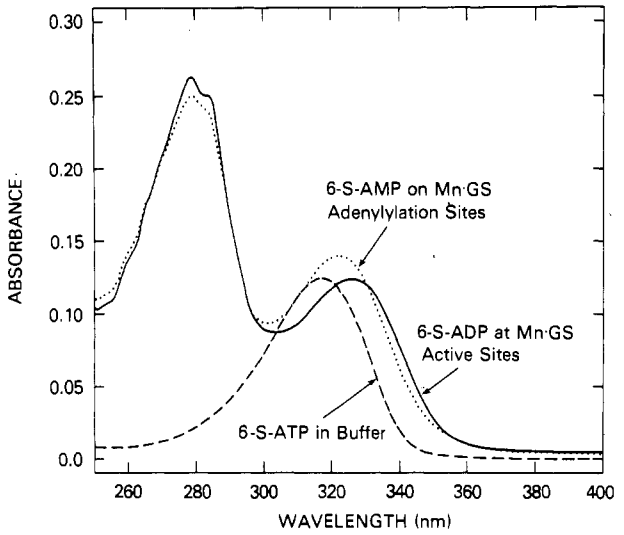

FIGURE 2: Spectral shifts of 6-mercaptopurine nucleotides on binding to specific sites of glutamine synthetase. Spectra were recorded at 25 °C in a buffer of 20 mM Hepes/KOH, 100 mM KCl, and 1 mM MnCl at pH 7.1. The dashed curve is the spectrum of free 6-S-ATP in this buffer (see Table II). The solid curve for "6-S-ADP at active sites" is the spectrum of unadenylylated glutamine synthetase (~0.33 mg/mL) after complete inactivation with 6-S-ATP, Mn^{2+}, and L-Met-(*S*)-sulfoximine and dialysis of the inactive enzyme. The dotted curve for "6-S-AMP on adenylylation sites" is the spectrum of manganese glutamine synthetase after adenylylation with 6-S-ATP, repurification, and dialysis; the final apparent adenylylation state of glutamine synthetase was 10.6 by the glutamyl transfer assay method ± Mg^{2+} at pH 7.57 of Stadtman et al. (1979).

Table II: Spectral Changes in Mercapto Nucleotides on Binding to Glutamine Synthetase[a]

species	metal present	λ_{max} (nm)	ϵ (M^{-1} cm^{-1})	$\Delta\epsilon_{max}(\lambda)$
8-S-ATP in buffer (pH 7.1)	±Mn^{2+}	297	27 000[b]	
8-S-ATP in buffer (pH 4.5)	0	297, 305	27 000[b]	
8-S-ADP on GS active site (pH 7.1)	±Mn^{2+}	303, 313	27 000	17 000 (316)
6-S-ATP in buffer (pH 7.1)	0	320	22 000	
	Mn^{2+}	317	19 400	
	Mg^{2+}	319	21 400	
6-S-ATP in buffer (pH 4.5)	±Mn^{2+}	321	22 000	
6-S-ADP on GS active site (pH 7.1)	±Mn^{2+}	326	22 000	9 000 (337)
6-S-AMP on GS adenylylation site (pH 7.1)	Mn^{2+}	322	~22 000	

[a] Spectral measurements were made at 25 °C in buffer at pH 7.1 containing 20 mM Hepes/KOH and 100 mM KCl in the absence or presence of 1.0 mM $MnCl_2$ or of 10 mM $MgCl_2$ (as indicated); measurements of the free nucleotide analogues were also made in 50 mM sodium acetate buffer at pH 4.5. Fully inactive glutamine synthetase (GS) derivatives (Figures 1 and 2) were dialyzed against buffer at pH 7.1 in the absence of Mn^{2+}; the adenylylated GS derivative (Figure 2) was dialyzed against buffer at pH 7.1 containing 1.0 mM $MnCl_2$. When protein was present, light scattering corrections, with either 340 or 400 nm as the wavelength of zero absorbance, were applied to spectra (Ginsburg et al., 1970). [b] Literature value (Ikehara et al., 1973).

in the inactive complexes after prolonged dialysis or storage at 4 °C for several months. Consequently, loosely bound or unbound nucleotide analogues were easily separated from the enzyme after inactivation reactions with ATP analogues and L-Met-(*S*)-sulfoximine.

The reactive thiol or amino groups of these ATP analogues were used to form adducts with a number of compounds that might serve as structural probes of the active site. In addition to the 8-S-AEDANS-ATP, adducts formed with trinitrobenzenesulfonate, dansyl chloride, (glycyl-L-methioninato)-platinum(II) (see below), [4-[[*p*-(dimethylamino)phenyl]-azo]phenyl]mercuric acetate (see below), *p*-mercuribenzenesulfonate, and several others were found to bind to the enzyme and to react with L-Met-(*S*)-sulfoximine to form the various inactive enzyme derivatives. By partially or fully inactivating glutamine synthetase with various ATP analogues in the presence of L-Met-(*S*)-sulfoximine and Mn^{2+}, we have been able to specifically "label" active sites of the enzyme with appropriate reporter groups.

The 8-(allylamino)-ADP was used to devise an analytical method for the separation of hybrid dodecamers of glutamine synthetase containing both unadenylylated and adenylylated subunits. The separation method was based on the fact that in the presence of arsenate (or orthophosphate) adenylylated subunits have ~2100-fold lower affinity for ADP than do unadenylylated subunits (Hunt et al., 1975). 8-(Allylamino)-ADP was incorporated into 5% polyacrylamide gels during polymerization. When glutamine synthetase was run in ADP-derivatized polyacrylamide gels in the presence of Mn^{2+} and arsenate at pH 7.8, a good separation of the fully adenylylated enzyme, the unadenylylated enzyme, and enzyme with an average state of adenylylation of 6 was obtained. Because the protein bands were diffuse, enzymes with small differences in states of adenylylation were not resolved, and efforts to optimize this procedure were not pursued. In the meantime, Chung & Rhee (1984) have obtained separation of glutamine synthetase species of different states of adenylylation by chromatography on monoclonal anti-AMP antibody affinity columns.

Spectral Shifts of Mercapto Nucleotides on Binding to Glutamine Synthetase. The absorbance spectra of 8-S-ATP and 6-S-ATP are substantially red-shifted upon binding to manganese glutamine synthetase at pH 7.1. Figure 1A shows spectra at pH 7.1 of free 8-S-ATP and of inactive complex with 8-S-ADP. The change in the extinction coefficient at 316 nm when 8-S-ATP or 8-S-ADP is bound to the active site of each subunit is 17 000 M^{-1} cm^{-1} (Table II). Figure 1B illustrates difference spectra obtained by adding 10–90 μM 8-S-ATP to the enzyme solution vs. the same amount of 8-S-ATP in buffer in the reference cuvette. At the end of the titration, the addition of excess L-Met-(*S*)-sulfoximine produced no further spectral change, showing that the enzyme was saturated with the nucleotide and that the spectral shifts are the same for the reversibly and the irreversibly bound analogues.

Figure 2 shows the shifts in the spectra of 6-mercapto nucleotides interacting with the Mn–enzyme at pH 7.1. A value of $\Delta\epsilon$ = 9000 M^{-1} cm^{-1} at 337 nm was obtained for both the reversible binding of 6-S-ATP and the irreversible binding of 6-S-ADP in the inactive complex (Table II). When 6-S-AMP was attached enzymatically to ~10 of the 11 available adenylylation sites of glutamine synthetase, the spectrum of 6-S-AMP was also red-shifted, but to a smaller degree than when 8-S-ADP was bound to the substrate site (Figure 2). The different absorbance maxima for 6-S-AMP on adenylylation sites (322 nm) and 6-S-ADP at active sites (326 nm) indicate that the environments of the 6-mercapto nucleotide are different at these sites. Earlier studies of Rhee et al. (1981) have shown that the fluorescence spectrum of aza-ϵ-AMP at the

adenylylation site is substantially different from that of free aza-ε-ATP. Thus, the local environment of the nucleotide base at the adenylylation site also differs from that of the solvent pool, although NMR studies of Villafranca et al. (1978) indicate that 5′-adenylate groups at adenylylation sites are free to rotate.

Table II summarizes spectral changes that occur when 8-S-ATP or 6-S-ATP binds to manganese glutamine synthetase at pH 7.1. Enzyme-bound mercapto nucleotides have acidic-like spectra (thioenol form), but the red shifts produced by binding either 8-S-ATP or 6-S-ATP to active sites are greater than those obtained by acidifying solutions of these nucleotide analogues. These spectral perturbations also apparently reflect an increased environmental hydrophobicity (or water exclusion) due to interactions between nucleotide bases and amino acid side chains proximal to active sites. Mercaptonucleotides also contribute to the protein absorbance at 280 nm (Figures 1 and 2).[3]

In the case of the binding of 6-S-ATP (but not of 8-S-ATP) to the enzyme in the presence of Mn^{2+}, a small portion of the observed spectral shift is due to the disruption of a complex between unbound Mn^{2+} and 6-S-ATP (Table II). The λ_{max} of 6-S-ATP in pH 7.1 buffer without Mn^{2+} is 320 nm, whereas in the presence of Mn^{2+} λ_{max} is 317 nm and the extinction coefficient is ~12% lower. In contrast, Mn^{2+} has no effect on the spectrum of 6-S-ADP bound to active sites at pH 7.1 and little effect on the spectrum of free 6-S-ADP at pH 4.5. These observations are consistent with 6-S-ADP or 6-S-ATP being in an acidic form with a decreased affinity for Mn^{2+} when bound to enzyme active sites, since the mercapto group of the bound nucleotide analogue is accessible (see below). Mg^{2+} (10 mM) produced a smaller blue shift in the spectrum of free 6-S-ATP at pH 7.1 (Table II) than that observed with 1 mM Mn^{2+}. In other experiments (not shown), the spectral shifts of 6-S-ADP and 8-S-ADP when bound to inactive complexes of unadenylylated magnesium glutamine synthetase were very similar to those observed with the manganese enzyme. Also, spectra of inactive complexes formed with the fully adenylylated, manganese enzyme and mercapto nucleotides were red-shifted.

Accessibility of the 8- and 6-Positions of the Nucleotide When Bound at Active Sites or at Adenylylation Sites. Table I shows that the active site of each subunit of glutamine synthetase also can accommodate large substituents attached to the 8-position of the adenine ring of ATP which is consistent with earlier studies showing that glutamine synthetase has a low specificity for binding nucleotides at the active site. Enzyme activity is expressed with nucleotides containing the purine bases adenine, guanine, and inosine (Rhee et al., 1976; Whitley & Ginsburg, 1980). The site at which the nucleotide base interacts with this enzyme therefore appears to be somewhat flexible. We wanted to know how accessible the nucleotide base bound at the active site is to reaction with exogenously added compounds. Because the protein sulfhydryl groups are buried and unreactive in the liganded enzyme, the 6- and 8-mercapto nucleotide inactive complexes were studied. We have found that both the 8- and 6-positions of these nucleotide analogues at enzyme active sites are sufficiently free to allow rapid and complete reaction of the nucleotide thiol with alkylating reagents (such as iodoacetamide and IAEDANS) and with mercurial reagents (such as PMPS and neohydrin).

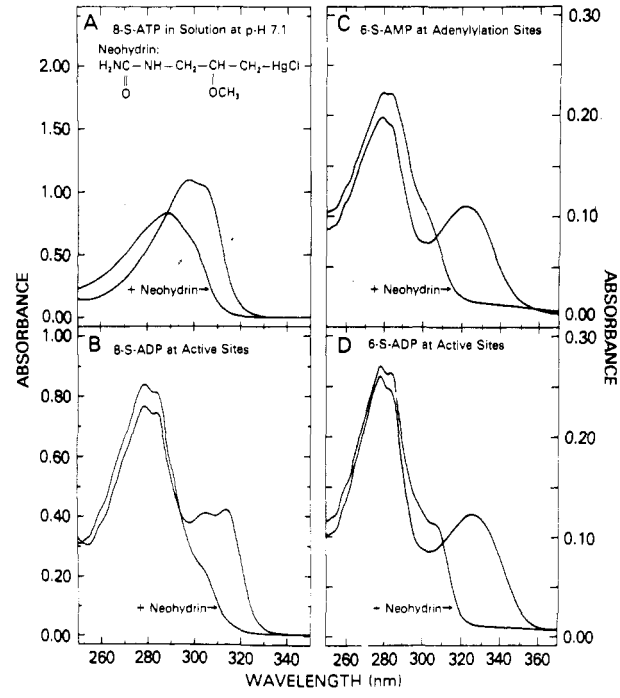

FIGURE 3: Accessibility of the 8- and 6-positions of enzyme-bound mercapto nucleotides: spectral shifts observed on reaction of the mercurial reagent neonhydrin with the thiol group of 8-S-ADP or 6-S-ADP at enzyme active sites (B and D), with free 8-S-ATP (A), or with 6-S-AMP attached to enzyme adenylylation sites (C). Spectra were recorded at 25 °C before and after addition of a 25-fold excess of neohydrin to mercapto nucleotide. For (A), (B), and (D), the buffer was 20 mM Hepes/KOH and 100 mM KCl, pH 7.1; for (C), the buffer also contained 1.0 mM $MnCl_2$. In (B) and (D), glutamine synthetase was completely inactivated with 8-S-ATP or 6-S-ATP, Mn^{2+}, and L-Met-(*S*)-sulfoximine and then exhaustively dialyzed. In (C), the enzyme with 6-S-AMP groups attached to adenylylation sites is described in the legend to Figure 2. In (A), the addition of stoichiometric amounts of neohydrin to free 8-S-ATP produced the maximum shift (see text).

Figure 3 shows that reactions of mercurial reagents with 8- or 6-mercapto-ATP can be detected by large blue shifts in the nucleotide spectra that accompany these reactions. Similar spectral shifts were produced by reaction of the mercurial reagent neohydrin at pH 7.1 with free 8-S-ATP (panel A), with 8-S-ADP at active sites of glutamine synthetase (panel B), with 6-S-AMP at adenylylation sites of glutamine synthetase (panel C), and with 6-S-ADP at active sites of the enzyme (panel D). Also, similar spectral shifts were observed when the mercurial compound was PMPS or the chromophore [4-[[*p*-(dimethylamino)phenyl]azo]phenyl]mercuric acetate used in fluorescence energy transfer experiments (see below). The spectral shifts of mercapto nucleotides produced by mercurial compounds (illustrated in Figure 3) were readily reversed by the addition of a 10-fold excess glutathione or 2-mercaptoethanol to displace the mercurial from the nucleotide thiol group (data not shown). Alkylating reagents such as iodoacetamide and IAEDANS also produced blue spectral shifts in the spectra of free and enzyme-bound mercaptonucleotides.

It was observed that excess neohydrin (~10-fold) to enzyme-bound mercaptonucleotides was required to produce the spectral shifts shown in Figure 3B,D, whereas a stoichiometric amount of neohydrin added to free 8-S-ATP or 6-S-ATP produced the maximum spectral change. This requirement for excess neohydrin appears to be due to nonspecific binding

[3] Whereas the native unadenylylated enzyme (containing 0.8 equiv of covalently bound 5′-adenylate groups per dodecamer) has a specific absorbance of $A^{0.1\%}_{280nm,1cm}$ = 0.738 (Ginsburg et al., 1970), enzyme inactivated with L-Met-(*S*)-sulfoximine, Mn^{2+}, and ATP, 8-S-ATP, or 6-S-ATP had corresponding 280-nm absorbance values of approximately 0.78, 0.94, or 1.04, respectively.

Table III: Quenching of Tryptophanyl Residue Fluorescence by Mercapto Nucleotides Bound at GS Active Sites with L-Met-(*S*)-sulfoximine Phosphate and Mn^{2+}[a]

(A) Inactivation with Varying [6-S-ATP] Followed by Inactivation with ATP

6-S-ADP/GS subunit	ΔA_{337nm}	% change in A_{337nm}	% activity loss	relative fluorescence after inactivation with ATP[b]	% of maximum fluorescence enhancement[b]	% fluorescence quench
0	0	0	0	96.5	100	0
0.25	0.046	26	23	79	73	27
0.50	0.091	47	42	62	46	54
0.75	0.135	74	60	49	25	75
1.20	0.201	100	96	33	0	100

(B) Effect of 100% Inactivation with ATP or with ATP Analogues

enzyme complex	additions	relative fluorescence (obsd)	% of maximum enhancement[b]	% quench
(a) Mn·GS (active)	±50 μM neohydrin	33	0	
(b) Mn·ADP·GS (inactive)	±50 μM neohydrin	100	100	0
(c) Mn·6-S-ADP·GS (inactive)	none	33	5	95
	50 μM neohydrin	70[c]	64	36
(d) Mn·8-S-ADP·GS (inactive)	none	47	33	67
	50 μM neohydrin	73[c]	79	21

[a] Glutamine synthetase (GS) tryptophanyl residue fluorescence was measured at 25 °C, 350-nm emission with 300-nm excitation, at 2.0 μM subunit in 20 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$ buffer at pH 7.1. In part A, GS was inactivated by incubation with 1 mM L-Met-(*S*)-sulfoximine and varying molar ratios of 6-S-ATP/subunit; the fluorescence was measured before and after excess ATP was added to completely inactivate the enzyme. The absorbance change at 337 nm was used to determine the amount of 6-S-ADP bound (see Figure 2), and activity measurements to determine the percent inactivation were made before ATP addition. In part B, the relative fluorescence of the native enzyme and of inactive GS complexes [containing L-Met-(*S*)-sulfoximine phosphate, Mn^{2+}, and nucleotide disphosphate] was measured before and after the addition of a 25-fold excess of neohydrin, which blue shifts the spectra of mercapto nucleotides (Figure 3). [b] Fluorescence measurements were corrected for the inner filter effect obtained by adding 2 μM free mercapto nucleotides to enzyme fully inactivated with ATP; corrections of 8% for 6-S-ATP and 15% for 8-S-ATP were used. [c] Effects of neohydrin on spectra or on fluorescence were reversed completely by the addition of 500 μM 2-mercaptoethanol (10-fold excess –SH to the mercurial reagent present), whereas 2-mercaptoethanol addition to the native enzyme had no effect on these properties.

of the mercurial to the protein since a titration of free 8-S-ATP (2 μM) in the presence of the inactive Mn–enzyme complex containing ADP at active sites (~2 μM in subunits) also required ~10-fold excess neohydrin. Since the sulfhydryl groups of the liganded enzyme are not available (Shapiro & Stadtman, 1967; Maurizi & Ginsburg, 1982b), it is possible that the mercurials bind to protein nitrogenous groups as reported for aspartate transcarbamoylase (Hunt et al., 1984). Because excess mercurial is required for complete reaction of enzyme-bound mercapto nucleotides, the mercurial reaction cannot be used to introduce the electron-dense Hg(II) at specific enzyme sites (see below).

Proximity of Tryptophanyl Residue(s) to the Subunit Active Site. The tryptophanyl residue fluorescence of the inactive glutamine synthetase complex (containing 2 equiv of Mn^{2+} and 1 equiv each of ADP and L-Met-(*S*)-sulfoximine phosphate per subunit; Maurizi & Ginsburg, 1982a) is ~2.5-fold enhanced over that of the native Mn enzyme (Table III, part B). This enhancement of intrinsic tryptophanyl fluorescence was shown previously to be a linear function of the extent of inactivation of the dodecameric enzyme by L-Met-(*S*)-sulfoximine, ATP, and Mn^{2+} (Maurizi & Ginsburg, 1982b), indicating that irreversible ligand binding to active sites does not perturb tryptophanyl fluorescence in active subunits adjacent to inactive subunits.

When the inactive complex is formed with 6-S-ATP, L-Met-(*S*)-sulfoximine, and Mn^{2+}, there is no enhancement of tryptophanyl residue fluorescence (Table III). When the enzyme was inactivated with varying substoichiometric amounts of 6-S-ATP in the presence of excess Mn^{2+} and L-Met-*S*-sulfoximine (Table III, part A), the amount of 6-S-ADP bound to active sites (measured from ΔA_{337nm}) paralleled activity loss. The enzyme was then further inactivated with ATP and L-Met-(*S*)-sulfoximine and the tryptophanyl fluorescence measured. The apparent quenching of tryptophan fluorescence by 6-S-ADP was determined from the decrease in maximal fluorescence enhancement, and the quench closely paralleled the amount of 6-S-ADP bound. Part B of Table III shows relative fluorescence values for the active Mn–enzyme and for fully inactive Mn–enzyme complexes containing ADP, 6-S-ADP, or 8-S-ADP at all active sites and the effects of a mercurial reagent (neohydrin) on the protein tryptophanyl fluorescence. The percent of maximum enhancement (100% for the inactive Mn·ADP·GS complex), corrected for inner filter effects, and the corresponding percent quench are shown for each enzyme complex. The presence of 6-S-ADP at active sites completely quenched the expected enhancement of tryptophanyl fluorescence (Table III, parts A and B) while 8-S-ADP at active sites results in a 67% quench of the expected enhancement.

The quench of intrinsic tryptophanyl residue fluorescence by the substitution of 6-S-ADP or 8-S-ADP for ADP in the inactive complex appears to be due to energy transfer from one or two tryptophanyl residues per subunit[4] to 6-S-ADP or 8-S-ADP rather than to differences in the enzyme conformation promoted by the binding of nucleotide analogues for the following reasons: (1) other analogues of ATP (e.g., 8-Br-ATP and 8-AE-ATP) that form inactive complexes at the active site with L-Met-(*S*)-sulfoximine and Mn^{2+} produce similar enhancements of tryptophan fluorescence. Thus, the final conformation of the inactive complex is somewhat independent of the ATP analogue used. (2) The overlap integral between the tryptophan emission (λ_{max} = 345 nm) and the absorption spectra of the mercapto nucleotides correlates well with the apparent quench observed: 67% for 8-S-ADP (λ_{max} = 315 nm) and ~100% for 6-S-ADP (λ_{max} = 326 nm). (3) Addition of mercurials, which have no effect on tryptophan

[4] Each subunit of the dodecamer contains two tryptophanyl residues (Levine, 1983) and one active site (Hunt et al., 1975). Studies are in progress to determine whether the binding of ATP or ATP analogues to the subunit active site influences the fluorescence properties of one or both tryptophans.

fluorescence of the inactive enzyme complex formed with ADP, shifts the absorption spectra of the mercapto nucleotides to the blue, thereby decreasing the overlap with the tryptophan emission and partially reversing the quench. Because there are two tryptophan residues per subunit and the relative contribution of each to the protein fluorescence is not yet known, it is not possible from these data to determine the distances between the nucleotides and the tryptophans. However, the extent of the quenching, especially with 6-S-ADP, suggests that at least one of the two tryptophanyl residues is very close to the nucleotide binding site at the active site.

Results with the fluorescent ATP analogue aza-ϵ-ATP confirm the proximity of one or two tryptophans to this nucleotide analogue bound to the subunit active site. The fluorescence excitation spectra of free aza-ϵ-ATP and aza-ϵ-ADP bound in an inactive complex on glutamine synthetase were recorded by monitoring the emission at 470 nm. Normalization of the spectra relative to the excitation maxima at 353 nm (for free aza-ϵ-ATP) and at 360 nm (for enzyme bound aza-ϵ-ADP) revealed a greater than 2-fold enhancement of emission for the enzyme-bound analogue when excitation was at 280 nm. This donor-dependent enhancement of acceptor fluorescence can only occur by energy transfer from a protein chromophore (probably tryptophan) to the aza-ϵ-ADP. Fluorescence polarization measurements of emission at 460 nm for enzyme-bound aza-ϵ-ADP showed that when excitation was at 360 nm the polarization was 0.32 ± 0.05, indicative of a partially immobilized ligand. Polarization measured with excitation at 300 nm, however, was <0.04. This apparent excitation wavelength dependence of the polarization of aza-ϵ-ADP fluorescence is expected if energy transfer occurs (at the lower wavelength excitation) between tryptophan and aza-ϵ-ADP. Rhee et al. (1981) have shown that energy transfer also occurs from tryptophan to aza-ϵ-AMP attached at the adenylylation site of the enzyme. We are currently attempting to take advantage of mercapto nucleotides and aza-ϵ-ATP to locate the tryptophan residues more precisely.[4]

In contrast to the above results obtained with aza-ϵ-ATP, there was no evidence of energy transfer from protein tryptophan to 8-S-AEDANS-ATP bound at active sites. The relative fluorescence of free and bound 8-S-AEDANS-ATP at 490-nm emission was the same with excitation at either 280 or 340 nm. This suggests that the AEDANS adduct at the 8-position of the bound nucleotide is farther from tryptophan residue(s) than aza-ϵ-ATP.

Evidence for Fluorescence Energy Transfer between Active Sites of Dodecameric Glutamine Synthetase. For the experiments in Figure 4, glutamine synthetase was 25% inactivated by incubation with L-Met-(*S*)-sulfoximine, Mn^{2+}, and a limiting concentration of 8-S-AEDANS-ATP, which had been prepared by alkylation of 8-mercapto-ATP with IAEDANS (Hudson & Weber, 1973). The fluorescence of this dialyzed enzyme derivative (containing an average of three 8-S-AEDANS-ADP groups per dodecamer) was measured at pH 7.1 in the absence and presence of ATP or Y-ATP [6-S-DABPM-ATP, a mercaptide adduct prepared by the reaction of 6-S-ATP with the chromophore [[4-[*p*-(dimethylamino)-phenyl]azo]phenyl]mercuric acetate]. ATP alone or ATP plus L-Met-(*S*)-sulfoximine binding to adjacent subunits has no effect on the fluorescence of AEDANS-ATP in the enzyme (data not shown). In fact, prior addition of ATP and L-Met-(*S*)-sulfoximine blocks the remaining active sites and prevents Y-ATP from binding (Figure 4A). The relatively small decrease in the fluorescence of the enzyme-bound 8-S-AEDANS-ADP in Figure 4A was due to the high absorbance of free Y-ATP ($\epsilon = 22\,000\ M^{-1}\ cm^{-1}$ at $\sim$460 nm); the dashed line in Figure 4 indicates this correction for an inner filter effect. As illustrated in Figure 4B, the fluorescence of the enzyme-bound 8-S-AEDANS-ADP was partially quenched when μM Y-ATP was added. The subsequent addition of ATP in Figure 4B reversed this effect of Y-ATP, which indicates that ATP competes with Y-ATP and that the quench produced by Y-ATP was due to the binding of Y-ATP to available active sites. When L-Met-(*S*)-sulfoximine was added to react with Y-ATP to form an inactive complex at available active sites (Figure 4C), the fluorescence of enzyme-bound 8-S-AEDANS-ADP was quenched to a greater extent and the subsequent addition of ATP caused no change since Y-ADP cannot be displaced from the inactive enzyme complex by ATP.

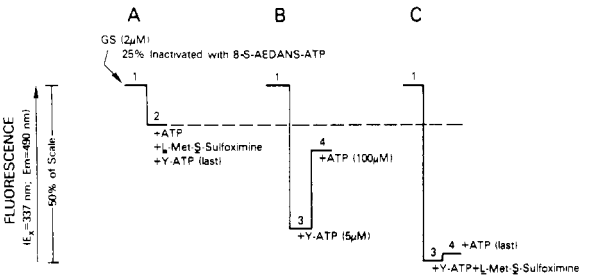

FIGURE 4: Evidence for fluorescence energy transfer between active sites on different subunits of dodecameric glutamine synthetase. For the experiments in this figure, glutamine synthetase was first 25% inactivated with 8-S-AEDANS-ATP, Mn^{2+}, and L-Met-(*S*)-sulfoximine and then dialyzed against buffer containing 20 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.1. Fluorescence of the enzyme-bound 8-S-AEDANS-ADP was measured at 25 °C with 490-nm emission and 337-nm excitation; enzyme subunit concentration was 2 μM. In (A), 100 μM ATP and 2 mM L-Met-(*RS*)-sulfoximine were added to the partially inactive enzyme (containing about three fluorescent donors per dodecamer) to block available active sites, and then 5 μM Y-ATP was added; the dashed line indicates the correction for long-distance energy transfer (inner filter effect) caused by the high absorbance of Y-ATP. In (B), 5 μM of Y-ATP was added to the partially inactive AEDANS-ADP–enzyme complex (B-3), and then 100 μM ATP was added in B-4. In (C), 5 μM Y-ATP was added with 2 mM (*RS*)-L-Met-sulfoximine to irreversibly bind Y-ADP to available active sites, and then 100 μM ATP was added (C-4).

The results outlined in Figure 4 show that the enzyme has a high affinity for Y-ATP ($K_D' < 20\ \mu M$), that ATP ($K_D' \simeq 1\ \mu M$) competes with the binding of Y-ATP, and that Y-ADP can be irreversibly bound to available active sites by phosphorylation of L-Met-(*S*)-sulfoximine in the presence of Mn^{2+}. Furthermore, it is clear from the results in Figure 4 that the quenching of the fluorescence of 8-S-AEDANS-ADP bound to active sites requires the binding of Y-ATP to adjacent active sites on the dodecamer and does not result from conformational changes promoted by additional ligand binding. Thus, most of the observed quenching of the fluorescence of enzyme-bound 8-S-AEDANS-ADP by the addition of Y-ATP in Figure 4 (B and C) was due to energy transfer from 8-S-AEDANS-ADP to Y-ATP or Y-ADP on adjacent subunits.

The observations of Figure 4 should, in principle (Stryer, 1978), allow a calculation of intramolecular distances between active sites of the dodecamer. For this purpose, Y-ADP should be introduced into all available active sites of the enzyme containing some 8-S-AEDANS-ADP groups, but this was not possible because addition of excess Y-ATP to the enzyme partially inactivated with 8-S-AEDANS-ATP caused aggregation of the protein. Additions of 3, 6, or 12 equiv of Y-ATP in the presence of L-Met-(*S*)-sulfoximine and Mn^{2+} gave activity losses substantially less than the amount of Y-ATP

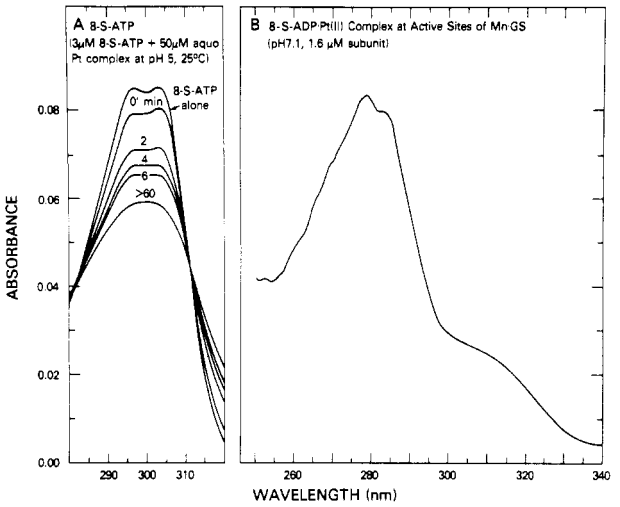

FIGURE 5: Specific labeling of active sites of glutamine synthetase with the electron-dense probe (glycyl-L-methioninato)platinum(II).[5] In (A), the time course of the reaction of 3 μM 8-S-ATP with 50 μM aquo(glycyl-L-methioninato)platinum(II) at 25 °C in 50 mM potassium phosphate/50 mM ammonium acetate buffer at pH 5.0 is shown. [The 0′ min scan at 60 nm/min was begun just after addition of the Pt(II) complex.] After isolation of the 8-S-ATP–(glycyl-L-methioninato)platinum(II) by ethanol precipitation (see Materials and Methods), the 8-S-ATP–Pt(II) adduct was introduced into all active sites of glutamine synthetase by reaction with L-Met-*S*-sulfoximine and Mn^{2+} at pH 7.1 (monitored by activity loss). After exhaustive dialysis against 20 mM Hepes/KOH and 100 mM KCl, pH 7.1 buffer, the spectrum was recorded and is shown in (B); Pt(II) analysis indicated that each subunit of the dodecamer contained 1.05 ± 0.05 equiv of Pt(II).

added. Because all unoccupied sites of glutamine synthetase could not be filled with Y-ATP and because we were concerned that a migration of the mercurial adduct on Y-ATP was possible, we made a similar acceptor chromophore by alkylation of 8-S-ATP with an iodoacetyl derivative. This more stable compound allowed the more rigorous fluorescence energy-transfer measurements presented in the accompanying paper (Maurizi et al., 1986).

Introduction of Electron-Dense Probes into Enzyme Active Sites or Adenylylation Sites for Structural Analysis. The mercapto nucleotides (6-S-ATP and 8-S-ATP) have been found to form stable complexes with (glycyl-L-methioninato)platinum(II).[5] In forming this complex, the thioenol form of 6- or 8-mercapto-ATP displaces water from the aquo-Pt(II) complex. The extent of reaction can be monitored by recording the change in absorbance of mercapto nucleotides upon formation of the sulfur–platinum bond. Figure 5A shows the time course for the reaction of a 16-fold excess of aquo(glycyl-L-methioninato)platinum(II) with 8-S-ATP at pH 5, 25 °C. The reaction of 6-S-ATP with aquo-(glycyl-L-methioninato)platinum(II) under the same condition produced an absorbance decrease of ~18% at 321 nm. As can be observed in Figure 5A, the formation of mercapto nucleotide–Pt(II) complexes was quite slow even in the presence of excess aquo(glycyl-L-methioninato)platinum(II). Rates of formation of mercapto nucleotide–Pt(II) complexes decreased with increasing pH; but by starting this reaction with higher concentrations of 8-S-ATP and the Pt reagent, it was possible to get a complete reaction at pH 7.1 in 3–4 h. Once the mercapto nucleotide–Pt(II) complexes were formed, they were quite stable and could be easily separated from nonbound aquo(glycyl-L-methioninato)platinum(II) by ethanol precipitation (see Materials and Methods).

The 8- or 6-mercapto-ATP–Pt(II) complexes could be specifically introduced into all active sites of dodecameric glutamine synthetase by using the mercapto–ATP–Pt(II) complexes to phosphorylate L-Met-(*S*)-sulfoximine in the presence of Mn^{2+}. After complete inactivation of the enzyme in the latter reactions, which occurred rapidly, the enzyme derivatives were exhaustively dialyzed against divalent cation-free buffer at pH 7.1; subsequent metal ion analysis and protein assays indicated 1.05 ± 0.05 equiv of Pt(II) per subunit and 2.0 equiv of Mn^{2+} per subunit.[6] The spectrum of dialyzed glutamine synthetase containing 1 equiv of the 8-S-ADP-Pt(II) complex per subunit is shown in Figure 5B.

In other experiments (not shown), it was found that aquo-(glycyl-L-methioninato)platinum(II) reacts with 6-S-AMP attached to adenylylation sites of glutamine synthetase. However, the aquo-Pt(II) complex does not readily react with 8-S-ADP bound at active sites with L-Met-(*S*)-sulfoximine phosphate and Mn^{2+}.

Scanning transmission electron microscopy (STEM) analysis of glutamine synthetase molecules together with improved methods for alignment of unstained particles is being performed at the Brookhaven National Laboratory (Hainfeld et al., 1984). The STEM can detect differences in electron scatter (which is proportional to the mass) with a resolution of 5–10 Å. A dense probe such as the Pt(II)–nucleotide complex bound at specific sites of glutamine synthetase will increase electron scatter at such points. Thus, for example, a comparison of the electron scatter from dodecameric particles inactivated with ATP or 8-S-ATP to that from dodecameric enzyme inactivated with the mercapto-ATP–Pt(II) complex can, in theory, be used to determine the location of Pt(II) bound at active sites. Also, Pt(II) at adenylylation sites should show increased electron scatter at these locations over that observed with the native enzyme. Such STEM analyses to locate these specific sites on glutamine synthetase are in progress.

Specific placements of a mercapto nucleotide–Pt(II) complex at each active site or at each adenylylation site of glutamine synthetase also provide electron-dense markers for X-ray structural determinations. Accordingly, this laboratory is in the process of preparing such derivatives of the dodecameric glutamine synthetase from *Salmonella typhimurium* (furnished by Dr. David Eisenberg) for the X-ray structural analysis being performed in that laboratory.[6]

CONCLUSIONS

Our results show that reaction of L-Met-(*S*)-sulfoximine with analogues of ATP at the active site of glutamine synthetase is a convenient method of introducing structural probes into the enzyme without covalent modification of the protein. The complex of L-Met-(*S*)-sulfoximine phosphate, ADP (or analogue), and two Mn^{2+} (or Mg^{2+}) resembles the intermediate formed in the biosynthetic reaction with ATP and L-glutamate; thus, the probes introduced in this manner

[5] The (glycyl-L-methioninato)platinum(II) chloride complex was generously supplied by J. J. Lipka at the Brookhaven National Laboratories for collaborative scanning transmission electron microscopy (STEM) studies with this group: J. F. Hainfeld, J. S. Wall, J. J. Lipka, and P. S. Furcinitti.

[6] Mercapto nucleotide–Pt(II) complexes at all active sites of the unadenylylated glutamine synthetase from *Salmonella typhimurium* also have been prepared in this laboratory by M. B. Blackburn, P. J. McFarland, and Ann Ginsburg for X-ray structural studies being conducted in the laboratory of David Eisenberg at the University of California at Los Angeles. Labeling the adenylylation sites of this enzyme with the 6-S-AMP–Pt(II) complex is currently under way.

reflect a catalytically significant conformation of the enzyme. Since the analogues bind in stoichiometric amounts to the enzyme, any number of subunits can be "labeled", and the extreme stability of the resulting inactive complexes allow studies to be performed in the absence of free ligands.

The three different types of nucleotide probes that have been described in this paper involved modifications of the purine base itself and adducts attached to the 6- or 8-position of the purine ring. Information about the local environment in three closely spaced but distinct regions of the nucleotide substrate binding site of each subunit therefore is possible. The difference in energy transfer from tryptophan to aza-ϵ-ATP or to 8-S-AEDANS-ATP illustrates the type of data that can be obtained pertaining to the enzyme structure and the orientation of the probe. The procedures described also allow the 12 active sites of the dodecameric enzyme to be labeled with different reporter groups. The application of these methods to determine fluorescence energy transfer between active sites of glutamine synthetase is presented in the accompanying paper. Since the inactivating transition-state complexes remain on the enzyme after partial dissociation into even-numbered oligomers of subunits (Maurizi & Ginsburg, 1982b), these same probes can be used in studying the assembly of the dodecameric enzymes and the interactions between active sites and subunit bonding domains. Some of the same nucleotide analogues also can be introduced as additional structural probes into the subunit adenylylation site, which is located quite near the active site.

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Registry No. 8-Br-ADP, 23600-16-0; IAEDANS, 36930-63-9; ATP, 56-65-5; 8-Br-ATP, 23567-97-7; 6-S-ATP, 27652-34-2; 8-S-ATP, 41106-66-5; 8-AE-ATP, 84498-09-9; 8-S-AEDANS-ATP, 99376-89-3; ADP, 58-64-0; 8-[(6-aminohexyl)amino]-ADP, 53602-89-4; 8-(allylamino)-ADP, 99397-60-1; 8-S-ADP, 59924-55-9; 6-S-ADP, 805-63-0; 6-S-AMP, 53-83-8; GS, 9023-70-5; L-Met-*S*-sulfoximine, 15985-39-4; diaminoethane, 107-15-3; diaminohexane, 124-09-4; tryptophan, 73-22-3.

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