Conformational Differences between Unadenylylated and Adenylylated Glutamine Synthetase from Escherichia coli on Binding L-Methionine Sulfoximine*

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L-Methionine-SR-sulfoximine (a proposed transition state analog for glutamine synthetase) produces tyrosyl residue perturbations on binding to unadenylylated but not to adenylylated glutamine synthetase from Escherichia coli, as evidenced by UV difference spectra. Binding L-methionine-SR-sulfoximine to complexes of unadenylylated glutamine synthetase (manganeseenzyme, magnesiumenzyme, and ADP-manganeseenzyme) produces red-shifted, UV difference spectra consistent with the burial of approximately 1 tyrosyl residue per unadenylylated subunit. This may involve primarily the tyrosyl residue at the subunit site of adenylylation because the binding of L-methionine-SR-sulfoximine to the fully adenylylated manganeseenzyme produces a red-shifted spectral perturbation of covalently bound 5'-adenylylated groups without a concomitant tyrosyl residue perturbation. Spectrophotometric results indicate also that both L-methionine-S-sulfoximine and L-methionine-R-sulfoximine in a mixture of the two isomers bind reversibly to glutamine synthetase and compete for a single subunit site. Hill plots of spectrophotometric titrations with L-methionine-SR-sulfoximine (treating the diastereoisomeric mixture as a single ligand) give for the unadenylylated enzyme: $[S]_{0.5} = 46 \mu M$ and $n_H = 0.9$ for manganeseenzyme; $[S]_{0.5} = 9 \mu M$ and $n_H = 1.0$ for ADP-manganeseenzyme complex; and $[S]_{0.5} = 1.9 \mu M$ and $n_H = 0.5$ for magnesium enzyme. For binding L-methionine-SR-sulfoximine to the fully adenylylated manganeseenzyme, $[S]_{0.5} = 0.19 \mu M$ and $n_H = 0.7$. The observed negative cooperativity ($n_H < 1$) on binding L-methionine-SR-sulfoximine to either the unadenylylated or fully adenylylated enzyme may involve homologous subunit interactions within the dodecamer.

Sedimentation velocity measurements show that unadenylylated and fully adenylylated manganeseenzymes have essentially the same conformation in the absence of L-methionine-SR-sulfoximine. However, the binding of L-methionine-SR-sulfoximine to these glutamine synthetase forms produces small but different alterations in hydrodynamic particle shape. The changes in sedimentation coefficients (corrected for buoyant weight increase due to ligand binding) are approximately +0.5% and -0.3% for L-methionine-SR-sulfoximine binding to the unadenylylated and fully adenylylated manganeseenzymes, respectively.

The binding of L-methionine-SR-sulfoximine to the

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zyme had 9 to 11 eq/dodecamer of L-methionine-S-sulfoximine phosphate tightly bound.

The interaction of L-methionine-SR-sulfoximine with manganese-glutamine synthetase from E. coli has been studied by NMR and EPR techniques (16–18). The EPR spectrum for Mn$^{1+}$ bound at n sites of the unadenylated enzyme is dramatically sharpened by the presence of L-methionine-SR-sulfoximine (16, 17). Spectral studies in this laboratory with a pyridoxylated derivative of unadenylated glutamine synthetase (19) showed that L-methionine-SR-sulfoximine produced difference spectra in the absorption regions of protein-bound pyridoxamine-P groups and of aromatic amino acid residues in the modified protein. The latter observation prompted the present study. UV spectral and sedimentation velocity measurements have been used in this study to demonstrate conformational differences between complexes of unadenylated and adenylylated glutamine synthetase with L-methionine-SR-sulfoximine.

**EXPERIMENTAL PROCEDURES**

Materials—L-Methionine-SR-sulfoximine was obtained from the Sigma Chemical Co. and was used without further purification. Concentrations were determined from dry weights and these were consistent with a modified ninhydrin colorimetric analysis (20). By the ninhydrin method (20), L-methionine-SR-sulfoximine gave a maximum color yield of 1.82 times that of L-leucine after 30 min of boiling. This is comparable to the value of 1.73 obtained by Manning et al. consistent with a modified ninhydrin colorimetric analysis (20).

Water systems of the Millipore Corp. from the Sigma Chemical Co. Ethylene glycol (reagent grade) was obtained from Eastman Organic Chemicals. Other reagents were as stated in the procedure of Woolfolk et al. (24) and stored as before. The latter observation was used to prepare and dilute solutions for protein difference spectra. All enzyme stock solutions and buffers were filtered through 0.45-μm Millipore filters. The ADP stock solutions were prepared in buffer (1.0 mM MnCl$_2$, 0.1 mM ADP) at pH 7.15 containing either 1.0 mM MnCl$_2$ or 40 mM MgCl$_2$. Protein final dilution was used as buffer in preparing and diluting solutions for protein difference spectra. All enzyme stock solutions and buffers were filtered through 0.45-μm Millipore filters. The ADP stock solutions were prepared in buffer (1.0 mM MnCl$_2$, 0.1 mM ADP) at pH 6.8, which was prepared and stored as described previously (8).

For difference spectra, enzyme sample solutions were prepared by dilution of a glutamine synthetase stock solution (7 mg/ml) with a solution of L-methionine-SR-sulfoximine and buffer (or ADP stock solution) to give a concentration of ~40 μM enzyme subunits (and 0.1 mM ADP). Enzyme reference solutions were prepared by dilution of the enzyme stock solution with buffer (or with buffer and the ADP stock solution) to give the same subunit concentration (and ADP concentration) as in the sample cell. The enzyme is >97% saturated with ADP (concentration of ADP in stock is 1.0 mM ADP (8)).

Difference spectra with unadenylated manganese enzyme recorded on the Cary 118 and Cary 15 spectrophotometers were identical. Peak-trough differences at 285.5 and 281.2 nm were used to monitor the amount of enzyme-ligand complex formed. The peak-trough differences for each solution were measured by scanning successively: 285.5, 281.2, 285.5, and 281.2 nm (for at least 3 min each) at 20°C with a Beckman model E analytical ultracentrifuge (equipped with a rotor temperature and control unit) using schlieren optics (with phase plate) and a speed of 40,000 rpm. Instrument calibrations, schlieren photographs, and density and viscosity measurements were as described previously (27). A two-place AND rotor and two cells containing 12 mm, 4° single sector Kel F center pieces were used; one of the two cells had a 1° positive wedge upper quartz window for nitrogen displacement and the other a reverse negative quartz window. The liquid column height approximated 0.05 cm lower than that of the cell with conventional plane quartz windows.

Before ultracentrifugation, unadenylated (GS) and the fully adenylylated (GS$_2$) enzymes were each dialyzed against several changes of 0.1 M Hepes (pH 7.15) containing 100 mM KCl, 1.0 mM MnCl$_2$, and of differential absorption. Fresh sample solutions for each titration point (with no serial dilution) gave the most reproducible difference spectra.

Protein solutions for difference spectra were prepared by dialyzing the enzyme at 4°C against several changes of 0.1 M Hepes (pH 7.15) containing either 1.0 mM MnCl$_2$ or 40 mM MgCl$_2$. Protein final dilution was used as buffer in preparing and diluting solutions for protein difference spectra. All enzyme stock solutions and buffers were filtered through 0.45-μm Millipore filters. The ADP stock solutions were prepared in buffer (1.0 mM MnCl$_2$, 0.1 mM ADP) at pH 7.15 containing either 1.0 mM MnCl$_2$ or 40 mM MgCl$_2$. Protein final dilution was used as buffer in preparing and diluting solutions for protein difference spectra. All enzyme stock solutions and buffers were filtered through 0.45-μm Millipore filters. The ADP stock solutions were prepared in buffer (1.0 mM MnCl$_2$, 0.1 mM ADP) at pH 6.8, which was prepared and stored as described previously (8).

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**Difference Spectra Measurements—**Difference spectra were obtained on a Cary model 15 spectrophotometer using the 0 to 0.1 slide wire and semimicro cells of 10-mm path length. All spectra were recorded at ambient temperature (~24°C) in triplicate in the region of differential absorption. Fresh sample solutions for each titration point (with no serial dilution) gave the most reproducible difference spectra.

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**Ultracentrifugation—**Sedimentation experiments were performed at 20°C with a Beckman model E analytical ultracentrifuge (equipped with a rotor temperature and control unit) using schlieren optics (with phase plate) and a speed of 40,000 rpm. Instrument calibrations, schlieren photographs, and density and viscosity measurements were as described previously (27). A two-place AND rotor and two cells containing 12 mm, 4° single sector Kel F center pieces were used; one of the two cells had a 1° positive wedge upper quartz window for nitrogen displacement and the other a reverse negative quartz window. The liquid column height approximated 0.05 cm lower than that of the cell with conventional plane quartz windows.

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(r) of the maximum ordinate of the schlieren peak as a function of time (t) and the sedimentation coefficient was calculated from the slope given by a linear least squares fit of the ln r versus t data. The menisci and rotor reference holes were used for exact plate alignment. Where necessary, the measured sedimentation coefficients were corrected to values (s_{20,w}) corresponding to a solvent with a viscosity and density of water at 20°C. Within the same run, the difference in sedimentation coefficients (Δs) was computed by the procedure of Howlett and Schachman (28), with a precision of at least ±0.1% in Δs/s, where s_{20,w} = 21.0 S for GS; and 21.2 S for GS_{21}. The changes in sedimentation coefficients were corrected for the density and viscosity contributions of unbound ligand (if necessary) and for the change in buoyant weight due to ligand binding as described by Kirschner and Schachman (29). For the latter correction, values for the partial specific volumes of unadenylylated glutamine synthetase (0.698 ml/g) and of AMP at pH 5.1 and 0.12 ionic strength (0.445 ml/g) are from Shapiro and Ginsburg (27). A value of 0.625 ml/g for the apparent specific volume of GS (unadenylylated enzyme, all difference spectra show a specific, red-shifted), broad minimum at -295 nm, and a small extremely large, broad maximum at -280 nm, a small (slightly blue-shifted), broad maximum at -295 nm, and a small extremely large, broad maximum at -315 nm.

**RESULTS**

**Difference Spectra**—L-Methionine-SR-sulfoximine produces protein difference spectra on binding to various glutamine synthetase complexes (unadenylylated, partially adenylylated, and fully adenylylated manganese-enzymes, unadenylylated magnesium-enzyme, and unadenylylated ADP-manganese-enzyme). Protein difference spectra at saturating levels of L-methionine-SR-sulfoximine for these enzyme complexes are given in Fig. 1 along with adenosine difference spectra produced by ethylene glycol solvent perturbation. The important observation is that the L-methionine-SR-sulfoximine promoted difference spectrum with unadenylylated glutamine synthetase is completely different from that with the fully adenylylated enzyme.

The shape and magnitude of the saturating spectra with the manganese and magnesium forms of unadenylylated glutamine synthetase are identical within experimental error; thus, a single spectrum is given in Fig. 1B. The saturating spectrum with the unadenylylated ADP-manganese enzyme (Fig. 1A) is very similar to these although the amplitude is slightly smaller and the extrema are slightly shifted. With adenylylated enzyme, all difference spectra show a specific, red-shifted, tyrosine perturbation (extrema at 285, 281, and 278 nm) and a small, red-shifted, tryptophan perturbation (extrema at 290, ~296, and ~303 nm). The tyrosine and tryptophan perturbations are compatible with the burial of approximate 0.1 tyrosyl side chain and 0.1 tryptophanyl side chain per subunit, respectively (30). The maximum at ~303 nm is probably an example of the atypical extremum found in difference spectra of some proteins that contain tryptophan (31); this has been attributed primarily to a change in the electrostatic environment of the tryptophan chromophore (31).

The spectrum produced by L-methionine-SR-sulfoximine binding to fully adenylylated manganese-enzyme (Fig. 1E) is strikingly different from that with the unadenylylated enzyme, the specific tyrosine perturbation is not observed. The saturating difference spectrum with GS_{21} is red-shifted with a large, broad maximum at ~280 nm, a small (slightly blue-shifted), broad minimum at ~295 nm, and a small extremely broad maximum at ~315 nm.

The small fraction of adenylylated subunits in GS_{21} preparations has no effect within experimental error on the difference spectrum produced by the binding of L-methionine-SR-sulfoximine to unadenylylated manganese subunits. The maximum contribution of the adenylylated subunits to the Δs_{280-296} value is 40 $M^{-1}cm^{-1}$ in Fig. 1B taking into consideration the somewhat lower affinity of adenylylated subunit for this ligand (Table I). The equality of the difference spectra with unadenylylated manganese and magnesium enzymes produced by the binding of L-methionine-SR-sulfoximine (Fig. 1B) suggests that the adenylylated subunits present in the latter case also have negligible effect. Thus, in Fig. 1, Δs values for complexes of GS_{21} preparations are expressed in terms of molar unadenylylated subunit, corresponding to difference spectra for GS_{21} enzyme complexes.

**Fig. 1.** Difference spectra of glutamine synthetase produced by the binding of L-methionine-SR-sulfoximine and difference spectra of adenosine produced by solvent perturbation with ethylene glycol. All protein spectra were at ~24°C in 50 mM Hepes/KOH and 100 mM KCl at pH 7.3 containing 1 mM MnCl2 (Spectra A to E) or 40 mM MgCl2 (Spectrum D). The spectra are given in terms of Δs (ΔA per m enzyme subunit or m adenosine per cm path length). Protein difference spectra are corrected to saturating levels of L-methionine-SR-sulfoximine (see "Results"). The enzyme subunit and adenosine concentrations were ~40 μM, and all spectra were recorded in cells of 1 cm path length. In the region of differential absorption (at wavelengths less than ~310 nm), the estimated error relative to the long wavelength base line is ±0.003 A; the estimated error in absorbance differences in this region taken to monitor extent of L-methionine-SR-sulfoximine binding (see Figs. 2 and 3) is ±0.002. The error of ±0.002 A is plotted at the top center in terms of Δs (±50 $M^{-1}cm^{-1}$) using vertical error flags. A, GS in the presence of saturating (0.1 mM free) ADP (ADP present in sample and reference cells, Δs expressed in molar unadenylylated subunit. B, GS_{21} in 1 mM MnCl2 or in 40 mM MgCl2, Δs expressed in molar unadenylylated subunit. C, GS_{21}, Δs expressed in molar total subunit. D, calculated GS_{21}; 56% of amplitude of Spectrum B + 50% of amplitude of Spectrum E, Δs expressed in molar total subunit. E, GS_{21}, Δs expressed in molar adenylylated subunit. F, Adenosine in 50 mM Hepes/KOH at pH 7.3 containing 100 mM KCl and 1 mM MnCl2 (before addition of ethylene glycol to sample cell and water to reference cell), perturbed with 56% (v/v) and 20% (v/v) ethylene glycol.
A difference spectrum of manganese-GS at 11 mM L-methionine-SR-sulfoximine is shown in Fig. 1C. No titration of GS was performed but 3.7 and 11 mM L-methionine-SR-sulfoximine produced the same difference spectrum within experimental error. In this case, Δ is expressed in terms of molar total (adenylylated + unadenylylated) subunit. Glutamine synthetase preparations in intermediate states of adenyllylation contain hybrid dodecamers, molecules having both unadenylylated and adenylylated subunits (3). In order to ascertain whether adenylylated and unadenylylated subunits in such hybrid dodecamers give rise to independent difference spectra, a saturating spectrum for GS enzyme manganese-enzyme was calculated (Fig. 1D) by taking the sum of 50% of that for GS (Fig. 1B) and 50% of that for GS (Fig. 1E). The measured and computed spectra for GS are the same within experimental error indicating that the two types of subunits within the same dodecamer at saturating levels of ligand show independent difference spectra.

Adenosine was used in these studies as an analog of the covalently bound AMP groups in adenylylated glutamine synthetase. Ethylene glycol at 20% (v/v) and 56% (v/v) was used to perturb adenosine, which was at the same concentration as that of enzyme subunit. The resulting difference spectra are illustrated in Fig. 1F. The spectrum of adenosine is red-shifted in ethylene glycol with a maximum at -275 nm; ethylene glycol also red shifts the spectra of aromatic amino acids (30). The shape of the adenosine difference spectrum (Fig. 1F) at wavelengths greater than -275 nm is similar to that produced by the binding of L-methionine-SR-sulfoximine to adenylylated subunit (Fig. 1E). In a control experiment, 21 mM L-methionine-SR-sulfoximine produced no adenosine difference spectrum (with 40 μM adenosine). These observations suggest that the difference spectrum observed with GS (Fig. 1E) is primarily due to perturbation of covalently bound 5'-adenylate groups by protein-bound L-methionine-SR-sulfoximine.

In other experiments, a small difference spectrum with manganese-GS was produced by 60 mM L-glutamate but not by 30 mM L-glutamate; the latter gives 80% saturation of the enzyme (23), whereas the former corresponds to ~90% saturation (16, 32). With L-glutamate, a specific tyrosine perturbation apparently is involved, with Δ285-289 ≈ 100 (μM subunit) cm⁻¹.

Saturation Curves—Subsaturating difference spectra were used to generate saturation curves for the binding of L-methionine-SR-sulfoximine to various glutamine synthetase complexes. Differences between absorbances (Δ expressed in molar subunit) at a maximum (λ₂) and minimum (λ₁) were taken...
as a measure of ligand binding. The titration data are presented in a Scatchard plot (Fig. 3) and Hill plots (Fig. 2) where 

\[ Y = \frac{\Delta E}{\Delta E_{\text{max}}} \] 

\( \Delta E_{\text{max}} \) is the maximum energy change for the unadenylylated enzyme complexes. The resultants [S] and [L] values and \( n_0 \) values from these Hill and Scatchard plots also are given in Table I, in parentheses, and are very similar to those obtained using the \( A_{285} - A_{280} \) differences. Thus, these parameters are independent of the stoichiometric factor differences used to measure ligand binding, and the small tryptophan perturbation follows that of tyrosine. Furthermore, complete difference spectra for each of the four complexes (Table I) were plotted from data measured at >90% and <90% saturation but corrected to full saturation with L-methionine-SR-sulfoximine and found to be identical within experimental error.

**Stoichiometry of Binding L-Methionine-SR-sulfoximine to Manganese-GS and to ADP-Manganese-GS**

The method of continuous variations of Job for determining the stoichiometry of equilibrium binding has been described and extended by Vosburgh and Cooper (33). This approach is valid when dealing with equivalent, noninteracting binding sites. The method of continuous variations consists of: (a) subjecting the system to a constraint on total concentrations of enzyme and ligand, e.g.,

\[ E_o + n \cdot L_o = C \]  

where \( E_o \) is the total molar enzyme concentration, \( L_o \) is the total molar ligand concentration, \( n \) is the assumed stoichiometry of binding, and \( C \) is the weighted sum of concentrations that is held constant throughout the experiment; (b) varying the mole fraction of enzyme, \( X_{E_o} \) (and of ligand, \( X_{L_o} \)), from 0 to 1 subject to the above constraint while monitoring the extent of enzyme-ligand complex formation. A plot of \( X_{E_o} \) (and of \( X_{L_o} \)) versus monitoring parameter shows an extremum. Vosburgh and Cooper (33) have discussed the relationship of the stoichiometry and \( K_o' \) values to the shape and amplitude of such plots.

The binding of L-methionine-SR-sulfoximine was investigated by the method of continuous variations in order to determine whether both L-methionine-S-sulfoximine and L-methionine-R-sulfoximine reversibly bind to enzyme in the absence of nucleotide substrate. Unadenylylated manganese-enzyme was chosen for this analysis in order to comply most closely with the requirement for equivalent, noninteracting binding sites (\( n_{o1} = 0.9 \)).

The initial assumption was that only the S diastereoisomer binds to glutamine synthetase (14); this corresponds to a stoichiometry of 2.0 eq of L-methionine-SR-sulfoximine/unadenylylated subunit. In this experiment, the values in the constraint equation (Equation 1) were: \( E_o \) = total molar concentration of unadenylated subunit, \( L_o \) = total molar concentration of L-methionine-SR-sulfoximine, \( n = 2.0 \), and \( C = 86 \mu M \). The data are plotted in Fig. 4 (A) as a function of mole fraction of unadenylated subunit and of S diastereoisomer (\( X_{GS} \) and \( X_{S} \) respectively). The intersection of lines tangent to the experimental points at low and high \( X_{GS} \) values occurs at \( X_{GS} = 0.62 \) (see dashed line-X in Fig. 4). This asymmetry suggests that the premise that only the S isomer binds is incorrect. Furthermore, the envelope of \( \Delta A \) (per cm path length) versus \( X \) was calculated (see Fig. 4, legend).
assuming that both isomers bind, but was plotted (Fig. 4, Curve A) in the same manner as were the experimental curves (i.e. \( \Delta A \) versus \( \bar{X} \)). The agreement between this calculated envelope and the experimental points indicates that both isomers bind.

Another experiment was performed in an analogous manner using a new constraint that is compatible with both isomers binding. The value of \( n \) in the constraint equation (Equation 1) was 1.0. The new data are plotted in Fig. 4 (A) as a function of mole fraction of unadenylylated subunit and of L-methionine-SR-sulfoximine (\( \bar{X}_{GS} \) and \( \bar{X}_{SR} \) isomers, respectively). The lines tangent to the experimental points cross at \( \bar{X}_{GS} \approx 0.52 \) (see solid line-X in Fig. 4) indicating the symmetry of the plot. The envelope for both isomers binding was computed (see Fig. 4, legend) and plotted (Fig. 4, Curve B) as \( \Delta A \) versus \( \bar{X}_{SR} \) isomers and is in agreement with the experimental data. The very slight asymmetry of the computed envelope (Fig. 4, Curve B) is due to the small amount of negative cooperativity taken into consideration (see Fig. 4, legend). Envelopes assuming normal, rectangular hyperbolic binding with \( K_p = 46 \mu M \) were calculated (Curves A' and B', not shown) and were very similar to those presented in Fig. 4 (Curves A and B) although Curve B' was perfectly symmetric.

Results of the study using the method of continuous variations unequivocally demonstrate that the stoichiometry of binding is 1.0 eq of L-methionine-SR-sulfoximine per unadenylylated subunit and we have equated this with both the S and R isomers binding, competing for a single subunit site. Because L-methionine-SR-sulfoximine is a 1:1 mixture of two diastereoisomers, an alternative interpretation for the observed stoichiometry is exclusive binding of one isomer that shows half-of-the-sites reactivity. This alternative interpretation can be eliminated on the basis of the following: (a) Weisbrod and Meister (14) showed that a 1 eq of L-[\( ^{15} \)N]-methionine sulfoximine phosphate/subunit was tightly bound to the inactivated *E. coli* enzyme; and (b) A direct interaction of bound L-methionine-SR-sulfoximine (saturating levels) with all Mn\(^{2+} \) bound at \( n = 1.0 \) metal ion sites of unadenylylated glutamine synthetase is suggested by the NMR studies of Villafranca et al. (16). (c) In recent Mn\(^{2+} \) binding studies, Hunt and Ginsburg (34) found that the presence of saturating concentrations of L-methionine-SR-sulfoximine produces a uniform enhancement in the affinity of all 12 \( n \) metal ion sites of the decameric Mn\(^{2+} \). Thus, the S and R isomers compete for a single subunit site or the unadenylylated manganese-enzyme and the enzyme has approximately the same affinity for each isomer in the mixture. If the affinities of the unadenylylated subunit for the two isomers were not approximately the same, a value of \( n \) between 1.0 and 2.0 in Equation 1 would be required in order to obtain a symmetric plot of \( \Delta A \) versus \( X \).

Unadenylylated ADP-manganese-enzyme has an affinity for L-methionine-SR-sulfoximine sufficiently great (\( K_p = 8.5 \mu M \)) to demonstrate that in this case also both the S and R isomers bind to enzyme. Up to a saturation level of 77% of the ADP-manganese-enzyme complex with L-methionine-SR-sulfoximine, calculations demonstrate that an insufficient amount of a single isomer is present to account for the amount of bound ligand, assuming that at complete saturation 12 eq of ligand/dodecamer are bound. The linearity of the Scatchard plot (Fig. 3) indicates that the affinity of the unadenylylated manganese-ADP-enzyme complex is approximately the same for the S and R isomers in the mixture. This result is essentially the same as that obtained by studying the binding of this ligand to unadenylylated manganese-enzyme by the method of continuous variations.

**Sedimentation Studies**—Table II shows the sedimentation coefficient changes of manganese-GS\(^{77} \) and manganese-GS\(^{79} \) produced by the binding of L-methionine-SR-sulfoximine. The effects of L-methionine-SR-sulfoximine on the sedimentation coefficients of GS\(^{77} \) and GS\(^{79} \) are different.

![Figure 4: Stoichiometry of binding L-methionine-SR-sulfoximine to unadenylylated manganese glutamine synthetase as determined by the method of continuous variations.](http://www.jbc.org/)
Sedimentation velocity experiments were performed at 20°C (40,000 rpm) and analyzed as described under "Experimental Procedures." The buffer was 20 mM Hapes/KOH, 100 mM KCl, and 1.0 mM MnCl₂ at pH 7.2. The concentration of L-methionine-SR-sulfoximine, Met(O)(NH), was 1.0 or 5.0 mM with the unadenylylated (GSı) or fully adenylylated (GS₂) enzyme, respectively. The value of \(\Delta s/s\) was calculated from the change in sedimentation coefficient \(\Delta s\) corrected for the density and viscosity of the unbound ligand if necessary and from \(s^0_{\text{ref}} = 21.0\) S for GSı or \(s^0_{\text{ref}} = 21.2\) S for GS₂. Values of \((\Delta s/s)_{\text{ref}}\) are averages of corresponding \((\Delta s/s)_{\text{obs}}\) values corrected for changes in buoyant weight due to ligand binding as described by Kirschner and Schachman (29). The precision in \((\Delta s/s)\) values is ±0.1%.

<table>
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<th>Protein concentration (mg/ml)</th>
<th>Sector 1 (sample)</th>
<th>Sector 2 (reference)</th>
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<th>((\Delta s/s)_{\text{corr}})</th>
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<td>GSı</td>
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</table>

* Ligand-promoted aggregation at this concentration of GS₂ was observed; the aggregates (~10% of the total mass) were well separated from the sedimenting boundary of the dodecamer.

† 5 mM Met(O)(NH) was present in these experiments with GSı in order to match the concentration of Met(O)(NH) needed to saturate GS₂.

The observed difference in sedimentation coefficient for GSı versus GSı (a control experiment; Table II) at a concentration of 2 mg/ml is zero within the estimated precision of ±0.1% in \((\Delta s/s)\) × 100 (28, 29, 35). At higher protein concentrations the error in \((\Delta s/s)\) × 100 is less than 0.1% (28), taking into account the molecular size of glutamine synthetase (27). The binding of L-methionine-SR-sulfoximine to the unadenylylated manganese-enzyme (GSı-Met(O)(NH) versus GSı in Table II) produces an increase in the sedimentation coefficient of this protein. It is necessary to correct \((\Delta s/s)_{\text{obs}}\) for the increase in the effective molecular weight due to ligand binding (29). Making this correction, \((\Delta s/s)_{\text{corr}}\), is about +0.5% for the binding of L-methionine-SR-sulfoximine to the unadenylylated manganese-enzyme. For L-methionine-SR-sulfoximine binding to the fully adenylylated manganese-enzyme (GSı-Met(O)(NH) versus GSı in Table II), \((\Delta s/s)_{\text{corr}}\), is about -0.3%.

In the experiments of Table II with GSı at a protein concentration of 4.1 mg/ml, L-methionine-SR-sulfoximine caused an aggregation of the adenylylated enzyme; this aggregation was not observed in the sedimentation experiments with GSı or at the lower concentration of GSı in Table II. These observations also indicate that conformations of the GSı-Met(O)(NH) and GSı-Met(O)(NH) complexes differ. The glutamine synthetase dodecamer is quite stable in the presence of Mn²⁺ and 0.1 M KCl; it does not dissociate into subunits or self-associate (1). The binding of L-methionine-SR-sulfoximine to GSı apparently exposes surface sites involved in intermolecular associations that are not accessible in the native enzyme (GSı or GSı) or in the GSı-Met(O)(NH) complex.

Earlier studies of Shapiro and Ginsburg (27) on the physical-chemical properties of glutamine synthetase from E. coli indicated that the manganese-enzymes of different adenylylation states had \(s^0_{\text{max}} = 20.3 ± 0.2\) S. This result was from several series of separate experiments (using an imidazole/HCl/NaCl buffer at pH 7.1), where sources of error (~1%) include rotor speed and temperature variations and optical and cell misalignment (35). In the present studies, apparent

\(s^0_{\text{max}}\) values are 21.0 S and 21.2 S for GSı and GSı, respectively. For the concentration dependence of the sedimentation coefficient \((\Delta s/s)\) of either GSı or GSı, \(k = -(1/s^2)(\Delta s/s)\), a characteristic value for nonassociating globular proteins (36). After correcting \((\Delta s/s)_{\text{obs}}\) for the effective molecular weight increase due to the covalently bound 5'-adenylylated groups, the sedimentation coefficients of GSı and GSı are the same (Table II). The absence of a detectable conformational difference between the unadenylylated and adenylylated forms of glutamine synthetase shown by \((\Delta s/s)_{\text{corr}}\) ≈ 0 for GSı versus GSı in Table II is similar to results from other studies utilizing optical techniques (1).

Sedimentation experiments were performed measuring the change in sedimentation coefficients for GSı-Met(O)(NH) versus GSı-Met(O)(NH) complexes also. The average value for \((\Delta s/s)_{\text{corr}}\), (correcting for covalently bound 5'-adenylylated groups in this case) is in good agreement with the difference between values from the separate experiments of GSı-Met(O)(NH) versus GSı and GSı-Met(O)(NH) versus GSı (Table II).

**DISCUSSION**

The studies reported here show that unadenylylated and adenylylated glutamine synthetase from E. coli undergo different conformational changes upon binding L-methionine-SR-sulfoximine, a proposed transition state analog (15). In addition, intrinsic protein fluorescent changes that occur when L-glutamate, ATP, and ammonia react with the unadenylylated enzyme (11, 37) were not observed in recent studies of Ubom et al. (38) when these substrates were added to the fully adenylylated enzyme. Both diastereoisomers of L-methionine-SR-sulfoximine might be expected to bind reversibly to glutamine synthetase on the basis of the modeling studies of Meister and co-workers (10, 15). The stoichiometry of binding L-methionine-SR-sulfoximine to manganese-GSı and to ADP-manganese-GSı is 1.0 eq/subunit, indicating that both diastereoisomers bind reversibly and compete for a single subunit site; furthermore, the affinity of each enzyme complex is approximately the
same for both isomers in the 1:1 diastereoisomeric mixture. The number of binding sites for L-methionine-SR-sulfoximine apparently is independent of the divalent cation present since the saturating difference spectra produced by the binding of this ligand to the manganese-\( \text{GS}_{\text{M}} \) and magnesium-\( \text{GS}_{\text{M}} \) complexes are identical (Fig. 1B). The number of binding sites for L-methionine-SR-sulfoximine also is independent of the adenyllylation state of the glutamine synthetase preparation (14, 34).

On binding L-methionine-SR-sulfoximine to each of the enzyme complexes studied, either diastereoisomer alone could be responsible for the observed UV-spectral perturbations (Fig. 1) or both isomers could give rise to similar or different spectral perturbations. Our studies can not distinguish among these alternatives. However, none of these possibilities would alter any of the conclusions inferred from the spectral saturation data or from the method of continuous variations. Since the unadenyllylated enzyme complexes show a specific tyrosine perturbation difference spectrum with sharp, well defined extrema and with an amplitude compatible with the burial of approximately a single tyrosine side chain per subunit, the observed perturbation may well arise from both diastereoisomers causing very similar if not identical perturbations with unadenyllylated subunit.

The half-saturation values from the spectrophotometric titrations of the unadenyllylated enzyme (Table I) are in very good agreement with those measured in other studies by different techniques. Measuring protein tryptophanyl residue fluorescent changes (which may relate to the tryptophanyl residue perturbation observed here), Timmons et al. (11) found \( k'_{D} = 2.6 \text{ mM} \) for the dissociation of L-methionine-SR-sulfoximine from the unadenyllylated magnesium-enzyme complex. From NMR studies, Villafranca et al. (16) report \( k'_{D} \) values of 30 \( \mu \text{M} \) and 9 \( \mu \text{M} \) for L-methionine-SR-sulfoximine dissociating from manganese-\( \text{GS}_{\text{M}} \cdot \text{Met(O)}(\text{NH}) \) and ADP-manganese-\( \text{GS}_{\text{M}} \cdot \text{Met(O)}(\text{NH}) \) complexes, respectively. The unusually high affinity of the manganese-enzyme for L-methionine-SR-sulfoximine relative to the affinities for the substrates L-glutamate and L-glutamine (18, 23, 32) possibly relates to this ligand being a transition state analog (10, 15). The 5-fold decrease in the \( [S]_{1/2} \) value for the unadenyllylated manganese-enzyme produced by saturating ADP (Table I) is indicative of synergism between the binding of ADP and L-methionine-SR-sulfoximine. Previously, synergism has been observed between the substrates ADP and L-glutamine with unadenyllylated manganese-enzyme (23) and between ATP and L-glutamate with unadenyllylated magnesium-enzyme (11).

For the fully adenylylated manganese-enzyme, \( [S]_{1/2} = 0.19 \text{ mM} \) for L-methionine-SR-sulfoximine, which is ~4-fold higher than that for the unadenyllylated manganese-enzyme. This difference in apparent affinities of the unadenylated and adenylylated manganese-enzyme for L-methionine-SR-sulfoximine could relate to the conformational differences between the manganese-\( \text{GS}_{\text{M}} \cdot \text{Met(O)}(\text{NH}) \) and manganese-\( \text{GS}_{\text{M}} \cdot \text{Met(O)}(\text{NH}) \) complexes (Fig. 1).

L-Methionine-SR-sulfoximine shows negative cooperativity \( (n_H < 1) \) on binding to three of the enzyme complexes studied (Table I). In each of these cases, at least one of the diastereoisomers must exhibit negative cooperativity on binding. Greater negative cooperativity is observed for L-methionine-SR-sulfoximine binding to magnesium-\( \text{GS}_{\text{M}} \) than to manganese-\( \text{GS}_{\text{M}} \); although identical saturating difference spectra are produced in the presence of \( \text{Mg}^{2+} \) and of \( \text{Mn}^{2+} \). Recent kinetic results of Rhee et al. (39) on the inactivation of unadenyllylated manganese-glutamine synthetase by ATP and L-methionine-SR-sulfoximine show that inactivated subunits retard the reactivity of neighboring subunits toward nucleotide or L-methionine-SR-sulfoximine or both. These and the present observations suggest that homologous subunit interactions within the dodecamer may be responsible for the negative cooperativity observed for the binding of L-methionine-SR-sulfoximine to glutamine synthetase. In previous studies of Denton and Ginsburg (40), a negative type of subunit interaction for the binding of ATP to the manganese-enzyme appeared to result from heterologous interactions between unadenylated and adenylylated subunits in hybrid glutamine synthetase dodecamers. Negative cooperativity also was observed (40) in substrate saturation functions for ATP, L-glutamate, and L-glutamine in steady-state analyses of the Mn"-supported biosynthetic activity of glutamine synthetase.

The difference spectra produced by L-methionine-SR-sulfoximine binding to unadenyllylated glutamine synthetase complexes (Fig. 1, A and B) show a specific tyrosyl perturbation. The magnitude of this red-shifted spectral change is equivalent to the burial of approximately 1 tyrosyl residue per subunit with a concomitant small tryptophan perturbation (30). The substrate L-glutamate produces a smaller tyrosyl residue perturbation with the unadenyllylated manganese-enzyme. In contrast, the substrate L-glutamine gives no spectral change on binding to glutamine synthetase.

Each glutamine synthetase subunit has 15 tyrosyl residues, one of which can be covalently modified by enzyme-catalyzed adenlylation (1-6). Adenylation does not change the UV-absorption (pH < 8) of the tyrosyl residue to which AMP is attached in phosphodiester linkage through the phenolic group (5). The 5'-adenylate groups of the adenylylated manganese-enzyme are fully exposed at the protein surface, as shown by the NMR data of Villafranca et al. (9) and by the reactivity of anti-AMP specific antibodies with adenylylated sites of glutamine synthetase (41). The absence of a detectable tyrosyl residue perturbation on binding L-methionine-SR-sulfoximine to the fully adenylylated enzyme suggests that this protein does not undergo the same conformational change as the diastereoisomers of L-methionine-SR-sulfoximine alone shows normal, rectangular hyperbolic binding with a stoichiometry of 1.0 eq/subunit and that they compete for a single subunit site, fractional saturation with the S isomer is 

\[
Y_S = K_a^S S_{\text{tot}}/(K_a^S S_{\text{tot}} + K_a^R R_{\text{tot}} + 1)
\]

where \( S_{\text{tot}} \) and \( R_{\text{tot}} \) are the free molar concentrations and \( K_a^S \) and \( K_a^R \) are the subunit association constants of the S and R isomers, respectively. The expression for \( Y_S \) is the same as the above with the S and R labels permuted. With the L-methionine-SR-sulfoximine preparation used, the total molar concentrations of the S and R isomers are approximately equal, i.e. \( S_{\text{tot}} \approx R_{\text{tot}} \). For the weak binding cases (with unadenyllylated magnesium and adenylylated manganese enzymes), \( S_{\text{tot}} \approx S \approx R \approx R_{\text{tot}} \). For the tighter binding case (with unadenyllylated manganese enzyme) where \( K_a^S \approx K_a^R \), the diastereoisomeric mixture, \( S_{\text{tot}} \approx S \approx R \approx R_{\text{tot}} \). In these weak and tight binding cases, the equations for \( Y_S \) and \( Y_R \) are simple, rectangular hyperbolas such that any weighted sum or difference of \( Y_S \) and \( Y_R \) (corresponding in general to different spectral perturbations for the isomers) also gives a simple, rectangular hyperbola, which corresponds to a normal, noncooperative \( (n_H = 1.0) \) binding isotherm. Thus, the observed negative cooperativity can not be explained in terms of normal, rectangular hyperbolic binding of each diastereoisomer; at least one isomer must exhibit negative cooperativity on binding.

\(^{a}\) Assuming that each diastereoisomer of L-methionine-SR-sulfoximine can interact with one protein molecule and that for each diastereoisomer, the binding isotherm is the same as the above with the S and R labels permuted. With the L-methionine-SR-sulfoximine preparation used, the total molar concentrations of the S and R isomers are approximately equal, i.e. \( S_{\text{tot}} \approx R_{\text{tot}} \). For the weak binding cases (with unadenyllyated magnesium and adenylylated manganese enzymes), \( S_{\text{tot}} \approx S \approx R \approx R_{\text{tot}} \). For the tighter binding case (with unadenyllyated manganese enzyme) where \( K_a^S \approx K_a^R \), the diastereoisomeric mixture, \( S_{\text{tot}} \approx S \approx R \approx R_{\text{tot}} \). In these weak and tight binding cases, the equations for \( Y_S \) and \( Y_R \) are simple, rectangular hyperbolas such that any weighted sum or difference of \( Y_S \) and \( Y_R \) (corresponding in general to different spectral perturbations for the isomers) also gives a simple, rectangular hyperbola, which corresponds to a normal, noncooperative \( (n_H = 1.0) \) binding isotherm. Thus, the observed negative cooperativity can not be explained in terms of normal, rectangular hyperbolic binding of each diastereoisomer; at least one isomer must exhibit negative cooperativity on binding.
that produced by the binding of this ligand to the unadenylated enzyme. The red-shifted UV-spectral change produced by the binding of L-methionine-SR-sulfoximine to adenylated glutamine synthetase (Fig. 1E) indicates that the environment of covalently bound S'-adenylated groups is altered by the binding of this ligand. Thus, the spectral results with GS1, GS2, and GS32 suggest that the binding of L-methionine-SR-sulfoximine at a subunit catalytic site influences the environment of a tyrosyl residue that is the subunit site of adenylylation. Additional evidence that this is the case has been obtained recently (34): The tight binding of L-methionine-SR-sulfoximine phosphate + ADP at the catalytic sites of glutamine synthetase blocks adenyltransferase-catalyzed adenylylation.

In both the absence and presence of ADP bound at the catalytic sites of unadenylated manganese-glutamine synthetase, the two diastereoisomers of L-methionine-SR-sulfoximine in the mixture bind with about equal affinity and a tyrosine perturbation occurs (Fig. 1, A and B). Nevertheless, only L-methionine-S-sulfoximine has the correct configuration when bound at the active site to be phosphorylated by ATP (10, 15). The distance between the n1 and n2 metal ion sites is shortened to ~8 Å by the binding of ADP at the n2 metal ion site (8, 16) and recent estimates of the distances within the subunit between the adenylylation site and the metal ion sites (n1 and n2) are only ~7 to 10 Å (9). Also, bound L-methionine-SR-sulfoximine interacts with the metal ion at the n1 site (16, 17, 34). Consequently, the spectral changes produced by L-methionine-SR-sulfoximine binding to the catalytic site could involve rather subtle conformational shifts. However, the results of Table II indicate that the binding of L-methionine-SR-sulfoximine to glutamine synthetase produces measurable changes in sedimentation coefficient.

The sedimentation coefficient of the manganese enzyme is unchanged by complete adenylylation, after correcting for the presence of covalently bound S'-adenylate groups in GS1E (Table II). However, saturation of the unadenylated manganese enzyme with L-methionine-SR-sulfoximine produces an effect on the overall hydrodynamic particle shape such that the frictional coefficient is apparently decreased. In contrast, the binding of L-methionine-SR-sulfoximine to the fully adenylylated manganese enzyme produces a slight apparent increase in the frictional coefficient. Furthermore, the binding of this analog to the adenylylated enzyme appears to expose sites involved in intermolecular associations.

Unadenylated and fully adenylylated manganese-glutamine synthetase have very similar conformations in the absence of L-methionine-SR-sulfoximine. The fact that these two enzyme forms exhibit marked differences in various catalytic properties (2-4, 6-8) may relate to the present observations. The spectrophotometric and sedimentation velocity results of this study indicate that the binding of L-methionine-SR-sulfoximine promotes different local and different gross structural changes in unadenylated and adenylylated enzymes. Thus, complexes of unadenylated and of fully adenylylated glutamine synthetase with this transition state analog have quite different conformations.

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