Investigating the effects of posttranslational adenylylation on the metal binding sites of *Escherichia coli* glutamine synthetase using lanthanide luminescence spectroscopy

LUIS P. REYNALDO,^{1,3} JOSEPH J. VILAFRANCA,² AND WILLIAM DEW. HORROCKS, JR.¹

¹The Pennsylvania State University, Department of Chemistry, University Park, Pennsylvania 16802

²Bristol-Myers Squibb Pharmaceutical Research Institute, Biomolecular Structure and Function Group,
P.O. Box 4000, Princeton, New Jersey 05843

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Abstract

Lanthanide luminescence was used to examine the effects of posttranslational adenylylation on the metal binding sites of *Escherichia coli* glutamine synthetase (GS). These studies revealed the presence of two lanthanide ion binding sites of GS of either adenylylation extrema. Individual emission decay lifetimes were obtained in both H₂O and D₂O solvent systems, allowing for the determination of the number of water molecules coordinated to each bound Eu³⁺. The results indicate that there are 4.3 ± 0.5 and 4.6 ± 0.5 water molecules coordinated to Eu³⁺ bound to the n1 site of unadenylylated enzyme, GS₀, and fully adenylylated enzyme, GS₁₂, respectively, and that there are 2.6 ± 0.5 water molecules coordinated to Eu³⁺ at site n2 for both GS₀ and GS₁₂. Energy transfer measurements between the lanthanide donor–acceptor pair Eu³⁺ and Nd³⁺, obtained an intermetal distance measurement of 12.1 ± 1.5 Å. Distances between a Tb³⁺ ion at site n2 and tryptophan residues were also performed with the use of single-tryptophan mutant forms of *E. coli* GS. The dissociation constant for lanthanide ion binding to site n1 was observed to decrease from $K_d = 0.35 \pm 0.09$ μM for GS₀ to $K_d = 0.06 \pm 0.02$ μM for GS₁₂. The dissociation constant for lanthanide ion binding to site n2 remained unchanged as a function of adenylylation state; $K_d = 3.8 \pm 0.9$ μM and $K_d = 2.6 \pm 0.7$ μM for GS₀ and GS₁₂, respectively. Competition experiments indicate that Mn²⁺ affinity at site n1 decreases as a function of increasing adenylylation state, from $K_d = 0.05 \pm 0.02$ μM for GS₀ to $K_d = 0.35 \pm 0.09$ μM for GS₁₂. Mn²⁺ affinity at site n2 remains unchanged ($K_d = 5.3 \pm 1.3$ μM for GS₀ and $K_d = 4.0 \pm 1.0$ μM for GS₁₂). The observed divalent metal ion affinities, which are affected by the adenylylation state, agrees with other steady-state substrate experiments (Abell LM, Villafranca JJ, 1991. *Biochemistry* 30:1413–1418), supporting the hypothesis that adenylylation regulates GS by altering substrate and metal ion affinities.

Keywords: adenylylation; glutamine synthetase; lanthanide luminescence; metal binding; posttranslational enzyme regulation

Glutamine synthetase (GS) [E.C. 6.3.1.2, L-Glutamate: ammonia ligase (ADP-forming)] is an enzyme that catalyzes the ATP-dependent conversion of glutamate to glutamine (Stadtman & Ginsburg, 1974). Crystallographic analysis reveals that the enzyme is a dodecamer consisting of 12 identical subunits (Almassy et al., 1986; Kinemages 1, 2). Kinetic and structural studies have shown that the enzyme requires two divalent metal ions per subunit for catalytic activity (Ginsburg, 1972; Hunt et al., 1975). The metal

ion sites are distinguished by their binding affinities, relative positions, ligand types, and substrate interactions. The metal ion site n1 is ligated by the amino acid side-chain carboxylates of glutamic acid residues at positions 131, 212, and 220. n1 is positioned closest to the molecule's C₆ axis of symmetry and provides a ligand for glutamate binding (Villafranca et al., 1976b). The metal ion at the site designated n2 is ligated by the side chains of glutamic acids at 129 and 357 and a histidine at 269. The n2 site provides ligation for the metal–ATP complex and activation of the ATP for catalysis (Hunt & Ginsburg, 1980; Kinemage 3).

An ordered kinetic mechanism has been established for *Escherichia coli* GS (Meek & Villafranca, 1980). Sequential binding of ATP and glutamate to form a γ-glutamyl phosphate intermediate is followed by the release of ADP (Fig. 1). Subsequent binding and nucleophilic attack by ammonia forms a tetrahedral intermediate,

Reprint requests to: William DeW. Horrocks, Jr., The Pennsylvania State University, Department of Chemistry, 152 Davey Laboratory, University Park, Pennsylvania 16802; e-mail: wdhw2@psuvm.psu.edu.

³ Present address: The Johns Hopkins University, Department of Chemistry, Remsen-Dunning Halls, 3400 North Charles Street, Baltimore, Maryland 21218; e-mail: reynaldo@jhunix.hcf.jhu.edu.

Fig. 1. Catalytic mechanism of GS.

which collapses in the forward direction, resulting in the release of glutamine and inorganic phosphate (Meek et al., 1982; Clark & Villafranca, 1985). The role of the divalent metal is to serve as a template for the charged nucleophile during phosphoryl transfer (Herschlag & Jencks, 1987). The metal ion also redistributes the excess charge buildup caused by the phosphoryl group and incoming ammonia nucleophile (Jencks, 1966).

The metal ions also play a role in maintaining the structural stability of the enzyme. Ginsburg and coworkers have defined the native, divalent metal-bound form of GS as the *taut* conformation (Kingdon et al., 1968). The metal-free form of the enzyme is termed the *relaxed* form. Upon relaxation of GS, a significant change occurs in (1) the UV difference spectrum, (2) its susceptibility toward sulfhydryl oxidation, (3) its susceptibility to denaturants such as urea, (4) its hydrodynamic properties, and (5) the kinetic properties of the catalyzed reaction (Shapiro & Ginsburg, 1968; Denton & Ginsburg, 1969). Another form, the *tightened* conformation, is produced when metal ion is added to the relaxed form. The tightened form is indistinguishable from the taut form on the basis of the enzyme's catalytic properties and resistance to denaturants or sulfhydryl oxidation. However, under certain conditions, the tightened form differs from the taut form in that it is much less soluble in dilute buffer solution (Valentine et al., 1968).

The amido group of glutamine is used as the nitrogen source in the biosynthesis of several molecules, including the amino acids histidine and tryptophan, the purines of guanine and adenine, cytidine triphosphate, and glucosamine 6-phosphate (Stadtman et al., 1980). Because glutamine is an essential molecule in nitrogen metabolism, its production is highly regulated at both the transcriptional and posttranslational levels (Magasanik, 1982). Stadtman has shown that each subunit of the dodecamer can be regulated at the posttranslational level by enzymatic adenylation of the tyrosine residue at position 397. Thus, GS has available a range of states from the fully unadenylylated (GS_0) to the fully adenylylated (GS_{12}) form of the enzyme (Stadtman & Ginsburg, 1974). Adenylation has the effect of increasing substrate K_m s and changing the metal ion specificity (Ginsburg et al., 1970; Abell & Villafranca, 1991a). The structural model of GS shows that the adenylylated tyrosine is exposed to the outer surface of GS and is positioned approximately 19 Å from either metal ion center, indicating that this mode of regulation does not involve simple steric hindrance by the adenyl group (Yamashita et al., 1989; Kinemages 1, 2). This highly developed regulatory mechanism allows GS to be an excellent model for the study of enzyme regulation.

The trivalent lanthanide ions (Ln^{3+}) are capable of luminescence in dilute aqueous solution at room temperature. A wealth of information is obtained in Eu^{3+} luminescence studies (Horrocks, 1993), including obtaining individual site binding isotherms and stoichiometries, determining the hydration number in the first coordination sphere, determining distances between bound metal ions in a multiple ion binding protein, and information as to the type of atoms coordinated to Eu^{3+} . Tb^{3+} luminescence can be induced by irradiating a protein to which Tb^{3+} is bound with light in the UV region. Some of the energy absorbed by the protein is transferred to the Tb^{3+} , over distances as far as 10 Å, causing the Tb^{3+} to luminesce. Brittain et al. (1976) observed this sensitized emission phenomena in a variety of proteins, and Horrocks and Collier (1981) have determined that a weak dipole-dipole coupling mechanism accounts for the energy transfer mechanism. Stoichiometric information and overall binding affinities can be determined from such studies, which complement the Eu^{3+} luminescence experiments. Additionally, experiments that quantitate the amount of energy transfer can be used to determine metal ion to protein chromophore distances.

In order to further our understanding of its complex regulatory mechanism, a study of the metal binding affinities of GS, as a function of regulatory state, was undertaken. Indeed, several chromophoric metal ions, including lanthanide ions, have been used in previous studies to probe the two metal binding sites of GS (Villafranca et al., 1976a; Lin et al., 1991a; McNemar et al., 1991). In this paper, luminescence studies of Eu^{3+} and Tb^{3+} binding to GS at the adenylation extrema are described. Information regarding the stoichiometry and binding affinities of Eu^{3+} are obtained and used in competition experiments to obtain Mn^{2+} ion affinities to GS as a function of adenylation state. Experiments that measured the distances between the two metal ions are also reported. GS contains two tryptophan residues, Trp-57 and Trp-158, per subunit, and earlier studies used these fluorophores for sensitized Tb^{3+} luminescence studies to determine binding affinities and fluorophore to Tb^{3+} distances (Lin et al., 1991b). Using the wild-type and several single-tryptophan mutant forms of GS, the present work also measures Tb^{3+} ion binding affinities as a function of GS's regulatory state and Tb^{3+} -tryptophan distances.

Results

Luminescence titration studies of Eu^{3+} and Tb^{3+} binding to wild-type and single-tryptophan mutant GSs

$^7F_0 \rightarrow ^5D_0$ Excitation spectrum

In agreement with earlier studies (Eads et al., 1985), the excitation spectrum obtained for Eu^{3+} bound to wild-type GS appears as a single broad, slightly asymmetric band centered at ~579.15 nm (Fig. 2). There are no significant differences in the excitation spectra obtained for Eu^{3+} bound to GS samples of either adenylation extrema. The intensity of this peak increases with Eu^{3+} concentration, as does the peak asymmetry, owing to a shoulder that grows in at 578.7 nm. Each excitation spectrum was decomposed into a set of hybrid Lorentzian-Gaussian functions. McNemar and Horrocks (1989) have shown that this hybrid peak is the best function for describing Eu^{3+} excitation spectral data of solutions. Throughout an excitation spectral titration, each spectrum is best described as the sum of two Lorentzian-Gaussian peaks at 579.15 nm [peak full width at half maximum intensity (fwhm) = 0.41 nm] and 579.05 nm (fwhm = 0.86 nm) for GS_0 .

Fig. 2. Curve resolved ${}^7F_0 \rightarrow {}^5D_0$ excitation spectra of Eu^{3+} bound to $10 \mu\text{M}$ (total subunit concentration) unadenylylated GS at 1.0 molar equivalents of Eu^{3+} fit with (A) one and (B) two Lorentzian-Gaussian peaks. Peak parameters: (A) $\lambda_1 = 579.13 \text{ nm}$, $\text{fwhm} = 0.42 \text{ nm}$; (B) $\lambda_1 = 579.15 \text{ nm}$, $\text{fwhm}_1 = 0.41 \text{ nm}$; $\lambda_2 = 579.05 \text{ nm}$, $\text{fwhm}_2 = 0.65 \text{ nm}$. Plots of the relative residuals ($100\% \times (\text{observed} - \text{predicted})/\text{observed}$) are shown below each fit. $\lambda_{em} = 614 \text{ nm}$.

Although an initial inspection may indicate that the excitation spectra up to 1.0 equivalents can be described by a single peak function, further analysis reveals the peak asymmetry. The theoretical fit to the excitation spectrum of Eu^{3+} bound to GS at $[\text{Eu}^{3+}]/[\text{GS}] = 1.0$ is improved greatly with the use of an additional peak (Fig. 2B).

Binding isotherms were generated by plotting the individual peak areas as a function of metal ion concentration. Shown in Figure 3A, the area of the peak centered at 579.15 nm for GS_0 increases as a function of the metal ion concentration up to 1.0 equivalents, after which the peak area does not change. The peak centered at 579.05 nm for GS_0 shows a binding curve displaying a lag in the peak area change until after one equivalent of Eu^{3+} has been added.

The two bands derived from the analysis of the excitation spectra indicate two unique Eu^{3+} ion environments. This result is consistent with the X-ray crystal structure (Almassy et al., 1986) and kinetic (Denton & Ginsburg, 1969; Abell & Villafranca, 1991b) and equilibrium binding studies (Ginsburg, 1972), which have also concluded that GS possesses two metal ion binding sites or requires two metal ions for catalytic activity. A relationship proposed by Albin and Horrocks (1985) correlates the formal ligand charge with the peak transition energy and can be used qualitatively in making site assignments for each peak. For wild-type GS_0 , the

excitation maxima of the two bands at 579.05 nm and 579.15 nm corresponds to a respective total ligand charge of -1.0 and -1.8 . Charges of -0.7 and -1.7 are calculated for the peaks at 579.02 nm and 579.13 nm , respectively, from wild-type GS_{12} . These charge determinations are also consistent with the X-ray crystal structure within the error estimated for the transition frequency/ligand charge of ± 1.0 charge unit. According to the crystal structure, a metal ion bound at site n2 is ligated by two carboxylates of residues E129 and E357 and a imidazole nitrogen from H269. The metal ion at site n1 is ligated by three carboxylates from E131, E212, and E220. Assuming that Eu^{3+} is ligated by the same residues in the crystal structure, the Eu^{3+} excitation peak transitions may be assigned to the GS metal binding sites. Thus, Eu^{3+} bound to n2 produces the peak transitions at 579.05 nm (GS_0) and 579.03 nm (GS_{12}), and the peaks at 579.15 nm and 579.13 nm are assigned to Eu^{3+} binding to n1 of GS_0 and GS_{12} , respectively.

${}^5D_0 \rightarrow {}^7F_2$ emission decay experiments

To complement the excitation spectral studies, time domain experiments were implemented to examine the Eu^{3+} binding sites in GS based on differences in their emission decay. Regardless of the adenylation state, the emission decay of Eu^{3+} bound to GS can be decomposed into a sum of two single-exponential components in both H_2O and D_2O solvent systems. The Eu^{3+} binding iso-

Fig. 3. A: Areas of the curve resolved ${}^7F_0 \rightarrow {}^5D_0$ excitation spectra of Eu^{3+} bound to $10 \mu M$ (total subunit concentration) unadenylylated GS as a function of Eu^{3+} concentration. The solid line is the theoretical fit to the data. Peak intensities for GS_0 (●) $\lambda_1 = 579.15$ nm, $K_d(Eu-n1) = 0.35 \mu M$, (■) $\lambda_2 = 579.05$ nm, $K_d(Eu-n2) = 3.8 \mu M$. B: Intensities at $t = 0$ ms (I_0) of the ${}^5D_2 \rightarrow {}^7F_0$ emission decay of Eu^{3+} bound to $10 \mu M$ (total subunit concentration) unadenylylated GS, GS_0 , as a function of Eu^{3+} concentration. $\lambda_{ex} = 579.25$ nm, $\lambda_{em} = 614$ nm. The solid line is the theoretical fit to the data. (▲) I_0 values for the 2.2-ms component, $K_d(Eu-n1) = 0.35 \mu M$, (◆) I_0 values for the 1.2-ms component, $K_d(Eu-n2) = 3.8 \mu M$.

therms were produced by plotting the intensity (I_0) at time = 0 s, of each lifetime component as a function of metal ion concentration. Figure 3B shows results clearly similar to the binding isotherms obtained from the excitation spectral data. The long lifetime component, 2.2 and 2.3 ms for GS_0 and GS_{12} , respectively, increases in intensity with the addition of Eu^{3+} ion. Whereas the shorter lifetime component (1.2 and 1.3 ms for GS_0 and GS_{12} , respectively) has a lag in emission intensity change as the first equivalent of Eu^{3+} is added. Using the similarities found in the binding isotherms from the emission decay and excitation spectral titrations, the lifetime components can be assigned to each Eu^{3+} environment. Thus, Eu^{3+} bound to site n1 has a decay lifetime of 2.2 and 2.3 ms in D_2O for GS_0 and GS_{12} , respectively, and Eu^{3+} bound to n2 has a decay lifetime of 1.2 and 1.3 ms in D_2O for GS_0 and GS_{12} , respectively.

Time-resolved spectrum

The presence of unique Eu^{3+} -binding environments can also be established by time-resolution of the excitation spectrum. A time-resolved spectrum is generated by observing the ${}^5D_0 \rightarrow {}^7F_2$ emission decay at several wavelengths along the excitation profile and then plotting the I_0 s of each lifetime component as a function of the excitation wavelength. Figure 4 shows the results of a time-resolved spectrum when 1.0 equivalent of Eu^{3+} is bound to GS_0 . Throughout the excitation wavelength ranges examined, two lifetime components of 1.2 ms and 2.2 ms were obtained. Excitation maxima and the peak's fwhm were calculated using the Lorentzian-Gaussian function used to deconvolute the excitation spectrum. The excitation band determined by the 2.2-ms lifetime component has the greater intensity peak maximum at 579.14 nm and a fwhm of 0.44 nm, whereas the lower intensity band determined by the 1.2-ms component has a peak maxima of 579.16 nm and a fwhm of 0.65 nm. The individual lifetime components may be assigned to the curve-resolved peaks from the excitation spectrum on the basis of the similarities found in the individual peak widths and relative intensities obtained in the time-resolved and excitation spectra. Thus, the 2.2-ms lifetime component (fwhm = 0.46 nm)

is assigned to the Eu^{3+} transition at 579.15 nm (fwhm = 0.41) and the 1.2-ms component (fwhm = 0.66 nm) is assigned to the Eu^{3+} transition at 579.05 nm (fwhm = 0.65 nm).

Hydration of the inner coordination sphere

Horrocks and Sudnick (1979) have shown that the number of water molecules, q , coordinated to the Eu^{3+} at each site is related

Fig. 4. Excitation and time resolved spectrum of 1 equivalent of Eu^{3+} bound to GS_0 . The intensities of the 2.2-ms (■) and 1.2-ms (●) ${}^5D_2 \rightarrow {}^7F_0$ emission decay components and sum (▲) are plotted as a function of excitation wavelength. The solid line is the Eu^{3+} ${}^7F_0 \rightarrow {}^5D_0$ excitation spectrum. Dotted lines are the single curve-resolved Lorentzian-Gaussian peak fitted to each lifetime component. $\lambda_{em} = 614$ nm.

to the difference in emission decay rates in H₂O and D₂O solvent systems by Equation 1:

$$q = 1.05(\tau_{\text{H}_2\text{O}}^{-1} - \tau_{\text{D}_2\text{O}}^{-1}), \quad (1)$$

where τ^{-1} is the emission decay rate (reciprocal lifetime) in ms⁻¹ of each Eu³⁺ environment in the solvent indicated by the subscript. In H₂O solvent systems, Eu³⁺ binding to GS₀ has lifetimes of 220 μ s and 300 μ s for sites n1 and n2, respectively. Eu³⁺ binding to GS₁₂ has lifetime components of 200 μ s and 300 μ s for Eu³⁺ at sites n1 and n2. These lifetimes were determined using decay traces obtained during the latter stages of the titration in H₂O. Lifetime components due to Eu³⁺ bound to the n1 site and that of free Eu³⁺ (116 ms) were held constant during the analysis. Hydration numbers can be calculated using the lifetime values for Eu³⁺ binding to GS₀ and GS₁₂ in D₂O solvent systems reported in the preceding section. Eu³⁺ bound to site n1 of GS₀ and GS₁₂ is coordinated by 4.3 ± 0.5 and 4.6 ± 0.5 water molecules, respectively, and, when bound to site n2 of GS₀ and GS₁₂, Eu³⁺ is coordinated by 2.6 ± 0.5 water molecules. The hydration number for Eu³⁺ binding to n1 is in excellent agreement with the value of 4.1 ± 0.5 water molecules determined previously by Eads et al. (1985) using Eu³⁺ emission decay data and magnetic resonance studies of Gd³⁺ bound to GS.

Wild-type—Sensitized Tb³⁺ emission and fluorescence emission titration

Tb³⁺ binding to metal-free wild-type GS has a profound effect on the protein fluorescence emission spectrum, as shown in Figure 5. As the Tb³⁺ concentration increases, there is a quenching of the intrinsic fluorophores, as seen in the decrease in intensity in the 310–450-nm region of the spectra. Plots of the intrinsic fluores-

cence intensity as a function of added Tb³⁺ show that protein fluorescence is quenched as a function of increasing Tb³⁺ concentration (Fig. 5, inset). In addition to the quenching, there is a broadening and shifting of the peak to longer wavelengths. Fluorescence emission titrations of metal-free GS with other lanthanide ions and the divalent metals Mg²⁺ and Mn²⁺ also induce quenching of comparable magnitudes. Concomitant to this quenching is the appearance of Tb³⁺ emission peaks at 490 nm and 543 nm. These peaks increase with the increasing Tb³⁺ concentration. Unlike the quenching effect observed for the intrinsic fluorophores, plots of the Tb³⁺-sensitized emission intensities as a function of Tb³⁺ concentration show a lag in intensity change during the addition of the first equivalent (Fig. 5, inset).

Tryptophan mutants—Sensitized Tb³⁺ emission titration

Binding isotherms similar to those obtained for wild-type GS were determined for the single-tryptophan mutants W57L and W158F (Fig. 6A). The Tb³⁺ binding isotherm of the double mutant, W57L, W158F also has a sigmoidal shape similar to the single-tryptophan mutants (data not shown). In sharp contrast to the results of the wild-type, single-, and double-tryptophan mutants is the hyperbolic shape of the sensitized emission titration curve of the triple mutant F49W, W57L, W158L (Fig. 6B). This suggests that the triple mutation affects the quantum yields of Tb³⁺ bound at either the n1 or n2 site or that the mutation may have created other changes in the Tb³⁺ binding environment.

Binding model

Metal ion binding isotherms were generated from the luminescence titration experiments described above. The dissociation constants were calculated from the normalized binding isotherms using the program EQUIL (Goldstein & Leung, 1990). For a macromol-

Fig. 5. Uncorrected fluorescence emission spectra of 20 μ M (total subunit concentration) wild-type GS₀ with Tb³⁺ to subunit ratios of 1.0, 2.0, 3.0, and 4.0 molar equivalents. $\lambda_{\text{ex}} = 295$ nm. Inset: (●) Relative fluorescence emission at 330 nm ($\lambda_{\text{ex}} = 295$ nm) of 20 μ M (total subunit concentration) unadenylylated wild type GS, GS₀, as a function of Tb³⁺ concentration. (■) Tryptophan-sensitized Tb³⁺-sensitized emission intensities at 545 nm ($\lambda_{\text{ex}} = 295$ nm) as a function of Tb³⁺ concentration added to 10 μ M (total subunit concentration) relaxed unadenylylated wild-type GS. The solid line through the data is the theoretical fit: GS₀ K_d (Tb-n1) = 0.27 μ M, K_d (Tb-n2) = 3.5 μ M.

Fig. 6. **A:** Tryptophan-sensitized Tb^{3+} -sensitized emission intensities at 545 nm ($\lambda_{\text{ex}} = 295$ nm) as a function of Tb^{3+} concentration added to 10 μM (total subunit concentration) relaxed unadenylylated W158F GS. The solid line through the data is the theoretical fit: $K_d(\text{Tb-n1}) = 0.29 \mu\text{M}$, $K_d(\text{Tb-n2}) = 7.7 \mu\text{M}$. **B:** Tryptophan-sensitized Tb^{3+} -sensitized emission intensities at 545 nm ($\lambda_{\text{ex}} = 295$ nm) as a function of Tb^{3+} concentration added to 10 μM (total subunit concentration) relaxed unadenylylated F49W, W57L, W158F GS. The solid line through the data is the theoretical fit: $K_d(\text{Tb-n1}) = 6.96 \mu\text{M}$, $K_d(\text{Tb-n2}) = 25.9 \mu\text{M}$.

ecule with two distinct binding sites, the macroscopic equilibria for each site can be expressed by Equations 2 and 3 with the corresponding equilibrium association constants given by Equations 4 and 5.

$$K_1 = \frac{[\text{LnGS}]}{[\text{GS}][\text{Ln}]} \quad (4)$$

$$K_2 = \frac{[\text{Ln}_2\text{GS}]}{[\text{LnGS}][\text{Ln}]} \quad (5)$$

In the most general binding model used, one that assumes a random binding scheme, there would be four microscopic configurations for metal binding: GS, Ln^1GS , Ln^2GS , and Ln_2GS , indicating the metal-free, n1-occupied, n2-occupied, and metal-saturated enzymes, respectively.

Also, assuming that the two sites are independent allows the microscopic binding equilibria, expressed by Equations 7 and 8, to be related to the macroscopic binding constants by Equations 9 and 10.

$$K'_1 = K'_3 = \frac{[\text{Ln}^1\text{GS}]}{[\text{GS}][\text{Ln}]} = \frac{[\text{Ln}_2\text{GS}]}{[\text{Ln}^2\text{GS}][\text{Ln}]} \quad (7)$$

$$K'_2 = K'_4 = \frac{[\text{Ln}^2\text{GS}]}{[\text{GS}][\text{Ln}]} = \frac{[\text{Ln}_2\text{GS}]}{[\text{Ln}^1\text{GS}][\text{Ln}]} \quad (8)$$

$$K_1 = K'_1 + K'_2 \quad (9)$$

$$K_2 = 1/(K'_3 + K'_4) \quad (10)$$

In the case where the metal ion affinity to n1 (K'_1) is greater than to n2 (K'_2) metal binding to GS is still independent, but effectively sequential (or ordered) and Equation 6 reduces to:

Ln^{3+} equilibrium binding constants

The assignment of an observed isotherm to a specific binding event is critical in the calculation of equilibrium metal binding constants. Assuming that both Tb^{3+} and Eu^{3+} bind to GS with approximately equal affinity, isotherms can be obtained from the above experiments that uniquely describe lanthanide ion binding to a specific site. Thus, Ln^{3+} binding to the n1 site is attributed to the isotherms generated by the peak at ~ 579.15 nm from the Eu^{3+} excitation spectral titration and the ~ 2 -ms lifetime component from the time-resolved Eu^{3+} emission decay titration. At the same time, n2 occupancy by a Ln^{3+} ion is attributed to the binding curve obtained in the sensitized Tb^{3+} emission titration. Both the Eu^{3+} binding isotherms generated by the ~ 1.2 -ms lifetime component in the Eu^{3+} emission decay titration and the peak at ~ 579.03 nm from the Eu^{3+} excitation spectral titration show a lag in intensity change in the titration of the first equivalent. Subsequent to n1 site saturation, the Eu^{3+} emission intensity attributable to binding at n2 increases and continues to do so well after this site is filled, a behavior consistent with contributions from free Eu^{3+} ion in the latter stages of the titration. For the sensitized Tb^{3+} emission titration, no significant intensity changes occur after n2 site saturation.

The equilibrium binding constants calculated by EQUIL employed a global analysis of the data from the three titrations (Eu^{3+} emission decay titration, Eu^{3+} excitation spectral titration, and sensitized Tb^{3+} emission titration) using the aforementioned signals to the data for the individual metal site isotherms. The theoretical isotherms for the Figures 3A, B and 5 (inset) are the result of the global fit to all three data sets using the independent sites model.

Clearly, the lag in metal ion binding to n2 indicates that metal ion binding to GS is an ordered process. Further, the binding model also shows that adenylation state has a profound effect on the metal affinity for site n1 because the affinity decreases approximately sixfold as the adenylation state decreases from fully adenylylated (GS_{12} $K_d(Ln-n1) = 0.06 \pm 0.02 \mu M$) to unadenylylated (GS_0 $K_d(Ln-n1) = 0.35 \pm 0.09 \mu M$). The affinity for lanthanide ions at site n2 remains unchanged as a function of the enzyme's regulatory state (GS_{12} $K_d(Ln-n2) = 2.6 \pm 0.07 \mu M$ and GS_0 $K_d(Ln-n2) = 3.8 \pm 0.09 \mu M$).

Mn²⁺ equilibrium binding constants determined via competition

Binding affinities for Mn^{2+} were determined by a competition titration with Eu^{3+} . Previous studies have demonstrated the utility of using metal ion competition to determine the metal ion dissociation constants for both multidentate ligands (Albin et al., 1984) and protein systems (Breen et al., 1985; Bruno et al., 1992; Burroughs et al., 1992), provided that the equilibrium dissociation constant for Eu^{3+} binding is known. In these experiments, the intensity of Eu^{3+} emission from the GS complex is compared in the presence and absence of the competing metal ion. The amount by which the Eu^{3+} signal intensity is reduced is proportional to the amount of Eu^{3+} that has been replaced by the competing Mn^{2+} ion.

For independent sites, Equations 12 and 13 apply to competition for sites n1 and n2, respectively, and the relative K_d -values for each site are obtained by Equations 14 and 15.

$$K(n1) = \frac{K_d(Eu - n1)}{K_d(Mn - n1)} = \frac{[Eu^{2+}][MnMnGS]}{[Mn^{2+}][EuMnGS]} \quad (14)$$

$$K(n2) = \frac{K_d(Eu - n2)}{K_d(Mn - n2)} = \frac{[Eu^{3+}][MnMnGS]}{[Mn^{2+}][MnEuGS]} \quad (15)$$

The competition emission decay titration was performed by titrating Eu^{3+} into a 10 μM sample of relaxed (metal-free) GS preincubated with 100 μM of Mn^{2+} and recording the $^5D_0 \rightarrow ^7F_2$ emission decay at each addition (0.2 equivalents Eu^{3+} per aliquot). Analysis of each emission decay revealed the existence of two single-exponential components of 2.1 ms and 1.2 ms, which were attributed to Eu^{3+} binding at sites n1 and n2, respectively. By use of the independent sites binding model, and given the total concentrations of Eu^{3+} , Mn^{2+} , and GS, and the Eu^{3+} equilibrium dissociation constants, EQUIL calculated the Mn^{2+} dissociation constants by simultaneously analyzing the isotherms of the 2.1- and 1.2-ms components (Fig. 7). Again, the adenylation state of GS has a distinct effect on the Mn^{2+} binding affinity at site n1, as evidenced by the sevenfold decrease in the dissociation constant as the adenylation state increases from 0 to 12: $K_d(Mn-n1) = 0.05 \pm 0.02 \mu M$ for GS_0 and $K_d(Mn-n1) = 0.35 \pm 0.02 \mu M$ for GS_{12} . This is the reverse of the trend in the binding affinity as a function of adenylation state that is observed for Ln^{3+} binding. The binding affinity of Mn^{2+} to n2 remains essentially unchanged as a function of adenylation state, $K_d(Mn-n2) = 5.3 \pm 1.3 \mu M$ for GS_0 and $K_d(Mn-n2) = 4.0 \pm 1.0 \mu M$ for GS_{12} .

Fig. 7. Intensities at $t = 0$ ms of the $^5D_2 \rightarrow ^7F_0$ emission decay of Eu^{3+} bound to 10 μM (total subunit concentration) unadenylylated GS as a function of Eu^{3+} concentration in the presence of 0.10 mM $MnCl_2$. The solid line is the theoretical fit to the data. Unadenylylated, GS_0 , (●) intensities for the 2.2-ms component, $K_d(Mn-n1) = 0.05 \mu M$; (■) intensities for the 1.2-ms component, $K_d(Mn-n2) = 5.3 \mu M$.

Metal-metal and metal-tryptophan distance measurements

Förster theory

In addition to providing information about metal-binding properties, time-resolution techniques may also be used to make metric inquiries about Eu^{3+} binding to GS. Distances between an energy donor-acceptor pair, consisting of either an intrinsic protein fluorophore (e.g., tryptophan) and Tb^{3+} , or between Eu^{3+} and Nd^{3+} can be determined from energy transfer measurements, which occur by a mechanism of weak dipole-dipole coupling (Förster, 1965). Studies from this laboratory have used this energy transfer mechanism in order to determine fluorophore-metal and metal-metal distances in a number of biomolecules (Horrocks & Collier, 1981; Bruno et al., 1992; Burroughs et al., 1994). In the present study, single-tryptophan mutants of GS are used to determine Trp- Tb^{3+} distances and the wild-type form of GS is used to determine the metal-metal distances.

E , the efficiency of energy transfer, represents the fraction of excited-state energy donor molecules that are de-excited by energy transfer to an acceptor. The relationship between E and the donor-acceptor distance (r) is given by Equation 16:

$$E = (1 + (r/R_0)^6)^{-1} \quad (16)$$

The distance at which $E = 0.5$ is also known as the critical or "Förster" distance:

$$R_0^6 = (8.78 \times 10^{-25}) \kappa^2 \phi_D n J, \quad (17)$$

where κ^2 is the orientation factor; ϕ_D is the quantum yield of the donor in the absence of the acceptor; n is the refractive index of the medium; and J is the spectral overlap integral:

$$J = \frac{\int F(\nu) \epsilon(\nu) \nu^{-4} d\nu}{\int F(\nu) d\nu}, \quad (18)$$

where $F(\nu)$ is the luminescence intensity of the donor and $\epsilon(\nu)$ is the molar absorptivity of the acceptor in units of $\text{M}^{-1} \text{cm}^{-1}$.

Intermetal ion distances

Intermetal ion distances were determined by measuring the effect on donor Eu^{3+} emission lifetimes by an acceptor Nd^{3+} ion. Equation 19 relates the efficiency of energy transfer to the lifetimes of the donor Eu^{3+} in the presence (τ_{da}) and absence (τ_d) of the acceptor, Nd^{3+} :

$$E = \left(1 - \frac{\tau_{da}}{\tau_d}\right). \quad (19)$$

The observed energy transfer is assumed to take place between the lanthanide ions residing at sites n1 and n2 of any subunit in the dodecamer. Distances between the active sites in adjacent subunits range from 40 to 50 Å and, due to the r^{-6} dependence of E , any intersubunit energy transfer would be negligible. A total of 2.0 equivalents of Ln^{3+} was added to relaxed GS in a 19:1, $\text{Nd}^{3+}:\text{Eu}^{3+}$ molar ratio. This ratio ensures that nearly every Eu^{3+} will have a Nd^{3+} as a nearest neighbor, thus minimizing any unaffected Eu^{3+} emission.

The observed Eu^{3+} emission decay lifetimes in both the presence and absence of the acceptor ion were decomposed into two lifetime components, reflecting the presence of Eu^{3+} in two environments (n1 or n2). The Eu^{3+} emission at each site is considerably quenched in the presence of Nd^{3+} , albeit at different efficiencies (Table 1). Such differences in the efficiency values are likely due to differences of the quantum yields, $\phi_{\text{Eu}^{3+}}^{\text{D}_2\text{O}}$, for each Eu^{3+} environment.

The estimation of the quantum yield of the donor Eu^{3+} , $\phi_{\text{Eu}^{3+}}$, presents a problem due to the lack of suitable reference standards. Haas and Stein (1971) reported the quantum yield of free Eu^{3+} in D_2O to be 0.78. In this study, it is assumed that $\phi_{\text{Eu}^{3+}} = 0.8$ in this solvent because the relatively long lifetimes found for Eu^{3+} bound to GS indicate that nonradiative decay is at a minimum. A J value of $8.56 \times 10^{-18} \text{ cm}^6 \text{ mol}^{-1}$ was determined previously using the corrected emission spectrum of Eu^{3+} bound to GS and the absorption spectrum of $\text{Nd}(\text{edta})^-$ (McNemar, 1989). This value of J was assumed to be the same for GS of either adenylation extrema. n was taken to be 1.36, an intermediate value of water ($n_{\text{H}_2\text{O}}$) and of organic molecules containing only first row elements (n_{organic}). Horrocks and Collier (1981) have shown that κ^2 can be taken to be 2/3, for intermetal energy transfer. Table 1 lists the distances and efficiency values determined for each GS adenylation state based on a calculated $R_0 = 10.3 \text{ Å}$.

Trp to Tb^{3+} distance measurements

In cases where the efficiency for energy transfer is very low, E can be determined from Equation 20 as defined by Yang and Söll (1974):

$$E = \frac{A_A}{A_D} \frac{\phi_D}{\phi_A}, \quad (20)$$

where A_D and A_A are the respective integrated areas of the corrected luminescence emission spectrum (on a cm^{-1} scale) of the donor (tryptophan) and the acceptor (Tb^{3+}), and ϕ_D and ϕ_A are their respective quantum yields in the absence of energy transfer.

The quantum yields of the tryptophan residues in the absence of an acceptor were determined by comparison of the areas of the

Table 1. Distances and parameters for Förster-type energy transfer distance measurements between the lanthanide binding sites in *Escherichia coli* GS

Enzyme	Site	τ_d (ms)	τ_{da} (ms)	E^a	r (Å)	r_{avg} (Å)
GS ₀	n1	2.2	1.72	0.22	12.7±0.5	12.1±0.5
	n2	1.2	0.8	0.33	11.5±0.5	
GS ₁₂	n1	2.3	1.8	0.22	12.7±0.5	12.2±0.5
	n2	1.3	0.9	0.31	11.7±0.5	

$$^a E = \left(1 - \frac{\tau_{da}}{\tau_d}\right) \text{ and } r = R_0 \left(\frac{1}{E} - 1\right)^{1/6} \text{ with } R_0 = 10.27 \text{ Å}.$$

corrected fluorescence emission spectra ($F_{\text{GS}}^{295\text{nm}}$ and $F_{\text{std}}^{280\text{nm}}$) and absorbance values ($A_{\text{GS}}^{295\text{nm}}$ and $A_{\text{std}}^{280\text{nm}}$) at the excitation wavelength between lanthanide-tightened GS and the standard, an aqueous L-tryptophan solution (ϕ_{std} at pH 6.0; Chen, 1967):

$$\phi_{\text{GS}} = \phi_{\text{std}} \frac{F_{\text{GS}}^{295\text{nm}}}{F_{\text{std}}^{280\text{nm}}} \frac{A_{\text{std}}^{280\text{nm}}}{A_{\text{GS}}^{295\text{nm}}}. \quad (21)$$

The corrected emission spectrum of Ln^{3+} -tightened GS in the absence of an acceptor used Lu^{3+} , a closed-shell nonacceptor ion, in place of Tb^{3+} . The quantum yields calculated at $[\text{Lu}^{3+}]/[\text{GS}_0] = 2.0$ for each mutant are: 0.11 (W158F), 0.06 (W57L), and 0.12 (F49W, W158F, W57L).

The quantum yield of Tb^{3+} ($\phi_{\text{H}_2\text{O}}^{\text{Tb}^{3+}}$) bound to GS at 2.0 equivalents was determined from the excited state lifetimes (Burroughs et al., 1994) in H_2O ($\tau_{\text{H}_2\text{O}}$) and D_2O ($\tau_{\text{D}_2\text{O}}$) using Equation 22 with ($\phi_{\text{D}_2\text{O}}^{\text{Tb}^{3+}} = 1.0$):

$$\phi_{\text{H}_2\text{O}}^{\text{Tb}^{3+}} = \frac{\tau_{\text{H}_2\text{O}}}{\tau_{\text{D}_2\text{O}}} \phi_{\text{D}_2\text{O}}^{\text{Tb}^{3+}}. \quad (22)$$

At 2.0 equivalents, the emission decay lifetimes of Tb^{3+} bound to the single-tryptophan mutants reveal a single-exponential component, indicative of a single Tb^{3+} environment. Using the assumption that a Tb^{3+} bound to n2 is the only luminescent Tb^{3+} , the quantum yields were determined as 0.36 and 0.43 for W158F and W57L, respectively. The lifetime of the emission decay from Tb^{3+} bound to the triple mutant is described by two single-exponential components. In this instance, a value of 0.26 was calculated as the mean quantum yield for Tb^{3+} at n1 and n2.

In principle, the spectral overlap integral, J , is calculated using the corrected emission spectrum of the donor tryptophan and the absorption spectrum of the acceptor Tb^{3+} . The corrected emission spectrum of the donor is obtained easily; however, the absorption spectrum of Tb^{3+} bound to GS is unavailable due to the very low absorptivities of the $\text{Tb}^{3+} f \rightarrow f$ transition. Bruno et al. (1992) have shown that the absorption spectra of Tb^{3+} complexed to various polyaminocarboxylate ligands are very similar, leading to a limited range of J values. The overlap of the corrected GS emission spectrum with the absorption spectrum of the $\text{Tb}(\text{edta})^{1-}$ complex is illustrated in Figure 8. R_0 values of the Tb-GS complexes at 2.0 equivalents were calculated using Equation 17 given the values of J , κ^2 , n , and ϕ_D determined as described above (Table 2).

The efficiency of energy transfer was calculated using Equation 20 and using the values of the quantum yields for tryptophan

Fig. 8. Overlap between the corrected fluorescence emission spectrum ($\lambda_{ex} = 295$ nm) of the donor tryptophan (dots) from 10 μ M (total subunit concentration) unadenylylated F49W,W57L,W158F GS and the absorption spectrum of the model acceptor (line), 0.1 M Tb(edta) $^{2-}$.

in GS and Tb $^{3+}$, as well as the areas of their respective corrected fluorescence emission spectra. When Tb $^{3+}$ is bound to GS, the emission bands are resolved poorly except at 545 nm. However, because the relative intensities of the Tb $^{3+}$ f $\rightarrow$ f transition bands vary little from system to system, a Tb(edta) $^{-1}$ emission spectrum was used to approximate the entire Tb $^{3+}$ spectrum and scaled to

match the observed intensity of the TbGS luminescence band at 545 nm.

At [Tb $^{3+}$]/[GS] = 2.0, the analysis is potentially complicated because the observed energy transfer from a single donor tryptophan must be partitioned to the two Tb $^{3+}$ acceptor ions in each subunit. For the single-tryptophan mutants, the situation is simplified to a single donor–single acceptor case because it is assumed no energy is transferred to the Tb $^{3+}$ ion bound to n1. Therefore, only the distance from the donor tryptophan to n2 is calculated for these mutants. This simplification cannot be made with the triple mutant F49W,W57L,W158F because sensitized emission experiments indicate that Tb $^{3+}$ bound to both sites n1 and n2 luminesce. In cases of a single donor and two equivalent equidistant acceptors, it has been demonstrated that $E \approx E_{obsd}/2$ when E_{obsd} values are less than a few percent (Horrocks & Collier, 1981; Bruno et al., 1992). Because the tryptophan in the F49W mutant is approximately equidistant from sites n1 and n2 (Table 2), this approximation can be used to determine a mean efficiency of energy transfer between W49 and each metal ion site. The distances from the metal ions to the tryptophans and the resulting E and R_0 values are summarized in Table 2.

Discussion

In earlier analyses of sensitized Tb $^{3+}$ emission titration experiments (Lin et al., 1991b), an independent-sites binding model was used to determine the equilibrium binding constants of metal ion binding to GS. Although the same binding model is used in the present work, the interpretation of the sensitized Tb $^{3+}$ emission results as it applies to the binding events is reevaluated. Previously, it was assumed that the sensitized Tb $^{3+}$ emission of the wild-type data involved a contribution from each bound Tb $^{3+}$, on the condition that the quantum yield of the Tb $^{3+}$ bound at n1 is much

Table 2. (Top) Distances and Förster-type energy transfer parameters between the bound Tb $^{3+}$ and tryptophan in *Escherichia coli* GS ([Tb $^{3+}$]/[GS] = 2.0) and (Bottom) distances between the same two groups derived from the crystallographic model of *Salmonella typhimurium* GS

Experimental results at [Ln $^{3+}$]/[GS $_0$] = 2.0 eq.						
Enzyme	Donor	Metal site	J^a	R_0 (Å)	E_{obsd} ($\times 10^{-4}$)	r (Å)
W158F	Trp 57	n2	2.64	3.84	6.22	13.1 $\pm$ 1.6
W57L	Trp 158	n2	2.77	3.76	7.66	12.4 $\pm$ 1.5
F49W,W158F,W57L	Trp 49	n1/n2	2.72	4.21	22.67	13.0 $\pm$ 1.6
Crystal structure distances						
	Subunit			Subunit		r (Å)
Mn $^{2+}$ at n1	A	Trp 57		B		17.3
Mn $^{2+}$ at n2	A	Trp 57		B		18.2
Mn $^{2+}$ at n1	A	Trp 158		A		21.0
Mn $^{2+}$ at n2	A	Trp 158		A		26.1
Mn $^{2+}$ at n1	A	Phe 49		B		12.4
Mn $^{2+}$ at n2	A	Phe 49		B		13.0
Mn $^{2+}$ at n1	A	Tyr 179		A		7.6
Mn $^{2+}$ at n2	A	Tyr 179		A		11.9

^a $J = (\times 10^{-19} \text{ cm}^6 \text{ mol}^{-1})$.

smaller than that of Tb^{3+} bound at site n2. In this study, it is hypothesized that there is no sensitized Tb^{3+} emission from Tb^{3+} ions bound to n1. The absence of sensitized Tb^{3+} emission for ions in site n1 may be due to an efficient quenching mechanism at that site or an unfavorable dipole orientation between the donor-acceptor pair in the relaxed and n1-tightened conformation, or both.

With the addition of Tb^{3+} to relaxed GS, a conformational change occurs that is manifested in the quenching of the fluorescence spectrum and changes in the absorption spectrum. Difference absorption (Ginsburg, 1972; Segal & Stadtman, 1972; Li, 1990; Reynaldo, 1995) and kinetic (Kingdon et al., 1968) experiments indicate that GS is in the fully tightened form when n1 is saturated, whereas the sensitized Tb^{3+} emission titration studies show that Tb^{3+} emission is extremely weak or nonexistent until one equivalent of Tb^{3+} has been added. An interpretation of the Tb^{3+} -sensitized emission data, which assumes a "silent" Tb^{3+} occupies the n1 site, consistently describes the observed binding isotherms obtained with wild-type and single- and double-tryptophan mutant GS. These experiments show a lag in intensity change as the first equivalent of Tb^{3+} is added, and imply that these isotherms describe metal ion binding to n2 exclusively.

The "anomalous" binding isotherm obtained from the Tb^{3+} emission titration of F49W, W57L, W158F can also be explained with the same binding model. Under neutral pH and high ionic strength, electron micrographs show that the relaxed form of GS maintains the quaternary structure of a dodecamer (Valentine et al., 1968), indicating that there are regions within the subunit that do not substantially change during the metal-induced conformational change. Placement of the tryptophan at such a position, provided that it is oriented favorably for energy transfer, would allow for Tb^{3+} emission at both n1 and n2. However, it is possible that a triple mutation may induce significant local conformational changes to the enzyme. One cannot rule out that such effects may alter significantly the metal binding mechanism from that found in the wild-type and in the single- and double-tryptophan mutants. Because the metal ions have crucial roles in the catalytic mechanism, and it is observed that activity is preserved with this mutant, such effects may be minimal in this triple mutant (Reynaldo, 1995).

The theoretical fits to the data used an independent-sites binding model. The data also indicates that metal ion binding to GS is an ordered process. However, a good fit alone is not sufficient to prove a model. The experiment must show not only a good fit to an accepted model, but also a poor fit to any plausible alternatives. Attempts to interpret the sensitized Tb^{3+} emission titration experiments as the combination of Tb^{3+} occupancy in sites n1 and n2 resulted in calculations of binding constants that failed to converge in EQUIL. Previous studies, which interpreted the binding isotherm as the sum of Tb^{3+} occupancy at both n1 and n2 sites, resulted in poor theoretical fits and the calculation of equilibrium binding constants for Tb^{3+} binding to GS of $K_d(\text{Tb-n1}) = 2.2 \times 10^{-11}$ M and $K_d(\text{Tb-n2}) = 3.0 \times 10^{-9}$ M (Lin et al., 1991b). Lin's reported value for Tb^{3+} binding to n1 indicates that metal binding is quantitative, a phenomena that is clearly not supported by the data presented in that paper or observed here. Also the literature value for the equilibrium dissociation constant for Tb^{3+} binding to n1 is well below the concentration level (16 μM) used to perform the experiment. The independent-sites metal binding model has been successful in elucidating the effects of the enzyme's regulatory state on metal ion affinity. Admittedly, the close

proximity of the divalent metals to each other implies to us that the present binding model is a naive description of metal binding to the enzyme. Indeed, previous kinetic inhibitor studies (Wedler et al., 1982) clearly indicate that subunit cooperativity does exist. However, even with our ability to obtain separate binding isotherms for each metal ion site, the present experiments are limited in their ability to distinguish between the various metal ion/conformational states that may occur in such a cooperative binding model.

The GS X-ray crystal structure from which the calculated tryptophan- Tb^{3+} distances are compared is derived from *Salmonella typhimurium* (Yamashita et al., 1989). This form differs from *E. coli* GS by ten amino acid substitutions (Almassy et al., 1986; Colombo & Villafranca, 1986); it is assumed that the structures of GS from the two sources are very similar. The distances determined from the crystal structure are taken from the center of the metal ion to the center of the indole ring system. Kinemage 3 illustrates the relative positions of the metal ions and tryptophans. Based on the results for the W158F mutant, the distances from Trp 57 to n2 are calculated to be 13.1 ± 1.6 Å. The X-ray model yields a distance of 18.2 Å. The difference between the two results may be attributed to the mobility of Trp 57, which is located on a loop on the outer surface of GS. Eisenberg indicated in his analysis of the crystallographic data that this loop has a high degree of flexibility and that the position of Trp 57 is not well defined (Yamashita et al., 1989). Rotation of Trp 57 around the $\text{C}_\alpha\text{-C}_\beta$ and $\text{C}_\beta\text{-C}_\gamma$ bonds of the residue results in distances as small as 14.9 Å. The mean distance calculated from the Tb^{3+} sites to Trp 49 of 13.0 ± 1.6 Å compares very favorably to the crystal structure, which has a metal at n2 to Phe 49 distance of 13.0 Å. However, the calculated distance of 12.4 ± 1.6 Å from the Tb^{3+} at n2 to Trp 158 in the W57L mutant is 13 Å shorter than the crystal structure distance of 26 Å. Based on the crystal data, an energy transfer efficiency of only 2% of the actual observed efficiency is predicted. This difference may be attributed to a unique conformation attained when binding a trivalent lanthanide ion. Additionally, Lin et al. (1991a) suggested that energy transfer from Tyr 179, which is normally quenched by Trp 57, may contribute to the observed energy transfer. Tyr 179 is located in a segment of the polypeptide chain containing the central loop, which extends into the central cavity (Kinemage 3). Despite the high absorptivity of tyrosine at 280 nm and its high quantum yield in aqueous solution, tyrosine fluorescence in proteins is generally much weaker than tryptophan fluorescence. Tyrosine fluorescence in proteins can be quenched by transfer of the hydroxyl proton to nearby uncharged amino acid groups or charged carboxylate groups during the excited state lifetime. Also, tyrosine to tryptophan energy transfer is expected to be efficient, because the Förster distance for tyrosine to tryptophan energy transfer is about 14 Å (Lackowicz, 1983). In the emission spectra of wild-type GS_0 (not shown) and W158F GS_0 (Fig. 9), tyrosine emission is very weak. However, tyrosine emission is very prominent in the emission spectrum of the W57L GS_0 mutant, indicating that Trp 57 may quench the tyrosine emission. Based on pK_a determinations of the tyrosine ionization using NBS-treated single-tryptophan mutants, Lin determined that a single tyrosine (from a total of 16 per subunit) is responsible for tyrosine emission. The tyrosine to Tb^{3+} distance measurements, made using the NBS-treated single-tryptophan mutants, of 7.6 and 11.9 Å also suggest that Tyr 179 may be the residue responsible for additionally sensitizing Tb^{3+} emission, along with Trp 158 in the W57L mutant (Lin et al., 1991a).

Fig. 9. Corrected fluorescence emission spectrum of tryptophan mutants at $[GS_0] = 10 \mu M$ (total subunit concentration) $\lambda_{ex} = 280$ nm.

The calculated intermetal distances of $12.1 \pm 1.5 \text{ \AA}$ for unadenylylated and $12.2 \pm 1.5 \text{ \AA}$ for fully adenylylated GS as determined by Eu^{3+} luminescence spectroscopy are also in good agreement with the Tb^{3+} – Ho^{3+} distances of $12 \text{ \AA}$ as calculated on the basis of earlier Förster energy transfer experiments (McNemar, 1989). However, the values determined by this study are longer than those reported by Lin et al. (1991b), who used the same energy transfer mechanism, but used the donor–acceptor pair of Tb^{3+} – Ho^{3+} and Tb^{3+} – Nd^{3+} ($7.9 \text{ \AA}$ and $6.8 \text{ \AA}$, respectively). In their determination of the intermetal distances, Lin et al. observed efficiencies of energy transfer approximately three times greater than the efficiencies observed in the present study. Rhee et al. (1981) also observed similar differences in the efficiency of energy transfer (E) as a function of the lanthanide donor–acceptor pair. In their study of carp parvalbumin, they observed that, when Eu^{3+} was used as an energy donor, E was found to be from three to seven times smaller than a donor–acceptor pair that used Tb^{3+} as the donor. These energy transfer efficiencies resulted in the calculation of intermetal distances that spanned a $3.8\text{-}\text{\AA}$ range.

However, these distances are still longer than the Mn^{2+} – Mn^{2+} separation of $5.6 \text{ \AA}$ in the *S. typhimurium* crystal structure (Almassy et al., 1986). This result may also suggest that the binding of lanthanide ion by the enzyme results in an active site conformation that is different from the catalytically active form imposed by the binding of a divalent metal ion such as Mg^{2+} or Mn^{2+} . Indeed, GS was found to be catalytically inactive in the presence of saturating levels of lanthanide ions. Also, manganese (II) EPR studies (Gibbs et al., 1984; Villafranca et al., 1985) in the presence of the transition-state inhibitor, methionine sulfoximine (MSOX), demonstrate that the enzyme's intermetal distances increases to $10 \pm 1 \text{ \AA}$ and $11 \pm 1 \text{ \AA}$ for unadenylylated and adenylylated GS, respectively. Although the binding of Eu^{3+} may cause the enzyme's active site to be perturbed, the Mn^{2+} competition experiments indicate that the lanthanides are being bound to GS by the same ligands that bind the divalent metals. We believe that the distance values obtained with the Eu^{3+} – Nd^{3+} donor–acceptor pair

provide an upper limit to the intermetal distances on Ln^{3+} -bound GS.

The complementary use of Tb^{3+} and Eu^{3+} luminescence experiments to study metal ion binding to GS has provided further insight to the relationship between the regulatory state of GS and the bound metal ions. Unlike previous studies, these experiments allowed for the separate study of metal ion binding to each site, n1 and n2. By obtaining a unique isotherm for metal ion binding to each site, we show that adenylylation changes the enzyme's affinity for metal ions significantly, regardless of the metal ion type. In the case of Mn^{2+} , the metal ion affinity decreases as the adenylylation state increases. Abell and Villafranca (1991a) have also shown, from steady-state kinetic measurements, that adenylylation also affects substrate binding with the K_m s for both ATP and glutamate, increasing as the adenylylation state increases. Therefore, we suggest that adenylylation of the Mn^{2+} -bound form of GS functions to regulate the *in vitro* enzyme activity by weakening metal ion binding and elevating the substrate K_m s above concentrations found *in vivo*.

Materials and methods

All chemicals were of the highest grade commercially available. Aqueous solutions were made using either ultrapure distilled deionized water or from commercially available D_2O (Aldrich Chemicals). $EuCl_3 \cdot 6H_2O$, $TbCl_3 \cdot 6H_2O$, and $MnCl_2$ were obtained from Aldrich Chemicals and used without further purification. The metal ions were standardized by either furnace atomic absorption spectroscopy or by titration with EDTA using arsenazo III indicator (Fritz et al., 1958).

Cell growth, enzyme purification, and sample preparation

Wild-type *E. coli* GS was expressed using the pGln35 plasmid as the expression vector. The tryptophan mutant forms GS were constructed using site-directed mutagenesis techniques and kindly provided by Drs. William Atkins (Atkins & Villafranca, 1992) and Toru Maruyama of the Villafranca laboratory. Fully unadenylylated and adenylylated forms of wild-type and mutant GS were obtained by transforming the plasmid into *E. coli* strains YMC21E and YMC21D, respectively (Sambrook et al., 1989). YMC21E, which can produce fully unadenylylated enzyme, lacks the adenylylation enzyme ATase, whereas YMC21D, which lacks the ATase modification enzyme UTase, allows for the full adenylylation of the enzyme. These cell strains were constructed and donated by J. Bowie of the UCLA Molecular Biology Institute. Both strains grew in minimal media by the method of Roseman and Levine (1987) and were harvested in stationary phase after 20 h of growth.

Wild-type and mutant *E. coli* GS were purified by the zinc precipitation method of Miller et al. (1974) with modifications. The harvested cell paste suspension was sonicated and then centrifuged to separate the cellular debris. The nucleic acids were precipitated by a streptomycin sulfate precipitation. GS was then extracted by a zinc-induced paracrystallization followed by exhaustive dialysis against EDTA to remove zinc ions. Further purification involved an acetone precipitation followed by an ammonium sulfate precipitation step. The centrifuged precipitate was resuspended and dialyzed in buffer containing 10 mM imidazole, pH 7.2, 100 mM KCl, and 1 mM $MnCl_2$. The enzyme was stored in solution at $4^\circ C$ until needed.

Metal-free samples of GS were prepared by concentration of the purified enzyme using a Centricon-30 (Amicon) centrifugal con-

centration apparatus followed by dilution with the metal free buffer. The sample was reconcentrated to a final volume of $\sim 50 \mu\text{L}$ and then transferred to a mini column containing a 1-mL bed of PolyMetal-B Sponge (Molecular Probes, Inc.) that was prewashed and equilibrated with the working metal-free buffer. The enzyme sample, incubated in the column for a period of 12–14 h, was recovered and reconcentrated to a final volume of 1 mL. Protein concentrations were determined using either the spectroscopic methods of Stadtman and Ginsburg (1974) or using the Pierce BCA protein assay. The standard curves for the BCA assay were based on a known quantity of wild-type GS. Absorption values were recorded using a Varian-Cary 210 Spectrophotometer.

Luminescence experiments

A photon-counting lanthanide luminescence spectrophotometer is used to collect Eu^{3+} emission decay and excitation spectrum. The excitation source consists of a pulsed Nd:YAG dye-pumped laser (Continuum, YG-581C and TDL-50). The laser operates at 10 Hz, producing between 60 and 90 mJ/pulse. The emitted photons are collected at 90 degrees to the incident laser beam and passed through a 0.2-m double monochromator (Instruments SA, DH-20-V-IR) set to 614 nm before reaching the photomultiplier tube (Hamamatsu, R928 HA). Photon counting uses a CAMAC (LeCroy Instruments) scalar unit was used for the high-speed counting of the signal from the photomultiplier tube. The CAMAC unit consists of a dual-channel scalar, two memory modules, and a IEEE-488 General Purpose Interface Bus (GPIB) for communication and to an external computer. This external computer consists of an MS-DOS 386SX computer (Swan Technologies) that is used for the preparation of lifetime and spectral histograms, analysis, and storage of the data. A mixture of Rhodamine 590 (Exciton) and Rhodamine 590 (Kodak) dyes was used to excite the ${}^7\text{F}_0 \rightarrow {}^5\text{D}_0$ transition of the Eu^{3+} ion. The ${}^5\text{D}_0 \rightarrow {}^7\text{F}_2$ emission band was monitored at 614 nm. Eu^{3+} emission decays were the sum of 3,000 transients (5-min collection time) with a time-base resolution of 10 or 40 μs /channel. Using the program PEAKFIT (Jandel Scientific), the analysis of the emission decays involved their decomposition into single-exponential components. The quality of the fit was judged by the plots of both the absolute and relative residuals between the experimental data and the sum of the single exponential. A fit was deemed acceptable if the residual plots exhibited random deviations from zero.

Fluorescence and Tb^{3+} luminescence experiments were measured on a SPEX Fluorolog-2 spectrofluorimeter with phosphorimeter accessory for recording emission decays or a Photon Technology International LS-100 spectrofluorimeter. Tb^{3+} -sensitized emission titration experiments were performed by exciting the combination of tyrosine and tryptophan residues or the tryptophan residues alone at 280 nm and 295 nm, respectively. The excitation and emission band pass was set to 3 and 6 nm, respectively. Following a 20-min incubation of the metal-enzyme complex, the emission intensity values at a given Tb^{3+} concentration were obtained by averaging the 545-nm emission intensity at 1-s intervals over a 60–120-s period. The Tb^{3+} emission decays obtained were analyzed for single-exponential components using the program PEAKFIT from Jandel Scientific. Fluorescence emission from the intrinsic tyrosine and tryptophan residues was monitored as a function of metal ion concentration by exciting at 280 or 295 nm and monitoring emission from 320 to 330 nm. The excitation and emission band pass was set to 2 and 4 nm, respectively. Except where

noted, the luminescence spectral measurements were uncorrected for monochromator dependencies. All luminescence experiments were performed at 25 °C with the spectral or intensity measurement taken 20 min after metal ion was added to ensure that any metal-induced conformational changes were complete. Buffers used in preparing the metal ion and protein solutions for all the titrations were 0.1 M Hepes, pH 7.0, 0.5 M KCl. All buffers were made from double-distilled deionized water.

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