to the two Tb(III) binding sites of GS.

Registry No. GS, 9023-70-5; L-Trp, 73-22-3; Tb, 7440-27-9.

REFERENCES

Almassy, R. J., Janson, C. A., Hamlin, R., Xuong, N.-H., & Eisenberg, D. (1986) *Nature 323*, 304–309.

Atkins, W. M., Stayton, P. S., & Villafranca, J. J. (1991) *Biochemistry* (first of four papers in this issue).

Beardsley, K., & Cantor, C. R. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 39–46.

Breen, P. J., Hild, E. K., & Horrocks, W. DeW., Jr. (1985) *Biochemistry 24*, 4991.

Chen, R. F. (1967) *Anal. Lett. 1*, 35–42.

Colombo, G., & Villafranca, J. J. (1986) *J. Biol. Chem. 261*, 10587–10591.

Denton, M. D., & Ginsburg, A. (1969) *Biochemistry 8*, 1714.

Eads, C. D. (1985) Ph.D. Thesis, The Pennsylvania State University.

Eads, C. D., Mulqueen, P., Horrocks, W. DeW., Jr., & Villafranca, J. J. (1985) *Biochemistry 24*, 1221.

Förster, T. (1948) *Ann. Phys. (Leipzig) 2*, 55–75.

Förster, T. (1965) in *Modern Quantum Chemistry* (Sinanoglu, O., Ed.) Part III, pp 93–137, Academic Press, New York.

Horrocks, W. DeW., Jr. (1982) *Adv. Inorg. Biochem. 4*, 201.

Horrocks, W. DeW., Jr., & Collier, W. E. (1981) *J. Am. Chem. Soc. 102*, 2856–2862.

Horrocks, W. DeW., Jr., & Sudnick, D. R. (1981) *Acc. Chem. Res. 14*, 384–392.

Hunt, J. B., Smyrniotis, P. Z., Ginsburg, A., & Stadtman, E. (1975) *Arch. Biochem. Biophys. 161*, 102.

Janson, C. A., Kayme, P. S., Almassy, R. J., Grunstein, M., & Eisenberg, D. (1986) *Gene 46*, 297–300.

Kunkel, T. A., Roberts, J. D., & Zabour, R. A. (1986) *Methods Enzymol. 154E*, 367–381.

Lin, W.-Y., Dombrosky, P., Atkins, W. M., & Villafranca, J. J. (1991a) *Biochemistry* (fourth of four papers in this issue).

Lin, W.-Y., Eads, C. D., & Villafranca, J. J. (1991b) *Biochemistry* (third of four papers in this issue).

Miller, R. E., Shelton, E., & Stadtman, E. R. (1974) *Arch. Biochem. Biophys. 163*, 155–171.

Reuben, J. (1971) *Biochemistry 10*, 2834.

Reuben, J. (1979) in *Handbook on the Physics and Chemistry of the Rare Earths* (Gscheidner, K. A., & Eyring, L., Eds.) North-Holland Publishing, Amsterdam.

Shapiro, B. M., & Stadtman, E. R. (1970) *Methods Enzymol. 17A*, 910–922.

Stadtman, E. R., & Ginsburg, A. (1974) *Enzymes (3rd Ed.) 10*, 755–807.

Villafranca, J. J., Ash, D. E., & Wedler, F. C. (1976) *Biochemistry 15*, 536, 544.

Willis, K. J., & Szabo, A. G. (1989) *Biochemistry 28*, 4902–4908.

Yamashita, M. M., Almassy, R. J., Janson, C. A., Cascio, D., & Eisenberg, D. (1989) *J. Biol. Chem. 264*, 17681–17690.

Yang, C.-H., & Joll, D. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2838–2842.

Fluorescent Probes for Measuring the Binding Constants and Distances between the Metal Ions Bound to *Escherichia coli* Glutamine Synthetase†

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ABSTRACT: TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate, has been used as a fluorescent probe to determine the binding constants of metal ions to the two binding sites of *Escherichia coli* glutamine synthetase (GS). TNS fluorescence is enhanced dramatically when bound to proteins due to its high quantum yield resulting from its interactions with hydrophobic regions in proteins. The fluorescence energy transfer from a hydrophobic tryptophan residue of GS to TNS has been detected as an excitation band centered at 280 nm. Therefore, TNS is believed to be bound to a hydrophobic site on the GS surface other than the active site and is located near a hydrophobic Trp residue of GS. GS binds lanthanide ions [Ln(III)] more tightly than either Mn(II) or Mg(II), and the binding constants of several lanthanide ions were determined to be in the range (2.1–4.6) × 10^{10} and (1.4–3.0) × 10^{8} M^{-1} to the two metal binding sites of GS, respectively. The intermetal distances between the two metal binding sites of GS were also determined by measuring the efficiencies of energy transfer from Tb(III) to other Ln(III) ions. The intermetal distances of Tb(III)–Ho(III) and Tb(III)–Nd(III) were 7.9 and 6.8 Å, respectively.

Escherichia coli glutamine synthetase (GS) is a dodecameric enzyme which catalyzes the formation of glutamine from glutamate, NH_4^+, and ATP. The enzyme contains two essential metal binding sites per subunit—a higher affinity n_1 site and a lower affinity n_2 site (Ginsburg, 1972; Hunt et al., 1975). The metal–metal interaction for the two binding sites of GS has been investigated by using different chromophoric metal ions [e.g., Mn(II), Co(II), Cr(III), and lanthanide ions (Ln(III))] and by different spectroscopic methods [e.g., NMR, EPR, fluorescence energy transfer, and sensitized Tb(III) luminescence] (Villafranca & Ash, 1976; Villafranca et al., 1977, 1978; Balakrishnan & Villafranca, 1979; Gibbs et al., 1984; Eads et al., 1985).

Tb(III) luminesces in solution at room temperature, and its luminescence is significantly enhanced when bound to proteins due to Förster-type energy transfer. The energy relay system

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of tryptophan → Tb(III) → other Ln(III) has been successfully applied to determine the metal–metal and metal–Trp distances in enzymes (Horrocks & Sudnick, 1981; Rhee et al., 1981; Horrocks et al., 1975; Horrocks & Collier, 1981). A similar approach has been attempted to determine the intermetal distances between the two binding sites of GS by assuming equal binding for the various lanthanide ions to GS. However, the calculated intermetal distances may be subject to a substantial error without knowledge of the binding constants of metal ions to the two binding sites of GS.

TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate, is a compound which does not fluoresce in water but fluoresces strongly in organic solvents and when bound to proteins (McClure & Edelman, 1966, 1967). Because TNS fluorescence is very sensitive to the local environment in a TNS–enzyme complex, binding of the substrate, inhibitor, or metal ions to the active site may cause a conformational change and hence induce a change in TNS fluorescence. Thus, in the present studies, TNS was used as a fluorescent probe to determine the binding constants of metal ions to GS.

Experimental Procedures

Materials. *E. coli* glutamine synthetase in a low state of adenylation was prepared by the method of Miller et al. (1974). Enzyme concentration and state of adenylation were determined spectrophotometrically (Shapiro & Stadtman, 1970). All enzyme concentrations were given as subunit concentrations. Metal-free enzyme was prepared by dialysis against 3 mM EDTA, 10 mM Hepes, and 100 mM KCl, pH 7.0, followed by extensive dialysis against the same buffer without EDTA. Lanthanide(III) solutions were prepared from the chloride salts obtained from Aldrich. $MgCl_2$ and $MnCl_2$ were purchased from Sigma. All experiments were carried out in 10 mM Hepes/100 mM KCl, pH 7.0 at 25 °C.

Determination of the Dissociation Constants of TNS and GS. The fluorescence experiments with Tb(III) and TNS were measured by using a Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with a differential corrected spectra unit and a variable-temperature cell holder.

TNS fluoresces strongly when bound to proteins. Analysis of a titration curve monitoring TNS fluorescence allows the evaluation of the dissociation constant of GS and TNS. The observed fluorescence of TNS, F, is related to the equilibrium concentration of TNS, [TNS], by

$$F = F_0 - K_D(F/[\mathrm{TNS}]) \quad (1)$$

where F_0 is the fluorescence of the GS–TNS complex. K_D is the dissociation constant of the GS–TNS complex and is defined as

$$K_D = [\mathrm{E}][\mathrm{TNS}]/[\mathrm{E{\cdot}TNS}] \quad (2)$$

[TNS] is related to the total concentrations of enzyme and TNS, $[E]_0$ and $[TNS]_0$, by

$$[\mathrm{TNS}] = [\mathrm{TNS}]_0 - (F/F_0)[\mathrm{E}]_0 \quad (3)$$

Determination of the Binding Constants of Metal Ions to GS. Fluorescent probes are analogous to adsorption indicators (Laurence, 1952), and their behavior may be studied by standard equilibrium methods. TNS is believed to bind to the enzyme at some site other than the active site. Thus, it does not compete with metal ions for the metal binding sites and is acting like an indicator.

There are four possible configurations of GS when considering the binding of metal ions to the two binding sites of GS: E(−−), E(M−), E(−M), and E(MM) for apo, n_1-occupied, n_2-occupied, and double-occupied enzymes, respectively.

Table I: Expressions Relating Binding Constants and Fluorescence Properties of Different Metal Ion Complexes of Glutamine Synthetase

configuration	fraction	fluorescence
E(−−)	$\phi_0 = 1/\Phi$	$\phi_0 F_0$
E(M−)	$\phi_1 = K_1[\mathrm{M}]/\Phi$	$\phi_1 F_1$
E(−M)	$\phi_2 = K_2[\mathrm{M}]/\Phi$	$\phi_2 F_2$
E(MM)	$\phi_3 = K_1K_2[\mathrm{M}]^2/\Phi$	$\phi_3(F_1 + F_2)$

Suppose that the binding constants of the metal ions at n_1 and n_2 are K_1 and K_2, respectively, and that the binding of the metal ion at one site does not affect the binding of the other; then K_1 and K_2 are given by

$$K_1 = [\mathrm{E(M-)}]/[\mathrm{E}][\mathrm{M}] = [\mathrm{E(MM)}]/[\mathrm{M}][\mathrm{E(-M)}] \quad (4)$$

$$K_2 = [\mathrm{E(-M)}]/[\mathrm{E}][\mathrm{M}] = [\mathrm{E(MM)}]/[\mathrm{M}][\mathrm{E(M-)}] \quad (5)$$

where [E] and [M] are the equilibrium concentrations of free enzyme and metal ion, respectively. The mass balance equation for the enzyme is given by

$$[\mathrm{E}]_T = [\mathrm{E}](1 + K_1[\mathrm{M}] + K_2[\mathrm{M}] + K_1K_2[\mathrm{M}]^2) = [\mathrm{E}]\Phi \quad (6)$$

where $[E]_T$ is the total concentration of the enzyme. The mole fractions and the fluorescence for the four configurations are given in Table I where F_0 is the TNS fluorescence in the absence of metal ions. F_1 and F_2 are the TNS fluorescence when n_1 or n_2 is fully occupied by M. The observed fluorescence (F), which is the sum of the fluorescence of the four configurations, is given by

$$F = \phi_0F_0 + (\phi_1 + \phi_3)F_1 + (\phi_2 + \phi_3)F_2 \quad (7)$$

To determine the binding constants K_1 and K_2, TNS fluorescence was measured as a function of the total metal ion concentration, $[M]_T$, at fixed concentrations of GS and TNS. For given values of K_1 and K_2, [M] can be estimated by successive approximation using eq 6 and the mass balance equation of the metal ion (eq 8)

$$[\mathrm{M}] = [\mathrm{M}]_T - (\phi_1 + \phi_2 + 2\phi_3)[\mathrm{E}]_T \quad (8)$$

where $[M]_T$ is the total concentration of the metal ion. For given values of K_1, K_2, and [M], the fraction for each configuration was calculated, and the values of F_0, F_1, and F_2 were also evaluated by a least-squares method according to eq 7. The binding constants were determined by varying the values of K_1 and K_2 until the best fit of the experimental TNS fluorescence titration curve by eq 7 was achieved.

Determination of the Binding Constants by Metal Competition. Suppose that there are two metal ions, M_A and M_B, competing for the two binding sites of the enzyme, n_1 and n_2, and the binding constants at n_1 and n_2 are K_{A1} and K_{A2} for M_A and K_{B1} and K_{B2} for M_B. If the binding of the metal ion at one site does not affect the binding of the other, then these binding constants are given by

$$K_{A1} = [\mathrm{E(M_A-)}]/[\mathrm{E}][\mathrm{M_A}] = [\mathrm{E(M_AM_A)}]/[\mathrm{M_A}][\mathrm{E(-M_A)}] \quad (9)$$

$$K_{A2} = [\mathrm{E(-M_A)}]/[\mathrm{E}][\mathrm{M_A}] = [\mathrm{E(M_AM_A)}]/[\mathrm{M_A}][\mathrm{E(M_A-)}] \quad (10)$$

$$K_{B1} = [\mathrm{E(M_B-)}]/[\mathrm{E}][\mathrm{M_B}] = [\mathrm{E(M_BM_B)}]/[\mathrm{M_B}][\mathrm{E(-M_B)}] \quad (11)$$

$$K_{B2} = [\mathrm{E(-M_B)}]/[\mathrm{E}][\mathrm{M_B}] = [\mathrm{E(M_BM_B)}]/[\mathrm{M_B}][\mathrm{E(M_B-)}] \quad (12)$$

where $[M_A]$ and $[M_B]$ are the equilibrium concentrations of free M_A and M_B, respectively. [E(XY)], where X, Y = M_A,

Table II: Expressions for Binding Constants for Competing Metal Ions Binding to Glutamine Synthetase and Related Fluorescence Properties

configuration	fraction	fluorescence
E(--)	$\phi_0 = 1/\Phi$	$\phi_0 F_0$
$E(M_A-)$	$\phi_1 = K_{A1}[M_A]/\Phi$	$\phi_1 F_{A1}$
$E(M_B-)$	$\phi_2 = K_{B1}[M_B]/\Phi$	$\phi_2 F_{B1}$
$E(-M_A)$	$\phi_3 = K_{A2}[M_A]/\Phi$	$\phi_3 F_{A2}$
$E(-M_B)$	$\phi_4 = K_{B2}[M_B]/\Phi$	$\phi_4 F_{B2}$
$E(M_AM_B)$	$\phi_5 = K_{A1}K_{B2}[M_A][M_B]/\Phi$	$\phi_5(F_{A1} + F_{B2})$
$E(M_BM_A)$	$\phi_6 = K_{A2}K_{B1}[M_A][M_B]/\Phi$	$\phi_6(F_{A2} + F_{B1})$
$E(M_AM_A)$	$\phi_7 = K_{A1}K_{A2}[M_A]^2/\Phi$	$\phi_7(F_{A1} + F_{A2})$
$E(M_BM_B)$	$\phi_8 = K_{B1}K_{B2}[M_B]^2/\Phi$	$\phi_8(F_{B1} + F_{B2})$

M_B, or empty, is the concentration of E(XY). The mass balance equation for the enzyme is given by

$$[E]_T = [E]\{1 + (K_{A1} + K_{A2})[M_A] + (K_{B1} + K_{B2})[M_B] + (K_{A1}K_{B2} + K_{A2}K_{B1})[M_A][M_B] + K_{A1}K_{A2}[M_A]^2 + K_{B1}K_{B2}[M_B]^2\} = [E]\Phi \quad (13)$$

There are nine possible configurations for this system; the mole fractions and fluorescence expressions are given in Table II, where F_{A1} and F_{A2} are the TNS fluorescence when the n_1 or n_2 sites are fully occupied by M_A while F_{B1} and F_{B2} represent the TNS fluorescence when the n_1 or n_2 sites are fully occupied by M_B. The observed fluorescence for this system is given by

$$F = \phi_0 F_0 + (\phi_1 + \phi_5 + \phi_7)F_{A1} + (\phi_3 + \phi_6 + \phi_7)F_{A2} + (\phi_2 + \phi_6 + \phi_8)F_{B1} + (\phi_4 + \phi_5 + \phi_8)F_{B2} \quad (14)$$

The mass balance equations for the metal ions are

$$[M_A] = [M_A]_T - (\phi_1 + \phi_3 + \phi_5 + \phi_6 + 2\phi_7)[E]_T \quad (15)$$

$$[M_B] = [M_B]_T - (\phi_2 + \phi_4 + \phi_5 + \phi_6 + 2\phi_8)[E]_T \quad (16)$$

The procedure for obtaining K_{B1} and K_{B2} from the known values of K_{A1} and K_{A2} is similar to that described in the previous section.

Determination of the Efficiency of Energy Transfer (E). Equation 14 can also be applied to Tb(III) luminescence titration of GS by mixtures of Tb(III)Ln(III) [where Ln(III) are the luminescence acceptors] with the following modifications: (1) $F_0 = 0$; (2) $F_{B1} = F_{B2} = 0$, since Ln(III) ions are nonluminescent; and (3) the luminescence for configurations E(TbLn) and E(LnTb) should be replaced by $\phi_5 F_{A1}(1 - E)$ and $\phi_6 F_{A2}(1 - E)$, respectively. Thus, the obtained Tb(III) luminescence for the titration of GS with mixtures of Tb/Ln is given by

$$F = [\phi_1 + \phi_7 + \phi_5(1 - E)]F_{A1} + [\phi_3 + \phi_7 + \phi(1 - E)]F_{A2} \quad (17)$$

If K_{A1}, K_{A2}, K_{B1}, and K_{B2} are all known, the fraction of each configuration can be calculated, and the efficiency of energy transfer can be evaluated by fitting eq 17.

Förster Theory. Intramolecular distances can be calculated from the efficiency of energy transfer assuming a mechanism of dipole–dipole radiationless energy transfer. The theory of this approach has been described in McNemar et al. (1991).

Results

Absorption, Excitation, and Emission Spectra of TNS. The absorption spectrum of TNS in aqueous solution has four absorption bands centered at 223, 263, 317, and 362 nm as shown in Figure 1. The fluorescence spectrum of TNS bound to GS (Figure 1) shows an emission band centered at 454 nm. McClure and Edelman (1966) have measured the emission spectra of TNS in several aqueous protein solutions and found that the emission maxima are in the range of 426–462 nm.

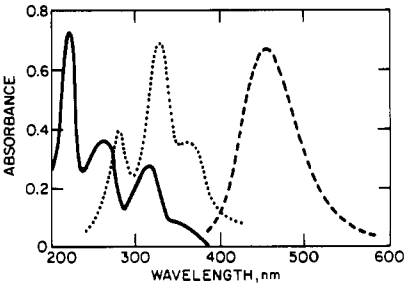

FIGURE 1: Absorption (—), fluorescence excitation [(···) λ_{em} = 454 nm], and fluorescence [(– – –) λ_{ex} = 328 nm] spectra of 1 μM GS and 40 μM TNS in 10 mM Hepes/0.1 M KCl, pH 7.0, at 25 °C.

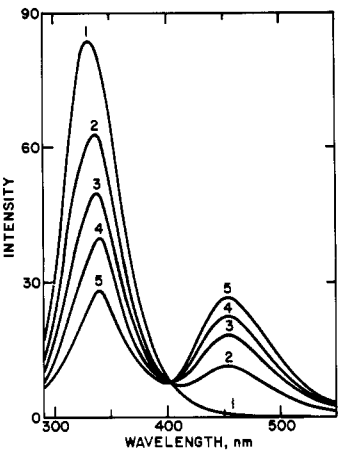

FIGURE 2: Fluorescence spectra (λ_{ex} = 280 nm) of 1 μM GS in the presence of (1) 0, (2) 4, (3) 8, (4) 12, and (5) 20 μM TNS in 10 mM Hepes/0.1 M KCl, pH 7.0, at 25 °C.

The excitation spectrum of TNS bound to GS has three bands centered at 280, 328, and 362 nm as shown in Figure 1. The excitation band at 280 nm, which is not seen in the absorption spectrum, is probably due to energy transfer from the aromatic amino acid residues of GS to TNS.

Fluorescence Spectra of TNS–GS Complexes at Various [TNS]. Figure 2 shows the fluorescence spectra of TNS–GS complexes, excited at 280 nm, of 1 μM GS with various concentrations of TNS. It is seen that as [TNS] increases, TNS fluorescence (λ_{max} = 454 nm) increases and concurrently the protein fluorescence (λ_{max} = 332 nm) decreases. The absorption spectrum of TNS bound to GS, centered at 317 nm (Figure 1), overlaps significantly with the emission spectrum of GS centered at 332 nm (Figure 2), and efficient energy transfer from fluorescent protein residues (mainly from tryptophan residues) to TNS is expected. The observation of efficient energy transfer from Trp residues to TNS implies that the binding site of TNS is located near the Trp residues. The spectra in Figure 2 show an isoemissive point at 403 nm. This suggests the same quantum yield of fluorescence for bound molecules over the range of [TNS] recorded (Anderson & Weber, 1965) and that binding of TNS is monotonic. It is to be noted that the emission maximum of TNS fluorescence does not change over the entire concentration range of TNS used while the emission maximum of the protein fluorescence is red-shifted from 332 to 340 nm.

Determination of the Dissociation Constant of the TNS–GS Complex. As shown in Figure 2, TNS fluorescence increases as the concentration of TNS increases at a fixed GS concentration. The dissociation constant can be evaluated from the titration curve of TNS fluorescence at various [TNS] according to eq 1 and 3. In determining K_D, an iteration procedure was employed using eq 1 and 3 until the best fit of the

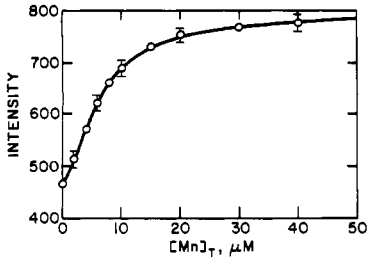

FIGURE 3: Titration of 2 μM GS and 2 μM TNS with Mn(II) in 10 mM Hepes/0.1 M KCl, pH 7.0, at 25 °C. The solid line is the theoretical curve calculated by eq 7, and the error bars are for ±10% in the value of each binding constant.

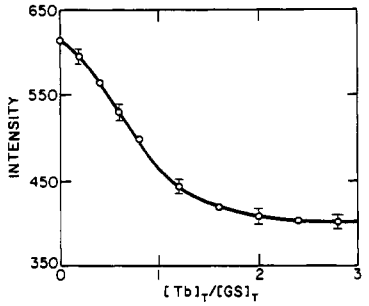

FIGURE 4: Titration of 1 μM GS, 1 μM TNS, and 20 μM Mn(II) with Tb(III) in 10 mM Hepes/0.1 M KCl, pH 7.0, at 25 °C. The solid line is the theoretical curve calculated by eq 14. The error bars represent ±10% in each binding constant.

titration curve was achieved. The final plot of F vs $F/[TNS]$ is linear (data not shown), and the calculated dissociation constant of the TNS–GS complex was 23 μM.

Determination of the Binding Constants of Metal Ions to GS. Since TNS fluorescence is very sensitive to its local environment in the TNS–enzyme complex, binding of metal ions, substrate, or inhibitor to the active site may cause a conformational change of the enzyme and hence induce a change in TNS fluorescence. Addition of Mn(II) and Mg(II) to the TNS–GS complex causes a dramatic increase in TNS fluorescence. Figure 3 shows the fluorescence titration of GS–TNS with Mn(II). This titration curve was fit to eq 7 according to the procedure described previously. The binding constants of Mn(II) to the two binding sites of GS were determined to be $K_1 = 5.7 \times 10^5$ M^{-1} and $K_2 = 4.7 \times 10^5$ M^{-1}. The binding constants of Mg(II) to GS were also determined by the same method to be $K_1 = 2.0 \times 10^4$ M^{-1} and $K_2 = 9.2 \times 10^3$ M^{-1}. Error bars for a variation of ±10% in each binding constant are given in the figure.

Determination of the Binding Constants by Metal Competition. Since the addition of lanthanide ions to TNS–GS complex causes only a small change in TNS fluorescence, the binding constants of these metal ions to GS were determined by metal competition. The TNS fluorescence for the solution of GS, TNS, and a saturating amount of Mn(II) was measured as it was titrated with Ln(III), and the experimental data were then fit to eq 14 by using the known values of K_{A1}, K_{A2}, F_0, F_1, and F_2 [M_A = Mn(II); M_B = Ln(III)] as described before. Figure 4 shows the changes in TNS fluorescence as a solution of 1 μM GS, 1 μM TNS, and 20 μM Mn(II) was titrated with Tb(III). The best fit of the titration is shown as the solid curve (error bars for ±10% in the binding constants are given). The binding constants of Tb(III) to the two binding sites of GS were determined to be $K_1 = 4.6 \times 10^{10}$ M^{-1} and $K_2 = 3.0 \times 10^8$ M^{-1}. The binding constants of other lanthanide ions to GS were also determined by the metal competition method, and the results are summarized in Table III.

Table III: Summary of the Binding Constants of Metal Ions Determined by Competition with Mn(II) to the Two Binding Sites of *E. coli* Glutamine Synthetase

metal ion	K_1 (M^{-1})	K_2 (M^{-1})
Mn(II)	5.7×10^5	4.7×10^5
Mg(II)	2.0×10^4	9.2×10^3
Tb(III)	4.6×10^{10}	3.0×10^8
Ho(III)	2.9×10^{10}	2.1×10^8
Nd(III)	2.1×10^{10}	1.6×10^8
Gd(III)	3.0×10^{10}	1.4×10^8

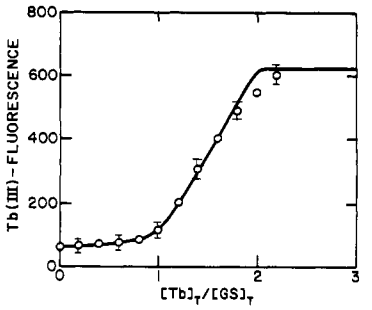

FIGURE 5: Titration of 16 μM GS with Tb(III) in 10 mM Hepes/0.1 M KCl, pH 7.0, at 25 °C. The solid line is the theoretical curve calculated by eq 18. The error bars represent ±10% in the binding constants.

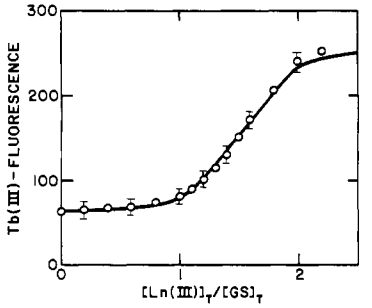

FIGURE 6: Titration of 16 μM GS with a 1:1 mixture of Tb(III)/Ho(III) in 10 mM Hepes/0.1 M KCl, pH 7.0, at 25 °C. The solid line is the theoretical curve calculated by eq 17. The error bars represent ±10% in each binding constant.

Determination of the Binding Constants for Tb(III) to GS from Tb(III) Fluorescence Experiments. Sensitized Tb(III) luminescence provides an alternative way to determine the binding constants of Tb(III)–GS complexes. Figure 5 shows the Tb(III) luminescence titration of 16 μM GS with Tb(III) in the absence of TNS. The equations derived for determining the binding constants of the Mn(II)–GS complexes in TNS luminescence experiments can also be used to determine the binding constants of the Tb(III)–GS complex. For Tb(III) fluorescence experiments, $F_0 = 0$ [i.e., the fluorescence of free Tb(III) ion in aqueous solution is very weak], and eq 7 becomes

$$F = (\phi_1 + \phi_3)F_1 + (\phi_2 + \phi_3)F_2 \qquad (18)$$

The titration data were fit to eq 18 by using the same procedure described above (same error analysis as before). The binding constants for Tb(III)–GS complexes were determined to be $K_1 = 4.1 \times 10^{10}$ M^{-1} and $K_2 = 3.0 \times 10^8$ M^{-1} which are in excellent agreement with the results obtained from the metal competition method.

Determination of the Efficiency of Energy Transfer. Since the binding constants of the different lanthanide ions have been determined, an accurate measure of the efficiencies of energy transfer from Tb(III) to other Ln(III) acceptors can be determined from the sensitized Tb(III) luminescence titration

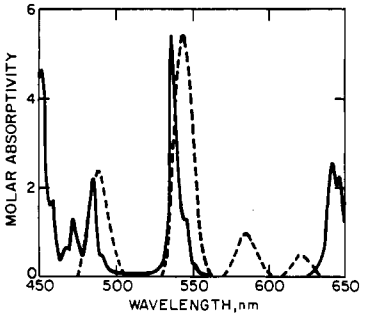

FIGURE 7: Spectral overlap of the emission spectrum [(---) λ_{ex} = 295 nm] of GS-bound Tb(III) and the absorption spectrum (—) of a 1:1 Ho(III)-EDTA complex. ϵ_{537nm} = 5.3 M^{-1} cm^{-1}.

of GS with mixtures of Tb/Ln. Figure 6 shows the changes in Tb(III) luminescence for the titration of 16 μM GS with a 1:1 mixture of Tb/Ho. The titration curve was fit to eq 14 by using the values of K_{A1}, K_{A2}, K_{B1}, and K_{B2} determined previously. The efficiency of energy transfer from Tb(III) to Ho(III) was determined to be 0.68. The same experiments were also carried out for the Tb/Nd, system and the efficiency of energy transfer for Tb(III)–Nd(III) was 0.84.

Determination of Inter/Metal Distances Using Measured Binding Constants of Ln(III) to GS. In order to calculate distances using the Förster equation, we need to evaluate the quantum yield of Tb(III), the overlap integral, and the efficiencies of energy transfer. The quantum yields of Tb(III) bound to GS were estimated to be 0.21 and 0.36 for 1 and 2 equiv of Tb(III) per subunit (McNemar et al., 1991) from the change in the luminescent exponential decay constant as the H_2O:D_2O ratio was varied.

The overlap in the fluorescence spectrum of Tb(III) bound to GS and the absorption spectrum of the Ho(III)–EDTA complex are illustrated in Figure 7. The Ho(III)–EDTA absorption spectrum was measured due to the weak absorption properties of Ho(III) which precludes use of the spectrum of Ho(III) bound to GS. The overlap integral was estimated from eq 17 to be 8.20 × 10^{-18} cm^6 mol^{-1}. The overlap integral for Tb–Nd was also determined from the fluorescence spectrum of Tb(III) ion bound to GS and the absorption spectrum of the Nd(III)–EDTA complex (data not shown) to be 8.34 × 10^{-18} cm^6 mol^{-1}.

The critical distance for 50% energy transfer, R_0, was calculated from eq 16. The orientation factor, κ^2, was taken as $^2/_3$, the value for an isotropic donor and an isotropic acceptor. According to Horrocks and Collier (1981), it is unlikely that the true value of κ^2 deviates from this value sufficiently to cause a significant error in the calculation of R_0. A value of 1.36 was used for the refractive index, n. The critical distances for Tb–Ho and Tb–Nd systems were calculated to be 8.95 and 8.97 Å, respectively. The Tb–Ln distances were then estimated by equiv 15 to be 7.9 and 6.8 Å for Tb–Ho and Tb–Nd, respectively.

Discussion

TNS is essentially nonfluorescent in aqueous solutions but fluoresces strongly when adsorbed to proteins. This is due to the high quantum yield of protein-bound TNS which results mainly from interactions between TNS and hydrophobic regions on the protein surface, consistent with the fact that TNS fluorescence is enhanced in solvents of low dielectric constant or of high viscosity (McClure & Edelman, 1966). As a result, TNS has been used as a probe for hydrophobic regions in proteins. It is evident from the excitation spectrum of the TNS–GS complex (Figure 1) that direct excitation of TNS at 328 or 362 nm will result in a strong TNS fluorescence at 454 nm. On the other hand, the observation of an excitation band at 280 nm, which is not seen in the absorption spectrum (Figure 1), is mainly due to energy transfer from fluorescent protein groups to TNS.

The fluorescence spectrum of GS (Figure 2) shows an emission maximum at 332 nm which is characteristic of Trp fluorescence in proteins. The fluorescence maximum of Trp and its derivatives is red-shifted as the solvent polarity increases (Lakowicz, 1983). As a result, the fluorescence maximum of a protein is dependent on the exposure of the Trp residues to the aqueous phase. Emission maxima at shorter wavelengths are expected for more hydrophobic Trp residues, and emission maxima at longer wavelengths are expected for Trp residues more exposed to the aqueous phase (Eftink & Ghiron, 1976). Inspection of the fluorescence spectra in Figure 2 shows that the fluorescence maximum of GS is red-shifted from 332 to 340 nm as the concentration of TNS increases. There are two Trp residues per subunit in GS (Colombo & Villafranca, 1986). It is likely that the remaining fluorescence at 340 nm is due to the Trp residue which is more exposed to the aqueous solution and the energy transfer to TNS is mainly from the more hydrophobic Trp residue.

As shown in Figure 3, TNS fluorescence almost doubled when 50 μM Mn(II) was added. This most likely indicates that TNS and Mn(II) are bound to GS at different sites. The binding constant for Mn(II)–GS (Table III) is larger than that of the TNS–GS complex (4.4 × 10^4 M^{-1}). If Mn(II) and TNS compete for the same site(s) and $[Mn(II)]_0$ is much greater than $[TNS]_0$, then most of the TNS would be displaced by Mn(II), and the fluorescence should be extremely weak. Thus, the increase in TNS fluorescence upon addition of Mn(II) and Mg(II) is consistent with the assumption that TNS is bound to GS at some hydrophobic site other than the active site and TNS is acting like an adsorption indicator. As the concentration of TNS increases, the intensity of TNS fluorescence increases, but the binding constants of metal ions to GS are not affected.

The binding constants given in Table III varied by several orders of magnitude for the different metal ions with the lanthanide ions binding most strongly to GS. Though the binding constants differed only by about 2-fold among the lanthanides, the fractional distributions of the Tb–Ln system are significantly different from those obtained by assuming equal binding of lanthanides. For example, the mole fractions for the configurations of E(TbNd), E(NdTb), E(TbTb), and E(NdNd) for the solution of 20 μM GS, 15 μM Tb(III), and 15 μM Nd(III) calculated by using the binding constants in Table III are 0.23, 0.22, 0.44, and 0.11, respectively, while the mole fraction is 0.25 for each complex if the binding constants of the lanthanides are assumed to be equal. This analysis takes into account the error ranges given in the figures; the conclusion, therefore, is that the efficiency of energy transfer determined by assuming equal binding of the lanthanides to GS results in a significant error in the calculation of the metal–metal distances.

The intermetal distances of Tb(III)–Ho(III) and Tb(III)–Nd(III) are in good agreement with each other. The distances are somewhat longer than the Mn(II)–Mn(II) distance of 5.8 Å from the crystal structure of *S. typhimurium* GS (Almassy et al., 1986) which differs by only 10 residues per subunit from *E. coli* GS (Colombo & Villafranca, 1986; Janson et al., 1986). The discrepancy may result from a slightly different conformation of GS in solution and in the crystal and/or a slightly different conformation for trivalent

Ln(III) binding compared to divalent Mn(II) binding. However, the Ln(III)–Ln(III) distances are in good agreement with metal–metal distances previously determined by EPR (Balakrishnan & Villafranca, 1978).

Since TNS fluorescence is very sensitive to its local environment, TNS is also a very sensitive probe for detecting the conformational changes in the enzyme. Addition of Mn(II) or Mg(II) to a TNS–GS solution causes a dramatic increase in TNS fluorescence, and thus significant conformational changes in GS are expected. These conformational changes may be necessary for the enzyme to be active. The effect of Ln(III) on the TNS fluorescence is quite different. There is essentially no fluorescence change when Ln(III) is bound to the n_1 site of GS while the TNS fluorescence decreases slightly when Ln(III) is bound to the n_2 site (data not shown). These results may suggest that the conformation of Ln(III)–GS is different from that of Mg(II)–GS or Mn(II)–GS, and it may also explain why Ln(III)–GS is catalytically inactive. In addition, the differences in conformations between Ln(III)–GS and Mn(II)–GS may lead to the internal distances being different. Analysis of the crystal structure of Ln(III)–GS should help resolve the differences among the active and inactive metal complexes of GS.

Registry No. GS, 9023-70-5; Mn(II), 7439-96-5; Mg(II), 7439-95-4; La(III), 7439-91-0; Tb(III), 7440-27-9; Ho(III), 7440-60-0; Nd(III), 7440-00-8; Gd(III), 7440-54-2.

References

Almassy, R. J., Janson, C. A., Hamlin, R., Xuong, N.-H., & Eisenberg, D. (1986) *Nature 323*, 304–309.

Anderson, S. R., & Weber, G. (1965) *Biochemistry 4*, 1948.

Balakrishnan, M. S., & Villafranca, J. J. (1978) *Biochemistry 17*, 3531–3538.

Balakrishnan, M. S., & Villafranca, J. J. (1978) *Biochemistry 18*, 1546–1551.

Colombo, G., & Villafranca, J. J. (1986) *J. Biol. Chem. 261*, 10587–10591.

Eads, C. D., Mulqueen, P., Horrocks, W. DeW., Jr., & Villafranca, J. J. (1985) *Biochemistry 24*, 1221–1226.

Eftink, M. R., & Ghiron, C. A. (1976) *Biochemistry 15*, 672.

Förster, T. (1948) *Ann. Phys. (Leipzig) 2*, 55–75.

Förster, T. (1965) in *Modern Quantum Chemistry* (Sinanoglu, O., Ed.) Part III, pp 93–137, Academic Press, New York.

Gibbs, E. J., Ransom, S. C., Cuppett, S., & Villafranca, J. J. (1984) *Biochem. Biophys. Res. Commun. 120*, 939.

Ginsburg, A. (1972) *Adv. Protein Chem. 26*, 1–79.

Horrocks, W. DeW., Jr., & Collier, W. E. (1981) *J. Am. Chem. Soc. 102*, 2856–2862.

Horrocks, W. DeW., Jr., & Sudnick, D. R. (1981) *Acc. Chem. Res. 14*, 383–392.

Horrocks, W. DeW., Jr., Holmquist, B., & Vallee, B. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4764–4768.

Hunt, J. B., Smyrniotis, P. Z., Ginsburg, A., & Stadtman, E. (1975) *Arch. Biochem. Biophys. 161*, 102.

Janson, C. A., Kayme, P. S., Almassy, R. J., Grunstein, M., & Eisenberg, D. (1986) *Gene 46*, 297–300.

Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Chapter 11, pp 341–382, Plenum Press, New York.

Laurence, D. J. R. (1952) *Biochem. J. 51*, 168.

McClure, W. O., & Edelman, G. M. (1966) *Biochemistry 5*, 1908.

McClure, W. O., & Edelman, G. M. (1967) *Biochemistry 6*, 559.

McNemar, L. S., Linn, W.-Y., Eads, C. D., Atkins, W. M., Dombrosky, P., & Villafranca, J. J. (1991) *Biochemistry* (second of four papers in this issue).

Miller, R. E., Shelton, E., & Stadtman, E. R. (1974) *Arch. Biochem. Biophys. 163*, 155–171.

Rhee, M.-J., Sudnick, D. R., Arkle, V. K., & Horrocks, W. DeW., Jr. (1981) *Biochemistry 20*, 3328–3334.

Shapiro, B. M., & Stadtman, E. R. (1970) *Methods Enzymol. 17A*, 910–922.

Villafranca, J. J., Ash, D. E., & Wedler, F. C. (1976) *Biochemistry 15*, 536–543.

Villafranca, J. J., Balakrishnan, M. S., & Wedler, F. C. (1977) *Biochem. Biophys. Res. Commun. 75*, 464–471.

Villafranca, J. J., Rhee, S. G., & Chock, P. B. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1255–1259.