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Comparative Study of Glutamine Synthetase Bound Lanthanide(III) Ions Using NMR Relaxation and Lanthanide(III) Luminescence Techniques†

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ABSTRACT: Changes in the intrinsic fluorescence intensity of glutamine synthetase induced by lanthanide(III) ion binding demonstrate the existence of three types of sites for these ions. The sites are populated sequentially during titrations of the enzyme, and the first two have a stoichiometry of 1 per enzyme subunit. The number of water molecules coordinated to Eu(III) bound to the first site was determined by luminescence lifetime techniques to be 4.1 ± 0.5. The hydration of Gd(III) bound to the same site was studied by magnetic field dependent water proton longitudinal relaxation rate measurements, and by water proton and deuteron relaxation measurements of one sample at single magnetic fields. The magnetic resonance techniques also yield a value of 4 for the hydration number.

The number of water molecules directly coordinated to an enzyme-bound metal ion must often be determined in order to assess the role of the metal ion in substrate binding and catalysis. Several methods for determining this parameter have therefore been developed, including water proton and deuteron NMR relaxation techniques (Burton et al., 1979), detection of ^{17}O superhyperfine coupling to paramagnetic metal ions using electron paramagnetic resonance (EPR)[1] (Reed & Leyh, 1980), luminescence lifetime measurements of Eu(III) and Tb(III) (Horrocks & Sudnick, 1981), and, most recently, EPR spin-echo envelope modulation measurements (Peisach et al., 1984).

The most frequently applied method for studying paramagnetic metal ions such as Mn(II) and Gd(III) is the NMR technique of measuring water proton relaxation rates (PRR) as a function of magnetic field and/or temperature. In general, data analysis is carried out in terms of the Solomon–Bloembergen–Morgan (SBM) equations (Solomon, 1955; Bloembergen & Morgan, 1961) which were developed to account for the relaxation behavior of aqueous solutions of paramagnetic metal ions. The reliability of hydration numbers determined by this type of analysis is subject to some well-founded concern stemming from two main sources. First, since the SBM theory was derived for aqueous ions, it may not be applicable to macromolecular systems. Second, since data analysis often involves the use of a multiparameter fitting procedure, the results are subject to the well-known ambiguities inherent in this method of analysis. It would be useful to study systems of known hydration number in order to assess the applicability of the theory to macromolecular systems.

Some members of the series of lanthanide(III) ions possess spectroscopic properties which allow determinations of the metal ion hydration number by more than one technique. In particular, measurements of the lifetime of the luminescent $^5D_0$ state of Eu(III) in H_2O and D_2O provide a direct and accurate measure of metal ion hydration (Horrocks & Sudnick, 1979, 1981). Gd(III), which has an $^8S_{7/2}$ ground state, makes an ideal probe for water proton and deuteron relaxation studies (Reuben, 1971). Moreover, the nearly identical sizes and chemical properties of these two ions make direct comparison of experiments valid.

Glutamine synthetase from *Escherichia coli* is a well-characterized enzyme which has been the subject of many investigations using metal ions as spectroscopic probes. The active form of the enzyme has 12 identical subunits, each of molecular weight 50 000 (Ginsburg, 1972). Each subunit has two essential divalent metal ion binding sites, designated n_1 and n_2, in the catalytically active region and a third set of sites thought to stabilize the catalytically active enzyme structure. For the past several years, one of our laboratories has been investigating structure–function relationships in this allosterically regulated enzyme. Understanding the role of divalent cations in the catalytic mechanism has been one of the primary goals of this work (Villafranca & Balakrishnan, 1979).

Water proton relaxation measurements have been used to study changes in the environment of Mn(II) at the n_1 site, which is near the glutamate binding site, upon substrate, product, and inhibitor binding (Villafranca et al., 1976a,b; Villafranca & Wedler, 1974). Similar studies of the n_2 site, which is involved in nucleotide binding, have also been carried out (Ransom, 1984), and the distance relationship between these metal ion sites has recently been investigated by EPR spectroscopy (Gibbs et al., 1984). In one of the early applications of lanthanide(III) ion probes of enzymes, Wedler & D'Auroa (1974) used changes in the absorption spectrum of Nd(III) in a qualitative study of substrate interactions with glutamine synthetase. Thus, with lanthanide(III) ions as probes, glutamine synthetase provides a system in which results

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[1] Abbreviations: SBM, Solomon–Bloembergen–Morgan; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EPR, electron paramagnetic resonance; PRR, proton relaxation rate(s); EDTA, ethylenediaminetetraacetic acid.

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of NMR relaxation experiments, which have previously been difficult to verify in systems of this nature, can be directly compared to results of an independent method, that of lanthanide luminescence.

In this report, we present a characterization of the interactions of lanthanide(III) ions with glutamine synthetase with regard to stoichiometry and types of sites. The hydration number of Eu(III) bound to a single site on this enzyme was determined by luminescence lifetime measurements. We compare this to the results of two different NMR relaxation studies for Gd(III) bound to the same site: (1) frequency-dependent water proton longitudinal relaxation between 6.8 and 50 MHz; and (2) water proton and deuteron longitudinal relaxation rates measured for one sample at single magnetic field strengths.

Materials and Methods

Materials. Glutamine synthetase in a low state of adenylylation (1.1–2.5 adenylylated subunits per dodecamer) was prepared by the method of Miller et al. (1974). Enzyme subunit concentration and state of adenylylation were determined spectrophotometrically (Shapiro & Stadtman, 1970). The buffer used for all experiments was 10 mM HEPES and 100 mM KCl, pH 7.0. Metal-free enzyme was prepared by dialysis against buffer containing 3 mM EDTA followed by extensive dialysis against buffer alone. Lanthanide(III) chloride salts were purchased from Aldrich. Solutions of enzyme in D_2O or in H_2O–D_2O mixtures were prepared by lyophilization of aqueous apoenzyme followed by replacement of H_2O with an equal volume of the desired solvent. Metal ions were added subsequently.

Intrinsic Enzyme Fluorescence. Intrinsic enzyme fluorescence was followed by using a Perkin-Elmer MPF-44B spectrofluorometer. Titrations were performed at 37 °C to hasten the slow conformational changes associated with metal ion binding.

Eu(III) Luminescence Studies. The laser apparatus used to study Eu(III) luminescence has been previously described (Horrocks & Sudnick, 1979, 1981). The transition between the ground $^7F_0$ and the excited $^5D_0$ state of Eu(III) involves two levels which are nondegenerate and therefore cannot be split by the ligand field. This transition thus consists of a single, unsplit line with a characteristic λ_{max} for each type of metal ion environment. The emissive nature of the $^5D_0$ level allows this transition to be monitored by observing the emitted luminescence from the $^5D_0 \rightarrow {}^7F_2$ transition near 614 nm while a tunable laser is scanned through the transition region. The number of water molecules, q, coordinated to the Eu(III) ion is given by

$$q = 1.05(\tau_H^{-1} - \tau_D^{-1}) \quad (1)$$

where τ is the luminescence lifetime in H_2O or D_2O (Horrocks & Sudnick, 1979).

NMR Relaxation Measurements. Longitudinal water proton relaxation times, T_1, in the 6.8–50-MHz range were measured by using the 180°–τ–90° null-point method. Data were obtained at 25 °C on a modified SEIMCO NMR spectrometer equipped with a home-built variable-frequency probe. Water proton and deuteron T_1's were measured at 25 °C on JEOL PFT 100, Bruker WP 200, and Bruker WH 360 NMR spectrometers by using the inversion recovery pulse sequence and fit by using computer programs supplied with the spectrometers. Deuterium was detected by using the lock channel of the proton probes of the Bruker WP 200 and WH 360 spectrometers, and with a deuterium probe on the JEOL PFT 100 spectrometer.

NMR Relaxation Theory. The paramagnetic contribution of Gd(III) to the longitudinal relaxation rate of a nucleus is given by the Solomon–Bloembergen–Morgan (SBM) equations:

$$1/T_{1p} = 1/T_1 - 1/T_{1d} = Pq/(T_{1m} + \tau_m) \quad (2)$$

$$1/T_{1m} = (C/r^6)f \quad (3)$$

$$f = 3\tau_c/(1 + \omega_i^2\tau_c^2) + 7\tau_c/(1 + \omega_s^2\tau_c^2) \quad (4)$$

$$1/\tau_c = 1/\tau_r + 1/\tau_m + 1/\tau_s \quad (5)$$

$$1/\tau_s = B[\tau_v/(1 + \omega_s^2\tau_v^2) + 4\tau_v/(1 + 4\omega_s^2\tau_v^2)] \quad (6)$$

Here, $1/T_1$ is the relaxation rate measured in a solution containing the paramagnetic probe, $1/T_{1d}$ is the same rate in a solution containing a suitable diamagnetic blank, P is the mole fraction of Gd(III), q is the number of water molecules interacting with the metal ion, τ_m is the coordination lifetime of the water molecule in the hydration shell of the metal ion, and $1/T_{1m}$ is the relaxation rate of the nucleus while in proximity to the metal ion. C is a collection of constants for the nucleus employed in the experiment (Burton et al., 1979), r is the Gd(III)–nuclear distance, and f is related to the spectral density of fluctuations in the electron–nuclear dipole–dipole interaction. ω_i and ω_s are the nuclear and electronic precession frequencies, respectively, τ_c is the correlation time characterizing modulation of the electron–nuclear dipole–dipole interaction, τ_r is the rotational correlation time of the complex, and τ_s is the electron spin relaxation time. B is a constant for a given metal ion environment, and τ_v is a correlation time which characterizes modulation of the zero-field splitting.

The temperature dependencies of the relevant correlation times are taken to be

$$\tau_v = \tau_v^0 \exp(E_v/RT) \quad (7)$$

$$\tau_r = \tau_r^0 \exp(E_r/RT) \quad (8)$$

$$1/\tau_m = (kT/h) \exp[(-\Delta H^\ddagger + T\Delta S^\ddagger)/RT] \quad (9)$$

where E_v and E_r are the activation energies for the appropriate processes and $\Delta H^\ddagger$ and $\Delta S^\ddagger$ are the enthalpy and entropy of activation, respectively, for the exchange process. Equations 2–9 are discussed in detail elsewhere (Burton et al., 1979; Dwek, 1973).

Error Analysis. Uncertainties in the parameters were estimated by using the standard propagation of errors method, where derivatives were estimated numerically if necessary. To estimate uncertainties in parameters derived from multiparameter curve fitting, a different technique was used. The parameter under analysis was fixed at values near the optimum, defined as the value which minimizes the error parameter $\epsilon^2 = \sum(r_m - r_c)^2$ where r_m is the measured value of the data point and r_c is that calculated on the basis of the values of the parameters. The remaining parameters were reoptimized to compensate for this constraint. ϵ^2 was plotted as a function of the fixed value of the parameter under analysis. The uncertainty range, which corresponds to the range of values of the parameter which give less than a 100% increase in the value of ϵ^2, was determined from these plots (Bevington, 1969).

Results and Discussion

Lanthanide Ion Binding to Glutamine Synthetase. Changes in the intrinsic fluorescence intensity of glutamine synthetase caused by the binding of various ligands provide a convenient parameter for following titrations (Timmons et al., 1974). Figure 1 is a plot of intrinsic enzyme fluorescence intensity

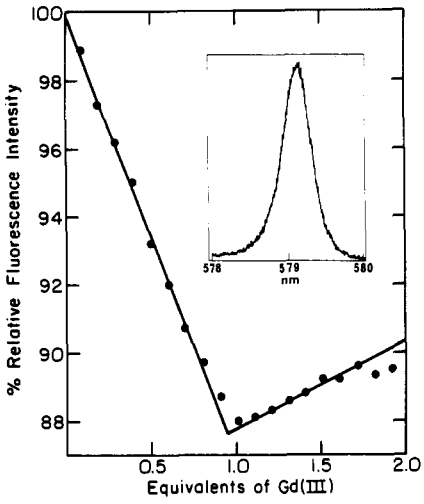

FIGURE 1: Titration of 16.5 μM apoglutamine synthetase with Gd(III) followed by intrinsic enzyme fluorescence. λ_{ex} = 287 nm, λ_{em} = 333 nm, T = 37 °C. Inset: Excitation spectrum of the $^7F_0 \rightarrow {}^5D_0$ transition of 60 μM Eu(III) bound to 103 μM glutamine synthetase.

(relative to apoenzyme) vs. the number of equivalents of Gd(III) added. The curve consists of two linear phases. The first phase represents binding to a site with a stoichiometry of 1 per enzyme subunit and is characterized by a 12.4% reduction in intrinsic enzyme fluorescence intensity. The second phase represents binding to a second site, also with a stoichiometry of 1 per subunit, characterized by a 2.4% increase in intrinsic enzyme fluorescence. Gd(III) added beyond 2 equiv binds to a site (or set of sites) of unknown stoichiometry and induces aggregation of some of the enzyme as detected by light scattering (data not shown). The deviation from linearity of the last few points of the second phase of Figure 1 corresponds to the onset of aggregation. The aggregation of glutamine synthetase induced by Zn(II) (Miller et al., 1974) may also result from binding of metal to these additional sites.

The binding behavior of lanthanide(III) ions to glutamine synthetase thus closely parallels that of the divalent metal ions which are required for activity. The n_1 and n_2 divalent metal ion sites differ enough in affinity that they are populated sequentially during titrations of the enzyme. This is also found for lanthanide(III) ions, although the lanthanide(III) ions bind more tightly. It is highly probable then that the first two lanthanide(III) binding sites are the n_1 and n_2 sites, respectively. A comparison of the interactions and spatial relationships of substrates and inhibitors with Gd(III) and with Mn(II) bound to the enzyme lends strong support to this conclusion (C. D. Eads and J. J. Villafranca, unpublished results).

The inset of Figure 1 is the excitation spectrum of the $^7F_0 \rightarrow {}^5D_0$ transition of Eu(III) bound to the first (n_1) site. The presence of a single peak centered at 579.15 nm (17 267 cm^{-1}) verifies that this metal ion experiences a single type of environment at metal:enzyme subunit ratios less than 1. M. Albin and W. DeW. Horrocks (unpublished results) have recently established a correlation between the total net charge, p, on the ligands in the first coordination sphere of Eu(III) complexes and the frequency, f in cm^{-1}, of the $^7F_0 \rightarrow {}^5D_0$ transition; namely, $f = -0.76p^2 + 2.29p + 17273$. Application of this relation to the present results predicts that the net charge is -1.7 ± 1, consistent with two negatively charge groups at the Eu(III) ion binding site.

Hydration Number Determination by Eu(III) Luminescence. A plot of the reciprocal lifetime of the $^5D_0$ state of Eu(III) bound to the first (n_1) site of glutamine synthetase

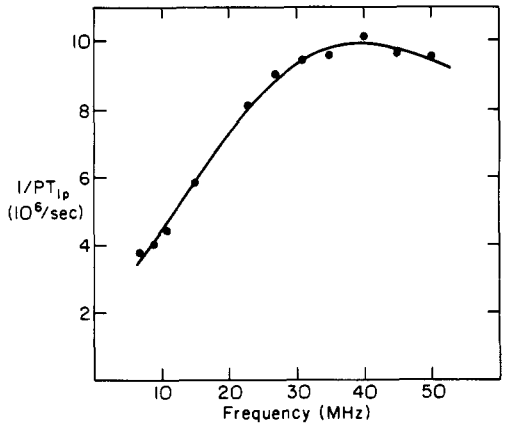

FIGURE 2: Frequency dependence of longitudinal water proton relaxation rates of a solution containing 0.157 mM glutamine synthetase and 0.146 mM Gd(III) at 25 °C. The diamagnetic control contained apoenzyme.

Table I: Parameters from Analysis of PRR Data of Gd(III)–Glutamine Synthetase in Figure 3[a]

	B ($\times 10^{19}$ rad/s)	τ_v (ps)	τ_m (ns)	q
best-fit value	3.26	13.2	2.62	3.71
range	3.0–3.7	9–18	1.6–4.8	3.2–4.8

[a] τ_r and r were assumed to be 2.0×10^{-7} s (Villafranca & Wedler, 1974) and 3.09 Å (Reuben, 1971), respectively.

as a function of the mole fraction of H_2O in H_2O–D_2O solutions was constructed. Extrapolation to pure H_2O and D_2O gives reciprocal excited-state lifetimes of 4.4 and 0.5 ms^{-1}, respectively. Use of eq 1 yields a value of 4.1 ± 0.5 for the number of water molecules coordinated to the metal ion.

Hydration Number Determination by Conventional PRR Measurements. The PRR due to Gd(III) bound to the first (n_1) site of glutamine synthetase is shown in Figure 2 for the 6.8–50-MHz range. The parameters B, τ_v, τ_m, and q of eq 2–6 were fit to these data as described under Materials and Methods by using a nonlinear least-squares optimization computer program developed in our laboratory. The line through the data is based on this analysis, and the values of the parameters are presented in Table I.

It is often possible to obtain two acceptable fits to a data set of this type, one corresponding to fast exchange ($\tau_m < T_{1m}$, eq 2) and one corresponding to intermediate or slow exchange ($\tau_m \geq T_{1m}$) conditions. These two cases can be distinguished by the PRR temperature dependence at 9 and 27 MHz presented as an Arrhenius plot (Figure 3). The positive slopes of these curves clearly demonstrate that relaxation is not exchange limited. The lines through the data are based on a least-squares analysis in terms of eq 2–7 (no contribution from τ_r was included). The parameters determined from this are E_v = 11 kJ/mol, $\Delta H^{\ddagger}$ = 8.3 kJ/mol, and $\Delta S^{\ddagger}$ = −49 J/(mol·K). The other parameters from this analysis are the same as in Table I within the indicated uncertainty.

Hydration Number Determination by the H/D Method. A more direct method for evaluating a limited number of parameters of the SBM equations involves the measurement of both solvent water proton and deuteron relaxation rates in mixed H_2O–D_2O solvent (H/D method) (Burton et al., 1977; Kushnir & Navon, 1984). Substitution of eq 3 into eq 2 gives the following:

$$PT_{1p} = (r^6/Cq)/f' + \tau_m/q \qquad (10)$$

Here, f' is taken to be

$$f' = 3\tau_c/(1 + \omega_i^2\tau_c^2) \qquad (11)$$

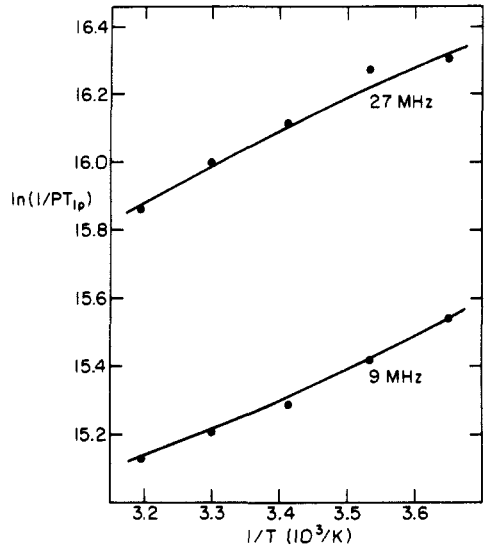

FIGURE 3: Temperature dependence of the longitudinal water proton relaxation rates at 9 and 27 MHz of the solution described in Figure 2.

where we prime the symbol f since it is an approximation of eq 4 and for reasons discussed below. Under fast exchange conditions, the last term of eq 10 drops out. Since the correlation times for water protons and deuterons are taken to be identical at a given magnetic field, measurement of PT_{1p} for both these nuclei in the same sample is sufficient to determine the two unknowns, q and τ_c.

Under conditions where the term involving τ_m cannot be neglected, additional measurements are required to determine the parameters in eq 10 and 11. The use of relaxation measurements made at a different field strength introduces only one additional unknown parameter, namely, τ_c at the different field (eq 5 and 6). Therefore, measurements of PT_{1p} for both protons and deuterons at each of two magnetic fields (four measurements) can be used to determine the four unknown parameters generated by eq 10 and 11 by simultaneous solution of the resulting system of equations (Burton et al., 1977).

The relaxation times determined for water protons and deuterons in a solution containing 1:1 H_2O–D_2O and 0.87 mM enzyme–Gd(III) [corrected for diamagnetic effects by using a similar sample containing La(III) instead of Gd(III)] are given in Table II. After the results given in Table I and Figure 3 which demonstrate fast exchange conditions were disregarded, an attempt was made to determine the unknown parameters in eq 10 and 11 by using relaxation measurements at pairs of magnetic fields. The results of this analysis for magnetic fields of 2.35 and 4.70 T, and the uncertainties determined by the standard propagation of errors method, are $q = 4.3 \pm 0.3$, τ_c(2.35 T) = 3.2 ± 0.4 ns, τ_c(4.70 T) = 2.6 ± 0.2 ns, and $\tau_m = 64 \pm 139$ ns. The uncertainty in τ_m exceeds the value of the parameter itself by a large amount. This demonstrates that the data contain no information concerning this parameter, as one would expect for fast exchange conditions. When other pairs of fields were used, this analysis gave physically unrealistic negative values of τ_m. In light of these results and the PRR analysis (Table I, Figure 3), it appears valid to ignore the last term of eq 10 and solve for q and τ_c from pairs of proton and deuteron T_1 measurements each made at a single magnetic field strength. The results of this analysis for all three fields are presented in Table II.

The trend of decreasing τ_c with increasing magnetic field strength seen in Table II contradicts the prediction of eq 5 and 6 and cannot be ascribed to experimental error. We consider

Table II: Parameters Determined from Measurement of the Paramagnetic Contribution to the Longitudinal Relaxation Times of Solvent Water Protons and Deuterons at 25 °C[a]

	field strength (T)		
	2.35	4.70	8.46
$PT_{1p}(^1H)$ ($\times 10^{-7}$ s)	2.20 ± 0.11	5.99 ± 0.30	10.4 ± 0.5
$PT_{1p}(^2H)$ ($\times 10^{-6}$ s)	1.90 ± 0.14	2.67 ± 0.21	6.45 ± 0.80
q	4.13 ± 0.16	4.26 ± 0.23	3.69 ± 0.25
τ_c ($\times 10^{-9}$ s)	3.34 ± 0.20	2.64 ± 0.17	1.17 ± 0.11

[a] Solvent water protons and deuterons were in a solution containing 0.892 mM glutamine synthetase subunits, 0.870 mM Gd(III), and 50% H_2O–50% D_2O solvent. The diamagnetic control for these measurements contained La(III) instead of Gd(III). Uncertainties were determined by propagation of errors starting with the assumption of a 5% uncertainty in all T_1 measurements. For this analysis, r was assumed to be 3.09 Å (Reuben, 1971).

below the effects of ignoring a possible outer-sphere contribution to the relaxation rates and of assuming an oversimplified model for the time dependence of the electron–proton magnetic interaction on this parameter.

In order to investigate the effects of a possible outer-sphere relaxation contribution (Kushnir & Navon, 1984), it was assumed (since fast exchange conditions are demonstrated for this system) that $1/PT_{1p} = 1/T_{1m} + 1/T_{1,os}$ where $T_{1,os}$ is the outer-sphere relaxation time.[2] The correlation time and hydration number, calculated by assuming no contribution from outer-sphere relaxation, were compared to the values of these parameters calculated from T_{1m} as the relative contribution of outer-sphere relaxation to $1/PT_{1p}$ was varied. It was found that for outer-sphere contributions of less than 10%, the parameters calculated by these two methods differed by less than 10%. In order to account for the coorelation time determined from the 8.46-T data compared to the results at 2.35 T, it was necessary to assume a >70% contribution from outer-sphere relaxation. Under these circumstances, the apparent hydration number would exceed the actual values by >60%. However, the hydration number determined at this field is in agreement with all the other determinations. Therefore, outer-sphere relaxation does not fully account for the trend in τ_c.

A more satisfactory explanation of this trend arises from considering the effect of assuming an empirical form, $J'(\omega_i)$, for the true spectral density function, $J(\omega_i)$. This leads to an empirical form, f', of the true function f. To this end, we rewrite eq 10 for both protons and deuterons in terms of both f' and f:

$$1/PT_{1p}^{H} = qC_H f(\omega_H) = qC_H K[3\tau_c/(1 + \omega_H^2\tau_c^2)] \quad (12)$$

$$1/PT_{1p}^{D} = qC_D f(\omega_D) = qC_D K[3\tau_c/(1 + \omega_D^2\tau_c^2)] \quad (13)$$

We introduce the field-dependent function K since it cancels when these equations are solved simultaneously for τ_c (eq 14),

$$\tau_c^2 = \frac{f(\omega_D) - f(\omega_H)}{\omega_H^2 f(\omega_H) - \omega_D^2 f(\omega_D)} = \frac{C_H T_{1m}^{H} - C_D T_{1m}^{D}}{C_D T_{1m}^{D}\omega_H^2 - C_H T_{1m}^{H}\omega_D^2} \quad (14)$$

either in terms of the measured relaxation rates[3] or in terms of the function f. Equation 14 shows that the correlation time

[2] It was also assumed that $T_{1,os}(^2H) = 42.4T_{1,os}(^1H)$. This assumption holds if the product of the outer-sphere correlation time and the nuclear precession frequency is less than 1, and if there is fast exchange from the outer sphere (Reuben, 1975).

[3] For the present conditions of fast exchange, $PT_{1p} = T_{1m}$ (eq 2).

determined by this technique will, in general, be a complicated function of magnetic field strength if $f'(\omega) \neq f(\omega)$ (i.e., if an inadequate form of the spectral density is assumed). This effect could well account for the trend in τ_c seen in Table II.

A model in which an internal rotation is included, such as rotation of a water molecule about its C_2 axis while bound to the metal ion, leads to a function f (eq 15) (Burton et al., 1979)

$$f(\omega_i) = 3[(1 - X)\tau_1/(1 + \omega_i^2\tau_1^2) + X\tau_2/(1 + \omega_i^2\tau_2^2)] \quad (15)$$

which can give an improved account of the data in Table II. Here, $0 < X < 1$, τ_1 is the correlation time for which internal rotational motions are not considered, $\tau_2^{-} = \tau_1^{-1} + \tau_z^{-1}$, and τ_z is the correlation time characterizing the internal rotational motion.

The parameters obtained by fitting[4] the data in Table II to eq 10, using eq 15 for f, are $\tau_1 = 5.6$ ns, $\tau_z = 0.59$ ns, $X = 0.42$, and $q = 4.7$. The average difference between the measured and calculated relaxation rates is 12%, which is somewhat larger than the uncertainty in the data. A fit to the same data using eq 11 for f gives $q = 3.7$, $\tau_c = 2.2$ ns, and a 32% average error. Use of eq 14 instead of eq 10 produces a better fit to the data, but this is certainly not proof of the validity of the internal rotation model since eq 14 has two additional adjustable parameters. This analysis is presented merely as one plausible explanation.

Thus, in the analysis of the hydration number (q) of bound Gd(III) using eq 12 and 13, the H/D method yields a value of τ_c which gives the correct *ratio* of the functions $f(\omega_H)$ and $f(\omega_D)$. However, use of the value of τ_c determined in this fashion can lead to a value of q which deviates from the actual value to the extent that the function $K = f/f'$ deviates from unity. The utility of the H/D method for determining the metal ion hydration number therefore depends on the ability of eq 11 to approximate the true function f for both protons and deuterons at a given magnetic field. A quantitative evaluation of the validity of eq 11 requires an accurate model describing the time dependence of the electron–proton magnetic interactions. Qualitatively, inspection of the values of q determined by Eu(III) luminescence, by conventional PRR measurements, and by the H/D method suggests that eq 11 provides a useful approximation of f for both nuclei at each field considered. Large deviations would produce corresponding deviations in the apparent value of q (eq 12 and 13), which are not observed. In spite of the many theoretical uncertainties, analysis of the water proton and deuteron relaxation data in terms of the SBM scheme has given an estimate of the metal ion hydration number in agreement with another technique.

Since the simple model assumed in eq 2–6 provides a good fit to the low-field PRR data, use of more complicated expressions involving more parameters is not justified.

In summary, the results of this investigation of the metal ion sites of *E. coli* glutamine synthetase indicate that there are three types of binding sites for lanthanide(III) ions of each subunit. This was also found for binding studies of divalent metal ions (Denton & Ginsburg, 1969). The first two sites each have a stoichiometry of 1 per subunit and are populated sequentially during titrations of the enzyme. The third type of site has an unknown stoichiometry, and lanthanide(III) binding to it induces aggregation of the enzyme. It is likely that the first two lanthanide(III) ion binding sites correspond to the n_1 and n_2 sites, respectively.

Two disparate physical techniques, lanthanide luminescence and NMR relaxation, were used to study the hydration of lanthanide(III) ions bound to the tightest of the sites. A comparison of the value of the hydration number determined by (1) Eu(III) luminescence lifetime measurements in H_2O and D_2O (4.1 ± 0.5), (2) PRR frequency dependence (Table I), and (3) water proton and deuteron relaxation measurements made at three different magnetic field strengths (Table II) shows that all determinations are in agreement. This result is gratifying since the Eu(III) luminescence technique is based on measurements of a series of model compounds of known hydration number (Horrocks & Sudnick, 1979). Lanthanide(III) ions have thus been characterized as useful probes of the Mg(II)-requiring enzyme glutamine synthetase. The luminescent and paramagnetic properties of these ions can now be used in further studies of the structure and involvement of metal ions in the catalytic mechanism of this enzyme.

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[4] The function minimized for this analysis was $\epsilon^2 = \sum[(r_m - r_c)/r_m]^2$. Fast exchange was assumed, and no frequency dependence was considered for τ_1.

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Protein-RNA Interactions in Belladonna Mottle Virus Investigated by Laser Raman Spectroscopy†

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ABSTRACT: Raman difference spectroscopy of the belladonna mottle virus (BDMV) and its separated RNA and protein components indicates that molecular interaction occurs between the single-stranded RNA genome and capsid subunits. The molecular interactions that stabilize the virion at pH 5.0 are altered or eliminated at pH 8.0, even though release of the RNA from the capsid is prevented by the addition of divalent metal cations (Ca^{2+}). From the perturbations that occur to Raman lines of cytosine and adenine rings of the encapsidated RNA molecule between pH values of 5.0 and 8.0, it is concluded that cytosines are protonated in significant numbers at the conditions which maintain the native virus structure and that the stacking of adenines is altered by changes in pH. The degree of protonation of RNA bases can be reduced by elevation of the pH to 8.0 for encapsidated RNA or by release of the RNA from the capsid at pH 5.0. Although the protein groups that interact with the viral RNA cannot be identified unambiguously from the Raman spectra, it is apparent that the molecular environments of aromatic amino acid side chains are altered with the same changes in pH (from 5.0 to 8.0) that perturb the cytosine and adenine ring structures. No significant change in secondary structures of the capsid subunit can be detected with changes in pH or with RNA release. On the other hand, the characteristic Raman lines of the phosphate groups of packaged RNA differ from those of naked RNA at all pH values examined, most likely as a result of specific electrostatic binding of divalent cations to RNA phosphates within the virus shell.

Belladonna mottle virus (BDMV) is a spherically shaped RNA plant virus of the tymovirus group. The T=3 icosahedral capsid, which has a diameter of 29 nm, contains 180 identical protein subunits of M_r 20 300 and a single RNA strand of M_r 1.9×10^6. The base composition of BDMV RNA, like that of the related TYMV RNA, is especially rich in cytosine residues: 37% C, 23% U, 23% A, and 17% G (Jankulowa et al., 1968). Both BDMV and TYMV are susceptible to loss of RNA when the viruses are isolated from infected plants. In vitro studies show, however, that the two tymoviruses differ significantly in susceptibility to diffusion of packaged RNA out of the capsid.

NMR and analytical ultracentrifugation studies indicate that BDMV RNA undergoes a structural transition within the capsid when the pH is increased from slightly acidic values to near neutrality (Virudachalam et al., 1983a,b). The NMR results suggest that below pH 6.5 the encapsidated RNA is relatively rigid, while above pH 6.8 the RNA achieves significantly greater mobility. A structural transition has also been observed for TYMV RNA over the same range of pH and has been attributed to the loss of specific protein-RNA interactions (Kaper, 1975). Such interactions may be a general feature of RNA plant viruses and may serve to restrict the mobility of the encapsidated RNA genome at lower pH.

At higher pH (>6.8), the RNA is readily released from the capsid of BDMV; however, a much greater increase of pH (>11.5) is required for release of TYMV RNA from its capsid. TYMV also preferentially resists release of its RNA for a variety of other structure-perturbing agents (Lyttleton & Matthews, 1958; Kaper, 1964, 1971; Jonard et al., 1972). The greater stability of TYMV vis-à-vis BDMV has been attributed to the presence of polyamines that are packaged in the native TYMV structure but not in the native BDMV structure. It has been proposed that repulsion between phosphate groups provides the driving force for ejection of RNA from the capsid and that such repulsive forces are neutralized by polyamines (Virudachalam et al., 1983a). This model is supported by the observation that addition of spermine, spermidine, and divalent cations to BDMV render the virus less susceptible to loss of RNA and more nearly identical with TYMV in its retention of RNA with increasing pH or with other structure-perturbing influences (Virudachalam et al., 1983b).

The specific interactions between RNA and protein that stabilize tymovirus structure and provide resistance to disassembly below pH 6.8 are not known. Various models, including abnormally titrating protein and RNA groups, have been proposed to account for the stability of RNA viruses at low pH, and some of these have been tested experimentally (Kaper, 1975). In a previous study of TYMV by Raman spectroscopy the presence of protonated cytidines and adenines (Cyt^+ and Ade^+) in the encapsidated RNA molecule was demonstrated for solution pH values well above the normal pK_a values of the bases (Hartman et al., 1978). Such protonated base residues have the potential for specific electro-

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