Identification of Nonprotein Ligands to the Metal Ions Bound to Glutamine Synthetase†

Charles D. Eads,‡ Russell LoBrutto,§ Alok Kumar,‡ and Joseph J. Villafranca*,‡

Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802, and Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

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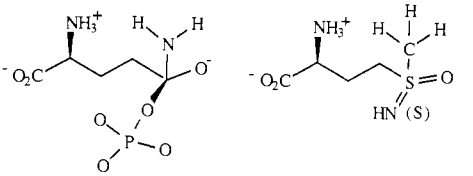
ABSTRACT: Electron paramagnetic resonance (EPR) was used to study the environment of Mn^{2+} bound to the tight (n_1) metal ion binding site of glutamine synthetase in the presence of analogues of the tetrahedral adduct, L-methionine (*S*)-sulfoximine [Met(O)(NH)-*S*] and L-methionine (*R*)-sulfoximine [Met(O)(NH)-*R*]. The Mn^{2+} EPR spectrum in the presence of Met(O)(NH)-*S* is identical with the previously published spectrum obtained from a mixture of isomers [Met(O)(NH)-*RS*] [Villafranca, J. J., Ash, D. E., & Wedler, F. C. (1976) *Biochemistry 15*, 544] and is characteristic of a highly octahedral metal ion environment with a small zero field splitting. The presence of Met(O)(NH)-*R* produces an EPR spectrum that appears characteristic of a more distorted metal ion environment, with a larger zero field splitting. These data demonstrate that the two isomers interact differently with the enzyme-bound Mn^{2+}. Broadening of the Mn^{2+} EPR spectrum in the presence of Met(O)(NH) is observed in ^{17}O-enriched water due to superhyperfine coupling of water to the metal ion. Deconvolution of the spectrum demonstrates the presence of at least a single water molecule in the inner coordination sphere of the metal ion. Superhyperfine coupling due to the ^{14}N nucleus of the imine nitrogen of the sulfoximine moiety of Met(O)(NH)-*S* but not of Met-(O)(NH)-*R* has been detected by electron spin-echo envelope modulation spectroscopy. Two intense peaks are evident in the presence of Met(O)(NH)-*S* with frequencies at 1.7 and 3.3 MHz. These peaks are absent when [^{15}N]imine-labeled Met(O)(NH) is used, indicating the presence of the sulfoximine nitrogen of Met(O)(NH)-*S* in the inner coordination sphere of the metal ion. Also supporting this conclusion is the fact that no peaks at 1.7 and 3.3 MHz were found when L-methionine sulfone or phosphinothricin was bound in place of Met(O)(NH). Taken together, these results suggest a model of the active site in which the metal ion is directly involved in the catalytic mechanism, serving to stabilize the tetrahedral adduct formed from ammonia and γ-glutamyl phosphate.

Glutamine synthetase catalyzes the synthesis of glutamine from glutamate, ammonia, and ATP. The bacterial enzyme consists of 12 identical subunits, each of M_r 51 814 (Colombo & Villafranca, 1986), arranged as two hexameric rings stacked face to face to form the catalytically active enzyme (Stadtman & Ginsburg, 1974). The X-ray crystal structure has recently been reported (Almassy et al., 1986). Glutamine synthetase has an absolute requirement for two divalent metal ions per subunit for catalysis (Hunt et al., 1975). The two types of metal ion binding sites can be distinguished on the basis of their metal ion affinities, their effects on the properties of the enzyme, and their interactions with substrates. The high-affinity site, designated the n_1 site, is near the substrate glutamate binding site (Villafranca et al., 1976a,b). The low-affinity site, designated the n_2 site, is involved in binding the metal–ATP complex (Hunt et al., 1975).

The synthesis of glutamine by glutamine synthetase is thought to proceed by a two-step mechanism (Clark & Villafranca, 1985; Meek et al., 1982; Midelfort & Rose, 1976). In the first step, nucleophilic attack on the terminal phosphate of ATP by glutamate produces γ-glutamyl phosphate (Krishnaswamy et al., 1962). In the second step, displacement of the phosphate by ammonia to yield the product glutamine proceeds via formation of a tetrahedral adduct (**1**) or transition state. L-Methionine sulfoximine [Met(O)(NH)], which has a tetrahedral moiety at the critical position, behaves as a transition-state analogue (Manning et al., 1969). Met(O)-(NH) has long been used in numerous biophysical investigations aimed at discerning the role of the n_1 site bound metal ion in the catalytic mechanism (Shrake et al., 1980; Gibbs et al., 1984; Wedler et al., 1982; Villafranca et al., 1976a,b; Hunt & Ginsburg, 1980; Balakrishnan & Villafranca, 1978; Ransom, 1984). The L,*S* isomer of Met(O)(NH) (**2**) can react with ATP on the surface of the enzyme in the presence of metal ions to form methionine sulfoximine phosphate, Met-(O)(NPO_3H) (Shrake et al., 1980). The enzyme–metal–Met(O)(NPO_3H)–ADP adduct thus formed is inactive since these molecules do not dissociate from the enzyme, even after extensive dialysis (Rowe et al., 1969; Maurizi & Ginsburg, 1982). Only when the pH is lowered can Met(O)(NPO_3H) and ADP be released from the enzyme, resulting in reactivation of the enzyme (Maurizi & Ginsburg, 1982).



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Using electron paramagnetic resonance (EPR) spectroscopy, our laboratory established that the binding of racemic Met-(O)(NH)-*RS* to glutamine synthetase with Mn^{2+} at the n_1 site results in an enzyme complex with Mn^{2+} in an environment of nearly octahedral symmetry (Villafranca et al., 1976b). Water proton relaxation rate studies indicate that Met(O)-(NH) binding reduces the solvent water accessibility of the

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‡The Pennsylvania State University.

§University of Pennsylvania School of Medicine.

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metal ion (Eads, 1985) and Met(O)(NPO_3H) formation entirely precludes the free exchange of water molecules from the n_1 site (Villafranca et al., 1976b; Ransom, 1984). These changes in the metal ion environment suggest that the metal ion may be directly involved in the catalytic mechanism. However, the nature of this involvement has not been established.

In this paper, studies of the role of the n_1 site bound metal ion were extended by examining in detail the mode of interaction of solvent water and of the resolved L,*S* and L,*R* isomers of Met(O)(NH) with Mn^{2+} bound to this site. Continuous-wave Mn^{2+} EPR spectroscopy was used to determine qualitative differences in the interactions of these isomers with the metal ion. Superhyperfine coupling of solvent water ^{17}O was used to quantitatively determine the extent of hydration of the n_1 site bound Mn^{2+} in the presence of Met(O)(NH), and electron spin-echo envelope modulation (ESEEM) spectroscopy was used to determine the proximity of Met(O)(NH) to the metal ion. The results of these experiments gave detailed information on the environment of the n_1 site bound metal ion and suggested a role for this ion in catalysis.

Materials and Methods

Materials. Glutamine synthetase in a low state of adenylylation (1.1 to 2.5 adenylylated subunits per dodecamer) was prepared by published procedures (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 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) containing 100 mM KCl, pH 7.0. Metal-free enzyme was prepared by dialysis against buffer containing 3 mM ethylenediaminetetraacetic acid (EDTA) followed by extensive dialysis against buffer alone. Matched samples of enzyme in ^{17}O-enriched water and in [^{16}O]water were prepared by dividing a stock solution of enzyme into two 50-μL aliquots. Each was lyophilized and redissolved in 50 μL of either ^{17}O-enriched water or [^{16}O]water.

L-Methionine (*SR*)-sulfoximine and L-methionine sulfone were obtained from Sigma. The resolved isomers of this compound were the kind gift of Dr. F. C. Wedler of The Pennsylvania State University. KOR Isotopes was the source for 60% ^{17}O-enriched, normalized water. DL-Phosphinothricin was a gift of Hoechst Chemical Co. All other reagents were obtained from standard chemical supply houses and used without further purification.

Synthesis of L-*Methionine* [^{15}N]*Sulfoximine.* The synthesis of L-methionine [^{15}N]sulfoximine was carried out by a modification of the procedure of Ronzio et al. (1969) as follows. A suspension of L-methionine sulfoxide (82 mg, 0.45 mmol) in chloroform (6.0 mL) and concentrated sulfuric acid (0.32 mL) was cooled in a round-bottom flask (100 mL). Sodium azide ($Na^{15}N_3$, 99% ^{15}N, 90 mg) was added to the reaction mixture and the flask immediately sealed to avoid the release of hydrazoic acid. The reaction mixture was stirred at 38–40 °C for 40 h, cooled, and then diluted by addition of water (20 mL) and chloroform (20 mL). The aqueous layer was separated, and additional product (methionine [^{15}N]sulfoximine) was extracted from the chloroform layer with water (3 × 30 mL). The pH of the aqueous solution was adjusted between 6.5 and 7.0 with saturated barium hydroxide solution and then filtered to remove barium sulfate. Water was then removed under reduced pressure at 55–60 °C, and the oily residue was dissolved in water (2.0 mL). This solution was chromatographed on Dowex 50W-X8 (1 × 30) cm, H^+ form). The column was first eluted with water (150 mL) to remove the salts and then with 1.5% ammonium hydroxide solution (125 mL). All ninhydrin-positive fractions were collected and lyophilized. L-Methionine [^{15}N]sulfoximine (82 mg, 90% yield) obtained as a crude product was crystallized with water–ethanol and gave 42 mg (46% yield) of pure L-methionine [^{15}N]sulfoximine. The product was checked for purity by 360-MHz NMR and paper chromatography, mp 207–209 °C dec.

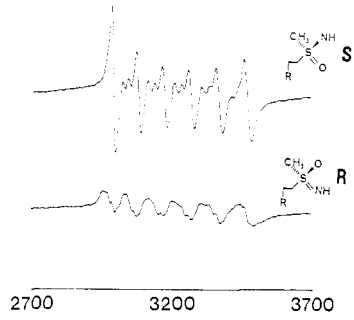


FIGURE 1: EPR spectra at 9.1 GHz. Solutions of Mn^{2+} bound to glutamine synthetase in the presence of Met(O)(NH)-*S* (upper) and Met(O)(NH)-*R* (lower). Conditions are given under Materials and Methods. The solutions contained 740 μM enzyme (subunit concentration), 440 μM Mn^{2+}, and 8.9 mM of each Met(O)(NH) isomer.

Methods. Continuous-wave EPR spectra were recorded on a Varian E-12 spectrometer at 9.1 GHz with an E-231 cavity and at 35 GHz with an E-266 cavity. The temperature was regulated to ±1 deg with an E-257 variable-temperature unit. Samples were held in quartz tubes (1-mm i.d. at 9.1 GHz, 0.1–0.2-mm i.d. at 35 GHz) capped with plastic plugs. Spectra were digitized and deconvoluted with a Teletek S-100 280A based computer utilizing a CalData D/A converter. The computer program for spectral manipulations was supplied by Center Computer Consultants, State College, PA.

Pulsed EPR spectroscopy was carried out at the University of Pennsylvania Department of Biochemistry and Biophysics in the laboratory of Dr. J. S. Leigh. The spectrometer and resonant probe employed in these experiments are described elsewhere (LoBrutto et al., 1986). ESEEM spectra were obtained at X-band at 5.5 K.

Results and Discussion

Continuous-Wave EPR Experiments. The continuous-wave EPR spectra of glutamine synthetase in the presence of Met(O)(NH)-*S* and Met(O)(NH)-*R* at room temperature are given at 9.1 GHz in Figure 1 and at 35 GHz in Figure 2. The spectra obtained with the Met(O)(NH)-*S* isomer are identical with the spectra obtained from racemic mixtures of the isomers (Villafranca et al., 1976b), demonstrating that the Met(O)(NH)-*S* isomer binds tightly enough to exclude the Met(O)(NH)-*R* isomer under these conditions. Indeed, Wedler et al. (1982) measured the binding constants of each isomer to glutamine synthetase, and the values are K_D(Met(O)(NH)-*S*) = 0.96 μM and K_D(Met(O)(NH)-*R*) = 42 μM. As discussed previously for spectra obtained from racemic Met(O)(NH) (Villafranca et al., 1976b), the spectrum for the Met(O)(NH)-*S* isomer is characteristic of a Mn^{2+} environment of high symmetry and low solvent accessibility, resulting in sharp lines due to a small zero field splitting (zfs).

The spectrum found with the Met(O)(NH)-*R* isomer can be rationalized in terms of an environment of lower symmetry than for Met(O)(NH)-*S* that also may have altered solvent accessibility. This results in higher values of the zfs and "broadened" lines. On the basis of the appearance of these

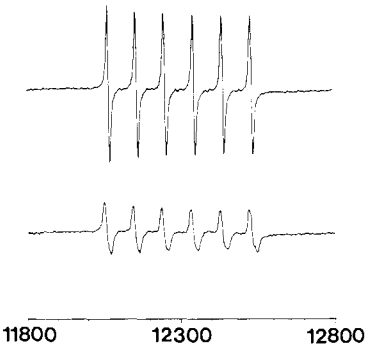


FIGURE 2: EPR spectra at 35 GHz. Conditions are the same as for Figure 1. Upper spectrum with Met(O)(NH)-*S* present and the lower spectrum with Met(O)(NH)-*R* present.

spectra, it is clear that the two isomers of Met(O)(NH) interact with the metal ion differently.

The EPR spectrum of enzyme-bound Mn^{2+} was obtained at 35 GHz in the presence of Met(O)(NH) containing ^{14}N or ^{15}N in the imine nitrogen. With the lowest field peak as a reference point, the peak-to-peak line width was 8 G for Met(O)(^{14}NH) and 7 G for Met(O)(^{15}NH) (data not shown). A more detailed description of these differences can be obtained by identifying the nonprotein ligands in the inner coordination sphere of the enzyme-bound metal ion by spin-echo EPR. These experiments are described in a later section of this paper.

^{17}O Superhyperfine Coupling Experiments. On the basis of the fairly large enhancement of water proton relaxation rates for solutions containing glutamine synthetase bound Mn^{2+} in the presence of Met(O)(NH), it appears that there is at least one water molecule in the inner coordination sphere of the metal ion for this complex. Reed and Leyh (1980) have developed a technique by which ^{17}O-containing ligands in the inner coordination sphere of Mn^{2+} can be identified. The technique involves the observation of superhyperfine coupling with the $S = {}^5/_2$ nucleus of ^{17}O. The effect of coupling to this nucleus is to split the EPR transition into six unresolved transitions, resulting in inhomogeneous broadening of the observed resonance line. Since the broadening is only a few gauss, the technique is applicable only to systems whose EPR line width is on the order of 15 G or less. We have therefore applied this technique to glutamine synthetase bound Mn^{2+} in the presence of racemic L-Met(O)(NH)-*RS*. As discussed above, use of this mixture results in exclusive binding of Met(O)(NH)-*S* to the enzyme, which gives sharp spectral transitions.

The upper spectra in Figure 3 show the two low-field hyperfine transitions of the 9.1-GHz EPR spectrum of the glutamine synthetase–Mn^{2+}–Met(O)(NH) complex for matched samples in [^{16}O]water and in 60% ^{17}O-enriched water. The observed broadening due to ^{17}O superhyperfine coupling establishes the presence of water in the inner coordination sphere of the metal ion. As described by Reed and Leyh (1980), it is possible to deconvolute the spectrum of the 60% ^{17}O-enriched sample into its ^{17}O-broadened and unbroadened components. Assuming that there is one water molecule in the inner coordination sphere of the enzyme-bound metal ion, then the spectrum obtained for the ^{17}O-enriched sample should have 60% ^{17}O character and 40% ^{16}O character. If there were two or three water molecules in the inner coordination sphere, then the broadened spectrum would have only 16% or 6.4% ^{16}O character, respectively. The lower spectrum of Figure 3 shows

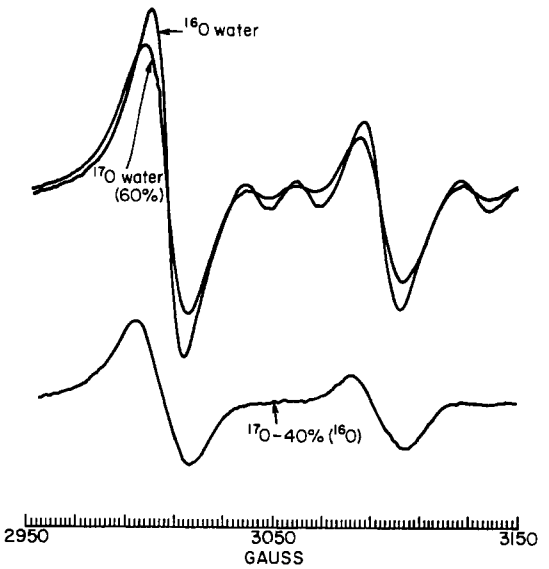


FIGURE 3: EPR spectra at 9.1 GHz with Met(O)(NH) present. The top spectra represent several of the low-field transitions of the glutamine synthetase–Mn^{2+}–Met(O)(NH) spectrum (see Figure 1 top) in the presence of 100% [^{16}O]water and 60% [^{17}O]water. The bottom spectrum was generated by computer subtraction of 40% of the spectrum obtained in [^{16}O]water from the spectrum in 60% [^{17}O]water.

the result of subtracting 40% of the unbroadened, ^{16}O-containing spectrum from the spectrum obtained in 60% ^{17}O-enriched water. If this value of 40%, corresponding to one coordinated water molecule, represented too large a fraction of the ^{17}O-broadened spectrum, then "gaps" in the deconvoluted spectrum would appear. Such gaps are not observed, demonstrating that for the best fit to the data there is at least a single water molecule coordinated to the enzyme-bound metal ion in the presence of Met(O)(NH). It should be noted that subtraction of 16% ^{16}O character also produced a smooth difference spectrum, so the data are consistent with either one or two coordinated water molecules. The same results were obtained at 35 GHz. An identical analysis was applied to samples containing, in addition to enzyme, Mn^{2+}, and Met(O)(NH), and saturating levels of MgADP and MgADP plus P_i [conditions as in Villafranca et al. (1976b)]. These molecules bind to the n_2 metal–nucleotide site and have little effect on the spectrum of the enzyme-bound Mn^{2+} at n_1 and produce no detectable change in the hydration of this metal ion site (data not shown).

Spin-Echo EPR Experiments. Electron–nuclear couplings that are much less than the EPR line width are difficult to detect with conventional EPR techniques. However, such weak coupling can often be detected by the electron spin-echo envelope modulation (ESEEM) technique (Rowan et al., 1965; Kevan, 1979). In this method, a series of two or three intense microwave pulses excites both "allowed" and "forbidden" transitions of an electron spin coupled to a nucleus of spin $I > 0$ (the "forbidden" transitions are those accompanied by a nuclear spin transition). Interference or "beating" due to the unequal precession frequencies of electrons in these two different environments causes periodic modulations of the electron spin-echo amplitude as a function of time between microwave pulses. Fourier transformation of the modulations produces a frequency spectrum very similar to those obtained by ENDOR (electron–nuclear double resonance). The frequency spectrum allows identification of the weakly coupled nuclei and, in some cases, gives a direct measure of the magnitude of the coupling as well.

The frequency-domain ESEEM spectrum of the enzyme complex with [^{14}N]imine-containing Met(O)(NH)-*S* is shown

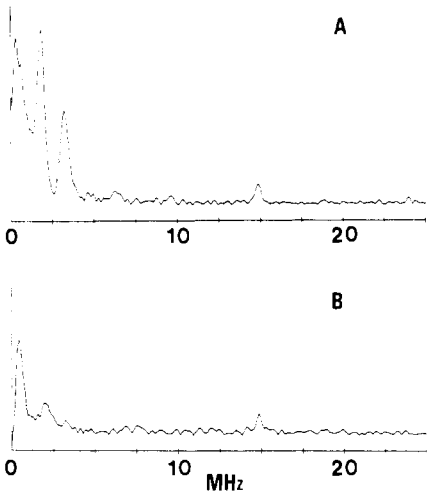
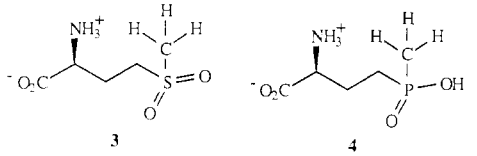


FIGURE 4: ESEEM spectra for glutamine synthetase and Mn^{2+} in the presence of isomers of Met(O)(NH). Solutions contained 517 μM glutamine synthetase, 465 μM Mn^{2+}, and 9 mM of each isomer of Met(O)(NH) ([*imine*-^{14}N]Met(O)(NH)-*S*, spectrum A; [*imine*-^{15}N]Met(O)(NH), spectrum B). The experimental conditions are H_0 = 3510 G, V_e = 9182 MHz, τ (time between pulses 1 and 2) = 230 ns, 90° pulse width = 20 ns, and T = 5.5 K. Spectra are the sum of 16 30-s sweeps at a 833-Hz repetition rate.

in Figure 4A. Two intense peaks are evident, at 1.7 and 3.3 MHz. There is no detectable shift of these peaks as H_0 is varied from the low-field edge to the high-field edge of the $+^1/_2 \leftrightarrow -^1/_2$ electronic transition, a range of 450 G. This is consistent with an assignment of the peaks to ^{14}N, since the predicted shift for that nucleus (about 0.14 MHz) is substantially smaller than the peak widths. Proton peak shifts would be much larger (about 14 times), and in any event, a proton frequency in this range would represent a coupling far too large to be detected by ESEEM. As ^{14}N and ^{1}H are the only non-zero-spin nuclei in the sulfoximine moiety, the peaks are certainly due to ^{14}N. The nuclear transitions that give rise to them have not been specifically assigned. Figure 4B shows the corresponding spectrum with [^{15}N]imine-labeled Met-(O)(NH). The two strong ^{14}N peaks have disappeared, indicating that they do in fact arise from the imine nitrogen of Met(O)(NH) and not from the protein itself. In Figure 5 are ESEEM data showing that these peaks are also absent in control samples prepared with [^{14}N]imine-containing Met-(O)(NH)-*R* (Figure 5A), with L-methionine sulfone (**3**)



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(Figure 5B) (in which the imine nitrogen of sulfoximine has been replaced by oxygen), and with phosphinothricin (**4**) (Figure 5C) [in which the sulfoximine moiety has been replaced by the $-P(CH_3)(OH)(O)$ moiety]. Because ESEEM is only sensitive to weak couplings of a few megahertz in magnitude, the absence of modulations in the Met(O)(NH)-*R* sample could indicate that there is no nitrogen coupling or that the coupling is too large to be measured.

Panels A and B of Figure 4 are presented on the same vertical scale, in order to emphasize the change produced by isotopic substitution. In Figure 5A, Met(O)(NH)-*R*, the weak peak near 2.3 MHz also occurs when L-methionine sulfone or phosphinothricin (panels B and C of Figure 5, respectively)

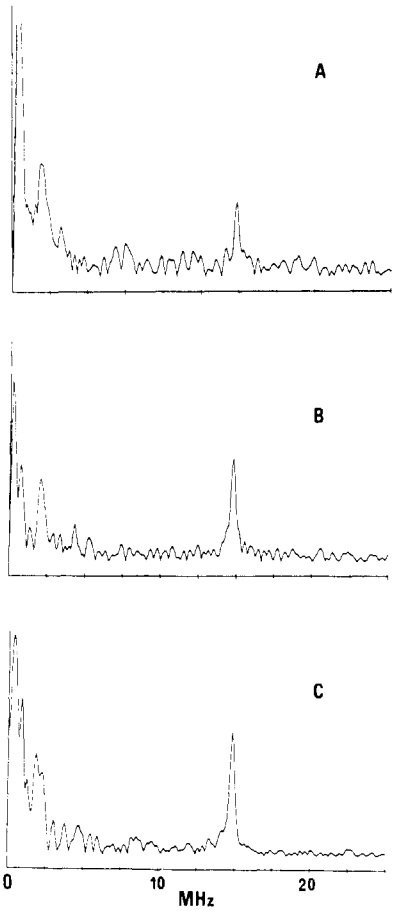

FIGURE 5: ESEEM spectra for glutamine synthetase and Mn^{2+} in the presence of substrate analogues. The experimental conditions are identical with those in the legend of Figure 4. (A) Same spectrum as Figure 4B, enzyme plus [*imine*-^{15}N]Met(O)(NH), but recorded at higher gain settings. (B) Same as (A) except in the presence of L-methionine sulfone (2 mM). (C) Same as (A) except in the presence of phosphinothricin (0.5 mM). The peak at 15 MHz is due to H_2O.

is substituted for Met(O)(NH). These peaks also exhibit no detectable frequency shift over the width of the $+^1/_2 \leftrightarrow -^1/_2$ transition and are likely due to an ^{14}N nucleus intrinsic to the protein. From the crystal structure of the enzyme, imidazole residues are near each of the metal ions (Almassy et al., 1986).

The absence of ^{15}N modulations in Figure 4B may be due to two effects. First, spin-echo modulations in general are shallower for smaller values of nuclear spin I ($I = {}^1/_2$ for ^{15}N). Second, the superhyperfine coupling due to ^{15}N is about 1.4 times larger than that for ^{14}N, and the modulation effect is often reduced for larger couplings. While ^{15}N does produce observable modulations in a number of systems [e.g., Mims and Peisach (1978) and LoBrutto (1986)], the loss of all modulations upon substituting ^{15}N for ^{14}N is not without precedent. Peisach et al. (1979) obtained a similar effect in a model compound of the type nitric oxide (NO)–heme–imidazole. The compound with [^{14}N]imidazole produced a rich spectrum of modulations in the three-pulse spectrum, while no modulations were apparent in the spectrum obtained with [^{15}N]imidazole, under the same conditions.

It is difficult to determine (1) whether the nitrogen that produces the modulations is a directly coordinated ligand to Mn^{2+}, (2) if the sulfoxyl oxygen atom is the ligand, or (3) if coupling arises from dipolar coupling to outer-sphere Met-(O)(NH). We have resolved this problem by comparison of Figure 4 (top) with data from model systems. It is well-known that an inner-sphere nitrogen ligand to Cu^{2+} does not produce observable modulations because the resulting coupling is too

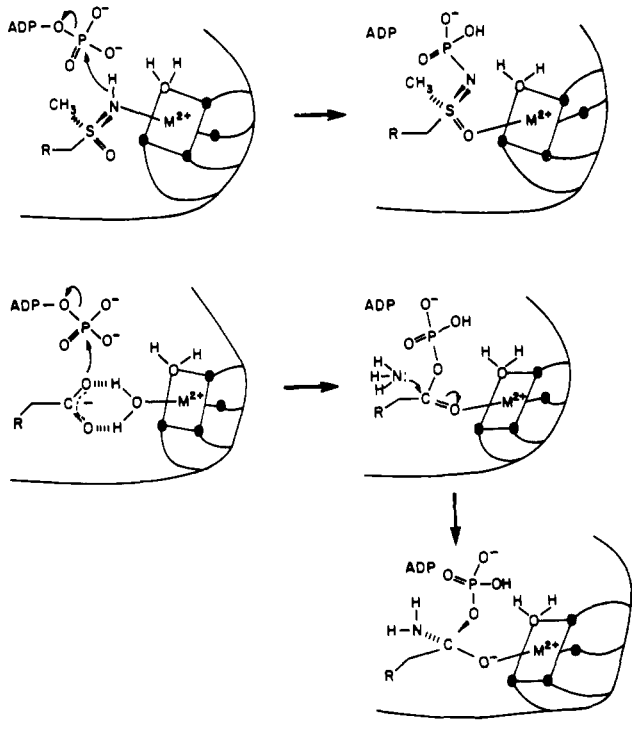

FIGURE 6: Models for the n_1 metal ion site of glutamine synthetase. (Top) Model for the binding of Met(O)(NH)-*S* to glutamine synthetase at the n_1 metal ion site. The reaction represents the phosphorylation of Met(O)(NH) by ATP. (Bottom) The sequence of reactions represents the normal biosynthetic reaction involving the n_1 metal ion, ATP, glutamate, and ammonia and is based in part on the EPR data in this paper.

large (Mims & Peisach, 1978). It is also well established that for imidazole (or histidine) ligands the nitrogen which is three bond lengths removed from the copper generally gives rise to substantial modulations, representing frequencies in the 0–4-MHz range (Mims & Peisach, 1978). However, a study of ^{14}N coupled to Mn^{2+} in protein complexes produced very different results (LoBrutto et al., 1986). For the complex Mn^{2+}–ADP–NCS–creatine in creatine kinase, modulations were detectable from the directly coordinated ^{14}N and ^{15}N atoms (independently obtained infrared data confirm the thiocyanate orientation in Mn^{2+} complexes; Reed et al., 1978). When nitrate, which cannot be N bonded to the metal, was substituted for thiocyanate, no modulations from either nitrogen isotope were detected. Furthermore, in the protein concanavalin A, modulations were detected from the directly coordinated nitrogen atom of the histidine ligand but not from the other nitrogen on the imidazole ring (LoBrutto et al., 1986). This conclusion was reached by considering that if the modulations were due to the nonligand nitrogen, then the coupling to the directly coordinated nitrogen would most likely be of the order of 10 G in magnitude. Even a 5–6-G coupling would produce an observable broadening or splitting in the relatively narrow (~8–10 G) Mn(II) lines ($I = 1$, so that the total line width would be at least twice the coupling). No such splitting was observed. These results show that only directly coordinated nitrogen atoms have produced modulations in the electron spin-echo envelope of Mn^{2+} and favor the assignment of the imine nitrogen of Met(O)(NH)-*S* as the direct ligand to Mn^{2+}.

The stereochemical result that the imine nitrogen of Met(O)(NH)-*S* is coordinated to the metal ion is of interest in view of previous studies. It has been established (Rowe et al., 1969) that the Met(O)(NH)-*S* isomer, in the presence of ATP and enzyme, becomes phosphorylated at the imine nitrogen position. This results in inactivation of the enzyme. When the imine nitrogen is coordinated to the Mn^{2+}, the p*K* of this group would be lowered, thus facilitating attack on the γ-P of ATP. After formation of Met(O)(NPO_3H), the Mn^{2+} could be coordinated to the oxygen (or both the oxygen and nitrogen or both the oxygen and phosphoryl group) resulting in tight, "irreversible" binding of this complex on the enzyme (Figure 6).

The present data can be related to previous NMR and EPR data with glutamine synthetase. The number of rapidly exchanging water molecules interacting with Mn^{2+} bound to the n_1 site is ~2 in the absence of substrates or substrate analogues (Eads, 1985; Ransom, 1984; Villafranca et al., 1976a,b) and drops to ~1 when glutamate or Met(O)(NH) is bound. These data are consistent with four protein ligands in all enzyme complexes. One water molecule would be displaced when Met(O)(NH) is coordinated to the Mn^{2+}, and the ^{17}O data and ESEEM data corroborate this idea. However, this may not be the case when glutamate is present. Previous NMR data suggested that the γ-carboxyl of glutamate (Villafranca & Balakrishnan, 1979) and the P atom of 2-amino-4-phosphonobutyric acid (Eads, 1985) were ~5 Å from the Mn^{2+} at the n_1 site. The $-CO_2^-$ or $-PO_3H^-$ groups of these compounds could be hydrogen bonded to a water molecule resulting in slow exchange of this ligand.

The X-ray crystal structure of glutamine synthetase (Almassy et al., 1986) shows that each metal ion has three or four ligands donated from the protein consistent with the data discussed above. Also, the crystal structure shows that each metal ion has at least one histidyl imidazole group in close proximity and this is indicated by the ESEEM data in Figure 5. The position of the Met(O)(NH) sulfur atom in the X-ray difference map shows that this atom is very close to the metal ions, consistent with the ESEEM data in this paper.

In the normal catalytic mechanism, displacement of the water molecule involved in hydrogen bonding the γ-carboxyl of glutamate may occur when the tetrahedral adduct is formed between ammonia and γ-glutamyl phosphate. To the extent that Met(O)(NH) is a good mimick of this step in catalysis, the ESEEM and ^{17}O data show one ligand to Mn^{2+} from the tetrahedral "center" and one or two water molecules. Thus the Mn^{2+} may serve in a catalytic role stabilizing the negative charge on the tetrahedral adduct, thereby lowering the energy barrier for its formation. Figure 6 presents a mechanism incorporating all of these ideas.

In conclusion, several biophysical methods have been used to study the involvement of the n_1 metal ion site in the reaction catalyzed by glutamine synthetase. The data demonstrate direct coordination of water molecules and one nitrogen atom from a substrate analogue in the enzyme complex, thereby suggesting that the enzyme supplies the other three or four ligands.

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Primary Structure of Mammalian Ribosomal Protein S6†

Richard E. H. Wettenhall,*,‡ Hans P. Nick,§,‖ and Trevor Lithgow‡
Department of Biochemistry, La Trobe University, Bundoora, Victoria 3083, Australia, and St. Vincent's Institute of Medical Research, Fitzroy, Victoria, Australia
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ABSTRACT: Ribosomal protein S6 was isolated from rat liver ribosomes by reversed-phase high-performance liquid chromatography (HPLC) and subjected to cyanogen bromide and proteolytic cleavages. The cleavage fragments were resolved by HPLC and sequenced by automated Edman degradation. The overall amino acid sequence of S6 (249 residues) was determined by alignment of the overlapping sequences of selected cyanogen bromide, chymotryptic, tryptic, and clostripain cleavage fragments. The only protein found to exhibit close homology with the S6 sequence is yeast ribosomal protein S10 (61% sequence identity). Previously, characterized phosphopeptide derivatives of S6 containing phosphorylation sites for adenosine 3′,5′-cyclic phosphate dependent and protease-activated protein kinases originate from the carboxy-terminal region of S6 encompassing residues 233-249.

Mammalian ribosomal S6 is the only ribosomal protein to be extensively phosphorylated at multiple sites in response to mitogenic and other polypeptide growth factors (Wool, 1979; Leader, 1980; Traugh, 1981; Thomas et al., 1982; Lastick & McConkey, 1981; Wettenhall et al., 1982, 1983; Perisic & Traugh, 1983; Tabarini et al., 1985; Martin-Perez & Thomas, 1983; Wettenhall & Howlett, 1979). This phenomenon is of interest because of the possibility that phosphorylation leads to increased protein synthetic activity in stimulated cells (Wettenhall & Howlett, 1979; Thomas et al., 1982; Duncan & McConkey, 1982; Traugh & Pendergast, 1986). S6 phosphorylation is catalyzed by various protein kinases, including adenosine 3′,5′-cyclic phosphate (cAMP)[1] dependent (Traugh, 1981; Wettenhall & Cohen, 1982), cGMP-dependent (Traugh, 1981), and Ca^{2+}/calmodulin-dependent (Padel & Soling, 1985) protein kinases, protein kinase C (Le Peuch et al., 1983; Parker et al., 1985), protease-activated protein kinases (Traugh, 1981; Perisic & Traugh, 1983; Donahue &

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* Address correspondence to this author.
‡ La Trobe University.
§ St. Vincent's Institute of Medical Research.
‖ Present address: Friedrich Miescher Institute, Basel, Switzerland.

[1] Abbreviations: RP-HPLC, reversed-phase high-performance liquid chromatography; Pth, 3-phenyl-2-thiohydantoin; GdmCl, guanidinium chloride; S2 and S4, solvents diethyl acetate and acetonitrile, respectively, used in the automated sequenator; TFA, trifluoroacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; cAMP, adenosine 3′,5′-cyclic phosphate; PDGF, platelet-derived growth factor; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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