Preparation and Characterization of Cobalt(III)- and Chromium(III)-Glutamine Synthetase Derivatives†

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ABSTRACT: Stoichiometric incorporation of Co(III) into the n_1 metal ion sites of *Escherichia coli* glutamine synthetase (unadenylylated) was carried out by hydrogen peroxide oxidation of enzyme-bound Co(II). This enzyme derivative was found to bind 1 mol of Mn(II) per subunit to the n_2 metal–nucleotide sites with a K_D value of 0.67 ± 0.11 mM. The Co(III)–enzyme was catalytically inactive (<0.1%) as judged by the γ-glutamyl transferase assay. Electron paramagnetic resonance (EPR) studies of Mn(II)–nucleotide complexes bound to the Co(III)–enzyme revealed differences in the environment of Mn(II) with ATP or ADP in the presence of the other substrates L-glutamate and NH_4^+. The ATP analogues AMPPCP and AMPPNP produced different enzyme-bound Mn(II) spectra than ATP. L-Methionine sulfoximine, a transition-state analogue for glutamine synthetase, gave an anisotropic Mn(II) spectrum with various nucleotides but the Mn(II) spectrum of Co(III)–enzyme–Mn(II) was quite distinct from the one that the sulfoximine produces when Mn(II) is in the n_1 metal ion site [Villafranca, J. J., et al. (1976) *Biochemistry 15*, 544]. Collectively these data demonstrate that the Mn(II) environment at the n_2 site is distinct from that previously observed for Mn(II) bound at the n_1 site. The paramagnetic Cr(III)–enzyme derivative (n_1 sites) was prepared by air oxidation of Cr(II)–enzyme. EPR studies were used to calculate a metal–metal distance between the n_1 and n_2 sites using Mn(II)–nucleotide complexes bound to the n_2 site. The metal–metal distance of 7 ± 2 Å is in good agreement with distances previously obtained for Mn(II)–enzyme–Cr(III)–nucleotide complexes (Mn(II) in n_1 and Cr(III)–nucleotide in n_2) by M. S. Balakrishnan & J. J. Villafranca [(1978) *Biochemistry 17*, 3531].

Glutamine synthetase from *Escherichia coli* is a dodecameric enzyme that catalyzes the reaction

$$\text{L-glutamate} + \text{ATP} + \text{NH}_3 \xrightleftharpoons{2M^{2+}} \text{L-glutamine} + \text{ADP} + \text{P}_i \quad (1)$$

For sometime it has been known that the enzyme has an absolute requirement for the binding of at least two metal ions per subunit for catalytic activity (Ginsburg, 1972). The metal ion sites are designated the n_1 and n_2 sites. Kingdon et al. (1968) showed that the metal ion free apoenzyme was catalytically inactive and Shapiro & Ginsburg (1968) demonstrated that binding of metal ions to the n_1 site produced an ultraviolet difference spectrum when compared with apoenzyme. Later Hunt et al. (1975) reported that the n_2 site was the metal–nucleotide site and thus the n_2 site is an intimate part of the active site.

Work from this laboratory has concentrated on biophysical studies of the two metal ion sites. Nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) studies have revealed that the two metal ion sites are in close proximity (5–7 Å) on the enzyme surface (Villafranca et al., 1975, 1976a,b, 1977, 1978; Balakrishnan & Villafranca, 1978) and that the n_1 site might indirectly be involved in substrate orientation.

Previous EPR studies of Mn(II) bound to the n_1 site showed that the environment of this metal ion was sensitive to bound substrates or inhibitors. However, since there are two metal ion sites, it is difficult to obtain information about Mn(II) at each site simultaneously from EPR experiments since the spectra overlap. Our approach to this problem was to prepare a suitable derivative of the n_1 site so that we could explore the n_2 site with Mn(II) as a probe.

Segal & Stadtman (1972) and Hunt & Ginsburg (1972) have conducted extensive studies on the properties of Co(II) activated glutamine synthetase. Villafranca et al. (1978) showed by fluorescence energy transfer experiments that Co(II) bound at both n_1 and n_2 were close to the adenylyl covalent modifier site on the enzyme. Balakrishnan & Villafranca (1978) reported that Co(II)–nucleotide complexes could bind to the n_2 site with Mn(II) at n_1.

It was the aim of experiments reported in this paper to have a diamagnetic metal ion bound at the n_1 site that would not interfere with the EPR spectrum of Mn(II) bound at the n_2 site. Cobalt in the trivalent oxidation state is ideal for the purpose since it is almost always diamagnetic in its complexes. There have been several reports on the oxidation of enzyme-bound Co(II) to Co(III), viz., carboxy peptidase (Kang et al., 1972, 1975; Van Wart & Vallee 1977, 1978), DNA-dependent RNA polymerase (Wu et al., 1977), aspartokinase (Ryzewski & Takahashi, 1975; Wright et al., 1976), carbonic anhydrase (Shinar & Navon, 1973), and alkaline phosphatase (Anderson & Vallee, 1975). These procedures were adapted for glutamine synthetase. Also, in this paper we report the first synthesis of a paramagnetic Cr(III)–enzyme derivative.

EPR studies in this paper reveal that when Co(III) or Cr(III) is bound to the n_1 site, Mn(II)–nucleotides bind to the n_2 site but the complexes are catalytically inactive. These EPR studies were useful in demonstrating differences in the environment of enzyme-bound Mn(II) when different substrates, substrate analogues, or inhibitors were bound.

Experimental Procedures

Materials. Glutamine synthetase was isolated from *Escherichia coli* in a state of low adenylylation as described previously (Woolfolk et al., 1966). L-Glutamate, ADP, ATP, L-methionine (*SR*)-sulfoximine, and L-methionine sulfone were products of Sigma. Miles Laboratories provided the β,γ-methyleneadenosine 5′-triphosphate and P-L Biochemicals supplied the adenylyl imidodiphosphate. All other chemicals were reagent grade. $^{57}CoCl_2$ and $^{51}CrCl_3$ were obtained from

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New England Nuclear Radio Chemicals. The protein concentration and Mn(II) content were determined as described in previous papers (Villafranca et al., 1976a,b).

Enzyme Assay. Glutamine synthetase activity was assayed by the γ-glutamyl transferase assay. The conditions of the assay are those described by Shapiro & Stadtman (1970). The enzymatic activity is expressed as the amount of enzyme catalyzing the synthesis of 1 μmol of γ-glutamyl hydroxamate per min per mg of protein at 37 °C.

Preparation of Co(III)-Glutamine Synthetase. Divalent cobaltous ion has been shown to bind to unadenylylated apoglutamine synthetase (Hunt & Ginsburg, 1972) and to also support the synthesis of glutamine from glutamate, ATP, and ammonia (Segal & Stadtman, 1972). A binding constant of 2×10^{-5} M was determined by Hunt & Ginsburg for Co(II) binding to the "tight" metal ion site but Co(II) binding to the second binding site was not determined. Villafranca et al. (1978) determined binding constants for Co(II) to the n_1 and n_2 metal ion sites with adenylylated glutamine synthetase and the values were $K_1 = 1.4 \times 10^{-5}$ and $K_2 = 2.5 \times 10^{-4}$ M.

Co(III)-glutamine synthetase was prepared by oxidation of Co(II)-glutamine synthetase similar to that adopted for Co(III)-carboxy peptidase A (Kang et al., 1975). Apoglutamine synthetase (0.18–0.23 mM subunit concentration) was equilibrated with a 1:1 stoichiometric amount of $CoCl_2$ that contained a suitable amount of ^{57}Co for radioassay (~1.4 × 10^5 cpm). Under these conditions ~70% of the n_1 site is occupied by Co(II) and ~3% of the n_2 site is occupied. To the above solution, 1.0–1.2 mM H_2O_2 was added and allowed to react at room temperature in 0.1 M KCl, 0.02 M Tris-Cl buffer at pH 7.2. The oxidation of Co(II) to Co(III) was followed by monitoring the increase in optical absorbance at 280 and 503 nm as well as by enzymatic assay. The time course of the increase in A_{503} was plotted by subtracting the absorbance value at infinite time, t_∞, from the value at other times, t. This value was divided by the difference in absorbance at zero and infinite times to obtain a first-order presentation of the data. After the activity of the enzyme had dropped to about 1% the solution was dialyzed for 8 h at 4 °C against three changes of a 50-fold excess of buffer. The extent of cobalt incorporation was determined by measurement of the γ-emission rate of ^{57}Co with a Baird Atomic Model 530 spectrometer. The molar ratio of cobalt to protein in the Co(III)-glutamine synthetase was usually in the range 0.95 to 1.05. In one experiment, a 0.6 to 1.0 ratio of Co(II) to enzyme was used to achieve less than complete incorporation. Control solutions of apoglutamine synthetase or of Mg(II)-glutamine synthetase were subjected to the same manipulations and assay conditions. For EPR measurements, Co(III)-glutamine synthetase was concentrated in a Collodion suction filtration apparatus to achieve a final enzyme subunit concentration of about 0.8 mM.

Preparation of Cr(III)-Glutamine Synthetase. The preparation of Cr(III)-glutamine synthetase required anaerobic conditions since a method had to be devised to prepare and manipulate Cr(II) for incubation with apoglutamine synthetase. A 50 mM solution of chromium(III) chloride in 0.1 mM hydrochloric acid was prepared that contained sufficient ^{51}Cr for radioassay. The solution was reduced to Cr(II) by zinc amalgam under nitrogen atmosphere. The reaction was easily followed as the green Cr(III) solution was reduced to sky blue Cr(II). Control experiments showed that, while reduction of Cr(III) to Cr(II) was slow in 0.1 mM HCl, later additions of this acidic solution to the apoenzyme did not lead to denaturation. Four microliters of the Cr(II) solution (8.5 × 10^4 cpm) was then transferred with an air-tight Hamilton syringe to a solution of apoglutamine synthetase (1.25 mL, 0.187 mM) which was previously degassed and kept under nitrogen atmosphere. The Cr(II) to enzyme subunit ratio was 1:1. After 20 min, the flask containing enzyme-Cr(II) was removed from nitrogen atmosphere and exposed to air. The instantaneous oxidation of Cr(II) to Cr(III) on the enzyme resulted in Cr(III)-glutamine synthetase. The incorporation of Cr(III) was followed by a determination of the γ-emission rate of ^{51}Cr. The Cr(III)-glutamine synthetase was then dialyzed for 8 h at 4 °C against three changes of a 20-fold excess of buffer. Chromium analysis as described above showed that the enzyme had 0.9 to 1.05 Cr per enzyme subunit.

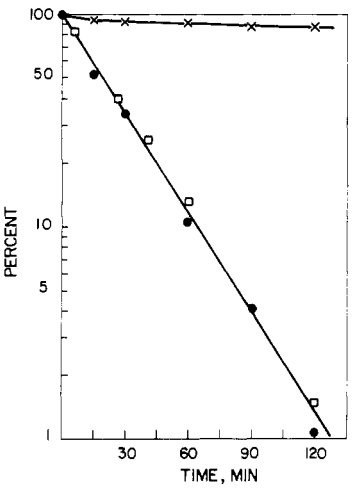

FIGURE 1: Plot of change in enzyme activity and optical absorption of enzyme during oxidation from Co(II) to Co(III). (●) The enzyme concentration was 0.18 mM (subunit concentration), 0.18 mM $CoCl_2$, and 1.0 mM hydrogen peroxide in a total volume of 2.1 mL. Aliquots (5.0 μL) were withdrawn at the time indicated and tested for enzyme activity by the transferase assay described in Experimental Procedures. The specific activity at zero time was 12 units/mg. (×) Same as above except the enzyme solution (0.13 mM subunit concentration) contained 60 mM $MgCl_2$. (□) The change in absorbance of the enzyme sample containing $CoCl_2$ was followed as a function of time at 503 nm as described in Experimental Procedures. The enzyme and $CoCl_2$ concentrations were as described above.

EPR Measurements. A Varian E-12 spectrometer was used to record spectra at 9 GHz. The instrument was equipped with an E-257 variable temperature accessory unit and the temperature was maintained within ±1 °C by heating precooled nitrogen gas that was passed through the Dewar assembly placed in an E-231 cavity. Aqueous samples of 20–25 μL were placed in quartz capillary tubing of 1.0 mm i.d. and the end of tubing was closed by using polyethylene tubing and a Teflon plug. A copper–constantan thermocouple was used to monitor cavity temperature throughout the runs. The binding of Mn(II) to Co(III)-glutamine synthetase was determined at pH 7.2, 25 °C, by methods previously used for glutamine synthetase (Villafranca et al., 1976a,b).

Results

Oxidation of Co(II)-Glutamine Synthetase by Hydrogen Peroxide. Apoglutamine synthetase was incubated with $CoCl_2$ and H_2O_2 as described in Experimental Procedures. Figure 1 shows a plot of percent change in enzyme activity with time during the oxidation of enzyme-bound Co(II) to Co(III). The loss of activity is first order and after 2 h the enzyme has about 1% of its original activity left. Also shown is the percent change in optical absorption of the Co(III)-enzyme. This change is identical with that observed for the loss of activity

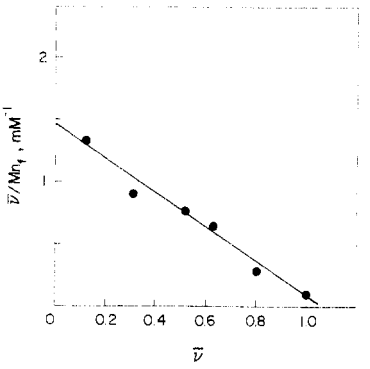

FIGURE 2: Scatchard plot of the interaction of Co(III)–glutamine synthetase with $MnCl_2$. The buffer was 100 mM KCl, 20 mM Tris-Cl, pH 7.2, 25 °C. The concentration of free and bound Mn(II), $(Mn)_f$, and $(Mn)_b$, respectively, was determined by EPR spectroscopy as described in Experimental Procedures. The subunit concentration of Co(III)–glutamine synthetase was 75 μM for each determination.

and is therefore consistent with the postulate that a loss of enzymatic activity occurs when enzyme–Co(II) is converted to enzyme–Co(III). There is a small increase in absorbance at 280 nm (~20%) during the course of the oxidation. This may be due to oxidation of aromatic amino acid residues or due to different conformations of the enzyme–Co(II) and enzyme–Co(III) derivatives. Such differences have been observed with other metal ions (Segal & Stadtman, 1972; Shapiro & Ginsburg, 1968). The enzyme was dialyzed and isolated as described in Experimental Procedures. Radioassay of ^{57}Co incorporated into the enzyme indicated that 1 mol of Co(III) was incorporated per subunit of glutamine synthetase. The EPR spectrum of enzyme–Co(II) at 6 K (Balakrishnan & Villafranca, 1978) was too broad to be useful for quantitative analysis of the disappearance of the Co(II)–enzyme spectrum during the oxidation as was done for Co(II)–carboxypeptidase (Van Wart & Vallee, 1978).

In a control experiment, enzyme–Mg(II) was subjected to identical conditions used for the oxidation of enzyme–Co(II) to enzyme–Co(III). Figure 1 shows that the enzyme with Mg(II) lost ~11% of its activity due to treatment with H_2O_2. When apoglutamine synthetase was treated with H_2O_2, ≥70% of the enzymatic activity was lost in 2 h.

Binding of Mn(II) to Co(III)–Glutamine Synthetase. The oxidation procedure described above resulted in stoichiometric incorporation of one Co(III) per subunit of glutamine synthetase. This metal ion is most likely bound to the n_1 site based on the initial concentrations of enzyme and Co(II) and the difference in binding constants of Co(II) to the n_1 and n_2 sites (see Experimental Procedures). EPR experiments were conducted to test whether Mn(II) could still bind to the n_2 site of enzyme–Co(III). Figure 2 shows a Scatchard plot resulting from these Mn(II) binding studies. Least-squares statistical analysis shows that one Mn(II) binds per subunit of enzyme–Co(III) with a binding constant of 0.67 ± 0.11 mM. This value is larger than the K_D value for Mn(II) binding to the n_2 site determined by Hunt et al. (1975) (K_D = 0.05 mM) and Villafranca et al. (1976a,b) (K_D = 0.045 mM). This experiment demonstrates that the Co(III)–enzyme binds Mn(II) but this enzyme derivative is catalytically inactive (≤0.1%) by enzymatic assay of the isolated protein.

EPR Spectra of Enzyme–Co(III)–Mn(II) Complexes. EPR spectra of Mn(II) bound to the n_1 "tight" metal ion site have previously been reported by Villafranca et al. (1976b). With Co(III) at the n_1 site, Mn(II) was shown above to bind to the enzyme, presumably, at the n_2 metal–nucleotide site. The EPR spectrum of E–Co(III) (0.77 mM) with Mn(II) (0.19 mM)

FIGURE 3: X-band EPR spectra for complexes of Co(III)–glutamine synthetase. All solutions contained 100 mM KCl, 20 mM Tris-Cl buffer, pH 7.2. (A) Cavity base line; (B) Co(III)–enzyme subunit concentration, 0.77 mM (0.770 mM enzyme sites, 0.765 mM Co(III); $MnCl_2$, 0.19 mM); (C) same as B with the addition of 37 mM L-glutamate and 3.6 mM ATP. The solution was incubated for 10 min at 25 °C prior to recording the spectrum; (D) same as C with the addition of 35 mM NH_4Cl; (E) same as B with the addition of 0.6 mM ADP. All solutions were recorded at 1 °C.

is shown in Figure 3B. Since the binding constant of Mn(II) to E–Co(III) was determined to be 0.67 mM, this spectrum would represent ~50% free Mn(II). The isotropic appearance of the spectrum confirms this. When L-glutamate (37 mM) was added there was no apparent change in the spectrum. However, when ATP was added the spectral intensity and shape changed to that shown in Figure 3C. Once again L-glutamate did not change the spectrum obtained with ATP but addition of NH_4^+ altered the intensities of some of the lines slightly (Figure 3D). Addition of ADP to a solution of E–Co(III)–Mn(II), at the same concentrations used for Figure 3B, produced the spectrum in Figure 3E. The spectral intensities are reduced from those in 3B and the appearance is altered from those in 3C and 3D. Thus the spectra in 3C, 3D, and 3E are indicative of enzyme bound Mn(II)–nucleotides and we conclude that the Mn(II) is bound at the n_2 site in accord with other published data (Hunt et al., 1975; Villafranca et al., 1976a).

As was shown by Villafranca et al. (1975, 1976b), methionine sulfoximine (a transition-state analogue) produced a very distinctive spectrum when added to E–Mn(II) where Mn(II) occupied the n_1 site. To test whether the Mn(II) added to E–Co(III) was in a site similar to n_1, two E–Co(III) enzyme preparations were made. In one, the final Co(III) to enzyme (subunit) ratio was 0.71:1.0, and in the other it was 0.99:1.0. The EPR spectrum of Mn(II) (0.17 mM) added to 0.46 mM of the preparation with 71% Co(II) is shown in Figure 4A. The spectrum reveals that there is substantial free Mn(II) under these conditions. This may be partially due to a weaker affinity of the H_2O_2-modified apoenzyme (29%) for Mn(II); however, the n_1 sites should still be partially occupied by Mn(II) as revealed below.

When methionine sulfoximine is added to the sample in Figure 4A, the characteristic sharp lines indicative of Mn(II) bound to n_1 in the presence of bound sulfoximine become apparent (Figure 4B). After addition of ADP to this solution,

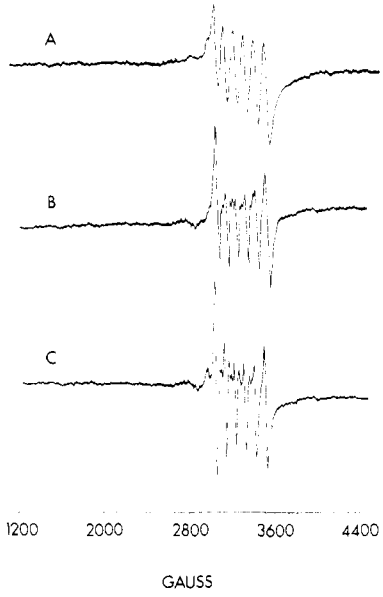

FIGURE 4: X-band spectra for Co(III)–glutamine synthetase complexes. (A) Enzyme sites, 0.46 mM, 0.31 mM bound Co(III), 0.17 mM $MnCl_2$; (B) same as A with the addition of 12 mM L-methionine (*SR*)-sulfoximine; (C) same as B with the addition of 0.6 mM ADP. T = 1 °C.

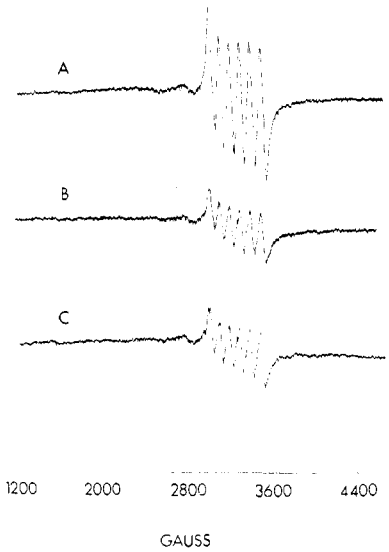

FIGURE 5: X-band spectra for complexes of Co(III)–glutamine synthetase and methionine sulfoximine. All solutions contained 20 mM Tris-Cl buffer, 100 mM KCl, 0.77 mM enzyme–Co(III) subunit concentration, 0.19 mM $MnCl_2$, pH 7.2, 1 °C. (A) Co(III)–enzyme–Mn(II) plus 10.3 mM L-methionine (*SR*)-sulfoximine; (B) solution A plus 0.94 mM ADP; (C) solution B plus 2.4 mM P_i.

further sharpening is observed (Figure 4C). This phenomenon has been demonstrated before (Villafranca et al., 1976b) and indicates changes in the Mn(II) environment of the 29% Mn(II) bound at the n_1 sites of the enzyme.

By contrast, when methionine sulfoximine is added to the enzyme with 99% Co(III) bound (Figure 5A), no sharpening is observed. This indicates that the Mn(II) is bound at the n_2 site and that the Mn(II) at this site does not change its environment in the same way as Mn(II) bound at n_1. Further addition of ADP (Figure 5B) and P_i (Figure 5C) to the E–Co(III)–Mn(II)–sulfoximine sample reduces the intensity of the free Mn(II). The individual spectra are similar to the one reported in Figure 3D for Mn(II)–ADP bound to E–Co(III) (99% Co(III) to enzyme). The addition of sulfoximine produces slight changes in the Mn(II) environment (Figures 5B and 5C) but these are quite different from that seen when Mn(II) is bound at n_1. Thus, it is concluded that, when enzyme is prepared with a 1:1 Co(III) to enzyme stoichiometry, the Co(III) is in the n_1 site and additional Mn(II) binds to the n_2 metal–nucleotide site.

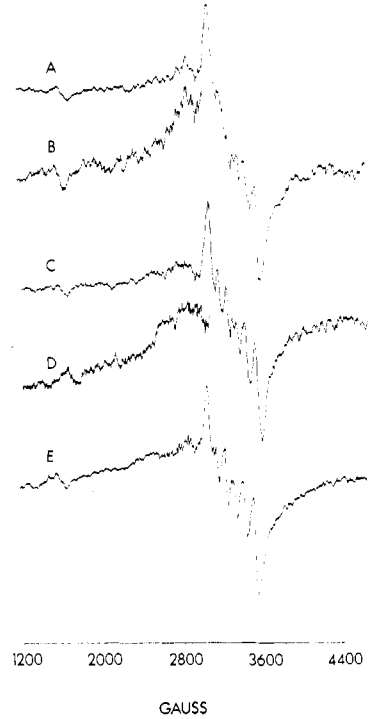

FIGURE 6: X-band spectra for Co(III)–glutamine synthetase complexes with adenine nucleotides and their analogues. All solutions contained 0.77 mM Co(III)–glutamine synthetase (subunit concentration), 0.19 mM $MnCl_2$. (A) Co(III)–enzyme–Mn(II) plus 3.7 mM ATP; (B) same solution at twice the gain setting as A; (C) Co(II)–enzyme–Mn(II) plus 6.7 mM AMPPCP; (D) same solution at twice the gain setting as C; (E) Co(III)–enzyme–Mn plus 3.3 mM AMPPNP. All the solutions for spectra A through E were incubated for 10 min at 25 °C and cooled to 1 °C before recording the spectrum.

The 1:1 E–Co(III) derivative was used to study the bindng of Mn–ATP and two ATP analogues, viz., AMPPCP and AMPPNP, to the n_2 site in greater detail. Figure 6A shows the spectrum of E–Co(III)–Mn(II)–ATP. The line shapes in the ~3200-G region suggest a distortion from cubic symmetry of the Mn(II). Also, additional poorly resolved transitions appear in the low-field portion of the spectrum when it is recorded at a higher gain setting (Figure 6B). It is difficult to assess whether the distortion is axial ($D \neq 0$, $E = 0$) or rhombic, i.e., both D and $E \neq 0$, for this Mn(II)–nucleotide complex.

The spectra for both AMPPCP and AMPPNP complexes with E–Co(III)–Mn(II) are presented in Figures 6C–6E. The more well-defined transitions in the ~3200-G region are indicative of less distortion (more isotropic line shape) than with the ATP complex. Thus, while ATP and the ATP analogues alter the Mn(II) environment, all three nucleotides produce different extents of distortion. More extensive studies should reveal whether the binding constants for the three nucleotides are also different. All the transitions observed are due to enzyme-bound Mn(II).

Additional transitions are seen in the low-field portions of the spectra for ATP (Figures 7A and 7B), AMPPCP (Figures 7C and 7D), and AMPPNP (Figures 7E and 7F) when methionine sulfoximine is added to the solutions. The low-field transitions of the spectrum taken with ATP are more intense (~2800 G) with sulfoximine bound to the protein and a distinct six-line pattern is seen in the spectrum of the E–

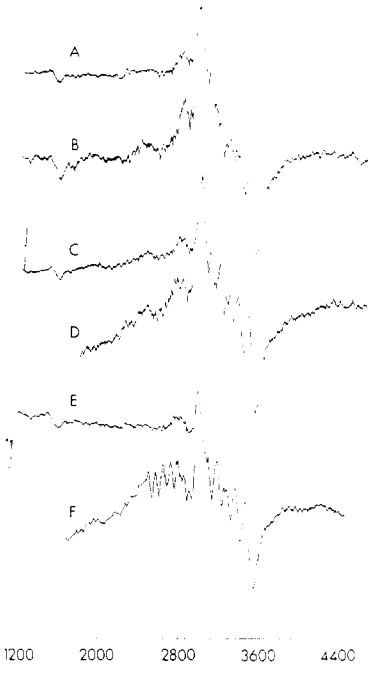

FIGURE 7: X-band spectra for complexes of Co(III)–glutamine synthetase with adenine nucleotides and their analogues in the presence of methionine sulfoximine. All solutions contained 0.77 mM Co(III)–enzyme sites, 0.19 mM $MnCl_2$, 10 mM L-methionine (*SR*)-sulfoximine. (A) Co(III)–enzyme–Mn(II)–methionine (*SR*)-sulfoximine plus 3.7 mM ATP; (B) same solution at twice the gain setting as A; (C) Co(III)–enzyme–Mn(II)–methionine (*SR*)-sulfoximine plus 6.7 mM AMPPCP; (D) same solution at twice the gain setting as C; (E) Co(III)–enzyme–Mn(II)–methionine (*SR*)-sulfoximine plus 3.3 mM AMPPNP; (F) same solution at twice the gain setting as E. All solutions for spectra A–F were incubated for 10 min at 25 °C and cooled to 1 °C before recording the spectrum.

Co(III)–Mn(II)–AMPPNP–sulfoximine complex. In the latter spectrum it can be estimated that $D = 0.05$–0.08 cm^{-1} and $E/D \sim 0.1$ from the diagrams in the papers of Aasa (1970) and Dowsing et al. (1969). Therefore, even though the Mn(II) is bound at the n_2 site in the above complexes, the addition of methionine sulfoximine, a transition-state analogue of the glutamate ammonia substrate site, produces conformational changes in the enzyme that alter the environment of the metal–nucleotide site. Synergistic interaction between the substrate sites has previously been reported using many other techniques (Timmons et al., 1974; Rhee & Chock, 1976).

EPR Spectra of Enzyme–Cr(III). Anaerobic solutions of apoglutamine synthetase and Cr(II) were mixed together as described in Experimental Procedures. An enzyme complex with ~1:1 stoichiometry of Cr(III) to enzyme subunit was prepared in this manner. Figure 8A presents the EPR spectrum of E–Cr(III) taken at 1 °C. The broad line at ~3200 G is characteristic of Cr(III) in a randomly oriented "powder spectrum" (cf. McGarvey, 1964, 1967). Unfortunately one cannot deduce very much information about the symmetry of the enzyme-bound Cr(III) or about the ligands bound to the metal ion. However, since other transitions are not observed at lower or higher field, the Cr(III) environment is probably very nearly isotropic with a small axial distortion ($D = 0.03$–0.1 cm^{-1}, McGarvey, 1967).

The E–Cr(III) complex was titrated with Mn(II) and ADP. Figure 8B shows a spectrum in which the added Mn(II) (0.125 mM) is bound to the enzyme as evidenced by the anisotropic nature of the spectrum. The enzyme-bound MnADP spectrum is superimposed over the Cr(III) transitions but nonetheless

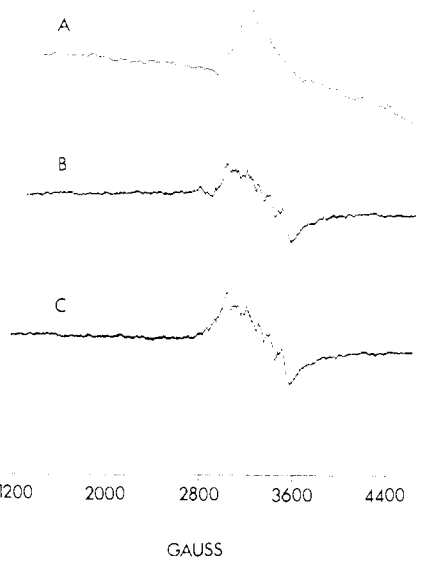

FIGURE 8: X-band spectra for Cr(III)–glutamine synthetase complexes. (A) Cr(III)–enzyme (0.27 mM); (B) 0.24 mM Cr(III)–enzyme, 0.13 mM $MnCl_2$, 5.3 mM ADP; (C) solution B plus 5.5 mM P_i. All solutions were prepared and manipulated at 1 °C.

the Mn(II) transitions sharpen slightly when P_i is added (Figure 8C) indicating a change in the Mn(II) environment. The spectra in Figures 8B and 8C look similar to the spectra obtained for enzyme-bound Mn(II) in the n_1 site after titration with Cr(III)–ADP (cf. Figure 3, Balakrishnan & Villafranca, 1978). For the E–Mn(II)–Cr(III)–ADP complex, there is an electronic spin–spin interaction between enzyme-bound Mn(II) and Cr(III). The result of this interaction is to reduce the spectral intensity of the bound Mn(II) by dipolar electronic relaxation. This phenomenon was used to calculate A distance of 7 Å between the bound metal ions at the n_1 and n_2 sites (Balakrishnan & Villafranca, 1978). To use the data in Figure 8 to calculate a Cr(III) to Mn(II) distance requires a few assumptions. First, the spectrum of Mn(II) must be the enzyme-bound spectrum. The data in Figure 8 are those of bound Mn(II) since addition of higher amounts of Mn(II) produced spectra that resembled those of free Mn(II). Second, to calculate the height of the "diminished" spectrum of Mn(II) due to Mn(II)–Cr(III) interaction, a diamagnetic control must be used. The appropriate control is E–Co(III)–Mn(II)–ADP and the peak heights of the lines in Figure 3E were used to arrive at a value for the Mn(II) spectrum in the absence of paramagnetic effects. Third, since the data for E–Co(III)–Mn(II)–ADP and E–Cr(III)–Mn(II)–ADP were gathered at the same spectrometer power settings, modulation amplitude and filter settings, the only normalization factor used to calculate the relative Mn(II) peak heights for both spectra was the Mn(II) concentration used in each, and the spectrometer gain settings; only the peak-to-peak heights of the six "major" transitions were measured.

The equation for calculating the Mn(II) to Cr(III) distance is

$$C = g\beta\mu^2\tau_c/\hbar r^6 \qquad (2)$$

where the constants g, β, and $\hbar$ have their usual meanings and μ, τ_c, and r are the magnetic moment of Cr(III), electron spin relaxation time of Cr(III), and distance between metal ions, respectively. C is the interaction coefficient for the spin–spin relaxation. Further details can be found in Balakrishnan & Villafranca (1978).

From the line width of E–Cr(III) of ~400 G, a lower limit of $\sim 1.8 \times 10^{-10}$ s was used for τ_c. The decrease in spectral

amplitude of the bound Mn(II) due to Cr(III) is ~66% and the Mn(II) to Cr(III) distance in the E–Cr(III)–Mn(II)–ADP complex is 7 ± 2 Å. This is very similar to the distance found for complexes in which Mn(II) is unambigously in the n_1 site and Cr(III)–nucleotides were bound at the n_2 site (Balakrishnan & Villafranca, 1978) and lead us to the conclusion that the complexes studied in this work have Cr(III) or Co(III) at the n_1 site.

Due to the small amount of E–Cr(III) available, studies with other substrates or inhibitors could not be performed.

Discussion

Studies of EPR spectra of enzyme-bound Mn(II) in the solution state have yielded unique information about changes in the coordination environment of the metal ion in response to substrate or inhibitor binding. Such studies have been reported for many enzymes including phosphoglucomutase (Reed & Ray, 1971), pyruvate kinase (Reed & Cohn, 1973), formyltetrahydrofolate synthetase (Buttlaire et al., 1975), and glutamine synthetase (Villafranca et al., 1976a,b).

The previous work from this laboratory reported changes in coordination environment of Mn(II) bound to the n_1 site and strikingly dissimilar spectra were found for complexes with the substrate, L-glutamate, and the transition-state analogue, L-methionine (*SR*)-sulfoximine. In this paper a diamagnetic metal ion derivative of glutamine synthetase in the n_1 site was prepared. Stoichiometric incorporation of Co(III) was accomplished by oxidation of enzyme–Co(II) with H_2O_2. The Co(III)–enzyme binds one Mn(II) and EPR experiments confirmed that the bound Mn(II) was most likely in the n_2 site. The EPR data in Figure 4 demonstrated that, when substoichiometric amounts of Co(III) were present, Mn(II) could still bind to the unoccupied n_1 sites and give the characteristic spectrum obtained previously with methionine sulfoximine (Villafranca et al., 1976b). Thus, the evidence leads to the conclusion that Mn(II) bound to enzyme–Co(III) (1:1 complex) is bound to a site other than n_1.

Since the EPR spectra of Co(III)–enzyme–Mn(II) with ATP, ADP, or ATP analogues are quite different from one another (Figures 3, 5–7), we conclude that the adenine nucleotides are bound to these metal ions and to the enzyme and that the EPR spectra reflect differences in metal ion coordination with these various nucleotides. Preliminary NMR data of the water relaxation rates with the above complexes (Villafranca, unpublished data) also support the idea that Mn(II) is bound to the n_2 metal–nucleotide substrate site in Co(III)–enzyme–Mn(II) complexes. From these data we conclude that we have prepared a useful derivative of glutamine synthetase permitting us to use the paramagnetic properties of Mn(II) to explore the n_1 and n_2 sites separately for substrate and inhibitor mapping studies.

In this paper we have also reported the preparation of a Cr(III)–enzyme derivative with Cr(III) bound stoichiometrically to the n_1 site. While the EPR properties of this derivative are limited in their applications, we have obtained initial data on the metal–metal distances between the n_1 and n_2 sites that confirm our previously published distance data (Balakrishnan & Villafranca, 1978). The Cr(III)–enzyme has also been used to obtain distances between the Cr(III) and the protons of L-glutamate by NMR methods and these data will be published later. Also since Cr(III) complexes are "substitution-inert", i.e., the ligands to the metal ion exchange very slowly, we have enzymatically hydrolyzed the Cr(III)–enzyme to obtain the peptide that has Cr(III) bound to it (Villafranca, Sinclair, & Vickroy, unpublished data). This application of substitution-inert derivatives of the n_1 metal ion site will lead to chemical determination of the amino acid ligands bound to the metal ion.

In conclusion, Co(III) and Cr(III) derivatives of the n_1 metal ion site of glutamine synthetase have been prepared and characterized. The usefulness of these derivatives lies in the diamagnetic (Co(III)) and paramagnetic (Cr(III)) properties of each metal ion. These metal ion derivatives are currently being used for further EPR and NMR studies of the catalytic and allosteric sites of *E. coli* glutamine synthetase.

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