DETERMINATION OF METAL-METAL DISTANCES IN *E. coli* GLUTAMINE SYNTHETASE BY EPR*

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SUMMARY: This paper reports the first determination of the distance between the two metal ions (per subunit) of *E. coli* glutamine synthetase. When Mn(II) is bound at the n_1 metal ion site its EPR spectrum is diminished in intensity but not broadened as Cr(III)-ATP or Cr(III)-ADP is bound to the enzyme. A paramagnetic spin-spin interaction is responsible for this phenomenon and a metal-metal distance of $\sim$7 Å is calculated for enzyme - Mn(II) - Cr(III)-ATP and $\sim$6 Å for enzyme - Mn(II) - Cr(III)-ADP. The metal-metal distance changes slightly when substrates or inhibitors are also bound to the enzyme demonstrating induced conformational changes in the protein at the metal ion sites.

Glutamine synthetase isolated from *Escherichia coli* has been shown to bind two divalent cations per subunit. (1-4). Both metal ions are required for the expression of catalytic activity. (5). The unadenylylated enzyme binds Mn(II) at these two sites with affinities that differ by two orders of magnitude (1,5). Recently, Hunt et al. (5) demonstrated that Mn(II) and ADP can bind to the enzyme sequentially or as the Mn-ADP complex thus establishing that one of the metal ion sites is involved in binding the nucleotide substrate. The other metal ion site was implicated as being near the glutamate binding site by electron paramagnetic resonance (EPR) studies (6,7).

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This paper presents the first distance determinations between the two metal ion sites. In these experiments, the interaction between the two enzyme-bound paramagnetic metal ions was monitored by EPR and the results were used for calculations of metal-metal distances employing the Leigh theory (8). The procedure involved binding of Mn(II) at one site and Cr(III)-ATP or Cr(III)-ADP at the metal-nucleotide site. These studies represent the first estimates of metal-metal distances on an enzyme using this technique.

EXPERIMENTAL

Materials and Methods

Glutamine synthetase was isolated in a state of low adenylylation ($E_{\overline{3.0}}$) from E. coli as described by Woolfolk et al. (9). All buffers, substrates and inhibitors were obtained from Sigma. Chromium(III)-ATP and Cr(III)-ADP were prepared and characterized by the method of DePamphilis and Cleland (10).

EPR Theory for Spin-Spin Relaxation

Cohn et al. (11) and Taylor et al. (12) demonstrated the use of EPR to measure the distance between a paramagnetic nitroxide spin label and Mn(II) on creatine kinase. The data analysis used Leigh's theory (8) of paramagnetic electron-electron relaxation effects. Briefly, this theory considers how the rigid lattice line shape of an EPR signal is influenced by a dipolar interaction of a second rigid spin. When the dipolar interaction is dominated by a correlation time, τ, that is short with respect to the rotation of the macromolecule (the rigid lattice into which the two spins are "imbedded"), the result is not an observed broadening of the epr signal, but instead only an "apparent" diminution in spectral amplitude. The epr line width of the observed spin is given by

$$\delta H = C(1-3\cos^2\theta_R')^2 + \delta H_o \qquad (1)$$

where δH_o is the line width in the absence of the second paramagnetic

spin, θ_R' is the angle between the applied magnetic field and the vector joining the two spins. The interaction coefficient C is given by

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

where r is the distance between the two spins, μ is the magnetic moment of the interacting paramagnetic ion ($\mu^2 = g^2\beta^2 S(S+1)$), and $\hbar$ is Planck's constant divided by 2π. The apparent loss in spin arises from the angular dependence of the first term in eq. 1. When two interacting spins are held in a fixed orientation on a macromolecule with the macromolecules randomly oriented with respect to the magnetic field, the result is a large dipolar contribution for most orientations (eq. 1) resulting in lines too broad to be observed ($\sim$2000 gauss). However, a small fraction of spins will be at $\theta_R' \simeq 54°$ for which $(3\cos^2\theta_R' - 1) = 0$ and these spins will have $\delta H \simeq \delta H_o$ resulting in little or no line broadening for this spin orientation. The spectral lines will then appear diminished in height but not broadened. It must be noted that this theory represents only one extreme in the very complicated interactions that can occur when two paramagnetic spins interact (see Abragam and Bleaney (13) and Schepler et al. (14)).

Results

When Mn(II) is bound to the tight metal ion site of glutamine synthetase, the EPR spectrum is isotropic. As demonstrated in a previous publication (6), the addition of L-methionine sulfoximine narrows the linewidths of the individual transitions of the enzyme-bound Mn(II). The resulting spectrum is unambiguously characteristic of the ternary enzyme-Mn(II)-sulfoximine complex and any additional spectral changes will be due to other interaction mechanisms. Titration of enzyme-Mn(II)-sulfoximine by Cr(III)-ATP drastically affects the amplitude of the signal without altering the linewidth (Fig. 1). This phenomenon was observed at both 9 and 35 GHz as seen in Fig. 1 and is precisely the

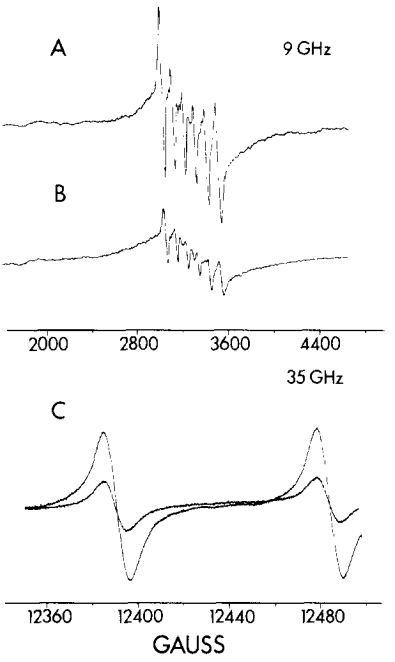

Figure 1: Effects of Cr(III)-ATP binding on the ERP spectrum of ternary Glutamine synthetase - Mn(II) - L-methionine-SR-sulfoximine complex. Spectrum A, Glutamine synthetase = 0.3 mM, Mn = 0.25 mM, Sulfoximine = 6 mM; Spectrum B, A plus 1.3 mM Cr(III)-ATP; 9.13 GHz, Temp = ±1°C, Modulation Amplitude = 8 Gauss, Power = 180 mW, Gain = 2.5×10^3. Spectrum C, Glutamine synthetase = 0.3 mM, Mn = 0.25 mM, Sulfoximine = 6 mM; 35.17 GHz, Temp = 25 ±1°C, Modulation Amplitude = 8 Gauss, Power = 7 dB, Gain = 6.3×10^2. The two lowest field transitions of the sextet are shown for clarity. The less intense spectrum is the result of the addition of 0.9 mM Cr(III)-ATP to the Glutamine synthetase - Mn(II) - methionine sulfoximine solution.

effect predicted by the theory of Leigh (8) for a paramagnetic spin-spin interaction. At saturating levels of Cr(III)-ATP, the amplitude of an individual transition decreases ~85%. A dipolar interaction coefficient (C) of 40 was evaluated according to Leigh (8) with S=3/2, g=2.0 and $\tau = 1.75 \times 10^{-10}$ sec for Cr(III). Using equation 2, a metal-metal distance of 6.8 Å was calculated (Table I).

Binding constants for Cr(III)-ATP to enzyme-Mn(II) and enzyme-Mn(II)-sulfoximine complexes were computed from titration data. The dissociation

TABLE I

Distances between Mn(II) and Cr(III)-nucleotides for various complexes of glutamine synthetase

Enzyme Complex[a]	C[b]	Distance, Å
Mn(II) - Cr(III)-ATP	30	7.1[c]
Mn(II) - Cr(III)-ATP - Glu	200	5.2
Mn(II) - Cr(III)-ATP - Glu - NH_4^+	90	5.9
Mn(II) - Cr(III)-ATP - γ-glutamyl hydroxamate	90	5.9
Mn(II) - Cr(III)-ATP - methionine sulfoximine	40	6.8
Mn(II) - Cr(III)-ADP	90	5.9
Mn(II) - Cr(III)-ADP - P_i	200	5.2
Mn(II) - Cr(III)-ADP - methionine sulfoximine	100	5.8
Mn(II) - Cr(III)-ADP - methionine sulfoximine - P_i	20	7.6

[a] In all experiments 0.3mM enzyme -Mn(II) was present. For each experiment titration with Cr(III)-nucleotide was performed until the diminution of the Mn(II) signal ceased. At this point the % decrease was calculated. The following saturating concentrations of the other components of each solution were present: glu, 50mM; NH_4^+, 50mM; γ-glutamyl hydroxamate, 35mM; methionine sulfoximine, 5.3mM; P_i, 50mM.

[b] Evaluated from a graph similar to that in Fig 3 of Leigh (8).

[c] Each distance is ± 0.3 Å when errors in the evaluation of C and τ are both considered.

constants were 0.20 and 0.30 mM respectively. These values are in good agreement with the binding constants for ATP (0.26 mM) with the Mg(II)-enzyme obtained by Timmons et al. (15) from fluorometric titrations. With Cr(III)-ADP as titrant, the amplitude of the enzyme-Mn(II) EPR signal was also diminished and the calculated K_D value was ∿0.015 mM.

EPR titrations of enzyme-Mn(II) with Cr(III)-ATP were also performed

in the presence of the substrate L-glutamate ($\pm NH_4^+$) and γ-glutamyl hydroxamate. Additional titrations with Cr(III)-ADP were performed with P_i or methionine sulfoximine present. The values of the interaction coefficients (C) at saturating levels of Cr(III)-nucleotides and the calculated distances for each complex are listed in Table I.

Discussion

The experiments described in this paper present the first estimation of metal-metal distances in *E. coli* glutamine synthetase. The values presented in Table I fall in the narrow range of 5-7 Å for the Mn(II) to Cr(III) distances. Some interesting trends are observed in these data that are consistent with other previously published experiments with glutamine synthetase. The Mn(II) to Cr(III) distance is shorter in the Cr(III)-ADP complex as compared to the Cr(III)-ATP complex. The K_D values are 0.015 and 0.20 mM respectively for these Cr(III)-nucleotide complexes and agree reasonably well with the binding constants for ATP (0.26 mM) and ADP(0.04 mM) with the Mg(II)-enzyme (15,16). Thus, both the difference in binding constants and the change in distance between the metal ion site are consistent with ADP and ATP stabilizing different conformational states of glutamine synthetase.

The addition of P_i to the Mn(II)-enzyme-Cr(III)-ADP complex results in a further decrease in metal-metal distance from 5.9 to 5.2 Å. Many previous experiments have demonstrated a synergism in ADP - P_i binding (7,16,17). Thus a result of this synergistic nucleotide - P_i interaction appears to be to move the two metal ion sites closer. Interestingly, there is no apparent difference in the metal-metal distance when the glutamate and ammonia sites are occupied by γ-glutamyl hydroxamate but the distance is lengthened by methioine sulfoximine.

The diminution of the individual EPR transitions of Mn(II) could result not only from a spin-spin dipolar interaction but also from a change

in zero field splitting (ZFS). An increase in ZFS resulting in a diminution in the - 1/2 ↔ 1/2 region could arise from a change in the environment of the bound Mn(II). This has been observed in our earlier work for some complexes of glutamine synthetase (7). However this mechanism can be ruled out for the complexes described in Table I since the spectra at 35 GHz would show pronounced shoulders or extra transitions due to an increase in ZFS (cf. Fig. 5 of Ref. 7). The 35 GHz spectrum in Fig. 1 of this paper demonstrates a diminution of intensity with no other observable changes. Thus, the spin-spin interaction seems to be the dominant mechanism producing the change in the spectrum.

Provided, the Cr(III)-nucleotides used in this study bind to the metal-nucleotide site of glutamine synthetase in the same manner as Mg(II) or Mn(II)-nucleotides (as suggested by the similarities in K_D values and kinetic data that exhibit a competitive inhibition pattern (Villafranca and Balakrishnan, unpublished data)), our results demonstrate that the two metal ion sites are in close proximity on the surface of the enzyme. These data are in accord with the previously suggested roles for the two metal ions in catalysis, i.e., one metal ion orienting the γ-carboxyl of glutamate and the other involved in metal-nucleotide binding and activation (4,5).

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