Distance Determinations between the Metal Ion Sites of *Escherichia coli* Glutamine Synthetase by Electron Paramagnetic Resonance Using Cr(III)–Nucleotides as Paramagnetic Substrate Analogues†

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ABSTRACT: The substitution-inert Cr(III)–nucleotides, CrADP and CrATP, were tested as inhibitors of unadenylylated *Escherichia coli* glutamine synthetase. Both compounds were linear competitive inhibitors vs. MgATP in the biosynthetic assay which consists of following the formation of glutamine from glutamate, ATP and ammonia. The K_I values were 9.6 ± 0.6 μM and 25 ± 1 μM for CrATP and CrADP, respectively. The paramagnetic property of the Cr(III)–nucleotides ($S = 3/2$) was used to study the interaction between Mn(II) ($S = 5/2$) bound at the n_1 "tight" metal ion site and Cr(III)–nucleotide bound at the n_2 metal ion site. Addition of a saturating amount of CrATP produces a 60% decrease in the electron paramagnetic resonance spectrum (EPR) of enzyme-bound Mn(II). This electronic spin–spin interaction between Mn(II) and Cr(III) was analyzed at both 9 and 35 GHz using the J. S. Leigh theory ((1970) *J. Chem. Phys. 52,* 2608) of dipolar electronic relaxation. Titration experiments with CrATP were conducted by following the decrease in the EPR spectral amplitude of enzyme–Mn(II) and a K_D value of 0.30 ± 0.04 mM was calculated. A distance of 7.1 Å between Mn and Cr was obtained by analysis of these EPR data using the Leigh theory. The above EPR experiments were also conducted in the presence of various substrates and inhibitors. With glutamate or glutamine present, the K_D and metal–metal distances were 0.28 mM and 5.2 Å and 0.055 mM and 5.9 Å, respectively. Substrates were thus shown to move the n_1 and n_2 metal ion sites closer together. These data correlate well with other data that demonstrate substrate induced conformational changes in the enzyme and synergism in substrate binding. Similar experiments were conducted with CrADP that also show synergistic interaction between substrate sites. The Mn to CrADP distances are also sensitive to which substrates are present. Additional data were gathered with substitution labile Co(II)–nucleotides. Titrations with both Co(II)–ADP and Co(II)–ATP in the presence of the inhibitor methionine sulfoximine produced a diminution of the EPR spectrum of enzyme-bound Mn(II). Thus a novel application of dipolar electron–electron relaxation between enzyme-bound metal ions has led to determination of distances between the n_1 and n_2 metal ion sites of *E. coli* glutamine synthetase. The two metal ions are in close enough proximity to both be involved in substrate binding and catalysis.

Glutamine synthetase from *Escherichia coli* is composed of 12 identical subunits and is known to catalyze several reactions in addition to the biosynthesis of glutamine from glutamate, ammonia, and ATP (Stadtman & Ginsburg, 1974). The enzyme shows an absolute requirement for two divalent cations per subunit for catalysis to occur (Hunt et al., 1975). The unadenylylated enzyme binds Mn(II) at these two sites with affinities that differ by approximately two orders of magnitude in the absence of substrates (Denton & Ginsburg, 1969; Hunt et al., 1975; Villafranca et al., 1976; Shrake et al., 1977). The first ("tight") metal ion site is known to produce conformational changes in the protein but may also be near the catalytic site (Villafranca et al., 1976). Hunt et al. (1975) demonstrated that the second metal ion site is the metal nucleotide site.

Recently, Cleland and co-workers have developed procedures for the synthesis and purification of substitution-inert Cr(III)–nucleotide complexes and have used them as dead-end inhibitors to study the kinetic mechanisms of several kinases (DePamphilis & Cleland, 1973; Janson & Cleland, 1974a,b; Danenberg & Cleland, 1975). Also, the paramagnetism and nonlability of the Cr(III)–nucleotide complexes make them useful for NMR experiments. Such studies have been conducted with pyruvate kinase to estimate the intersubstrate distances between CrATP and substrates (Gupta et al., 1976).

The data presented in this paper represent the beginning of a project designed to explore the metal–nucleotide site of glutamine synthetase by the use of substitution-inert metal–nucleotide analogues. CrATP and CrADP were used in preliminary kinetic studies to test their suitability as analogues of MgATP and MgADP with glutamine synthetase. Also, since there is evidence that implicates both metal ions at the catalytic site of glutamine synthetase (Villafranca et al., 1976) and since both divalent cations are essential for the phosphoryl transfer reaction, an EPR study was conducted to directly determine the distance between the two metal ion sites. This paper presents the novel use of an EPR method to measure metal–metal distances on a protein and establishes that the two metal ion sites on glutamine synthetase are 5–7 Å apart. A preliminary account of this work has appeared (Villafranca et al., 1977). These newly obtained data for the distances between the two metal ion sites provide evidence that the metal ion sites change their spatial relationship with respect to one another in response to the binding of the other substrates (or inhibitors) to the enzyme.

Experimental Section

Materials. Glutamine synthetase was isolated from *Escherichia coli* in a state of low adenylylation ($E_{\overline{1.0}}$ or $E_{\overline{3.0}}$) as described previously (Woolfolk et al., 1966). The subscript refers to the average number of adenylyl groups per 12 sub-

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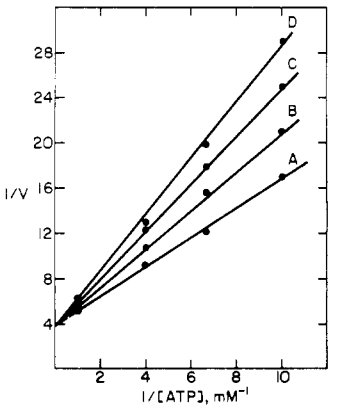

FIGURE 1: Competitive inhibition of initial velocities of CrATP vs. MgATP. Glutamate, 50 mM; NH_4^+, 50 mM. CrATP: (A) 0; (B) 2.9 μM; (C) 5.8 μM; (D) 8.7 μM.

units. All chemicals were products of Sigma. The enzyme activity was determined by the procedure of Hunt et al. (1975), while the Mn(II) content was determined as described in an earlier paper (Villafranca et al., 1976). Protein concentration was measured by the method of Lowry et al. (1951) and by spectral measurements as described by Ginsburg et al. (1970).

Chromium(III)-Nucleotide Complexes. A blue 100 mM hexaaquochromium(III) solution was used as starting material for the preparation of chromium(III)-nucleotide complexes. CrATP was prepared by heating a 100-mL solution containing 10 mM hexaaquochromium(III) and 10 mM Na_2ATP for 12 min at 80 °C and then cooling, according to the method of DePamphilis & Cleland (1973). The formation of CrATP was monitored as a change in color from blue to green upon heating. The solution was then placed on a column (1.5 × 15 cm) of Dowex 50-X2 H^+, 100-200 mesh, eluted by extensive washing with distilled water followed by lithium formate, 0.1 M, pH 3.5 at 4 °C, and further purified according to DePamphilis & Cleland (1973). The CrATP concentration was determined by measuring the UV absorption at 260 nm using a millimolar extinction coefficient of 15.4. By this method the predominant isomer of CrATP produced is the α,β,γ-tridentate complex. CrADP was prepared by heating hexaaquochromium(III) and NaADP in the same fashion as the CrATP followed by isolation according to DePamphilis & Cleland (1973). Total chromium was determined in both CrATP and CrADP after conversion to chromate in 0.5 M NaOH, 1.5% H_2O_2, by heating at 100 °C for 4 min and then cooling in ice water (Postmus & King, 1955). Absorption at 375 nm was linear with chromium concentration (ϵ = 4815) over several orders of magnitude.

The UV spectra of Cr(III)-nucleotides were the same as those reported by DePamphilis & Cleland (1973). The EPR spectrum of CrATP at liquid nitrogen temperature shows an axial appearance with g = 1.97 and g = 4.7.

EPR Measurements. A Varian E-12 spectrometer was used to record spectra at both 9 GHz (X-band) and 35 GHz (K-band). The spectrometer was equipped with an E-257 variable temperature accessory unit. The temperature was maintained within ±1 °C by heating precooled nitrogen gas which was passed through the Dewar assembly placed in E-231 cavity operating at 9 GHz. Temperature was controlled at 35 GHz at 25 ± 1 °C in the same manner as at 9 GHz except that the Dewar assembly surrounded an E-266 cavity. A copper-constantan thermocouple was used to monitor cavity temperature throughout the runs. Aqueous samples of 25-50 μL were placed in quartz capillary tubing of 1.0 mm i.d. and the end of the tubing was closed by using polyethylene tubing and a Teflon plug. Samples run at K band were placed in quartz tubing which had been drawn to 0.1-0.2 mm i.d. A polyethylene plug was used to close the end of the capillary tube after 1-3 μL of solution was drawn into the capillary tube. A single capillary tube was used at either 9 or 35 GHz for a titration experiment. Reproducibility of spectra was checked at several points in an experiment. Amplitude of a single transition could be reproduced to ≤3% at both 9 and 35 GHz. EPR spectra of the Co^{2+}-enzyme were obtained at 9 GHz at 6-7 K using the Heli-tran LTD 3-110 liquid helium transfer system.

Kinetic Assays and Data Analysis. Kinetic assays were run in 1-mL total volume in 1-cm cuvettes by measuring absorbance changes at 340 nm with a Gilford spectrophotometer and a 10 mV recorder at 25 °C. The assay mixture for kinetic studies included (per cuvette) 33 mM imidazole, 33 mM 2-methylimidazole, and 33 mM 2,4-dimethylimidazole buffer, 100 mM KCl, pH 7.15, 16 μg of each of the coupling enzymes, pyruvate kinase and lactic dehydrogenase, 1 mM phosphoenolpyruvate, 0.16 mM NADH, 1.5 μg of glutamine synthetase, 1-5 mM free Mg^{2+} (as $MgCl_2$), 50 mM glutamate, 50 mM NH_4^+, and MgATP as specified. Kinetic data were plotted graphically to determine the pattern and then fitted to the rate equation for competitive inhibition by a least-squares method using the Fortran program of Cleland (1967).

$$V = \frac{VA}{K(1 + I/K_{is}) + A}$$

The points on the graphs are the experimental data and the lines are from the fits to the above equation.

The unadenylylated enzyme cannot be assayed, as described above, in the presence of Mn^{2+} (Stadtman & Ginsburg, 1974).

Results

Kinetic Inhibition Patterns of CrATP and CrADP vs. MgATP. Since the substitution-inert coordination complexes of Cr(III)-nucleotides have been shown to be dead end inhibitors in kinetic studies with various kinases (DePamphilis & Cleland, 1973; Danenberg & Cleland, 1975), the inhibition patterns of CrATP and CrADP vs. MgATP were determined with glutamine synthetase. The biosynthetic assay for glutamine synthetase involves following the formation of ADP from ATP, glutamate, and ammonia using the pyruvate kinase-lactic dehydrogenase coupled assay system. The lack of strong inhibition of pyruvate kinase by CrATP makes this assay feasible. Also CrATP and CrADP are stable complexes under the conditions of this assay, pH 7.15, with the temperature at 25 °C (DePamphilis & Cleland, 1973).

When CrATP was used as an inhibitor vs. MgATP and the reaction started by the addition of glutamine synthetase, the resulting pattern of initial velocities was competitive (Figure 1) with a K_{is} value of 9.6 ± 0.6 μM. Similarly with CrADP as inhibitor, competitive inhibition was also observed with a K_{is} value of 25 ± 1 μM. The weaker inhibition by CrADP compared with CrATP has also been observed with hexokinase (Danenberg & Cleland, 1975). By these kinetic criteria, CrATP and CrADP are good analogues for metal-nucleotides with *E. coli* glutamine synthetase.

Interaction of Cr(III)-Nucleotides with Enzyme-Mn(II). Of the two metal ion binding sites on glutamine synthetase, the "tight" metal ion sites can be selectively populated under conditions where [enzyme] ≥ [Mn(II)], since the binding constants of Mn(II) to the "tight" and "weak" metal ion sites per subunit of unadenylylated glutamine synthetase are 5 × 10^{-7} and 4.5 × 10^{-5} M, respectively (Hunt et al., 1975; Vil-

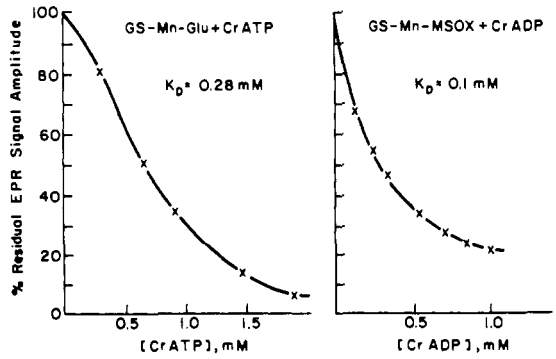

FIGURE 2: Titration of glutamine synthetase bound Mn(II) with CrATP and CrADP. (Left) Saturating level of L-glutamate (50 mM); (right) saturating level of L-methionine (*SR*)-sulfoximine (12 mM). The amplitude of the lower field line of Mn(II) sextet (35 GHz) is plotted vs. CrATP and CrADP concentrations. The solution for both titrations contained Mn(II)-enzyme, 0.3 mM (subunit concentration); pH 7.1 and T = 25 °C.

lafranca et al., 1976). The EPR spectrum of Mn(II) bound only at the "tight" site was shown to be relatively isotropic; i.e., the zero field splitting is small (Villafranca et al., 1976). Addition of CrATP or CrADP produces a decrease in the spectral amplitude of the isotropic sextet signal of bound Mn(II). This decrease in the amplitude of the EPR spectrum is due to the electronic spin–spin interaction between bound Mn(II)- and Cr(III)-nucleotides.

In a control experiment in the absence of enzyme, a 0.2 mM Mn(II) solution was titrated with CrATP (or CrADP) to determine if the two paramagnetic species could interact by dipolar spin–spin relaxation resulting in a decrease in the Mn(II) signal. No decrease in the signal occurred until the concentration of CrATP (or CrADP) exceeded 3 mM. However, the decrease in amplitude was still <5% at 15 mM CrATP. For enzyme-bound Mn(II), a 60% decrease in signal occurred at a molar ratio of CrATP to enzyme-Mn(II) of 5:1 (1.5 mM CrATP, 0.3 mM enzyme-Mn(II)). In the absence of enzyme both paramagnetic species are tumbling randomly in solution and occasionally interact to produce dipolar relaxation. However, when the two paramagnetic species are rigidly bound at a fixed distance on an enzyme surface, they can interact strongly with each other (Leigh, 1970).

At saturating concentrations of CrATP, the amplitude of the enzyme bound Mn(II) signal decreases by ~60%. CrATP can be displaced from the enzyme-Mn(II)-CrATP complex by addition of ATP or ADP (not greater than stoichiometric with enzyme concentration) resulting in an increase in the Mn(II) signal amplitude. This is not due to the release of free Mn(II) since the spectral shape of free Mn(II) is quite different from that of enzyme-Mn(II) and could be detected quite easily. This result indicates that CrATP binds at the nucleotide site of glutamine synthetase, a conclusion also reached from the kinetic experiments which displayed a competitive inhibition pattern.

The diminished amplitude of the enzyme-Mn(II) EPR signal is a function of the concentration of the bound Cr(III)-nucleotides. The binding constants of Cr(III)-nucleotides to the enzyme-Mn(II) could therefore be determined by titrations with CrATP or CrADP.

The following equations were used to fit the titration data with Cr-nucleotides (Cr-Nuc):

$$E_0 + Mn \overset{K_1}{\rightleftharpoons} EMn \quad (1)$$

$$EMn + Cr\text{-}Nue \overset{K_2}{\rightleftharpoons} EMnCr\text{-}Nuc \quad (2)$$

TABLE I: Dissociation Constants from EPR Titration.[a]

	K_d (μM)
CrATP + enzyme-Mn(II)	300
CrATP + enzyme-Mn(II)-glutamate	280
CrATP + enzyme-Mn(II)-glutamate-NH_4^+	61
CrATP + enzyme-Mn(II)-glutamine	55
CrATP + enzyme-Mn(II)-methionine sulfoximine	200
CrADP + enzyme-Mn(II)	15
CrADP + enzyme-Mn(II)-glutamine	145
CrADP + enzyme-Mn(II)-glutamine-P_i	38
CrADP + enzyme-Mn(II)-methionine sulfoximine	100

[a] All data were obtained at pH 7.0, 25 °C, 10 mM imidazole–100 mM KCl buffer.

$$EMn + S \overset{K_3}{\rightleftharpoons} EMnS \quad (3)$$

$$EMnS + Cr\text{-}Nuc \overset{K_4}{\rightleftharpoons} EMnSCr\text{-}Nuc \quad (4)$$

$$I_{obsd} = (I_0[EMn] + I_R[EMnCr\text{-}Nuc])/[E_0] \quad (5)$$

where S is substrate or inhibitor, I_0 is the initial EPR signal, I_R is the signal at saturating Cr-Nuc, and I_{obsd} is the observed signal at any point in the titration. When S was added prior to titration with Cr-Nuc, EMn and EMnCr-Nuc in eq 5 were replaced by EMnS and EMnSCr-Nuc, respectively. In the initial fit to the data, K_2 or K_4 was calculated at each data point, while K_1 or K_3 was the literature value (Rhee & Chock, 1976; Stadtman & Ginsburg, 1974; Villafranca et al., 1976; Timmons et al., 1974). The average K_2 or K_4 value was then used to generate a titration curve and the deviation between the data points and the theoretical curve was minimized by successive iterations. Titration data for CrATP and CrADP were also obtained in the presence of glutamate and methionine sulfoximine and are presented in Figure 2. The solid lines in Figure 2 are theoretical curves drawn for a particular binding constant.

The values of the dissociation constants of Cr(III)-nucleotides from the corresponding ternary or quaternary enzyme complexes are listed in Table I. The dissociation constants were 0.20 ± 0.03 and 0.30 ± 0.04 mM for CrATP in the presence and absence of methionine sulfoximine, and 0.28 ± 0.03 mM in the presence of glutamate. The binding constant obtained by Timmons et al. (1974) from fluorometric titrations for ATP to the Mg(II) enzyme is 0.26 mM. With CrADP as titrant, the calculated K_D values were 0.015 ± 0.006 mM and 0.10 ± 0.02 mM in the absence and presence of methionine sulfoximine.

Calculation of Distances between Mn(II) and Cr(III). Since CrATP (or CrADP) produces a dramatic decrease in the spectral amplitude without any observable line broadening of the sextet, the final relative amplitude of the Mn(II) spectrum in the presence of a saturating level of Cr(III)-nucleotides could be used to calculate the distance between the two paramagnetic centers on glutamine synthetase. The detailed theoretical treatment of this phenomenon has been dealt with by Leigh (1970) and a preliminary report has appeared elsewhere (Villafranca et al., 1977). Briefly, Leigh's theory of the dipolar interaction between two dissimilar electron spins bound to the same macromolecule gives the following equation for the line width ($2\delta H$) at half maximal amplitude of the observed EPR signal. For the case of Mn(II) the lowest field line was used to compute the values of C and K_D but these values did not change by more than 5% if the sum of all the lines was used.

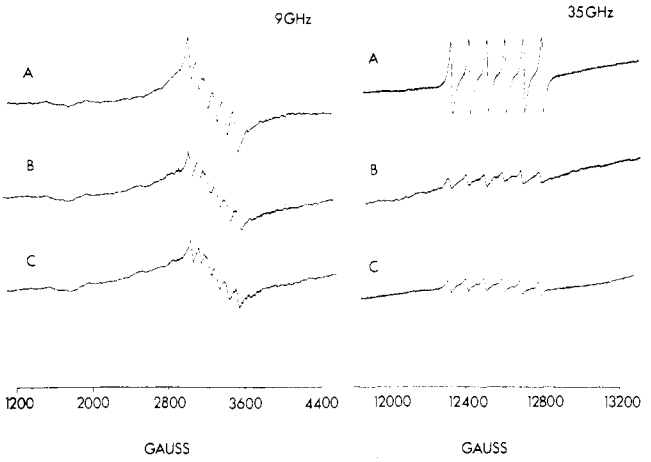

FIGURE 3: Comparison of the effect of CrATP on the EPR spectra of Mn(II)-glutamine synthetase in the presence and absence of glutamate and NH_4^+, at 9 and 35 GHz. The initial and final spectra (at saturating levels of CrATP) are shown. (A) Enzyme-Mn(II)-glutamate; (B) enzyme-Mn(II)-glutamate-CrATP; (C) enzyme-Mn(II)-glutamate-NH_4^+-CrATP pH 7.1 and T = 25 °C. The enzyme-Mn(II) subunit concentration is 0.3 mM.

$$\delta H = C(1 - 3\cos^2\theta_R')^2 + \delta H_0$$
$$C = g\beta\mu^2\tau_c/\hbar r^6 \qquad (6)$$

where the constants g, β, and $\hbar$ have their usual meanings, μ and τ_c are the magnetic moment and the electron spin relaxation time of the paramagnetic ion, Cr(III) in this case. r is the distance between Mn(II) and Cr(III). θ_R' is the angle between a position vector and the magnetic field direction, and δH_0 is the natural line width in the absence of dipolar broadening.

The apparent "loss" of signal amplitude arises from the angular dependence of the interaction of the two spins. For most angular orientations, the dipolar contribution is large compared with the unperturbed line width and the resulting lines are too broad (~2000 G) to be observed. At $\theta_R' \simeq 54°$, the line width is unperturbed and represents the observed "diminished" signal. From the EPR signals of enzyme-Mn(II) in the presence and absence of Cr(III)-nucleotides, the value of C, the interaction coefficient and thus the distance, r, between the two paramagnetic ions can be calculated.

Interaction of CrATP with Enzyme-Mn(II) in the Absence and Presence of Substrates and Inhibitors. It is possible to determine changes in the distance between the two metal ion sites in the presence of substrates (glutamate and NH_4^+) or inhibitor (methionine sulfoximine) by using Cr(III)-nucleotides and the method outlined above for measuring metal-metal distances. Titrations of enzyme-bound Mn(II) were conducted as outlined previously with (1) enzyme-Mn(II), (2) enzyme-Mn(II)-glutamate, and (3) enzyme-Mn(II)-glutamate-NH_4^+. Titration data were obtained at both 9 and 35 GHz. The initial and final spectra (at saturating levels of Cr-nucleotides) are presented in Figure 3. The individual transitions of bound Mn(II) at both 9 and 35 GHz remain isotropic but decreased in amplitude after additions of saturating concentrations of CrATP. This indicates that only the dipolar spin-spin interaction causes the decrease in amplitude of the EPR signal and not a drastic change in the zero-field splitting of Mn(II) (cf. Villafranca et al. (1976) for EPR spectra of glutamine synthetase with altered zero-field splitting). The percentage of decrease in amplitude of EPR signal varied considerably in each case depending on which substrate(s) were present. The percentage decrease in amplitude was greater in the presence of glutamate than with the enzyme-Mn(II) or enzyme-Mn(II)-glutamate-NH_4^+ complex.

TABLE II: Distances between Mn(II) and Cr(III)- or Co(II)-Nucleotides for Various Complexes of Glutamine Synthetase.

enzyme complex[a]	C[b]	distance (Å)
Mn(II)-CrATP	30	7.1[c]
Mn(II)-CrATP-glutamate	200	5.2
Mn(II)-CrATP-glutamate-NH_4^+	90	5.9
Mn(II)-CrATP-glutamine	90	5.9
Mn(II)-CrATP-γ-glutamyl hydroxamate	90	5.9
Mn(II)-CrATP-methionine (*SR*)-sulfoximine	40	6.8
Mn(II)-CrADP	90	5.9
Mn(II)-CrADP-P_i	200	5.2
Mn(II)-CrADP-glutamine	70	6.2
Mn(II)-CrADP-glutamine-P_i	350	4.8
Mn(II)-CrADP-methionine (*SR*)-sulfoximine	100	5.8
Mn(II)-CrADP-methionine (*SR*)-sulfoximine-P_i	20	7.6
Mn(II)-Co(II)-methionine (*SR*)-sulfoximine	12	7.3[d]
Mn(II)-Co(II)-ADP-methionine (*SR*)-sulfoximine	25	6.5[d]
Mn(II)-Co(II)-ADP-methionine (*SR*)-sulfoximine-P_i	140	4.9[d]
Mn(II)-Co(II)-ATP-methionine (*SR*)-sulfoximine	90	5.2[d]

[a] In all experiments 0.3 mM enzyme-Mn(II) was present. For each experiment, titration with metal-nucleotide was carried out 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: glutamate, 50 mM; NH_4^+, 50 mM; γ-glutamyl hydroxamate, 35 mM; methionine sulfoximine, 5.3 mM; P_i, 50 mM; glutamine, 15 mM. [b] Evaluated from a graph similar to that in Figure 3 of Leigh (1970). [c] Each distance is ±0.5 Å when errors in the evaluation of C and τ_c are both considered (see Results). C is from eq 6. [d] An effective "g" value of 4 for Co^{2+} was used (see Results).

Also it is interesting to note that the percentage decrease in amplitude for the quinary complex, enzyme-Mn(II)-glutamate-NH_4^+-CrATP falls between those of the ternary enzyme-Mn(II)-CrATP and quaternary enzyme-Mn(II)-glutamate-Cr(III)-ATP complexes. The dipolar interaction coefficient, C, and the distance, r, were obtained as outlined before using $S = 3/2$, $g = 2.0$, and $\tau_c = 1.75 \times 10^{-10}$ s for Cr(III). The value of τ_c is from the line width of the enzyme-Mg(II)-CrATP (or CrADP) complex (375 G) determined at 9 or 35 GHz. It is assumed that $T_{1e} \simeq T_{2e}$ for Cr(III)-nucleotides as was found by Gupta et al. (1976). The C and r values are listed in Table II.

The value of r depends on the ratio of τ_c/C. Since C changes from 30 to 200 in the first two complexes of Table II, τ_c would have to change from 1.75×10^{-10} to 1.17×10^{-9} s for r to remain at 7.1 Å for both complexes. This would correspond to a decrease in line width of CrATP to ~56 G and should be easily observable by EPR. Since this change is not observed, we can set an upper limit to the change in τ_c of a factor of two. This corresponds to a change in r of ±0.5 Å. The other errors arise in reproducibility of spectral measurements (see Methods) and evaluation of C. The combined error in these values is ≤10%. Thus, the largest error in determining r is in assuming that τ_c is similar for each complex. The changes in r for the various complexes in Table II are quite large in many instances and lie outside the generous error range of ±0.5 Å. Therefore the changes in metal-metal distances are significant for many of the complexes studied in Table II.

EPR titrations were also conducted in the presence of L-glutamine and L-γ-glutamyl hydroxamate. It is interesting to note that the final extent of change in the relative amplitude

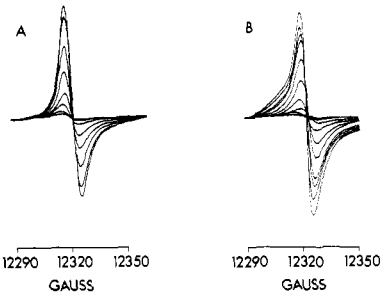

FIGURE 4: EPR titration at 35 GHz of the lowest field line of the glutamine synthetase bound Mn(II) at constant level of L-methionine (*SR*)-sulfoximine. (A) CrATP as titrant, (B) Co(II)-ATP as titrant, pH 7.1 and $T = 25$ °C.

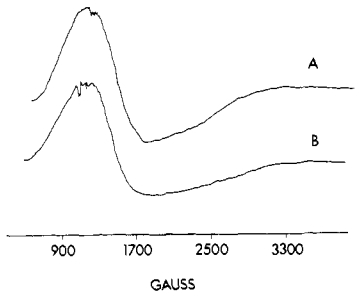

FIGURE 5: EPR spectra of cobalt(II)-glutamine synthetase at 12 K. The concentration of enzyme is 1.0 mM (subunit concentration). (A) Co(II)-enzyme; [Co(II)] = 0.9 mM. (B) Co(II)-enzyme-ATP; [Co(II)] = 1.8 mM; [ATP] = 2 mM. Instrument settings for the two spectra were as follows: frequency = 9.18 GHz; microwave power = 10 mW; time constant = 0.3 s; receiver gain = 1.6×10^3; and modulation amplitude = 20 G.

and thus the value of the dipolar interaction coefficient were nearly the same as for the enzyme-Mn(II)-CrATP complexes with L-glutamine, L-γ-glutamyl hydroxamate, or L-glutamate plus NH_4^+ present.

In the presence of saturating concentrations of L-methionine (*SR*)-sulfoximine, a transition state analogue of the enzymic reaction, an earlier report from this laboratory demonstrated that the line widths of the individual transitions of Mn(II) bound to the enzyme are narrowed (Villafranca et al., 1976). The resulting spectrum is unambiguously characteristic of the ternary enzyme-Mn(II)-methionine sulfoximine complex. The narrow lines of the individual transitions make observation of the decrease in EPR amplitude by the addition of CrATP easier. In addition, changes in zero-field splitting would be more apparent. Titration of this complex with CrATP drastically reduces the spectral amplitude (Figure 4) without line broadening and the spectrum remains isotropic. Interestingly, a similar addition of MgATP instead of CrATP to the ternary enzyme-Mn(II)-methionine sulfoximine produces an anisotropic spectrum with D and $E \neq 0$ (Villafranca et al., 1976). This anisotropy produced with MgATP was not observed in the quaternary enzyme-Mn(II)-methionine sulfoximine-CrATP complex. Thus the interpretation that the spin-spin interaction between Mn(II) and Cr(III) produces the effects seen in this paper seems justified. Values of C and r for various complexes with methionine sulfoximine are listed in Table II.

Interaction of CrADP with Various Enzyme-Mn(II) Complexes. Similar to titrations with CrATP, enzyme-Mn(II) was titrated with CrADP in the absence and presence of methionine sulfoximine with and without P_i. From the titration pattern, it is evident that CrADP produces a considerably larger decrease in amplitude of the EPR signal compared with that produced by CrATP. Also, the binding constants obtained for CrADP are tighter, while the percentage of decrease in the EPR signal amplitude is ~85% in the quaternary enzyme-Mn(II)-CrADP-P_i complex. By contrast, MgADP produces an *increase* in the signal amplitude (Villafranca et al., 1976), a further indication that all of the phenomena described in this paper are due to spin-spin interaction between paramagnetic Cr(III) and Mn(II). The other ternary enzyme-Mn(II)-CrADP, quaternary enzyme-Mn(II)-methionine sulfoximine-CrADP and enzyme-Mn(II)-glutamine-CrADP complexes have intermediate relative amplitude values. The C and r values are listed in Table II.

Interaction of Co(II) and Co(II)-Nucleotides with Enzyme-Mn(II)-Methionine Sulfoximine Complex. Similar to the paramagnetic Cr(III)-nucleotides, the dipolar spin-spin interaction was also observed between Mn(II) at the "tight" site and Co(II)-nucleotide bound to the metal-nucleotide site of glutamine synthetase. Addition of Co(II) to enzyme-Mn(II) displaces the bound Mn(II) as evidenced by a large increase in Mn(II) signal amplitude characteristic of free Mn(II). Hunt & Ginsburg (1972) also showed that Co(II) and Mn(II) can compete for the "tight" metal ion site. In order to avoid the displacement of Mn(II) from the tight sites by Co(II), the EPR titrations with Co(II) and Co(II)-nucleotides were performed in the presence of methionine sulfoximine. Under these conditions Mn(II) is not displaced from the n_1 sites (Villafranca et al., 1976).

Titration of enzyme-Mn(II)-methionine sulfoximine with Co(II) alone results in a decrease in the EPR spectral amplitude (~50% maximum) without any observable line broadening. Also, since the titrations were performed at 25 °C, the EPR signal of Co(II) alone would not interfere. The decrease in EPR spectral amplitude in the presence of ADP (i.e., as Co(II)-ADP) was approximately 60%. This was further reduced in the presence of a saturating concentration of P_i. The relative amplitudes were calculated and the C and r values are listed in Table II. The addition of Co(II)-ATP to the ternary enzyme-Mn(II)-methionine sulfoximine produces a decrease of ~70% (Figure 4). The significance of these data will be discussed later.

To be able to use eq 1 for the calculation of the distance between Mn(II) and the paramagnetic probe Co(II), one must know the effective "g" value of Co(II) bound to the metal nucleotide sites of glutamine synthetase. The effective "g" value could be obtained from the EPR spectra of enzyme-Co(II) and of enzyme-Co(II)-Co(II)ATP recorded at 6-7 K (Figure 5). The EPR spectra reveal the presence of an anisotropic **g** tensor. A broad signal appears distinctly in the region of $g \simeq 6$-7. The other g values which cannot be accurately determined due to the broadness of the lines are between 4 and 2. This is typical of high spin ($S = 3/2$) Co(II) (McGarvey, 1966). Splittings are observed in the $g = 6$-7 region of the spectra. Similar observations were made in the Co(II) complexes of pyruvate kinase (Melamud & Mildvan, 1975) and were attributed to a distorted octahedral coordination of Co(II). From these values, only an estimate of the effective g value can be made. A value of $\langle g \rangle_{av}$ of 4 was chosen for calculations. Using this value and the C values from titrations, distances between Mn(II) and Co(II) were obtained (Table II).

An interesting observation in the data in Figure 5 is that the areas of the broad peak at $g \simeq 6$-7 are nearly identical. The difference in the enzyme solutions used to obtain these spectra is in the concentration of Co(II) present, i.e., 0.9 mM for the top spectrum and 1.8 mM for the bottom spectrum. An observation of this type is expected based on the previously reported data in this paper concerning metal-metal dipolar re-

TABLE III: Binding Constants for Various Substrates to Unadenylylated Glutamine Synthetase ($E_{1.0}$).

	K_{diss} (μM)	ref
ADP-Mn(II) + enzyme	2.86	*b*
ADP-Mn(II) + enzyme[a]	0.035	*b*
ADP + enzyme-Mn(II)	4.6 ± 1.5	*b*
ADP + enzyme-Mn(II)-glutamine	2.4 ± 1.0	*c*
ADP + enzyme-Mn(II)-P_i	0.05 ± 0.03	*b,d*
P_i + enzyme-Mn(II)	1400 ± 300	*c*
P_i + enzyme-Mn(II)-ADP	30 ± 10	*d*
ADP + enzyme-Mg(II)	40 ± 5	*d*
P_i + enzyme-Mg(II)	5000 ± 500	*c*
ADP + enzyme-Mg(II)-P_i	30 ± 10	*d*
ATP + enzyme-Mg(II)	260	*e*
ATP + enzyme-Mg(II)-glutamate	40	*e*

[a] Under assay conditions, with arsenate and glutamine. [b] Hunt et al., 1975. [c] Rhee & Chock, 1976. [d] Rhee et al., 1976. [e] Timmons et al., 1974.

laxation between bound metal ions on glutamine synthetase. However, evaluation of the Co(II) EPR data using the Leigh theory to determine a distance between the two bound interacting Co(II) ions is not theoretically sound (see Discussion). It is important though that this observation of "diminished" spectral density in the EPR spectrum when two Co(II) ions are bound to the enzyme is similar to that observed for the other metal ion pairs tested, viz., Mn(II)-Cr(III) and Mn(II)-Co(II).

Discussion

The results from two experimental approaches in this paper provide strong evidence that Cr(III)-nucleotides bind at the metal-nucleotide substrate site of *E. coli* glutamine synthetase. Thus, kinetic experiments clearly demonstrate competitive inhibition of CrADP and CrATP vs. MgATP, while EPR experiments show that ADP displaces CrATP in an enzyme-Mn(II)-CrATP complex. The method of preparation of CrATP used by us gives predominantly the tridentate α,β,γ-coordinated complex. Since there may be 10-15% of other isomers of CrATP, we have made the presumption that the predominant α,β,γ complex is the isomer that binds tightly to glutamine synthetase. Also α,β,γ-coordinated CrATP and α,β-coordinated CrADP are mixtures of diastereoisomers and it is not yet known whether only one or more isomers bind to this system.

Nonetheless, the strong interaction observed by EPR between enzyme-Mn(II) and CrATP or CrADP permits calculation of an upper limit to the binding constants of these analogues to glutamine synthetase. Since the value of C calculated from the dipolar relaxation effect is independent of K_D (at saturating concentration of CrATP or CrADP), the value of r is not affected by the above considerations. Also, since metal ions can bind to the n_2 metal-nucleotide site in the absence of nucleotides as shown by Hunt et al. (1975), and since it is unlikely that the Cr(III) moiety of Cr(III)-nucleotides binds directly to the enzyme (due to slow ligand exchange of the water molecules bound to Cr(III)), the binding specificity of CrADP and CrATP to glutamine synthetase is most likely through the adenosine group. Thus, the metal-metal distances determined by EPR in this paper may be 1-2 Å displaced from the "true" metal ion site of the metal-nucleotide active site of glutamine synthetase. This estimate is based on model-building studies assuming the triphosphate moiety of CrATP binds in the same "region" as that of MgATP.

As shown in this paper, the decrease in the EPR signal of Mn(II) due to the binding of Cr(III)-nucleotides can be used to obtain the binding constants of Cr(III)-nucleotides to the enzyme. A compilation is given in Table III of a number of binding constants of ATP and ADP to glutamine synthetase with Mg(II) or Mn(II). The K_D values for CrATP from the enzyme-Mn(II)-CrATP (0.30 mM), enzyme-Mn(II)-CrATP-glutamate (0.28 mM) and enzyme-Mn(II)-CrATP-methionine sulfoximine (0.20 mM) complexes are in good agreement with the binding constants for ATP (Table III) with Mg(II) enzyme obtained by Timmons et al. (1974) from fluorometric titrations. Thus in the presence of glutamate and methionine sulfoximine, the binding of CrATP is slightly tighter. Timmons et al. (1974) found that the binding constant of ATP to enzyme-Mg(II)-glutamate was 40 μM and the K_I value (determined under kinetic conditions) in the present paper is 9.6 μM.

The dissociation constant of CrADP from the ternary enzyme-Mn(II)-CrADP is 0.015 mM and is in the same range of binding constants for ADP (0.040 mM) to enzyme-Mg(II) and for ADP (0.0046 mM) to enzyme-Mn(II) (Rhee & Chock, 1976) (Table III).

The apparent discrepancy between the K_I value of 9.6 μM given above obtained with enzyme-Mg(II) and the K_D value obtained by EPR titrations of 61 μM for the most closely analogous complex, i.e., enzyme-Mn(II)-glutamate-NH_3-CrATP, may be due to slightly different conformations of the enzyme at the n_2 site induced by binding Mn(II) at n_1 and CrATP at n_2. However, the K_D for CrADP (38 μM) from the enzyme-Mn(II)-glutamine-CrADP-P_i complex is very close to the K_I value (25 μM) determined kinetically. Very few studies have been conducted with one metal ion at n_1 and another at n_2 and the exact manner in which the binding of Cr(III)-nucleotides is affected by changes induced at n_1 by binding different metal ions is one of the future objectives of this work. In a notable experiment Hunt & Ginsburg (1972) demonstrated that, when glutamine synthetase is saturated with either Mn(II) or Mg(II) at n_1, further addition of Mn(II) results in preferential binding of this extra Mn(II) to n_2 rather than the extra Mn(II) displacing Mg(II) from n_1. This is due to a kinetic phenomenon of metal ion binding at n_2 and may not reflect the true binding constants of additional metal ions at n_2. In the present paper, a stable metal-nucleotide complex is bound at the n_2 site and previous discussion suggests that the primary mode of binding of Cr(III)-nucleotides is through the adenosine moiety. This latter consideration may be the reason that ADP alone will displace CrATP from the nucleotide site.

The metal-metal distances between Mn(II) bound at the "tight" site and Cr(III) at the metal-nucleotide site on the enzyme presented in Table II fall in the narrow range of 5-7 Å. Certain trends are observed in these data that can be placed in context with other previously published experiments with glutamine synthetase. The distance of 7.1 Å between Mn(II) in the "tight" site and the Cr of CrATP is lowered to 5.2 Å in the presence of glutamate. Addition of the other substrate NH_4^+ changes this distance to 5.9 Å. Both the experiments reported herein and the fluorescence experiments with enzyme-Mg(II)-ATP-glutamate (Timmons et al., 1974) demonstrate enzyme conformational changes produced when metal-nucleotides bind. The conformational changes produced upon binding nucleotides precede the first chemical event, i.e., formation of a γ-glutamyl phosphate intermediate as shown by ^{18}O experiments of Midelfort & Rose (1976). Such an intermediate could also be formed in the quaternary enzyme-Mn(II)-glutamate-CrATP complex, resulting in an enzyme-Mn(II)-γ-glutamyl-P-CrADP complex. Preliminary

experiments suggest this (Balakrishnan & Villafranca, unpublished results), but the rate of this reaction is many orders of magnitude slower than the biosynthetic reaction. CrATP has been shown to be a substrate for enzymatic reactions (Danenberg & Cleland, 1975).

Data in this paper indicate that the Mn(II)-Cr(III) distance changes as a result of glutamate binding. When NH_4^+ binds, another conformational change may result that produces the observed increase in the distance between the metal ion centers. By comparison, Timmons et al. (1974) showed that there was a slow decrease in fluorescence resulting from addition of NH_4^+ to the enzyme-glutamate-ATP complex. The second chemical step in the overall mechanism most likely involves ammonia adding to the γ-glutamyl-P. At present the above result showing an increase in distance between metal ion centers may also parallel the conformation change observed by Timmons et al. upon addition of NH_4^+.

In the quaternary enzyme-Mn(II)-methionine (*SR*)-sulfoximine-CrATP complex, the Mn(II) to Cr(III) distance (6.8 Å) is similar to the distance in the absence of this analogue. However, this metal-metal distance is greater than that in the presence of L-glutamate (5.2 Å), L-glutamine (5.9 Å), or L-γ-glutamyl hydroxamate (5.9 Å). At first glance this would seem to be an anomaly since the sulfoximine is a transition-state analogue for the enzymatic reaction. But these data might simply reflect the fact that the same enzyme conformer that is stabilized by sulfoximine and ATP to produce sulfoximine phosphate and inactivate the enzyme is not the conformer produced with CrATP and sulfoximine. Presently studies are underway to see if CrATP and sulfoximine will quantitatively inactivate glutamine synthetase. Preliminary results suggest an inactivation rate much slower than with MgATP (Balakrishnan & Villafranca, unpublished data). For the enzyme-Mn(II)-CrADP-sulfoximine-P_i complex, in which the metal-metal distance is 7.6 Å, electrostatic repulsion between CrADP and P_i might be maximized resulting in the furthest distance observed between the two metal ion centers.

Selected Co(II) complexes of enzyme-Mn(II) were investigated in order to determine the essential differences involved in using a substitution inert paramagnetic dead-end inhibitor and using a divalent, catalytically active paramagnetic ion as probes. Co(II) alone when added to the ternary enzyme-Mn(II)-methionine sulfoximine gave a value of 7.3 Å for the distance between Mn(II) and Co(II). The presence of ADP produces a decrease of 0.8 Å in the distance bringing the two metal ions to within 6.5 Å. In the presence of Co(II)-ADP-P_i, the synergistic interaction between ADP and P_i also observed from fluorometric titrations (Rhee & Chock, 1976)) apparently brings the two metal ions much closer (4.9 Å). This is in contrast to that observed with CrADP-P_i where the distance increases. Also the metal-metal distance in the quaternary enzyme-Mn(II)-Co(II)-ATP-methionine sulfoximine complex was 5.2 Å, again shorter than the distance observed with CrATP. These results demonstrate that in the catalytically competent complexes formed with CrATP the metal ion centers are closer as the result of protein conformational changes. These changes may be produced prior to or during the various bond-making and -breaking steps.

A comment should be made about the use of the Leigh's theory to calculate distances between unlike spins on an enzyme surface. Originally Leigh applied this to the problem of Mn(II) interacting with the unpaired spin of a covalently bound nitroxyl spin label on creatine kinase. Recently this approach has also been used to estimate a distance between a nitroxyl spin-labeled compound and Fe(III) on transferrin (Najarian et al., 1978) and between a nitroxyl spin-labeled hapten and Gd(III) on an antibody Fv fragment (Dwek et al., 1975). We have for the first time applied the theory to two paramagnetically interacting metal ions on a protein surface. The basic criterion for applicability of Leigh's theory is that the longitudinal electron spin relaxation time T_{1e} of the perturbing spin (in this work Cr(III) or Co(II)) be short compared with the transverse electron spin relaxation time T_{2e} of the observed spin (Mn(II) in this paper). For glutamine synthetase, T_{2e} for bound Mn(II) is from 2.2×10^{-9} to 6.6×10^{-9} s in the various complexes and T_{1e} for Cr-nucleotides is $\simeq 1.8 \times 10^{-10}$ s (assuming $T_{1e} \simeq T_{2e}$ for Cr(III), this work; Gupta et al., 1976). $T_{1e} \simeq T_{2e}$ for Co(II) is $\simeq 6 \times 10^{-12}$ s (this work; Melamud & Mildvan, 1975). Thus in both cases it appears valid to use Leigh's theory to compute metal-metal distances. However, in the opposite cases, i.e., observation of changes in the EPR spectra of Cr(III) or Co(II) upon addition of Mn(II), Leigh's theory as presented would not strictly apply for distance calculations.

The diminution of the individual EPR transitions of Mn(II) could result not only from a spin-spin dispolar interaction but also from a change in zero-field splitting (ZFS). An increase in ZFS resulting in a diminution in the $-\frac{1}{2} \leftrightarrow +\frac{1}{2}$ 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 (Villafranca et al., 1976). However, this mechanism can be ruled out for the complexes described in Table II since the spectra at 35 GHz would show pronounced shoulders or extra transitions due to an increase in ZFS. The 35-GHz spectra of Figure 4 of this paper demonstrate a diminution of spectral amplitude with no other observable change. Thus, the dipolar spin-spin interaction seems to be the dominant mechanism producing the change in the spectra.

In summary, the presence of dipolar spin-spin interaction between two paramagnetic probes bound to an enzyme provides a useful method of determining the metal-metal distances between the two sites. These data yield unique information on the spatial relationship between the two metal ion sites and changes in the distance between these sites associated with the addition of substrates and inhibitors. Additionally, titrations based on the dipolar interaction provide a direct measurement of the binding constants of metal-nucleotides in the presence and absence of substrates or inhibitors. Our results demonstrate clearly that the two metal ion sites of *E. coli* glutamine synthetase are in close proximity (i.e., 5-7 Å) on the surface of the enzyme.

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Studies on the Mechanism of Action of Plasma Amine Oxidase†

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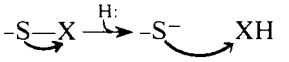
ABSTRACT: The product released when plasma amine oxidase catalyzes the oxidation of *p*-hydroxybenzylamine is *p*-hydroxybenzyaldehyde. No free imine can be detected. However, when this oxidation is carried out in the presence of NaB^3H_4, stereospecifically tritiated *S*-[α-^{3}H]-*p*-hydroxybenzylamine can be isolated. We concluded that oxidation of the substrate leads to the formation of an enzyme-bound imine, which is then hydrolyzed by the enzyme prior to release. In the presence of $NaBH_4$, some of the enzyme-bound imine (approximately 10%) was trapped. When the enzyme is reduced under anaerobic conditions with ethylglycinate in ^3H_2O or ethyl[α-^{3}H]glycinate in H_2O, no nonexchangeable ^{3}H is introduced into the reduced enzyme. The failure to incorporate nonexchangeable hydrogen into the reduced enzyme and the intermediate formation of an imine make the proposal very unlikely that pyridoxal-phosphate, or a structurally related molecule, is a cofactor which is directly reduced by the substrate. When the enzyme is inactivated by bromoethylamine, a suicide inactivator which probably reacts with the reduced enzyme, a number of functional groups, including a cysteine SH group, are labeled. When the enzyme is first reduced by substrate and then denatured, one cysteine SH group can be labeled in the nonreduced enzyme. A peptide map of the NEM-labeled enzyme shows that a single peptide is predominantly labeled in the reduced enzyme. We concluded that, upon reduction of the enzyme, one SH group is released per subunit. In the oxidized enzyme, the SH group is bonded to an as yet unidentified molecule (X). The reduction of the enzyme involves:



Plasma amine oxidase catalyzes the oxidation of primary amines:

$$RCH_2NH_3^+ + O_2 + H_2O \rightarrow RCHO + NH_4^+ + H_2O_2 \quad (1)$$

When substrate is added to the enzyme in the absence of O_2, a stoichiometric amount of product is formed (Oi et al., 1970; Reed & Swindell, 1969), and the enzyme becomes reduced as indicated by the altered absorption spectrum of the enzyme (Yamada & Yasunobu, 1962). The reduced enzyme also differs from the oxidized enzyme in that it is unaffected by several "suicide" inactivators such as 2-bromoethylamine (Neumann et al., 1975) and phenylglycinate (Maycock et al., 1975; Suva, 1978).

The identity of the group at the active site which becomes reduced is presently unknown. Since the enzyme contains Cu^{2+}, it appears possible that Cu^{2+} is reduced, but ESR studies show that the oxidation state of Cu^{2+} is unchanged in the reduced enzyme (Yamada et al., 1963). It has also been frequently proposed (Von Werle & Von Rechman, 1949; Yamada & Yasunobu, 1963) that pyridoxal functions as electron acceptor. This proposal, first made in 1949, is based on the spectral properties of the enzyme and the sensitivity of the enzyme to carbonyl reagents. However, pyridoxal cannot be resolved from the enzyme and all attempts to isolate a pyridoxal derivative from the enzyme have been unsuccessful. This negative evidence has cast doubt on the conclusion that plasma amine oxidase is a pyridoxal enzyme (Yasunobu et al., 1976; Tsurushiin et al., 1975). The possibility remains that a structurally modified form of pyridoxal, possibly covalently attached to the protein, is involved and therefore cannot be readily identified.

In this paper, we described a number of experiments designed to test the pyridoxal mechanism and to elucidate the structure of the active site.

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