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A Nuclear Magnetic Resonance Study of the Topography of Binding Sites of *Escherichia coli* Carbamoyl-phosphate Synthetase†

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ABSTRACT: Two paramagnetic probes, viz., Mn^{2+} and Cr^{3+}-ATP, were used to map distances to various loci on carbamoyl-phosphate synthetase by using NMR measurements. The paramagnetic influence of Mn^{2+} on the ^1H of L-glutamate and L-ornithine was measured at 200 and 360 MHz. On the basis of these data, a correlation time for the paramagnetic interaction was determined (2×10^{-9} s) and used to compute distances. These were in the range 7–9 Å. Distances were also calculated from Mn^{2+} to the ^{13}C-5 atom of glutamate (8.6 Å), to the monovalent cation site (~8 Å), and to the phosphorus atoms of ATP in the $Co(NH_3)_4ATP$ complex. For studies of the monovalent cation site relaxation rates of $^6Li^+$, $^7Li^+$, and $^{15}NH_4^+$ were measured. With $Cr^{3+}ATP$ as a paramagnetic substrate analogue, Cr^{3+} to ^{13}C distances were measured with the substrates HCO_3^- and [5-^{13}C]glutamate. These NMR data provide the first topographical map of the arrangement of substrates, metal ion activators, and allosteric modifiers on the *Escherichia coli* carbamoyl-phosphate synthetase dimer.

Carbamoyl-phosphate synthetase from *Escherichia coli* catalyzes the formation of an important metabolite, carbamoyl phosphate from CO_2, ATP, and a nitrogen source (NH_3 or glutamine depending on the organism). Carbamoyl phosphate is an important precursor for pyrimidine and arginine biosynthesis, and carbamoyl-phosphate synthetase is allosterically regulated in most organisms. In *E. coli*, IMP stimulates activity while UMP inhibits catalysis. Ornithine acts as a potent activator of this enzyme and can overcome inhibition by UMP (Trotta et al., 1973). Thus, regulation of the activity of carbamoyl-phosphate synthetase is crucial for the biosynthesis of nucleic acids and proteins. In higher organisms this enzyme is also important in expulsion of nitrogen via the urea cycle which has arginine as an essential component.

Meister's laboratory (Anderson & Meister, 1966; Trotta et al., 1973) explored the enzymic mechanism in detail and demonstrated that the enzyme requires two molecules of ATP for catalysis and that the overall reaction is given by eq 1.

$$2MgATP + HCO_3^- + \text{L-glutamine} \rightarrow 2MgADP + P_i + \text{L-glutamate} + \text{carbamoyl phosphate} \quad (1)$$

Recent data from our laboratory (Raushel et al., 1978; Raushel & Villafranca, 1979, 1980a,b) and from Wimmer et al. (1979) have established the kinetic mechanism of the enzyme and provided kinetic evidence for the formation of intermediates in the reaction mechanism. These data establish that the intermediates proposed by Anderson & Meister (1965) do indeed form on the enzyme in a kinetically competent manner.

The enzyme has a unique structure in that it is composed of two nonidentical subunits (Matthews & Anderson, 1972; Trotta et al., 1971), the small subunit (M_r ~48 000) possessing the glutamine binding site while the large subunit (M_r ~130 000) contains sites for the other substrates and the allosteric modifiers (Trotta et al., 1971). We have begun a program to map the substrate and allosteric modifier sites by magnetic resonance and fluorescence techniques. Our initial work focused on establishing binding constants for metal ions and substrate analogues as well as labeling specific sulfhydryl

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groups (Raushel et al., 1979). The present report deals with an NMR[1] investigation of the substrate sites in relation to a structural metal ion site and the monovalent cation activator site. The results of this work are used to construct an initial map of the location of these sites relative to one another and to their location on each subunit. The following paper presents fluorescence energy transfer experiments using covalently attached fluorescent labels to establish distances between sulfhydryl sites and active site components (Kasprzyk et al., 1983).

Materials and Methods

Materials. Carbamoyl-phosphate synthetase (CPS) was isolated from *E. coli* by the method of Matthews & Anderson (1972). $^{15}NH_4Cl$ and $K_2{}^{13}CO_3$ were supplied by Merck, and ^6LiOH was from Oak Ridge National Laboratory. DL-[5-^{13}C]Glutamic acid was obtained from Pro Chem. CrATP was prepared according to the procedure of DePamphilis & Cleland (1973) and Dunaway-Mariano (1978). $Co(NH_3)_4ATP$ was made according to Cornelius et al. (1977). All other materials were obtained from commercial sources at the highest purity available.

Nuclear Magnetic Resonance Measurements. Spin–lattice relaxation times (T_1) for the various ligands of carbamoyl-phosphate synthetase were determined by using a 180°–τ–90° pulse sequence with a Brüker WP-200 or WM-360 or a JEOL PS-100 multinuclear NMR spectrometer. The T_1 values and their standard errors were calculated by using a computer program supplied by the Nicolet Instrument Corp. All experiments were performed at 20 ± 1 °C in either 5- or 10-mm NMR tubes. Delay times of at least $5T_1$ were used in all experiments. Broad-band proton decoupling was used in the measurement of $^6Li^+$ and $^{15}NH_4{}^+$ to take advantage of the nuclear Overhauser enhancement.

The following experimental conditions were used to measure the paramagnetic effect on the spin–lattice relaxation rates of the ligands of CPS at the indicated NMR frequencies. *Mn^{2+}–L-[^{1}H]Ornithine* (200 and 360 MHz): 20 mM L-ornithine, 100 mM KCl, 99% D_2O, 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5, 82 μM CPS, and 26 μM Mn^{2+}. *Mn^{2+}–L-[^{1}H]Glutamate* (200 and 360 MHz): 25 mM L-glutamate, 100 mM KCl, 99% D_2O, 10 mM Tris, pH 7.5, 1.0 mM L-ornithine, 67 μM CPS, and 56 μM Mn^{2+}. *Mn^{2+}–DL-[^{13}C]Glutamate* (25 MHz): 25 mM DL-[5-^{13}C]-glutamate, 100 mM KCl, 1 mM L-ornithine, 20% D_2O, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.5, 37 μM CPS, and 12.5 μM Mn^{2+}. *Mn^{2+}–[^{31}P]CoATP* (81 MHz): 5.0 mM $Co(NH_3)_4$ ATP, 100 mM KCl, 1 mM L-ornithine, 20% D_2O, 50 mM Hepes, pH 7.5, 33 μM CPS, and 6.0 μM Mn^{2+}. *Mn^{2+}–^{15}N* (10 MHz): 100 mM $^{15}NH_4Cl$, 20% D_2O, 50 mM Tris, pH 7.5, 47 μM CPS, and 56 μM Mn^{2+}. *Mn^{2+}–^6Li* (29 MHz): 100 mM $^6Li^+$, 50 mM Tris, pH 7.5, 40 μM CPS, 5.0 mM L-ornithine, 40 μM Mn^{2+}, and 20% D_2O. *Mn^{2+}–^7Li* (39 MHz): 100 mM LiCl, 50 mM Tris, pH 7.5, 5.0 mM ornithine, 20% D_2O, 46 μM CPS, and 26 μM Mn^{2+}. *Mn^{2+}–^7Li* (78 MHz): 100 mM LiCl, 50 mM Tris, pH 7.5, 5 mM ornithine, 52 μM CPS, 20% D_2O, and 46 μM Mn^{2+}. *CrATP–DL-[^{13}C]Glutamate* (25 MHz): 0.35 mM CrATP, 50 mM 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.5, 0.80 mM EDTA, 5.0 mM $MgCl_2$, 25 mM DL-[5-^{13}C]glutamate, 100 mM KCl, 5.0 mM L-ornithine, and 43 μM CPS. *CrATP–[^{13}C]HCO_3^-* (25 MHz): 0.35 mM CrATP, 50 mM Pipes, pH 7.5, 5.0 mM $MgCl_2$, 25.0 mM [^{13}C]HCO_3^-, 1.0 mM EDTA, 5.0 mM L-ornithine, and 43 μM CPS.

Distance Measurements. The distances between enzyme-bound Mn^{2+} or CrATP and the various ligands of CPS were determined by measuring the effect of E–Mn^{2+} or E–CrATP on the spin–lattice relaxation times of the nuclei of the ligands. A modified form of the Solomon–Bloembergen equation (Solomon & Bloembergen, 1956) was used to compute the distance.

$$r = C[T_{1M}f(\tau_c)]^{1/6} \quad (2)$$

In this equation r is the distance between the two sites (in angstroms), T_{1M} is the paramagnetic contribution of the enzyme–Mn^{2+} or enzyme–CrATP complex to the spin–lattice relaxation rate of the nuclei of the various ligands, and $C = [(2/15)\gamma_I{}^2g^2\beta^2S(S+1)]^{1/6}$ where γ_I is the gyromagnetic ratio, S is the electron spin, and β is the Bohr magneton. The values of C for the Mn^{2+} interaction with ^{1}H, ^{6}Li, ^{7}Li, ^{13}C, ^{15}N, and ^{31}P are 812, 429, 593, 512, 379, and 601, respectively. The value of C for the CrATP interaction with ^{13}C is 445. $f(\tau_c)$ is calculated by

$$f(\tau_c) = 3\tau_c/(1 + \omega_I{}^2\tau_c{}^2) \quad (3)$$

where ω_I is the nuclear Larmor precision frequency and τ_c is the correlation time for the dipolar interaction. The paramagnetic contribution to the observed relaxation rate is calculated by

$$\frac{1}{pT_{1p}} = \frac{1}{T_1(\text{E-para})} - \frac{1}{T_1(\text{E})} - \frac{1}{T_1(\text{para})} \quad (4)$$

where $1/T_1$(E-para) and $1/T_1$(E) are the observed relaxation rates in the presence of enzyme–Mn^{2+} (or enzyme–CrATP) and enzyme alone, respectively, and p is the mole fraction of ligand that is bound to the enzyme–Mn^{2+} or enzyme–CrATP complex. $1/T_1$(para) is the contribution due to free Mn^{2+} or CrATP in solution and is calculated from the difference in relaxation rates between samples of ligand in buffer with and without Mn^{2+} (or CrATP) in the absence of enzyme. This last correction was made because the binding of both Mn^{2+} and CrATP to CPS is weak, which results in significant amounts of Mn^{2+} or CrATP free in solution. The amount of total Mn^{2+} that was bound to the enzyme was either determined from its known dissociation constant with CPS (35 μM) (Raushel et al., 1979) or determined directly by using EPR to evaluate the fraction of Mn^{2+} that is free in solution (Cohn & Townsend, 1954). The concentration of enzyme was determined from its absorbance at 280 nm ($E^{0.1\%} = 0.700$) (Wellner et al., 1973) by using a molecular weight of 180 000 (Matthews & Anderson, 1972). The amount of CrATP bound to CPS was calculated from its kinetically determined K_i value of 0.45 mM (Raushel et al., 1979).

In the analysis of the data, the assumption was made that $1/(pT_{1p}) \simeq 1/T_{1M}$, which is the case for fast exchange. This will be justified later.

Results

Effect of CPS·Mn^{2+} and CPS·CrATP on the Spin–Lattice Relaxation Rates of Ligands of Carbamoyl-phosphate Synthetase. The values for the spin–lattice relaxation rates of the nuclei of various ligands of CPS are shown in Table I. As can be seen from Table I, a larger increase in the relaxation rates of ^{1}H, ^{13}C, ^{31}P, and Li nuclei occurs when ligands bind to paramagnetic metal ion complexes with the enzyme as compared to binary metal ion–ligand complexes. This suggests

[1] Abbreviations: NMR, nuclear magnetic resonance; Pipes, 1,4-piperazinediethanesulfonic acid.

Table I: Relaxation Rates of Ligands in Various Solutions with and without Carbamoyl-phosphate Synthetase[a]

ligand	nucleus	frequency (MHz)	relaxation rate (s^{-1}) $1/T_1$(E-para)	$1/T_1$(E)	$1/T_1$(para)
	Relaxation by E·Mn^{2+}				
L-ornithine[b]	^{1}H-α	200	1.4	0.83	0.21
	^{1}H-β	200	2.5	1.5	0.08
	^{1}H-γ	200	2.3	1.5	0.07
	^{1}H-δ	200	1.8	1.0	0.02
L-glutamate[c]	^{1}H-α	200	5.3	0.59	3.6
	^{1}H-β	200	4.4	1.2	2.0
	^{1}H-γ	200	5.0	1.0	2.4
DL-glutamate[d]	^{13}C-5	25	0.14	0.036	0.057
$Co(NH_3)_4ATP$[e]	^{31}P-αβ	81	2.4	0.58	1.2
	^{31}P-γ	81	4.2	0.84	2.6
Li^+	^{7}Li[f]	39	0.18	0.07	0.002
	^{7}Li[g]	78	0.16	0.08	0.006
	^{6}Li[h]	29	0.025	0.010	0.0005
	Relaxation by E·CrATP				
DL-glutamate[i]	^{13}C-5	25	0.15	0.034	0.082
HCO_3^-	^{13}C	25	0.360	0.023	0.020

[a] The following dissociation constants were used in the calculation of ligands bound to E·Mn^{2+}: L-ornithine, 0.1 mM (Anderson, 1977); L-glutamate, 2.0 mM (P. M. Anderson, unpublished experiments); $Co(NH_3)_4ATP$, 1.7 mM (Raushel et al., 1979); Li^+, 80 mM (F. M. Raushel et al., unpublished experiments); HCO_3^-, 2.0 mM (Raushel et al., 1978). [b] The mole fraction (p) of ligand bound to E-Mn^{2+} was 8.5×10^{-4} in these experiments. [c] For these experiments, $p = 9.2 \times 10^{-4}$. [d] For these experiments, $p = 2.6 \times 10^{-4}$. [e] For these experiments, $p = 4.2 \times 10^{-4}$. [f] For these experiments, $p = 7.1 \times 10^{-5}$. [g] For these experiments, $p = 1.2 \times 10^{-4}$. [h] For these experiments, $p = 8.8 \times 10^{-5}$. [i] The mole fraction (p) of ligand bound to E-CrATP was 7.6×10^{-4} for these experiments.

that the paramagnetic contributions to the T_1 values in the enzyme complexes are significant enough to use the Solomon–Bloembergen equation to calculate internuclear distances. The standard error on all T_1 measurements was less than 10%. The value of p has been determined from the known dissociation constant of E·Mn^{2+} (Raushel et al., 1979) and from the dissociation constant of CPS with each of the other bound ligands. When the data in Table I are used in eq 4, significant effects are seen with both Mn^{2+} and CrATP as the paramagnetic metal ion. Attempts were also made to measure the paramagnetic effects of E·Mn^{2+} on the spin–lattice relaxation rates of two other allosteric modifiers, IMP and UMP. However, since both of these nucleotides bind Mn^{2+} fairly tightly ($K_D \sim 10^{-5}$ M), most of the observed relaxation was due to binary complexes of IMP·Mn^{2+} and UMP·Mn^{2+}.

Effect of CPS·Mn^{2+} on the Relaxation Rates of $^{15}NH_4^+$. Measurements of the effect of E·Mn^{2+} on the relaxation rates of $^{15}NH_4^+$ were also made. These experiments were complicated by the fact that NH_4^+ (or NH_3) can bind to a number of different sites on the enzyme. NH_4^+ is known to replace K^+ as the monovalent activator (Anderson & Meister, 1966); it is a substrate for the reaction involving ATP and HCO_3^- and thus may bind to sites on both the large and small subunits. Also, NH_4^+ binds to the ornithine allosteric site (Anderson, 1977). The binding of NH_4^+ at the substrate site most likely does not occur with our experimental design since the K_m for NH_4^+ is 170 mM (Raushel et al., 1978). Additionally CPS has an ordered kinetic mechanism with NH_4^+ being the third substrate that adds to the enzyme. Thus, no binding should occur in the absence of the other substrates.

The various contributions to the observed relaxation rates of $^{15}NH_4^+$ with enzyme and Mn^{2+} were determined in the following manner: a sample of enzyme, Mn^{2+}, and $^{15}NH_4^+$ was made up as described under Materials and Methods and the T_1 of $^{15}NH_4^+$ was 10.3 ± 0.3 s. This includes contributions from binding to both the ornithine site and the monovalent cation site. $^{15}NH_4^+$ was displaced from the ornithine site by the addition of 100 mM L-ornithine. Since the dissociation constant of ornithine from E–ornithine is 1×10^{-4} M (Anderson, 1977) and the activation constant for $^{15}NH_4^+$ at the ornithine site is 25 mM, effectively all of the NH_4^+ is displaced from this site on the enzyme. The T_1 of $^{15}NH_4^+$ after the addition of ornithine was 12.2 ± 0.4 s. The calculated value of $1/T_{1M}$ is 88 s^{-1} by using $p = 1.7 \times 10^{-4}$. The $^{15}NH_4^+$ was then displaced from the monovalent cation site by the addition of 400 mM KCl. The dissociation constants for K^+ and NH_4^+, determined from their activation of CPS, are 20 mM[2] for both of these monovalent cations, and thus 80% of the $^{15}NH_4^+$ at the K^+ site will have been displaced by the added KCl. The T_1 for $^{15}NH_4^+$ after the addition of K^+ was 17.4 ± 0.4 s. The calculated value of $1/T_{1M}$ is 170 by using $p = 1.45 \times 10^{-4}$. The final value for the T_1 of $^{15}NH_4^+$ is the same as that for a solution of enzyme and $^{15}NH_4^+$ in the absence of Mn^{2+}, suggesting that binding at other sites on the protein is insignificant.

Table II: Distances from Mn^{2+} to ^{1}H of L-Ornithine and L-Glutamate in Complexes with Carbamoyl-phosphate Synthetase[a]

ligand	nucleus	$1/(pT_{1p})$ (s^{-1}) 200 MHz	360 MHz	r (Å)
L-ornithine	^{1}H-α	420	230	8.5 ± 0.6
	^{1}H-β	1080	510	7.3 ± 0.5
	^{1}H-γ	860	370	7.6 ± 0.6
	^{1}H-δ	920	400	7.5 ± 0.6
L-glutamate	^{1}H-α	1210	500	7.1 ± 0.5
	^{1}H-β	1300	520	7.1 ± 0.5
	^{1}H-γ	1740	670	7.0 ± 0.5

[a] Calculated from eq 2 by using $\tau_c = 2 \times 10^{-9}$ s.

Calculation of τ_c and Distances. In order to use the data in Table I to calculate internuclear distances from the Solomon–Bloembergen equation, a value for τ_c must first be determined. Three methods were used to evaluate τ_c. The value of τ_c for the enzyme–Mn^{2+} system was calculated from the ratio of pT_{1p} values for $^7Li^+$ at frequencies of 78 and 39 MHz by assuming that there is no magnetic field dependence on τ_c. The second method involved calculating τ_c from the frequency dependence of pT_{1p} for the ^{1}H of L-glutamate and L-ornithine. From the data in Table II τ_c ranged from 0.7×10^{-9} to 1.2×10^{-9} s for various ^{1}H in these molecules. The third method involves determining the ratio of pT_{1p} for $^6Li^+$ and $^7Li^+$ at the same magnetic field strength (Raushel & Villafranca, 1980a,b). This latter method determines a unique value for τ_c at this magnetic field strength since the correlation time and r must be the same for both $^6Li^+$ and $^7Li^+$. The ratio of pT_{1p} for $^7Li^+$ at 78 and 39 MHz is 2.4 which gives a τ_c value of 4.0×10^{-9} s. The ratio of pT_{1p} for $^6Li^+$ and $^7Li^+$ is 3.6 at a magnetic field strength of 47 kG, which results in a calculated correlation time of 2.4×10^{-9} s. For all experiments a ratio >1 means that the assumption $1/(pT_{1p}) = 1/T_{1M}$ is valid (Dwek, 1973). The τ_c values calculated by all the methods given above are of the magnitude expected for the electron spin relaxation time of Mn^{2+} in the various enzyme complexes (Mildvan & Gupta, 1978; Villafranca, 1982).

To compute the Mn^{2+}–nuclei distances an average τ_c value of 2×10^{-9} s was used for Mn^{2+}–^{1}H data (Table II) while a

[2] F. M. Raushel, unpublished observations.

Table III: Distances from Mn^{2+} and CrATP to Ligand Nuclei in Complexes of Carbamoyl-phosphate Synthetase[a]

ligand	nucleus	$1/(pT_{1p})$ (s^{-1})	r (Å)
	Mn^{2+}–Ligand Distances		
glutamate	^{13}C-5	320[b]	8.6 ± 0.6
$Co(NH_3)_4ATP$	^{31}P-$\alpha\beta$	1480	<6.6 ± 0.4
	^{31}P-γ	1810	<6.4 ± 0.4
Li^+	^{7}Li	1520	7.5 ± 0.4
	^{7}Li	620	7.6 ± 0.4
	^{6}Li	170	8.0 ± 0.5
NH_4^+, monovalent cation site	^{15}N	170	7.4 ± 0.5
NH_4^+, ornithine site	^{15}N	88	8.2 ± 0.6
	CrATP–Ligand Distances		
glutamate	^{13}C-5	41	7.0 ± 0.5
HCO_3^-	^{13}C	420	4.7 ± 0.3

[a] Calculated from eq 2 by using $\tau_c = 3.2 \times 10^{-9}$ s for enzyme-Mn^{2+} and $\tau_c = 2.0 \times 10^{-10}$ s for enzyme–CrATP. [b] Data were corrected for binding of only the L-glutamate isomer of DL-glutamate.

τ_c value of 3.2×10^{-9} s was used for all other calculations (Table III). The errors in the distances listed in Tables II and III spans this small range in τ_c values for Mn^{2+}–nuclear interactions plus an error of 25% in pT_{1p} values. The latter errors include errors in T_1 measurements (±10%) and in the computation of the mole fraction of bound ligands from K_D values.

An estimate for the correlation time in the CrATP system is more difficult to obtain. Gupta et al. (1976) have determined a lower limit on the electron spin relaxation time of 1×10^{-10} s for CrATP from the peak to peak width in the EPR spectrum. Gupta et al. also measured the frequency dependence of the enhancement of water protons by pyruvate kinase bound CrATP and obtained a τ_s value of $(1.2\text{–}2.3) \times 10^{-10}$ s. Balakrishnan & Villafranca (1978) calculated a value of τ_c of 1.75×10^{-10} s for CrATP bound to glutamine synthetase from the EPR spectra of enzyme-bound CrATP. In the absence of more direct data with carbamyl-phosphate synthetase and since it seems that τ_s does not vary significantly in different enzyme systems, we used a value of 2×10^{-10} s for τ_c.

Discussion

The objective of our current study was to establish the topographical relationships among several loci on *E. coli* carbamoyl-phosphate synthetase. The sites investigated were the glutamate, metal–ATP, HCO_3^-, ornithine, monovalent cation, and free divalent cation sites. The enzyme has been shown to require a free divalent cation for activity in addition to utilizing metal–nucleotide complexes as substrates (Raushel et al., 1979). Activity with Mg^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, and Zn^{2+} have been demonstrated, while Ca^{2+} and Ni^{2+} are not active in the overall reaction. Kinetic data showed that addition of Mg^{2+}_{free} and MgATP is equilibrium ordered with free metal binding prior to metal–nucleotide. Thus, the free metal ion site must be populated for catalysis and free metal is an absolute requirement for enzymatic activity and not just a positive allosteric effector. The binding constant for Mn^{2+} at the free metal ion site (35 μM) has previously been determined (Raushel et al., 1979).

For the current study we utilized the paramagnetic properties of Mn^{2+} ($S = {}^5/_2$) and of Cr^{3+}ATP ($S = {}^3/_2$), an analogue of the metal–nucleotide substrate, in NMR studies of the relaxation rates of several nuclei. Our data demonstrate significant paramagnetic effects of Mn^{2+} (bound to the free divalent cation site) on the $1/T_1$ values of the ^{1}H's of glutamate and ornithine. Enzyme-bound Mn^{2+} also significantly influenced the longitudinal relaxation rates of the phosphorus atoms

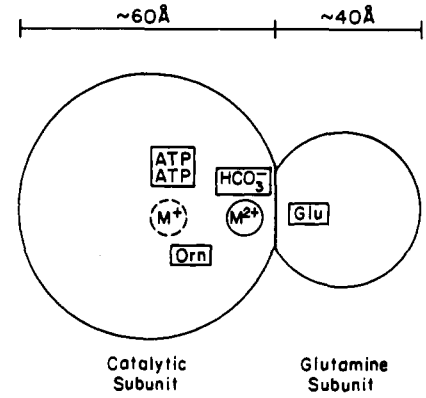

FIGURE 1: Schematic drawing of the topographical relationship among substrate, metal ion, and allosteric sites on carbamoyl-phosphate synthetase as determined by NMR measurements. The exact location of the monovalent cation site, i.e., to which subunit it binds, is not known and this is indicated by the dashed circle. Distances are omitted for clarity, and thus the binding sites are not to scale but are relative locations.

of Co^{3+}ATP, a diamagnetic substrate analogue, and the C-5 carbon of glutamate. These NMR data were used to calculate distances between Mn^{2+} and the various nuclei by use of the Solomon–Bloembergen equation. The theoretical justification and limitations of this technique have been discussed by several authors (Dwek, 1973; Mildvan & Gupta, 1978; Villafranca, 1982). Specific limitations applicable to the study of Co^{3+}ATP and Cr^{3+}ATP have been recently discussed (Villafranca, 1982).

The distances to the ^{13}C-5 of glutamate must be viewed as upper limits since the experiments were done with DL-[5-^{13}C]glutamate. The binding constant of D-glutamate to CPS is unknown although it is probably weaker than that of L-glutamate. If the binding constant of D-glutamate is significantly weaker to CPS than is L-glutamate, then the measured T_1 value in the presence of E·Mn^{2+} will be dominated by the D-glutamate that is unbound and not significantly relaxed by enzyme–Mn^{2+}. This complication would reduce the calculated value of $1/T_{1M}$ and thus the distances must be upper limits. This same argument also applies to β,γ-$Co(NH_3)_4ATP$ since this compound is a mixture of diastereomers and we have not measured the relative binding constants of the Λ and Δ isomers to the enzyme.

In addition to a metal ion site for free divalent cations, carbamoyl-phosphate synthetase is activated by monovalent cations (Trotta et al., 1973; Lusty, 1978). NH_4^+ as well as K^+ are thought to be positive allosteric modifiers of the enzyme's activity. We were able to measure distances from the free Mn^{2+} site to the monovalent cation site utilizing $^{15}NH_4^+$, $^6Li^+$, and $^7Li^+$ isotopes. The M^{2+}–M^+ distance was ~7.5 Å. The Mn^{2+}–NH_4^+ data were obtained in the presence of saturating ornithine. NH_4^+ can also bind to the L-ornithine site and the M^{2+}–NH_4^+ (ornithine site) distance was ~8.2 Å. From these experiments we conclude that the monovalent cation site and the site for binding the R–NH_3^+ moiety(s) of ornithine are different sites. Thus, the positive allosteric effects elicited by L-ornithine and M^+ are most likely distinct phenomena. However, an extensive kinetic study of the allosteric behavior of the enzyme in terms of V_{max} and K_m effects on the substrates has not yet been reported.

A model for the three dimensional relationships among the various sites is presented in Figure 1. A precise three-dimensional model is not given since results from NMR data are represented only as a series of point-to-point vectors between a metal ion site and the various nuclei. Triangulation is possible for the Mn^{2+} and Cr^{3+} to ^{13}C-5 distances of glu-

tamate, and these distances can be represented as a planar surface. However, the exact relationship between Mn^{2+} and Cr^{3+} is not known but can be estimated from the Mn^{2+} to ^{31}P distances obtained with the $Co(NH_3)_4ATP$ complex (assuming the $Cr^{3+}ATP$ and $Co^{3+}ATP$ complexes occupy the same site). On the basis of the fact that glutamine and thus glutamate bind to the small subunit, and ATP, HCO_3^-, and ornithine bind to the large subunit, we have arranged the molecules according to the relative distances reported in this paper. The absolute distance relationships still remain to be determined. However, since Mn^{2+} is nearly equidistant to the nuclei of glutamate (small subunit) and ornithine (large subunit), the Mn^{2+} site must be near the subunit interface as shown in Figure 1. Also, the relative relationship between the ATP, HCO_3^-, and glutamine sites must be nearly correct considering the stepwise nature of the enzymic mechanism. The NMR data, the distance relationships obtained by fluorescence energy transfer reported in the following paper (Kasprzyk et al., 1983) and the previously reported EPR data (Raushel et al., 1979) are all brought together in schematic form in the following paper (Kasprzyk et al., 1983).

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Registry No. Mn, 7439-96-5; CrATP, 69381-95-9; $Co(NH_3)_4ATP$, 63915-26-4; Li, 7439-93-2; ammonium, 14798-03-9; bicarbonate, 71-52-3; glutamic acid, 56-86-0; ornithine, 70-26-8; carbamoylphosphate synthetase, 37233-48-0.

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