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Spatial Relationships among the Active and Allosteric Sites of Carbamoyl-Phosphate Synthetase[1]

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NMR experiments were conducted to map distances among various loci on *Escherichia coli* carbamoyl-phosphate synthetase. Three paramagnetic probes, viz., Mn^{2+}, Cr^{3+}-ATP, and nitroxide spin-labels were used in experiments designed to measure the $1/T_1$ (longitudinal relaxation rate) of various nuclei in enzyme complexes with these paramagnetic species. The distance between the monovalent cation activator site and enzyme-bound Cr^{3+}-ATP was determined using three different monovalent cations, $^{133}Cs^+$, $^{15}NH_4^+$, and Li^+ (^6Li and ^7Li). Substantial paramagnetic effects were observed on the $1/T_1$ values for all four nuclei and the M^+ to Cr^{3+} distance was ~4 Å. Additional NMR data with $^{133}Cs^+$ and Mn^{2+} were used to obtain the distance between the two cation activator sites, monovalent and divalent, and a Mn^{2+} to Cs^+ distance of 8.0 Å was calculated, corroborating earlier work [F. M. Raushel, P. M. Anderson, and J. J. Villafranca (1983) *Biochemistry* **22**, 1872–1876]. Three separate sulfhydryl sites on carbamoyl-phosphate synthetase were spin-labeled with 3-maleimido-2,2,5,5-tetramethylpyrrolidinyl-1-oxy. Each of these enzyme–nitroxide complexes was used to examine the paramagnetic influence on the ^1H of L-glutamate and L-ornithine and also the ^1H and ^{31}P of IMP and UMP. Small paramagnetic effects were observed on these nuclei and only lower limits on the distance from each nitroxide could be obtained. Thus both L-ornithine and L-glutamate are >11 Å from each sulfhydryl site while IMP and UMP are >15 Å from these sites. A topographical map is presented based on these data and data from our previous NMR studies that show the spatial relationship among the active-site components of carbamoyl-phosphate synthetase. © 1985 Academic Press, Inc.

INTRODUCTION

The biosynthesis of carbamoyl-phosphate in *Escherichia coli* is accomplished by glutamine-dependent carbamoyl-phosphate synthetase. The enzyme exists as a dimer of two nonidentical subunits (*1, 2*). The small subunit ($M_r \simeq 42{,}000$) is involved in binding glutamine, while the large subunit ($M_r \simeq 130{,}000$), for which the DNA sequence has recently been published (*3*), has separate binding sites for the other substrates and the allosteric modifiers, ornithine, IMP, and UMP (*1, 4*). However, recent data from Meister's laboratory suggests that the IMP and UMP sites overlap (*5*). As established by Meister's laboratory (*6–8*), the stoichiometry

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of the reaction involves two molecules of ATP, and one each of HCO_3^- and glutamine, to produce carbamoyl-phosphate, P_i, glutamate, and two molecules of ADP.

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

The kinetic mechanism has been determined (*9*) and substantial experimentation has established that the reaction proceeds in a stepwise manner with the initial formation of carboxyphosphate (*10–13*) followed by phosphorylation of enzyme-bound carbamate (*6, 8, 11*). The enzyme has both monovalent and divalent cation activator sites (*8, 14*), and we have focused our recent efforts on using NMR, EPR, and fluorescence energy transfer methods to map the spatial relationships among the cation activator, substrate, allosteric, and sulfhydryl (*15*) sites of carbamoyl-phosphate synthetase (*14, 16, 17*). These data related distances among (i) the sulfhydryl sites, (ii) the sulfhydryl to active-site components, and (iii) selected distances from a structural Mn^{2+} site (*14*) to the ornithine, glutamate, ATP, and monovalent cation activator sites.

This paper presents a recently obtained unique set of NMR data that was used to determine distances between Cr^{3+}-ATP bound at the active site and the monovalent cation activator site. Additional NMR data defines limits on the spatial relationships among three spin-labeled sulfhydryl sites and the allosteric (ornithine, IMP, and UMP) and glutamate sites. These data are placed in the context of our initial topographical map (*16, 17*) defining relationships among the molecules that bind at the active site and those that bind to the allosteric sites. A three-dimensional model of the active-site structure, based on the composite NMR data, was generated using molecular modeling computer software.

EXPERIMENTAL PROCEDURES

Materials. The method of Matthews and Anderson (*2*) was used to purify carbamoyl-phosphate synthetase isolated from *E. coli* to a specific activity of 150–200 μmol h^{-1} mg^{-1}. 3-Maleimido-2,2,5,5-tetramethylpyrrolidinyl-1-oxy (MTPO)[3] was purchased from Syva Research Chemicals. ^{6}LiOH was purchased from Oak Ridge National Laboratory. α,β,γ-Cr^{3+}-ATP was prepared according to the procedure of DePamphilis and Cleland (*18*) and Dunaway-Mariano (*19*). All other materials were obtained from commercial sources at the highest purity available.

Enzyme assays. A Hitachi Model 100-80 UV/VIS Spectrometer was used to spectrophotometrically follow the activity of carbamoyl-phosphate synthetase at 340 nm using a pyruvate kinase–lactate dehydrogenase coupling system (*9*). Specific conditions for the assays conducted at 37°C are given in our earlier work (*17*).

Preparation of spin-labeled carbamoyl-phosphate synthetase. The spin-labeled NEM derivatives of carbamoyl-phosphate synthetase were prepared by using the

[3] Abbreviations used: MTPO, 3-maleimido-2,2,5,5-tetramethylpyrrolidinyl-1-oxy, Pipes, 1,4-piperazinediethanesulfonic acid, Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

procedures of Foley *et al.* (*15*) and Matthews and Anderson (*2*) for the three different sulfhydryl groups on the enzyme. These derivatives have been named E-$MTPO_1$, E-$MTPO_2$, and E-$MTPO_3$. The methods employed were nearly identical to those from our previous work with these spin-labeled derivatives (*14*). After preparation each enzyme sample was subsequently lyophilized for use in the NMR experiments. The enzyme was stable throughout this procedure. The stoichiometry of labeling was determined by comparing the integrated EPR spectrum of free label in 8 M urea with that of E-MTPO in 8 M urea using a computer program supplied by Centre Computer Consultants. The stoichiometry of labeling was found to be between 0.9 and 1.0 for all the derivatives.

EPR spectroscopic measurements. A Varian E-12 spectrometer operating at 9 GHz was used to record all EPR spectra. Capillary tubes of 1 mm i.d. were used for all samples. A modulation amplitude of 4 G and a power level of 100 mW were used for all spectra. Data were accumulated in an S-100 microcomputer and double integration was performed to determine the stoichiometry of labeling.

NMR measurements. A Bruker WP-200 or a JEOL PS-100 multinuclear NMR spectrometer was used to measure spin-lattice relaxation times (T_1) for the various compounds and ions interacting with carbamoyl-phosphate synthetase. A 180°-τ-90° pulse sequence was employed to measure T_1 values. Delay times of at least 5 T_1 were used throughout. The standard errors for the T_1 values were determined by computer analysis. Either 5- or 10-mm NMR tubes were used in all experiments, which were conducted at an ambient temperature of 20 ± 1°C. To take advantage of the nuclear Overhauser enhancement, broad-band proton decoupling was used in the measurement of $^6Li^+$ and $^{15}NH_4^+$ relaxation times.

A list of enzyme samples and experimental conditions are presented below for the various NMR experiments:

(1) E-$MTPO_{1,2, \text{ or } 3}$–L-[^{1}H]ornithine (100 and 200 MHz): 5.0 mM L-ornithine, 5.0 mM $MgCl_2$, 100 mM KCl, 99% ^2H_2O, 10 mM Tris, pH 7.5, 0.15 mM E-MTPO.

(2) E-$MTPO_{1,2, \text{ or } 3}$–L-[^{1}H]glutamate (100 and 200 MHz): 5.0 mM L-glutamate, 5.0 mM $MgCl_2$, 100 mM KCl, 99% ^2H_2O, 10 mM Tris, pH 7.5, 0.15 mM E-MTPO.

(3) E-$MTPO_{1,2, \text{ or } 3}$–[^{1}H]UMP (100 MHz): 5.0 mM UMP, 5.0 mM $MgCl_2$, 1.0 mM EDTA, 100 mM KCl, 99% ^2H_2O, 10 mM Tris, pH 7.5, 0.15 mM E-MTPO.

(4) E-$MTPO_{1,2, \text{ or } 3}$–[^{1}H]IMP (100 MHz): 5.0 mM IMP, 5.0 mM $MgCl_2$, 1.0 mM EDTA, 100 mM KCl, 99% ^2H_2O, 10 mM Tris, pH 7.5, 0.15 mM E-MTPO.

(5) E-$MTPO_{1,2, \text{ or } 3}$–[^{31}P]UMP (40 and 81 MHz): 5.0 mM UMP, 5.0 mM $MgCl_2$, 1.0 mM EDTA, 100 mM KCl, 99% ^2H_2O, 10 mM Tris, pH 7.5, 0.15 mM E-MTPO.

(6) E-$MTPO_{1,2, \text{ or } 3}$–[^{31}P]IMP (40 and 81 MHz): 5.0 mM IMP, 5.0 mM $MgCl_2$, 1.0 mM EDTA, 100 mM KCl, 99% ^2H_2O, 10 mM Tris, pH 7.5, 0.15 mM E-MTPO.

(7) Cr^{3+}-ATP–^{6}Li (29 MHz): 100 mM $^6Li^+$, 50 mM Pipes, pH 6.5, 5.0 mM L-ornithine, 5.0 mM $MgCl_2$, 50 μM enzyme, 20% ^2H_2O, 0.35 mM Cr^{3+}-ATP.

(8) Cr^{3+}-ATP–^{7}Li (78 MHz): 100 mM $^7Li^+$, 50 mM Pipes, pH 6.5, 5.0 mM L-ornithine, 5.0 mM $MgCl_2$, 50 μM enzyme, 20% ^2H_2O, 0.35 mM Cr^{3+}-ATP.

(9) Cr^{3+}-ATP–^{15}N (10 MHz): 100 mM $^{15}NH_4Cl$, 50 mM Pipes, pH 6.5, 5.0 mM $MgCl_2$, 50 μM enzyme, 20% ^2H_2O, 0.20 mM Cr^{3+}-ATP.

(10) Cr^{3+}-ATP–^{133}Cs (26 MHz): 100 mM CsCl, 50 mM Pipes, pH 6.5, 5.0 mM L-ornithine, 5.0 mM $MgCl_2$, 50 μM enzyme, 20% ^2H_2O, 0.2 mM Cr^{3+}-ATP.

(11) Mn^{2+}-^{133}Cs (26 MHz): 10 mM CsCl, 50 mM Tris, pH 7.5, 5.0 mM L-ornithine, 50 μM enzyme, 20% ^2H_2O, 50 μM Mn^{2+}.

Data analysis. Various enzyme complexes (E-Mn^{2+}, E-Cr^{3+}-ATP, or E-MTPO) were used to measure paramagnetic effects on the spin-lattice relaxation rates of the nuclei of the compounds or ions bound to carbamoyl-phosphate synthetase. The Solomon-Bloembergen (*20*) equation (Eq. [2]) was used to compute the distances between the paramagnetic centers and the nuclei.

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

In Eq. [2], C is a collection of constants for each nucleus and paramagnetic species [see Ref. (*21*) for these values and a definition of $f(\tau_c)$].

Equation 3 presents an expression for the paramagnetic contribution to the observed longitudinal relaxation rate:

$$1/T_{1p} = 1/T_{1(\text{E-para})} - 1/T_{1(\text{E})} - 1/T_{1(\text{para})}, \qquad [3]$$

where $1/T_{1(\text{E-para})}$ is the observed relaxation rate in the presence of E-Mn^{2+}, E-Cr^{3+}-ATP, or E-MTPO, and $1/T_{1(\text{E})}$ is for enzyme alone. The contribution due to free Mn^{2+} or Cr^{3+}-ATP in solution interacting with the molecules whose T_1 values are being measured is $1/T_{1(\text{para})}$ and is calculated from the difference in relaxation rates between samples of ligands in buffer with and without Mn^{2+} or Cr^{3+}-ATP in the absence of enzyme. This latter correction was made because of the magnitude of the binding constants of both Mn^{2+} and Cr^{3+}-ATP to carbamoyl-phosphate synthetase which results in small but significant amounts of Mn^{2+} and Cr^{3+}-ATP free in solution. The amount of total Mn^{2+} or of Cr^{3+}-ATP that was bound to the enzyme was determined from their known dissociation constants with carbamoyl-phosphate synthetase which are, respectively, 35 and 450 μM (*14*). For Mn^{2+}, the amount of bound Mn^{2+} was also determined by EPR (*14*). These corrections were not required for E-MTPO because all of the label is covalently bound. The concentration of enzyme was determined from its absorbance at 280 nm ($\varepsilon^{0.1\%} = 0.7$) (*22*) using a molecular weight of 180,000.

Equation [4] describes the relationship between the experimentally obtained $1/T_{1p}$ value (Eq. [3]), the paramagnetic dipolar electron–nuclear relaxation time (T_{1M}) in the enzyme complex, and the lifetime of this complex, τ_m:

$$1/T_{1p} = pq/(T_{1M} + \tau_m). \qquad [4]$$

In this expression, p = [ligand in paramagnetic complex]/[ligand total] and q is the number of ligands in the complex, generally 1. In the analysis of the data the assumption was thus made that $1/pT_{1p} \simeq 1/T_{1M}$, which is the case for fast exchange where $T_{1M} \gg \tau_m$. Limitations in the analysis of NMR data such as those presented in this paper have been discussed in detail elsewhere (*21, 23*) and will not be repeated here.

Computer modeling. A three-dimensional model of various components of the active site of carbamoyl-phosphate synthetase was generated using distance relationships generated by the NMR experiments and crystallographic data. A computer program for molecular modeling that is part of the ADAPT structure–activity software of Jurs and co-workers (*24–26*) was used to generate a

TABLE 1

RELAXATION RATES OF LIGANDS IN VARIOUS SOLUTIONS WITH AND WITHOUT CARBAMOYL-PHOSPHATE SYNTHETASE[a]

Ligand	Nucleus	Frequency (MHz)	$1/T_{1(E\text{-}para)}$	$1/T_{1(E)}$	$1/T_{1(para)}$
Relaxation by E-Cr^{3+}-ATP					
Li^+	^6Li	29	0.091	0.008	0.053
	^7Li	78	0.602	0.085	0.310
Cs^+	^{133}Cs	26	0.224	0.111	0.048
NH_4^+	^{15}N	10	0.045	0.034	0.001
Relaxation by E-Mn^{2+}					
Cs^+	^{133}Cs	26	0.153	0.120	0.005

[a] The following dissociation constants (*16*) were used in the calculations of ligands bound to E-Cr^{3+}-ATP and E-Mn^{2+}; Li^+, 80 mM; NH_4^+, 20 mM; and Cs^+, 100 mM (lower limit calculated from a fluorescence titration; data not shown). The standard error in each T_1 determination was $\leq 10\%$. For E-Mn^{2+}, $p = 2.0 \times 10^{-4}$ and for E-Cr^{3+}-ATP, $p = 7.3 \times 10^{-5}$.

three-dimensional representation of the chemical structure as it would exist in its lowest energy conformation. The crystal structure coordinates including bond lengths, bond angles, and torsional angles of the six-member ring structure of Cr-pyrophosphate determined by Merritt *et al.* (*27*) was used along with standard bond lengths for bicarbonate to generate the initial model. The final geometry of the complex was optimized by using an intermolecular strain energy function. The NMR distance data from the paramagnetic species to the various nuclei were then used to establish the overall final model.

RESULTS

Effect of enzyme–Cr^{3+}–ATP and enzyme–Mn^{2+} on the spin-lattice relaxation rates of monovalent cations bound to carbamoyl-phosphate synthetase. Previous NMR studies from this laboratory reported on the distances between the monovalent and divalent cation activator sites. This report expands our studies to explore the spatial relationship between the metal–ATP site(s) and the monovalent cation site. The values for the spin-lattice relaxation rates of various monovalent cations that bind to carbamoyl-phosphate synthetase are shown in Table 1. As can be seen from the data, a substantial increase in the relaxation rates of $^6Li^+$, $^7Li^+$, $^{15}NH_4^+$, and $^{133}Cs^+$ occurs when these ions bind to the E-Cr^{3+}-ATP complex as compared to the rates in the absence of Cr^{3+}-ATP. From our earlier kinetic studies (*10, 11*), we assumed that Cr^{3+}-ATP binds to only one ATP site since this compound is a linear competitive inhibitor vs. MgATP. Also in these NMR experiments, Cr^{3+}-ATP can be displaced by P^1,P^5-di(adenosine-5′)pentaphosphate, reducing the paramagnetic effects to values observed in the absence of enzyme.

The paramagnetic contributions to the T_1 values in the enzyme complexes com-

TABLE 2

DISTANCES FROM Cr^{3+}-ATP AND Mn^{2+} TO MONOVALENT CATIONS IN COMPLEXES OF CARBAMOYL-PHOSPHATE SYNTHETASE

Ligand	Nucleus	$1/pT_{1p}$ (s^{-1})	r (Å)
Cr^{3+}-ATP-ligand distances[a]			
Li^+	^6Li	250	4.3 ± 0.4
	^7Li	1725	4.3 ± 0.4
Cs^+	^{133}Cs	890	3.4 ± 0.4
NH_4^+	^{15}N	92	4.5 ± 0.4
Mn^{2+}-ligand distance[b]			
Cs^+	^{133}Cs	140	8.0 ± 0.5

[a] Calculated from Eq. [2] using $\tau_c = 2.0 \times 10^{-10}$ s.

[b] Calculated from Eq. [2] using $\tau_c = 2.0 \times 10^{-9}$ s.

puted from the data in Table 1 (using Eqs. [3] and [4]) are significant enough to calculate the Cr^{3+} to M^+ internuclear distances using Eq. [2]. The value of p, the mole fraction of bound species, was determined from (i) the known K_D value of E-Cr^{3+}-ATP (*14*) and (ii) the individual binding constants for each monovalent cation (Table 1). Also, an estimate for the correlation time in the Cr^{3+}-ATP system was calculated from the pT_{1p} ratio for $^6Li^+$ and $^7Li^+$ at one magnetic field strength using the method of Raushel and Villafranca (*28*). The experimentally determined ratio of pT_{1p} for $^6Li^+$ and $^7Li^+$ is 6.9 at a magnetic field strength of 47 kG, which results in a correlation time of $\leq 2.0 \times 10^{-10}$ s. This value is similar to that determined by Gupta *et al.* (*29*) of $\sim 2 \times 10^{-10}$ s obtained from the frequency dependence of the enhancement of water protons by pyruvate kinase-bound Cr^{3+}-ATP. With glutamine synthetase, Balakrishnan and Villafranca (*30*) determined a value of τ_c of 1.75×10^{-10} s for Cr^{3+}-ATP bound to this enzyme. A value of 2.0×10^{-10} s was thus used for τ_c to compute the Cr^{3+}-M^+ distances given in Table 2.

T_1 data were also obtained for $^{133}Cs^+$ interacting with E-Mn^{2+} to corroborate our earlier NMR study of monovalent cation binding to carbamoyl-phosphate synthetase (*16*). From the T_1 data listed in Table 1 and using the correlation time for E-Mn^{2+} of 2×10^{-9} s previously determined by Raushel *et al.* (*16*), a Mn^{2+} to Cs^+ distance was calculated and this value is given in Table 2. This distance is in agreement with our previous study consistent with the conclusion that Cs^+ binds to the same site as Li^+ and NH_4^+.

Effect of enzyme–MTPO$_{1,2\ or\ 3}$ on the spin-lattice relaxation rates of various nuclei of ornithine, glutamate, UMP, and IMP. We have not previously reported attempts to obtain distances by NMR measurements between the three sulfhydryl sites and the allosteric L-ornithine, UMP, or IMP sites or the L-glutamate site. To do this, the spin-lattice relaxation rates of the ^1H of L-ornithine and L-glutamate, and the ^1H and ^{31}P of IMP and UMP were obtained in solutions containing enzyme alone and enzyme samples labeled with the spin-label MTPO at each of three

TABLE 3

RELAXATION RATES OF LIGANDS IN VARIOUS SOLUTIONS WITH AND WITHOUT CARBAMOYL-PHOSPHATE SYNTHETASE–SPIN-LABEL$_1$[a]

Ligand	Nucleus	Frequency (MHz)	Relaxation rate (s^{-1})	
			$1/T_{1(E\text{-}para)}$	$1/T_{1(E)}$
L-Ornithine	^1H-β[b,c]	100	1.12	1.12
	^1H-δ	100	0.93	0.87
	^1H-β	200	1.24	1.23
	^1H-δ	200	0.98	0.93
L-Glutamate	^1H-γ[d]	100	1.02	0.99
	^1H-β	100	0.98	0.92
	^1H-γ	200	1.02	0.99
	^1H-β	200	1.15	1.12
IMP	^1H-2[e]	100	0.69	0.68
	^1H-2′	100	0.44	0.42
	^{31}P	40	0.120	0.121
	^{31}P	81	0.139	0.127
UMP	^1H-4′[f]	100	0.57	0.53
	^1H-5′	100	0.34	0.31
	^{31}P	40	0.099	0.095
	^{31}P	81	0.139	0.131

[a] The following dissociation constants (*16*) were used in the calculation of ligands bound to E-MTPO$_1$: L-ornithine, 0.1 mM; L-glutamate, 2.0 mM; IMP, 0.2 mM; UMP, 5 μM.

[b] The symbols after the ^1H refer to the specific protons.

[c] For these experiments, $p = 2.94 \times 10^{-2}$.

[d] For these experiments, $p = 2.13 \times 10^{-2}$.

[e] For these experiments, $p = 2.88 \times 10^{-2}$.

[f] For these experiments, $p = 3.00 \times 10^{-2}$.

TABLE 4

RELAXATION RATES OF LIGANDS IN VARIOUS SOLUTIONS WITH AND WITHOUT CARBAMOYL-PHOSPHATE SYNTHETASE–SPIN-LABEL$_2$[a]

Ligand	Nucleus	Frequency (MHz)	Relaxation rate (s^{-1})	
			$1/T_{1(E\text{-}para)}$	$1/T_{1(E)}$
L-Ornithine	^1H-β	100	1.14	1.12
	^1H-δ	100	0.87	0.87
	^1H-β	200	1.26	1.23
	^1H-δ	200	0.94	0.93
L-Glutamate	^1H-γ	100	0.91	0.81
	^1H-β	100	1.00	0.92
	^1H-γ	200	1.05	0.99
	^1H-β	200	1.16	1.12
IMP	^{31}P	40	0.132	0.121
	^{31}P	81	0.134	0.127
UMP	^{31}P	40	0.098	0.095
	^{31}P	81	0.136	0.131

[a] The dissociation constants, symbols, and p (mole fraction) values are the same as those used in Table 3.

TABLE 5

RELAXATION RATES OF LIGANDS IN VARIOUS SOLUTIONS WITH AND WITHOUT CARBAMOYL-PHOSPHATE SYNTHETASE–SPIN-LABEL$_3$[a]

Ligand	Nucleus	Frequency (MHz)	Relaxation rate (s^{-1})	
			$1/T_{1(E\text{-para})}$	$1/T_{1(E)}$
L-Ornithine	^{1}H-β	100	1.14	1.12
	^{1}H-δ	100	0.88	0.87
	^{1}H-β	200	1.26	1.23
	^{1}H-δ	200	0.95	0.93
L-Glutamate	^{1}H-γ	100	0.90	0.81
	^{1}H-β	100	1.02	0.92
	^{1}H-γ	200	1.00	0.99
	^{1}H-β	200	1.20	1.12
IMP	^{31}P	40	0.123	0.121
	^{31}P	81	0.128	0.127
UMP	^{31}P	40	0.098	0.095
	^{31}P	81	0.136	0.131

[a] The dissociation constants, symbols, and p (mole fraction) values are the same as those used in Table 3.

TABLE 6

DISTANCES FROM SPIN-LABEL$_{1,2, \text{ or } 3}$ TO ^{1}H OF L-ORNITHINE, L-GLUTAMATE, IMP, AND UMP AND TO ^{31}P OF IMP AND UMP WITH CARBAMOYL-PHOSPHATE SYNTHETASE[a]

Ligand	Nucleus	Frequency (MHz)	$1/pT_{1p}$[b] (s^{-1})	r (Å)
L-Ornithine	^{1}H-β	100	4.42	≥14
	^{1}H-δ	100	3.40	≥15
	^{1}H-β	200	4.76	≥11
	^{1}H-δ	200	2.38	≥13
L-Glutamate	^{1}H-γ	100	4.22	≥14
	^{1}H-β	100	4.69	≥14
	^{1}H-γ	200	5.16	≥11
	^{1}H-β	200	6.10	≥11
IMP	^{1}H-2	100	2.78	≥15
	^{1}H-2′	100	1.74	≥16
	^{31}P	40	0.49	≥19
	^{31}P	81	0.49	≥16
UMP	^{1}H-4′	100	2.00	≥16
	^{1}H-5′	100	1.33	≥17
	^{31}P	40	0.33	≥20
	^{31}P	81	0.50	≥16

[a] Calculated from Eq. [2] using $\tau_c = 5.0 \times 10^{-9}$ s.

[b] $1/pT_{1p}$ was calculated using a 10% difference in T_1 in the presence of E-MTPO and enzyme alone.

specific sites. The $1/T_1$ data were obtained at two magnetic field strengths for most of the studies and are given in Tables 3, 4, and 5. From these data, one can see that the covalently attached spin-labels have no discernible effect within experimental error on the measured $1/T_1$ relaxation rates of the nuclei of each molecule. Thus, only limits of the internuclear distances can be calculated from these data. This was done using Eq. [2] and assuming a 10% maximum difference in T_1 values, i.e., the relative error in each T_1 measurement. Since no paramagnetic effect was observed with the three spin-labels, the frequency dependence of the T_{1p} data could not be used to determine τ_c. Instead, the indirect method using EPR linewidth measurements was used, giving a lower limit of τ_s of 5.0×10^{-9} s for all three of the E-MTPO systems (*23*). This value was used to calculate a limit for the E-MTPO$_{1,2, \text{and } 3}$ nuclei distances and these are given in Table 6.

DISCUSSION

Carbamoyl-phosphate synthetase has been shown to require a free divalent cation as an absolute requirement for activity in addition to metal–ATP as a substrate. Also, this enzyme is activated by monovalent cations (*8*), and NH_4^+ as well as K^+ are considered to be positive allosteric modifiers of the enzyme's activity. Determining the spatial relationship between the monovalent cation activator site and the metal–ATP site was one of the goals of our current studies. The longitudinal relaxation rate of four cations, viz., $^6Li^+$, $^7Li^+$, $^{15}NH_4^+$, and $^{133}Cs^+$, were measured. For the lithium isotopes and ammonium, the M^+-Cr^{3+} distance was ~4.4 Å, while a shorter distance (3.4 Å) was obtained with Cs^+. Due to the uncertainty in these distance measurements, all the cations may form the same structural complex on the enzyme surface. However, it should be noted that the crystal ionic radii of Li^+ and Cs^+ are 0.86 and 1.84 Å, respectively, which may lead to subtle differences in the binding of these cations to the enzyme. The NMR data are not sensitive enough to detect small changes in structure of ~1 Å. The distance between the M^+ site and Cr^{3+} determined by our studies suggests that these ions are separated by one or two water molecules. Since the water molecules in the hydration shell of Cr^{3+} do not rapidly exchange, these water molecules may form hydrogen bonds with the water molecules of the M^+ ions. The important result is that the monovalent cation is close enough to the γ-P of ATP (*16*) that it may serve as a binding site for HCO_3^- and orient this molecule for attack on ATP in the first step of the reaction. Figure 1 presents a molecular model generated as described under Experimental Procedures using a computer fit to the distance data from this study and from our previous NMR data.

Based on the results of our earlier NMR study (*16*), the aim of which was to determine distance relationships among components of the substrate sites, we designed several new experiments to map the distance relationships among the active and allosteric sites. The approach we used for the experiments in this paper exploited the paramagnetic properties of the nitroxide spin-label MTPO ($S = 1/2$) which could be attached to each of three specific sites on the enzyme (*14, 15*). The rationale for these NMR experiments was predicated on our earlier results that

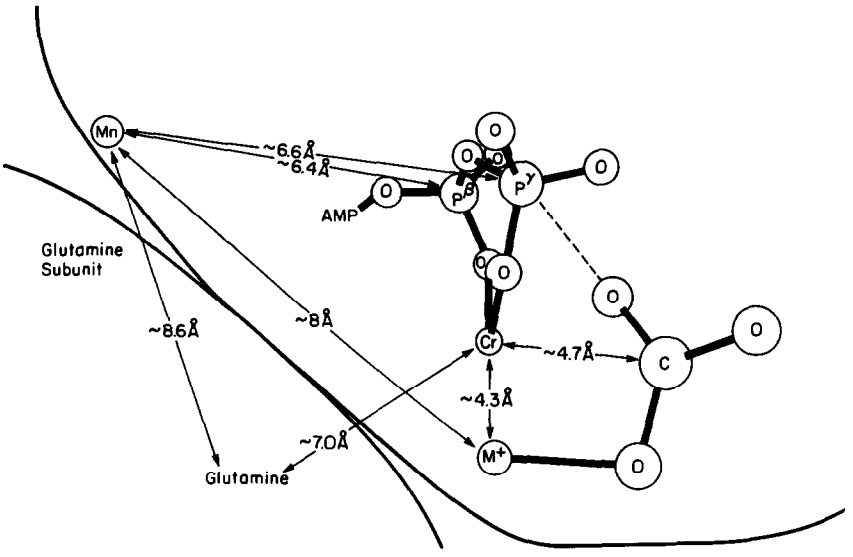

FIG. 1. Schematic drawing of carbamoyl-phosphate synthetase. The relative relationship of the various sites is based on magnetic resonance data and the crystallographic coordinates of Cr-pyrophosphate (*27*) as a model of Cr-ATP. The enzyme molecule is viewed with the glutamine binding subunit and the catalytic subunit along with the other components of the active site. The relative relationships among the glutamate, HCO_3^-, ATP, M^+, and M^{2+} sites are based on the NMR measurements in this paper and those in Ref. (*16*).

demonstrated that the sulfhydryl sites were ≥20 Å from the structural divalent cation site, but these data did not define the location of the ornithine site relative to these sites. Model building predicted that the ornithine site could be ~10 Å from either or both of the sulfhydryl sites on the large subunit and also predicted the closest possible distance relationships between these sites. Also, the glutamate site could be ~9 Å from the sulfhydryl sites based on model building. Thus, our experiments with spin-labeled probes could provide one of two types of positive results, i.e., (i) paramagnetic effects of the spin-labels on the nuclei of substrates and allosteric molecules that would lead to the calculation of inter-site distances, or (ii) lack of paramagnetic effects giving distance limits among the various sites.

The relaxation rate data with the MTPO spin-labels covalently attached to three different sulfhydryl groups show very small (if any) paramagnetic effects on the $1/T_1$ values of nuclei of ornithine, glutamate, IMP, or UMP. Thus, precise distances could not be measured and only limits of the distances between nitroxide labels and various nuclei were obtained. A 10% difference in the T_1 values was used as the minimum measurable change that could be determined and this value was used to calculate a minimum distance between loci. The glutamate and ornithine sites were found to be >11 Å from all three sulfhydryls, while the allosteric modifiers IMP and UMP were >15 Å from the nitroxides attached to the three sulfhydryl groups. From our previous fluorescence energy transfer experiments

(*17*) and also from our earlier EPR work (*14*), the three sulfhydryls were found to be >20 Å from the metal-ATP site(s) and the divalent cation site. From these results it was proposed that the sulfhydryl groups were on one "side" of the dimer [diameter of the large subunit is ~60 Å and diameter of the small subunit is ~40 Å (*16*)] and the active site was on the other "side." This conclusion is further substantiated by the current set of NMR experiments which suggests that the allosteric sites are spatially removed from the sulfhydryl sites and are quite likely on the active site "side" of the dimer. Similar NMR experiments like those described above were used by DeWolf *et al.* (*31*) to conclude that the catalytic and allosteric sites of AMP nucleosidase are >25 Å apart and thus spatially distinct.

In sum, the objective of these NMR studies with *E. coli* carbamoyl-phosphate synthetase was to continue our study to establish the topographical relationships among several loci at the active, allosteric, divalent, and monovalent cation sites utilizing various physical methods. The data in the present paper add new distance relationships between the monovalent cation activator site and the metal–ATP site(s) and define distance limits to the allosteric sites. Since several of these intersubunit sites are >20 Å apart and a crystal structure of this enzyme is not available, other probes will have to be employed (e.g., fluorescent probes for energy transfers measurements) to accomplish this goal. Our laboratory is engaged in such studies.

REFERENCES

1. Trotta, P. P., Burt, M. E., Haschemeyer, R. M., and Meister, A. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 2599–2603.
2. Matthews, S. L., and Anderson, P. M. (1972) *Biochemistry* **11,** 1176–1183.
3. Nyunoya, H., and Lusty, C. J. (1983) *Proc. Natl. Acad. Acad. Sci. USA* **80,** 4629–4633.
4. Anderson, P. M. (1977) *Biochemistry* **16,** 587–593.
5. Boettcher, B., and Meister, A. (1982) *J. Biol. Chem.* **257,** 13971–13976.
6. Anderson, P. M., and Meister, A. (1965) *Biochemistry* **4,** 2803–2809.
7. Anderson, P. M., and Meister, A. (1966) *Biochemistry* **5,** 3157–3163.
8. Trotta, P. P., Pinkus, L. M., Wellner, V. P., Estis, L., Haschemeyer, R. H., and Meister, A. (1973) *in* The Enzymes of Glutamine Metabolism (Prusiner, S., and Stadtman, E. R., eds.), pp. 431–482, Academic Press, New York.
9. Raushel, F. M., Anderson, P. M., and Villafranca, J. J. (1978) *Biochemistry* **17,** 5587–5591.
10. Raushel, F. M., and Villafranca, J. J. (1979) *Biochemistry* **18,** 3424–3429.
11. Raushel, F. M., and Villafranca, J. J. (1980) *Biochemistry* **19,** 3170–3174.
12. Wimmer, W. J., Rose, I. A., Powers, S. G., and Meister, A. (1979) *J. Biol. Chem.* **254,** 1854–1859.
13. Powers, S. G., and Meister, A. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 3020–3024.
14. Raushel, F. M., Rawding, C. J., Anderson, P. M., and Villafranca, J. J. (1979) *Biochemistry* **18,** 5562–5566.
15. Foley, R., Poon, J., and Anderson, P. M. (1971) *Biochemistry* **10,** 4562–4569.
16. Raushel, F. M., Anderson, P. M., and Villafranca, J. J. (1983) *Biochemistry* **22,** 1872–1876.
17. Kasprzyk, P. G., Anderson, P. M., and Villafranca, J. J. (1983) *Biochemistry* **22,** 1877–1882.
18. DePamphilis, M. L., and Cleland, W. W. (1973) *Biochemistry* **12,** 3714–3724.
19. Dunaway-Mariano, D. (1978) *Fed. Proc. Biol* **37,** 1420.
20. Solomon, I., and Bloembergen, N. (1956) *J. Chem. Phys.* **25,** 261–266.
21. Villafranca, J. J. (1982) *in* Methods in Enzymology (Packer, L., ed.), Vol. 87, pp. 180–197, Academic Press, New York.

22. WELLNER, V. P., ANDERSON, P. M., AND MEISTER, A. (1973) *Biochemistry* **12,** 2061–2066.
23. DWEK, R. A. (1973) *In* Nuclear Magnetic Resonance in Biochemistry, Oxford Univ. Press (Clarendon), London.
24. STUPER, A. J., BRUGGER, W. E., AND JURS, P. C. (1979) *in* Computer Assisted Studies of Chemical Structure and Biological Function, pp. 83–90, Wiley-Interscience, New York.
25. BRUGGER, W. E., AND JURS, P. C. (1975) *Anal Chem.* **47,** 781–784.
26. STUPER, A. J., AND JURS, P. C. (1976) *J. Chem. Inf. Comp. Sci.* **16,** 199–205.
27. MERRITT, E. A., SUNDARALINGAM, M., AND DUNAWAY-MARIANO, D. (1981) *J. Amer. Chem. Soc.* **103,** 3565–3567.
28. RAUSHEL, F. M., AND VILLAFRANCA, J. J. (1980) *J. Amer. Chem. Soc.* **102,** 6618–6619.
29. GUPTA, R. K., FUNG, C. H., AND MILDVAN, A. S. (1976) *J. Biol. Chem.* **251,** 2421–2430.
30. BALAKRISHNAN, M. S., AND VILLAFRANCA, J. J. (1978) *Biochemistry* **5,** 3157–3163.
31. DEWOLF, W. E., JR., MARKHAM, G. D., AND SCHRAMM, V. L. (1980) *J. Biol. Chem.* **255,** 8210–8215.