Paramagnetic Probes for Carbamoyl-Phosphate Synthetase: Metal Ion Binding Studies and Preparation of Nitroxide Spin-Labeled Derivatives†

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ABSTRACT: Carbamoyl-phosphate synthetase from *Escherichia coli* has been shown to require a free divalent cation for activity in addition to the metal ions needed to complex the nucleotides used in the reaction. Activity with Mg^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, and Zn^{2+} has been demonstrated, but no activity could be shown with Ca^{2+} or Ni^{2+} in the overall reaction. A double-reciprocal plot of velocity vs. [MgATP] at various $[Mg^{2+}]_{free}$ is equilibrium-ordered with Mg^{2+} binding before MgATP. Thus, the free metal ion site must be populated for catalysis to occur and therefore free Mg^{2+} is an absolute requirement for enzymatic activity and not just a positive allosteric effector. The dissociation constant for free Mg^{2+} from this kinetic study is 4.2 ± 0.7 mM. Metal ion binding studies to the free metal ion site were conducted by using electron paramagnetic resonance (EPR) techniques. These studies show one tight Mn^{2+} site with a dissociation constant of 35 ± 3 μM and some nonspecific weaker sites. By competition experiments with Mn^{2+}, the binding constants at pH 7.5 for the other metal ions are the following: Mg^{2+}, 5.2 mM; Co^{2+}, 270 μM; Ni^{2+}, 960 μM; Zn^{2+}, 220 μM; Cd^{2+}, 220 μM; Ca^{2+}, 9.4 mM; Gd^{3+}, 140 μM. Since the dissociation constant for Mg^{2+} from the enzyme is in reasonable agreement in both the kinetic and binding experiments, they most likely refer to the same site. The substitution-inert ATP analogues Cr^{3+}ATP and $Co^{3+}(NH_3)_4$ATP are competitive inhibitors vs. MgATP at pH 6.5. Three different sulfhydryl groups on carbamoyl-phosphate synthetase have been derivatized with 3-maleimido-2,2,5,5-tetramethylpyrrolidinyl-1-oxy. Two of the sulfhydryl are located on the large subunit, and one is located on the small glutamine binding subunit. The EPR spectra of the two spin-labels located on the large subunit are insensitive to additions of substrates or allosteric modifiers. The mobility of the spin-label on the glutamine subunit is restricted upon the binding of glutamine and enhanced upon the binding of MgATP. The effect by MgATP shows that binding of substrates at one subunit can cause conformational changes at the other subunit. By use of the method of Leigh [Leigh, J. S. (1970) *J. Chem. Phys. 52*, 2680], it has been shown that all three nitroxide groups are ≥20 Å away from the Mn^{2+} and MnATP sites.

Carbamoyl-phosphate synthetase from *Escherichia coli* catalyzes the reaction

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

The enzyme has a molecular weight of ~180 000 and is composed of two nonidentical subunits (Matthews & Anderson, 1972; Trotta et al., 1971). The smaller subunit of molecular weight 48 000 contains the binding site for glutamine. The larger subunit of molecular weight 130 000 contains the binding sites for the rest of the substrates and allosteric modifiers (Trotta et al., 1971).

Because *E. coli* carbamoyl-phosphate synthetase catalyzes a reaction with four substrates and five products, it has been an interesting enzyme to study by using kinetic techniques (Raushel et al., 1978; Raushel & Villafranca, 1979). Since this enzyme also has at least 10 separate ligand binding sites for substrates, activators, and allosteric modifiers, it presents a challenging problem to map out these sites by using magnetic resonance techniques. However, to use these techniques there must be a number of paramagnetic centers. This communication describes the preparation and the usefulness of a number of such probes.

Matthews & Anderson (1972) and Foley et al. (1971) have shown that three different sulfhydryl groups can be modified with *N*-ethylmaleimide by using slightly different reaction conditions. Two of these sulfhydryl groups are on the large subunit and one is on the small subunit. In this report these sulfhydryl groups have been successfully modified with nitroxide spin-labels in order to probe the mode of subunit–subunit interactions and allosteric regulation. They have also been used as paramagnetic centers for distance determinations.

Preliminary evidence has indicated the requirement for a free divalent cation that is essential for full activity of the enzyme (Raushel et al., 1978). The metal ion specificity and mechanism of kinetic activation have now been determined by using EPR and kinetic approaches.

Materials and Methods

Carbamoyl-phosphate synthetase was isolated from *E. coli* according to the method of Matthews & Anderson (1972) to a specific activity of 180–210 μmol/h. 3-Maleimido-2,2,5,5-tetramethylpyrrolidinyl-1-oxy was purchased from Syva Research Chemicals. CrATP was prepared according to the procedure of DePamphilis & Cleland (1973) & Dunaway-Mariano (1978). $Co(NH_3)_4$ATP was made according to Cornelius et al. (1977).

Enzyme Assays. Carbamoyl-phosphate synthetase activity was measured spectrophotometrically by using a pyruvate kinase–lactate dehydrogenase coupling system as previously described (Raushel et al., 1978). When Zn^{2+} was used as the sole divalent cation, the activity was measured by stopping the reaction with CCl_4 and then determining the ADP concentration by using pyruvate kinase and lactate dehydrogenase in a system containing Mg^{2+}.

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Data Analysis. The kinetic data were analyzed by using the Fortran programs of Cleland (1967). Data conforming to an equilibrium-ordered initial velocity pattern were fit to eq 2 and competitive inhibition patterns to eq 3.

$$v = \frac{VAB}{K_{ia}K_b + K_bA + AB} \quad (2)$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \quad (3)$$

Preparation of Spin-Labeled Carbamoyl-Phosphate Synthetase. The spin-labeled derivatives of carbamoyl-phosphate synthetase were prepared by using the procedures of Foley et al. (1971) and Matthews & Anderson (1972) for the preparation of *N*-ethylmaleimide derivatives of three different sulfhydryl groups on the enzyme. In accordance with the nomenclature of Foley et al. (1971), these derivatives have been named CPS–spin-label$_1$, CPS–spin-label$_2$, and CPS–spin-label$_3$.

CPS–Spin-Label$_1$. In a volume of 0.50 mL, carbamoyl-phosphate synthetase (5.1 mg/mL) was incubated with 130 mM potassium phosphate buffer (pH 7.5), 13 mM L-ornithine, and 0.35 mM 3-maleimido-2,2,5,5-tetramethylpyrrolidinyl-1-oxy at 17 °C for 2.0 h. The reaction mixture was then extensively diafiltered at 4 °C with an Amicon ultrafiltration apparatus using 40 mM Hepes[1] (pH 7.5), 100 mM KCl, and 10 mM ornithine to remove the phosphate and unreacted spin-label. The enzyme retained 100% of its original activity.

CPS–Spin-Label$_2$. For derivatization of the second sulfhydryl group with a spin-label, the first sulfhydryl had to be reacted with *N*-ethylmaleimide according to the procedure of Matthews & Anderson (1972). In a volume of 0.50 mL, carbamoyl-phosphate synthetase (8.6 mg/mL) was incubated with 130 mM potassium phosphate buffer (pH 7.5), 10 mM ornithine, and 0.44 mM *N*-ethylmaleimide for 1 h at 17 °C. The unreacted *N*-ethylmaleimide was removed by diafiltration using 130 mM potassium phosphate and 10 mM ornithine with the Amicon ultrafiltration apparatus. The enzyme at this stage retained 100% of its original activity. Then in a volume of 1.0 mL, 3.4 mg of the *N*-ethylmaleimide-derivatized enzyme was incubated with 10 mM ATP, 10 mM $MgCl_2$, 10 mM HCO_3^-, and 0.30 mM 3-maleimido-2,2,5,5-tetramethyl-pyrrolidinyl-1-oxy for 2 h at 17 °C and then diafiltered with 40 mM Hepes (pH 7.5), 100 mM KCl, and 10 mM ornithine. This derivative was 6% as active as the unreacted carbamoyl-phosphate synthetase compared with 14% for the *N*-ethylmaleimide derivative as reported by Foley et al. (1971).

CPS–Spin-Label$_3$. For derivatization of the third sulfhydryl with a spin-label, two SH groups were first reacted with *N*-ethylmaleimide according to the procedure of Foley et al. (1971) and the enzyme was then incubated with the nitroxide spin-label. In a volume of 1.0 mL, carbamoyl-phosphate synthetase (5.1 mg/mL), 130 mM potassium phosphate (pH 7.5), 10 mM ornithine, 10 mM ATP, 10 mM HCO_3^-, 10 mM $MgCl_2$, and 0.75 mM *N*-ethylmaleimide were incubated for 1 h at 17 °C and then diafiltered with 130 mM potassium phosphate, pH 7.5. In a volume of 1.0 mL, 4.1 mg of this modified enzyme derivative was incubated with 130 mM potassium phosphate, pH 7.5, and 0.75 mM 3-maleimido-2,2,5,5-tetramethylpyrrolidinyl-1-oxy for 2 h at 17 °C followed by diafiltration with 40 mM Hepes (pH 7.5), 10 mM ornithine, and 100 mM KCl at 4 °C. Two percent of the original synthetase activity remained.

[1] Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; CPS, carbamoyl-phosphate synthetase.

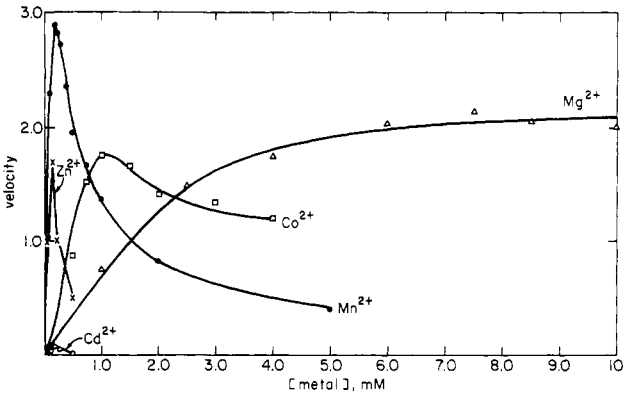

FIGURE 1: Variation of enzymatic activity with various concentrations of added divalent cations. Experimental conditions: 0.1 mM ATP, 10 mM HCO_3^-, 10 mM glutamine, 10 mM ornithine, 50 mM Hepes (pH 7.5), 100 mM KCl. Additional details are given in the text. Added divalent cation: Mg^{2+} (△), Mn^{2+} (●), Co^{2+} (□), Cd^{2+} (○), Zn^{2+} (×). The velocities are in arbitrary units.

EPR Spectra. All EPR spectra were taken on a Varian E-12 spectrometer operating at a frequency of 9 GHz. Samples were placed in capillary tubes of 1-mm i.d., and the temperature was controlled at 25 ± 1 °C with a Varian temperature controller. For all spectra a modulation amplitude of 4 G and a power level of 100 mW were used.

Results

Activation by Divalent Cations. Shown in Figure 1 is a plot of activity vs. amount of added divalent cations. The ATP concentration used in these experiments is 0.10 mM. With Mg^{2+} as the divalent cation the enzyme is not saturated until at least 3.0–6.0 mM. This suggests that the enzyme requires a free divalent cation in addition to the metal ion that binds ATP since the ATP should be fully complexed with Mg^{2+} at less than 1.0 mM ($K_d \simeq 26$ μM at 25 °C) (Sillen & Martel, 1971). The optimal level for activity with Co^{2+}, Zn^{2+}, and Mn^{2+} occurs at much lower concentrations than for Mg^{2+}, suggesting tighter binding of these metal ions to carbamoyl-phosphate synthetase. Since the dissociation constants of Co^{2+} and Mn^{2+} to ATP are 22 and 13 μM, respectively (Sillen & Martel, 1971), the binding of metal ion to nucleotide cannot be a factor in these experiments. Cd^{2+} gave slight activity with the enzyme, and Ni^{2+} was inactive under these conditions.

From the above experiments it appears that carbamoyl-phosphate synthetase requires both a free divalent cation and a divalent cation to complex the nucleotide substrate(s). Therefore, it should be possible to design an experiment in which the ATP is complexed with Mg^{2+} and the enzymatic activity is stimulated by adding low levels of other more tightly binding divalent cations. This was accomplished with a concentration of 0.1 mM ATP and 1.0 mM Mg^{2+}, and Figure 2 shows the results of adding Cd^{2+}, Zn^{2+}, or Mn^{2+}. Figure 3 presents the results of adding Ni^{2+}, Ca^{2+}, Mg^{2+}, or Co^{2+} in the presence of the Mg^{2+} and ATP levels as described above. All of the divalent cations that activated the enzyme alone (without Mg^{2+}) also stimulated activity in these mixed metal ion experiments at low levels of Mg^{2+}. Ni^{2+} and Ca^{2+} did not activate but did inhibit.

Initial Velocity Pattern. Since free Mg^{2+} is required for maximal activity and its binding constant to free enzyme is weaker than to ATP (see next section), it is possible to determine its mode of activation through the use of an initial velocity study. Shown in Figure 4 is the double-reciprocal plot of velocity vs. [MgATP] at various $[Mg^{2+}]_{free}$. The pattern is of the equilibrium-ordered type (Cleland, 1970) with free

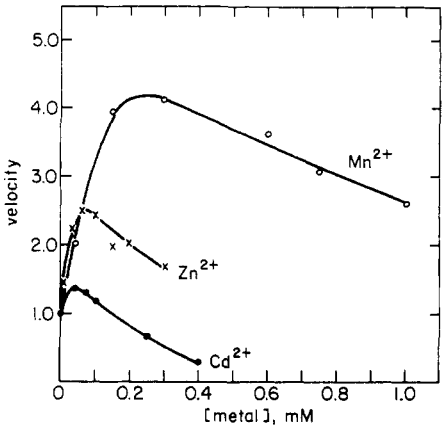

FIGURE 2: Stimulation of enzymatic activity upon addition of various divalent cations. Experimental conditions: 0.1 mM ATP, 1.0 mM Mg^{2+}, 10 mM HCO_3^-, 10 mM glutamine, 10 mM ornithine, 50 mM Hepes (pH 7.5), 100 mM KCl. Added divalent cation: Mn^{2+} (○), Zn^{2+} (×), Cd^{2+} (●). Additional details are given in the text. The velocities are in arbitrary units.

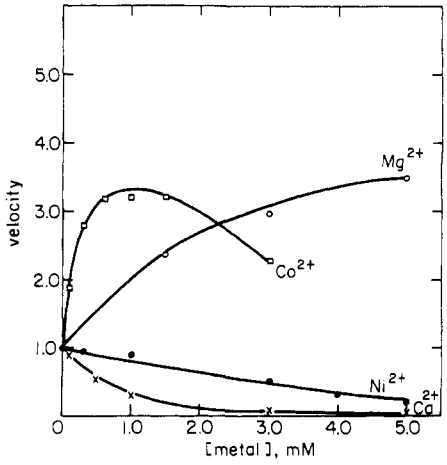

FIGURE 3: Stimulation or inhibition of enzymatic activity upon addition of various divalent cations. Experimental conditions are the same as in Figure 2. Added divalent cations: Mg^{2+} (○), Co^{2+} (□), Ni^{2+} (●), Ca^{2+} (×). The velocities are in arbitrary units.

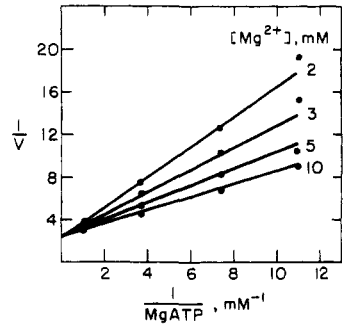

FIGURE 4: Variation of enzymatic activity with concentration of Mg^{2+}_{free} and MgATP. The data were fit to eq 2. The velocities are in arbitrary units.

Mg^{2+} binding before MgATP. From a fit to eq 2, the K_{ia} for $[Mg^{2+}]_{free}$ is 4.2 ± 0.7 mM.

Binding of Mn^{2+}. The dissociation constant for the binding of Mn^{2+} to the enzyme was determined by the EPR technique of Cohn & Townsend (1954). Shown in Figure 5 is the Scatchard plot for the binding of Mn^{2+} to carbamoyl-phosphate synthetase at pH 7.5. Analysis of the data shows approximately one metal ion binding site with a dissociation constant of 35 ± 3 μM. Data taken at higher Mn^{2+} concentrations (data not shown) demonstrate additional nonspecific

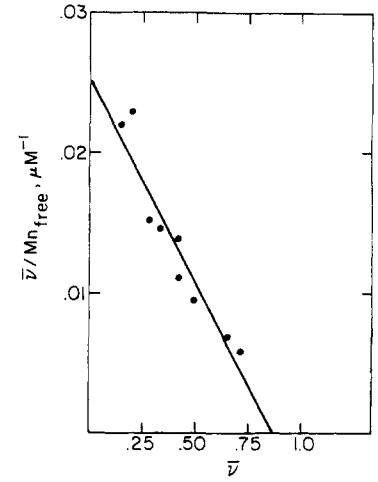

FIGURE 5: Scatchard plot for the binding of Mn^{2+} to carbamoyl-phosphate synthetase. Expermental conditions: 50 mM Hepes (pH 7.5), 100 mM KCl, 10 mM ornithine, 50 μM carbamoyl-phosphate synthetase. $\bar{\nu}$ is moles of Mn^{2+} bound divided by moles of enzyme, and Mn_{free} is the concentration of free Mn^{2+}.

Table I: Dissociation Constants of Metal Ions to Carbamoyl-Phosphate Synthetase

metal ion	K_D (μM)
Mn^{2+}	35 ± 3
Mg^{2+}	5200 ± 400, 4200 ± 700[a]
Co^{2+}	270 ± 20
Ni^{2+}	960 ± 14
Zn^{2+}	220 ± 14
Cd^{2+}	220 ± 10
Ca^{2+}	9400 ± 700
Gd^{3+}	140 ± 16

[a] From the initial velocity pattern in Figure 4.

(probably inhibitory) weaker binding by Mn^{2+} at other sites. The binding constant was also determined at pH 6.5 in 50 mM Pipes buffer and is 112 ± 18 μM.

Binding of Other Metal Ions. The binding constants of other metal ions to carbamoyl-phosphate synthetase were determined by competition between these metal ions and Mn^{2+} for the enzyme. The data were gathered by using the previously described EPR technique and analyzed by the method of Gupta & Benovic (1978). The binding constants for these other metal ions to carbamoyl-phosphate synthetase are shown in Table I.

Inhibition by CrATP and CoATP. The substitution-inert analogues of MgATP, bi- and tridentate CrATP, and β,γ-bidentate $Co(NH_3)_4ATP$ were tested as competitive inhibitors of MgATP with carbamoyl-phosphate synthetase. CrATP is paramagnetic and $Co(NH_3)_4ATP$ is diamagnetic, and each is useful for structural studies of ATP-utilizing enzymes. The analogues were found to be good competitive inhibitors vs. MgATP at pH 6.5 in 50 mM Pipes buffer. The K_{is} for both the bi- and tridentate CrATP was 0.45 ± 0.05 mM while the K_{is} for $Co(NH_3)_4ATP$ was 1.7 ± 0.2 mM. The data were fit by using eq 3. The K_m for MgATP under these conditions is 0.20 mM.

Modification of Carbamoyl-Phosphate Synthetase by 3-Maleimido-2,2,5,5-tetramethylpyrrolidinyl-1-oxy. Shown in Figure 6 are the EPR spectra of carbamoyl-phosphate synthetase after modification (see Materials and Methods) by reaction with 3-maleimido-2,2,5,5-tetramethylpyrrolidinyl-1-oxy. All three spin-labeled derivatives show characteristically "strongly immobilized" spectra (Dwek, 1973) with rotational correlation times, τ_R, that are $\geq 5 \times 10^{-9}$ s. The separation

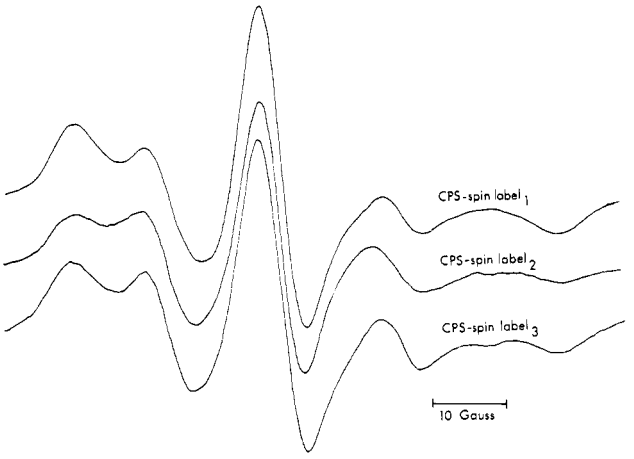

FIGURE 6: EPR spectra for the three spin-labeled derivatives of carbamoyl-phosphate synthetase. Experimental conditions: carbamoyl-phosphate synthetase, 3.0 mg/mL; 40 mM Hepes, pH 7.5; 100 mM KCl; 10 mM ornithine. Additional details are given in the text.

of the outermost peaks is ~65 G. For spin-labeled derivatives 1 and 3 there is no change in the EPR spectrum upon addition of Mg^{2+}, glutamine, ATP, ADP, UMP, P_i, Mn^{2+}, MgATP, or MnATP. There was also no effect on the EPR spectrum of CPS–spin-label$_2$ upon addition of P_i, UMP, ATP, ADP, Mn^{2+}, or Mg^{2+}. However, as shown in Figure 7, the addition of 10 mM MgATP substantially alters the spectrum of CPS–spin-label$_2$. The highest field peak is lost and the center peak increases in height by ~36%. The opposite effect is seen with the addition of glutamine or glutamate (spectrum not shown). The center peak diminishes in height and the outermost peaks are increased slightly in intensity. MnATP produces the same effect as MgATP. As will be discussed later, the paramagnetic effect of Mn^{2+} on the amplitude of the nitroxide spin-label spectrum can be used to calculate distances between Mn^{2+} and a spin-label (Leigh, 1970).

Discussion

Divalent Metal Ion Dependence. The dependence of enzyme activity on Mg^{2+} concentration at low ATP concentration (Figure 1) clearly demonstrates that carbamoyl-phosphate synthetase requires a free divalent cation for full activity in addition to the metal ion needed to complex the nucleotide(s) substrate. The initial velocity pattern of $[Mg^{2+}]_{free}$ vs. [MgATP] is of the equilibrium-ordered type, which indicates binding of Mg^{2+} first followed by the binding of MgATP (Cleland, 1970). At saturating MgATP the K_m for Mg^{2+} is essentially zero since MgATP is able to trap Mg^{2+} on the enzyme. These results suggest that free Mg^{2+} is an absolute requirement for enzymatic activity and not just a positive allosteric effector. Elliot & Tipton (1974) have also shown an equilibrium-ordered pattern for $[Mg^{2+}]_{free}$ vs. [MgATP] with the beef liver carbamoyl-phosphate synthetase.

A site for an uncomplexed divalent cation is confirmed by the observation of one tight binding site for Mn^{2+} by using EPR techniques. The dissociation constant for Mg^{2+} to this site is 4–5 mM, as determined by both kinetic experiments (Figure 4) and by competition with Mn^{2+} using EPR. Thus, this site has an approximately 100-fold preference for Mn^{2+} (K_D = 35 μM) over Mg^{2+}. The tight binding of Mn^{2+} also shows up in the kinetics since at low ATP concentration (0.1 mM) the enzyme is saturated with Mn^{2+} at ~0.2 mM. With Mn^{2+} as the sole divalent cation there is inhibition of enzyme activity at higher concentrations of Mn^{2+}. This inhibition is probably caused by binding to some additional inhibitor metal ion sites. These binding sites are also evident in the EPR experiments with Mn^{2+} and appear to be nonspecific with much higher dissociation constants.

Other metal ions such as Co^{2+}, Zn^{2+}, and Cd^{2+} also bind at both the divalent cation site and the nucleotide site. All of these metal ions have dissociation constants of ~250 μM to the free divalent cation site of carbamoyl-phosphate synthetase. It was also interesting to find Zn^{2+} as an activator of carbamoyl-phosphate synthetase since Zn^{2+} is usually not a good activator of kinases (Morrison & Heyde, 1972). It has, however, been found to be active with pyridoxal kinase (White & Demsey, 1970; Neary & Diven, 1970) and phosphoglycerate kinase (Larsson-Raznikiewicz, 1970).

At present it is not possible to determine the function of this divalent cation site. However, magnetic resonance studies are now in progress with the aim of mapping the active and allosteric binding sites, and possibly once the placement of this divalent cation site relative to the other sites is known, a function for this divalent cation can be established.

Spin-Labeled Derivatives of Carbamoyl-Phosphate Synthetase. Three different spin-labeled derivatives of carbamoyl-phosphate synthetase have been made by reacting maleimide spin-labels with three different sulfhydryl groups on the enzyme. The exact labeling conditions and subunit location of the reactive sulfhydryls have been extensively studied by Matthews & Anderson (1972) and Foley et al. (1971) by using *N*-ethylmaleimide. In this report it is assumed that the spin-labeled maleimides react in an analogous manner to *N*-ethylmaleimide. There is no evidence at present to indicate that the spin-labeled maleimide reacts any differently than *N*-ethylmaleimide.

The first sulfhydryl group modified is located on the large subunit (M_r 130 000) and is derivatized in the presence of L-ornithine (CPS–spin-label$_1$). The EPR spectrum for this derivative can be characterized as "strongly immobile" (Dwek, 1973). There is no change in the spectrum upon addition of any of the substrates or modifiers of the enzyme, suggesting that any conformational changes produced by the ligands are not manifest at the spin-labeled site. However, strongly immobile spectra such as these are not very sensitive to small conformational changes (Taylor et al., 1971).

Leigh (1970) has pointed out that the distance between the spin-labeled site and a second paramagnetic center can be determined by measuring the diminution of the spin-label spectrum upon the addition of Mn^{2+} or some other paramagnetic species. When Mn^{2+} or MnATP was added there was no detectable diminution of the EPR spectrum of CPS–spin-label$_1$ when compared to an identical solution with Mg^{2+} or MgATP, respectively. By use of the equations derived by Leigh (1970) assuming (1) that the correlation time for Mn^{2+} in the enzyme complex is $\geq 3 \times 10^{-10}$ s and (2) that a 7% reduction of the center peak height could be detected, the Mn^{2+} to nitroxide distance is greater than 20 Å for both the free Mn^{2+} and MnATP sites. For a correlation time of 3×10^{-9} s [found for many enzyme–Mn^{2+} complexes (Dwek, 1973)], the distance is ≥27 Å.

The second modified sulfhydryl group is located on the small (glutamine binding subunit) subunit (M_r 48 000). This sulfhydryl group is totally unreactive in the absence of MgATP plus HCO_3^-. The EPR spectrum for this derivative (CPS–spin-label$_2$) is again "strongly immobilized", but it is not identical with the other derivatives. Significant changes in the EPR signal are detected on binding of MgATP to the large subunit. The resulting spectrum is only "moderately immobilized", which is due to the nitroxide having more rotational freedom (McConnell & McFarland, 1970). This is

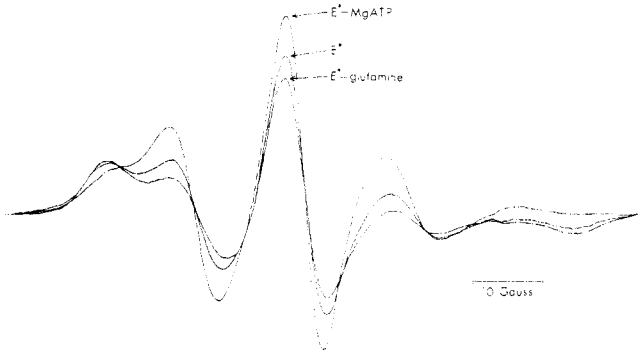

FIGURE 7: EPR spectra for CPS–spin-label$_2$ upon addition of MgATP and glutamine. Experimental conditions: CPS–spin-label$_2$ (3.0 mg/mL) with no additions (E'); CPS–spin-label$_2$ (3.0 mg/mL) plus 10 mM MgATP and 10 mM Mg^{2+} (E'–MgATP); CPS–spin-label$_2$ (3.0 mg/mL) and 20 mM glutamine (E'–glutamine). Additional details are given in the text.

fully consistent with the conditions needed to derivatize this sulfhydryl. The addition of MgATP is needed to expose or make more accessible this sulfhydryl group which in turn should affect the rotational ability of the nitroxide once it is covalently attached to the protein.

The addition of L-glutamine (which binds to the small subunit) to CPS–spin-label$_2$ has the opposite effect (Figure 7). The center line diminishes and the outermost peaks intensify slightly. The effect by MgATP shows that binding of ligands to one subunit can cause conformational changes on the other subunit, as has been observed by others (Anderson & Carlson, 1975; Trotta et al., 1974). These conformational changes probably make it possible for ammonia transfer to occur without uncoupling of the two sites.

There was also no diminution of the EPR spectrum upon the addition of Mn^{2+} to CPS–spin-label$_2$, and the effect on adding MnATP was similar to adding MgATP. Thus, the Mn^{2+} and MnATP sites are greater than 20 Å away from this nitroxide group. However, conformational changes are detected with this enzyme derivative over a long distance.

The third sulfhydryl group that was modified is located on the large subunit (Matthews & Anderson, 1972). For modification of this sulfhydryl group, the first two SH groups were derivatized with *N*-ethylmaleimide in the presence of ornithine and MgATP. The third sulfhydryl group was then modified with nitroxide in the presence of UMP (Matthews & Anderson, 1972). The EPR spectrum shows a "strongly immobilized" nitroxide group, and there is no effect on adding any substrates or allosteric modifiers. This SH group must also be >20 Å from the Mn^{2+} and MnATP sites. Foley et al. (1971) have shown that when the third sulfhydryl group is modified with *N*-ethylmaleimide the rate of synthesis of ATP from ADP and carbamyl phosphate catalyzed by the enzyme is increased twofold although the rate for the forward reaction is greatly reduced. This does, however, show that the substrates will bind to enzyme with the third sulfhydryl group modified and indicates that the absence of any detectable effect on the EPR spectrum of CPS–spin-label$_3$ by the various ligands is not caused by lack of binding. We have also attempted to modify a fourth SH group. This sulfhydryl group is located on the small subunit in the glutamine binding site. This SH group is thought to react with glutamine to form a thioester linkage (Wellner et al., 1973). The labeling of this sulfhydryl group with a nitroxide spin-label would then permit the determination of the distance between the two binding sites on the large and small subunits. Thus far, all attempts to modify this sulfhydryl group have been unsuccessful.

In summary, *E. coli* carbamoyl-phosphate synthetase has been shown to have an absolute requirement for a free divalent cation for activity. Mn^{2+}, Co^{2+}, Cd^{2+}, Zn^{2+}, and Mg^{2+} are active with the enzyme at both the free divalent cation site and the metal–nucleotide site(s). The enzyme was shown to bind 1 equiv of Mn^{2+} with a dissociation constant of ~35 μM. Mg^{2+} binds to this site approximately 100-fold weaker.

Three different sulfhydryl groups have been modified with a nitroxide spin-label. By use of Leigh's (1970) theory, it has been demonstrated that all three spin-labels are >20 Å away from the Mn^{2+} and MnATP sites. The SH group on the glutamine binding subunit is sensitive to the binding of MgATP and glutamine. MgATP increases the mobility of the spin-label, and glutamine decreases the mobility.

The nitroxide groups are stable free radicals and can be used as paramagnetic probes in NMR experiments for distance measurements. Such experiments are now underway with Mn^{2+}, CrATP, and the nitroxides as multiple paramagnetic probes to map the spatial relationships among the substrate and allosteric sites on this allosteric enzyme.

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