Goldsack, D. E., Eberlein, W. S., & Alberty, R. A. (1966) *J. Biol. Chem. 241*, 2653–2660.
Imamura, T., Baldwin, T. O., & Riggs, A. (1972) *J. Biol. Chem. 247*, 2785–2797.
Izatt, R. M., Christensen, J. J., Pack, R. T., & Bench, R. (1962) *Inorg. Chem. 1*, 828.
Job, D., Zeba, T., Puppo, A., & Rigaud, J. (1980) *Eur. J. Biochem. 107*, 491–500.
Kandler, R. L., & Satterlee, J. D. (1983) *Comp. Biochem. Physiol., B: Comp. Biochem. 75B*, 499–503.
Kandler, R. L., Constantinidis, I., & Satterlee, J. D. (1984) *Biochem. J. 226*, 131–138.
Kassner, R. J., Kykta, J. G., & Cusanovich, M. A. (1985) *Biochim. Biophys. Acta 831*, 155–158.
Padlan, E. A., & Love, W. E. (1974) *J. Biol. Chem. 249*, 4067–4078.
Parkhurst, L. J., Sima, P., & Goss, D. J. (1980) *Biochemistry 19*, 2688–2692.
Seamonds, B. (1971) in *Probes of Structure and Function of Macromolecules and Membranes* (Chance, R., Yonetani, T., & Mildvan, A. S., Eds.) Vol. II, pp 317–320, Academic, New York.
Seamonds, B., & Forster, R. E. (1972) *Am. J. Physiol. 223*, 734–738.
Seamonds, B., Forster, R. E., & George, P. (1971) *J. Biol. Chem. 246*, 5391–5397.
Seamonds, B., McCray, J. A., Parkhurst, L. J., & Smith, P. D. (1976) *J. Biol. Chem. 254*, 2579–2583.
Smith, R. M., & Martell, A. E. (1976) *Critical Stability Constants*, Vol. 4, pp 26, Plenum, New York.
Vega-Catalan, F. J., Odeyemi, O. J., & Okonjo, K. O. (1986) *J. Biol. Chem. 261*, 10576–10581.

Interactive Binding between the Substrate and Allosteric Sites of Carbamoyl-Phosphate Synthetase†

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ABSTRACT: The interaction between *Escherichia coli* carbamoyl-phosphate synthetase (CPS) and a fluorescent analogue of an allosteric effector molecule, 1,N^6-ethenoadenosine 5′-monophosphate (ε-AMP), has been detected by using fluorescence techniques and kinetic measurements. From fluorescence anisotropy titrations, it was found that ε-AMP binds to a single site on CPS with K_d = 0.033 mM. The nucleotide had a small activating effect on the rate of synthesis of carbamoyl phosphate but had no effect on the K_m for ATP. To test whether ε-AMP binds to an allosteric site, allosteric effectors (UMP, IMP, and CMP), known to bind at the UMP/IMP site, were added to solutions containing the ε-AMP–CPS complex. With addition of these effector molecules, a progressive decrease of the fluorescence anisotropy was observed, indicating that bound ε-AMP was displaced by the allosteric effectors examined. From these titrations, the dissociation constants for UMP, IMP, CMP, ribose 5-phosphate, 2-deoxyribose 5-phosphate, and orthophosphate were determined. When MgATP, a substrate, was employed as a titrant, the observed decrease in anisotropy was consistent with the formation of a ternary complex (ε-AMP–CPS–MgATP). The effect of ATP binding, monitored at the allosteric site, was magnesium dependent, and free magnesium in solution was required to obtain a hyperbolic binding isotherm. Solvent accessibility of ε-AMP in binary (ε-AMP–CPS) and ternary (ε-AMP–CPS–MgATP) complexes was determined from acrylamide quenching, showing that the base of ε-AMP is well shielded from the solvent even in the presence of MgATP. Using the theory of ligand binding [Weber, G. (1975) *Adv. Protein Chem. 29*, 1–83], we computed the free energy of coupling between MgATP and ε-AMP to be +0.48 kcal/mol. Thus, the experiments described in this paper provide evidence for antagonism (anticooperative interaction) between the substrate and allosteric sites and suggest that this interaction provides a method of regulating the physiological activity of CPS.

The first step of the biosynthesis of pyrimidine nucleotides in *Escherichia coli* is the formation of carbamoyl phosphate from glutamine, bicarbonate, and two molecules of ATP. Since carbamoyl phosphate is also utilized by the arginine pathway, the enzyme that performs its synthesis, carbamoyl-phosphate synthetase (CPS),[1] is regulated by a number of metabolites; these include ammonia, potassium ions, ornithine, and IMP which activate CPS and UMP which is an allosteric inhibitor of the enzyme.

The enzyme is composed of two nonidentical subunits (Matthews & Anderson, 1972). The small subunit (M_r 48 000) has the glutamine binding site whereas the large subunit (M_r 130 000) possesses binding sites for ATP, bicarbonate, and allosteric effectors (Trotta et al., 1971; Matthews & Anderson, 1972).

NMR, EPR, and fluorescence energy-transfer experiments in this laboratory have established a partial topographical map of the enzyme based on point-to-point vectors between various binding loci (Raushel et al., 1979, 1983; Kasprzyk et al., 1983).

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[1] Abbreviations: ε-AMP, 1,N^6-ethenoadenosine 5′-monophosphate; CPS, carbamoyl-phosphate synthetase; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate.

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However, the distance between any of the three reactive thiols (Foley et al., 1971) and the UMP binding site was out of the range of these methods, and only a lower limit could be obtained (Kasprzyk et al., 1985). The spatial relationship of this allosteric binding site with respect to other loci on the enzyme and the molecular mechanism by which the interactive binding of ATP and UMP take place are unknown. It has been suggested that the IMP binding site may overlap with the UMP binding site (Boettcher & Meister, 1981, 1982) yet IMP activates and UMP inhibits the enzyme.

The regulation of the enzyme activity is accomplished by a direct influence exerted on CPS by a number of inhibitors and activators (Anderson & Meister, 1966; Trotta et al., 1974; Anderson, 1977a) and by a reversible polymerization–depolymerization process that involves ligand-dependent shifting of the equilibrium between several active enzyme forms (Anderson, 1977a, 1984, 1986; Powers et al., 1980).

In this paper, we present the results of applying a unique method of monitoring the interaction between the ATP and the UMP/IMP binding sites utilizing fluorescence properties of 1,N^6-ethenoadenosine 5′-monophosphate (ϵ-AMP). Using this probe, we were able to provide details regarding the energetics of the interaction between the ATP binding site and the allosteric effector sites.

Experimental Procedures

Materials and Methods. Carbamoyl-phosphate synthetase was isolated from *Escherichia coli* according to the method of Matthews and Anderson (1972). Its specific activity was 150–200 μmol h^{-1} mg^{-1}. Electrophoresis-grade acrylamide was purchased from Bio-Rad. Other nucleotides, substrates, and coupling enzymes were obtained from Sigma. All solutions were passed through Amicon 0.45-μm microporous filters before use.

Enzyme Assays. The activity of carbamoyl-phosphate synthetase was measured by using a pyruvate kinase–lactate dehydrogenase system; the reaction was followed at 340 nm with the use of a Hitachi Model 100-80 UV/VIS spectrophotometer. Each 1-mL cuvette contained 50 mM Hepes buffer, pH 7.5, 100 mM KCl, 10 mM L-Gln, 20 mM $KHCO_3$, 1 mM phospho(enol)pyruvate, 0.2 mM NADH, 40 μg each of pyruvate kinase and lactate dehydrogenase, 10–12 μg of CPS, and ATP and $MgCl_2$ as described in the text. All assays were performed at 25 °C.

Fluorescence Measurements. Fluorescence measurements were carried out with a Perkin-Elmer MPF-44 fluorescence spectrometer equipped with a holder for 4 × 4 mm microcuvettes. A Perkin-Elmer polarization accessory consisting of a holder and two Polaroid HNP′B polarizers was used to measure the polarization. Polarization, p, was calculated according to Azumi and McGlynn (1962) and subsequently converted to anisotropy, A:

$$A = 2p/(3 - p) \tag{1}$$

It has to be noted that for a heterogeneous system the measured polarization is not a simple function of the fractional fluorescence intensities and polarizations of the systems' components and, consequently, anisotropy was used to describe ligand binding equilibria. [For an alternative approach, see Rajkowski and Cittanova (1981).] The anisotropy values were corrected for the presence of the scattered light from protein solutions.

Fluorescence titrations were done according to the following procedure: 250 μL of CPS solution in 50 mM Hepes and 100 mM KCl, pH 7.5, was placed in a 4 × 4 mm fluorescence cuvette thermostated at 25 °C. Using a Hamilton microsyringe, we added 1 μL of ϵ-AMP followed by gentle stirring of the contents with a small Teflon paddle. The initial anisotropy was determined, and 1–5-μL aliquots of a ligand were successively added, each followed by anisotropy determination, usually in duplicate or triplicate. The excitation and emission wavelengths were 320 and 420 nm, respectively. When acrylamide was used to quench ϵ-AMP fluorescence, intensity rather than anisotropy was measured according to this procedure.

Data Analysis. Fluorescence polarization was used to measure the binding parameters for interaction of ϵ-AMP and CPS. This method offers a definitive advantage for this system since it does not require a physical separation of bound and free fluorophore [for a review and equations applicable to this and more complex cases, see Dandliker et al. (1981), Rajkowski and Cittanova (1981), and Malencik and Anderson (1988)].

In our case, the number of binding sites, n, and the dissociation constant, K_d, were obtained as follows. First, the anisotropy for the completely bound ϵ-AMP, A_b, was determined from polarization titration of ϵ-AMP with the enzyme. Then, a titration of a constant amount of CPS with ϵ-AMP was conducted, yielding an experimentally observed A value for each, total ϵ-AMP concentration. For each point, the fraction of ϵ-AMP bound to the protein, α, was calculated from eq 2 [see Teichberg and Shinitzky (1973)]. Equation 2 applies

$$\alpha = (A - A_f)/(A_b - A_f) \tag{2}$$

only to cases where there is no quenching or enhancement of the ligand fluorescence upon binding. The number of moles of ϵ-AMP bound per 1 mol of CPS, r, can be expressed as

$$r = \alpha L_t/[P] \tag{3}$$

where [P] is the protein concentration. Knowing r and the concentration of free ligand, $L_f = (1 - \alpha)L_t$, one can construct a Scatchard plot:

$$r/L_f = (-1/K_d)r + n/K_d \tag{4}$$

from which n and K_d can be obtained directly.

Dissociation constants from displacement titrations were calculated by a least-squares fit of the data to eq 5 where ΔA

$$\Delta A = \Delta A_{max} L_f/(K_d + L_f) \tag{5}$$

$= \Delta A_0 - A$; i.e., ΔA is the difference between the initial anisotropy A_0 and the anisotropy at ligand concentration L_f, and ΔA_{max} is the value of ΔA at saturation. Since for polarization experiments CPS was used at a high concentration (~50 μM), the total ligand concentration is significantly different from its free value. We applied an iterative procedure to obtain the true value of K_d and ΔA_{max}: Initially, K_d and A_{max} were calculated by using L_t instead of L_f, and free ligand concentration was computed for each experimental point:

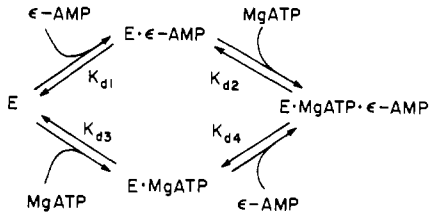
$$L_f = L_t - [P]\Delta A/\Delta A_{max} \tag{6}$$

Equation 6 is valid for $n = 1$. Then, the values of L_f were used to compute a new set of binding parameters from eq 5, and the procedure was repeated until a convergence had been reached, i.e., when K_d and ΔA_{max} did not change by more than 0.01% in a subsequent iteration.

Fluorescence Quenching. Dynamic quenching of a fluorophore in a heterogeneous two-component system (free and bound ϵ-AMP) is described by a generalized Stern–Volmer equation (Eftink & Jameson, 1982):

$$\frac{F_0}{F} = \left[\frac{1 - f_b}{1 + k_f\tau_{0f}[Q]} + \frac{f_b}{1 + k_b\tau_{0b}[Q]}\right]^{-1} \tag{7}$$

Scheme I



F_0 and τ_0 denote the fluorescence intensity and the lifetime of a fluorophore in the absence of quenching, F is the fluorescence intensity observed in the presence of the quencher at concentration [Q], k is the quenching rate constant, and f_b is the fraction of ligand bound. The indexes f and b denote free and bound species, respectively. Since in our case there is no change in the fluorescence intensity upon binding of ε-AMP to CPS, we have assumed that $\tau_{0f} = \tau_{0b}$. The parameters for the quenching rate constant for the free ligand can easily be determined; thus, knowing f_b, one can calculate k_b, the quenching rate constant for bound ligand.

Rapid Equilibria. To calculate anisotropy changes in Figure 5, the equations were used (the symbols refer to Scheme I):

$$[E{\cdot}\epsilon\text{-AMP}]/E_0 = K_{d2}K_{d3}[\epsilon\text{-AMP}]/D \quad (8)$$

$$[E{\cdot}\epsilon\text{-AMP}{\cdot}MgATP]/E_0 = K_{d3}[\epsilon\text{-AMP}][MgATP]D \quad (9)$$

where D is defined as

$$D = K_{d1}K_{d2}K_{d3} + K_{d2}K_{d3}[\epsilon\text{-AMP}] + K_{d1}K_{d2}[MgATP] + K_{d3}[\epsilon\text{-AMP}][MgATP] \quad (10)$$

and E_0 stands for the total enzyme concentration. The fraction of bound ε-AMP, f_b, is calculated as

$$f_b = ([E{\cdot}\epsilon\text{-AMP}] + [E{\cdot}\epsilon\text{-AMP}{\cdot}MgATP])/[\epsilon\text{-AMP}]_{tot} \quad (11)$$

and the change in the anisotropy can be expressed as

$$\Delta A = f_b A_b - A_0 \quad (12)$$

where A_b is the anisotropy for bound ε-AMP and A_0 is the initial anisotropy, i.e., when [MgATP] = 0. It follows then:

$$-\Delta A/A_0 = (A_0 - f_b A_b)/A_0 \quad (13)$$

RESULTS

Binding of ε-AMP to Carbamoyl-Phosphate Synthetase. The binding of ε-AMP to CPS is not accompanied by either a change in quantum yield of the nucleotide or a shift in its fluorescence maximum. The K_d and the stoichiometry of the interaction were measured by using fluorescence anisotropy titrations. To determine A_b, the anisotropy of the bound ε-AMP, a small amount of the nucleotide was titrated with the enzyme (Figure 1A). From this titration, an A_b value of 0.176 ± 0.017 was obtained. To determine the K_d and the number of binding sites, n, a constant amount of CPS was titrated with ε-AMP. From these data, a Scatchard plot was constructed from which a K_d of 0.033 ± 0.007 mM and n = 1.02 ± 0.07 were obtained (Figure 1B). A nonlinear least-squares fit of the data to a hyperbola (Bevington, 1969) yielded K_d = 0.027 ± 0.017 mM and n = 0.97 ± 0.12.

The A_b value determined as described above can be compared to A_b computed from the Perrin equation:

$$A_0/A_b = 1 + 3\tau_0/\rho_0 \quad (14)$$

where ρ_0 is the rotational relaxation time. For a spherical molecule of 180 000 molecular weight, and lifetime, τ_0, of the attached chromophore of 23.4 ns (Secrist et al., 1972; Kasprzak & Kochman, 1981), ρ_0 is equal to 142.5 ns. The limiting Anisotropy A_0 for ε-AMP derivatives excited at 320 nm is 0.27 (Secrist et al., 1972). Thus, from eq 14 A_b = 0.18.

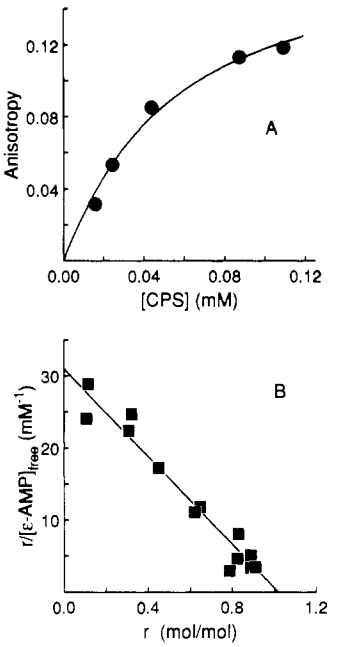


FIGURE 1: Binding parameters of ε-AMP to carbamoyl-phosphate synthetase. (A) Anisotropy titration of a constant amount of ε-AMP (15 μM) with the enzyme. The line was computed by using A_b = 0.176 and K_d = 0.04 mM. The size of the data points indicates the experimental error. (B) Scatchard plot obtained from titration of a constant amount of the enzyme (51.5 μM) with ε-AMP. The line corresponds to K_d = 0.033 mM and n = 1.0. See Data Analysis section for further details.

This value is almost identical with the one experimentally determined (A_b = 0.176) and shows that ε-AMP has virtually no motion with respect to the protein when bound to CPS.

ε-AMP as an Allosteric Effector of CPS. ε-AMP was tested as an inhibitor of the carbamoyl phosphate synthesis reaction with respect to ATP at a concentration of 2.8 mM in the presence of 15 mM excess of Mg^{2+}. The fluorescent nucleotide had only a small activating effect (approximately 10%); the K_m(ATP) remained unchanged and was 0.41 ± 0.03 mM. The effect of ε-AMP on the UMP inhibition was also examined at an ε-AMP concentration of 0.05–1 mM and a UMP concentration of 2 μM. At a concentration of 1 mM, ε-AMP relieved 31% of the inhibition caused by UMP.

Displacement Titration of the ε-AMP–CPS Complex with Nucleotide Analogues. Several compounds that bind to CPS were studied to test whether they displace or interact with bound ε-AMP. Figure 2 presents data showing a progressive decrease of the fluorescence anisotropy upon addition of these compounds. With the exception of MgATP, the initial anisotropy drops by 85–95% at saturating concentrations of the various compounds, approaching the anisotropy for free ε-AMP; the fluorescence intensity remained constant. The change in anisotropy can be explained by a displacement of ε-AMP from the enzyme by the added compounds.

Specifically, Figure 2A shows a titration of the ε-AMP–CPS complex with UMP. The observed fluorescence anisotropy reached its "buffer" value at an [UMP]/[CPS] ratio of 0.96 mol/mol. This finding is in agreement with the kinetic experiments strongly suggesting that the single ε-AMP binding site is identical or at least partially overlaps with the allosteric effector site of CPS.

The decrease in the fluorescence anisotropy was used for determining the dissociation constants for a number of nucleotides, sugar phosphates, and P_i (Table I). For all the ligands that brought the initial anisotropy to almost zero, a competitive mechanism of displacement was assumed. The

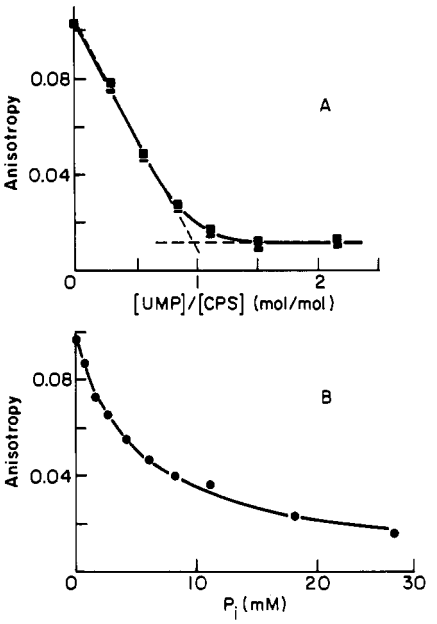


FIGURE 2: Titration of ϵ-AMP–CPS complex with UMP and P_i. (A) Change of the fluorescence anisotropy caused by UMP; [CPS] = 52.4 μM, [ϵ-AMP] = 25 μM. The line was simulated wth K_d(app) = 5.14 mM and $\Delta A_{max}/A_0$ = 95.6%. (B) Titration with P_i, conditions as above. The line was computed for K_d(app) = 3.1 mM and $\Delta A_{max}/A_0$ = 92%.

Table I: Dissociation Constants for the Interaction of Nucleotides and Their Analogues with Carbamoyl-Phosphate Synthetase at pH 7.5 and 25 °C

ligand	K_d ± SD (mM)[a]	ligand	K_d ± SD (mM)[a]
ϵ-AMP	0.033 ± 0.007	2-deoxyribose-5-P	1.4 ± 0.21
UMP	<0.005	P_i	2.9 ± 0.25
IMP	0.012 ± 0.002	MgATP[b]	0.64 ± 0.08
CMP	0.81 ± 0.08		0.54 ± 0.12[c]
ribose-5-P	1.2 ± 0.11		

[a] Mean ± SD for 2–5 independent determinations. [b] Not competive with ϵ-AMP. [c] Kinetic value of K_m in the presence of 10 mM excess Mg^{2+}.

apparent K_d values, computed as described under Data Analysis, were further corrected for the competitive effect of the probe by using

$$K_d = K_d^{app}/[1 + [\epsilon\text{-AMP}]/K_d(\epsilon\text{-AMP})] \quad (15)$$

The correction was never large since the concentration of ϵ-AMP used, typically 9.9 μM, was well below the K_d(ϵ-AMP) of 33 μM.

Magnesium ions (up to 15 mM) did not have any effect on the fluorescence anisotropy of the bound ϵ-AMP. With uridine, at high concentrations (>5 mM), an increase in polarization was observed. We have demonstrated in a separate experiment (data not shown) that the observed increase in anisotropy was due to quenching of the fluorescence of ϵ-AMP by uridine and, consequently, a shortening of the τ_0 for ϵ-AMP, which according to eq 14 leads to an increase in the steady-state anisotropy.

Effect of MgATP. When MgATP was used as a titrant, the observed anisotropy changes were qualitatively different from those found for other ligands. First, the titration curves were magnesium dependent, and, second, the anisotropy was not brought down to zero at saturation but leveled off at about 50% of its initial value (Figure 3B). Similar results were obtained from steady-state kinetic experiments in which carbamoyl phosphate synthesis was measured as a function of MgATP concentration (Figure 3A). When 10 mM excess

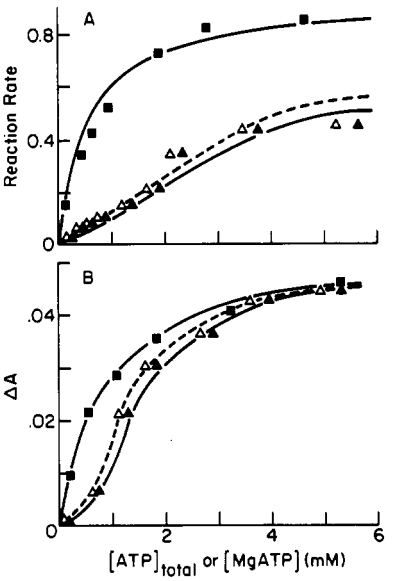


FIGURE 3: Influence of Mg^{2+} on the interaction of ATP with CPS. (A) Steady-state activity of CPS as a function of total [ATP] or [MgATP] concentration. The assays were conducted as described under Experimental Procedures. The reaction rate is given in arbitrary units; (■) reaction rates in the presence of 10 mM excess of Mg^{2+} over ATP; the line was computed for K_m = 0.54 mM; (▲) with equimolar [Mg^{2+}] to [ATP] concentrations plotted as a function of total [ATP]; (△) as (▲) but plotted as a function of [MgATP]. (B) The effect of [MgATP] on the anisotropy change of ATP. CPS and ϵ-AMP concentrations were 49.3 and 10 μM, respectively. Excitation wavelength, 320 nm; emission wavelength, 420 nm. ΔA denotes the difference between the initial and current anisotropy. (■) ΔA in the presence of 10 mM excess of $MgCl_2$. The solid line was computed for K_d = 0.64 mM and ΔA_{max} = 0.0488; (▲) equimolar [Mg^{2+}] to [ATP] concentrations plotted as a function of total [ATP]; (△) as (▲) but plotted as a function of [MgATP].

of Mg^{2+} was present, the interaction of MgATP with CPS could be described as a simple hyperbola with K_m = 0.54 ± 0.11 mM; a K_d value of 0.64 ± 0.07 mM was determined from anisotropy titrations (Figure 3B). However, when Mg^{2+} and ATP were used at equimolar concentration, the dependence followed a sigmoidal shape (Figure 3). There is a qualitative difference between the fluorescence and the kinetics curves: at high Mg^{2+} concentration, the anisotropy curve reached the same limiting value as that obtained in the presence of a large excess of magnesium whereas the reaction rate did not.

When plotted as a function of the total ATP concentration, the data presented in Figure 3 contain three parameters which varied during the titrations: the concentrations of Mg^{2+}, free ATP, and MgATP. Using eq 5 in Kasprzak and Kochman (1980), we calculated the amount of MgATP for each experimental point and replotted the data as a function of a single variable, viz., the concentration of MgATP complex (Figure 3, dashed lines). For experiments done at equimolar [Mg^{2+}] and [ATP], both kinetic and anisotropy curves were slightly shifted toward lower values when the results were plotted as a function of [MgATP]. However, the shape and the magnitude of the deviation from hyperbolic dependence remained unchanged.

When the experiments were conducted at 10 mM excess Mg^{2+} over ATP, the dependence of the anisotropy on the total [ATP] followed a simple hyperbola. Under these conditions, the concentration of the MgATP complex was practically equal to the total [ATP], and the free ATP concentration was negligibly small (0.29% at 0.2 mM total [ATP] and 0.30% at 5 mM); the concentration of free magnesium ions was high and approximately constant (10 mM). The results from anisotropy titrations and kinetic experiments and the fact that

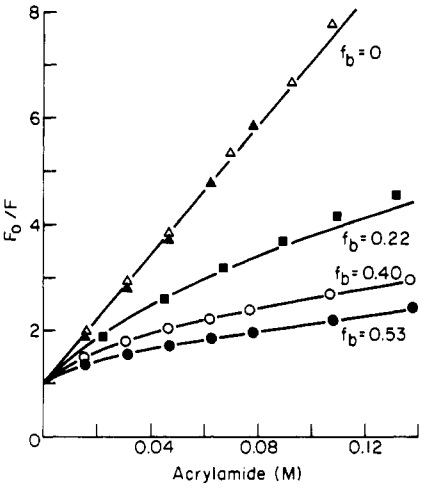


FIGURE 4: Stern–Volmer plots for the acrylamide quenching of ϵ-AMP–CPS fluorescence. Emission and excitation wavelengths were 420 and 320 nm, respectively. Quenching curves for the following: (△, ▲) free ϵ-AMP (25 μM); the line was computed with $k_f = 3 \times 10^9$ M^{-1} s^{-1}; (●) ϵ-AMP in the presence of CPS (50 μM); the line was simulated by using eq 7 with $k_b = 1.2 \times 10^8$ M^{-1} s^{-1} and $f_b = 0.526$; (○) in the presence of CPS (50 μM) and MgATP (1 mM); the line was computed for $k_b = 1.2 \times 10^8$ M^{-1} s^{-1} and $f_b = 0.395$. (■) as (○) but in the presence of 10 mM excess Mg^{2+} over ATP; [MgATP] = 3.6 mM, [CPS] = 37.1 μM. The line corresponds to $k_b = 1.2 \times 10^8$ M^{-1} s^{-1} and $f_b = 0.22$.

binding of MgATP is dependent on the concentration of free magnesium ions indicate that MgATP and Mg^{2+} bind simultaneously to the enzyme and the binding is cooperative; i.e., binding of one ligand increases the affinity for the other, corroborating the earlier data of Raushel et al. (1979). When the enzyme is saturated with Mg^{2+}, the titration curve corresponds to the binding of MgATP to the Mg–enzyme complex. The apparent sigmoidal shape (Figure 3) is presumably caused by plotting the data as a function of one of the substrates while varying the concentrations of MgATP and Mg^{2+} simultaneously.

Since in fluorescence experiments the enzyme was used at relatively high concentration, the ATPase activity of CPS had to be taken into account. We have found that the amount of ATP hydrolyzed after a 30-min incubation of CPS at 25 °C with the highest ATP concentration used during the titrations (5.5 mM) was 7–9%. The concentration of the reaction products during an actual titration experiment was much lower because most of the time the enzyme was exposed to subsaturating ATP concentrations. Also, if the reaction products had an influence on the binding curves, some time dependence of the anisotropy values would be expected. This, however, has never been seen. Therefore, we conclude that Figure 3 presents the binding of MgATP to CPS undistorted by the presence of ADP and P_i.

Acrylamide Quenching of the Fluorescence of ϵ-AMP Bound to CPS. Acrylamide is a very efficient quencher of the ϵ-AMP fluorescence (Eftink & Ghiron, 1975). When the nucleotide is free in solution, the linear Stern–Volmer plot in Figure 4 indicates that the quenching is purely dynamic (collisional); the bimolecular quenching rate constant, k_f, was calculated to be 3×10^9 M^{-1} s^{-1}. When CPS was present, a heterogeneous population of fluorophores was generated (free and bound ϵ-AMP) giving rise to a nonlinear Stern–Volmer plot (Figure 4). Using the values of f_b from the fluorescence anisotropy titrations and the value of τ_0, we found the value of k_b, the quenching rate constant for the bound fluorophore, to be 1.2×10^8 M^{-1} s^{-1}. Thus, the base moiety of ϵ-AMP is shielded from the solvent by the protein because its quenching rate cosntant decreases 25-fold when the fluorophore is bound to the protein.

The presence of MgATP, when a partial release of ϵ-AMP has occurred, made the Stern–Volmer plots steeper (Figure 4, closed squares). However, even at a 3.6 mM concentration of ATP, a significant fraction of the probe was bound to the protein and was protected from quenching.

Discussion

The experiments described in this paper indicate that ϵ-AMP can be used as a fluorescence probe to monitor allosteric interactions in carbamoyl-phosphate synthetase. On the basis of anisotropy titrations and kinetic measurements, we conclude that the ϵ-AMP binding site is identical with or at least partially overlaps with the UMP/IMP allosteric binding site of CPS. This characteristic makes ϵ-AMP useful in monitoring direct interactions with the allosteric site as well as an indirect influence of one or both of the ATP sites. The procedure of determining the affinity for some of the CPS ligands by following changes in the fluorescence anisotropy, developed in the present work, is especially valuable when interactions of weakly bound effectors are studied, since the use of equilibrium dialysis or column chromatography to determine the K_d would require impractically high enzyme concentrations.

It has been suggested that the UMP binding site possesses several functional groups in fixed positions that are in contact with the specific loci of the interacting nucleotide (Boettcher & Meister, 1982). The lack of the 2′-hydroxyl group in dUMP must account for its 10-fold lower, compared to UMP, affinity to CPS (Boettcher & Meister, 1982). However, as seen in Table I, ribose-5-P and deoxyribose-5-P bind with affinities identical within experimental error. In the absence of the nucleotide base, the ribose phosphates may interact with different enzymic groups, and the interaction with the 2′-hydroxyl may not be significant.

Anderson (1977) reported that phosphate buffer decreased the binding of IMP and UMP. Boettcher and Meister (1981) suggested that there is a site for phosphate on the enzyme with a K_d of about 3 mM. In this work, we have presented evidence of phosphate binding presumably to or near the allosteric site of the enzyme with a K_d of 2.9 mM. While kinetic experiments revealed that P_i is a competitive inhibitor vs MgATP (Raushel et al., 1978), the present evidence suggests that there exist at least two different sites for phosphate. However, it is still not clear how P_i binding to any of these sites can *enhance* the inhibition by UMP (Anderson, 1977b; Boettcher & Meister, 1981, 1982). Further work is required to explain this point.

Our results have important implications for understanding several aspects of the interaction between the ATP and UMP binding sites in CPS. It is clear that a ternary complex, ϵ-AMP–CPS–MgATP, is formed in which ϵ-AMP has a reduced affinity for the enzyme. It is worthwhile to consider a mechanistic model of such interactions. One of the possibilities is that in the presence of MgATP some groups on ϵ-AMP have reduced interaction with the enzyme and do not contribute to the binding energy. However, the acrylamide quenching experiments and the results of the anisotropy titrations with MgATP exclude the *nucleotide base* from participating in such a mechanism: When MgATP and ϵ-ATP form a ternary complex with CPS, the base of ϵ-AMP does not change its solvent accessibility as compared to the binary ϵ-AMP–CPS complex; in both cases, the base is well shielded from the solvent.

It has been postulated that free magnesium ions are necessary for the full activity of CPS (Raushel et al., 1979). From Figure 3, it is clear that magnesium not only affects the rate

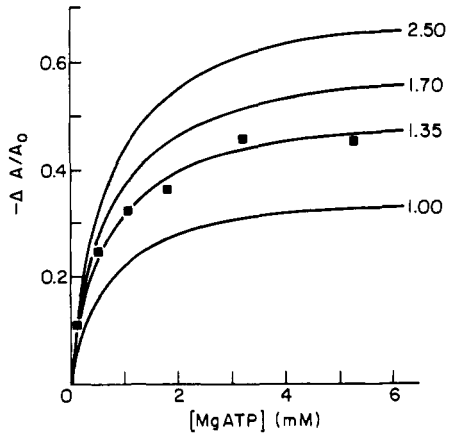


FIGURE 5: Dependence of the relative change in the fluorescence anisotropy on the value of K_{d2}. Numbers next to the curves indicate the assumed value of K_{d2}. Experimental points (■) are as in Figure 3B in the presence of 10 mM excess of Mg^{2+} over ATP.

of the catalytic reaction of CPS (at equimolar ATP to magnesium, the reaction rate never reaches maximum) but also alters the *binding* of ATP to the enzyme. However, since we have not seen any affect of Mg^{2+} alone on the affinity of ϵ-AMP, a direct energetic coupling between the divalent cation site and the ϵ-AMP site seems unlikely.

Scheme I is consistent with our findings concerning the formation of ternary complexes between CPS and MgATP and ϵ-AMP. For the MgATP binding, the equilibria correspond to the binding of the nucleotide to either the "first" or the "second" binding sites, *provided that the enzyme is saturated with* Mg^{2+}. The fact that the anisotropy of ϵ-AMP decreases to only 50% of its initial value upon saturation of the enzyme with MgATP is also consistent with this scheme. Incomplete displacement of the fluorescent nucleotide is expected when a ternary complex of CPS, ϵ-AMP, and MgATP is formed. When CPS is saturated with MgATP, the K_d for ϵ-AMP increases approximately 2.3 times (from 0.033 to 0.075 mM). Since ϵ-AMP is used at a concentration well below its K_d, one can expect that the concentration of bound ϵ-AMP (and the observed anisotropy) will be reduced by the same factor. This is in good agreement with the experimental results. Thus, the fluorescence anisotropy of bound ϵ-AMP attained at saturation is a sensitive function of K_{d2}, the dissociation constant for the interaction of MgATP with the ϵ-AMP–CPS complex.

The value of K_{d1} is equal to 0.033 mM; the "true" value of K_{d3} must be close to its apparent value obtained at 10 mM excess Mg^{2+} over ATP because the concentration of ϵ-AMP was kept below its K_d value. On the basis of the fluorescence and kinetic data, we have used a value of 0.6 mM for K_{d3}. Using the equations shown in the Data Analysis section, we have calculated the relative change of the anisotropy, $-\Delta A/A_0$, for several values of K_{d2} (Figure 5). The best agreement of the simulated curves with the experimental data was obtained when K_{d2} = 1.35 mM; this implies that K_{d4} = 0.075 mM.

One fact which is not consistent with this scheme is the apparent insensitivity of the K_m for ATP to the presence of the effector. This can be explained by assuming that interactions of ϵ-AMP with the enzyme and the transmission of the effect of its binding to the ATP binding site(s) may be modulated by glutamine or bicarbonate which were present during kinetic assays but were absent during fluorescent titrations.

The free energy of coupling between MgATP and ϵ-AMP, ΔG_{XY}, is defined as the difference between standard free energy changes associated with the dissociation of MgATP from the ϵ-AMP–CPS–MgATP and the CPS–MgATP complexes, respectively (Weber, 1975). Using the dissociation constants presented above, we have obtained a value of +0.48 kcal/mol for ΔG_{XY}; the positive sign signifies the "antagonistic" (anticooperative) interaction between ligands. This value is almost identical with a ΔG of +0.5 kcal/mol obtained for the binding of CTP and succinate to aspartate transcarbamylase (Changeux et al., 1968) and is slightly lower than +0.8 kcal/mol for the interactive binding of phenylalanine and Mn^{2+} to pyruvate kinase (Kayne & Price, 1972). On the other hand, the antagonism in binding of CPS observed for ϵ-AMP and MgATP is much weaker than the coupling between oxygen and 2,3-diphosphoglycerate interaction with hemoglobin for which a value +1.3 kcal/mol has been measured (Tyuma et al., 1971).

The significance of the observed coupling between the allosteric and the MgATP binding sites could be best demonstrated with a physiologically important ligand such as UMP, provided that a set of dissociation constants for its interaction with CPS has been obtained. Such data are not yet available. Nevertheless, since ϵ-AMP and UMP occupy the same or overlapping sites (from data in this paper), we can conclude that the ΔG_{XY} for UMP and MgATP binding is similar to that for ϵ-AMP and MgATP.

When the concentration of ϵ-AMP is much below its K_d, the binding of MgATP will partially reverse the formation of the ternary complex by releasing some of the bound ϵ-AMP. For the nonphysiological ligand, the reaction rate remains almost unchanged because the ligand itself has little effect on the CPS activity. However, for a physiologically relevant ligand, such as UMP, the reduction by 50% of the concentration of the allosteric effector bound to the enzyme would undoubtedly result in a significant relief of the inhibition. On the other hand, when the concentration of the allosteric inhibitor is much greater that its K_d, the effect of MgATP binding is negligible. Thus, the degree of saturation of the allosteric site of the enzyme is modulated by MgATP but only when the concentration of the allosteric effector is low, i.e., approximating "normal" physiological conditions. Besides the reversible dissociation–association equilibria of CPS (Anderson, 1984, 1986) such a mechanism of altering the sensitivity of the enzyme to binding of allosteric effectors may provide an important additional mode of regulation of the CPS activity.

REFERENCES

Anderson, P. M. (1977a) *Biochemistry 16*, 583–586.
Anderson, P. M. (1977b) *Biochemistry 16*, 587–593.
Anderson, P. M. (1984) *Biochemistry 23*, 3346–3353.
Anderson, P. M. (1986) *Biochemistry 25*, 5576–5582.
Anderson, P. M., & Meister, A. (1986) *Biochemistry 25*, 3164–3169.
Azumi, T., & McGlynn, S. P. (1962) *J. Chem. Phys. 37*, 2413.
Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, p 204, McGraw-Hill, New York.
Boettcher, B. B., & Meister, A. (1981) *J. Biol. Chem. 256*, 5977–5980.
Boettcher, B. B., & Meister, A. (1982) *J. Biol. Chem. 257*, 13971–13976.
Changeux, J.-P., Gerhard, J. C., & Schachman, H. K. (1968) *Biochemistry 7*, 531–539.
Dandliker, W. B., Hsu, M.-L., Levin, J., & Rao, R. B. (1981) *Methods Enzymol. 74*, 3–28.
Eftink, M. R., & Ghiron, C. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3290–3294.

Eftink, M. R., & Jameson, D. M. (1982) *Biochemistry 21*, 4443-4449.
Foley, R., Poon, J., & Anderson, P. M. (1971) *Biochemistry 10*, 4562-4569.
Kasprzak, A. A., & Kochman, M. (1980) *Eur. J. Biochem. 104*, 443-450.
Kasprzak, A. A., & Kochman, M. (1981) *J. Biol. Chem. 256*, 6127-6133.
Kasprzyk, P. G., Anderson, P. M., & Villafranca, J. J. (1983) *Biochemistry 22*, 1877-1882.
Kasprzyk, P. G., Whalen-Pederson, E., Anderson, P. M., & Villafranca, J. J. (1985) *Bioorg. Chem. 13*, 98-109.
Kayne, F. J., & Price, N. C. (1972) *Biochemistry 11*, 4415-4420.
Malencik, D. A., & Anderson, S. R. (1988) *Biochemistry 27*, 1941-1949.
Matthews, S. L., & Anderson, P. M. (1972) *Biochemistry 11*, 1176-1183.
Powers, S. G., Meister, A., & Haschenmeyer, R. H. (1980) *J. Biol. Chem. 255*, 1554-1558.
Rajkowski, K. M., & Cittanova, N. (1981) *J. Theor. Biol. 93*, 691-696.
Raushel, F. M., Anderson, P. M., & Villafranca, J. J. (1978) *Biochemistry 17*, 5588-5591.
Raushel, F. M., Rawding, C. J., Anderson, P. M., & Villafranca, J. J. (1979) *Biochemistry 18*, 5562-5566.
Raushel, F. M., Anderson, P. M., & Villafranca, J. J. (1983) *Biochemistry 22*, 1872-1876.
Secrist, J. A., III, Barrio, J. R., Leonard, N. J., & Weber, G. (1972) *Biochemistry 11*, 3499-3506.
Teichberg, V. I., & Shinitzky, M. (1973) *J. Mol. Biol. 74*, 519-531.
Trotta, P. P., Burt, M. E., Haschenmeyer, R. H., & Meister, A. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2599.
Trotta, P. P., Estis, L. F., Meister, A., & Haschenmeyer, R. H. (1974) *J. Biol. Chem. 249*, 482-491.
Tyuma, I., Shimizu, K., & Imai, K. (1971) *Biochem. Biophys. Res. Commun. 43*, 423.
Weber, G. (1975) *Adv. Protein Chem. 29*, 1-83.

Protein Components of Human Tracheobronchial Mucin: Partial Characterization of a Closely Associated 65-Kilodalton Protein†

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ABSTRACT: A high-density mucin glycoprotein was isolated from human tracheobronchial secretions substantially free of contaminating protein, low-density glycoprotein, proteolytic enzymes, and lipid. A closely associated 65-kDa protein was discovered while investigating the effect of 2-mercaptoethanol treatment on the purified mucin glycoprotein. It has been established that the 65-kDa protein is neither α_1-antichymotrypsin nor human serum albumin, two proteins of similar molecular weight which are found in crude tracheobronchial secretions. This protein lacks cross-reactivity with antibodies directed against serum components and is presumably comparable to the 65-kDa protein similarly isolated from canine tracheal pouch secretions [Ringler et al. (1987) *Biochemistry 26*, 5322-5328]. Although both the presence of sulfhydryl groups and the ability to be reassociated with the mucin molecule have been established, it is not clear whether its association is due to direct disulfide bonding, hydrophobicity, or entrapment. It was found that ^{14}C-methylated methemoglobin was an inappropriate substrate for measurement of proteolytic activity in mucin preparations due to inherent entrapment and clearance capabilities of mucin molecules.

Secretory products of the upper airway epithelial cells provide the components necessary for control of the physical properties of the respiratory mucus. Since this material must be translocated in an upward direction for clearance, and simultaneously provide a coating on cellular surfaces, its rheological properties must be tightly regulated. These are largely defined by a mucin-type glycoprotein with a covalent molecular weight in the range of 10^6. The ability of this macromolecule to aggregate is an important feature of the overall system as is its capacity to bind lipid and cations. Detailed studies of the chemical structure of the glycoprotein have been performed in several laboratories, and a composite analytical profile has been defined (Roberts, 1976; Creeth et al., 1977; Sachdev et al., 1980; Rose et al., 1984; Slayter et al., 1984; Woodward et al., 1982, 1987). A recent study of material isolated from canine tracheal mucus indicated that a nonglycosylated protein of M_r 65K was closely associated with the mucin glycoprotein (Ringler et al., 1987). The present report details the properties of a comparable protein from human secretions and the role of disulfide bonds in the associative behavior of the two macromolecules.

Materials and Methods

Isolation of Delipidated Tracheobronchial Mucin. Tracheobroncial aspirates were collected from patients hospitalized for nonpulmonary illness, and the major mucin glycoprotein was isolated as before with minor modifications (Woodward et al., 1982). Briefly, upper respiratory tract aspirates were

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