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A Functional Analysis of the Allosteric Nucleotide Monophosphate Binding Site of Carbamoyl Phosphate Synthetase

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The catalytic activity of carbamoyl phosphate synthetase (CPS) from *Escherichia coli* is allosterically regulated by UMP, IMP, and ornithine. Thirteen amino acids within the domain that harbors the overlapping binding sites for IMP and UMP were mutated to alanine and characterized. The four residues that interact directly with the phosphate moiety of IMP in the X-ray crystal structure (K954, T974, T977, and K993) were shown to have the greatest impact on the dissociation constants for the binding of IMP and UMP and the associated allosteric effects on the kinetic constants of CPS. Of the four residues that interact with the ribose moiety of IMP (S948, N1015, T1017, and S1026), S1026 was shown to be more important for the binding of IMP than UMP. Five residues (V994, I1001, D1025, V1028, and I1029) were mutated in the region of the allosteric domain that surrounds the hypoxanthine ring of IMP. With the exception of V994A, these mutations had a modest influence on the binding and subsequent allosteric effects by UMP and IMP.

Key Words: **carbamoyl phosphate synthetase; allosteric regulation; nucleotide monophosphate; feedback inhibition.**

Carbamoyl phosphate synthetase (CPS)[2] from *Escherichia coli* catalyzes the formation of carbamoyl phosphate from glutamine, bicarbonate, and ATP, according to the following reaction:

$$2\ \mathrm{ATP} + \mathrm{HCO_3^-} + \text{glutamine} \rightarrow 2\ \mathrm{ADP} + \mathrm{P_i} + \text{glutamate} + \text{carbamoyl-P}. \quad [1]$$

Carbamoyl phosphate is subsequently used in the distinct biosynthetic pathways for arginine and pyrimidine nucleotides (1). In addition to the overall reaction, the large subunit of CPS catalyzes two partial reactions: (i) a bicarbonate-dependent ATPase reaction (Eq. [2]) and (ii) an ATP synthesis reaction (Eq. [3]) (2):

$$\mathrm{MgATP} + \mathrm{H_2O} \rightarrow \mathrm{MgADP} + \mathrm{P_i}, \quad [2]$$

$$\mathrm{MgADP} + \text{carbamoyl-P} \rightarrow \mathrm{MgATP} + \mathrm{NH_2CO_2^-}. \quad [3]$$

The enzyme isolated from *E. coli* is a large multidomain heterodimeric protein. The smaller subunit is responsible for the hydrolysis of glutamine, whereas the larger subunit catalyzes a series of consecutive reactions leading to the overall production of carbamoyl phosphate. The large subunit contains independent binding sites for the two ATP molecules utilized in the overall synthesis of carbamoyl phosphate and also the binding sites for the allosteric effectors UMP, IMP, and ornithine (3). As a key enzyme in the initiation of two biosynthetic pathways, CPS from *E. coli* is feedback-inhibited by UMP, the end product of the pyrimidine pathway, and activated by ornithine (4–6). UMP and ornithine achieve their regulatory effects by binding to separate allosteric sites (7, 8), localized in the extreme C-terminal domain of the large subunit of CPS (8–12). UMP and IMP are believed to have a single or overlapping binding site on the protein (13–15). UMP is a strong inhibitor of the enzyme while IMP is a mild activator or inhibitor that depends on the temperature and other specific assay conditions (6, 13, 16–18). The binding of IMP and that of UMP are

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[2] Abbreviations used: CPS, carbamoyl phosphate synthetase; G6PDH, glucose-6-phosphate dehydrogenase.

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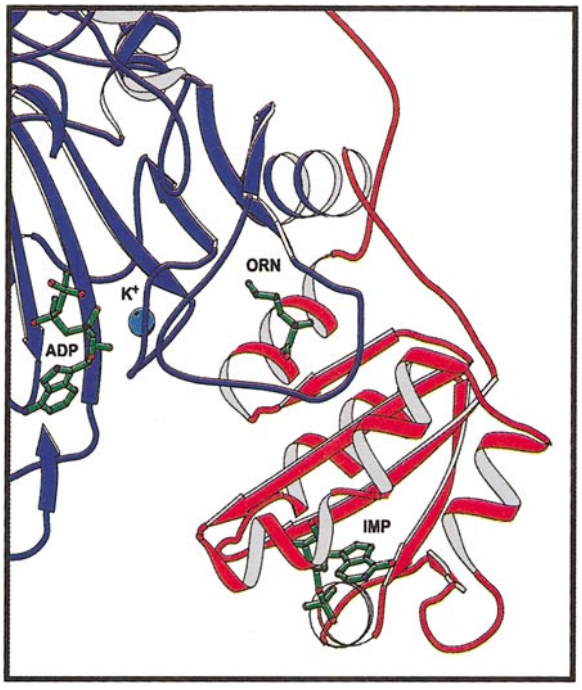

FIG. 1. Ribbon representation of the allosteric domain (red) and a portion of the carbamoyl phosphate domain (blue) of CPS (19). The substrate ADP and the effector molecules of IMP and ornithine are represented in a ball-and-stick format at their respective binding sites within the CPS structure. IMP is buried within the allosteric domain, while ornithine is located at the interface between the two domains. The sphere represents a potassium ion found between the ornithine binding site and the active site.

strictly competitive with one another, while ornithine and either IMP or UMP can bind simultaneously to CPS. The allosteric effects of ornithine completely dominate those of UMP when both allosteric effectors are bound simultaneously to the allosteric domain (16). The allosteric effectors exert their perturbations primarily on the ATP bound to the carbamate domain, since UMP and ornithine have a rather modest effect on the kinetic parameters of the partial bicarbonate-dependent ATPase reaction that occurs in the absence of glutamine (6, 8).

A ribbon representation of the allosteric domain (red) and a portion of the carbamate domain (blue) of the large subunit of CPS is presented in Fig. 1 (19). The molecules of ornithine and IMP are approximately 14 and 26 Å, respectively, from the ATP-binding site within the carbamate domain of CPS (19). Ornithine is localized at the interface between the two domains, whereas IMP is well buried within the allosteric domain, at the C-terminal portion of a five-stranded parallel β sheet. The side chains of the amino acids K954, T974, T977, K993, N1015, and T1017 are apparently responsible for anchoring the IMP molecule to the protein via the phosphate and the ribose moieties of this nucleotide (19).

Among the mutations previously reported within the allosteric nucleotide-binding pocket, S948A/F, K954A, T974A, T977A, and K993A produced the greatest changes on IMP/UMP binding and the nucleotide-mediated regulation of CPS activities (8, 20–22). However, it is currently unknown how the enzyme makes a structural distinction between IMP and UMP and how the allosteric signal is transmitted from the IMP/UMP binding site to the enzyme active site. In order to further elaborate the regulatory mechanism for the allosteric control of CPS by UMP and IMP, we report here a detailed mutational analysis of the allosteric nucleotide-binding domain of the large subunit of *E. coli* carbamoyl phosphate synthetase.

MATERIALS AND METHODS

Materials. Vent DNA polymerase, Magic Miniprep DNA purification kits, and Magic PCR Preps DNA purification kits were purchased from Promega. The QuikChange site-directed mutagenesis kit was purchased from Stratagene. Oligonucleotide synthesis and DNA sequencing reactions were done by the Gene Technologies Laboratory, Texas A&M University.

Preparation of mutant forms of CPS. Site-directed mutagenesis was performed using the plasmid pMS03, carrying the *car*AB genes, as the initial DNA template (23). The mutants, T974A, K993A, T1017A, and S1026A, were constructed as previously described for the preparation of T977A (8), using the polymerase chain reaction and the overlap extension method of Ho *et al.* (24). The other modified forms of CPS were made with the QuikChange site-directed mutagenesis kit from Stratagene. The mutant plasmids were transformed in the RC50 cell line (a generous gift from Dr. Carol J. Lusty) for the expression of CPS. The wild-type and the mutant enzymes were purified to homogeneity as previously described (25). The identity of the mutant plasmids was conformed by DNA sequencing.

Allosteric parameters. Kinetic parameters were obtained in the absence or presence of saturating amounts of IMP, UMP, or ornithine. The concentrations of IMP and UMP never exceeded 10 mM to avoid direct interference with the binding of ATP at the active sites of CPS. The rate of ATP hydrolysis in the absence of glutamine was measured at 25°C by coupling the production of ADP to pyruvate kinase and lactate dehydrogenase while monitoring the loss of

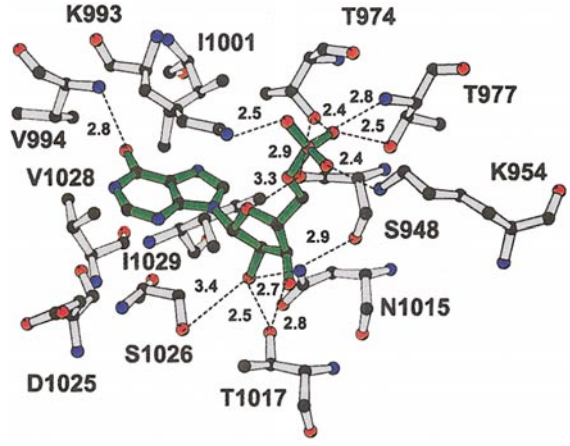

FIG. 2. Ball-and-stick representation of the 13 residues within the IMP binding pocket of CPS targeted for mutagenesis (19).

TABLE I

Kinetic Parameters for the Bicarbonate-Dependent ATPase Reaction[a]

	None		IMP		UMP		Ornithine	
Enzyme	K_a (mM)	k_{cat} (s^{-1})	K_a (mM)	k_{cat} (s^{-1})	K_a (mM)	k_{cat} (s^{-1})	K_a (mM)	k_{cat} (s^{-1})
Wild type	0.031	0.76	0.038	0.82	0.08	0.54	0.011	0.66
S948A[b]	0.12	0.20	0.12	0.16	0.09	0.12	0.09	0.15
	4.0	0.47	2.0	0.46	2.7	0.47	3.2	0.43
K954A	nd	nd	nd	nd	nd	nd	nd	nd
T974A	0.073	0.14	0.091	0.16	0.09	0.14	0.049	0.13
T977A	0.073	0.35	0.086	0.41	0.13	0.34	0.026	0.38
K993A	0.056	0.29	0.073	0.41	0.19	0.34	0.018	0.25
V994A[c]	0.020	1.40	0.023	1.48	0.11	1.46	0.010	1.30
	6×10^{-4}[d]		6×10^{-4}[d]		2×10^{-4}[d]		5×10^{-5}[d]	
I1001A	0.037	0.86	0.040	0.73	0.05	0.81	0.010	0.58
N1015A[b]	0.060	0.07	0.10	0.10	0.07	0.07	0.059	0.06
	3.1	0.33	3.3	0.37	3.1	0.35	2.2	0.34
T1017A	0.057	0.29	0.055	0.29	0.11	0.26	0.012	0.24
D1025A	0.036	1.07	0.062	1.25	0.12	0.98	0.011	0.97
S1026A	0.027	0.37	0.033	0.35	0.06	0.26	0.013	0.48
V1028A	0.030	0.68	0.036	0.76	0.06	0.35	0.010	0.58
I1029A	0.056	0.54	0.043	0.52	0.11	0.43	0.016	0.48

Note. nd, not determined.

[a] Reaction conditions: pH 7.5, 25°C, 20 mM bicarbonate, 10 mM $MgCl_2$, 100 mM KCl, and saturating concentration of IMP, UMP, or ornithine. Concentrations of UMP and IMP were 6- to 100-fold higher than the apparent K_d for the effector and never exceeded 10 mM. Ornithine was fixed at 10 mM.

[b] Parameters determined from Eq. [5] for nonlinear double reciprocal plots.

[c] Parameters determined from Eq. [7] for nonlinear double reciprocal plots.

[d] Units are in mM^2.

NADH at 340 nm. A typical reaction mixture contained 50 mM Hepes, pH 7.5, 1.0 mM phosphoenolpyruvate, 0.2 mM NADH, 100 mM KCl, 10 mM $MgCl_2$, 20 mM bicarbonate, 33 μg pyruvate kinase, 33 μg lactate dehydrogenase, and variable amounts of ATP (0.01 to 4.0 mM). The reactions were started by the addition of 75–140 μg of CPS in a final volume of 2.5 ml. The glutamine-dependent ATPase activity was measured in the same way as the bicarbonate-dependent ATPase reaction, with the addition of 10 mM glutamine and ATP varying between 0.01 and 8.0 mM. The reactions were initiated by the addition of 13–94 μg of CPS in a final volume of 2.5 ml. The rate of ATP formation from ADP and carbamoyl phosphate was measured at 25°C by coupling the synthesis of ATP to hexokinase and glucose-6-phosphate dehydrogenase (G6PDH) and monitoring the appearance of NADH at 340 nm. The typical reaction mixture contained 50 mM Hepes, pH 7.5, 100 mM KCl, 0.75 mM NAD^+, 1.0 mM glucose, 15 mM $MgCl_2$, 78 μg hexokinase, 16.4 μg G6PDH, and variable amounts of ADP (0.01 to 4.0 mM). Carbamoyl phosphate was added just before the enzyme at a final concentration of 2.0 mM. The reaction was started by adding 0.2 to 0.4 mg of CPS in a final volume of 2.0 ml. Allosteric parameters of wild-type and mutant CPS were determined using the same reactions, except that the substrate concentration (ATP or ADP) was fixed at 0.2 mM, while the IMP, UMP, or ornithine concentrations were varied between 0 and 10 mM. The synthesis of carbamoyl phosphate was determined, as previously described (23), by measuring the rate of citrulline formation in a coupled assay containing ornithine transcarbamoylase and ornithine (26).

Statistical analysis of kinetic data. The kinetic parameters, V_m and K_a, were determined by fitting the data to Eq. [4] in which ν is the initial velocity, A is the varied substrate concentration, V_m is the velocity at saturating substrate concentration, and K_a is the Michaelis constant. Nonlinear double-reciprocal plots were fit to Eq. [5], in which A is the substrate concentration, K_1 and K_2 are the Michaelis constants at low and high substrate concentrations, respectively, while V_1 and V_2 are the maximal velocities at low and high substrate concentrations, respectively. Other nonlinear double reciprocal plots were fit to Eq. [7], with K_1 in mM and K_2 in mM^2. The effect of the allosteric ligands was determined by fitting the data to Eq. [6], in which L is the allosteric ligand, ν is the initial velocity, ν_o is the initial rate in absence of the effector, α is the ratio of ν at infinite L to ν_o, and K_d is the apparent dissociation constant for the effector L. The error limits in all of the reported kinetic constants are ≤20% of the stated values.

$$\nu = (V_m A)/(K_a + A), \quad [4]$$

$$\nu = [(V_1 A)/(K_1 + A)] + [(V_2 A)/(K_2 + A)], \quad [5]$$

$$\nu = \nu_o(\alpha L + K_d)/(L + K_d), \quad [6]$$

$$\nu = (V_m A^2)/(A^2 + K_1 A + K_2). \quad [7]$$

RESULTS

Thirteen residues within the allosteric nucleotide-binding domain of CPS were selected for mutation to alanine, based on the known three-dimensional structure of IMP bound to CPS (19). This allosteric binding site interacts directly with IMP through three distinct

TABLE II

Kinetic Parameters for the Glutamine-Dependent ATPase Reaction[a]

	None		IMP		UMP		Ornithine	
Enzyme	K_a (mM)	k_{cat} (s^{-1})	K_a (mM)	k_{cat} (s^{-1})	K_a (mM)	k_{cat} (s^{-1})	K_a (mM)	k_{cat} (s^{-1})
Wild type	0.81	12.1	0.78	12.4	1.4	3.3	0.068	10.4
S948A	0.76	0.6	0.89	0.75	1.1	0.59	0.72	0.60
K954A	1.21	1.5	0.97	2.0	1.1	1.0	0.19	1.4
T974A	0.44	0.4	0.49	0.48	0.4	0.43	0.09	0.47
T977A	1.41	2.8	1.2	3.0	1.4	1.9	0.16	3.2
K993A	0.74	1.9	0.79	1.9	0.75	0.81	0.071	1.6
V994A	0.18	2.5	0.19	2.8	0.27	1.9	0.027	2.4
I1001A	0.86	6.2	0.70	7.1	1.4	5.1	0.087	6.2
N1015A	1.36	0.6	1.03	0.48	0.95	0.57	1.1	0.42
T1017A	1.17	2.6	1.1	3.4	1.08	1.6	0.10	3.6
D1025A	0.44	4.0	0.57	5.1	0.46	2.0	0.062	5.4
S1026A[b]	0.03	0.4	0.02	0.29	0.05	0.37	0.011	0.68
	2.4	2.3	1.8	2.6	6.8	0.78	0.14	1.5
V1028A	0.97	8.3	0.79	9.9	1.7	3.2	0.082	8.5
I1029A	1.3	5.2	0.93	6.3	1.6	2.8	0.14	6.1

[a] Reaction conditions: pH 7.5, 25°C, 20 mM bicarbonate, 10 mM $MgCl_2$, 100 mM KCl, 10 mM glutamine, and saturating concentration of IMP, UMP, or ornithine. Concentrations of UMP and IMP were 6- to 100-fold higher than the K_d for the effector and never exceeded 10 mM. Ornithine was fixed at 10 mM.

[b] Parameters determined from Eq. [5] for nonlinear double reciprocal plots.

regions of the nucleotide. The amino acids selected for mutagenesis were K954, T974, T977, and K993 in the phosphate region; S948, N1015, T1017, and S1026 in the ribose region; and V994, I1001, D1025, V1028, and I1029 in the vicinity of the aromatic base (see Fig. 2). The kinetic parameters, K_m and k_{cat}, obtained for the wild-type and mutant enzymes, are presented in Tables I–III. The allosteric parameters, K_d and α, obtained for the wild-type and mutant enzymes, are provided in Tables IV and V, while Table VI summarizes the ability of the wild-type and selected mutant enzymes to catalyze the synthesis of carbamoyl phosphate.

Bicarbonate-dependent ATP hydrolysis. With the wild-type enzyme, the kinetic parameters for the partial ATPase reaction were only slightly perturbed by the allosteric effectors UMP and ornithine, whereas IMP did not show any significant effect (Table I). Moreover, the mutations constructed within the allosteric nucleotide monophosphate binding site did not affect significantly the activity of the bicarbonate-dependent ATPase reaction, except for T974A and V994A, which displayed a fivefold decrease and a twofold increase in k_{cat}, respectively. However, S948A, K954A, and N1015A exhibited nonlinear double reciprocal plots at variable concentrations of ATP (Table I).

Glutamine-dependent ATP turnover. UMP was a strong inhibitor of the wild-type enzyme, producing a 2-fold increase in K_{ATP} and a 4-fold decrease in k_{cat} (Table II). At 0.2 mM ATP, UMP decreased the turnover of the wild-type enzyme by 75%, with an apparent K_d of ~1 μM (Table IV and Fig. 3). V1028A was the only mutant in which the allosteric effects of UMP were similar to those found with the wild-type enzyme (Fig. 3). With most mutants, UMP inhibited the reaction, but to a lesser extent than with the wild-type protein (Fig. 3). The residues, which upon mutation to alanine produced the greatest increase in the apparent K_d for UMP, are localized in the phosphate region, with K954A, T977A, and K993A increasing the apparent K_d for UMP by 350- to 1400-fold. In the ribose region, S948A increased the K_d for UMP by a factor of 100. In the vicinity of the aromatic base, V994A and I1029A increased the K_d for UMP by ~30-fold. IMP had a relatively small overall effect on the kinetic parameters of the wild-type and mutant enzymes. No alteration in the catalytic activity by IMP could be measured for 5 of 13 mutants (Table IV). With I1029A, T977A, T1017A, and S1026A, the apparent K_d for IMP increased 13- to 74-fold. With the exception of I1029A, the residues in the vicinity of the aromatic base played a minor role in the binding of IMP to CPS.

The synthesis of carbamoyl phosphate was perturbed in several of the mutant enzymes. The number of molecules of ADP produced to synthesize one molecule of carbamoyl phosphate varied, from 2 with the wild-type enzyme to 5 with T974A and S1026A, 9 with V994A, and 18 with S948A. With N1015A, the synthesis of carbamoyl phosphate was not detectable (Table VI).

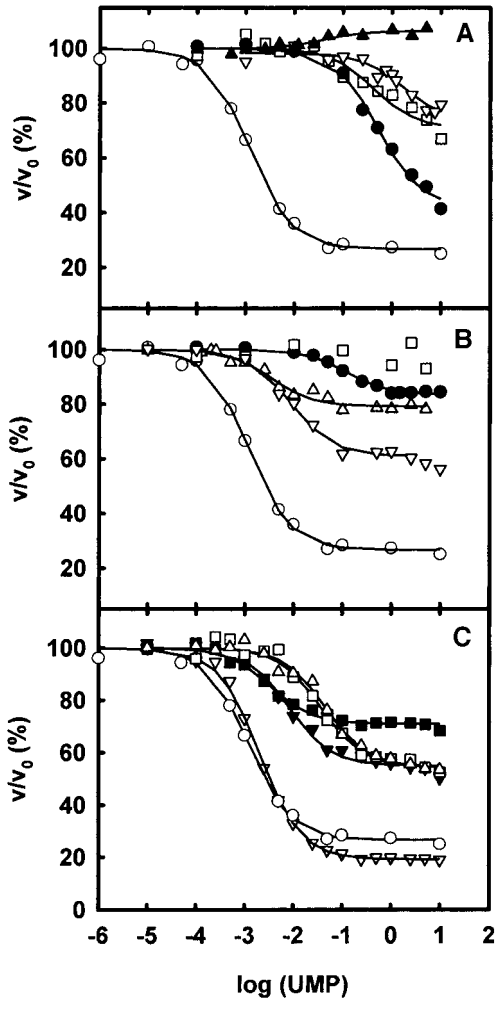

FIG. 3. Influence of UMP on the velocity of the glutamine-dependent ATPase reaction of wild-type and mutant forms of CPS (25°C, 0.2 mM ATP). Velocities are expressed relative to v_o, the velocity in the absence of effector. (A) Phosphate region: (○) wild type; (□) K954A; (▲) T974A; (▽) T977A; (●) K993A. (B) Ribose region: (○) wild type; (●) S948A; (□) N1015A; (▽) T1017A; (△) S1026A. (C) Base region: (○) wild type, (△) V994A; (■) I1001A; (▼) D1025A; (▽) V1028A; (□) I1029A. The lines are fits of the data to Eq. [6].

ATP synthesis reaction. With the wild-type enzyme, the binding of IMP induced a slight increase in K_{ADP} for the partial back reaction (Table III), and, hence, a weak inhibition of the catalytic activity by ~30% with an apparent K_d of 27 μM (Table V, Fig. 4). With T974A, IMP produced a 2-fold increase in K_{ADP} and an unusual 2-fold decrease in k_{cat} (Table III). At 0.2 mM ATP, IMP decreased the maximal activity of T974A by 65%, thus producing a greater inhibition than with the wild-type enzyme. Conversely, with N1015A, T1017A, and V1028A, IMP decreased K_{ADP} by ~2-fold. The saturation curves confirmed that IMP was a weak activator of the partial back reaction with K954A, T1017A, and V1028A, while IMP had no observable effect on N1015A (Table V and Fig. 4). All of the other mutants were inhibited by IMP with values for α close to the value obtained for the wild-type enzyme (Table IV). T977 and S1026, and to a lesser extent S948, T974, and T1017, were important residues for the binding of IMP to CPS, as mutation of these residues to alanine increased the apparent K_d for IMP by factors ranging from 16 to 60. All of these residues are in the vicinity of the phosphate or the ribose moiety of IMP. In the aromatic base region of the allosteric binding site, V994A and I1001A increased moderately the binding constant for IMP by 5- to 10-fold.

With the wild-type enzyme, UMP was a powerful inhibitor of the partial back reaction, as it produced a 14-fold increase in K_{ADP} and a 5-fold decrease in k_{cat} (Table III). The allosteric effects of UMP on K_{ADP} and k_{cat} were reduced slightly with S1026A and V1028A. With V994A, I1001A, and D1025A, UMP induced an 8- to 10-fold increase in K_{ADP}, but had little (V994A, D1025A) or no (I1001A) effect on k_{cat} (Table III). At a fixed concentration of ATP, UMP induced a 50% inhibition in the rate of the back reaction with T974A and T977A (Table V). The mutants K954A, I1001A, N1015A, T1017A, and I1029A also reduced significantly the extent of inhibition by UMP. The rest of the mutants displayed moderate (S948A, K993A, V994A, D1025A) or minor (S1026A, V1028A) effects on the degree of inhibition by UMP. The significant residues for the binding of UMP to CPS were in the phosphate region (K954, T974, T977, and K993) and in the ribose region (S948 and N1015) (Table V). Among the other mutations, only V994A in the aromatic base region resulted in a significant increase in the K_d for UMP (Table V).

DISCUSSION

Ornithine, IMP, and UMP allosterically regulate carbamoyl phosphate synthetase from *E. coli*. The regulatory effects by these modulators of catalytic activity are found primarily, but not exclusively, on the Michaelis constant for the substrate MgATP. The binding of ornithine to CPS enhances the catalytic activity, whereas UMP diminishes substrate turnover. The allosteric effects exhibited by IMP are much smaller than those observed with either UMP or ornithine and very much dependent on specific assay conditions. In addition to the effects on catalytic activity, the binding of either ornithine or IMP to CPS induces the oligomerization to a tetramer, while the binding of UMP promotes the formation of a dimer (27). However, the differences in catalytic activity do not appear to be a direct consequence of the changes in the state of oligomerization (27).

TABLE III

Kinetic Parameters for the ATP Synthesis Reaction[a]

	None		IMP		UMP		Ornithine	
Enzyme	K_a (mM)	k_{cat} (s^{-1})	K_a (mM)	k_{cat} (s^{-1})	K_a (mM)	k_{cat} (s^{-1})	K_a (mM)	k_{cat} (s^{-1})
Wild type	0.32	0.32	0.37	0.32	4.4	0.06	0.019	0.32
S948A	0.26	0.34	0.33	0.36	1.7	0.19	0.016	0.27
K954A	0.32	0.08	nd	nd	1.0	0.06	nd	nd
T974A	0.53	0.16	1.04	0.082	0.55	0.11	0.26	0.14
T977A	0.45	0.18	0.42	0.151	0.67	0.12	0.034	0.16
K993A	0.47	0.12	0.54	0.146	1.7	0.03	0.022	0.18
V994A	0.18	0.41	0.21	0.38	1.42	0.26	0.019	0.43
I1001A	0.19	0.53	0.27	0.50	1.6	0.53	0.015	0.53
N1015A	0.55	0.022	0.21	0.01	1.0	0.02	0.41	0.02
T1017A	0.81	0.25	0.47	0.30	1.3	0.13	0.021	0.21
D1025A	0.20	0.51	0.38	0.65	2.0	0.34	0.026	0.69
S1026A	0.27	0.51	0.44	0.70	2.6	0.14	0.019	0.53
V1028A	0.45	0.55	0.27	0.51	3.7	0.2	0.015	0.53
I1029A	0.23	0.26	0.19	0.25	0.63	0.10	0.023	0.32

Note. nd, not determined.

[a] Reaction conditions: pH 7.5, 25°C, 2.0 mM carbamoyl phosphate, 15 mM $MgCl_2$, 100 mM KCl, and saturating concentration of IMP, UMP, or ornithine.

The three-dimensional structure of CPS has been solved in the presence of ornithine and IMP. These structures have shown that the binding site for each of these allosteric effectors is localized to a domain at the extreme C-terminus of the large subunit. An X-ray structure of CPS in the presence of UMP has not been solved but kinetic and mutagenic experiments are consistent with a model for the UMP binding site that is identical or overlapping with the site for IMP (6, 13–16, 21, 22). However, it remains unclear how the protein discriminates between IMP and UMP or how the allo-

TABLE IV

Allosteric Parameters for the Glutamine-Dependent ATPase Reaction[a]

	IMP		UMP		Ornithine	
Enzyme	α	K_d (μM)	α	K_d (μM)	α	K_d (μM)
Wild type	1.2	11	0.27	1.2	3.4	140
S948A	na	na	0.83	120	1.1	25
K954A	na	na	0.71	410	2.2	220
T974A	na	na	1.1	50	1.6	870
T977A	1.1	230	0.75	1600	4.3	1500
K993A	na	na	0.46	420	3.0	160
V994A	1.1	32	0.54	46	1.4	110
I1001A	1.4	19	0.71	3.3	3.3	86
N1015A	na	na	na	na	na	na
T1017A	1.4	250	0.61	8.7	5.4	190
D1025A	1.1	17	0.55	8.2	2.7	190
S1026A	1.3	780	0.79	3.4	4.0	950
V1028A	1.4	33	0.19	2.1	3.5	180
I1029A	1.7	140	0.54	37	4.5	1200

Note. na, not attainable.

[a] The α and K_d parameters were determined by fitting the effector saturation curves to Eq. [6]. Reaction conditions: pH 7.5, 25°C, 0.2 mM ATP, 20 mM bicarbonate, 10 mM $MgCl_2$, 100 mM KCl, and effector concentration varying between 0 and 10 mM.

TABLE V

Allosteric Parameters for the ATP Synthesis Reaction[a]

	IMP		UMP		Ornithine	
Enzyme	α	K_d (μM)	α	K_d (μM)	α	K_d (μM)
Wild type	0.70	27	0	0.8	2.4	70
S948A	0.71	430	0.12	140	1.7	46
K954A	1.20	10	0.24	170	1.7	60
T974A	0.35	590	0.56	1700	1.1	170
T977A	0.60	1600	0.46	520	1.8	500
K993A	0.82	46	0.12	120	3.3	160
V994A	0.78	260	0.15	26	1.9	120
I1001A	0.74	130	0.24	2.0	2.0	54
N1015A	na	na	0.24	1500	na	na
T1017A	1.6	330	0.30	4.2	4.0	57
D1025A	0.66	27	0.10	4.4	2.0	75
S1026A	0.67	1100	0.03	0.3	1.6	14
V1028A	1.20	3	0.05	1.4	2.6	100
I1029A	na	na	0.21	4.3	2.6	230

Note. na, not attainable.

[a] The α and K_d parameters were determined by fitting the effector saturation curves to Eq. [6]. Reaction conditions: pH 7.5, 25°C, 0.2 mM ADP, 2.0 mM carbamoyl phosphate, 15 mM $MgCl_2$, 100 mM KCl, and effector concentration varying between 0 and 10 mM.

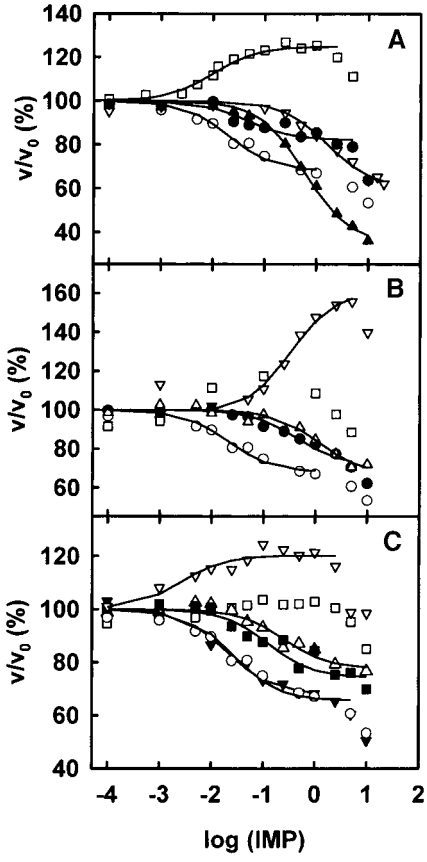

FIG. 4. Influence of IMP on the partial ATP synthesis reaction for the wild-type and mutant forms of CPS (0.2 mM ADP, 2.0 mM carbamoyl phosphate). The rates of reaction are expressed relative to v_o, the velocity in the absence of effector. (A) Phosphate region: (○) wild type; (□) K954A; (▲) T974A; (▽) T977A; (●) K993A. (B) Ribose region: (○) wild type; (●) S948A; (□) N1015A; (▽) T1017A; (△) S1026A. (C) Base region: (○) wild type; (△) V994A; (■) I1001A; (▼) D1025A; (▽) V1028A; (□) I1029A. The lines were calculated from fits of the data to Eq. [6].

steric signals are transmitted to the active site of CPS. Thirteen residues within the allosteric binding domain of CPS were mutated in an effort to identify the specific interactions involved in the recognition of nucleotide monophosphate effectors and the subsequent alteration in catalytic activity. This analysis will concentrate on the measured effects on the partial ATP synthesis reaction.

Direct interactions with phosphate. In the three-dimensional structure of the IMP-bound complex of CPS, the phosphate moiety of the nucleotide interacts with the protein through electrostatic contacts to the side chains of four residues. The hydroxyl groups of T974 and T977 are 2.4–2.5 Å away from the nearest phosphoryl oxygen of the bound IMP, while the ε-amino groups of K954 and K993 are 2.4–2.5 Å away from the phosphate substituent of IMP. Since the value of α for IMP is very close to unity, the apparent K_d value for IMP is more difficult to measure accurately than it is for UMP. Nevertheless, the apparent dissociation constant for UMP (0.8 μM) from CPS is significantly lower than it is for IMP (27 μM) with the wild-type enzyme. Mutation of any of the four amino acid residues that make direct electrostatic contact with the phosphate moiety of the bound nucleotide monophosphate effector results in an elevation in the value of α and also in the apparent K_d for the binding of UMP to CPS. With UMP there is a direct correlation in the elevation of the apparent K_d and the diminution of the maximum allosteric effect. For example, the T974A mutant had the greatest increase in K_d (to 1700 μM) and the greatest increase in the magnitude of the value of α (to 0.56) for UMP. Thus, the diminished binding of UMP to these mutants also resulted in a weaker allosteric effect at saturating concentrations of UMP.

The trends with IMP are not so easily interpreted. For example, the apparent K_d for IMP with the T974A mutant is elevated but the value of α is lowered substantially. Therefore, the maximum inhibitory effect at saturating concentrations of IMP is enhanced with T974, relative to the effects exerted by IMP with the wild-type enzyme. Moreover, the K954A mutant is an activator rather than an inhibitor of the partial back reaction with very little change in the apparent K_d value.

Interactions with ribose. There are four residues in the X-ray crystal structure of the IMP–CPS complex that make direct molecular interactions with the ribose moiety of IMP. The side-chain hydroxyl of S1026 is within hydrogen bonding distance to the 2′-hydroxyl of the bound IMP. The removal of the hydroxyl group in S1026A has a relatively small effect on the allosteric properties possessed by UMP since the S1026A mutant has an apparent K_d and value of α for UMP that are very close to those of the wild-type protein. However,

TABLE VI

Synthesis of Carbamoyl Phosphate by Wild-Type and Mutant CPS[a]

Enzyme	ADP production (μmol/min/mg)	CP synthesis (μmol/min/mg)	ADP/CP
Wild type	3.9	1.7	2.3
S948A	0.2	0.011	18
T974A	0.23	0.043	5.3
V994A	1.2	0.14	9.0
N1015A	0.2	<0.01	—
S1026A	0.5	0.1	5.0

[a] Reaction conditions: pH 7.5, 25°C, 4 mM ATP, 20 mM bicarbonate, 10 mM $MgCl_2$, 100 mM KCl, 10 mM ornithine, 10 mM glutamine.

the removal of the hydroxyl group from S1026 significantly increased the apparent K_d for IMP. Therefore, the contribution of the side chain from S1026 to the binding of IMP with CPS is significantly greater than it is to the binding of UMP. The interaction of this residue contributes to the distinction between IMP and UMP in the allosteric binding pocket.

The other three residues that interact with the ribose moiety of IMP are S948, N1015, and T1017. The hydroxyl group of T1017 is within hydrogen bonding distance from the ribose hydroxyls of IMP. The mutation of T1017 to an alanine had a relatively modest effect on the allosteric properties possessed by UMP but, curiously, the T1017A mutant was activated, rather than inhibited, by IMP. This inversion of the allosteric effect by IMP, from a mild inhibitor to a mild activator of the ATP synthesis reaction, was reported for the wild-type enzyme by a change in the assay temperature (17). Previous experiments by Boettcher and Meister (13, 14) also showed that a change in structure at the 2′-carbon of the ribose moiety of UMP reversed the allosteric effect, from inhibition with UMP to activation with *ara*-UMP and dialdehyde-UMP. Boettcher and Meister (13, 14) have proposed a model in which the discrimination between IMP and UMP occurs via a differential orientation of the ribose moiety within the bound IMP–CPS complex. However, none of the mutations that have been characterized in the ribose region of the allosteric domain of CPS could reverse the effect of UMP from inhibition to activation. The side chain of S948 interacts with the C4′-oxygen of IMP. Removal of the hydroxyl group of S948 elevated the K_d for both UMP and IMP as observed previously (20, 22). A significant change in the catalytic activity was observed upon mutation of N1015. This mutant was unable to synthesize carbamoyl phosphate and the allosteric effects were nearly abolished for all of the reactions that could be measured. Clearly the loss of N1015 has caused a significant perturbation to the allosteric domain that has, in turn, disrupted the catalytic core of the large subunit.

Interactions with aromatic base. IMP and UMP differ only by their aromatic base. Therefore, it was anticipated that the residues in the vicinity of the base within the IMP/UMP binding site would play a key role in the discrimination between IMP and UMP. Previous reports on mutations within the vicinity of the nucleotide base have included R1030A and R1031A (8), H995A (15), and V994A, G997A, G1008A, R1020A, and R1021A (22). Except for V994A, these mutations had a rather modest influence on the binding and subsequent allosteric effects exhibited by UMP and IMP. The structure of CPS with IMP revealed that the hypoxanthine ring of IMP is packed against a fairly hydrophobic pocket formed by the side chains of I1001, V1028, and I1029. The only direct electrostatic contact with the enzyme occurs via the backbone of V994 (19). In the present study, five mutations were created in the vicinity of the base: V994A, I1001A, D1025A, V1028A, and I1029A. Our results confirmed that V994 plays a significant role in the binding and the allosteric effects of UMP and IMP (Tables III and V), but V994A did not suppress altogether the allosteric regulation by the nucleotides. This suggests that this residue is not as essential for the transduction of the allosteric signal as previously proposed (22). The mutants I1001A, V1028A, and I1029A reduced the hydrophobic constraints of the binding domain for the aromatic base. The mutation of V1028 produced little change in the allosteric properties compared to the wild-type enzyme. I1029A affected the binding and the allosteric effects of both UMP and IMP. I1001A had little affect on the binding of UMP and IMP, as well as on the allosteric effects of IMP. However, I1001A completely suppressed the effect of UMP on k_{cat}, and accordingly, the K_d for UMP was significantly increased. These results support the conclusion that the hydrophobic pocket, identified in the aromatic binding domain of the nucleotide (19), plays a role in the allosteric regulation of CPS activities by IMP and UMP, but is not directly responsible for the discrimination between IMP and UMP.

Allosteric effects of ornithine. Since all of the mutations reported here are directly concerned with residues in the nucleotide-binding pocket, it was anticipated that the binding and allosteric effects exhibited by ornithine would remain relatively unchanged. Except for T977A and I1029A, for which the apparent K_d for ornithine increased by about an order of magnitude, the binding of ornithine to CPS was largely unaffected. However, the activation by ornithine of the forward reaction was reduced in K954A, T974A, and V994A and suppressed in S948A and N1015A. S948A and V994A showed the lowest activation of the ATPase reaction by glutamine and had the largest ratio of ATP hydrolyzed per molecule of carbamoyl phosphate produced. With S948A and V994A, the observed decrease in the activation by ornithine of the glutamine-dependent ATPase reaction can be explained by an uncoupling of the partial reactions of CPS. The activation effect of ornithine in the partial back reaction was barely visible in T974A and N1015A. In agreement with our finding, T974A was previously reported to display a lower activation by ornithine (22). These results support the proposal that the allosteric regulations by UMP/IMP and ornithine are not entirely independent of one another, but are coupled (16, 20). The residues that contribute to the binding of ornithine and the subsequent transmission of the allosteric signals to the carbamate domain of the large subunit are identified in the companion paper (28).

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