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Dissection of the Conduit for Allosteric Control of Carbamoyl Phosphate Synthetase by Ornithine

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Ornithine is an allosteric activator of carbamoyl phosphate synthetase (CPS) from *Escherichia coli*. Nine amino acids in the vicinity of the binding sites for ornithine and potassium were mutated to alanine, glutamine, or lysine. The residues E783, T1042, and T1043 were found to be primarily responsible for the binding of ornithine to CPS, while E783 and E892, located within the carbamate domain of the large subunit, were necessary for the transmission of the allosteric signals to the active site. In the K loop for the binding of the monovalent cation potassium, only E761 was crucial for the exhibition of the allosteric effects of ornithine, UMP, and IMP. The mutations H781K and S792K altered significantly the allosteric properties of ornithine, UMP, and IMP, possibly by modifying the conformation of the K-loop structure. Overall, these mutations affected the allosteric properties of ornithine and IMP more than those of UMP. The mutants S792K and D1041A altered the allosteric regulation by ornithine and IMP in a similar way, suggesting common features in the activation mechanism exhibited by these two effectors. © 2002 Elsevier Science (USA)

Key Words: **carbamoyl phosphate synthetase; allosteric control; ornithine.**

In the companion paper (26), several key amino acids were identified within the allosteric binding site for IMP/UMP from carbamoyl phosphate synthetase (CPS)[2] that are responsible for the discrimination and recognition between the nucleotide monophosphate effectors. The residues that interact directly with the phosphate and ribose moieties of IMP had the greatest impact on the binding of the allosteric nucleotide monophosphate effectors and on the alterations to the kinetic constants of CPS induced by the binding of either UMP or IMP. One residue, S1016, that hydrogen bonds to the ribose moiety of IMP influenced the binding of IMP more than the binding of UMP, suggesting a potential role in the discrimination between UMP and IMP. However, the residues surrounding the hypoxanthine ring of IMP in the X-ray crystal structure had only a modest influence on the binding constants for the monophosphate nucleotides and the allosteric effects exhibited by either UMP or IMP. The largely hydrophobic residues in the IMP binding site, shown to interact with the base of the nucleotide monophosphate, appear not to be responsible for the ultimate distinction between UMP and IMP.

Ornithine, the first substrate in the biosynthetic pathway for arginine, is an allosteric activator of CPS from *Escherichia coli* (1–4). Competitive binding experiments have demonstrated that ornithine does not associate physically with the same site as either UMP or IMP (5–7). Ornithine activates CPS by increasing the affinity of the enzyme for the substrate MgATP and this activation phenomenon dominates the inhibitory effects induced by the binding of UMP (6). The specific binding site for the positive allosteric effector ornithine was first identified in the X-ray crystal structure of CPS (8, 9). In this protein complex ornithine acts as a bridge between the allosteric and the carbamate domains and a pictorial view of the binding pocket for ornithine is illustrated in Fig. 1 (9). Five residues interact directly with ornithine in the bound complex, either through the backbone amide groups of D791, Y1040, and T1042 or through the side chains of E783, E892, and T1042 (9).

The concept of a *conduit* for the propagation of conformational effects from an allosteric ligand binding site to an active site has been formulated for aspartate transcarbamoylase (10–12) and has been the subject of structure-based computational analyses and molecular

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[2] Abbreviation used: CPS, carbamoyl phosphate synthetase.

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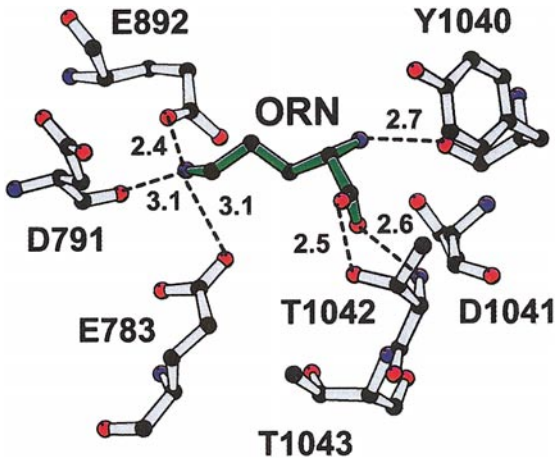

FIG. 1. Ball-and-stick representation of the molecular interactions between ornithine and the amino acid residues in the allosteric and carbamate domains of CPS. The distances between heteroatoms are indicated in angstroms.

modeling (13, 14). However, a general model of the structural basis for the transmission of allosteric effects within regulated enzymes is not well understood. The binding of ornithine and IMP/UMP to the allosteric domain of CPS may be communicated to the catalytic active sites via a series of hydrogen bonds (15). A hydrogen-bonded pathway from the 2′-hydroxyl group of the ribose moiety of IMP through ornithine to the terminal phosphoryl group of ADP has been previously proposed on the basis of the X-ray structure of CPS bound with ornithine and IMP (15).

The amino acid residues that participate in the binding sites for potassium, IMP, ornithine, and ADP are shown in Fig. 2 (15). The potassium ion is coordinated to CPS by the side chains of E761, H781, and S792, in addition to the backbone carbonyl oxygens of Q784 and V787, to form a *K loop* (8, 9). The mutant E761A, in the K loop from the carbamate domain, alters the overall allosteric effects of ornithine and UMP on the kinetic parameters (16). Moreover, the mutation of T1042 to isoleucine in the ornithine binding site impairs the allosteric activation of CPS by ornithine and diminishes the allosteric effects by UMP on enzyme activity (17). Taken together, these results suggest that the signal transduction pathways for the allosteric effects of ornithine, IMP, and UMP may have common or overlapping segments. In this paper, a comprehensive mutational analysis of the binding pocket for ornithine and the K loop of CPS within the carbamate domain of CPS is reported. This study was conducted in order to elucidate the essential residues for the allosteric effects by ornithine and to identify the potential pathways for the propagation of the induced conformational alterations.

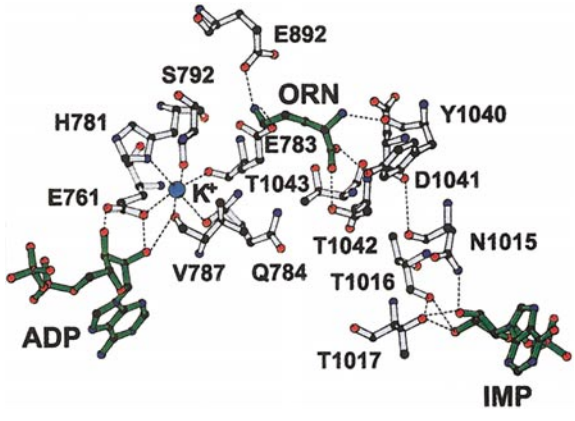

FIG. 2. Ball-and-stick representation of the amino acid residues from CPS that form a putative pathway of linked hydrogen-bonded residues that extend from the ribose hydroxyl of IMP through the ornithine and the potassium binding sites to the ribose hydroxyl of ADP.

TABLE I

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

	Ornithine		UMP		IMP	
Enzyme	α	K_d (mM)	α	K_d (μM)	α	K_d (μM)
Wild type	3.4	0.14	0.27	1.2	1.2	11
E761A[b]	na[c]	na[c]	na	na	na	na
H781A	2.1	0.07	0.31	1.5	1.2	20
H781K	2.1	1.8	0.43	0.6	na	na
E783Q	1.3	11	0.25	2.3	nd	nd
E783A	na[c]	na[c]	0.33	0.9	1.2	20
E783K	na[c]	na[c]	0.42	2.4	nd	nd
Q784A	5.9	0.54	0.44	2.7	0.9	70
S792A	4.7	0.11	0.32	0.7	1.1	6
S792K	0.70	10	0.44	1.7	0.7	200
E892Q	2.0	1.0	0.30	0.7	nd	nd
E892A	na[c]	na[c]	0.29	4.4	1.1	60
E892K	na	na	0.24	5.3	nd	nd
D1041A	14	0.10	0.44	1.6	3.9	100
T1042A	3.9	14	0.45	0.43	1.6	90
T1043A	4.2	14	0.48	1.9	1.2	70
T1043K	na	na	0.27	10	0.48	2100

Note. nd, not determined; na, not attainable.

[a] The values of α and K_d were determined by fits of the effector saturation curves to Eq. [2]. Reaction conditions: pH 7.5, 25°C, 0.2 mM ATP, 20 mM bicarbonate, 10 mM $MgCl_2$, and 100 mM KCl.

[b] Reactions measured at 200 mM bicarbonate and 0.05 mM ATP.

[c] Inhibition at concentrations of ornithine above 10 mM was observed.

MATERIALS AND METHODS

Materials. Restriction enzymes were from New England Biolabs and Life Technologies. The QuikChange site-directed mutagenesis kit was purchased from Stratagene. The Magic Miniprep DNA purification kit was purchased from Promega. Oligonucleotide synthesis

TABLE II

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

	None		Ornithine		UMP		IMP	
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	0.07	10.4	1.35	3.3	0.78	12.4
E761A	0.06	6.7	0.07	6.7	0.05	6.7	nd	nd
H781A	0.70	9.8	0.19	10.1	1.11	3.6	0.56	9.7
H781K	0.52	0.7	1.17	2.6	0.23	0.3	0.53	0.64
E783Q	0.52	7.9	0.37	7.3	1.39	3.2	nd	nd
E783A	1.45	6.3	1.67	4.2	0.32	0.7	1.58	7.9
E783K	0.52	0.85	0.49	0.6	0.14	0.3	nd	nd
Q784A	1.36	6.7	0.19	11.3	0.90	1.7	1.36	5.7
S792A	1.56	6.2	0.75	17.2	0.28	0.6	1.52	6.3
S792K	0.54	0.63	0.40	0.3	0.23	0.2	0.52	0.5
E892Q	0.46	6.5	0.10	7.2	1.46	3.1	nd	nd
E892A	0.18	9.3	0.24	9.2	0.81	8.0	0.20	9.9
E892K	0.28	8.1	0.45	7.7	1.43	4.8	nd	nd
D1041A	2.2	7.6	0.10	14.3	1.10	1.4	0.87	14.1
T1042A	1.77	7.1	0.48	9.4	2.2	3.9	1.2	9.0
T1043A	1.64	4.9	0.48	7.4	1.86	2.6	1.71	5.6
T1043K	1.47	6.9	1.35	6.7	1.27	1.3	1.22	3.7

Note. nd, not determined.

[a] The kinetic constants were determined from a fit of the data to Eq. [1]. 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.

and DNA sequencing were done by the Gene Technologies Laboratory, Texas A&M University.

Mutagenesis, expression, and purification of proteins. The mutant E761A (16) and the mutants E783Q, E783K, E892Q, and E892K were constructed using the method of overlap extension (18). The remaining mutant plasmids were created using the QuikChange site-directed mutagenesis kit from Stratagene. Mutant enzymes were expressed and purified from the RC50 mutant strain of *E. coli* that is defective in wild-type CPS activity (a generous gift from Dr. Carol Lusty). The mutant proteins were purified following the procedure previously described (19). The identity of the mutant plasmids was confirmed by DNA sequencing.

Kinetic and allosteric parameters. The glutamine-dependent ATPase and the ATP synthesis reactions were measured as previously described (20). Briefly, the allosteric parameters (K_d, α) were first estimated by measuring the enzyme activity at various concentrations of effector at a fixed nonsaturating concentration of substrate (0.2 mM ATP or 0.2 mM ADP and 2.0 mM carbamoyl phosphate). The kinetic parameters for the substrate (K_a, k_{cat}) were measured in the absence and presence of a saturating concentration of the allosteric ligand. The glutamine-dependent ATPase reaction was initiated by the addition of 12–450 μg of enzyme per 2.5 ml reaction volume. The ATP synthesis reaction was initiated by adding 0.2–2.0 mg of enzyme in a final volume of 2.0 ml. For certain mutant enzymes, the ATP synthesis reaction contained either 10 (H781K, E783K, S792K) or 20 mM (E761A) carbamoyl phosphate, and the effector saturation curves were obtained at 0.5 (E783K) or 1.0 mM (E761A, H781K, S792K) ADP. Saturating concentrations of UMP and IMP were 0.1 and 1.0 mM, respectively, except for E761A, H781K, S792K, D1041A, T1042A, and T1043K, for which IMP was utilized at 2.5–5.0 mM. The synthesis of carbamoyl phosphate was determined, as previously described (20), by measuring the rate of formation of citrulline in a coupled assay with ornithine and ornithine transcarbamoylase (21).

Statistical analysis of kinetic data. The kinetic parameters V_m and K_a were determined by fitting the data to Eq. [1], in which ν is the initial velocity, A is the substrate concentration, V_m is the initial velocity at saturating substrate concentration, and K_a is the Michaelis constant of CPS for substrate A. The allosteric parameters were determined by a fit of the data to Eq. [2], in which L is the allosteric ligand concentration, ν is the initial velocity, ν_o is the initial rate in absence of 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:

$$\nu = V_m A/(K_a + A), \quad [1]$$

$$\nu = \nu_o(\alpha L + K_d)/(L + K_d). \quad [2]$$

RESULTS

The three-dimensional protein structure of CPS with ADP and ornithine bound to the enzyme was used as a guide for the functional dissection of the ornithine and potassium binding sites within the carbamate and the allosteric domains of CPS (8, 9, 15). The residues selected for site-directed mutagenesis to alanine within the binding pocket for ornithine included E783, E892, D1041, T1042, and T1043, whereas those involved in the direct coordination of the potassium ion included E761, H781, E783, Q784, and S792. In order to modulate the electrostatic charge within the ornithine and the potassium binding sites, certain residues were also mutated to glutamine or lysine. Each mutant was tested for its ability to be regulated by the allosteric

TABLE III

Allosteric Parameters for the ATP Synthesis Reaction[a]

	Ornithine		UMP		IMP	
Enzyme	α	K_d (mM)	α	K_d (μM)	α	K_d (μM)
Wild type	2.4	0.07	<0.02	0.8	0.70	27
E761A[b]	na[c]	na[c]	0.80	6	na	na
H781A	1.9	0.08	0.06	1.7	0.64	40
H781K[b]	2.3	4.0	0.67	1.7	na	na
E783Q	1.4	12	0.04	1.6	0.81	60
E783A	na[c]	na[c]	0.04	1.1	1.17	8
E783K[b]	na[c]	na[c]	0.21	2.9	nd	nd
Q784A	4.4	0.41	0.10	2.2	0.44	50
S792A	5.5	0.27	0.20[b]	1.8[b]	0.64	70
S792K[b]	0.7	1.7	0.66	4.2	0.65	250
E892Q	1.6	0.8	0.05	1.0	nd	nd
E892A	na	na	0.08	3.6	0.76	28
E892K	na	na	0.05	2.2	nd	nd
D1041A	3.2	0.02	<0.02	0.7	2.02	33
T1042A	1.7	6	0.03	0.4	1.21	25
T1043A	2.8	13	0.26	1.1	1.14	5
T1043K	na[c]	na[c]	0.17	2.0	0.23	200

Note. nd, not determined; na, not attainable.

[a] The α and K_d parameters were determined from a fit of the effector saturation curves to Eq. [2]. Reaction conditions: pH 7.5, 25°C, 0.2 mM ADP, 2.0 mM carbamoyl phosphate, 15 mM $MgCl_2$, and 100 mM KCl.

[b] Reaction at 10 (E783K, H781K, S792K) or 20 mM (E761A) carbamoyl phosphate and 0.5 (E783K) or 1.0 mM (E761A, H781K, S792A, S792K) ADP.

[c] Concentrations of ornithine above 10 mM inhibited the reaction.

ligands ornithine, UMP, and IMP. The allosteric parameters, α and K_d, obtained for the wild-type and the mutant enzymes are presented in Tables I and III. The kinetic parameters, K_a and k_{cat}, obtained for the wild-type and the mutant enzymes are provided in Tables II and IV. The ability of the wild-type and selected mutant enzymes to catalyze the synthesis of carbamoyl phosphate is given in Table V.

Effect of mutation on catalytic turnover. In the absence of allosteric ligands, most of the mutations constructed for this investigation did not affect the enzyme turnover, relative to the wild-type enzyme, with the exception of H781K, E783K, and S792K. With these mutants the catalytic activities of CPS were diminished by one to two orders of magnitude relative to the wild-type enzyme (Tables II and IV). The mutants E761A, E783K, and S792K were particularly inefficient in the synthesis of carbamoyl phosphate and the turnover of ATP was poorly enhanced by the addition of glutamine to the assay solutions (Table V).

Effect of mutation on allosteric properties. Ornithine activated the glutamine-dependent ATPase and the ATP synthesis reactions of the wild-type CPS with an apparent K_d of 70–140 μM (Tables I and III and Fig. 3). Ornithine activated the wild-type enzyme by increasing the affinity of CPS for its nucleotide substrate (ATP or ADP) by about one order of magnitude, while not affecting the value of k_{cat} (Tables II and IV). With the mutant enzymes, the effect of ornithine on the Michaelis constant for ATP (Table II) was increased (D1041A), reduced (E783Q, H781A, E892Q, T1042A, T1043A, S792A), suppressed (S792K, E761A, E783A, E783K, E892A, T1043K), or reversed (H781K, E892K). With some mutants, ornithine also induced an unusual increase (T1042A, T1043A, H781K, Q784A, S792A, D1041A) or decrease (S792K, E783K) in the value of k_{cat} for the forward reaction (Table II). Remarkably, ornithine was a stronger activator with D1041A than with the wild-type enzyme (Tables II and IV and Fig. 3). In contrast, ornithine had no effect on the activity of E761A, E783A/K, E892A/K, and T1043K (Fig. 3). Moreover, S792K converted ornithine into a mild inhibitor (Fig. 3). The residues that contributed the most to the binding of ornithine to CPS were E783, T1042, and T1043 and, to a lesser extent, E892 (Tables I and III and Fig. 3).

UMP is bound more tightly to the wild-type CPS than is IMP. UMP is a strong inhibitor of the catalytic activity of CPS, whereas IMP induces more modest effects (Tables I and II and Figs. 4 and 5). The mutations constructed at the ornithine and the potassium binding sites did not alter significantly the binding of UMP and IMP, with the exception of T1043K and S792K, which affected the binding of IMP more than UMP (Tables I and III). In general, these mutations had a greater impact on the allosteric effects of IMP than of UMP (Tables I and III and Fig. 4). However, the inhibition of the ATP synthesis reaction by UMP was particularly reduced in E761A, H781K, and S792K (Table III and Fig. 5).

DISCUSSION

In the present study, we have attempted to identify those amino acid residues within the large subunit of CPS that are important for the binding and/or the allosteric effects of ornithine and to probe the influence of the residues forming the potassium loop on the allosteric regulation of CPS. In the initial part of this analysis, we will concentrate primarily on the direct impact of mutations within the ornithine and potassium binding sites. We will conclude with a discussion of the effects of these mutations on the transmission of the conformational signals from the ornithine and IMP/UMP binding sites. For this purpose, residues in the vicinity of the binding sites for ornithine (E783, E892, D1041, T1042, T1043) and potassium (E761, H781, E783, Q784, S792) were mutated to alanine and, in certain cases, to glutamine or lysine. Kinetic characterization of each mutant enzyme was conducted in

TABLE IV

Kinetic Parameters for the ATP Synthesis Reaction[a]

	None		Ornithine		UMP		IMP	
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	0.02	0.32	4.4	0.065	0.37	0.32
E761A[b]	0.33	0.03	0.35	0.03	0.3	0.019	0.30	0.02
H781A	0.61	0.34	0.10	0.26	1.7	0.08	0.85	0.31
H781K[b]	0.61	0.02	1.52	0.05	0.3	0.006	0.50	0.01
E783Q	0.24	0.56	0.07	0.44	2.3	0.163	0.32	0.49
E783A	1.67	0.21	1.67	0.16	0.5	0.016	1.88	0.18
E783K[b]	0.13	0.02	0.07	0.01	0.3	0.011	nd	nd
Q784A	0.83	0.28	0.09	0.38	1.0	0.047	1.37	0.20
S792A	1.43	0.08	0.47	0.30	0.5	0.010	1.40	0.06
S792K[b]	0.65	0.03	0.28	0.02	0.3	0.017	0.33	0.02
E892Q	0.15	0.44	0.03	0.39	2.0	0.155	nd	nd
E892A	0.061	0.34	0.07	0.32	0.7	0.133	0.097	0.32
E892K	0.13	0.39	0.20	0.29	1.8	0.16	nd	nd
D1041A	0.46	0.56	0.02	0.54	3.9	0.080	0.14	0.62
T1042A	0.60	0.33	0.06	0.28	3.5	0.107	0.23	0.36
T1043A	0.52	0.17	0.08	0.16	1.5	0.075	0.61	0.18
T1043K	0.37	0.32	0.40	0.25	0.4	0.073	1.01	0.17

Note. nd, not determined.

[a] The kinetic constants were determined from a fit of the data to Eq. [1]. 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.

[b] Reaction at 10 (H781K, E783K, S792K) or 20 mM (E761A) carbamoyl phosphate.

the presence or the absence of the allosteric ligands ornithine, UMP, and IMP.

Binding site for ornithine. The X-ray crystal structure of CPS revealed that the binding site for ornithine is located at the interface between the carbamate and the allosteric domains (9, 22). The δ-amino group of ornithine is ion paired to the side chain carboxylate groups of E783 and E892, while the hydroxyl group from T1042 interacts with the α-carboxylate of ornithine. Additional interactions between CPS and ornithine occur through the backbone amides of D791, Y1040, and T1042. Since the neutralization of the negatively charged binding pocket by the δ-amino group of ornithine might play a role in regulation, we attempted to mimic the effect of ornithine with the mutants E783Q and E892Q. Neither E783Q nor E892Q was able to imitate the allosteric effect of ornithine with an increased affinity of CPS for ATP or ADP. Therefore, simple electrostatic perturbations cannot explain the allosteric properties of ornithine with CPS. However, the neutralization of the negative charge contributed by the side chain of E783 had a greater impact on the binding of ornithine and the subsequent allosteric effects than the modification of the side chain from E892. The allosteric effects of ornithine were suppressed after mutating either of the two glutamates to alanine or lysine, indicating a significant role for these side chains in the allosteric activation mechanism of ornithine. In a sequence alignment of several bacterial CPS enzymes (data not shown), E892 is conserved in bacteria harboring the allosteric regulation by ornithine (23), but not in the *Bacillus* sp. enzymes that are insensitive to this effector (23, 24).

Ornithine activated the enzymatic reactions catalyzed by T1042A at a level similar to that of the wild-type enzyme, indicating that T1042 is not essential for the transmission of the allosteric signal from ornithine to the active site, in contrast to a previous report with T1042I (17). In wild-type CPS, ornithine is a K-type activator that increases the affinity of CPS for its sub-

TABLE V

Glutamine Activation of the ATPase Reaction and Formation of Carbamoyl Phosphate[a]

Enzyme	Gln activation (fold)[b]	ADP production (μmol/min/mg)	CP synthesis (μmol/min/mg)	ADP/CP
Wild type	16	3.9	1.7	2.3
E761A	2.3	2.5	0.02	125
H781K	nd	0.8	0.23	3.3
E783K	1.7	0.3	0.02	15
S792K	2.4	0.13	0.013	10

[a] Reaction conditions: pH 7.5, 25°C, 4.0 mM ATP, 20 mM bicarbonate, 10 mM $MgCl_2$, 100 mM KCl, 10 mM ornithine, and 10 mM glutamine.

[b] Ratio of ATPase activity in presence or in absence of 10 mM glutamine.

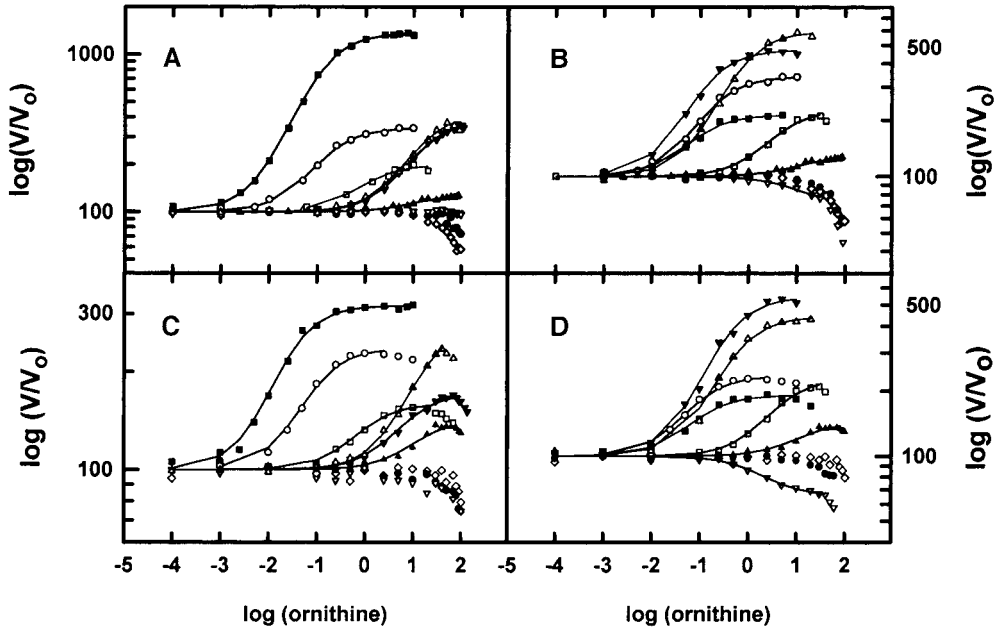

FIG. 3. Influence of ornithine on the velocity of the glutamine-dependent ATPase reaction (A and B) and the ATP synthesis reaction (C and D) for the wild-type and mutant enzymes. (○) Wild type. A and C illustrate residues within the ornithine binding pocket: (▲) E783Q; (◇) E783A; (□) E892Q; (●) E892A; (■) D1041A; (▼) T1042A; (△) T1043A; and (▽) T1043K. B and D illustrate residues within the K loop: (●) E761A; (■) H781A; (□) H781K; (▲) E783Q; (◇) E783A; (△) Q784A; (▼) S792A; and (▽) S792K.

strate ATP. The mutant D1041A was evaluated because the carboxylate side chain of this residue is ~3.5 Å from the α-amino group of ornithine, but D1041 apparently does not contribute directly to the binding of ornithine. In the forward reaction, ornithine was a stronger activator of D1041A than with the wild-type enzyme and became a V-type activator by increasing the value of k_{cat}. The side chain of T1043 extends toward the carbamate domain and is ~4.5 Å from the carboxylate group of E783. T1043K was constructed in an attempt to forge an alternative electrostatic link from the allosteric domain to the carbamate domain, in a manner similar to the binding of ornithine. However, T1043K did not mimic the action of ornithine on the affinity of CPS for ATP but it did suppress the allosteric effect of ornithine. The mutation T1043A confirmed that this residue is involved in the binding of ornithine to CPS, in a manner similar to T1042 and E783. However, T1043 is not critical for the allosteric effects induced by ornithine.

Potassium loop. In the structure of CPS with IMP and ornithine, E761 and E783 are two key residues that are found at the interface of two binding pockets (15). The carboxylate side chain of E761 hydrogen bonds with the ribose hydroxyl of ADP and coordinates with the potassium ion. E783 interacts with the δ-amino group of ornithine through the carboxylate side chain and also coordinates the potassium ion via the backbone carbonyl oxygen. Other residues contributing to the K loop include the side chains of H781 and S792 and the carbonyl backbone oxygens of Q784 and V787. The primary ligands to the potassium ion were deleted by mutating E761, H781, and S792 to alanine. The allosteric effects of ornithine were suppressed in

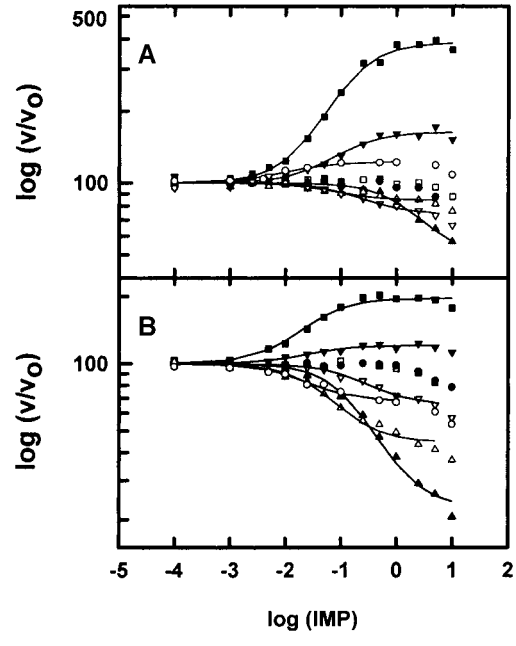

FIG. 4. Influence of IMP on the velocity of the glutamine-dependent ATPase reaction (A) and the ATP synthesis reaction (B) of the wild-type and mutant enzymes. (○) Wild type; (●) E761A; (□) H781K; (△) Q784A; (▽) S792K; (■) D1041A; (▼) T1042A; and (▲) T1043K.

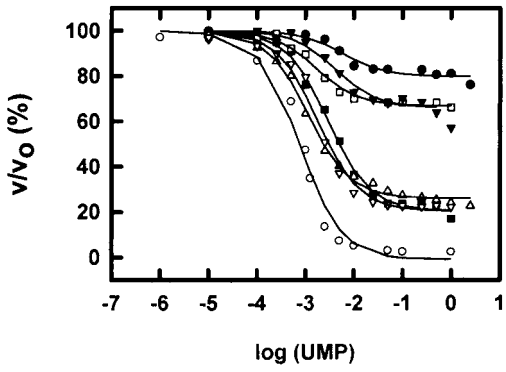

FIG. 5. Influence of UMP on the velocity of the ATP synthesis reaction of the wild-type and mutant enzymes. (○) Wild type; (●) E761A; (□) H781K; (■) E783K; (▽) S792A; (▼) S792K; and (△) T1043A.

E761A but were maintained in H781A and S792A, indicating that only E761 is crucial for the transmission of the allosteric signal from ornithine. H781K and S792K were constructed in an attempt to replace or displace the potassium ion from the K loop. S792K converted ornithine from a K-type activator to a V-type inhibitor of the reactions catalyzed by CPS. With H781K, ornithine had unusual K-type inhibitor and V-type activator effects. The mutation of H781 and S792 to lysine perturbed the binding of ornithine more than the mutation to alanine, suggesting a more global modification on the overall structure of the K loop.

Transmission of allosteric signal. The mutation of residues within the ornithine binding site demonstrated that E783 and E892 are important for the allosteric activation by ornithine, but that D1041, T1042, and T1043 are not required. Therefore, the two primary protein ligands (E783 and E892), located within the allosteric ornithine binding site on the carbamate domain of the large subunit, are necessary for signal transduction from ornithine to the active site. The mutational analysis of the primary ligands to the potassium ion showed that E761 was essential for the transmission of the allosteric effect of ornithine to the active site, but not H781 or S792. The alteration of the allosteric properties possessed by ornithine in H781K and S792K can, in part, be explained by a modification of the overall structure of the K loop, possibly via a displacement of the potassium ion itself, which in turn may have adversely affected the binding site for ornithine.

The presence of IMP bound to the allosteric domain of CPS may be transmitted to the active site within the carbamate domain via a series of hydrogen bonds. The identification of an electrostatic pathway was probed through mutagenesis of amino acid side chains in this region of the enzyme. One putative conduit begins at the 2′-hydroxyl group of the ribose moiety of IMP, passes through N1015, D1041, and T1042, and ultimately winds it way to the ATP binding site via the potassium loop. With the wild-type enzyme, IMP has minor effects on the kinetic parameters of reactions catalyzed by CPS. At the interface between the IMP and the ornithine binding sites, D1041A, and to a lesser extent T1042A, converted IMP into a V- and K-type activator. The mutation of D1041 increased the extent of CPS activation by both IMP and ornithine. This result is consistent with a common or overlapping component(s) in the mechanism of signal transduction by these two allosteric ligands.

In contrast, T1043A had little effect on the allosteric properties of IMP. However, T1043K increased significantly the K_d for IMP and converted IMP from a weak effector in the wild-type enzyme to a strong inhibitor. The side chain of Q784 is ~4 Å from T1043. The mutant Q784A was made in an attempt to identify an alternative allosteric signal transduction pathway from the IMP binding site. Q784A did not disrupt the allosteric signal from IMP but produced modifications on the allosteric effects by IMP similar to those of T1043K. The deletion of the primary ligands of the potassium ion did not alter the allosteric effects of IMP, with the exception of E761A. However, the stronger alterations on the allosteric effects of IMP by the mutants H781K and S792K suggest a more profound modification on the structure of the K loop that may in turn have affected the allosteric signal pathway from IMP.

The allosteric inhibitory signal of UMP was maintained in all of the mutants, with the exception of E761A, on which UMP had little or no effect. In the wild-type enzyme, UMP had a complex effect, being a K- and V-type inhibitor. With the exception of E892A, these mutations altered the K-type inhibitor effects of UMP more than the V-type inhibitor effects. Curiously, several mutations significantly affected the inhibition by UMP in the ATP synthesis reaction (large increases of α) but not in the full forward reaction. This discrepancy may be due to the already stronger K-type inhibitory effect of UMP in the back reaction of the wild-type enzyme, compared to the forward reaction. In the case of the ATP synthesis reaction of H781K, E783K, and S792K, the large increase in the value of α for UMP may also be explained by a broader modification of the structure of the K loop.

It has been reported that the mutant T1042I reduced the effects of UMP and IMP on the enzyme activity by 5-fold and increased the apparent dissociation constants of CPS for IMP and UMP by 7- and 30-fold, respectively, although this residue is not at the UMP/IMP binding site (16). The mutant E761A suppresses the effects of ornithine and UMP on the kinetic parameters in the overall biosynthetic reaction (16). In addition, certain mutations within the IMP/UMP binding site impaired the ability of CPS to be activated by

ornithine. When ornithine is bound to the wild-type enzyme, the positive allosteric effects dominate the effects exhibited by the simultaneous binding of either UMP or IMP (6). Consistent with these reports, the mutational analyses presented in this paper of the ornithine and the potassium binding sites strongly support the proposal for a coupling between the allosteric transduction signal pathways for UMP, IMP, and ornithine.

CONCLUSIONS

The allosteric activation of CPS by ornithine could be completely suppressed with the mutations E783A/K, E892A/K, and T1043K, by preventing the binding of ornithine or disrupting the allosteric signal transduction pathway. The mutants E783Q, E892Q, T1042A, and T1043A show that the side chains of these amino acids are important for the binding of ornithine to CPS. In good agreement, the three-dimensional structure of CPS reveals that these residues form hydrogen bonds to ornithine, with the exception of T1043 (9, 15, 22). This study identified E783 and E761 as key residues in the allosteric signal transduction pathway from ornithine to ATP. The mutations at the ornithine and potassium binding sites altered the allosteric mechanisms of ornithine and IMP more than UMP, with the exception of E761A. Nevertheless, several mutations increased, reduced, suppressed, or reversed the allosteric effects of ornithine, IMP, and UMP on the kinetic parameters of the CPS reactions, thus supporting the proposal for a coupling between the ornithine and the nucleotide binding sites (6, 17). Moreover, the activation mechanism by ornithine and IMP are likely to have common features since S792K and D1041A altered the allosteric regulation by ornithine and IMP in the same way. This study identified residues of the binding site for ornithine but also demonstrated how mutations away from the allosteric ligand binding sites can alter significantly the transmission of the allosteric signal to the active site of the enzyme. In this respect, the residues at the interface between the IMP and the ornithine binding sites, as well as those forming the structure of the K loop, play a significant role in the transmission of the allosteric signal from the allosteric binding sites of ornithine, IMP, and UMP.

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