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Mechanism of oligomerization of *Escherichia coli* carbamoyl phosphate synthetase and modulation by the allosteric effectors. A site-directed mutagenesis study

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Abstract **We use site-directed mutagenesis to clarify the role of effector-mediated oligomerization changes on the modulation of the activity of *Escherichia coli* carbamoyl phosphate synthetase (CPS) by its allosteric activator ornithine and its inhibitor UMP. The regulatory domain mutations H975L, L990A and N992A abolished, and N987V decreased CPS oligomerization. The oligomerization domain mutation L421E prevented tetramer but not dimer formation. None of the mutations had drastic effects on enzyme activity or changed the sensitivity or apparent affinity of CPS for ornithine and UMP. Our findings exclude the involvement of oligomerization changes in the control of CPS activity, and show that CPS dimers are formed by the interactions across regulatory domains, and tetramers by the interactions of two dimers across the oligomerization domains. A mechanism for effector-mediated changes of the oligomerization state is proposed.**

Key words: Carbamoyl phosphate synthetase; Oligomerization; Allosteric regulation; Site-directed mutagenesis; Pyrimidine biosynthesis; Arginine biosynthesis

1. Introduction

Escherichia coli carbamoyl phosphate synthetase (CPS), a heterodimer of 41.4 and 117.7 kDa subunits, catalyzes the first step in the routes of arginine and pyrimidine biosynthesis, and is feed-back inhibited by UMP and activated by IMP and ornithine [1]. Although the mechanism of the CPS reaction, catalyzed by the large subunit, is relatively well understood, consisting of two analogous steps of bicarbonate and carbamate phosphorylation and an intermediate step of carbamate synthesis from carboxyphosphate and ammonia, little is known about the mechanism of modulation of the activity by the allosteric effectors [1]. UMP and IMP bind at the same site in the C-terminal 20-kDa domain of the large subunit [2–7], called the regulatory domain, and ornithine binds at the boundary between this domain and the carbamate phosphorylation domain [5]. The sites of binding of the effectors are far from the sites of carbamate and bicarbonate phosphorylation or from the intramolecular tunnel that allows the migration of carbamate between the phosphorylation centers [5–7]. Since the enzyme heterodimers associate into oligomers and the effectors strongly influence the oligomerization state of the enzyme, UMP favoring dimers and ornithine favoring tetramers (the term monomer refers to the CPS heterodimer; dimer, trimer and tetramer refer to the association of 2, 3 or 4 CPS heterodimers), there has been discussion on the possibility that the effectors modulate enzyme activity by altering CPS oligomerization [1,8–11]. In the crystal structures of CPS in the presence of ornithine or of ornithine and IMP, the enzyme is a tetramer in which contacts exist between the regulatory domains of each pair of enzyme molecules, involving residues that are near the binding site for the effector nucleotide (Fig. 1A–C) [6–7]. Additional intermolecular contacts are provided by hydrophobic residues of the oligomerization domain of the large CPS subunit [6]. In the tetramer two given enzyme molecules are connected either through the regulatory domain or through the oligomerization domain, but not through both types of contacts [6]. We have prepared CPS mutants with amino acid changes in the regulatory or the oligomerization domain that would be expected to hamper oligomer formation. Leu421 has been replaced by glutamate (L421E), to create repulsive forces that may interfere with the association of the oligomerization domains (Fig. 1D) [6], and hydrophobic interactions or hydrogen bridges linking the regulatory domains of interacting CPS molecules (Fig. 1A–C) [6] have been eliminated by changing Leu990 or Asn992 to alanine, His975 to leucine or Asn987 to valine. The double and triple mutants H975L/N987V and L421E/H975L/N987V have also been prepared and analyzed. Our findings exclude the involvement of oligomerization changes in effector control of CPS activity, and show that CPS dimers are formed by the interactions across regulatory domains, and tetramers by the interactions of two dimers across the oligomerization domains. A mechanism for effector-mediated changes of the oligomerization state is proposed.

As this paper was being submitted a publication appeared [14] reporting the effects on oligomerization and enzyme activity of the mutations N987D and L421E. The results in that paper are discussed on the light of our present results.

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Abbreviations: CPS, carbamoyl phosphate synthetase

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2. Materials and methods

2.1. Site-directed mutagenesis, expression and purification of mutant enzymes

Standard protocols were used for transformation of *E. coli* and for isolation and digestion of DNA [15]. pLLK12 [16] which carries the *car*AB genes inserted in pUC19 (*car*A and *car*B encode the small and large CPS subunits) was mutated at will using the Transformer Site-Directed Mutagenesis Kit (from Clontech) [17] and appropriated mutagenic primers. After mutant plasmid synthesis with a high fidelity T4 polymerase (from Ecogen), and transformation of BMH 71-18 *mut*S *E. coli* cells (mismatch repair deficient; from Clontech), plasmids carrying the desired mutation(s) were isolated from ampicillin-resistant colonies. The mutations were confirmed by DNA sequencing (Servicio de Sequenciación, IBMCP-CSIC, Valencia, Spain), and in all cases retromutation to the wild type was used to prove that the effects observed were due to the mutation. Wild type and mutant CPS forms were expressed from the corresponding plasmids in *E. coli* strain L814, which has a deletion of *car*AB [16], and were purified as described [4], yielding preparations of 85–95% purity (based on Coomassie-stained SDS–PAGE).

2.2. Gel filtration chromatography

Purified CPS, either wild type or mutant, was incubated 15 min at 24°C in the column solution, at the indicated concentration, prior to application in 0.05 ml to a Superdex 200 HR 10/30 column that was equilibrated and eluted at 24°C with 0.1 M KPi, pH 8.0, containing when indicated 0.1 mM UMP or 10 mM ornithine. Molecular masses were estimated from plots of K_{av} values versus the molecular masses of marker proteins (Fig. 2B). $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume (determined by protein assay at OD_{280} and by SDS–PAGE), V_o is the excluded volume (determined with blue dextran) and V_t is the column bed volume.

3. Results and discussion

3.1. Influence of the mutations on CPS oligomerization

Wild type and mutant CPS forms were soluble and represented 20–40% of the soluble bacterial protein. As expected [1,8–11], at high concentration and in the presence of ornithine, wild type CPS was a tetramer, as shown from its molecular mass of 640 kDa, estimated by gel filtration (Table 1; Fig. 2). At low concentrations or in the presence of UMP, CPS was dimeric, whereas at high concentration, in absence of effectors, it behaved as a dissociating system possibly reflecting the dimer–tetramer equilibrium (Table 1). Of the regulatory domain mutations (Fig. 2; Table 1) L990A, N992A and H975L (alone or as a part of the multiple mutants H975L/N987V and L421E/H975L/N987V) abolished oligomer formation even at high CPS concentration or in the presence of ornithine (mass estimates, 140–170 kDa). Among regulatory domain mutants, only N987V was reminiscent of wild type CPS, (Fig. 2; Table 1), since at high concentration it aggregated in the presence of ornithine, it formed dimers in the

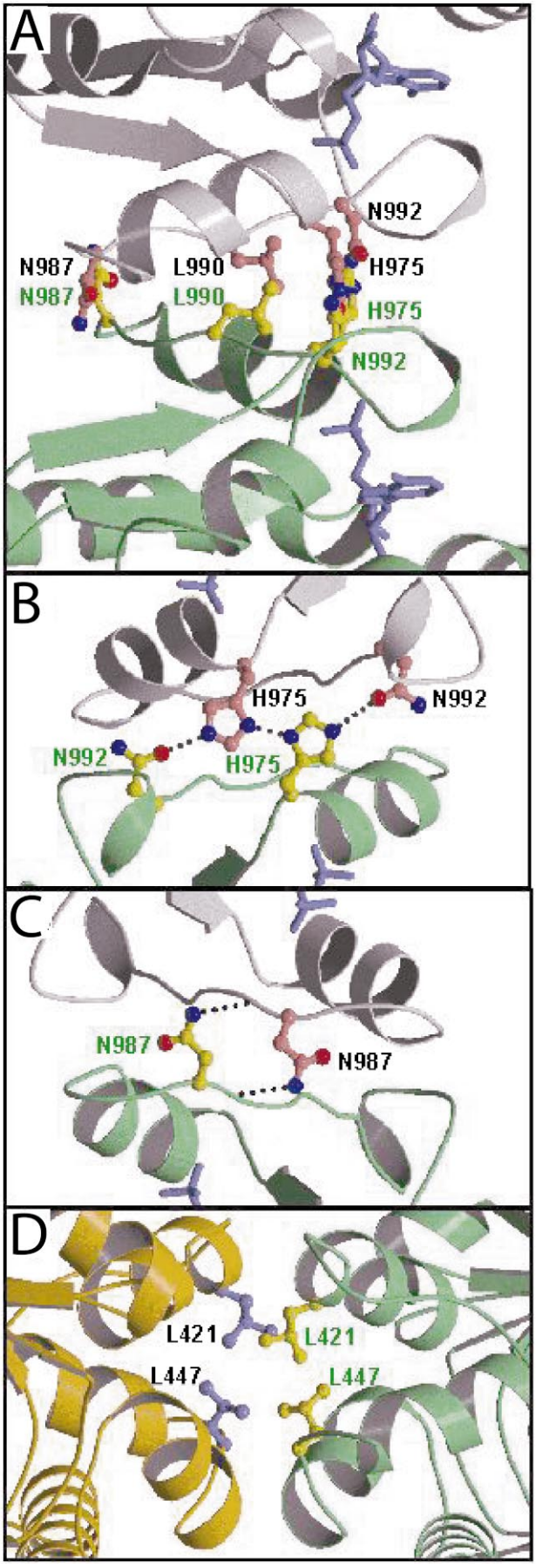

Fig. 1. Ribbon representation of the interface between regulatory domains (A) and details thereof (B and C), and of the interface between oligomerization domains (D). Interacting residues are shown in ball and stick representation and are labeled with the residue number. Colors are used to differentiate the polypeptide chains and residues of different enzyme molecules. Bound IMP (which shares the binding site with UMP, [4]; UMP–CPS complexes have not been reported) is colored blue in stick representation. In B and C hydrogen bonds are detailed; those in C link main chain oxygens (not represented) to the side chain of N987. Drawn with MOLSCRIPT [12] and Raster3D [13], using the coordinates deposited for CPS (PDB 1ce8) [7].

presence of UMP, and its mass exceeded that of the monomer without effectors. Nevertheless, the estimated masses were lower for N987V than for wild type CPS, and thus this mutation also hampered somewhat oligomerization. Judged from the mass estimates, the oligomerization domain mutation L421E reduced the aggregation form of concentrated CPS in the presence of ornithine to dimers (instead of the tetramer observed with wild type CPS). In the presence of UMP or without effectors the effects of L421E were less clear, although the elution positions suggest that dimers may also predominate at high protein concentration (Table 1).

Overall, as expected, all the mutations decreased the oligomerization of CPS. The regulatory domain mutations that abolished the central hydrophobic interaction mediated by Leu990 (Fig. 1A) (L990A) or the three-hydrogen bond network mediated by His975 and Ans992 (Fig. 1B) had the greatest effect. The substantial oligomerization capacity of the N987V mutant indicates a less important role of the two hydrogen bonds mediated by Asn987 (Fig. 1C), or their partial replacement by new contacts between the hydrophobic side chains of the valine residues introduced by the mutation. Since regulatory domain mutations can render the enzyme unable to form any kind of oligomer, oligomerization domain interactions appear too weak to form stable dimers. In contrast, the oligomerization domain mutation L421E decreased tetramer but did not prevent dimer formation, indicating that the dimer is formed by interactions of two enzyme molecules across their regulatory domains. Thus, binding must be stronger across the regulatory than across the oligomerization domains, in agreement with the larger number of contacts mediated by the regulatory domain (Fig. 1). The ability of the oligomerization domain to mediate aggregation of preformed dimers to tetramers, but not of monomers to dimers, is understandable, since the free energy of binding of two preformed dimers to form the tetramer, given by the expression $\Delta G^{0(\mathrm{dimer})} = 2\,\Delta G^{0(\mathrm{olig})} + \Delta G^{S}$, should be considerably more negative than the free energy of binding across the oligomerization domain of two enzyme monomers, $\Delta G^{0(\mathrm{olig})}$. In the expression for $\Delta G^{0(\mathrm{dimer})}$, ΔG^{S} is the 'connection Gibbs energy' [18], which reflects the very high effective concentration (typically $\gg 100$ M for small molecules; see for example [19]) of the second pair of interacting oligomerization domains after the first pair has bound.

Since UMP prevents association of CPS dimers into tetramers, its binding into the regulatory domain must hamper the interactions across the oligomerization domain, despite the fact that this domain is very far from the regulatory domain [5]. Perhaps UMP, by bridging the interaction interface of the regulatory domain to the remainder of the enzyme molecule (Fig. 1A), alters the orientation of the latter with respect to the interface and thus the position of the oligomerization domain, resulting in the misalignment of the two oligomerization domains of one dimer for simultaneous contact with the corresponding domains of another dimer. Ornithine, by binding at the boundary between regulatory and carbamate phosphorylation domains [5], may also influence the relative orientation of the bulk of the enzyme molecule with respect to the regulatory domain, and therefore it may act as UMP but in an opposite sense. The determination of the crystal structure of CPS–UMP complexes will be essential to test these proposals.

Table 1
Molecular mass estimation by gel filtration through Superdex 200 HR 10/30 of wild type and mutant CPS forms[a]

Effector	None		UMP 0.1 mM		Ornithine 10 mM	
Enzyme (mg/ml)	0.5	30	0.5	30	0.5	30
Mutants	molecular mass (kDa)					
Wild type	290	490	300	315	320	640
L421E	280	400	285	380	235	370
H975L	142	145	165	160	145	145
N987V	190	315	180	295	160	440
L990A	145	145	165	165	145	145
N992A	145	160	160	165	145	158
H975L/N987V	140	150	160	155	145	140
L421E/H975L/N987V	140	150	170	165	140	140

[a]Methodological details are given in Section 2.

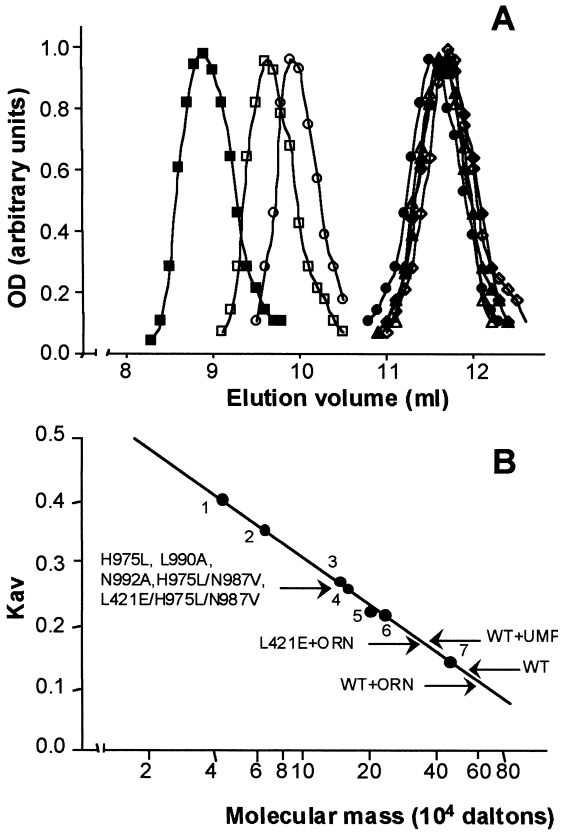

Fig. 2. Gel filtration chromatography. A: Chromatographic profiles of wild type and mutant enzymes (30 mg/ml) loaded on a Superdex 200 HR column and eluted in the presence of 10 mM ornithine. (■) Wild type; (□) N987V; (○) L421E; (●) N992A; (△) H975L; (▲) L990A; (◇) H975L/N987V; (♦) L421E/H975L/N987V. B: Plot of K_{av} as a function of the mass of standard proteins: (1) ovoalbumin (43 kDa); (2) bovine serum albumin (67 kDa); (3) alcohol dehydrogenase (150 kDa); (4) aldolase (158 kDa); (5) β-amylase, (200 kDa); (6) catalase (232 kDa); and (7) ferritin (440 kDa). The position in the plot of CPS, either wild type (WT) or with the indicated mutation(s) is indicated by the arrows.

3.2. *Influence of the mutations on the catalytic activity and allosteric regulation*

None of the mutations, including those that prevented completely enzyme oligomerization, or even the triple mutation affecting both the regulatory and the oligomerization domains, had an important effect on the enzyme activity in the

absence of effectors (Table 2), the rate of the reaction catalyzed by the mutants differing from the wild type by a factor < 2. The stoichiometry of the reaction, judged from the production of ADP and carbamoyl phosphate, agreed within experimental error with the expectation for the normal reaction (2 ATP molecules used per carbamoyl phosphate molecule formed) [1]. Thus, the association between heterodimers is not required for activity, and the results agree with the inference derived from the 3-D CPS structure [5–7] that neither the oligomerization domain or the regulatory domain is involved in the catalysis of the enzyme reaction or in the transfer of intermediates between the two phosphorylation sites. Furthermore, the low magnitude of the differences in activity between wild type and mutant CPS forms strongly suggests that the oligomerization state has little influence on the enzyme activity. This suggestion is supported also by the observation of constant specific activity of wild type CPS irrespective of the enzyme concentration (range tested, 0.015–0.4 mg/ml) or of the elution position of the enzyme in the gel filtration system under conditions at which different oligomeric forms predominate (data not shown; activity was assayed for 1–5 min at 24°C and, for column fractions, immediately after elution).

Since the modest rate decrease observed with the different mutations was not paralleled by a similar decrease in the rates of the ATPase or ATP synthesis partial reactions (Table 2) that reflect the steps of bicarbonate and carbamate phosphorylation, respectively (except for mutant N987V, for which the complete and the ATPase reactions decreased similarly), the mutations may interfere somewhat with a reactional step or process that is not reflected in the partial reactions, such as the reaction of carboxyphosphate and ammonia or the migration of the resulting carbamate to the carbamate phosphorylation site. In fact, most mutations increased somewhat the bicarbonate-dependent ATPase partial reaction, as expected if water had an easier access to the enzyme-bound carboxyphosphate intermediate than in wild type CPS.

Fig. 3 shows that the effects of UMP and of ornithine are essentially the same for all the mutations tested, irrespective of the influence of the mutation on the oligomerization state of the enzyme and also irrespective of the enzyme concentration in the assay (0.015 or 0.4 mg/ml). Under the assay conditions used, ornithine increased in all cases enzyme activity to a maximum of approximately 1.75-fold the activity in the absence of ornithine, with a K_a value of approximately 70 μM. Similarly, under these assay conditions, UMP decreased the activity in all cases to a minimum of approximately 6% of the activity in the absence of any effectors, and the K_i was approximately 4 μM. The identical effects of UMP and ornithine

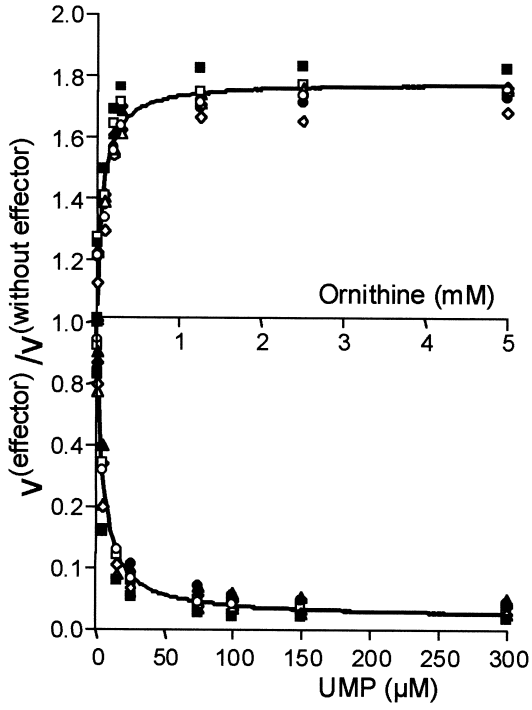

Fig. 3. Influence of ornithine and UMP on carbamoyl phosphate synthesis. Velocities are expressed as a fraction of the velocity of the same enzyme form in the absence of effectors. The hyperbola drawn for ornithine activation corresponds to a velocity at infinite ornithine of 1.77 and a K_a^{Orn} of 70 μM; and that for UMP inhibition to a velocity at infinite UMP of 0.06 and a K_i^{UMP} of 4 μM. Activity was assayed at 24°C in a mixture of 40 mM KPi, pH 8.0, 0.1 M KCl, 20 mM $NaHCO_3$, 10 mM glutamine, 4 mM $MgSO_4$, 2 mM ATP, 5 mM phosphoenolpyruvate and 10 U/ml pyruvate kinase, measuring carbamoyl phosphate production [4]. The results given are those for 0.015 mg/ml enzyme concentrations; essentially identical results were obtained at 0.4 mg/ml enzyme. (■) Wild type; (□) N987V; (●) N992A; (○) L421E; (△) H975L; (▲) L990A; (◇) H975L/N987V; (◆) L421E/H975L/N987V.

Table 2
CPS activity and partial bicarbonate-dependent ATPase and ATP synthesis reactions catalyzed by the wild type and mutant forms of the enzyme[a]

Activity	CPS		HCO_3^- ATPase	ATP synthesis
Production of (μmol/min/mg)	CP	ADP	ADP	ATP
Wild type	3.0	5.4	0.22	0.42
L421E	2.5	4.5	0.31	0.39
H975L	1.9	3.7	0.23	0.43
N987V	1.9	3.7	0.43	0.28
L990A	2.1	4.1	0.29	0.39
N992A	2.5	4.9	0.30	0.39
H975L/N987V	1.7	3.5	0.28	0.44
L421E/H975L/N987V	2.0	3.7	0.21	0.45

[a]CPS activity was assayed at 37°C in a solution containing 0.1 M Tris–HCl, pH 8.0, 0.1 M KCl, 20 mM $NaHCO_3$, 10 mM glutamine, 7 mM $MgSO_4$ and 5 mM ATP, determining after 10 min carbamoyl phosphate or ADP [4] production. To assay the HCO_3^--dependent partial ATPase activity, glutamine was omitted and 2.5 mM phosphoenolpyruvate, 0.25 mM NADH, 0.04 mg/ml pyruvate kinase and 0.025 mg/ml lactate dehydrogenase (both ammonia-free) were added, and the production of ADP was monitored at 340 nm. The partial reaction of ATP synthesis from ADP and carbamoyl phosphate was assayed at 37°C in a solution containing 0.1 M Tris–HCl, pH 8.0, 0.1 M KCl, 15 mM $MgSO_4$, 15 mM glucose, 5 mM ADP, 5 mM carbamoyl phosphate, 1 mM NADP, 0.1 mg/ml hexokinase and 0.025 mg/ml glucose-6-phosphate dehydrogenase, by monitoring the change in OD_{340}.

with the wild type and mutant forms of CPS and the small magnitude of the activity changes caused by the mutations compared with the inhibition caused by UMP clearly indicate that the activity changes triggered by the effectors cannot result from changes in the oligomerization state of the enzyme. Since most of the mutants are always monomeric, the CPS monomer (the α/β-heterodimer) can exhibit different degrees of activity depending on whether ornithine, IMP or UMP are bound. Clearly, the 3-D structure of the complex of the enzyme with UMP should be determined and compared with the corresponding structures with ornithine and/or IMP to try to determine why UMP is such an strong inhibitor of the enzyme.

A paper reporting the effects on CPS activity and oligomerization, monitored by ultracentrifugation at 0.05–3 mg CPS/ml, of mutations affecting two of the presently studied residues, N987D and L421E, either separately or as the double mutant, has appeared [14] as our manuscript was being prepared for submission. In that report N987D prevented oligomerization, and thus, in our mutant N987V the hydrophobic interactions between the valine residues must compensate in part for the intermolecular hydrogen bonds that are eliminated by the mutation, as proposed above. The results with L421E agree in the two studies, although the effects of the mutations on the apparent affinity of CPS for the allosteric effectors or on the bicarbonate-dependent ATPase partial reaction were not studied in [14], as is done here. However, in Kim and Raushel's report [14] the effects of the mutations on the kinetic parameters for ATP in the overall reaction and for ADP in the partial reaction of ATP synthesis, in the absence and at fixed high concentration of each effector, are examined, and the equilibrium constants for oligomer formation are estimated. Overall, the experimental data in the present report, in which the effect of mutations affecting a larger number of residues is analyzed, and in the study of Kim and Raushel are generally in agreement and complementary.

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