In the presence of CTP, UTP becomes an allosteric inhibitor of aspartate transcarbamoylase

(regulation/pyrimidine biosynthesis/nucleotides/feedback inhibition)

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ABSTRACT   The allosteric control of aspartate transcarbamoylase (ATCase, EC 2.1.3.2) of *Escherichia coli* involves feedback inhibition by both CTP and UTP rather than just CTP alone. It has been known that CTP functions as a heterotropic inhibitor of catalysis; however, the inhibition by CTP alone is incomplete (50–70% at various aspartate concentrations) and there is only a partial occupancy of the allosteric binding sites by CTP at saturating concentrations. The logic of these allosteric characteristics can now be understood in that UTP is a synergistic inhibitor of ATCase in the presence of CTP even though UTP has no independent effect at pH 7.0. When saturating concentrations of CTP are present, the concentration of substrate required for half-maximal activity ($S_{0.5}$) of the native holoenzyme for aspartate increases from 5 to 11 mM. When CTP and UTP are both present, the aspartate requirement increases further ($S_{0.5} = 17$ mM). At aspartate concentrations <5 mM, the heterotropic inhibition of ATCase is 90–95% in the presence of both pyrimidine nucleotides. UTP does enhance the binding of CTP to the holoenzyme but the number of tight binding sites does not change ($n = 3$). The binding of UTP is stabilized in the presence of CTP although its binding characteristics are not as strong as those of CTP. The recent crystallographic studies of Kim *et al.* [Kim, H.K., Pan, Z., Honzatko, R.B., Ke, H.M. & Lipscomb, W.N. (1987) *J. Mol. Biol.* 196, 853–875] have described a structural asymmetry across the molecular two-fold axis that is consistent with these CTP/UTP interactions. The synergistic inhibition of ATCase by both CTP and UTP provides a satisfying logic for ensuring a balance of endogenous pyrimidine nucleotide pools.

The aspartate transcarbamoylase (ATCase, EC 2.1.3.2) of *Escherichia coli* provides a classic example of an enzyme subject to allosteric control by the end product of its biosynthetic pathway (1, 2). The holoenzyme of ATCase is composed of two separable catalytic trimers (c_3) and three regulatory dimers (r_2) that interact through a variety of specific protein–protein interfaces (3, 4). These interactions provide for specific conformational transitions (4–7) of the holoenzyme, $2(c_3):3(r_2)$, which result in changes in the catalytic rates and ligand affinities (8–10). Upon binding of substrates and/or substrate analogues the enzyme appears to make a concerted transition from an inactive "T conformation" (condensed form of the enzyme) to an expanded, more active "R conformation" (5–7). CTP affects catalysis by increasing the concentration of aspartate required to produce maximal activity. In contrast, ATP decreases the substrate requirement without altering the V_{max} of the enzyme (1, 2). Both nucleotide effectors have been shown to competitively bind at the same allosteric sites even though they induce different heterotropic effects on catalysis (11–14). Other pyrimidine nucleotides, specifically UTP and TTP, do not

have any effect. GTP does promote a significant inhibitory effect under some conditions (25–30%) but less than that of CTP (50–70%). 8-Bromoguanosine 5'-triphosphate, as well as gadolinium complexes of ATP, CTP, and GTP, binds to the regulatory subunits, whereas UTP does not appear to bind to the native enzyme (11–13). These allosteric controls have been promoted for their inherent logic in balancing endogenous purine and pyrimidine nucleotide pools; however, there has always been a quantitative paradox relative to the incomplete inhibition by CTP (10, 14, 15). In addition, it appears that there is discrete heterogeneity in CTP binding as half of the allosteric sites are capable of providing for high-affinity binding of CTP whereas three additional, low-affinity sites have been identified in some studies (16–19).

A potential structural basis for this "half-site reactivity" has been provided by recent studies of Lipscomb and coworkers (20) in which a molecular asymmetry has been observed across the "two-fold axis of symmetry" of the CTP-liganded enzyme. In the presence of saturating concentrations of CTP, one of the allosteric sites of each dimer is fully occupied, whereas the second has an occupancy factor of <0.4. This asymmetry is translated into differences in the positioning of Arg-54 in the active sites and changes in the interface of the regulatory and catalytic subunits. Thus when the three CTP-binding sites on one side of the two-fold axis of symmetry are filled, the catalytic sites in that half assume a different conformation than the catalytic sites in the other half of the holoenzyme. Similarly, the partially filled allosteric binding sites on the opposing half of the enzyme adopt a different conformation than the completely filled CTP-binding sites. Even though both opposing sets of active sites and allosteric sites experience structural changes relative to the unliganded holoenzyme, the nature of those rearrangements is not identical. These differences appear to be divided between the opposing catalytic subunits of the oligomer such that the sites in each subunit are similar to one another but distinct from those in the other subunit. In the same manner, the allosteric sites in one half of the enzyme are occupied by CTP, whereas those in the other half are only partially occupied yet different from those observed in the unliganded enzyme.

The observations presented in these studies identify a role for UTP in completing the allosteric inhibition of ATCase by pyrimidine nucleotides and permit the development of a more complete model for metabolic regulation of pyrimidine biosynthesis.

MATERIALS AND METHODS

Enzyme Purification and Assay. The ATCase of *E. coli* was overproduced from the plasmid KUR1278 (derived from

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Abbreviations: ATCase, aspartate transcarbamoylase; $S_{0.5}$, concentration of substrate required for half-maximal activity; CPSase, carbamoylphosphate synthetase.
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pPBh105 by transferring the *Pst* I–*Sal* I fragment into pUC8; ref. 21) and expressed in *E. coli* EK1104 (22). To obtain homogeneous holoenzyme preparations it was necessary to utilize the heat step (72°C) and a single pI precipitation (pH 5.9) of the purification procedures described by Gerhart and Holoubek (23). The architectural purity of the enzyme was verified by nondenaturing activity gel analysis and/or chromatography on DEAE-Sephadex (24). Enzymatic activity was determined as described (25, 26) in the presence of saturating carbamoyl phosphate (4.8 mM) and varying concentrations of aspartate depending upon the experimental purpose. The assays were all performed at pH 7.0, 28°C with nucleotide effector concentrations varying from 0 to 12 mM.

Equilibrium Dialysis. The equilibrium binding of nucleotide triphosphates was determined according to the general procedures of Tauc *et al.* (15) as modified to match the assay conditions used to analyze the synergism of CTP and UTP utilized in these studies (25). Nucleotide concentrations were adjusted from 1 to 500 μM, and the specific radioactivities of the [3H]-labeled NTPs (ICN-Biochemicals) were adjusted to obtain $\approx$1000 cpm per μl. The linearity of the equilibrium binding was evaluated over concentrations of ATCase from 0.03 to 30.0 mg/ml, and 3.0 mg/ml (10 μM) was utilized for studies with [14C]CTP binding and 30 mg/ml was used for UTP binding studies. Equilibrium was ensured by evaluation after 36 and 72 hr at 4°C. The purities of the radioactively labeled nucleotides were determined by HPLC analysis as described (27).

EXPERIMENTAL RESULTS

Relative Activities for Combinations of Nucleotide Effectors. Table 1 summarizes the relative activities for various purine and pyrimidine nucleotide effectors independently and in combination. Saturating concentrations for each nucleotide (2 mM) were used throughout the studies and nucleotide effects were determined at the concentration of substrate required for half-maximal activity ($S_{0.5}$) of the unliganded enzyme (5 mM aspartate) and one-half that value (2.5 mM aspartate). In each study, the effect of the nucleotides was amplified at the lower substrate concentration. When saturating concentrations of nucleotides were mixed, as previously reported (27), CTP reversed the activation by ATP. Other nucleotides had very little effect on the ATP-induced

activation of ATCase; neither UTP nor GTP counteracted the ATP effect but actually promoted slight increases in activity. The most interesting effects were observed from the combination of CTP and UTP, which resulted in a 90–95% inhibition of ATCase at both aspartate concentrations. This cooperative effect of CTP and UTP was maintained in the presence of either ATP or GTP. It should be noted that CTP was capable of maintaining inhibition in the presence of either purine nucleotide alone. When all four nucleotides were present simultaneously, slight inhibition (10–40%) was observed. These results were most important because they revealed a synergism between CTP and UTP in which UTP could contribute to the allosteric inhibition of ATCase even though it had no independent effect. In the presence of ATP, GTP contributed to the activation of the enzyme, in spite of its independent inhibitory effect, and provided a synergistic companion for ATP to compete with CTP and CTP/UTP combinations.

Synergistic Inhibition of CTP and UTP. To evaluate whether the effect of combinations of CTP and UTP was a nucleotide-specific synergism or a concentration-dependent phenomenon, various overlapping effector response curves were analyzed. The results presented in Fig. 1 demonstrate that the UTP/CTP synergism was dependent upon both CTP and UTP and could not be mimicked by high concentrations of either nucleotide alone. Individual nucleotides demonstrated first-order saturation kinetics relative to their allosteric effects. When the CTP concentration was maintained at 2 mM and increasing concentrations of UTP were added, catalytic activity was reduced to 5–10% of the control values. Thus, it is clear that combinations of both CTP and UTP were required to effect maximal inhibition of the native ATCase from *E. coli* at pH 7.0. This effect is not as pronounced at pH 8.3 when UTP does have some independent inhibitory effect ($\approx$15–20%) (data not shown).

Saturation Kinetics of Nucleotide Effectors. Fig. 2 demonstrates that ATP appears to have a lower apparent binding affinity than CTP based on kinetic saturation responses, whereas UTP had no inhibitory effect at concentrations up to 12 mM. The half-saturating concentration for ATP activation was 0.8 mM, whereas CTP-induced inhibition required levels that were decreased by a factor of $\approx$5 (0.15 mM). This weaker

Table 1. Relative specific activities for combinations of nucleotide effectors

Nucleotide effector*	Aspartate[†]	
	2.5 mM	5.0 mM
ATP	1.86	1.35
CTP	0.31	0.43
GTP	0.57	0.71
UTP	0.95	0.95
ATP/CTP	0.70	0.85
ATP/GTP	1.98	1.58
ATP/UTP	1.96	1.52
CTP/GTP	0.41	0.58
CTP/UTP	0.05	0.06
GTP/UTP	0.66	0.84
A/C/G[‡]	1.78	1.60
A/C/U[‡]	0.07	0.11
A/G/U[‡]	1.95	1.70
C/G/U[‡]	0.05	0.11
A/C/G/U[‡]	0.63	0.92

*Effector concentrations are 2 mM for each nucleotide.
[†]Relative activities represent specific activities with effector divided by specific activity of ATCase without nucleotide ligands at the indicated aspartate concentrations.
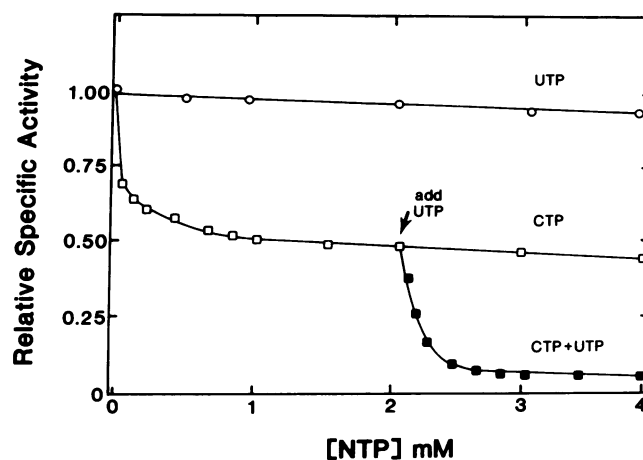[‡]A = ATP; C = CTP; G = GTP; U = UTP.

FIG. 1. Synergistic inhibition of CTP and UTP. The saturation kinetics of the effects of CTP and UTP on the activity of ATCase were determined under standard assay conditions employing saturating carbamoyl phosphate concentrations and 5 mM aspartate (pH 7.0). Percentage relative activity was measured at increasing concentrations of nucleotide (NTP). Independent effects of UTP (○) and CTP (□) are depicted up to 4 mM. The synergistic effect of CTP and UTP was determined by adding increasing concentrations of UTP to saturating (2 mM) concentrations of CTP (■).
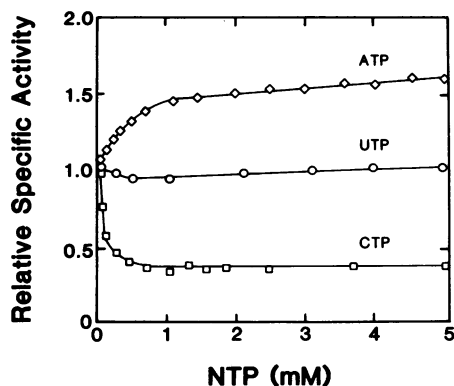
FIG. 2. Comparative saturation kinetics of ATCase by nucleotide effectors. The saturation kinetics for ATP, CTP, and UTP were determined as described in the legend to Fig. 1.

binding of ATP has been described by others (19, 20) and would explain the dominance of CTP inhibition over ATP activation. The nature of this competitive advantage for CTP can be described in a slightly different manner by evaluating competitive saturation curves (data not shown). In these studies, increasing concentrations of either CTP or ATP were used to counteract saturating (2 mM) concentrations of the other allosteric effector. The concentrations of nucleotide required to overcome the effect of its antagonistic nucleotide (to adjust the relative activity of the reaction to that of the unaffected enzyme) were ≈0.8 mM CTP and ≈3.6 mM ATP. These results are consistent with the competition of ATP and CTP for the same binding sites with higher CTP-binding affinities.

Synergistic Interaction of CTP and UTP in Effecting Allosteric Inhibition. Saturation studies with combinations of CTP and UTP were performed to evaluate the nature of the synergistic interaction between the two nucleotides (Fig. 3). In the presence of saturating concentrations of either CTP or UTP (2 mM), the kinetic development of synergistic inhibition was more rapid than the partial inhibition induced by CTP alone. The requirement for maximal CTP inhibition was lower by a factor of ≈10 in the presence of UTP. Although the concentration of CTP required for half-maximal inhibition was ≈0.15 mM at subsaturating concentrations of aspartate, the requirement in the presence of UTP was <0.02 mM. In fact, 0.02 mM concentrations of either UTP or CTP were adequate to effect 50% inhibition in the presence of 2 mM concentrations of the other nucleotide. Therefore, it appears
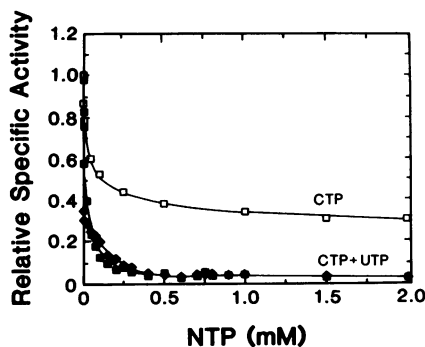

FIG. 3. Enhancement of heterotropic inhibition of CTP by UTP. Two millimolar concentrations of UTP were present while the effect of increasing concentrations of CTP (□) on the relative specific activity of ATCase was determined. Similarly, saturating concentrations of CTP (2 mM) were augmented by increasing concentrations of UTP (■) or saturating concentrations of UTP were augmented with increasing concentrations of CTP (▲).
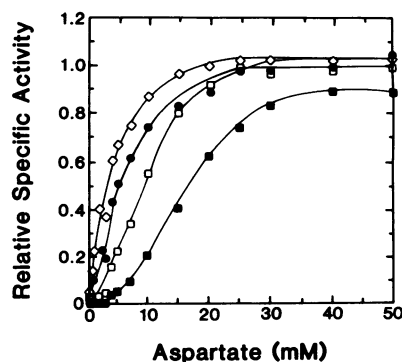

FIG. 4. Aspartate saturation kinetics in the presence of nucleotides. The aspartate saturation of ATCase activity was determined in the presence of 2 mM concentrations of ATP (◊), CTP (□), and CTP + UTP (■) as compared to the saturation kinetics in the absence of nucleotide (●). Standard assay conditions were used except for varying aspartate concentrations.

that the CTP/UTP synergism served to improve the inhibitory effects of CTP as well as enhancing overall heterotropic inhibition.

Aspartate Saturation in the Presence of Nucleotide Effectors. Fig. 4 presents the kinetics of aspartate saturation in the presence of ATP, CTP, and CTP + UTP. The $S_{0.5}$ concentration of aspartate in the absence of allosteric effectors was ≈5.0 mM, whereas ATP reduced the value to 3.0 mM and CTP elevated the concentration to 11.0 mM while the values for V_{max} did not change. UTP had no independent effect on either $S_{0.5}$ or maximal activity. Millimolar concentrations of both CTP and UTP demanded higher concentrations of aspartate for activity and the $S_{0.5}$ was ≈17.0 mM. Although the effects of other nucleotides could be completely overcome by higher aspartate concentrations, the CTP/UTP affected enzyme did not appear to be able to regain the V_{max} of the unliganded enzyme (only 85% at 50 mM aspartate). The kinetic parameters determined in the presence of various nucleotide effectors are summarized in Table 2.

Equilibrium Binding of Nucleotide Triphosphates. Saturation equilibrium binding studies demonstrated that 10 μmol of protein binds ≈30 μmol of CTP (three sites per holoenzyme) in the presence or absence of UTP at pH 7.0. A Scatchard plot of the data (Fig. 5) indicated that $n = 2.8$–3.0 in both cases. Addition of varying concentrations of UTP (20–300 μM) or phosphonacetyl-L-aspartate (100 μM) did not affect the number of tight binding sites for CTP. The binding constants in the presence of phosphonacetyl-L-aspartate were estimated from the data in Fig. 5 and it appears that UTP enhances CTP binding 30–40% (K_S: CTP alone = 3×10^5 M^{-1}; with 2 mM UTP = 4.3×10^5 M^{-1}). Independent binding

Table 2. Kinetic parameters of ATCase in the presence of nucleotide effectors

Nucleotide*	V_{max}[†]	$S_{0.5}$[‡]	n_{app}[§]	Effect[¶]
None	7.2	5.5	2.1	100
ATP	7.4	3.0	1.7	140
CTP	7.1	11.0	2.8	60
UTP	7.2	5.5	2.1	95
CTP/UTP	6.3	17.0	3.0	5

*Nucleotide concentrations were 2 mM for each effector.
[†]Maximal velocity estimated at saturating concentrations of substrates (maximal velocity reported as μmol/hr per μg of protein).
[‡]Concentration of aspartate required to produce half-maximal velocity.
[§]Hill coefficient (±5%) estimated from aspartate saturation kinetics (determined by slope of log $v/[V_{max} - v]$ vs. log S) (26).
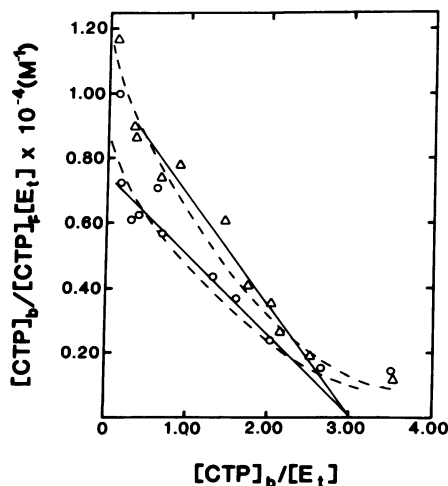[¶]Percentage relative activity at 5 mM aspartate.

FIG. 5. Scatchard representation of CTP equilibrium dialysis binding to native ATCase holoenzyme in the presence (△) and absence (○) of 2 mM UTP. [E_t], total enzyme concentration.

studies with UTP demonstrated that 4–12% of the total label was associated with protein dependent on the concentration of UTP, but it was not possible to complete a saturation study due to the insensitivity of the binding at higher concentrations. In this sense, UTP demonstrated the weak binding characteristic of ATP association rather than the tight binding of CTP. The presence of 2 mM CTP did not detectably enhance the binding of UTP to the ATCase holoenzyme (data not shown).

DISCUSSION

The ATCase of *E. coli* provides one of the classic models for allosteric regulation of catalytic activity in which the end product of a biosynthetic pathway controls the first unique enzyme of the pathway. Several detailed reviews have been published in recent years and describe an elegant enzyme subject to homotropic and heterotropic ligand responses (8–10). As the endogenous CTP pools increase, the heterotropic inhibition of ATCase would directly modulate the *de novo* synthesis of pyrimidine nucleotides. Furthermore, pyrimidine nucleotide pools could be coordinated with purine nucleotide pools through the competition of ATP and CTP for the regulatory control of ATCase (11, 12, 14, 27), resulting in an activation or inhibition of *de novo* pyrimidine biosynthesis. From a metabolic perspective, it would seem that the intracellular chemistry of the bacterial cell is poised to maximize these allosteric interactions since the endogenous metabolic pool for aspartate approximates the $S_{0.5}$ of ATCase (5 mM) and the nucleotide pools are ≈0.5–1.0 mM for CTP and ≈2–3 mM for ATP (27) under conditions of steady-state growth in *E. coli*. These physiological conditions would provide the most sensitive responses for the allosterically regulated enzyme since endogenous ligand concentrations approximate the half-saturating values required for inducing homotropic and heterotropic effects *in vitro*.

Despite this textbook characterization of ATCase, there are a series of subtle incongruities that have been reported relative to the enzyme from *E. coli*:

(*i*) CTP only inhibits the enzyme 50–70% under various physical conditions, such as altered pH, substrate concentrations, or effector concentrations (1, 2, 10, 14, 27).

(*ii*) CTP appears to have only three tight binding sites although it could be expected that there should be one equivalent binding site for each of the six regulatory polypeptides. This half-site reactivity has been described by several authors (16–19).

(*iii*) Recent structural refinement of the CTP·ATCase has suggested that there is an asymmetry across the molecular, two-fold axis of symmetry such that the active sites and r·c interfaces of opposing halves of the enzyme are configured differently. The mechanistic significance of the asymmetry has not been determined (20).

The studies presented in this research provide a unifying hypothesis for all of these observations. Thus it can be postulated that CTP binding to half of the regulatory sites of ATCase effectively restricts the catalytic efficiency of one of the catalytic trimers, whereas the three sites of the other subunit retain catalytic competency. Upon the tight binding of CTP to the first set of allosteric sites, a structural reorientation of the remaining binding sites could become permissive for UTP or CTP binding, thus completing the inactivation of ATCase. The binding of UTP did not appear to facilitate the equivalent occupancy of all six allosteric sites by CTP since there were only three tight binding sites for CTP in the presence or absence of UTP. Nonetheless, the binding of CTP was affected by the presence of UTP in that the K_i appeared to increase ≈20–30% (from 3×10^5 to 4×10^5 M^{-1}). CTP did not appear to affect the binding of UTP at pH 7.0 in either the presence or absence of phosphonacetyl-L-aspartate. On the other hand, kinetic studies with combinations of nucleotides indicate that CTP and UTP do possess a direct, nucleotide-specific synergism that can be counteracted by high concentrations of ATP (>8 mM). Therefore, it appears that the inhibitory synergism of CTP and UTP is the result of enhanced CTP interactions at three regulatory sites accompanied by some additional, permissive inhibition by UTP. Detailed studies of the synergistic effects and structural consequences of UTP and CTP binding are necessary.

This model is quite compelling for its inherent metabolic logic, which would lead to an endogenous balancing of the pyrimidine nucleotide pools (Fig. 6). Elevated levels of intracellular pools of CTP alone would exert two simultaneous regulatory functions, as a partial inhibitor for the entire *de novo* pathway through ATCase (1, 2) and for direct control over its own synthesis through competitive inhibition of CTP synthetase (28). Since CTP alone only partially inhibits
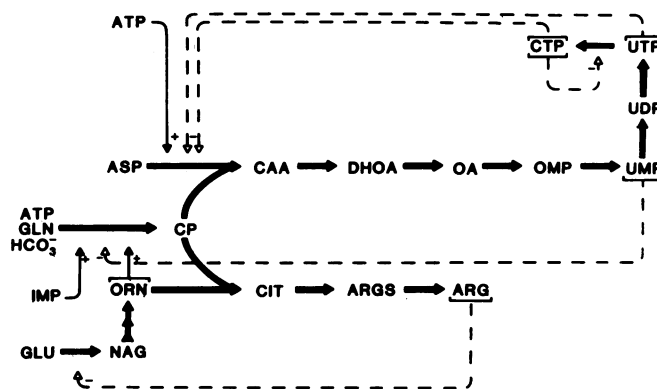

FIG. 6. Allosteric control patterns for *de novo* pyrimidine and arginine biosynthesis in *E. coli*. CTP and UTP provide synergistic feedback inhibition of ATCase, whereas ATP provides competitive heterotropic activation. The feedback inhibition of carbamoylphosphate synthetase (CPSase) by UMP is subject to antagonism by ornithine (ORN) and the enzyme is activated by IMP. *N*-Acetylglutamate synthase is subject to feedback inhibition by L-arginine (ARG). The biosynthetic intermediates of the *de novo* pyrimidine pathway are glutamine (GLN), carbamoylphosphate (CP), aspartate (ASP), carbamoyl aspartate (CAA), dihydroorotic acid (DHOA), orotic acid (OA), orotidine-5′-monophosphate (OMP), and the common uridylates (UMP, UDP, and UTP). The identified intermediates of the arginine biosynthetic pathway are glutamate (GLU), *N*-acetylglutamate (NAG), ornithine (ORN), citrulline (CIT), arginosuccinate (ARGS), and arginine (ARG).

ATCase, significant pyrimidine synthesis would continue; however, this would primarily serve to increase the UTP pool since the conversion of UTP to CTP would be restricted at CTP synthetase. Ultimately, CTP and UTP pools would be elevated, thus permitting the thorough inhibition of ATCase. Concomitant with the increasing triphosphate pools, an expanding UMP pool would provide for the inhibition of CPSase and reduce the production of carbamoylphosphate for pyrimidine and arginine biosyntheses (29, 30). To ensure that elevated pyrimidine nucleotide pools do not result in the depletion of carbamoyl phosphate for arginine biosynthesis, ornithine specifically antagonizes the UMP inhibition of CPSase and is capable of independent activation (30). When adequate levels of arginine are available, the ornithine pool decreases as the result of feedback of the first enzyme of the *de novo* pathway, *N*-acetylglutamate synthase (31). In addition to the heterotropic controls emanating from the *de novo* pathways, various purine nucleotides (particularly, IMP and ATP) provide for the allosteric activation of CPSase and ATCase (1, 2, 29, 30). Thus elevated purine nucleotide pools would stimulate the *de novo* synthesis of pyrimidine nucleotides. The combination of CTP and UTP provides a synergistic control that effects the inhibition of ATCase in the presence of purine nucleotides, and IMP is capable of competitive activation of CPSase relative to UMP. In this manner, CPSase and ATCase are able to preside over the balancing of pyrimidine nucleotide pools with their purine counterparts in *E. coli*.

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