Carbamyl-phosphate synthetase domain of the yeast multifunctional protein Ura2 is necessary for aspartate transcarbamylase inhibition by UTP

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Abstract In *Saccharomyces cerevisiae*, the first two reactions of pyrimidine biosynthesis are catalyzed by the multifunctional protein Ura2 carrying both carbamyl-phosphate synthetase (CPSase) and aspartate transcarbamylase (ATCase) enzyme activities. In order to study how UTP regulates both of these activities mutant strains were constructed: one strain which expressed the Ura2 protein fused to the green fluorescent protein, and two strains expressed truncated Ura2 proteins. These strains exhibited a phenotype associated with a modified regulation of the pyrimidine pathway. Results presented in this report provide arguments in favor of a single UTP binding site located on the CPSase domain, and support a model in which ATCase activity is inhibited by UTP only when it can interact with the CPSase domain.
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Key words: Pyrimidine nucleotide; *URA2*; Multifunctional protein; UTP regulation; *Saccharomyces cerevisiae*

1. Introduction

In *Saccharomyces cerevisiae*, the first two steps of pyrimidine biosynthesis are catalyzed by the Ura2 protein. The Ura2 polypeptide chain (2212 aa), encoded by the gene *URA2*, carries four different domains, three of which are enzymatically active: GATase (glutamine amidotransferase), CPSase (carbamyl phosphate synthetase) and ATCase (aspartate transcarbamylase). Located between CPSase and ATCase, the fourth domain has no known activity in the Ura2 protein, although it catalyzes the third reaction of the pathway (dihydroorotase (DHOase)), in CAD, the mammalian counterpart of Ura2. This intermediary domain has been called DHOase-like in Ura2 since its sequence presents homologies with CAD [1]. CPSase synthesizes carbamyl phosphate (CP) from bicarbonate, ATP-Mg and glutamine. ATCase uses CP and aspartate to synthesize ureido-succinate. Both enzymatic steps are subject to feedback regulations by UTP which take place at two levels: repression of *URA2* gene transcription, and inhibition

by UTP of both enzymatic activities [2]; the inhibition of the first two activities of the pyrimidine pathway is specific for the yeast enzyme and does not take place in CAD in which only the CPSase activity is inhibited by UTP [3].

Mutations which affect repression of the *URA2* gene have never been found although they were looked for very carefully by F. Lacroute's group; in contrast, mutations which affect inhibition of Ura2 activities were found to map on the *Ura2* gene: they result in a loss of sensitivity towards UTP of either CPSase, ATCase or both activities. Some of these mutations have been shown to lead to the excretion of uracil responsible for a 5-fluorouracil resistant (FUR) phenotype used for screening such mutant cells [4]. Recently *FUR2* point mutations ('2' since they are allelic to *URA2*) have been mapped: they were shown to be gathered on the Ura2 protein sequence either on the ATCase domain or on the CPSase domain [5]. These results suggested either a regulation which could depend on two UTP independent regulatory sites, or a regulation based on interactions between domains one of which would carry the UTP binding site.

The respective contribution of each domain to the mechanism of ATCase inhibition by UTP was investigated in this work, with the help of different in vitro *URA2* constructions expressing modified Ura2 protein: a Ura2 protein fused to a GFP (green fluorescent protein) domain, and two different truncated Ura2 proteins. Results support the fact that a single UTP binding site is located on the CPSase domain and provide arguments for a model in which ATCase activity would be feedback-inhibited only when it can interact with the CPSase domain.

2. Materials and methods

2.1. Materials

Na[^{14}C]aspartate 200 mCi/mmol and Na[^{14}C]bicarbonate 55 mCi/mmol were from Amersham SA. Yeast extract, yeast nitrogen base and peptone were from Difco Laboratories. UTP was from Pharmacia, ATP and L-aspartate from Sigma-Aldrich. All other products were from Prolabo.

2.2. Yeast strains and growth conditions

Two strains derived from FL100 wild type (ATCC 28383) and W303.1A *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 can 1-100* (R. Rothstein UMDNJ-New Jersey Medical School, Newark USA) were used to express recombinant *TRP1* vectors carrying the modified versions of *URA2*: MD 296.16A (α *CPA2 ura2Δ::HIS3 trp1-1*) and MD 293.9A (α *cpa2 ura2Δ::HIS3 trp1-1*). In both strains, the *URA2* chromosomal gene was totally deleted by a *HIS3* insertion (*ura2Δ::HIS3*) made according to [6]. Transformed strains were called by the name of the plasmid preceded by *CPA2* or *cpa2*, depending upon the recipient strain used, respectively MD 293.16A or MD 293.9A.

Precultures were made in YPD (yeast extract 1%, peptone 2%,

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glucose 2%) supplemented with arginine and uracil 40 mg/l. Precultures of transformed cells only were done in YNB (yeast nitrogen base 0.67 g/l buffered with sodium citrate/citric acid to pH 5.6) supplemented with uracil and a mixture of amino acids and bases which included arginine, uracil and adenine but not tryptophan. Cultures were made in YNB buffered as above and supplemented as mentioned in legends. One OD_{660} unit corresponds to about $2-4 \times 10^7$ haploid cells/ml.

Plasmid constructions are presented in Fig. 1. Plasmid pRA5N (p5N) is the reference centromeric plasmid, derived from pNC160 [7], it carries the wild type *URA2* gene under its own promoter. Plasmid pRA21 (p21) carries a 3′ fusion of *URA2* with the GFP coding sequence; this vector was constructed by inserting in the last *Msc*I site of the *URA2*-ORF a PCR product which contains the GFP sequence from *Aequorea victoria* (mutation pS65T-C1, from Clontech). This amplification also introduced a small sequence encoding a 5 amino acid linker between Ura2p and GFP. Plasmids pRAΔCPS or [pΔCPS] and pRAΔATC or [pΔATC] were both constructed by inverse PCR, the selected domain sequence being deleted directly on the final plasmid ([8] and publication submitted).

2.3. CPSase and ATCase enzyme activities

CPSase and ATCase enzyme activities were measured by permeabilization as in [9] using conservation buffer (Tris-acetate 50 mM, pH 7.4, glycerol 10% v/v, ethylene glycol 30% v/v, EGTA 2 mM, pepstatin 2 μM, PMSF 1 mM). ATCase activity was measured as in [10], Na[^{14}C]aspartate (about 5×10^4 dpm/μmol) being the substrate. Specific activity was expressed in μmol of ureido-succinate/h/10^{10} cells. CPSase activity was measured as in [10,11] using Na[^{14}C]bicarbonate (about 7×10^5 dpm/μmol). Specific activity was expressed in μmol of ureido-succinate/h/10^{10} cells. Both activities were determined using three increasing volumes of cell suspension, alternatively determinations were done in triplicate.

3. Results

It should be recalled that in *S. cerevisiae*, CP is produced not only by the CPSase-ura (encoded by *URA2*), but also by the CPSase of the arginine pathway (CPSase-arg encoded by genes *CPA1* and *CPA2*) [12]. Thus, if UTP inhibition of ATCase can be measured in a *CPA2* background, regulation of the CPSase-ura can only be measured in a *cpa2* background (i.e. deprived of CPSase-arg activity). Typically, a *cpa2 URA2* strain (i) grows on YNB minimum medium because its CPSase-ura provides CP for both uracil and arginine pathways (i.e. it does not strictly channel CP to pyrimidine synthesis); but (ii) cannot grow when uracil is added to the YNB medium because the repleted pool of UTP represses *URA2* and inhibits CPSase-ura activity, CP synthesis is thus stopped and arginine is no longer made.

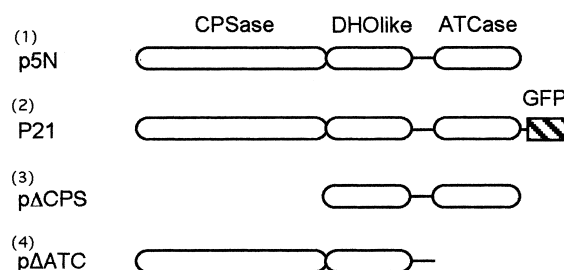Strain *cpa2* [p5N], which carries wild type *URA2* under its

Fig. 1. Presentation of the four plasmids used in this study and which express from p5N, (1) the control Ura2 protein; from p21, (2) the fusion protein Ura2-GFP; from pΔCPS, (3) a truncated Ura2 protein lacking the CPSase domain; from the pΔATC, (4) a truncated Ura2 protein lacking the ATCase domain.

own promoter, has been used as a control in this work since it has been shown to exhibit the same characteristics as described above for *cpa2 URA2* strains.

A 3′-terminal fusion of *URA2* with the GFP sequence and two partial deletions of *URA2* have been constructed; the corresponding proteins have been analyzed in this study (Fig. 1).

3.1. The Ura2-GFP fusion protein carries CPSase and ATCase activities, both less sensitive to UTP inhibition than the control protein

Cells *CPA2* [p21], which express the Ura2-GFP fusion, were found to grow on minimal medium and to excrete uracil. They were able to grow in the presence of 10 mM of a toxic analog of uracil, 5-fluorouracil (5FU), a phenotype called *FUR2* (fluorouracil resistant), which suggested an active ATCase no longer regulated by UTP [4]. The ability of cells *cpa2* [p21] to grow on minimal medium (Fig. 2) revealed that CPSase-ura of the Ura2-GFP fusion protein was enzymatically active; however, since these cells were able to grow slowly in the presence of uracil, this CPSase was apparently not regulated as tightly as the control enzyme. Thus, both activities present on the fusion protein seemed not to be regulated properly. Repression and inhibition of CPSase and ATCase activities expressed in *cpa2* [p21] cells were measured as explained and presented in Table 1. A similar level of repression was found for both activities in *cpa2* [p21] and in *cpa2* [p5N] cells, although specific activities were slightly higher in the latter. This repression was confirmed by quantification of the *URA2* messenger RNA on Northern blots (data not shown). In con-

Table 1
ATCase and CPSase activities measured during exponential phase growth of four strains which express (1) the control Ura2 protein encoded by p5N, (2) the fusion protein Ura2-GFP encoded by p21, (3) a truncated Ura2 protein lacking the CPSase domain encoded by pΔCPS, and (4) a truncated Ura2 protein lacking the ATCase domain encoded by pΔATC

Strain	Protein expressed	Growth conditions	ATCase		CPSase	
			Specific activity	Inhibition by 2 mM UTP (%)	Specific activity	Inhibition by 2 mM UTP (%)
(1) *cpa2* [p5N]	Wild type	YNB	40	55	2.6	58
		YNB+uracil 200 mg/l	14	43	1.0	50
(2) *cpa2* [p21]	Ura2-GFP	YNB	34	3	2.3	13
		YNB+uracil 200 mg/l	10	0	1.1	0
(3) *cpa2* [pΔCPS]	DHO-ATC	YNB+uracil 200 mg/l	21	0	–	–
(4) *cpa2* [pΔATC]	CPS-DHO	YNB+uracil 200 mg/l	–	–	1.3	0

Both specific activities are expressed in μmol of ureido-succinate/h/10^{10} cells. Repression of the *URA2* gene was estimated, when possible, by the ratio between activities measured in cells grown in the presence of 200 mg/l of uracil, and activities of cells grown on minimal medium. Sensitivity of both activities towards UTP was routinely estimated by the ratio between activities measured in the presence and in the absence of 2 mM UTP in the assay.
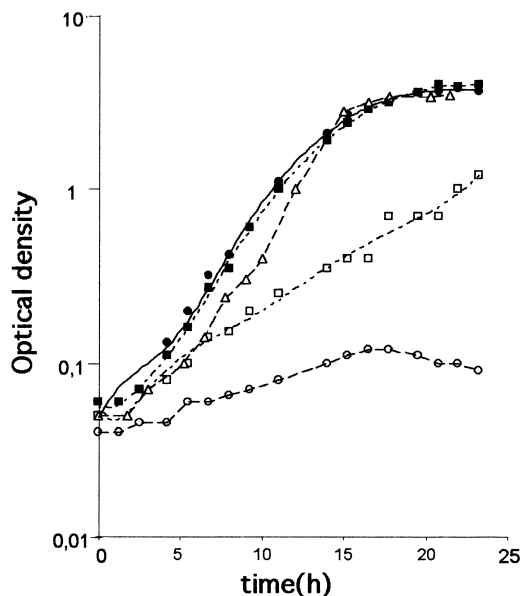
Fig. 2. Growth curves of the different mutant strains in comparison with the control strain in minimal medium plus 200 mg/l uracil (open symbols) or without uracil (closed symbols). Symbols for the three strains: cpa2[p5N] (circles); cpa2[p21] (squares); cpa2[ΔATC] (triangle).

3.2. The Ura2 protein deprived of the CPSase domain exhibits an ATCase totally insensitive to UTP inhibition

In order to determine the role of each domain in the feedback inhibition of both activities, two domain-specific deletions were made on *URA2* (Fig. 1): a deletion of the ATCase domain (carried on plasmid pΔATC), and a deletion which will be referred to as ΔCPS although it overlaps both GATase and CPSase domains; it is carried by plasmid pΔCPS.

cpa2 [pΔATC] cells have no ATCase activity. They were found to grow on minimal medium plus arginine and uracil, but also in the presence of 200 mg/l uracil alone, suggesting a CPSase-ura still active but not properly regulated. Measurements of ΔATC-CPSase activity were made only in cells grown in the presence of uracil, and, since these cells do not grow on minimal medium, it was not possible to estimate the level of repression. Results presented in Table 1 show that ΔATC-CPSase has specific activity roughly similar to the control measured in cells grown in the same conditions; we thus estimated that the repression level was likely not changed in the mutant cells *cpa2* pΔATC. However, this CPSase activity had lost part of its sensitivity to UTP, and the kinetics of its inhibition when UTP concentrations increased was found to be totally different from the control kinetics: activity decreased exponentially while the control followed a sigmoidal curve (Fig. 3B).

cpa2 [pΔCPS] have no CPSase activity at all. As above, it was not possible to estimate the level of repression in these cells since they do not grow on minimal medium. ΔCPSase-ATCase-specific activity was measured in cells grown in the presence of uracil, and it was found to be slightly higher than the control measured in cells grown in the same conditions.

CPA2 [pΔCPS] cell growth on minimal medium (Fig. 2) indicates the presence of a functional ATCase domain, even in the absence of the whole CPSase domain; their growth in the presence of 5FU reveals a *FUR2* phenotype associated with an ATCase insensitive to UTP inhibition, as shown in Table 1. Actually, ATCase activity appeared to be activated when UTP concentrations were increased (Fig. 3A); nevertheless, this activation was considered not to be specific since other nucleotides besides UTP – such as UMP, CTP and

trast, both activities of the fusion protein exhibited a lower sensitivity to UTP than the control enzyme. ATCase activity was poorly inhibited even in the presence of high concentrations of UTP (Fig. 3A): the [UTP]$_{0.5}$ (concentration of UTP necessary to inhibit half of the maximum activity) was approximately an order of magnitude higher for the chimeric protein (12 mM) than for the wild type enzyme (1 mM). However, as shown in Fig. 3B, cpa2 [p21] CPSase activity, which remained insensitive to UTP up to a concentration of 1–2 mM, was finally inhibited to the same extent as the control enzyme when higher concentrations of UTP were used; the [UTP]$_{0.5}$ was about 4 mM for the p21-CPSase, compared to 2 mM for the control.
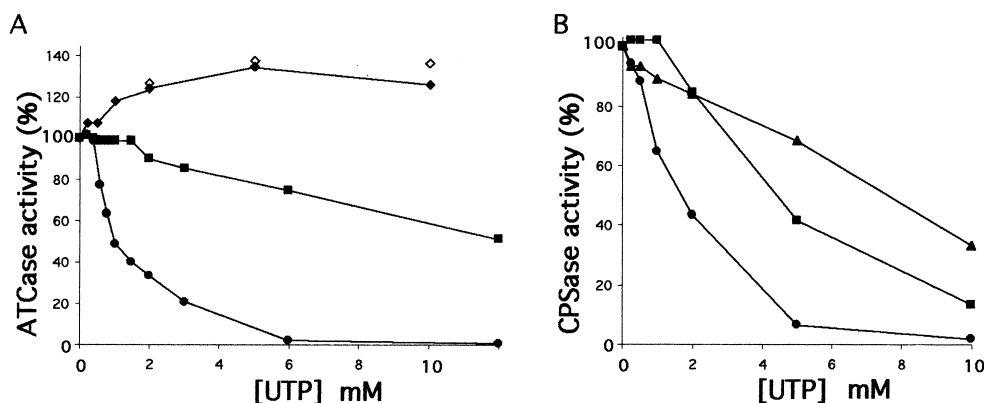

Fig. 3. Impact of increasing amount of UTP on the two Ura2 activities. A: Variations of the ATCase activity due to (●) the control Ura2 protein encoded by p5N; (■) the fusion protein Ura2-GFP encoded by p21; (♦) a truncated Ura2 protein lacking the CPSase domain encoded by pΔCPS; (◇) ATCase activity of pΔCPS assayed in the presence of 2, 5 and 10 mM of ATP, CTP and UMP. (Similar values were found for all three nucleotides.) B: Variations of CPSase activity due to (●) the control Ura2 protein encoded by p5N; (■) the fusion protein Ura2-GFP encoded by p21; (▲) a truncated Ura2 protein lacking the CPSase domain encoded by pΔATC. All plasmids were expressed in a *cpa2 ura2Δ* background; *cpa2*[p5N] and *cpa2*[p21] were grown on YNB, *cpa2*[pΔCPS] in YNB supplemented with 200 mg/l uracil and 40 mg/l arginine, *cpa2*[pΔATC] in YNB supplemented with 200 mg/l uracil.

ATP – were found to have a similar inhibitory effect on ATCase activity. Triphosphate compounds could be responsible for this positive interference, since phosphate ions have been found to interfere with ATCase activity [13].

4. Discussion

This study was mainly focused on the way inhibition by UTP of ATCase and CPSase activities takes place in the yeast multifunctional protein Ura2. Nevertheless, repression by pyrimidines of the *URA2* gene was also estimated in *cpa2* [p5N] and in *cpa2* [p21] strains: specific activities as well as messenger RNA expression were measured in cells grown in the presence and in the absence of uracil. The level of repression by pyrimidines was found to be similar to that previously measured in wild type cells, i.e. about 1.5–3. Repression was thus considered not to be modified in the *cpa2* [21] strain. Truncated Ura2 protein-specific activities were measured in repression conditions only (i.e. in the presence of 200 mg/l uracil); data were found to be similar to (for *cpa2* [pΔATC] CPSase), or slightly higher (for *cpa2* [pΔCPS] ATCase) than, the control. Repression was thus assumed not to be significantly modified when truncated Ura2 proteins were expressed; a conclusion consistent with the fact that, even when no Ura2 polypeptide is made, a normal level of repression by pyrimidines of the *URA2* promoter has been found by Potier [14], as well as by us (i.e. the synthesis of the GFP protein directly fused to the *URA2* promoter and expressed in an *ura2* deletion strain was normally repressed by pyrimidines).

Inhibition by UTP of Ura2 ATCase and CPSase activities was analyzed in a Ura2-GFP fusion protein and in two truncated Ura2 proteins. The two domain-specific deletions cover either the ATCase or the CPSase coding region but do not include the intermediary DHOase-like region. Each of the corresponding truncated Ura2 proteins was found to be active in the absence of the other domain. However, as proposed before by Roelants [15], it cannot be excluded that the DHOase-like region of the polypeptide contributes to the normal folding of the catalytic domains. Deletion of the ATCase domain resulted in a CPSase which remained sensitive to UTP, but less so than the wild type enzyme. This result suggests that the UTP binding site might be located either on the CPSase domain (as shown for *E. coli* [16] or CAD CPSase [17]), or on the DHOase-like region also present on this truncated protein. On the other hand, deletion of the CPSase domain allowed the expression of an ATCase totally insensitive to UTP; thus UTP has no inhibitory effect on the ATCase activity when the whole GATase-CPSase domain is absent. Two sets of experiments support the idea that the intermediary DHOase-like region does not hold the UTP regulatory site: (i) none of the *FUR2* mutations located by Jaquet [5] was found to be localized in this region; (ii) preliminary studies on an Ura2 protein truncated of its DHOase-like region have shown that this protein exhibited both activities, but CPSase – whose specific activity was low – responded to UTP inhibition while ATCase was totally insensitive to UTP (data not shown). All these results led us to the conclusion that only one UTP binding site exists which is located on the CPSase domain.

These results also point to the importance of the association between domains in the regulation of both activities by UTP.

(a) The results obtained with truncated proteins show that independent ATCase species (from pΔCPS) are not sensitive to UTP while CPSase species (pΔATC) remain sensitive to the inhibitor. CPSase activity can thus be inhibited independently of ATCase, probably through conformational changes inside the isolated CPSase domain, as suggested by the hyperbolic decrease of this CPSase activity when UTP concentrations increase.

(b) The results obtained with the Ura2-GFP fusion protein (from [p21]) have shown that the presence of the GFP at the C-terminal end of the protein affects neither CPSase nor ATCase activities, even if the latter is known to be active as a trimer. The GFP domain interferes with both enzyme responses to UTP: it affects ATCase sensitivity to UTP which is dramatically reduced while CPSase remains sensitive to higher concentrations of UTP. However, the sigmoidal kinetics of inhibition of CPSase could reflect a possible cooperativity between sites on which UTP binds (thus CPSase domains), a conclusion which cannot be drawn from CPSase-[pΔATC] data which support no cooperativity.

Thus, taking into account the significant differences which appear between the mechanisms involved in the specific inhibition of ATCase or CPSase activities, we suggest the following model.

On the one hand CPSase, always found to be – more or less – sensitive to UTP, even in the absence of the ATCase domain (Ura2ΔATC), would be covalently linked to the UTP binding site; its inhibition would be induced by conformational changes associated, or not, with a cooperative fixation of UTP on its regulatory site(s); on the other hand, ATCase sensitivity to UTP – as observed with modified Ura2 proteins (Ura2-GFP, Ura2-ΔCPS or Ura2-ΔDHO) – would only be regulated through non-covalent (weak) interactions with the CPSase domain holding the UTP regulatory site. Previous observations, made on the native Ura2 protein, can also take place within this model: an ATCase species no longer sensitive to UTP has already been found when dissociation of the native Ura2 protein (carrying both activities sensible to UTP) occurred, either on sucrose density gradients [10] or upon its heat denaturation [18,19], while in both cases, CPSase species still remained sensitive to UTP inhibition [18].

Structural analyses have suggested that a defined area on the ATCase domain could be involved in interactions: structural properties of the ATCase catalytic domain from CAD [20] and from Ura2 [21] have been deduced from *E. coli* ATCase crystallographic data [21–23]. Both sequences were in agreement with a trimeric structure for the ATCase catalytic domain, the active site(s) being located at the interface between two monomers. The amino acids known to be involved in interactions with regulatory subunits of *E. coli* ATCase were not found on the eukaryotic sequences, but new hydrophobic residues – absent on *E. coli* ATCase trimer – appeared on the surface of the eukaryotic trimers, on the other side of the catalytic sites. The authors suggested that this surface could be involved in interactions with another protein or with another domain of the same protein [21]. Some *FUR2* mutations which have been described are actually located on this surface: this is the case for one group of Jaquet's *FUR2* point mutations whose location, when transferred on the ATCase trimeric structure, maps in this area; another set of Jaquet's *FUR2* mutations (which also induced a specific loss of ATCase inhibition) was localized on the second half of the

CPSase domain [5]. The bipolar distribution of these *FUR2* mutations strongly supports the idea that interactions between ATCase and CPSase domains would be responsible for ATCase feedback inhibition.

Similar conclusions were obtained with the fusion protein whose GFP domain (fused to the terminal residue of the Ura2 sequence known to emerge on the trimer surface opposite the catalytic site) most probably points out on this surface, impairing its normal interactions. Thus, UTP inhibition of ATCase activity is likely to involve interactions between the ATCase trimer surface opposite the catalytic site and the second half of the CPSase domain which carries the UTP binding site.

Interactions have also been proposed to explain a mechanism which differs from those involved in inhibition by UTP, but which takes place in the yeast Ura2 [24] as well as in the mammalian CAD [25] multifunctional proteins: channeling of CP between the CPSase and ATCase domains. Specific interactions proposed to be involved in this mechanism should allow alignment of catalytic domains in order to facilitate the transfer of CP from one catalytic site to the other [25]. Residues involved in such interactions should thus be on the catalytic side of the ATCase trimer, unlike those proposed to be involved into UTP inhibition. Studies carried out on the yeast Ura2 protein suggested that channeling occurs between domains carried by the same polypeptide chain [24]. Work is in progress to investigate whether ATCase feedback inhibition would, or would not, require such a specific contribution of the Ura2 polypeptide chains.

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