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Integrated allosteric regulation in the *S. cerevisiae* carbamylphosphate synthetase – aspartate transcarbamylase multifunctional protein

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Abstract

Background: The *S. cerevisiae* carbamylphosphate synthetase – aspartate transcarbamylase multifunctional protein catalyses the first two reactions of the pyrimidine pathway. In this organism, these two reactions are feedback inhibited by the end product UTP. In the present work, the mechanisms of these integrated inhibitions were studied.

Results: The results obtained show that the inhibition is competitive in the case of carbamylphosphate synthetase and non-competitive in the case of aspartate transcarbamylase. They also identify the substrate whose binding is altered by this nucleotide and the step of the carbamylphosphate synthetase reaction which is inhibited. Furthermore, the structure of the domains catalyzing these two reactions were modelled in order to localize the mutations which, specifically, alter the aspartate transcarbamylase sensitivity to the feedback inhibitor UTP. Taken together, the results make it possible to propose a model for the integrated regulation of the two activities of the complex. UTP binds to a regulatory site located in the vicinity of the carbamylphosphate synthetase catalytic subsite which catalyzes the third step of this enzyme reaction. Through a local conformational change, this binding decreases, competitively, the affinity of this site for the substrate ATP. At the same time, through a long distance signal transmission process it allosterically decreases the affinity of the aspartate transcarbamylase catalytic site for the substrate aspartate.

Conclusion: This investigation provides informations about the mechanisms of allosteric inhibition of the two activities of the CPSase-ATCase complex. Although many allosteric monofunctional enzymes were studied, this is the first report on integrated allosteric regulation in a multifunctional protein. The positions of the point mutations which specifically abolish the sensitivity of aspartate transcarbamylase to UTP define an interface between the carbamylphosphate synthetase and aspartate transcarbamylase domains, through which the allosteric signal for the regulation of aspartate transcarbamylase must be propagated.

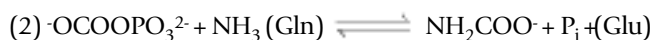
Background

Although numerous allosteric enzymes were studied, much less information is available concerning the coordinated regulation of activities in multienzymatic complexes. Two feedback inhibited multienzyme complexes were studied in *Saccharomyces cerevisiae*, the N-acetylglutamate synthase/N-acetyl glutamate kinase [1] and the carbamylphosphate synthetase – aspartate transcarbamylase [2] complexes.

In prokaryotes the first three reactions of the pyrimidine biosynthetic pathway are catalyzed by independent enzymes namely carbamylphosphate synthetase (CPSase, EC 2.7.2.9), aspartate transcarbamylase (ATCase, EC 2.1.3.2), and dihydroorotase (DHOase, EC 3.5.2.3). *Escherichia coli* CPSase is feedback inhibited by UMP [3] and ATCase by CTP and UTP [4] (Figure 1). In contrast, in mammals these three reactions are catalyzed by an hexameric 240-kDa multifunctional protein, the protein CAD, made of covalently linked domains, each one catalyzing one of these reactions [5-7]. In this case, only CPSase is regulated, being feedback inhibited by UTP [8]. An interesting intermediary organisation is observed in *S. cerevisiae*. In this case a 240-kDa bifunctional protein encoded by the URA2 locus possesses the CPSase and ATCase activities [2,9,10] but lacks the DHOase activity, although it contains an inactive pseudo-DHOase (pDHO) domain [10,11] homologous to the functional DHOases [5-7]. In this organism both CPSase and ATCase are feedback inhibited by UTP [12,13] (Figure 1). Similarly to what is observed in CAD, this yeast multifunctional protein is organized into four major functional domains:

-The glutaminase (GLNase) domain which hydrolyzes glutamine and transfers ammonia to the carbamylphosphate synthetase domain [10].

-The CPSase domain which catalyzes the synthesis of carbamylphosphate from two molecules of ATP, bicarbonate and ammonia in a stepwise fashion that involves three partial reactions: the activation of bicarbonate by ATP, the reaction of the activated species, carboxyphosphate, with ammonia to form carbamate and the ATP-dependent phosphorylation of carbamate to form carbamylphosphate [3,14]:



-The inactive pDHO domain.

-The ATCase domain which catalyzes the reaction of carbamylphosphate and aspartate to form carbamylaspartate.

The N- and C-halves of CPSases from all organisms examined so far, show a significant degree of sequence similarity [10,15-20], an observation which was interpreted to mean that the genes coding for these enzymes evolved through a process of gene duplication, fusion, and differentiation [15,16]. The two domains corresponding to these two halves are called CPS-A and CPS-B. Unexpectedly, it was discovered that each of these two domains of the mammalian CAD CPSase are able to independently catalyze the formation of carbamylphosphate provided that they dimerize [21,22]. In the same way, a truncated yeast bifunctional protein lacking the GLNase and CPS-A domains ($C_B\text{ApD}$) was shown to possess the CPSase activity regulated by UTP [23]. In contrast, the ATCase domain was no longer sensitive to this nucleotide, indicating that the two catalytic activities are controlled by distinct mechanisms [23].

In order to identify amino acid residues implicated in the feedback inhibition by UTP, genetics were used to positively select *in vivo* and characterize missense mutations in the URA2 gene, which specifically affect the feedback – inhibition of ATCase [24]. In these mutants ATCase is no longer inhibited by UTP although CPSase retains full sensitivity to this effector, indicating again that UTP affects the activities of CPSase and ATCase by different mechanisms [25].

In the present work the use of *S. cerevisiae* mutants in which single amino acid replacements abolish the sensitivity of ATCase to UTP allowed to study specifically the process of feedback inhibition of the CPSase domain by this nucleotide. In addition, the reaction step affected by the feedback inhibitor UTP was identified in both the entire complex and in the truncated protein ($C_B\text{ApD}$). Moreover a computational approach was used to predict the structures of the CPSase and ATCase domains. The results obtained provide informations about the integrated allosteric regulation of the two enzymatic activities of the complex, and indicate that the regulatory site is located in the CPS-B domain. The modelling defines an interface between the CPSase and ATCase domains for the transmission of the allosteric signal.

Results

Inhibition of the CPSase and ATCase activities of the complex

UTP inhibition of the coupled reaction

The influence of the feedback inhibitor UTP on the *S. cerevisiae* coupled CPSase-ATCase reaction catalyzed by the complex was determined using the wild type complex

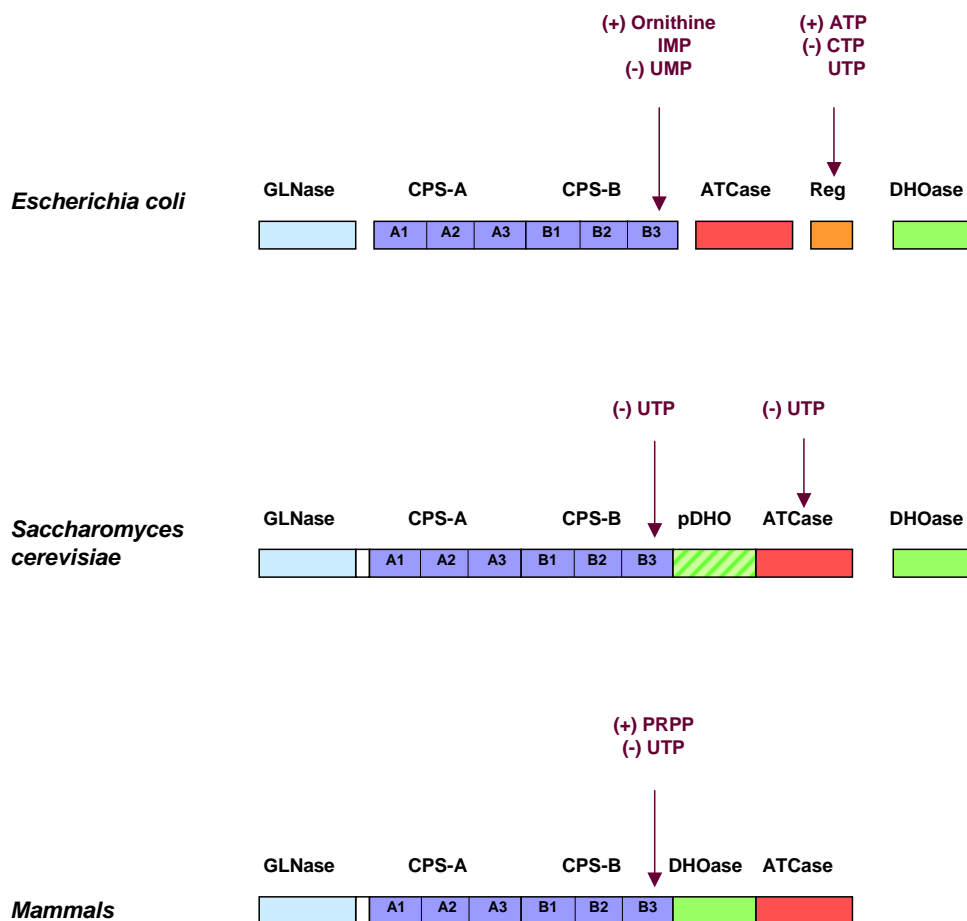


Figure 1
Organization and allosteric regulatory properties of the enzymes catalyzing the first three reactions of the pyrimidine pathway in *E. coli*, *S. cerevisiae* and mammals. This scheme shows the functional domains that catalyze the first steps in the *de novo* pyrimidine biosynthetic pathway, the amidotransferase or glutaminase domain (GLNase), the CPSase synthetase domain consisting of two subdomains (CPS-A and CPS-B), the dihydroorotase domain (DHOase), and the ATCase domain. The activities are associated with separate polypeptide chains in *E. coli*, whereas in mammals (CAD) all are consolidated on a single multifunctional protein. In yeast, the CPSase and ATCase domains are carried by a single polypeptide, but in this case the active DHOase that is encoded by a separate gene is replaced with an inactive DHOase homologue (pDHO). Eucaryotes ATCases lack the regulatory subunit (Reg) found in the *E. coli* protein. The scheme also shows the allosteric effectors that regulate the activity of these proteins and the localization of the regulatory sites represented by arrows.

present in the URA2-EK1104 strain, and is shown in Figure 2. Under the conditions used, complete inhibition is observed for UTP concentrations superior to 3 mM, and half inhibition of the overall reaction is observed in the presence of 0.8 mM. The specific inhibition by UTP of each of the two individual reactions was then investigated.

UTP inhibition of ATCase

The ATCase activity of the complex can be specifically tested without interference of the CPSase activity by providing carbamylphosphate and aspartate to the complex.

The influence of UTP on this activity was determined using the same strain than above. The result obtained is shown in Figure 3. Complete inhibition cannot be obtained and under the conditions used (50 mM aspartate) one observes a 20% residual activity. Similar results were previously reported [12,25,26], the value of the plateau of inhibition varying with the aspartate concentration [26]. Such a pattern is characteristic of a process of partial competitive inhibition in which the binding of the inhibitor to a site distinct from the catalytic site decreases the affinity of the active site for the substrate through a

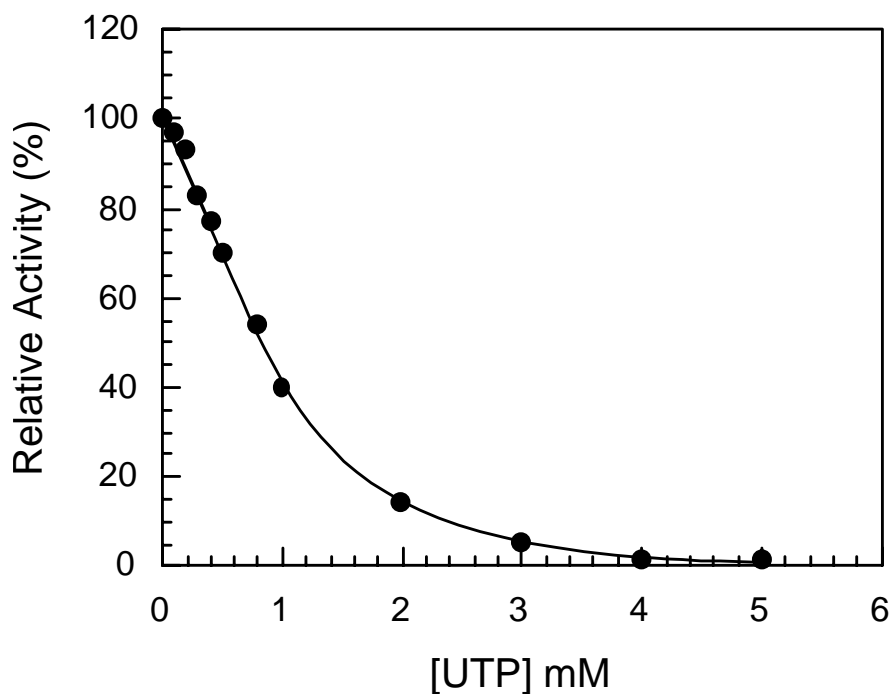


Figure 2

UTP inhibition of the *S. cerevisiae* CPSase-ATCase coupled reaction. The influence of UTP on the coupled CPSase-ATCase reaction was determined as indicated in the Methods using the dialyzed crude extract of the *E. coli* EKI 104 strain transformed by pC4-URA2, in the presence of 50 mM aspartate.

conformational change. This result is consistent with the previously reported observation that towards carbamyl-phosphate the inhibition by UTP is of uncompetitive type [26].

UTP inhibition of CPSase

Since the CPSase assay involves its coupled reaction with ATCase, in order to study specifically the influence of the feedback inhibitor on the CPSase reaction, it was necessary to use mutants in which the ATCase reaction is insensitive to this effector. Such mutants were previously described [25] and it was shown that this specific desensitization could result from single missense mutations located either in the ATCase domain or in the CPSase domain of the complex [25]. Three of these mutants were used in this study: Asn1094→Asp located in the CPSase domain; Glu1933→Lys and Glu2182→Lys located in the ATCase domain [25] (Table 1). Complete inhibition of the CPSase reaction by UTP was observed in the three cases, in accordance with the complete inhibition of the coupled reaction reported above. In order to investigate the nature of this inhibition and to identify the substrate(s) whose binding or utilization is altered by the effector, the influence of UTP was measured in the pres-

ence of varying concentrations of the three substrates of CPSase: ATP, glutamine, and bicarbonate. The three mutants provide the same results which are presented here in the case of Glu2182→Lys. These results are shown in Figure 4, where double reciprocal plots are presented on the left, and Dixon plots on the right. Taken together, these results show unambiguously that UTP acts as a competitive inhibitor towards ATP and as a non competitive inhibitor towards bicarbonate and glutamine, a pattern which indicates that UTP acts by decreasing specifically the apparent affinity of the catalytic site for ATP with a K_i of 3 ± 1 mM. These results indicate a process of absolute competitive inhibition toward ATP.

The UTP inhibition curves of the complex and of the individual CPSase and ATCase reactions exhibit a slight cooperativity with a Hill number of 1.2 ± 0.1 . This is shown in Figure 5 in the case of the Glu2182→Lys mutant.

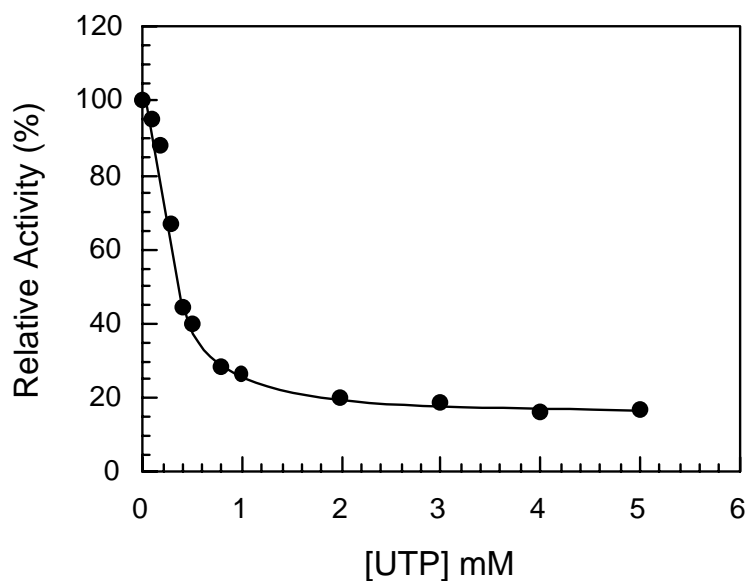


Figure 3

UTP inhibition of the *S. cerevisiae* ATCase reaction of the CPSase-ATCase complex. The influence of UTP on the ATCase reaction of the bifunctional complex was determined as indicated in the Methods in the presence of 50 mM aspartate and 10 mM carbamylphosphate using dialyzed crude extract of the *E. coli* EK1104 strain transformed by pC4-URA2.

Determination of the CPSase reaction step influenced by the feedback inhibitor UTP

Wild type complex

As indicated in the Background section, the reaction catalyzed by CPSase is complex and consists in three partial reactions. Two of them involve a molecule of ATP and these two steps, (1) and (3), can be specifically tested: step (1) on the basis of the bicarbonate dependent ATP/ADP exchange and step (3) in the presence of carbamylphosphate and ADP measuring the ATP production using a coupled reaction with hexokinase and glucose 6-phosphate dehydrogenase (see Methods). The influence of UTP on these two partial reactions was investigated and the results obtained are shown in Figure 6, which shows clearly that it is strictly the third partial reaction catalyzed by the CPS-B domain, which is specifically inhibited by UTP.

C_βApD mutant

In the C_βApD mutant it is a dimer of CPS-B domain (see Background and Fig. 7a) which catalyzes the three steps of the CPSase reaction [23]. Since in the wild type complex it is only the CPS-B domain which is sensitive to UTP, it was interesting to determine whether in this mutant the

step 1 catalyzed by this domain becomes also sensitive to the feedback inhibitor. Thus, the putative influence of UTP on the partial reaction 1 was investigated in comparison with the partial reaction 1 catalyzed by CPS-A in the wild type complex. The results obtained are shown in Figure 7b where it appears that in this mutant, both steps 1 and 3 are feedback inhibited by UTP, indicating that the regulatory site for UTP is effective for the inhibition of step 1 when catalyzed by the CPS-B where it is located. This observation is consistent with the lack of a regulatory site in the CPS-A domain [27,28].

Modelling of the CPSase and ATCase domains and localization of the missense mutations affecting the sensitivity of ATCase to UTP

Amino acid sequences alignment

In order to model the CPSase domain of the complex, we used FASTP to compare the amino acid sequences of *E. coli* CPSase and the *S. cerevisiae* CPSase domain of the CPSase-ATCase complex (Fig. 8). These two homologous proteins have approximately the same size, and show very similar sequences with few insertions and deletions. The degree of identity between the *E. coli* enzyme (residues 2–1073) and the yeast CPSase domain (434–1502) is 42%.

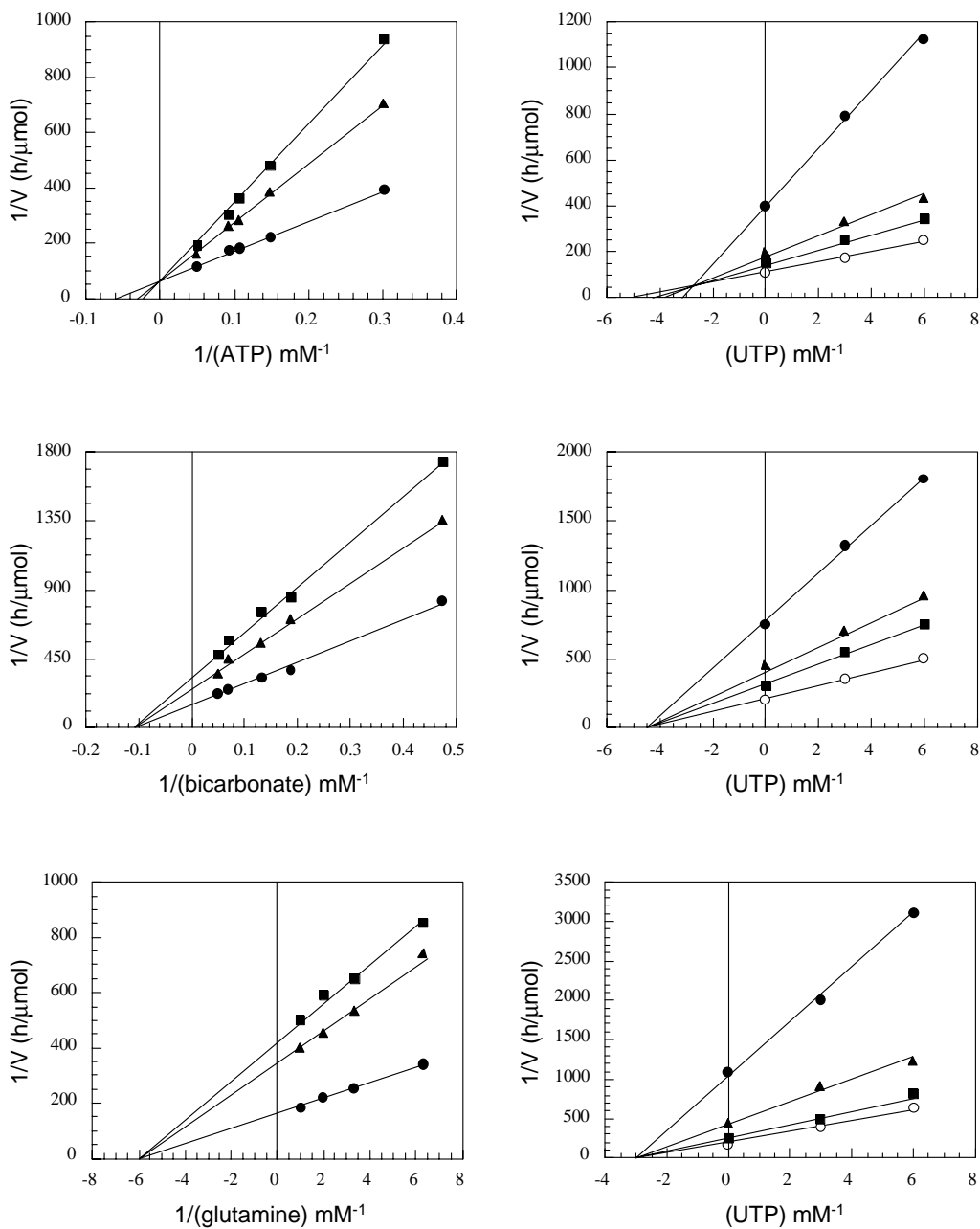


Figure 4

Substrate saturation curves of the CPSase from the mutant Glu 2182→Lys in presence or absence of UTP. The CPSase activity was measured as indicated in the Methods. On the left are represented the double reciprocal plots in the presence of (●) 0 mM, (◆) 3 mM, and (■) 6 mM UTP. On the right are presented the corresponding Dixon plots. The concentrations used were (●) 6.7 mM, (◆) 9.3 mM, (■) 10.7 mM, and (○) 33 mM in the case of ATP; (●) 2.1 mM, (◆) 5.3 mM, (■) 7.5 mM, and (○) 20 mM in the case of bicarbonate; (●) 0.03 mM, (◆) 0.07 mM, (■) 0.3 mM, and (○) 1 mM in the case of glutamine.

Table 1: Position of the missense mutations affecting the sensitivity of ATCase to UTP.

Mutants	Domain
Arg1076 →Ser	CPSase
Asn1094 →Asp	
Tyr1096 →Cys	
Trp1112 →Leu	
Asp1220 →Tyr	
Phe1924 →Val	ATCase
Glu1933 →Lys	
Glu1933 →Gly	
Glu2052 →Lys	
Gly2055 →Ser	
Asn2058 →Asp	
Asn2157 →Lys	
Glu2182 →Lys	
Asp2186 →Asn	

All the mutants carry missense mutations leading to a single amino acids replacement. Five of them are located in the CPSase domains while the others are in the ATCase domain. In these mutants, ATCase is no longer inhibited by UTP although CPSase retains full sensitivity to this effector.

This alignment involves 10 small deletions and 6 insertions in the yeast sequence (Fig. 8). None of these insertions and deletions occur in highly conserved regions or within regions of well-defined secondary structure (not shown). Moreover, all the amino acid residues of the catalytic site which were identified in the X-ray structure of the *E. coli* CPSase are conserved in the yeast sequence. The same method was used to align the sequences of the ATCase domain of the *S. cerevisiae* complex and the ATCase catalytic chain of *E. coli*. The result obtained is identical to that previously reported from the use of the Needleman and Wunsch algorithm [29,30]. The degree of identity is 42% and that involves the presence of 4 minor deletions and 3 insertions. Here also all the amino acid residues which were previously shown to interact with the substrates in the catalytic site of *E. coli* ATCase are present in *S. cerevisiae* [30,31].

Modelling of the *S. cerevisiae* CPSase and ATCase domains

The similarity of the *S. cerevisiae* CPSase domain and the *E. coli* CPSase in sequence and kinetic properties prompted us to model the structure of this yeast domain using the X-ray coordinates of the *E. coli* CPSase [32] as a tertiary template as indicated in Methods. The model obtained is shown as a Swiss-PdbViewer representation in Figure 9. It appears clearly that these two structures are very similar. In the same way and for the sake of homogeneity, the same procedure was used to model the *S. cerevisiae* ATCase domain on the basis of the crystallographic structure of the homologous *E. coli* enzyme [33]. Again,

the two structures appear to be strikingly identical (not shown) in accordance with the observation previously made using a different procedure [30].

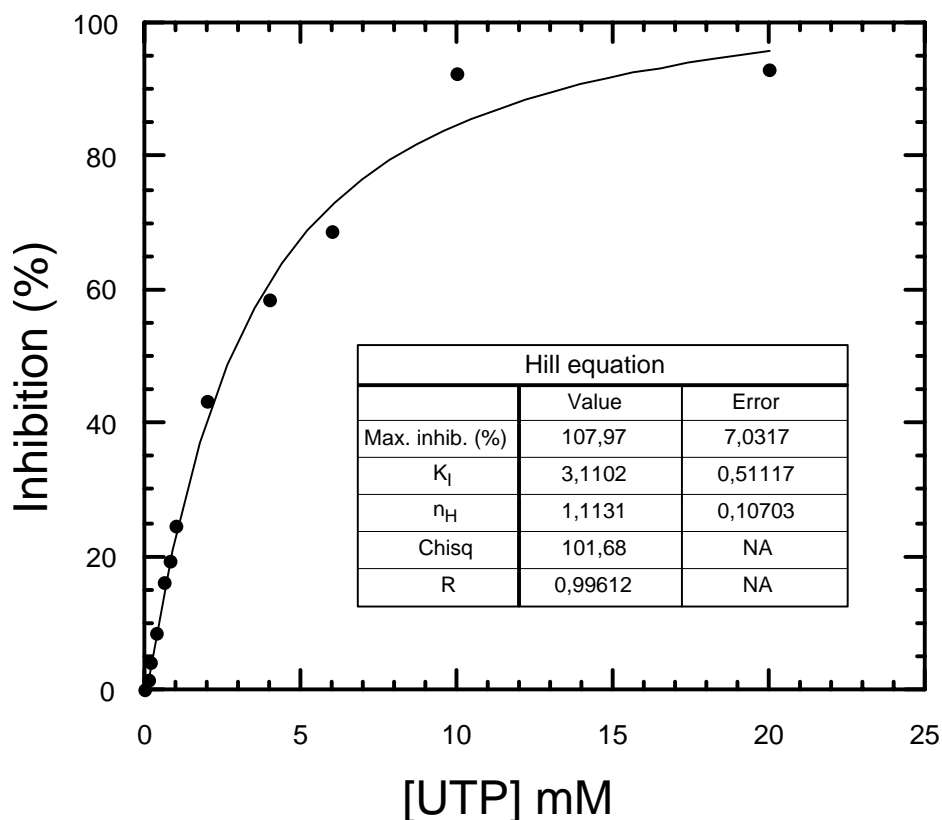
Localization of the ATCase desensitizing mutations

As reported above a series of missense mutants of the *S. cerevisiae* complex were obtained in which the ATCase activity is insensitive to the feedback inhibitor UTP, the CPSase activity remaining fully sensitive to this nucleotide [25]. Interestingly, these point mutations are located either in the ATCase or in the CPSase domains (Table 1). Thus, it was of interest to localize these point mutations in the modelled structures of the two domains. The result is shown in Figure 10. Most interestingly, it appears that these mutants are clustered in a particular region of the surface of the two domains, suggesting that these two regions constitute an interface between the two domains, through which the regulatory signal from the allosteric UTP binding site located in the CPSase domain to the catalytic site of the ATCase domain should be transmitted. However one of these mutations (Phe1924→Val) is located close to the catalytic site of ATCase suggesting that this residue would be rather involved in the transmission of the regulatory signal from the interface to the ATCase catalytic site.

Discussion

In the *S. cerevisiae* CPSase-ATCase complex the two activities are feedback inhibited by the end-product UTP [12,13]. In the case of CPSase, the results reported here indicate that the substrate whose binding is altered by this effector is ATP as shown previously in the case of *E. coli* CPSase [34]. In the case of yeast ATCase, UTP decreases the affinity for the substrate aspartate [26]. Taken together with previously published observations, the results reported here show that the feedback inhibition of the yeast complex is of absolute competitive nature in the case of CPSase and of partial competitive nature in the case of ATCase. This last behavior is characteristic of a process of allosteric inhibition in which the effector binds to a regulatory site distinct from the catalytic site. In the case of CPSase the absolute competition indicates that UTP binds close enough to directly prevent the binding of ATP to this site through a local conformational change.

Several lines of evidence show that the regulatory site where UMP (procaryote CPSases) or UTP (eukaryote CPSases) binds is localized in the B3 subdomain (Fig. 1) [27,28,35]. Among the two steps of the CPSase reaction which, each, use a molecule of ATP (steps 1 and 3) only the third one is inhibited by UTP (Fig. 6). This partial reaction is specifically catalyzed by the B2 subdomain. Thus, it appears that the UTP binding site is located in the CPS-B domain which catalyzes the partial reaction which is specifically inhibited by this nucleotide, a feature which

**Figure 5**

UTP inhibition curve of the CPSase from the mutant Glu 2182→Lys. The percentage of inhibition by UTP was tested under the standard conditions as indicated in the Methods in the presence of increasing concentrations of UTP. The kinetic data were fit to the Hill equation :

$$v = \frac{(\% \text{ of inhibition})_{\max} \cdot (\text{UTP})^{n_H}}{(K_i)^{n_H} + (\text{UTP})^{n_H}}$$

relates to the absolute competitive inhibition reported above. In the *E. coli* enzyme this regulatory site and the CPS-B catalytic site are distant by approximately 20 Å [36]. However, in the yeast complex this distance might be lower as the result of interactions with the ATCase and pDHOase domains. Alternatively, UTP might act through a different mechanism in the yeast complex and might provoke the competitive inhibition through binding to the CPS-B ATP binding site.

The first partial reaction catalyzed by the domain A is not affected by the presence of UTP. It was shown previously that the dimer of the isolated B domain is able to catalyze the synthesis of carbamylphosphate [23]. Interestingly, in this case, the first step of the CPSase reaction becomes sensitive to UTP (Fig. 7). Thus, the reaction normally catalyzed by the A domain becomes sensitive to UTP when it

is catalyzed by the B domain which contains the allosteric UTP binding site. Taken together, these observations indicate that although it is homologous to the B3 domain, the A3 subdomain is unable to bind UTP.

As far as the feedback inhibition of the ATCase activity is concerned, several lines of evidence show that the presence of the CPSase domain of the *S. cerevisiae* complex is necessary for the ATCase domain to be sensitive to UTP, and that a single UTP binding site is located in the CPSase domain. Separation of the CPSase and ATCase domains by limited proteolysis [37] or genetic engineering [23-25,38] leads to the desensitization of the ATCase domain. Taken together with the results reported here concerning the partial competitive character of the ATCase feedback inhibition, the requirement of the CPSase domain

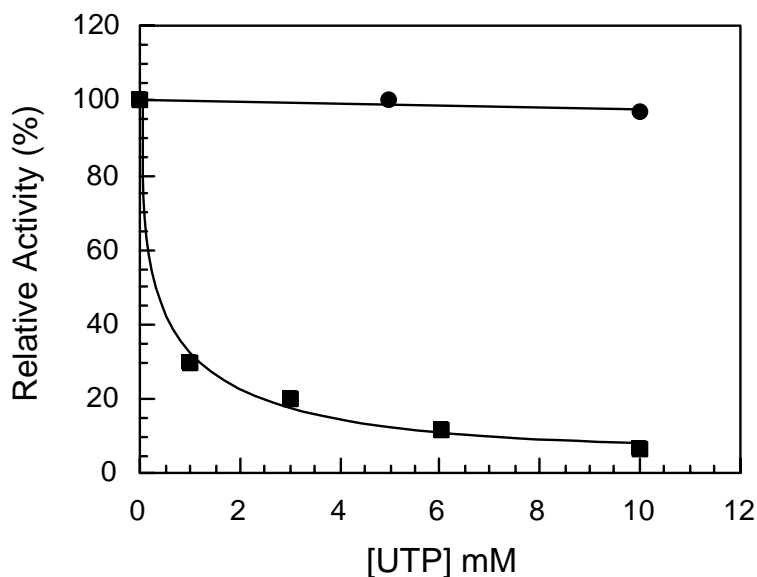


Figure 6

Influence of UTP on the partial reactions 1 and 3 of the CPSase reaction of the wild type complex. The influence of UTP on steps 1 and 3 was determined as indicated in the Methods using dialyzed crude extract of the *E. coli* EK1104 strain transformed by pC4-URA2. (●) partial reaction 1, and (■) partial reaction 3.

strongly suggests that the CPSase and the ATCase catalytic sites are both under the influence of the UTP binding regulatory site located in the B3 subdomain.

Conclusions

The results obtained provide informations about the mechanisms of allosteric inhibition of the two activities of the CPSase-ATCase complex. It is of particular interest that the mutations which specifically abolish the sensitivity to UTP of the ATCase reaction, without altering the CPSase inhibition, are clustered on the surface of either the CPS-B domain or the ATCase domain. This observation strongly suggests that these two regions constitute the interface between the two domains, interface through which the regulatory signal must be transmitted from the CPSase regulatory site to the catalytic site of the ATCase domain. This transmission could involve either a specific path between this interface and the catalytic site of ATCase, or a more global conformational change of this ATCase domain leading to a decrease of the affinity of this site for aspartate.

This is an original example of integrated allosteric regulation in a multifunctional complex that the catalytic

domain of one activity is the allosteric site for the other activity.

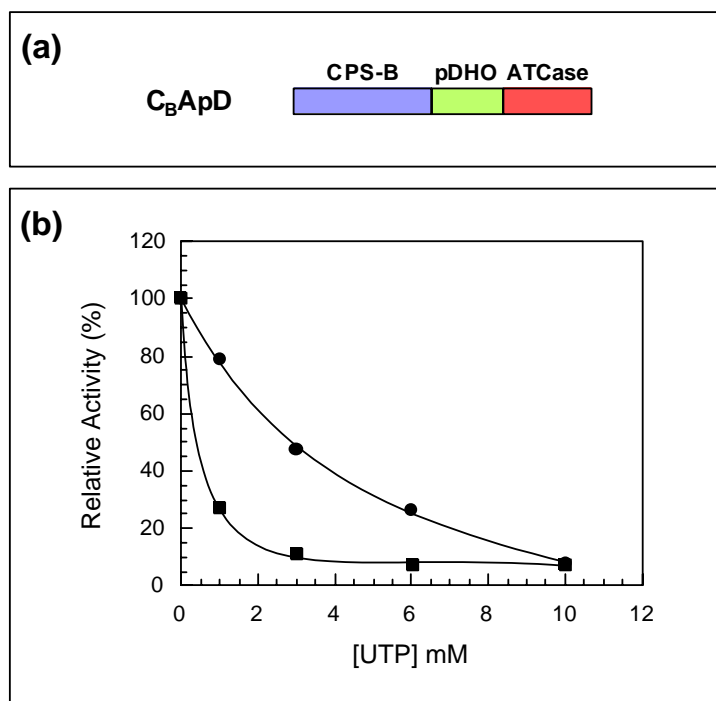
Methods

Plasmids and strains

The 14.0-kb plasmid pC4-URA2 contains the yeast *ura2* gene encoding the bifunctional CPSase-ATCase complex [25]. The pSV-C_BApD recombinant plasmid encodes a protein C_BApD, that possesses the C-terminal half of CPSase (C_B), linked to the pDHO (pD) and the ATCase (A) domains [23].

The *S. cerevisiae* LJ5 strain was transformed by pC4 carrying a wild-type or mutated *ura2* allele [25]. The LJ5 recipient strain was chosen because it is devoid of endogenous CPSase and ATCase activities.

The *E. coli* mutant L673 strain [39], defective in *carA* and *carB*, as well as the Lon-protease, was a gift from Dr. Carol Lusty (Public Health Research Institute of the City of New York). The genes *carA* and *carB* encode the small and large subunits of *E. coli* carbamoyl phosphate synthetase, respectively. The *E. coli* host strain EK1104 [40] lacks the

**Figure 7**

UTP influence on the partial reactions 1 and 3 of the CPSase reaction catalyzed by C_BApD . (a) The truncated complex C_BApD possess CPS-B, pDHO and ATCase domains. (b) The influence of UTP on steps 1 and 3 was determined as indicated in the Methods using dialyzed crude extract. (●) partial reaction 1, and (■) partial reaction 3.

pyrB1 genes. *E. coli* EK1104 and L673 cells were transformed with pC4-URA2 and pSV- C_BApD respectively.

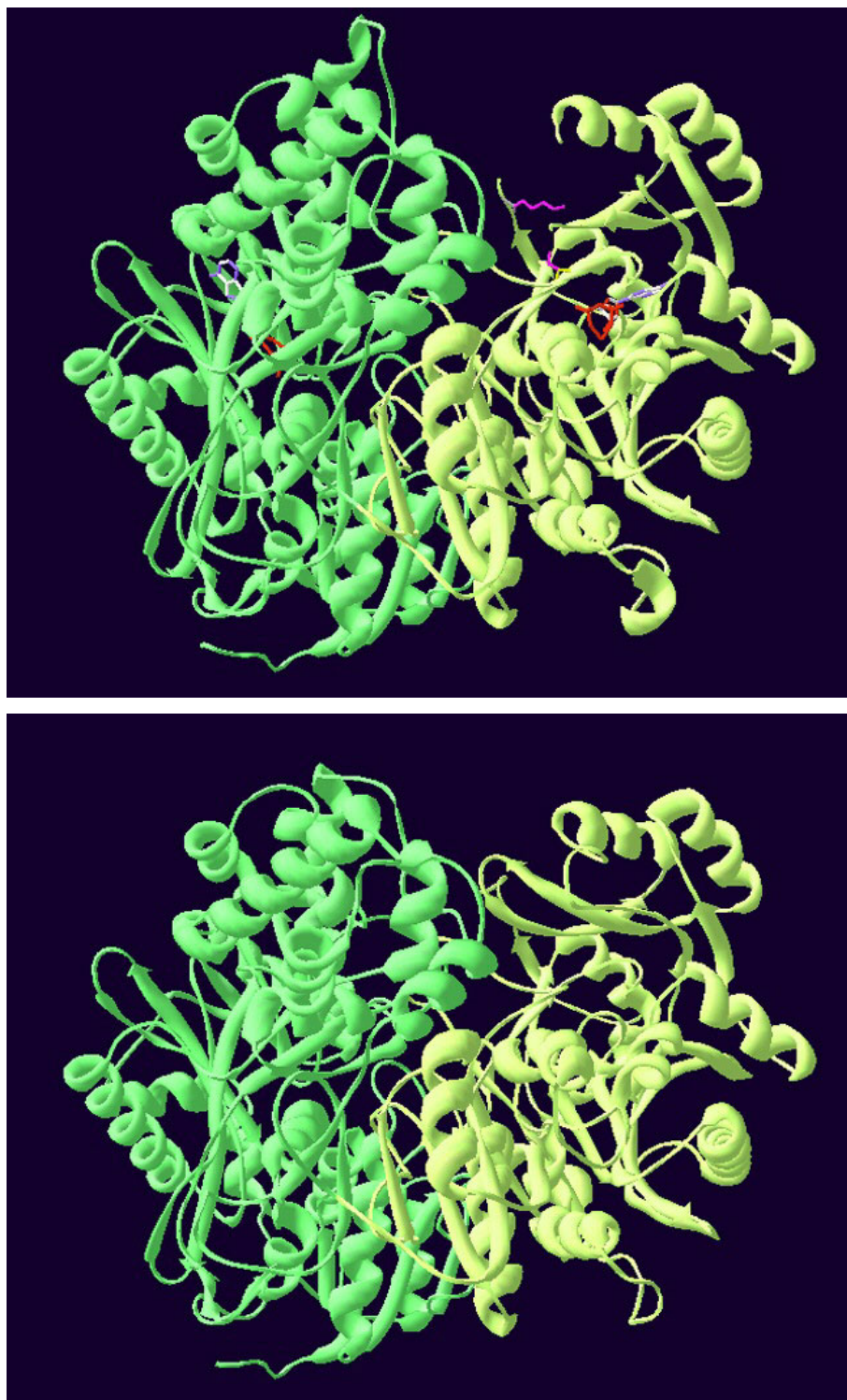
Cell growth and preparation of cell-free extracts

S. cerevisiae LJ5 strain harboring the recombinant plasmids was grown on YNB (6.7 g yeast nitrogen base / 1.2% glucose) at 30°C. Supplements were added to a 50 mg/ml final concentration. *E. coli* EK1104 and L673 cells harboring the recombinant plasmids were routinely grown from a single colony in 2xYT media supplemented with 100 µg/ml ampicillin. For induction of recombinant proteins under control of the pyrB1 promoter, the EK1104 and L673 cells were grown in a minimal media consisting of 6 g/l Na_2HPO_4 , 3 g/l KH_2PO_4 , 1 g/l NH_4Cl , 5 g/l casamino acids, 4 g/l glucose, 0.5 mg/l $ZnSO_4 \cdot 7H_2O$, 0.1 mM $CaCl_2$, 1 mM $MgSO_4 \cdot 7H_2O$, 10 mg/l tryptophan, supplemented with 12 µg/ml uracil and 100 µg/ml ampicillin. Under these conditions, there is sufficient uracil to sustain growth for about 19 to 21 hours, after which time, uracil is exhausted, growth is slowed, and the recombinant protein is expressed. Growth was monitored spectrophotometrically at 600 nm. The cells were harvested in late exponential phase or early stationary phase, by centrifugation at 3000 g for 30 minutes in a Centrikon T-124

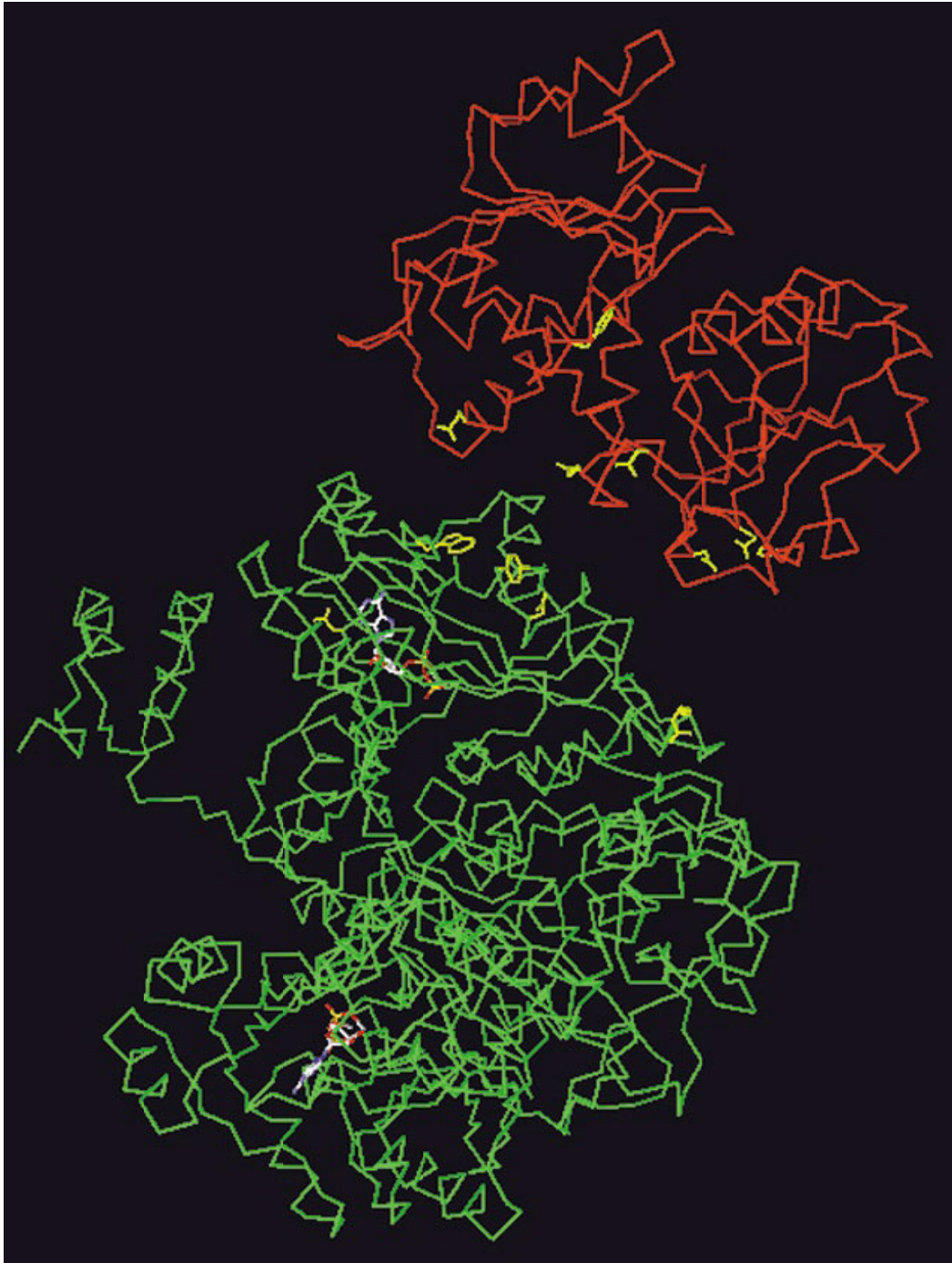
centrifuge. The cells were resuspended in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, and disrupted by sonication three times for one minute on ice, using a Biosonik III sonifier set at 20,000 kHz. The sonicate was cleared by centrifugation at 12,000 g for 30 minutes at 4°C. These extracts were dialyzed in order to eliminate all the metabolites, including nucleotides, which might interfere with enzyme assays. Protein concentrations were assayed by the Lowry method [41].

Enzymatic activities

Enzymatic activities were tested on crude dialyzed extracts as described by Penverne & Hervé [26]. The ATCase activity was tested as described by Denis-Duphil *et al.* [37]. The standard conditions used were 30 mM (^{14}C)aspartate (0.03 µCi/µmol), 10 mM carbamylphosphate, and 50 mM Tris-HCl, pH 7.5. The assays were conducted at 30°C for 10 minutes. The CPSase activity of the yeast CPSase-ATCase and the C_BApD complexes were assayed in the presence of 5 µg of *E. coli* ATCase catalytic subunits to efficiently trap all the unstable carbamylphosphate formed. The standard conditions used were 50 mM Tris-Ac, pH 7.5, 100 mM KCl, 100 mM NH_4Cl , 150 mM (^{14}C)sodium bicarbonate (0.168 µCi/µmol), 20 mM mag-

**Figure 9**

Modelled structure of the *S. cerevisiae* CPSase domain of the CPSase-ATCase complex. Top: crystallographic structure of the *E. coli* CPSase [32]. Bottom: modelled structure of the *S. cerevisiae* CPSase domain of the bifunctional protein obtained as indicated in the Methods. The CPS-A and CPS-B domains are in green and light-green respectively. The two catalytic subsites of the *E. coli* CPSase are indicated by two molecules of ADP. Lysine992 and serine948 located in the UMP binding site of the *E. coli* CPSase are in magenta.

**Figure 10**

Probable interface between the CPSase and ATCase domains of the *S. cerevisiae* bifunctional complex. In green: the yeast CPSase domain. In red: the yeast ATCase domain. In yellow: point mutations which specifically abolish the sensitivity of the ATCase activity to UTP (see Table 1). From top to bottom: Asp1220, Trp1112, Tyr1096, Asn1094, Arg1076 in the CPSase domain; Glu1933, Glu2052, Phe1924, Asn2058, Asp2186, Glu2182, Asn2157 in the ATCase domain. The CPSase catalytic subsites are indicated by two molecules of ADP.

nesium acetate, 10 mM ATP, and 50 mM aspartate. The assays were conducted at 30°C for 30 minutes. The extracts samples were extensively dialyzed immediately before the enzymatic tests in order to eliminate all the metabolites (including nucleotides) potentially able to interfere with the activities.

The overall carbamylphosphate synthetase – aspartate transcarbamylase activity was tested as described by Penverne *et al.* [42] without the addition of *E. coli* ATCase catalytic subunits. The partial reaction 1 of CPSase was assayed at 25°C by coupling the production of MgADP to the oxydation of NADH through the inclusion of pyruvate kinase, PEP, and lactate dehydrogenase in the assay mixture. Disappearance of NADH was followed continuously by monitoring the decrease in absorbance at 340 nm with a strip chart recorder. Each cuvette contained in a final volume of 1.0 ml the following: 50 mM Tris-Ac, pH 7.5, 100 mM KCl, 20 mM MgCl₂, 0.2 mM NADH, 1 mM PEP, 50 mM NaHCO₃, 10 mM L-glutamine, 10 mM MgATP, 0.1 mg of pyruvate kinase, and 0.15 mg of lactate dehydrogenase. The inhibitor UTP, if present, was 10 mM. The reaction was started with the addition of 100 µl of dialyzed crude extract. The partial reaction 3 of CPSase was measured at 25°C by coupling MgATP production with the reduction of NADP with hexokinase, glucose, and glucose-6-phosphate dehydrogenase. All cuvettes contained 50 mM Tris-Ac, pH 7.5, 100 mM KCl, 20 mM MgCl₂, 1 mM NADP, 10 mM glucose, 1 unit each of hexokinase and glucose-6-phosphate dehydrogenase, 6 mM of MgADP (≈ Km), and 10 mM carbamoyl phosphate. The inhibitor UTP, if present, was 10 mM. The reaction was started by the addition of 100 µl of dialyzed crude extract. It was verified that the CPSase reaction was fully dependent on the presence of the three substrates. In the case of the L673 strain these controls were already published [23]. The same full requirement was observed in the case of the EK1104 strain. The bicarbonate ATPase-dependent reaction was undetectable in absence of bicarbonate.

Assay for UTP inhibition

The sensitivity of CPSase and ATCase to the feedback inhibitor UTP was assayed under the standard conditions described above in the presence of varying concentrations of this effector.

Sequence alignments

Sequences of *E. coli* CPSase (CARB, SwissProt P00968), *E. coli* ATCase catalytic chain (PYRB, SwissProt P00479) and *S. cerevisiae* CPSase-ATCase complex (PYR1, SwissProt P07259) were used. The sequences were aligned using the BIONET program FASTP (BLOSUM 50 matrix, ktup = 2) and Protein Information Ressource program ALIGN. Input parameters were chosen empirically.

Homology modelling of the *S. cerevisiae* CPSase and ATCase domains

The three-dimensional structure of the *S. cerevisiae* CPSase and ATCase domains were modelled by comparative protein modelling methods and energy minimization using the program SWISS-MODEL [43] in the optimized mode. The 2.10 Å coordinate set for the CPSase from *E. coli* [32] was used as the template for modelling the yeast CPSase monomer. The 2.5 Å structure of the *E. coli* ATCase complexed with the bisubstrate analogue N-(phosphonoacetyl)-L-aspartate [33] was used as the template for modelling the yeast ATCase monomer. Swiss-PdbViewer 3.5 [43] was used to analyse and visualize the structures.

List of abbreviations

The abbreviations used are: CPSase, carbamylphosphate synthetase; ATCase, aspartate transcarbamylase; GLNase: glutamine amidotransferase; DHOase, dihydroorotase; pDHO, the yeast domain that exhibits sequence similarity to functional DHOases but which lacks activity; C_BApD, the truncated yeast complex consisting of the CPS-B domain fused to the ATCase domain via the pDHO domain; CPS-A, the subdomain corresponding to the amino half of the CPSase synthetase domain or subunit; CPS-B and C_B, the subdomain corresponding to the carboxy half of the CPSase synthetase domain or subunit.

Authors' contributions

VS performed the kinetic analysis with the participation of BP, the molecular modelling and the construction of the truncated C_BApD protein in collaboration with HG and DE. SP and JLS provided the mutants. GH coordinated the investigations. All authors read and approved the final manuscript.

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