Synergistic Inhibition of *Escherichia coli* Aspartate Transcarbamylase by CTP and UTP: Binding Studies Using Continuous-Flow Dialysis†

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ABSTRACT: The allosteric control of *Escherichia coli* aspartate transcarbamylase (ATCase) involves feedback inhibition by both CTP and UTP, although it is only in the presence of CTP that UTP appears to inhibit the activity of the enzyme. In order to better understand the parts played by both pyrimidine nucleotides in this synergistic inhibition, binding studies were performed by continuous-flow dialysis and ultracentrifugation methods. The results obtained show that UTP binds to ATCase in the absence of CTP. Nevertheless, this binding does not induce any inhibition unless CTP is present. The mutual influence of CTP and UTP on their respective binding constants suggests that they bind to the same regulatory sites. However, the results obtained cannot be satisfactorily explained by a simple competition between the nucleotides, and it is shown that reciprocal affinity enhancements play a fundamental role. CTP enhances the affinity of UTP for the regulatory sites 80-fold, and conversely, UTP enhances the affinity of CTP 5-fold. Interestingly, the isolated regulatory subunits bind the two pyrimidine nucleotides following the same pattern as the entire enzyme. These observations indicate that the synergistic inhibition mechanism relies entirely on interactions between the two adjacent allosteric sites which belong to the same regulatory dimer.

Escherichia coli aspartate transcarbamoylase (ATCase, EC 2.1.3.2) is one of the best-known allosteric enzymes [for review, see Allewell (1989), Hervé (1989), Perutz (1989), and Kantrowitz and Lipscomb (1990)]. However, more than 30 years after the first studies on this enzyme (Gerhart & Pardee, 1962), many fundamental aspects of its catalytic and regulatory properties remain largely unknown.

ATCase catalyses the first committed step of pyrimidine biosynthesis, i.e., the carbamylation of the amino group of aspartate by carbamyl phosphate to produce *N*-carbamyl-L-aspartate and inorganic phosphate. This enzyme consists of two catalytic trimers and three regulatory dimers (Wiley & Lipscomb, 1968), which can be dissociated by the use of mercurials (Gerhart & Holoubek, 1967). ATCase shows homotropic cooperative interactions between the catalytic sites for aspartate binding and possibly catalysis. This phenomenon is explained by a concerted transition of quaternary structure, from a tight "T state" that has a low affinity for aspartate to an expanded "R state" that has a high affinity for this substrate. This transition is coupled to changes in the tertiary structure of the catalytic chains. The two extreme conformations have been clearly identified and their structures have been solved by X-ray crystallography with a resolution of 2.4 Å (Honzatko et al., 1982; Ke et al., 1984, 1988; Krause et al., 1987).

CTP, the end product of the pathway, inhibits the ATCase activity, while ATP, the end product of the purine pathway, activates the enzyme. This antagonism is assumed to contribute in maintaining a balance between the intracellular pools of purine and pyrimidine nucleotides. CTP and ATP affect the ATCase activity by respectively increasing and decreasing the concentration of aspartate required to produce half the maximal velocity of the enzyme. Although there still is some debate about the precise mechanism of action of these nucleotides (Eisenstein et al., 1990; Xi et al., 1991), their effect is best explained by the combination of a primary and a secondary effect (Thiry & Hervé, 1978; Tauc et al., 1982): The primary effect consists of local tertiary conformational changes that alter the affinity for aspartate of the nearest catalytic site. The secondary effect consists of a modification of the T/R ratio resulting from the variation of the proportion of sites occupied by aspartate induced by the primary effect. Both nucleotide effectors bind competitively to the same allosteric sites in the N-terminal domain of the regulatory chains, approximately 60 Å away from the nearest catalytic site. Therefore, the primary signal has to be transmitted over this distance, and various site-directed mutagenesis studies have shown that the CTP and the ATP signals are transmitted through different pathways (Xi et al., 1990, 1991; Van Vliet et al., 1991). This observation can contribute to explain how their effects are discriminated. On the other hand, crystallographic studies have shown that CTP and ATP have a slight but significant effect on the quaternary structure of both the T and R states of the enzyme (Gouaux et al., 1990; Stevens et al., 1990): this effect is very small compared to that involved in the T ↔ R transition. Upon ATP binding, the distance between the two catalytic trimers is slightly increased, although none of the hydrogen bonds or other polar interactions which are characteristic of the T state are disrupted. It has recently been suggested that both these slender indirect quaternary structure changes and the previously described direct primary effect are part of the mechanism of CTP and ATP effects (Stevens & Lipscomb, 1992).

The binding of both nucleotides shows a heterogeneous pattern that has been interpreted in terms of three high-affinity and three low-affinity sites, whose binding constants differ by approximately a 20-fold factor (Gray et al., 1973; Matsumoto & Hammes, 1973; Tauc et al., 1982). Generally, negative cooperativity between the two regulatory sites that belong to the same dimer is considered to be the most likely explanation of this phenomenon (Tondre & Hammes, 1974; Issaly et al., 1982). However, the preexistence of two independent classes

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of binding sites cannot be totally ruled out: a slight molecular asymmetry has been observed across the 2-fold axis of symmetry in the CTP- and ATP-liganded enzyme crystals (Kim et al., 1987; Stevens et al., 1990), although it is most probably due to crystal packing and hence should not exist in solution (Kim et al., 1987).

Recently, Wild et al. (1989) have shown that the combination of UTP and CTP inhibits the enzyme more efficiently than CTP alone. However, discrepancies arose between this report and that of Zhang and Kantrowitz (1991) regarding the mutual influence of UTP and CTP on their respective binding. In the present work, continuous-flow dialysis and ultracentrifugation methods were used to study the binding of UTP, CTP, and ATP, alone or in combination, as well as their influence on the ATCase activity. Furthermore, the binding of these nucleotides to the isolated regulatory subunits was investigated.

EXPERIMENTAL PROCEDURES

Materials

Enzyme. Native ATCase was overproduced from the plasmid pEK2 and expressed in *E. coli* strain EK1104 (Nowlan & Kantrowitz, 1985). It was then purified to homogeneity by a method similar to that described by the aforementioned authors, although a further chromatography step on Sepharose CL-6B was found to be necessary, prior to the isoelectric precipitation at pH 5.8, in order to eliminate totally the remaining nucleic acids and some impurities. Isolated regulatory subunits were prepared as described by Gerhart and Holoubek (1967).

Chemicals. Carbamyl phosphate (dilithium salt), L-aspartate, CTP and ATP (sodium salts), and *p*-hydroxymercuribenzoic acid (pHMB) were purchased from Sigma Chemical Co. UTP (sodium salt) and dextran T40 were obtained from Pharmacia, imidazole and Titriplex (EDTA, ethylenedinitrilotetraacetic acid) from Merck, 2-mercaptoethanol from Kodak, and Aquasol liquid scintillation cocktail from NEN Research Products. [2-^{3}H]ATP (23 Ci/mmol), [5-^{3}H]CTP (23 Ci/mmol), and [5-^{3}H]UTP (10.2 Ci/mmol) (ammonium salts) were from Amersham, while L-[U-^{14}C]-aspartate (300 mCi/mmol) was from CEA-Saclay.

Both labeled and unlabeled triphosphate nucleotides were controlled for purity by the method described by Beck and Howlett (1977). Spectrophotometry was used to determine the exact concentration of unlabeled solutions: at pH 7.0, ϵ(ATP) = 15 400 at 259 nm, ϵ(CTP) = 9000 at 271 nm, and ϵ(UTP) = 10 000 at 262 nm.

Buffer. All continuous-flow dialysis, ultracentrifugation, and enzyme assay experiments involving ATCase were performed in buffered solutions containing 5×10^{-2} M imidazole (pH 7.0), 10^{-3} M 2-mercaptoethanol, and 10^{-4} M EDTA. For the binding studies with isolated regulatory subunits, 10^{-3} M zinc acetate was added, in order to prevent dissociation of the dimers and subsequent aggregation.

Methods

Measurement of Ligand Binding: (A) Continuous-Flow Dialysis. The principle of the continuous-flow dialysis apparatus used was similar to that first described by Colowick and Womack (1969). However, various modifications were made in order to increase its efficiency and accuracy: a schematic diagram of its main features is shown in Figure 1. It consists of a dialysis cell with a 1-mL upper chamber, containing the enzyme and labeled substrate, separated by a

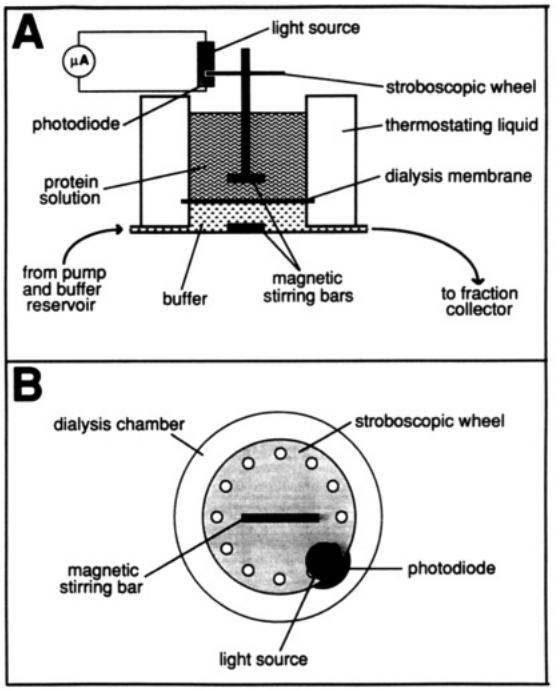

FIGURE 1: Principal features of the continuous-flow dialysis apparatus. (A) Schematic general diagram (μA: microamperemeter). (B) Detail of the stroboscopic device used to monitor the stirring rate. The light source is above the stroboscopic wheel while the photosensible diode, the magnetic stirring bar, and the dialysis chamber are below.

Spectra/Por 6 membrane (cutoff MW 25 000) from a 150-μL lower chamber, through which buffer is pumped at a constant rate (2 mL/min) and from which the effluent is sampled. The dialysis chambers and the tubing are carefully thermostated using a Haake D3 apparatus. Both the upper and the lower chamber are magnetically stirred in order to ensure their homogeneity. Previous experiments (data not shown) demonstrated that it is crucial that the stirring rate remains constant. A stroboscopic device (Figure 1B) was therefore added to the apparatus: it consists of a wheel with a regularly placed holes, fused with the upper-chamber stirring system, and linked to a light source. A photosensitive diode, connected to a microamperemeter, recovers the light across the holes and hence allows one to measure the induced current, in order to monitor the stirring rate and keep it constant all along the experiment.

The dialysis rate of free ligand reaches a steady state within 3 min under the conditions used. Additional rate measurements are made after successive additions of small volumes of concentrated unlabeled ligand in the upper chamber. Owing to the fact that dialysis equilibrium is rapidly reached after each addition, a full 10-point Scatchard plot can be obtained in less than 2 h with a single enzyme sample. The enzyme concentrations used were 2–10 mg/mL (6.5–32 μM) for CTP binding experiments, 10 mg/mL in the case of ATP, and 40 mg/mL (130 μM) in the case of UTP. For studies involving isolated regulatory subunits, the concentration used was 1–7 mg/mL (29–206 μM). Under these conditions, maximal binding of the nucleotides did not exceed 95%. The final addition of unlabeled ligand provides a measure of the dialysis rate, at a total ligand concentration for which the proportion of bound ligand in the upper chamber is negligible. In order to avoid triphosphate nucleotides binding to the active sites of the enzyme (Matsumoto & Hammes, 1973), 15 mM carbamyl phosphate was always added to both the enzyme sample and the circulating buffer. During the experiments,

1-mL fractions of effluent were collected and their radioactivity was measured after addition of 4 mL of water and 8 mL of Aquasol.

(*B*) *Ultracentrifugation.* Ultracentrifugation was used in order to confirm the results obtained in the case of the entire enzyme. Although this method is faster and more handy than continuous-flow dialysis, it provides less accurate results. Its principle was previously described by Howlett et al. (1978). Samples (100 μL) were centrifuged for 1 h at 70 000 rpm (approximately 280000*g*) in a Beckman TL-100 tabletop ultracentrifuge, using a TL-100.1 fixed-angle rotor. To avoid convective stirring of the sedimented protein into the supernatant, 2 mg/mL dextran T40 was added to each sample (Howlett et al., 1978), and the deceleration option was chosen so that the rotor stops in approximately 5 min. Under the conditions used, less than 0.5% of the initial protein was found in the upper 20 μL of the supernatant. This small volume was carefully removed and its radioactivity was measured in order to estimate the concentration of free labeled nucleotides. Enzyme was found to retain full activity after centrifugation. The protein, nucleotide, and carbamyl phosphate concentrations were the same as for the continuous-flow dialysis experiments.

Curve Fitting. Scatchard plots were fitted by EZ-fit, a computer program developed by F. W. Perrella from the Medical Products department of E. I. du Pont de Nemours & Co. Saturation by the ligand could be considered alternatively to follow three possible mechanisms: "Michaelis–Menten", "Hill" (cooperative), or "isoenzyme" (two independent classes of sites). The parameters were first adjusted by a simplex routine and then minimized by classical Gauss/Newton least-squares iterations. The aspartate saturation curves were fitted both by KaleidaGraph, developed by Abelbeck Software for Macintosh, and by a program written in our laboratory by P. Tauc: these two programs use least-squares minimization procedures.

Enzyme Assay Using [^{14}C]Aspartate. The ATCase activity was determined as previously described (Perbal & Hervé, 1972), in the presence of the above-mentioned buffer and 10^{-2} M carbamyl phosphate. The specific activity is expressed as micromoles of *N*-carbamyl-L-aspartate formed per hour per milligram of protein.

Protein Concentration. The enzyme concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard, taking into account the 20% overestimate given by this method for the native enzyme and the 30% underestimate for isolated regulatory subunits (Kerbiriou et al., 1977).

RESULTS

(*1*) *Binding of UTP to ATCase in the Presence and Absence of CTP.* Previous studies lead to contradictory conclusions concerning the possible influence of CTP on the binding of UTP to ATCase. On one hand, Wild et al. (1989) found that, at 4 °C, UTP binds to the enzyme in the absence of CTP and that the presence of saturating amounts of CTP does not detectably affect the binding of UTP. On the other hand, Zhang and Kantrowitz (1991) showed that, at 25 °C, UTP binding was insignificant in the absence of CTP and that it was improved more than 50-fold in its presence.

The binding of UTP was investigated, at 4 and 25 °C, in the presence and absence of CTP, using continuous-flow dialysis and ultracentrifugation methods. Due to the low affinity of UTP for the enzyme (K_d of about 1 mM at 4 °C) and to technical limitations of the methods used, the binding could not be measured along the entire saturation curve. However, the Scatchard plots obtained over the range of UTP concentrations used (from 5×10^{-6} to 3×10^{-3}M) were linear enough to be extrapolated to saturation, using the EZ-fit computer program described in the Experimental Procedures section.

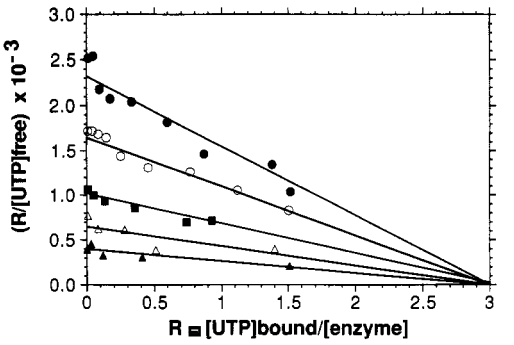

FIGURE 2: Scatchard plot of the binding of UTP to native ATCase. The binding was measured at 4 °C, as indicated in Experimental Procedures, in the presence (○) or absence (●) of 1 mM CTP and in the presence of 2 mM ATP (■). At 25 °C, it was determined in the presence (▲) or absence (△) of 2 mM CTP.

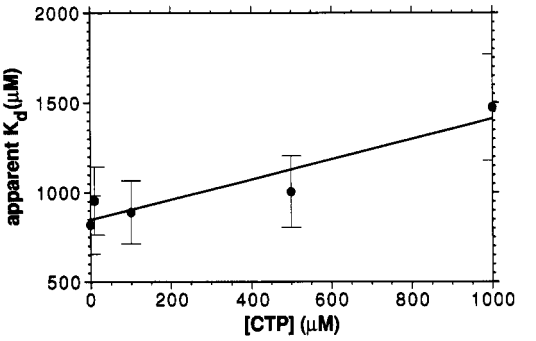

FIGURE 3: Influence of CTP on the binding of UTP to ATCase at 4 °C. Values of apparent dissociation constants for UTP are the mean of at least two measures, by continuous-flow dialysis and ultracentrifugation methods. Uncertainties on the values obtained are indicated by error bars and were approximately 20%.

Table I: Nucleotide Binding to ATCase: Apparent Dissociation Constants at 4 °C[a]

		K_d 1 (μM)	K_d 2 (μM)
UTP binding	alone	810 ± 200	nd[b]
	1 mM CTP	1460 ± 250	nd
	2 mM ATP	1830 ± 350	nd
CTP binding	alone	9 ± 0.5	260 ● 65
	2 mM UTP	4.8 ● 0.2	220 ± 70
ATP binding	alone	65 ± 6	1250 ± 400
	2 mM UTP	100 ● 10	3100 ± 900

[a] Values were obtained by fitting the Scatchard curves with the program described in the Experimental Procedures section, choosing the isoenzyme option. K_d1 and K_d2 indicate respectively the dissociation constants for the high-affinity and the low-affinity sites. [b] nd, not detectable.

The results obtained at 4 and 25 °C are presented in Figure 2. The Scatchard plots extrapolate to three UTP binding sites, whether or not CTP is present. Moreover, saturating CTP concentrations appear to have a slight negative effect on the apparent dissociation constant for UTP, although the variation is small in view of the uncertainties of the measured values (Figure 3 and Table I). Thus, CTP does not seem to have a significant influence on the apparent affinity of UTP for the regulatory sites, either at 4 or at 25 °C. The exact meaning of this observation is analyzed further.

(*2*) *Influence of UTP on the Binding of CTP to ATCase.* As previously mentioned, CTP shows a heterogeneous pattern

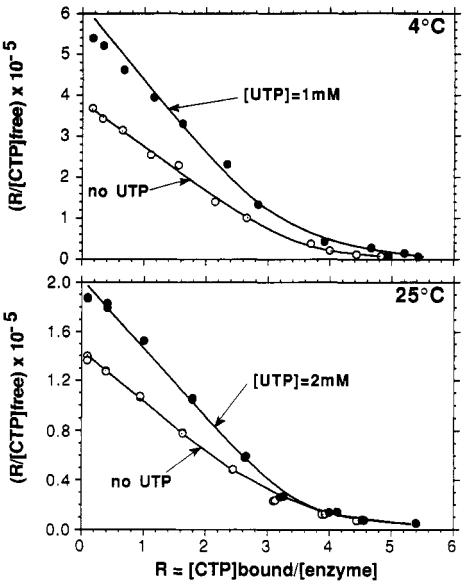

FIGURE 4: Scatchard plot of the binding of CTP to native ATCase. The binding was measured at 4 and 25 °C, in the presence (●) or absence (○) of UTP. The values of K_d1 and K_d2, calculated from the binding curves at 4 °C, are reported in Table I.

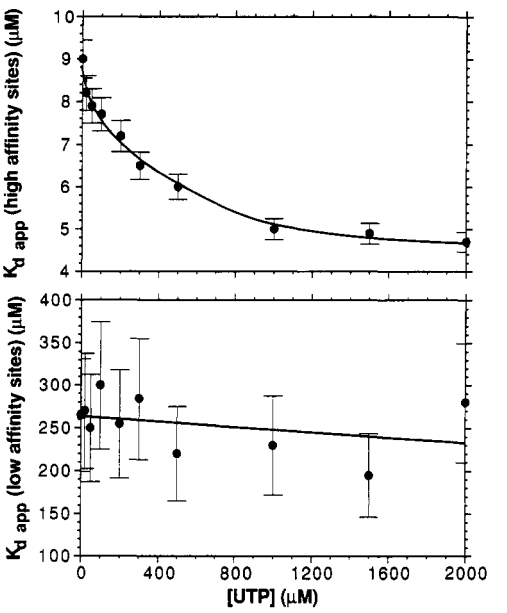

FIGURE 5: Influence of UTP on the binding of CTP to ATCase at 4 °C. Uncertainties on the measured values of the apparent dissociation constants for CTP are indicated by error bars and were approximately 5% for the high-affinity and 25% for the low-affinity sites.

of binding to ATCase, which has been analyzed in terms of two classes of three binding sites each, whose dissociation constants differ by approximately a 20-fold factor. UTP has been reported to enhance the binding of CTP to the enzyme: Wild et al. (1989) showed that, at 4 °C, 2 mM UTP improves the apparent binding constant of CTP to the high-affinity sites by about 30%. On the other hand, Zhang and Kantrowitz (1991) found that, at 25 °C, 10 mM UTP (providing approximately the same degree of saturation as 2 mM at 4 °C) increases the apparent binding constant of CTP by more than 250%. In addition, according to the latter, saturating UTP almost totally suppresses the binding of CTP to the low-affinity sites.

In the course of the present investigation, both continuous-flow dialysis and ultracentrifugation were used to measure CTP binding, at 4 and 25 °C, in the presence and absence of UTP. Figure 4 shows that subsaturating UTP improves the affinity of CTP for the enzyme, at both temperatures. This is mainly due to a decrease of approximately 40% in the apparent dissociation constant for the high-affinity sites (Table I).

In order to clarify the influence of UTP on the binding of CTP, the variation of the apparent dissociation constants for both the high- and the low-affinity sites was analyzed as a function of UTP concentration, at 4 °C (Figure 5). A concentration of 1 mM UTP was sufficient to obtain the full effect on the apparent dissociation constant for the first class of sites. In contrast, the binding of CTP to the low-affinity sites was not significantly altered in the presence of saturating UTP.

(*3*) *Competition between ATP and UTP.* Although the binding sites for CTP and ATP have been unambiguously identified and described in detail by X-ray crystallography (Stevens et al., 1990; Gouaux et al., 1990), there is, at present, no similar information on the binding sites for UTP. In order to know whether UTP binds to the same allosteric sites as the other nucleotides, the reciprocal influence of UTP and ATP on their respective binding was investigated by continuous-flow dialysis. It must be emphasized, however, that competitive binding of two ligands cannot be considered as an absolute proof that these ligands bind to the same sites.

Table I shows that 2 mM UTP has a significant influence on the binding of ATP to both its high- and low-affinity sites, although it would seem that competition is stronger for the second class of sites. Conversely, saturating ATP increases the apparent dissociation constant of UTP by about 100% (Figure 2).

(*4*) *Binding of UTP and CTP to the Isolated Regulatory Subunits of ATCase.* So far, no UTP binding studies have been carried out on the isolated regulatory subunits. In view of the fact that there was no available information regarding the location of the UTP binding sites, and in order to better understand the nature of the reciprocal influences of CTP and UTP, it was essential to investigate the putative binding of UTP to the isolated regulatory dimers, a more simple structure than the entire ATCase molecule (three regulatory dimers and two catalytic trimers).

Therefore, the binding of UTP and CTP was studied under the same conditions as for the entire enzyme. The results obtained are very similar to those reported above for the native enzyme. UTP binds indeed to the isolated regulatory subunits (Figure 6A). The linear Scatchard plot extrapolates to one binding site per regulatory dimer. As in the case of the entire enzyme, the apparent dissociation constant for this nucleotide is decreased by about a factor of 2 in the presence of 1 mM CTP (Table IIA).

CTP binding to the isolated regulatory dimers shows a heterogeneous pattern, which can be ascribed to two independent sites whose dissociation constants differ by a factor of 40 (Table IIA). In addition, as in the case of the native enzyme, 1 mM UTP increases the binding to the high-affinity site but does not seem to alter significantly binding to the low-affinity site.

It is important to stress that the results fit much better with the negative cooperativity hypothesis (Figure 6B): the sum of the standard squared deviations determined by the program EZ-fit is more than twice lower if this option is chosen instead of that of two independent sites. Interestingly, the presence of UTP does not appear to modify the calculated Hill coefficient (n_H) of the regulatory dimers, whereas the half-

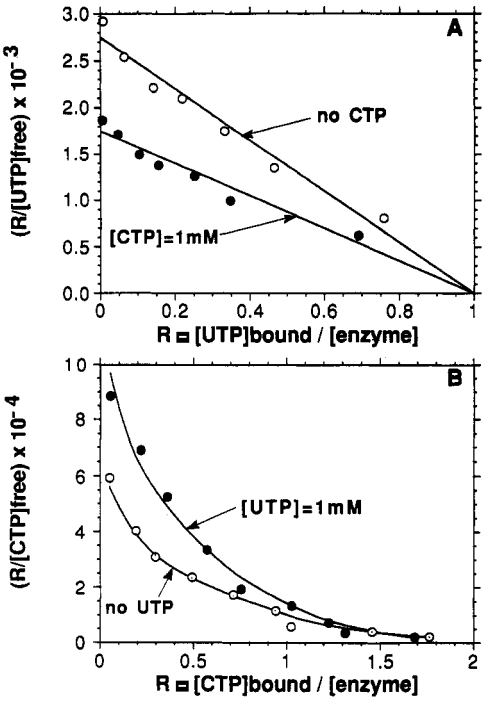

FIGURE 6: Binding of UTP and CTP to the isolated regulatory subunits at 4 °C. (A) Scatchard plot of UTP binding in the presence (●) or absence (○) of 1 mM CTP. (B) Scatchard plot of CTP anticooperative binding in the presence (●) or absence (○) of 1 mM UTP.

Table II

(A) UTP and CTP Apparent Dissociation Constants for the Isolated Regulatory Subunits at 4 °C[a]

		K_d 1 (μM)	K_d 2 (μM)
UTP binding	alone	350 ± 75	nd[b]
	1 mM CTP	620 ± 150	nd
CTP binding	alone	22 ± 3	420 ± 120
	1 mM UTP	10 ± 1.5	350 ± 150

(B) Anticooperative Binding of CTP to the Isolated Regulatory Subunits[c]

		$L_{0.5}$[d] (μM)	n_H[e]
CTP binding	alone	90 ± 12	0.76 ● 0.05
	1 mM UTP	39 ± 6	0.78 ± 0.09

[a] Values were obtained as indicated in Table I. [b] nd, not detectable. [c] Values were obtained by fitting the Scatchard curves with the program described in the Experimental Procedures section, choosing the Hill option. [d] $L_{0.5}$ = concentration required for half-maximal binding. [e] n_H = calculated Hill coefficient.

saturating concentration of CTP decreases by more than a factor of 2 (Table IIB).

(5) *Effect of UTP on the Activity of ATCase.* As was initially observed by Wild et al. (1989), at 37 °C, the combination of CTP and UTP inhibits ATCase activity more than CTP alone (Figure 7); this phenomenon was described as being a synergistic effect of the two pyrimidine nucleotides, as it cannot be mimicked by high concentrations of CTP. Since most of the binding experiments reported above were performed at 4 °C, it was important to determine whether this synergistic behavior is also observed at that temperature. Figure 7 shows that such is the case, although CTP inhibition is slightly attenuated at low temperature. Furthermore, as expected, the nucleotide concentrations required to obtain the maximal effects are lower at 4 °C than at 37 °C.

Under the conditions used, UTP alone appears to increase significantly the ATCase activity (maximal activation of about 30% at 37 °C and 10% at 4 °C). In order to better understand

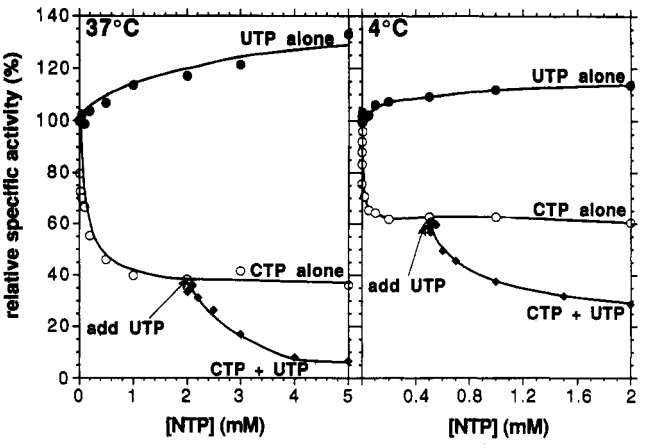

FIGURE 7: Influence of CTP and UTP on the activity of native ATCase at 4 and 37 °C. Assays were performed as described in Experimental Procedures, in the presence of 5 mM aspartate and varying total nucleotide concentrations ([NTP]): CTP alone (○), UTP alone (●), and constant CTP plus varying UTP (◆).

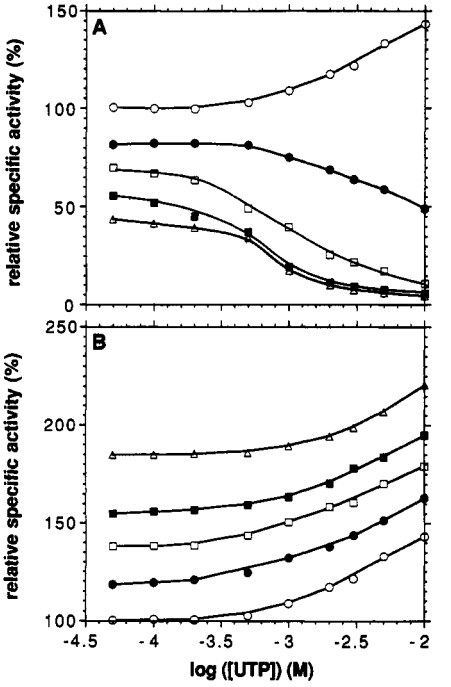

FIGURE 8: Influence of CTP and ATP on the effect of UTP. (A) ATCase activity at 37 °C as a function of UTP concentration, in the presence of no CTP (○), [CTP] = 0.02 mM (●), 0.1 mM (□), 0.5 mM (■), and 2 mM (△). (B) ATCase activity as a function of UTP concentration in the presence of no ATP (○), [ATP] = 0.05 mM (●), 0.2 mM (□), 1 mM (■), and 5 mM (△).

the effect of UTP, it was analyzed as a function of the CTP concentration, at 37 °C. Figure 8A shows that, as CTP concentration increases, the synergistic inhibition progressively appears, concealing the activation due to UTP alone. On the contrary, it is shown in Figure 8B that, whatever the concentration of ATP, the effect of UTP remains qualitatively the same.

(6) *Effect of UTP on the Aspartate Saturation Curve.* In order to investigate which kinetic parameters of ATCase are affected by UTP, aspartate saturation curves were determined in the presence of CTP alone, UTP alone, and CTP + UTP (Figure 9). The calculated parameters (Table III) show that whereas CTP increases the $S_{0.5}$ for aspartate and the calculated Hill coefficient (n_H), without altering V_{max}, UTP has an effect only on V_{max}. The combination of CTP and UTP alters all three parameters. It is interesting to note that V_{max} is the

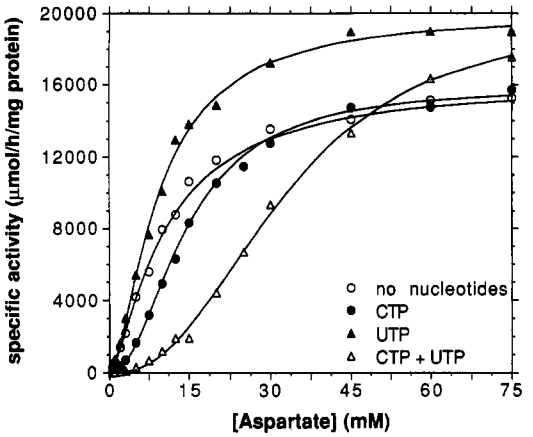

FIGURE 9: Aspartate saturation curves in the presence of nucleotides. The ATCase activity was assayed as described in Experimental Procedures, at 37 °C, in the absence of nucleotides (○) and in the presence of alternatively 1 mM CTP (●), 5 mM UTP (▲), and 1 mM CTP plus 5 mM UTP (△).

Table III: Kinetic Parameters of ATCase in the Presence and Absence of Nucleotide Effectors[a]

nucleotide	V_{max}[b] [μmol h^{-1} (mg of protein)$^{-1}$]	$S_{0.5}$[c] (mM)	n_H[d]
none	16 600 ± 600	10 ± 0.8	1.4 ± 0.1
CTP	16 800 ± 600	15 ± 0.8	2.1 ± 0.2
UTP	20 100 ± 500	10 ± 0.8	1.4 ± 0.1
CTP + UTP	20 000 ± 1200	33 ± 2.5	2.5 ± 0.25

[a] The ATCase activity was assayed as described in Experimental Procedures, in the absence of nucleotides and in the presence of alternatively 1 mM CTP, 5 mM UTP, and 1 mM CTP plus 5 mM UTP. [b] V_{max} = maximal velocity. [c] $S_{0.5}$ = concentration of aspartate required to produce half-maximal velocity. [d] n_H = calculated Hill coefficient.

same as in the case of UTP alone (about 25% higher than in the case of the free or CTP-liganded enzyme). On the contrary, the increase of both $S_{0.5}$ and n_H is much larger in the presence of CTP + UTP than when CTP is alone.

DISCUSSION

The results reported here show that UTP binds significantly to ATCase in the absence of CTP and that CTP binds to two apparent classes of sites whether or not UTP is present. These observations are in agreement with the results of Wild et al. (1989). However, they are not entirely consistent with those of Zhang and Kantrowitz (1991): on one hand, according to these authors, it is only in the presence of CTP that significant UTP binding could be observed, and on the other hand, in the presence of saturating UTP, CTP virtually bound to only one class of sites.

The experimental conditions used for binding studies in these two approaches differ by two parameters: the temperature (4 °C in the first report, 25 °C in the second) and the nature of the buffer (stoichiometric mixture of Bis-Tris, Tris, and CAPS in the first case, HEPES in the second). The experiments described here were performed at both temperatures, and no difference in the mutual influences of the two nucleotides could be revealed. This observation is in agreement with the results of Wedler and Gasser (1974), which indicate that although a discontinuity is observed in the Arrhenius plot of ATCase at about 15 °C, CTP does not affect the enzyme activity in a markedly different manner at 5 and 35 °C. Therefore, temperature does not appear to be a valid explanation of the above-mentioned discrepancies. The only remaining difference in the experimental conditions used is the nature of the buffer. It was observed in the course of the present study that the binding of UTP to the isolated regulatory subunits is 3 times weaker in the buffer used by Zhang and Kantrowitz (1991) than in the imidazole buffer used herein (data not shown). Therefore, HEPES might interfere with the binding of the pyrimidine nucleotides, although such a hypothesis requires further investigation.

ATCase shows a heterogeneous pattern for CTP binding which can be analyzed in terms of two classes of three sites each, whose dissociation constants differ by a factor of 20. On the other hand, UTP binding appears to be limited to three sites. In order to interpret correctly these observations, it was of great importance to investigate the binding of UTP and CTP to the isolated regulatory dimers. Interestingly, the pattern observed is the same as in the case of the entire enzyme: while CTP binds to both allosteric sites of the regulatory subunit with affinities whose ratio is approximately 20, UTP appears to bind to only one site out of two. The Scatchard plots obtained in this simple situation, in which there are two potential binding sites instead of six in the entire enzyme, can be more readily interpreted. In particular, the pattern of CTP binding is shown to fit better with the negative cooperativity model than with that of two preexisting dissimilar sites. Negative cooperativity can also be suspected for UTP; although the Scatchard plots could be linearly fitted to one site per dimer in a satisfactory way, the slight incurvation of these curves (Figure 6A) suggests that UTP might also bind to a second site but with an extremely weak affinity.

The exact location of the UTP binding sites would require complementary experiments, such as affinity labeling (currently under way) and crystallography. However, as UTP and CTP have very similar structures, UTP most likely binds to the same allosteric sites as CTP and ATP. The fact that competitive binding can be observed between UTP and ATP makes this hypothesis even more probable.

However, it is particularly noteworthy that the influence of CTP or ATP on the apparent K_d of UTP (and vice versa) is not as important as what would be expected in the case of a simple direct competition, such as that observed between ATP and CTP (Changeux et al., 1968). For instance, if simple competition is assumed between CTP and UTP, the apparent K_d for UTP in the presence of 1 mM CTP should be more than 5×10^{-2} M, according to

$$K_{d(UTP/CTP)} = K_{d(UTP)}\left(1 + \frac{[CTP]}{K_{d(CTP)}}\right) \qquad (1)$$

in which $K_{d(UTP)}$ is the dissociation constant for UTP alone, $K_{d(CTP)}$ is that for CTP, and $K_{d(UTP/CTP)}$ is the apparent dissociation constant for UTP in the presence of CTP. Instead, the observed value is 1.46×10^{-3} M (Table I), i.e., more than 1 order of magnitude lower than the theoretical value. This result is most simply explained by assuming that the competition phenomenon is compensated by positive site–site interactions: the binding of CTP to one site of a regulatory dimer enhances the affinity for UTP of the second site, although in this case this enhancement is concealed by competition. The same remark applies to the mutual influence of UTP and ATP on their respective binding.

The influence of UTP on the binding of CTP is more striking. In the presence of 2 mM UTP, the apparent K_d values for the high-affinity and the low-affinity sites of CTP ($K_{d(CTP/UTP)}$, calculated in the same way as in eq 1 should be respectively 30 μM and 800 μM if simple direct competition is assumed; in reality, the observed values are 4.8 μM and 220 μM (Table I), i.e., values that are even lower than those measured in the absence of UTP (9 μM and 260 μM). Thus, in this case, the

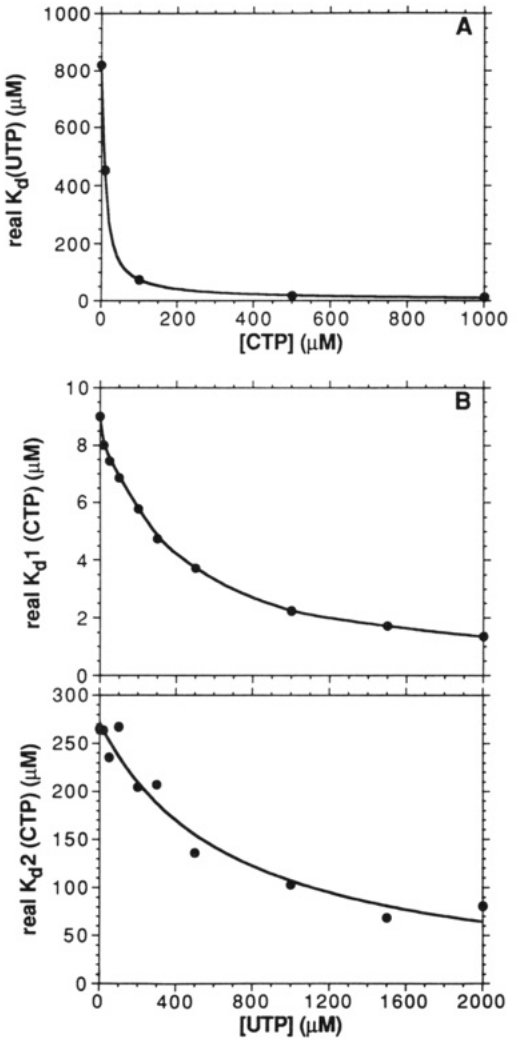

FIGURE 10: Variation of the real dissociation constants of UTP and CTP for ATCase. (A) Influence of CTP on the real dissociation constant for UTP. The real values were calculated from the apparent values using eq 2, as described in the text. (B) Influence of UTP on the real dissociation constants of CTP for its high- and low-affinity sites.

positive site–site interactions are important enough to overcome the competition phenomenon, and therefore an enhancement of the CTP affinity for the enzyme by UTP is actually observed.

It is possible to calculate the extent of these affinity enhancements by correcting the apparent dissociation constants measured by continuous-flow dialysis, taking into account the competition phenomenon. For instance, the real dissociation constant for UTP in the presence of CTP can be obtained from the apparent dissociation constant (shown in Figure 3), using

$$\text{real } K_{d(\text{UTP/CTP})} = \frac{\text{apparent } K_{d(\text{UTP/CTP})}}{1 + ([\text{CTP}]/K_{d(\text{CTP})})} \qquad (2)$$

A similar equation was used to calculate the real K_d of CTP in the presence of UTP. The results of these calculations (Figure 10) show that the real dissociation constant for UTP is very much improved in the presence of CTP (from 800 μM in the absence of CTP to approximately 10 μM in the presence of saturating CTP). The same phenomenon occurs with the real dissociation constants of CTP for both its high- and low-affinity sites (respectively from 9 μM and 260 μM in the absence of UTP to 1.7 μM and 70 μM in the presence of 2 mM UTP).

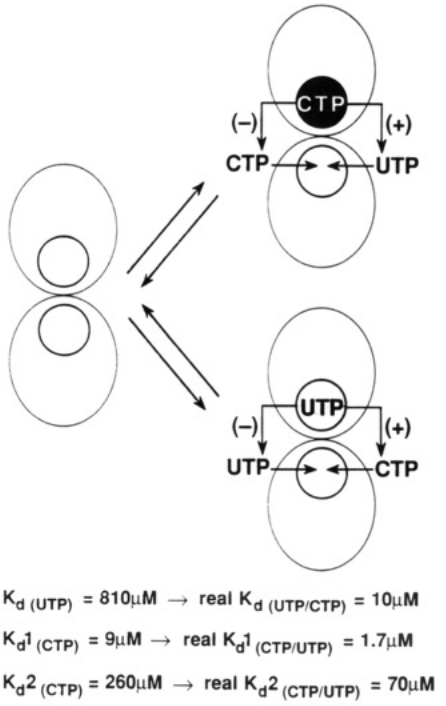

FIGURE 11: Model of site–site interactions within the regulatory dimer. The regulatory dimer and the two allosteric sites are schematically represented. A description of the model is provided in the text. (–) indicates a decrease in affinity (negative cooperativity), while (+) corresponds to an affinity enhancement (positive site–site interaction). The calculations described in the Discussion section show that CTP enhances the affinity of UTP 80-fold and that UTP enhances the affinity of CTP 5-fold.

In order to better understand the nature of these positive interactions, the same binding experiments were performed on the isolated regulatory subunits. The mutual influence of UTP and CTP on their respective apparent dissociation constants remains the same as for the native enzyme, clearly indicating that the above-mentioned reciprocal affinity enhancements rely entirely on site–site interactions within the regulatory dimer and do not involve communication between dimers.

Taken together, the results lead to the following model (Figure 11): the binding of CTP to one regulatory site decreases the affinity of this nucleotide for the complementary site of the dimer (negative cooperativity) and increases the affinity of UTP for this second site (positive site–site interaction). Conversely, the binding of UTP to one site decreases its own binding and increases that of CTP to the second site. This model can be extended to UTP–ATP mutual influences but not to CTP–ATP ones, which are purely competitive (Changeux et al., 1968).

In order to understand how UTP participates in the regulation of ATCase activity, the above-described binding pattern must be correlated with the enzyme kinetics. The main conclusion is that it is only in the presence of CTP that the binding of UTP induces an increased inhibition of the enzyme activity. Two possible mechanisms can be proposed to explain this phenomenon. On one hand, CTP binding could induce a rearrangement of the regulatory site that would enable UTP to act as an inhibitor. On the other hand, CTP could simply become more efficient in the presence of UTP, without UTP becoming directly an inhibitor. It must be kept in mind that the synergistic effect of CTP and UTP cannot be mimicked by saturating concentrations of CTP: therefore, the increased efficiency of CTP in this second hypothesis cannot be explained solely by an increased affinity of this nucleotide. In either

case, the cooperative behavior of CTP and UTP can be formally designated as synergistic, on the basis of the terminology proposed by Segel (1975).

Surprisingly, it is shown here that, under the experimental conditions used, UTP alone slightly activates the ATCase activity by increasing the V_{max} of the enzyme without affecting n_H or $S_{0.5}$ for aspartate. It must be noted that such an increase was not observed in previous studies (Wild et al., 1989; Zhang & Kantrowitz, 1991). It seems unlikely that this phenomenon is due to impurities present in the commercial preparations of UTP that were used: indeed, their purity was checked by the method described by Beck and Howlett (1977), and they all yielded the same result. It is noteworthy to point out that the activation was almost nonexistent when the kinetic experiments were performed in Tris-HCl buffer, at pH = 8 (data not shown): pH and the nature of the buffer might therefore be an explanation of this phenomenon. The UTP activation cannot be considered to be analogous to the ATP activation (which only affects n_H and $S_{0.5}$ without altering V_{max}). Furthermore, the UTP-induced positive effect on the V_{max} is not suppressed when saturating concentrations of CTP are added, indicating that UTP alone acts through a mechanism distinct from that of the other two allosteric effectors.

More detailed studies are necessary to understand precisely the nature of the above-described positive site–site interactions and of the rearrangements of the allosteric sites on which they rely. Some information has already been made available by the study of mutants of the enzyme in which the synergistic CTP–UTP inhibition is lost. Three such examples of substitutions of residues in the regulatory subunits have been reported in the literature: Lys56 → Ala (Corder & Wild, 1989), Asp19 → Ala (Zhang & Kantrowitz, 1991), and His20 → Ala (Zhang & Kantrowitz, 1992). In the case of the two latter modifications, it was shown that UTP binding still occurs but that the UTP-induced enhancement of the CTP affinity is reduced. Thus, it appears clearly that, in such mutants, positive site–site interactions within the regulatory dimer have been altered. Interestingly, the three modified residues all have in common the fact that they belong to the allosteric site and that they have been shown, by crystallographic studies, to interact with the γ-phosphate of CTP. Therefore, interactions with this terminal phosphate appear to be crucial for the synergistic inhibition and, hence, for the communication between the allosteric sites. These matters are currently being investigated.

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