Substitutions in hamster CAD carbamoyl-phosphate synthetase alter allosteric response to 5-phosphoribosyl-α-pyrophosphate (PRPP) and UTP

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CPSase (carbamoyl-phosphate synthetase II), a component of CAD protein (multienzymic protein with CPSase, aspartate transcarbamylase and dihydro-orotase activities), catalyses the regulated steps in the *de novo* synthesis of pyrimidines. Unlike the orthologous *Escherichia coli* enzyme that is regulated by UMP, inosine monophosphate and ornithine, the mammalian CPSase is allosterically inhibited by UTP, and activated by PRPP (5-phosphoribosyl-α-pyrophosphate) and phosphorylation. Four residues (Thr^{974}, Lys^{993}, Lys^{954} and Thr^{977}) are critical to the *E. coli* inosine monophosphate/UMP-binding pocket. In the present study, three of the corresponding residues in the hamster CPSase were altered to determine if they affect either PRPP activation or UTP inhibition. Substitution of the hamster residue, positionally equivalent to Thr^{974} in the *E. coli* enzyme, with alanine residue led to an enzyme with 5-fold lower activity and a near loss of PRPP activation. Whereas replacement of the tryptophan residue at position 993 had no effect, an $Asp^{992} \rightarrow Asn$ substitution yielded a much-activated enzyme that behaved as if PRPP was present. The substitution $Lys^{954} \rightarrow Glu$ had no effect on PRPP stimulation. Only modest decreases in UTP inhibitions were observed with each of the altered CPSases. The results also show that while PRPP and UTP can act simultaneously, PRPP activation is dominant. Apparently, UTP and PRPP have distinctly different associations within the mammalian enzyme. The findings of the present study may prove relevant to the neuropathology of Lesch–Nyhan syndrome.

Key words: allosteric, carbamoyl-phosphate synthetase II (CPSase), Lesch–Nyhan syndrome, pyrimidine.

INTRODUCTION

In animals, CPSase (carbamoyl-phosphate synthetase II; EC 6.3.5.5) is the first and rate-limiting step in the *de novo* synthesis of pyrimidines. This enzyme and the enzymes catalysing the subsequent two steps in the pathway (aspartate transcarbamylase and dihyro-orotase) are covalently linked as domains of the multienzymic protein, CAD [1–3]. CPSase comprises several domains including catalytic domains involved in the hydrolysis of glutamine, syntheses of carboxyphosphate and carbamoyl phosphate [4–6]. At the end of the CAD CPSase is a regulatory domain comprising approx. 137 amino acids [5] (Figure 1). The enzyme is inhibited by UTP, the end-product of the pathway, and is activated by PRPP (5-phosphoribosyl-α-pyrophosphate), a substrate for the synthesis of both purines and pyrimidines [7].

The orthologous *Escherichia coli* enzyme responds allosterically to inhibition by UMP and to activation by IMP (inosine monophosphate) and ornithine [8,9]. UTP and PRPP appear to have no effect on the bacterial enzyme [4]. The regulatory domain of the bacterial CPSase, similar to the animal CAD CPSase, is at the C-terminal end of the enzyme [5,10]. When the bacterial regulatory domain is replaced by the hamster regulatory domain, the chimaeric enzyme responds to UTP inhibition and PRPP activation [4]. UMP, IMP and ornithine no longer affect this enzyme. These results indicate that the hamster regulatory domain contains all the necessary elements for binding its allosteric effectors and for transmitting a signal for inhibition or activation. Additional studies [11,12] have found that activation and inhibition are mediated through changes in apparent affinity for MgATP at the adjacent domain where carbamoyl phosphate is synthesized.

The hamster CPSase shows an overall 40 % amino acid sequence identity to the *E. coli* enzyme [4]. The sequences of the regulatory domains of the hamster and bacterial enzymes show only 24 % sequence identity (Figure 1), suggesting a significant divergence in structure and function. Presumably, the lower sequence identity reflects a difference in the structure of the effector-binding sites of the two enzymes.

Recently, the *E. coli* CPSase has been crystallized with inorganic phosphate [5] or IMP [13], and the protein structures were resolved. When inorganic phosphate is included in the crystal, it appears bound to the regulatory domain through four amino acids: Lys^{954}, Thr^{974}, Thr^{977} and Lys^{993} [5]. IMP binds these same four residues [13]. Other studies [14–17] on the *E. coli* enzyme have demonstrated that UMP and IMP appear to compete for the same or nearly the same binding site in the regulatory domain, whereas ornithine binds to a completely separate site. Site-directed mutagenesis studies [18–20] further showed that alteration to alanine residue of any of the four residues that co-ordinate to the inorganic phosphate seen in the crystal structure abolish UMP and IMP binding but have little or no effect on ornithine activation. It has been deduced that Lys^{954}, Thr^{974}, Thr^{977} and Lys^{993} probably play important roles in co-ordinating with the 5′-phosphoribosyl moiety of UMP and IMP. Since UMP and IMP share an identical 5′-phosphoribosyl structure, it makes sense that both effectors would fit to the same binding pocket and show competitive binding kinetics. Additional residues around Lys^{993} may also affect UMP/IMP binding or signalling in response to binding these allosteric effectors [21].

The regulatory domain, in the absence of allosteric effectors, may also have a role in bacterial CPSase activity. Changing Val^{994} to an Ala results in an inactive CPSase [19]. Furthermore,

Abbreviations used: CPSase, carbamoyl-phosphate synthetase II; CAD, multienzymic protein with CPSase, aspartate transcarbamylase and dihydro-orotase activities; IMP, inosine monophosphate; PRPP, 5-phosphoribosyl-α-pyrophosphate.

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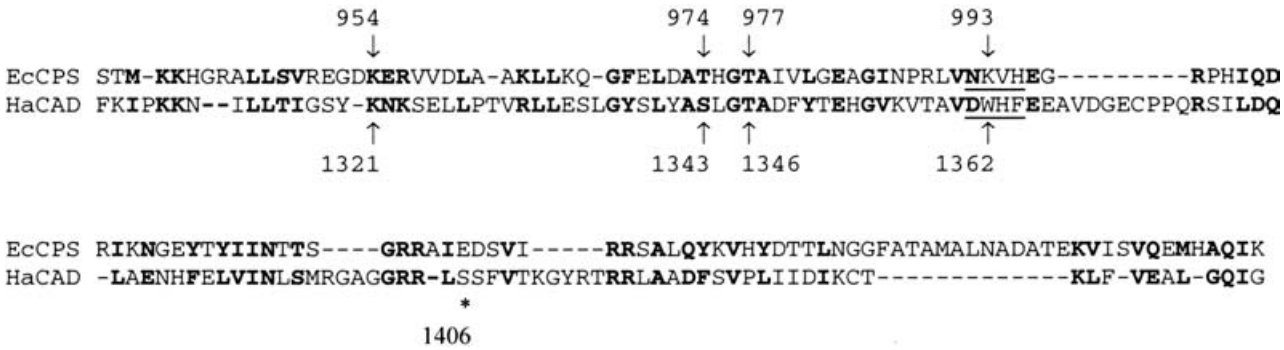

Figure 1 Amino acid sequence comparison between the allosteric domains of the *E. coli* and hamster CPSases

Positions of the residues of the bacterial and hamster enzymes are shown above and below respectively. Arrows indicate the four key positions that bind phosphate and IMP in the bacterial CPSase. The asterisk (∗) indicates serine residue phosphorylated in mammalian protein. Boldface letters represent conserved residues.

truncation of as few as 14 amino acids from the C-terminus leads to significantly reduced catalytic activity [12].

Much less is understood about the regulatory domain of the CAD CPSase. C-terminal truncations of the CAD CPSase regulatory domain exhibit activity that is essentially insensitive to UTP and PRPP [22,23]. Phosphorylation of the hamster residue Ser^{1406} (Figure 1) by cAMP-dependent protein kinase A leads to a more active enzyme [11]. A similar result is observed when Ser^{1406} is replaced by a glutamic residue [24], an alteration that should mimic a phosphoserine. In both cases, UTP inhibition is reduced, but PRPP activation remains unaffected. Such a result suggests that PRPP and UTP do not share an identical binding site within the regulatory domain [24]. Moreover, neither ends of the PRPP and UTP molecules share a similar structure. UTP does share the same pyrimidine base as UMP, and PRPP shares the 5′-phosphoribosyl structure found in both UMP and IMP [17]. Other changes outside the regulatory domain of CAD affect the response of the CPSase to allosteric effectors. These include phosphorylation of mammalian CAD by mitogen-activated protein kinase [25], and autophosphorylation [26] and mutation in *Drosophila* called *Suppressor of black* [6]. The mechanisms by which these other changes exert their effects are unknown.

The focus of the experiments in the present study was to examine, by site-directed mutagenesis of the hamster CAD CPSase, three of the sites shown to be important to UMP/IMP binding in the *E. coli* enzyme. The three sites by the *E. coli* nomenclature are Lys^{954} (identical residue in both species), Ser^{974} (threonine in the bacterial sequence) and Trp^{993} (lysine in the bacterial sequence). In addition, the residues around 993 of the hamster CPSase were replaced by those found in the *E. coli* CPSase ($Asp^{992}Trp^{993}His^{994}Phe^{995} \rightarrow Asn^{992}Lys^{993}Val^{994}His^{995}$) as these were also shown to be important to CPSase activity and to UMP or IMP binding in *E. coli* [19]. The actual residue positions in hamster CAD CPSase are Lys^{1321}, Ser^{1343}, Trp^{1362} and positions 1361–1364 ($Asp^{1361}Trp^{1362}His^{1363}Phe^{1364}$) (Figure 1), but the *E. coli* nomenclature will be used to avoid confusion. Results of the present study provide important information about how PRPP and UTP interact with the regulatory domain. The results demonstrate significant differences in the CPSase effector binding sites of hamster and *E. coli*, but indicate that PRPP interacts with a binding pocket somewhat similar to that used by UMP and IMP in the bacterial enzyme. Finally, the dominance of PRPP activation over UTP inhibition may explain nucleotide imbalances observed in a transgenic mouse model of Lesch–Nyhan disease [27].

MATERIALS AND METHODS

Construction

Site-directed mutagenesis was performed with either the Quik Change kit (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's instruction or by PCR overlap extension [28]. The template DNA, pHE-A12^{e}B3^{m}, was kindly provided by Dr D. R. Evans (Wayne State University School of Medicine, Detroit, MI, U.S.A.) [23]. The forward primer 5′-GTCAAGGTGACAGCTGTGAACAAGGTCCATGAAGAGGCTGTGG-3′ (mutation underlined) and reverse primer 5′-CCACAGCCTCTTCATGGACCTTGTTCACAGCTGTCACCTTGAC-3′ were used to mutate $Asp^{992}Trp^{993}His^{994}Phe^{995} \rightarrow Asn^{992}Lys^{993}Val^{994}His^{995}$. The oligonucleotides for creating $Ser^{974} \rightarrow Ala$ were 5′-GCCTCTACGCCGCCCTGGGTAC-3′ (forward) and 5′-GTACCCAGGGCGGCGTAGAGGC-3′ (reverse). To generate $Lys^{954} \rightarrow Glu$, the mutagenic primers were 5′-CCATCGGCAGCTACGAGAACAAAAGTGAGCTG-3′ and a primer corresponding to complementary strand. The oligonucleotide, 5′-GGTGACAGCTGTGAACTGGCACTTTGAAGAGGCTG-3′ and its complementary strand were used to make $Asp^{992} \rightarrow Asn$. PCR amplifications were performed with *Pfu Turbo* DNA polymerase (Stratagene). The entire B3 domain was then sequenced to verify the mutation and confirmed no other unwanted mutations. The mutagenic DNA was then introduced into the final construct, pCIN-His-CAD [29], by two steps of sequential subcloning with *Bpu*1102I–*Sal*I and *Not*I–*Bam*HI. Plasmid DNA was purified by using Qiagen columns. Restriction digestion, ligation, DNA fragment purification and transformation were performed according to standard procedures [30]. All mutations and surrounding sequences were again confirmed by DNA sequencing.

Cells and transfection

A derived Chinese-hamster ovary-K1 cell line, G9C, which is deficient in the endogenous CAD gene [22], was used for transfections. G9C cells were maintained in Ham's F-12 medium containing 3 μM uridine and 10% (v/v) foetal bovine serum. The wild-type and mutated constructs were stably transfected by calcium phosphate method [30]. All transfectants were selected and maintained on Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, U.S.A.) with 10% foetal bovine serum, 1 mg/ml G418 (Geneticin; Gibco BRL), 10 μM thymidine, 0.5 mM proline and 3 μM uridine.

Protein purification

Cells were collected from 10 mm × 100 mm near-confluent plates and lysed by sonication in a buffer [50 mM Hepes (pH 7.9 at 4 °C)/150 mM NaCl/5 % (w/v) glycerol/10 mM imidazole/10 mM 2-mercaptoethanol]. The lysate was then clarified and loaded on to a 0.3 ml Ni^{2+}-nitrilotriacetate column (Qiagen, Valencia, CA, U.S.A.). The column was washed with 50 mM Hepes (pH 7.9 at 4 °C)/150 mM NaCl/5 % glycerol/10 mM 2-mercaptoethanol, with increasing concentrations of imidazole: 10, 20 and 40 mM. The His_6-tagged CAD protein was then eluted with 150 mM imidazole in the same buffer as above. After the purification, the enzyme was immediately brought to a final concentration containing 30 % (v/v) DMSO, 5 % glycerol, and stored at − 70 °C. Through this method, wild-type and substituted proteins were purified to near homogeneity at a yield of approx. 200 μg. Protein concentrations were determined by the method of Bradford [31], with the Bio-Rad protein assay dye.

Enzyme assays

CPSase activity was measured essentially as described by Irvine et al. [32] and by Simmons et al. [6], in which the unstable product, carbamoyl phosphate, was converted into urea. Ammonia was used as the nitrogen donor for the reactions. The basic assay mixture contained 100 mM Hepes (pH 7.4 at 37 °C)/7.5 % DMSO/2.5 % glycerol/30 mM KCl/20 mM NH_4Cl/10 mM bicarbonate (including 2.5 μCi [^{14}C]bicarbonate)/0–8 mM ATP with $MgCl_2$ in excess of ATP at all concentrations by 2 mM. The reactions were initiated by 25–45 μl of enzyme and performed at 37 °C for 30 min. Magnesium-balanced 2 mM UTP and 1 mM PRPP were included in the assays with allosteric effectors.

To investigate further the UTP and PRPP regulation, the basic CPSase assays containing different concentrations of UTP · Mg^{2+} (from 0 to 4 mM) or PRPP · Mg^{2+} (from 0 to 1 mM) were performed. For the reactions in which both UTP and PRPP simultaneously appeared, UTP concentrations varied from 0 to 2 mM, whereas PRPP was held constant at 1 mM. Alternatively, PRPP concentrations were also varied between 0 and 1 mM, whereas UTP was held constant at 2 mM.

RESULTS

All mutations were introduced into the full-length CAD cDNA rather than a cDNA encoding only the CPSase domain. This was to reduce the chance of artifacts such as increased susceptibility to proteolysis. Also, inter-domain and inter-enzymic interactions should remain essentially undisturbed. CPSase assay results do vary from day-to-day even when assays are performed on the same batch of purified CAD protein. To reduce the impact of such variations, CAD from tissue-cultured cells with wild-type and mutant genes were purified in parallel and, when assayed for CPSase activity, were assayed on the same day. In this way, even though a particular altered CPSase varied in activity on different days, its relative activity when normalized to wild-type CPSase did remain constant.

The first site-directed mutation studied biochemically was $Ser^{974} \rightarrow Ala$. CPSase activities of the wild-type and $Ser^{974} \rightarrow Ala$ enzymes were measured first as a function of ATP concentration as shown in Figure 2. In the absence of allosteric effectors, $Ser^{974} \rightarrow Ala$ CPSase showed a reduction in activity of approx. 5-fold over the wild-type enzyme. Under conditions of saturating UTP (2 mM), both enzymes showed similar degrees of inhibition. At saturating levels of 2 mM PRPP, an 88 % reduction in activation was observed with the $Ser^{974} \rightarrow Ala$ enzyme.

To investigate further allosteric responsiveness, ammonia-dependent catalytic activities were measured in the presence of 2 mM ATP but with different concentrations of PRPP and UTP respectively (Figure 3). The results from $Ser^{974} \rightarrow Ala$ show that PRPP activation was almost completely abolished at 0.05 mM PRPP, a concentration where near-maximal activation is observed with normal enzyme. UTP inhibition was slightly decreased at concentrations of up to 2 mM UTP (Figure 3). Since IMP and UMP are competing allosteric effectors in the *E. coli* CPSase, we examined whether activation by PRPP would compete with inhibition by UTP in the mammalian enzyme. Assays containing both 1 mM PRPP and different concentrations of UTP (Table 1), and 2 mM UTP and various concentrations of PRPP (results not shown) were performed. The results from the wild-type demonstrated that PRPP activated the CPSase to the same degree regardless of the UTP concentration. The $Ser^{974} \rightarrow Ala$ enzyme shows significantly less activation in the presence of PRPP and UTP. Moreover, enzyme activity decreased with increasing concentrations of UTP. These results show that PRPP activation is dominant, protecting the wild-type enzyme from UTP inhibition. But, with the substitution, $Ser^{974} \rightarrow Ala$, the severe decrease in PRPP activation revealed that UTP could still inhibit the enzyme. This finding indicates that UTP and PRPP do not compete for or share an identical binding site on the allosteric subdomain. To confirm further this conclusion, the enzymes were preincubated with PRPP or UTP for 10 min on ice, then these enzymes were used in CPSase reactions with the other effector (results not shown). The results revealed that preincubation of PRPP led to full activation in the wild-type, whereas preincubation with UTP could not block PRPP activation. For the $Ser^{974} \rightarrow Ala$ substituted enzyme, the results were similar to those where no preincubation was performed.

The hamster allosteric domain corresponding to the region around the *E. coli* residue Lys^{993} is longer and not well conserved (Figure 1). The sequence $Asp^{992}Trp^{993}His^{994}Phe^{995}$ of hamster was replaced by the *E. coli* sequence, $Asn^{992}Lys^{993}Val^{994}His^{995}$. We examined whether such a significantly altered enzyme would still respond to UTP and PRPP. The ammonia-dependent CPSase activity of the substituted enzyme was assayed as a function of ATP concentration (Figure 4a). Remarkably, the enzyme showed only reduced activation in response to PRPP and only slightly less sensitivity to UTP inhibition. To examine more closely the response to PRPP or UTP, CPSase activity was measured as a function of allosteric effector concentrations (Figure 4b). These results confirmed that the altered enzyme exhibits reduced PRPP activation, but only at lower concentrations of PRPP (< 0.5 mM). At higher concentrations of PRPP, activation of the altered enzyme is similar to that for the enzyme from the wild-type. UTP inhibition was decreased slightly at all UTP concentrations.

When PRPP and UTP are co-incubated with the $Asn^{992}Lys^{993}Val^{994}His^{995}$ substituted enzyme, the activity is increased as if UTP was not present (results not shown) in a similar manner as observed for the enzyme from wild-type (Table 1). The possibility that the $Asn^{992}Lys^{993}Val^{994}His^{995}$ substitution made the hamster enzyme responsive to the *E. coli* enzyme effectors, IMP, UMP or inorganic phosphate was tested. None of these three compounds had any effect on the modified hamster enzyme or on its response to PRPP or UTP.

Three single-residue substitutions were also made in this region. $Trp^{993} \rightarrow Phe$ and $Trp^{993} \rightarrow Lys$ had essentially no effect in the modulation of the hamster enzymes' allosteric regulation (results not shown), a result in stark contrast with the *E. coli* substitution of $Lys^{993} \rightarrow Ala$ [18,19]. However, the substitution, $Asp^{992} \rightarrow Asn$, gave an enzyme that behaves as if it is already activated by PRPP

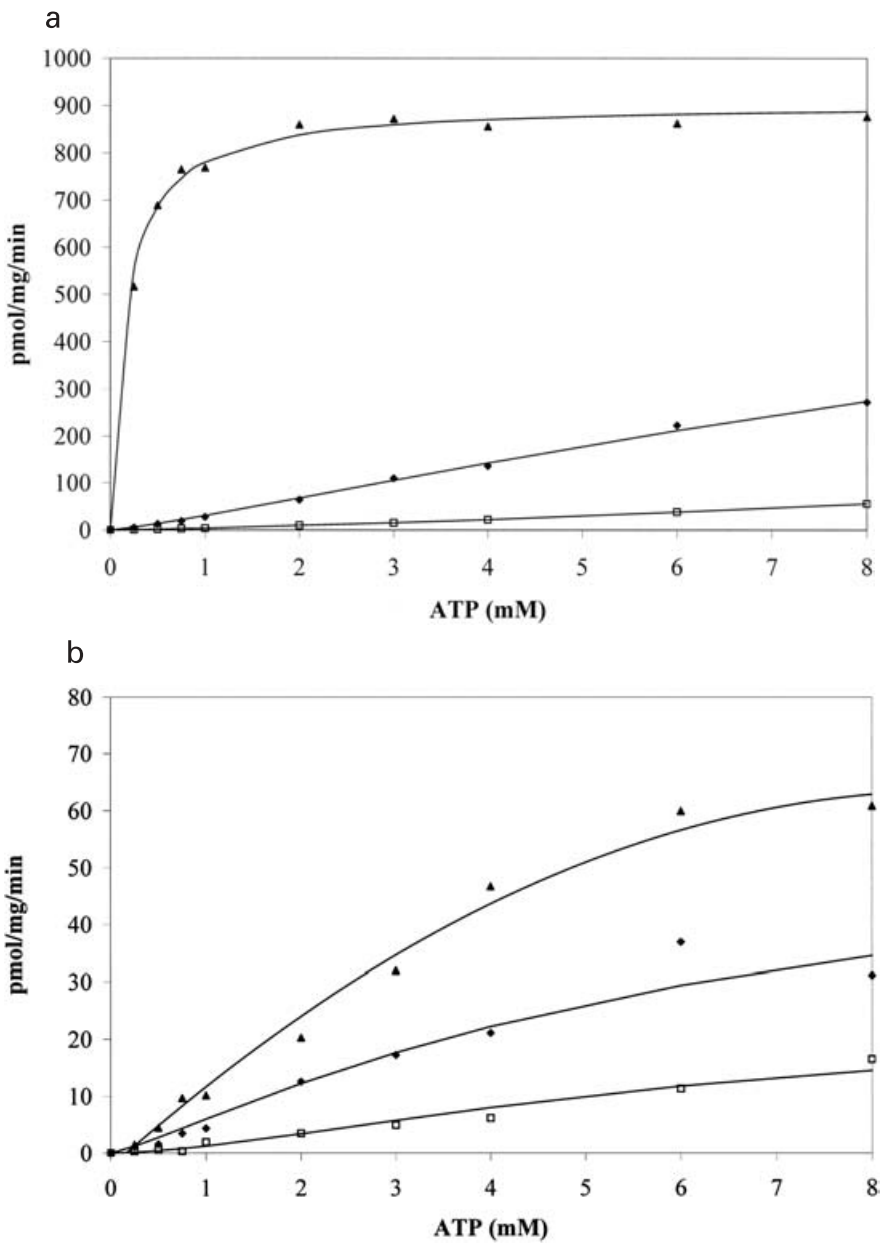

Figure 2 CPSase activities of the wild-type (a) and Ser^{974} → Ala CAD (b) measured as a function of ATP concentration, in the presence or absence of allosteric regulators

The concentration of ATP was as indicated, and Mg^{2+} concentration was in excess of the ATP at all concentrations by 2 mM. The assay was performed with no effectors (◆), with 1 mM PRPP (▲) or 2 mM UTP (□): (a) wild-type CAD, (b) Ser^{974} → Ala CAD.

(Table 2). Addition of PRPP leads to even further activation of the enzyme.

Finally, a charge-reversed substitution, Lys^{954} → Glu, was created and its ATP saturation curve was determined (Figure 5). Enzyme activity in the absence of effectors was slightly lower when compared with the enzyme from wild-type. UTP inhibition was reduced. Overall, this substituted enzyme behaved much like the wild-type enzyme (Table 2).

DISCUSSION

Understanding the allosteric regulation of CPSase has been complicated and challenging for many years. A major advancement in this field was the release of an X-ray crystal structure of *E. coli* CPSase provided by Raushel and co-workers [5]. Not only did this work disclose the fine structure of the catalytic domains and a 96 Å (1 Å = 0.1 nm) interior channel, but it also revealed a binding site for inorganic phosphate located within the allosteric regulatory domain. The side chains of Lys^{954}, Thr^{974}, Thr^{977} and Lys^{993} were shown to interact with this inorganic phosphate. Kinetic studies [14,17] indicate that an inorganic phosphate interferes with the allosteric responsiveness to IMP/UMP, and both IMP and UMP strictly compete with each other for the same binding site. Furthermore, the recent disclosure of the three-dimensional architecture of *E. coli* CPSase with IMP confirmed that the IMP-binding site is identical with the

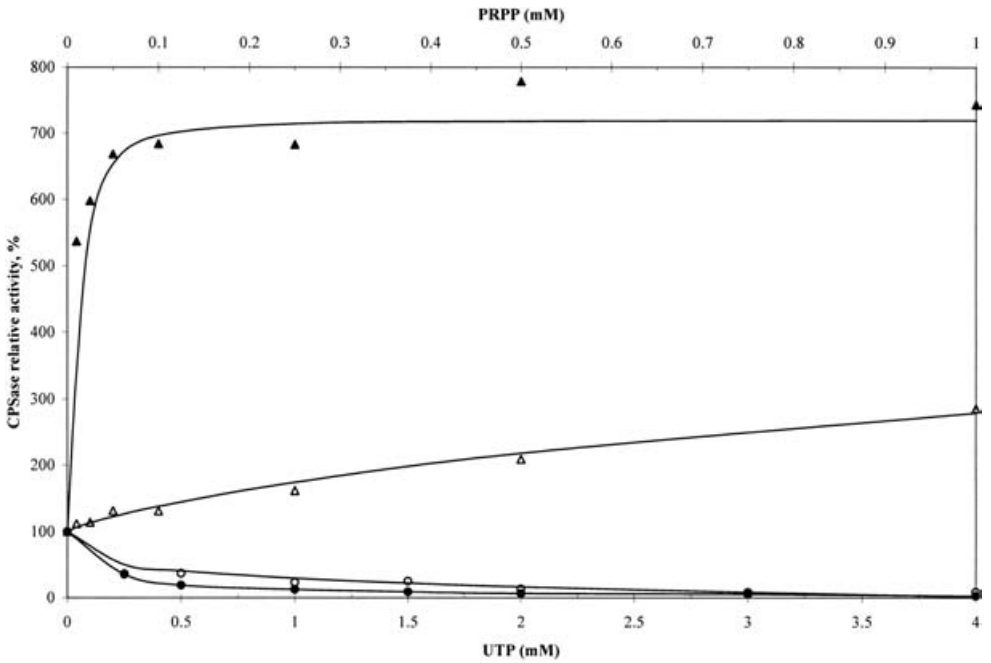

Figure 3 Effects of UTP or PRPP concentration on CPSase activity

CPSase activities from wild-type CAD (▲) and $Ser^{974} \rightarrow Ala$ CAD (△) were measured as a function of PRPP concentration, and wild-type CAD (●) and $Ser^{974} \rightarrow Ala$ (○) were measured as a function of UTP concentration. The ATP concentration was held constant at 2 mM. Magnesium concentration was in excess of the ATP, UTP or PRPP concentrations by 2 mM.

Table 1 Allosteric effector interactions for wild-type and $Ser^{974} \rightarrow Ala$

CPSase activities were measured with 2 mM ATP and various amounts of UTP as indicated, in the absence and presence of 1 mM PRPP. Results without PRPP were standardized to results obtained with no UTP added. Results with PRPP were standardized against results obtained with no UTP and no PRPP.

	Wild-type (%)		$Ser^{974} \rightarrow Ala$ (%)	
UTP (mM)	No PRPP	PRPP	No PRPP	PRPP
0	100	534	100	202
0.5	19	551	36.9	63
1	12	505	23	42.5
2	6.4	426	13	29.7

previously determined phosphate-binding site. These studies with *E. coli* CPSase hinted that the corresponding residues of the mammalian CPSase may interact with the phosphate groups of the eukaryotic allosteric effectors in a similar manner.

The hypothesis was that UTP or PRPP might bind in a pocket formed by the corresponding hamster residues Lys^{954}, Ser^{974}, Thr^{977} and Trp^{993}. Amino acid substitutions in Lys^{954}, Ser^{974} and Trp^{993} had only modest effects on UTP inhibition, ruling out the possibility that these residues are part of a binding pocket for UTP. Further work will be necessary to elucidate the residues in the allosteric domain that do form the UTP-binding pocket. On the other hand, substitutions of Ser^{974} with alanine residue did have an effect on PRPP activation. Ser^{974} in the hamster CPSase corresponds to one of the key residues in the IMP-binding pocket of the *E. coli* enzyme. The *E. coli* Thr^{974} is the closest residue that forms a direct hydrogen bond with the phosphoryl oxygen of IMP.

Encouraged by the importance of Ser^{974}, we speculated a superposition of PRPP binding in the hamster enzyme within the IMP-binding pocket of the *E. coli* enzyme. Unfortunately, the results are split on this proposal. Substitutions at residues Trp^{993} and Lys^{954} had little effect on PRPP activation. Interestingly, the residue next to Trp^{993}, namely Asp^{992}, when replaced by an asparagine residue, showed an almost 4-fold activation in the absence of PRPP. Indirectly, this indicates that this residue may have a role in binding PRPP or in communicating a response to allosteric effectors to the CPSase. Replacing the hamster residues $Asp^{992}Trp^{993}His^{994}Phe^{995}$ by the corresponding *E. coli* residues Asn^{992} $Lys^{993}Val^{994}His^{995}$ did increase the $[S]_{0.5}$ for PRPP by 4-fold (Table 2) and concomitantly decreased PRPP activation of the altered CPSase at PRPP concentrations below 0.4 mM. Although the results are imperfect, they do suggest that PRPP may interact with a similar binding site in the hamster CPSase allosteric domain as does IMP/UMP in the *E. coli* enzyme. Such a finding is consistent with the fact that all three effectors share a phosphoribosyl moiety.

The activity of wild-type hamster CPSase in response to various PRPP concentrations is best described as hyperbolic (Figures 3 and 4b). However, a hyperbolic response does not describe the two altered CPSases with lower PRPP activation (Figures 3 and 4b). How to explain these changes in kinetic properties is still not clear.

A previous study [33] on a 171-residue deletion from the carboxyl end of *E. coli* CPSase showed a complete loss of enzyme activity. Similar results were obtained from our laboratory [22] proving that a truncated CPSase lacking the C-terminal 129 amino acids caused lethality in transfected mammalian cells lacking CPSase activity. Further evidence by Liu et al. [4] showed that a hybrid CPSase containing the *E. coli* catalytic domain and the mammalian allosteric domain appeared to have kinetic properties resembling the mammalian enzyme even in the absence of allosteric ligands. These previous studies suggested that the allosteric domain is required to serve not only in regulating CPSase activity but also in enzyme catalysis. Perhaps,

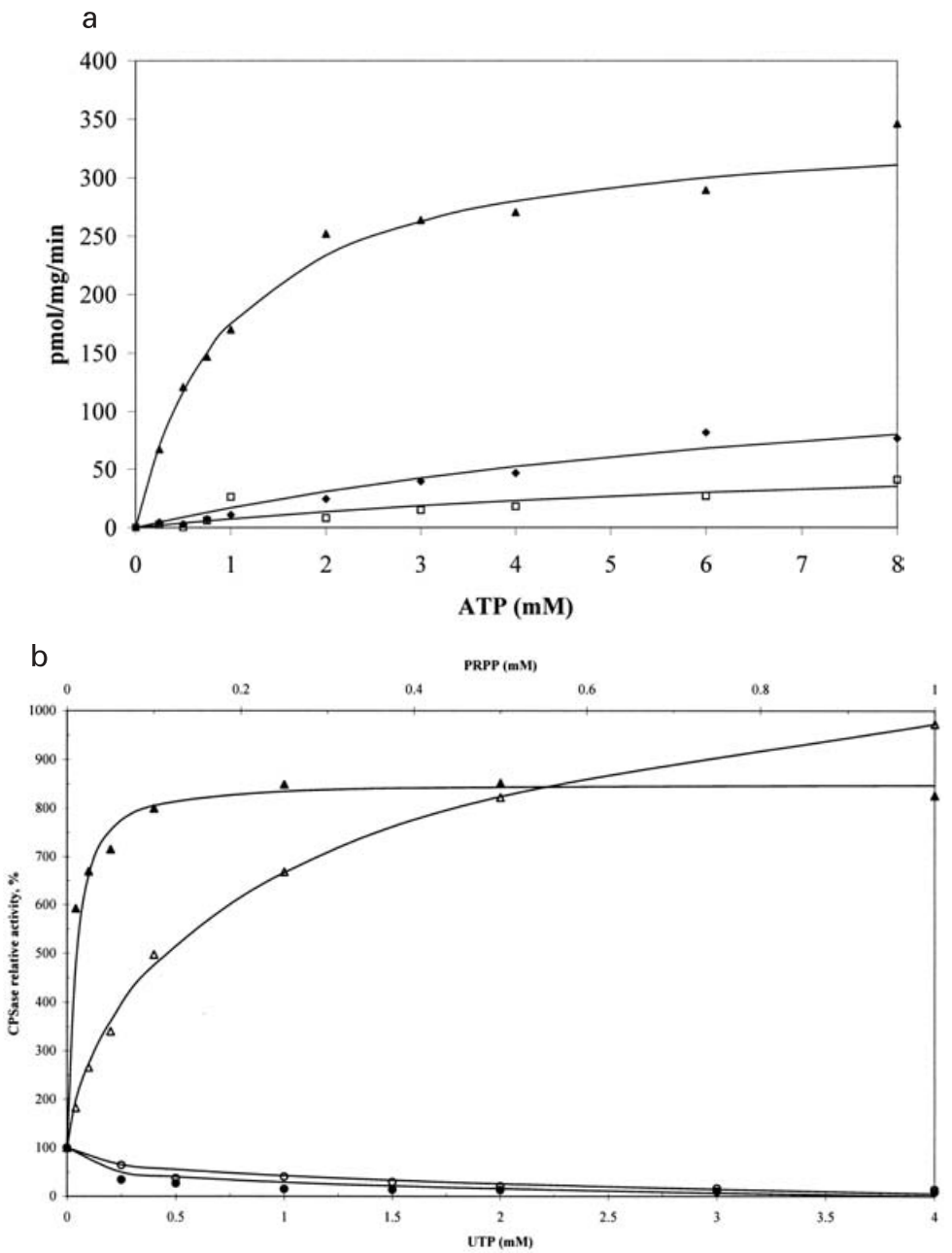

Figure 4 Analysis of CAD substituted at residues 992–995 with those of the *E. coli* CPSase

(**a**) ATP saturation curves of CPSase activity from $Asn^{992}Lys^{993}Val^{994}His^{995}$ in the presence and absence of effectors. The concentration of ATP varied as indicated, and Mg^{2+} concentration was in excess of the ATP at all concentrations by 2 mM. The assay was performed with no effectors (◆), with 1 mM PRPP (▲) or 2 mM UTP (□). (**b**) Effects of UTP or PRPP on CPSase activity. CPSase activity was measured as a function of PRPP for wild-type CAD (▲) and $Asn^{992}Lys^{993}Val^{994}His^{995}$ (△). Effects of UTP are shown as wild-type CAD (●) and $Asn^{992}Lys^{993}Val^{994}His^{995}$ (○). ATP concentration was 2 mM.

a communication between the allosteric domain and the catalytic region, specifically the B2 active site, is required for the catalytic and regulatory activities. Such a proposal may provide an explanation for why the altered enzymes studied here exhibit diverse catalytic activities even in the absence of effectors. This is consistent with the kinetic study of several substitutions and deletions of the *E. coli* CPSase [12]. For $Ser^{974} \rightarrow$ Ala enzyme or the *E. coli* $Thr^{974} \rightarrow$ Ala enzyme [18,20], there is currently no structural data to explain why these enzymes have reduced activity and much less responsiveness to the allosteric effectors, PRPP and UMP respectively.

Results presented here are consistent with the model proposed by Evans and co-workers [4,23] that the allosteric activator and inhibitor trigger different conformational changes within the allosteric domain, which are directed towards the catalytic domain. For the wild-type, the $[S]_{0.5}$ of ATP is decreased by 19-fold when PRPP is present. Hence, once PRPP binds to the enzyme, the activation signal is transmitted to the catalytic domain, leading to an increased affinity for ATP and an increased catalytic activity for the CPSase. The inhibitor, UTP, has an opposite effect on the enzyme. Our results indicate how PRPP and UTP interact. The activator PRPP and the inhibitor UTP can both

Table 2 Kinetic parameters of wild-type and substituted hamster CAD

Kinetic parameters of CPSase activity represent the S.E.M. for three determinations. V_{max} (pmol · mg^{-1} · min^{-1}) is defined as the maximal observed specific activity and $[S]_{0.5}$ (mM) represents the apparent affinity for ATP.

	Wild-type		$Ser^{974} \rightarrow Ala$		$Asn^{992}Lys^{993}Val^{994}His^{995}$		$Asp^{992} \rightarrow Asn$		$Lys^{954} \rightarrow Glu$	
Ligand	$[S]_{0.5}$	V_{max}	$[S]_{0.5}$	V_{max}	$[S]_{0.5}$	V_{max}	$[S]_{0.5}$	V_{max}	$[S]_{0.5}$	V_{max}
None	3.8 ± 0.4	270 ± 2	3.0 ± 0.1	34 ± 3	2.8 ± 0.1	79.0 ± 3.5	3.8 ± 0.2	1048 ± 27	3.0 ± 0.1	144 ± 4
1 mM PRPP	0.20 ± 0.01	884 ± 11	2.7 ± 0.2	63 ± 2	0.80 ± 0.03	312 ± 10	0.20 ± 0.01	2600 ± 90	0.20 ± 0.01	892 ± 14
2 mM UTP	4.7 ± 0.8	56.0 ± 0.7	3.8 ± 0.1	15.0 ± 0.4	3.00 ± 0.08	36 ± 3	3.60 ± 0.12	310 ± 10	2.8 ± 0.1	62 ± 2

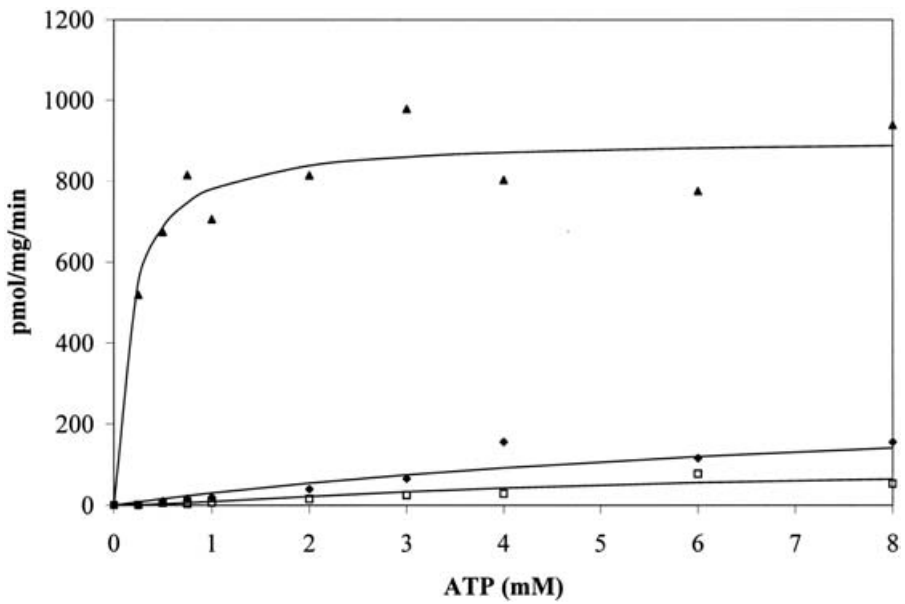

Figure 5 CPSase activities of $Lys^{954} \rightarrow Glu$ CAD measured as a function of ATP concentration, in the presence or absence of allosteric regulators

The concentration of ATP was as indicated, and Mg^{2+} concentration was in excess of the ATP at all concentrations by 2 mM. The assay was performed with no effectors (◆), with 1 mM PRPP (▲) or 2 mM UTP (□).

bind to the enzyme simultaneously. PRPP and UTP appear to have non-competing-binding sites on the allosteric domain. Results of the present study for the wild-type enzyme indicate that, in the presence of both effectors, PRPP activation is dominant.

Such findings appear to disagree with the two-state modelling of CPSase [11,34] in which the inhibitor binds only to an inactive T-state form, whereas activating ligands stabilize an active R-state conformation. In contrast, our results appear to agree with Raushel's model [13] that both activating and inhibiting ligands bind to *E. coli* CPSase simultaneously. With the near loss of PRPP activation in the $Ser^{974} \rightarrow Ala$ enzyme, the inhibitory effect of UTP is revealed even in the presence of PRPP.

Whereas mutations in the gene encoding the purine salvage enzyme hypoxanthine–guanine phosphoribosyltransferase are known to cause Lesch–Nyhan syndrome, the cause for the associated neurological manifestations are unknown. A previous study [27] found that astroglial cultures derived from hypoxanthine–guanine phosphoribosyltransferase-deficient mice have increased levels of both PRPP and UTP along with accelerated *de novo* purine biosynthesis [27]. Prior to the work reported here, it was unclear why UTP would increase in astroglial cells. However, this now makes sense based on our conclusion that PRPP plays a dominant role over UTP in regulating CPSase activity. Pelled et al. [27] proposed that the neurological deficiencies associated with Lesch–Nyhan may be due to the imbalance in purine and pyrimidine levels. Perhaps, to reduce the increase in UTP levels in patients with Lesch–Nyhan syndrome, it would be beneficial to consider UTP analogues that decrease CPSase activity in the presence of increasing PRPP concentrations.

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