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Review Article

Initial Steps of *De Novo* Pyrimidine Nucleotide Biosynthesis in Parasites and Mammalian Tissues: Purification, Regulation, Adaptation, and Evolution

TAKASHI AOKI

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Abstract

The *de novo* pathway for pyrimidine nucleotide biosynthesis represents one of the oldest metabolic pathways, and six sequential steps leading to the production of UMP remain intact throughout the evolution, although the molecular organizations of enzyme proteins deviate significantly in prokaryotes, protozoa, fungi, and animals. Comparative aspects of the initial three enzymes of the pathway, their purification, regulation, inhibition, and inactivation, are described in this article. Implications in possible molecular adaptation and evolution of the enzymes are also discussed.

Key words: carbamoyl-phosphate synthetase II (EC 6.3.5.5), aspartate carbamoyltransferase (EC 2.1.3.2), *de novo* pyrimidine biosynthesis, metabolic regulation, adaptation, evolution

Introduction

This article mainly deals with our own work on the initial steps of *de novo* pyrimidine nucleotide biosynthesis in helminth and protozoan parasites and in mammalian tissues including rat hepatomas. Pyrimidine and purine nucleotides are, of course, essential units for nucleic acid structure and function as genetic information. A number of parasites are incapable of synthesizing purines *de novo* and thus rely on salvaging performed purine bases and nucleosides. In contrast, most organisms possess the *de novo* biosynthetic pathway for pyrimidine nucleotides. This fact may indicate a more basic implication of the *de novo* pyrimidine pathway in the balanced, continued pyrimidine and purine supply for nucleic acid synthesis. Exceptions that anaerobic protozoa of the genera *Entamoeba*, *Giardia*, and *Tritrichomonas* possess no capacity for *de novo* pyrimidine synthesis (Jarroll *et al.*, 1987) have not been verified by the absence of genes encoding enzymes of the pathway.

The *de novo* pyrimidine pathway represents one of the oldest metabolic pathways, and six sequential steps leading to the production of UMP (Fig. 1) remain intact throughout the evolution. However, shortly before we started this work, Kurelec (1972, 1973) reported that parasitic helminths were unable to synthesize pyrimidines *de novo*. Our first experiment was thus to examine the incorporation of C^{14}-bicarbonate, as a radiolabeled tracer, into uridine nucleotides in tissues and intact worms of *Ascaris suum* and *Schistosoma mansoni* (Aoki, 1979). The purified uridine nucleotides had radioactivity, providing evidence that the *de novo* pathway is operative in these helminth parasites. Similar results were reported by Kobayashi *et al.* (1978) for liver and lung flukes *Clonorchis sinensis* and *Paragonimus ohirai*.

Department of Parasitology, Juntendo University School of Medicine, Hongo, Bunkyo-ku, Tokyo 113, Japan.
青木　孝（順天堂大学医学部寄生虫学教室）

Abbreviations frequently appearing in the text: CPS II, carbamoyl-phosphate synthetase II; ACT, aspartate carbamoyltransferase; DHO, dihydroorotase; CAD, CPS II-ACT-DHO (multifunctional protein); GAT, glutamine amide transfer (subunit or domain); CPS, carbamoyl-phosphate synthesis (subunit or domain)

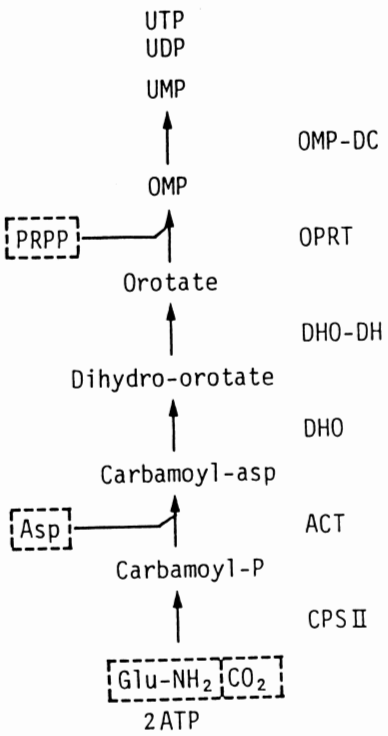


Fig. 1 Six steps of the *de novo* pyrimidine biosynthetic pathway. CPS II links amide nitrogen (N) of L-glutamine and bicarbonate (C), then ACT binds (C) with L-aspartate, and finally DHO closes the 6-member pyrimidine ring. Thus, the initial three enzyme reactions provide the basic structure for pyrimidine bases. DHO-DH, dihydroorotate dehydrogenase; OPRT, orotate phosphoribosyltransferase; OMP-DC, orotidine 5'-phosphate decarboxylase. Carbamoyl-P, carbamoyl phosphate; Asp, L-aspartate; Carbamoyl-asp, carbamoyl-L-aspartate; OMP, orotidine 5'-phosphate; PRPP, 5-phosphoribosyl 1-pyrophosphate.

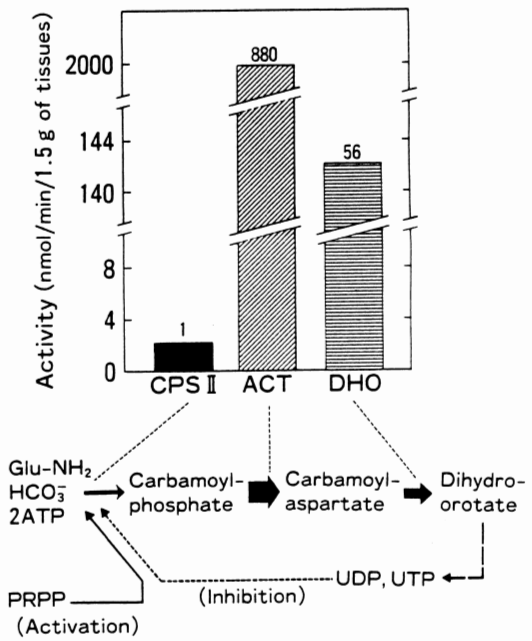


Fig. 2 Initial three steps and regulation of *de novo* pyrimidine biosynthesis in *Ascaris suum*. 1:880:56, approximate relative activities of CPS II, ACT, and DHO.

Purification of CPS II, ACT, and DHO from *Ascaris suum*

Other evidence for the capacity of parasitic helminths in synthesizing pyrimidines *de novo* was provided by the detection, purification, and characterization of CPS II, which catalyzes the first and key step of the pathway (Fig. 1), from *A. suum* (Aoki *et al.*, 1975). This was the first report of the presence of CPS II in parasites. To stabilize this enzyme, as well as the mammalian CPS II, cryoprotectants such as glycerol and dimethyl sulfoxide were required during its purification, assay, and storage.

Ascaris CPS II was copurified with ACT and DHO, the second and third enzymes of the pathway (Aoki *et al.*, 1975; 1980). The relative activities of the three enzymes remained nearly constant, 1:850-890:50-60, throughout the purification (Fig. 2). Sucrose gradient centrifugation of the purified fraction resulted in co-sedimentation of these enzymes. It is thus concluded that CPS II probably limits the rate of *de novo* pyrimidine synthesis and occurs as a multienzyme complex (CAD) together with ACT and DHO in *A. suum*. Conclusions of the same kind were obtained for the initial steps of pyrimidine synthesis in *S. mansoni* (Aoki and Oya, 1979) and two other flukes (Kobayashi *et al.*, 1978).

Control of *de novo* pyrimidine biosynthesis through regulation of CPS II activity in helminth and protozoan parasites

In *A. suum*, the activity of the rate-limiting CPS

II was subjected to feedback inhibition by UDP and UTP, while it was stimulated by 5-phosphoribosyl 1-pyrophosphate (PRPP) (Fig. 2). The second enzyme, ACT, had very high activity with no apparent regulatory function. These findings indicate that CPS II plays a key role in the control of pyrimidine biosynthesis in *Ascaris suum* (Aoki *et al.*, 1975; 1980), as it does in mammalian tissues (Tatibana and Shigesada, 1972).

Most of the catalytic and regulatory properties of *Ascaris* CPS II are similar to those of the mammalian enzyme. A notable feature of the parasite enzyme is that its activity is more strongly inhibited by nucleoside diphosphates, UDP, dUDP, dADP, and CDP, than by corresponding nucleoside triphosphates, UTP, dUTP, dATP, and CTP (Aoki *et al.*, 1975; 1980). Such a unique property was also observed in *S. mansoni* (Aoki and Oya, 1979) and in two other species of flukes (Kobayashi *et al.*, 1978). Later, we found this particular mechanism for the regulation of CPS II activity in a trypanosomatid *Crithidia fasciculata* (Aoki and Oya, 1987b), indicating that the mechanism is widely distributed in parasitic protozoa, nematodes, and trematodes, irrespective of their habitats in host animals. In contrast, mammalian CPS II, as well as yeast and *Drosophila* enzyme, is most sensitive to inhibition by UTP (Tatibana and Shigesada, 1972), and *Escherichia coli* enzyme is to that by UMP. We prefer therefore to consider that the higher susceptibility of parasite CPS II to inhibition by UDP and other nucleoside diphosphates may have resulted from adaptation of organisms to their parasitic lives, although it is impossible to exclude the possibility that this specific mechanism for CPS II regulation is closely associated with the phylogeny of parasitic organisms. To prove this hypothesis, determination and comparison of endogenous nucleotide pools as negative effectors and of primary structures at the negative effector-binding site(s) in CPS II from various sources are needed.

Initial steps of pyrimidine biosynthesis in r at liver and hepatomas

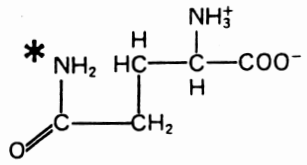
Our biochemical studies on initial steps of *de novo* pyrimidine synthesis were extended to subcutaneously transplanted Morris hepatomas, with a wide range of growth rates, and rat liver used as control (Aoki and Weber, 1981; Aoki *et al.*, 1982a). The rate of growth was defined as time required for tumor to reach a diameter of 1.5 cm, and ranged from 0.5 to more than 10 months. In 13 hepatomas, the increases in CPS II specific activities correlated significantly with the tumor growth rates, ranging from nearly the control value to an about 10-fold elevated value. Rapidly growing hepatomas also showed the increased levels of enzyme protein and mRNA (Reardon and Weber, 1986). A rough correlation was also recognized between the specific activity of CPS II and growth rate in *E. coli* (high), various protozoan parasites and rapidly growing hepatomas (medium), and rat tissues and animals (low) including *A. suum* and *S. mansoni* (Aoki and Oya, 1987a). These observations imply that the high level of CPS II activity is a prerequisite factor for rapid growth. This may also imply that blockage of tumor CPS II activity brings about a fatal effect on neoplasms.

Inactivation by acivicin of CPS II *in vivo*

To test this possibility, rats bearing a rapidly proliferating hepatoma received intraperitoneal injections of acivicin [L-($\alpha S,5S$)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid], an L-glutamine antagonist (Fig. 3) (Aoki *et al.*, 1982b; Denton *et al.*, 1982). Rat liver and hepatomas were excised and determined for the specific activities of CPS II, yielding the more markedly decreased CPS II activity in hepatoma than in liver. We attempted to restore the CPS II activity lowered by acivicin *in vivo*. Neither treatment by gel filtration on Sephadex G-25, purification nor extensive dialysis of the hepatoma and liver CPS II recovered the inhibited activity, suggesting that the enzyme was irreversibly inactivated by acivicin *in vivo*. In rat liver and hepatoma, the activities of ACT, which resides in the same polypeptide with CPS II and DHO, were not affected; i.e., acivicin action was selective.

Mechanisms of inhibition and inactivation by acivicin of CPS II examined *in vitro*

Figure 3 shows the structures of L-glutamine and acivicin, an L-glutamine analog which has been



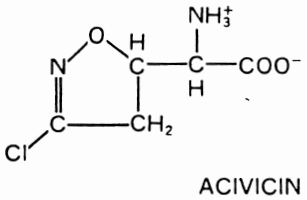


Fig. 3 Structures of L-glutamine and acivicin. Amide group (*) of glutamine is biochemically equivalent to NH_3. It is transferred by glutamine amide transfer (GAT) domain to NH_3-dependent carbamoyl-phosphate synthesis (CPS) domain in CPS II. Generally, exogenously added NH_3 can also be utilized as alternative nitrogen donor for glutamine-utilizing enzymes, but much less efficiently than amide nitrogen of glutamine. Acivicin has no free amide group to be liberated and instead Cl^- is replaced by an active site of the GAT domain, resulting in a covalently bound acivicin-GAT complex.

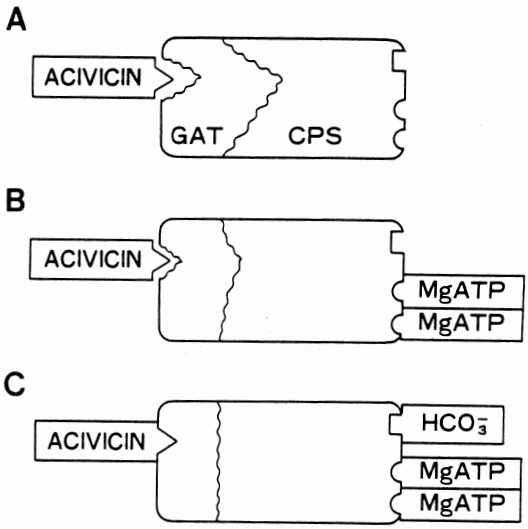


Fig. 4. Mechanism of inactivation by acivicin of L-glutamine-dependent CPS II activity: Acceleration of inactivation velocity by binding of substrates. In the absence of glutamine, acivicin directly interacted with the glutamine-binding site of GAT domain and yielded slow inactivation (A). Inactivation velocity was accelerated markedly by MgATP (B) and most extensively by MgATP plus bicarbonate (C). Bicarbonate alone was marginally stimulative. Wavy lines postulate conformational changes in glutamine-binding site and in GAT domain. Possible conformational changes in CPS domain are disregarded.

tested for antitumor action. CPS II is a glutamine-utilizing enzyme which also requires bicarbonate and MgATP as substrates. Ammonia also can serve as nitrogen donor for this enzyme, which, however, has a 1000-times higher affinity for glutamine than for ammonia (Aoki *et al.*, 1982a).

In the presence of L-glutamine, acivicin competitively inhibited the liver and hepatoma CPS II activities. In the absence of glutamine, on the other hand, acivicin directly interacted with the glutamine-binding site of the enzyme and brought about an irreversible, selective, and time-dependent inactivation of the glutamine-dependent activities of liver and hepatoma CPS II (Aoki *et al.*, 1982b). The ammonia-dependent CPS II activity was not affected. Figure 4 shows the modulation of inactivation where addition of other substrates, MgATP and MgATP plus bicarbonate, accelerated the inactivation velocity 5-fold and 18-fold, respectively, presumably due to conformational changes of the enzyme. In conclusion, acivicin most probably brings about an irreversible inactivation by active site-directed affinity labeling of the glutamine-binding site of the enzyme *in vitro* and *in vivo*, and the affinity labeling is accelerated by conformational changes induced in the enzyme by binding of substrates.

Protection from inactivation by acivicin of glutamine-dependent activities of liver and hepatoma CPS II was also examined *in vitro*, and it was shown that L-glutamine was most effective (Aoki *et al.*, 1982b). Rat liver had a 9-times higher concentration of L-glutamine (6 μmol/g tissue) than hepatoma (Sebolt and Weber, 1984), probably participating in *in vivo* protection of liver CPS II from attack by acivicin. In other words, the lower L-glutamine

concentration in hepatoma may have afforded lesser protection to the tumor enzyme. Acivicin may have thus exerted greater cytotoxicity to hepatoma than to normal liver (Weber *et al.*, 1984).

Effect of acivicin on a trypanosomatid CPS II *in vitro* and *in vivo*

In vitro and intracellular actions of acivicin on CPS II from *C. fasciculata,* which we employed as a model for trypanosomatids, were analyzed under the conditions comparable to those used for rat liver and hepatomas. A summary of the results, in comparison with those obtained for the mammalian tissues, is shown in Table 1 (Aoki and Oya, 1987c; 1988). Acivicin *in vitro* competitively inhibited the trypanosomatid CPS II activity, with a K_i value of 2 μM, indicating that the parasite enzyme was more sensitive to inhibition by acivicin. Conversely, the parasite enzyme had a lower affinity for a physiological substrate L-glutamine. The sum of these effects must be a greater susceptibility to acivicin for the parasite CPS II than for the mammalian enzyme. As suspected, the *in vitro* determination of inactivation half-time by 10 μM acivicin showed much faster inactivation for the parasite CPS II than for the mammalian enzyme. Similarly, intracellular inactivation by acivicin of CPS II activity appeared to be faster in *Crithidia* than in mammalian tissues (Table 1). There must be a critical step at which acivicin is delivered into the cytoplasm of the cells and develops its efficacy, i.e., the plasma membrane barrier or transport system. Although the mechanism(s) of translocating acivicin and glutamine were unknown in trypanosomatids, the drug was actually taken up even at a low concentration and in the presence of a high concentration of glutamine, as revealed by the fact that intracellular inactivation by acivicin of CPS II activity took place. We examined *in vitro* the efficacy of acivicin on the growth of *C. fasciculata, L. mexicana,* and *Trypanosoma cruzi* in a serum-free medium GIT; IC_{50} values, the concentrations at which 50% growth inhibition was observed, were 1.5 μM, 10 nM, and less than μM order, respectively (Aoki, unpublished data).

Table 1 Differential action of acivicin on mammalian and protozoan CPS II

Action of acivicin	Mammal	*Crithidia*
In vitro inhibition		
K_i for acivicin (μM)	7	2
K_m for glutamine (μM)	20	270
In vitro inactivation		
$t_{1/2}$ at 10 μM acivicin (min)	8	3
In vivo inactivation		
$t_{1/2}$ at 10 μM acivicin (min)	ND	41
100 μM acivicin (min)	21	ND

$t_{1/2}$, inactivation half-time, the time required for 50% decrease in the glutamine-dependent CPS II activity. ND, not determined.

Purification and characterization of ACT from *Leishmania mexicana*

ACT was purified 148-fold, with 15% recovery of the activity, to near homogeneity from the extract of culture forms of *L. mexicana*, a causative agent of South American cutaneous leishmaniasis (Table 2). The results were presented previously in a preliminary form (Aoki *et al.*, 1990). Ammonium sulfate fractionation of the crude extract resulted in the separation of CPS II and ACT, implying that these two enzymes do not constitute a multifunctional CAD protein but exist as independent proteins. The ACT was further purified by high performance liquid chromatography (HPLC) using a gel filtration column of TSK Gel G4000SW, DEAE-Sepharose column chromatography, and again HPLC (TSK Gel G3000SW). SDS-PAGE of the purified enzyme revealed only 2 protein bands, 68 and 59 kDa; the former was thought to correspond to leishmanial ACT, as the peaks of ACT activity in two gel filtration profiles were located close to the position of bovine serum albumin (66 kDa). The molecular weight of around 34 kDa was reported as the ACT domain in yeast, *Drosophila*, and mammalian multienzyme complexes, as the catalytic subunit in *E. coli* ACT (see Fig. 6), and as an independent enzyme in *Leishmania donovani* (Mukherjee *et al.*, 1988). To gain more severe separation of our 68 kDa polypeptide, we carried out SDS-PAGE in the pres-

Table 2 Purification of ACT from *Leishmania mexicana*

Fraction	Protein (mg)	Total act. (nmol/min)	Spec. act. (nmol/min/mg)	Purification (-fold)	Yield (%)
Extract	134.4	4594.9	34.2	1	100
Ammonium sulfate	16.5	3962.5	240.2	7.0	86
HPLC (G4000SW)	5.3	2243.7	423.3	12.4	49
DEAE-Sepharose	0.30	702.3	2341.0	68.5	15
HPLC (G3000SW)	0.14	706.7	5047.9	147.6	15

ence of 7 M urea, with no alteration of the electrophoretic pattern.

The activity of our purified ACT was insensitive to various nucleotides including CTP, an allosteric inhibitor for *E. coli* ACT. Thus, leishmanial ACT, as well as *Ascaris* and *Schistosoma* enzyme, may not play a key regulatory role in *de novo* pyrimidine synthesis. Table 3 shows a comparison of kinetic parameters of ACT from *E. coli, L. mexicana,* and higher animals. Purification and kinetic analysis indicated unusual properties of *L. mexicana* ACT, i.e., high molecular weight, high K_m for a substrate, carbamoyl phosphate, and concomitantly high inhibition constant (K_i) for PALA. Since the affinities of the enzyme for carbamoyl phosphate and PALA were quite low, we examined the reversibility of activity inhibited by PALA. Exhaustive dialysis in the presence of a high concentration of L-aspartate enhanced the decreased activity to some extent, indicating a partially reversible binding of PALA to *Leishmania* ACT. Whether these unusual properties are reflections of particular regions of the primary structure of the enzyme protein is awaited to be analyzed.

Table 3 Comparison of kinetic parameters of ACT from various sources

Parameter	*E. coli*	*Leishmania*	Animals
K_m for carbamoyl-P (mM)	0.03	0.16	0.02
K_m for L-aspartate (mM)	20	17	21
K_i for PALA (nM)	27	3500	0.1–10

PALA, *N*-(phosphonacetyl)-L-aspartate, is a transition-state analog, i.e. a very tightly binding inhibitor possessing structural features of both substrates, carbamoyl phosphate and L-aspartate, in one molecule.

Possible evolution of CPSs and initial steps of *de novo* pyrimidine biosynthesis

As well as *Leishmania* CPS II and ACT, 50%-saturated ammonium sulfate precipitated *Crithidia* CPS II activity, leaving ACT activity in the supernatant (Aoki and Oya, 1987a). Similarly, these enzymes occur as distinct forms in various protozoan parasites (Asai *et al.*, 1983; Tampitag and O'Sullivan, 1986; Mukherjee *et al.*, 1988; Krungkrai *et al.*, 1990), as they do in *E. coli.* To identify similarities and differences between the trypanosomatid and other organisms' CPS II, kinetic parameters were compared (Table 4) (Aoki and Oya, 1987a). The *Crithidia* CPS II shared the same levels of K_m values for L-glutamine, NH_3, and bicarbonate with *E. coli* enzyme. However, the trypanosomatid CPS II had an exceptionally low K_m value for MgATP of 1.7 mM. This may relate to unusual regulatory properties, one of which has been discussed earlier, since the affinity for MgATP is under flexible and stringent regulation in response to negative and positive effectors in most CPS II

Table 4 Comparison of kinetic parameters of CPS II from various sources

Source	K_m (mM)			
	L-glutamine	NH_3	MgATP	HCO_3^-
Animals including helminth parasites	0.02	15	10	20
Crithidia fasciculata	0.3	30	1.7	1.7
Escherichia coli	0.4	90	8.0	1.2

(Aoki *et al.*, 1982a). From these observations, we expected a phylogenetically close relationship between the protozoan and prokaryotic CPS II, and also expected an exclusively unique property to the parasite enzyme.

As shown in Fig. 5, protozoan CPS II may stand at a very important position in the evolution of initial steps of *de novo* pyrimidine synthesis. *E. coli* possesses only one type of CPS II which provides carbamoyl phosphate for both arginine and pyrimidine synthesis. From this type of prokaryotic enzyme, a monofunctional pyrimidine-specific CPS II may have diverged in an unicellular eukaryote, and may then have been subjected to further evolution to a bifunctional protein carrying CPS II and ACT activities in a yeast-type organism. There are however contradictory hypotheses on this point, as described later. The CPS II-ACT complex may have evolved to a trifunctional protein CAD bearing CPS II, ACT, and DHO activities in multicellular animals. The multienzyme complex known to exist in helminth parasites belongs to the last category. In this postulated evolutionary course of pyrimidine-specific CPS II, the protein structures were mark-

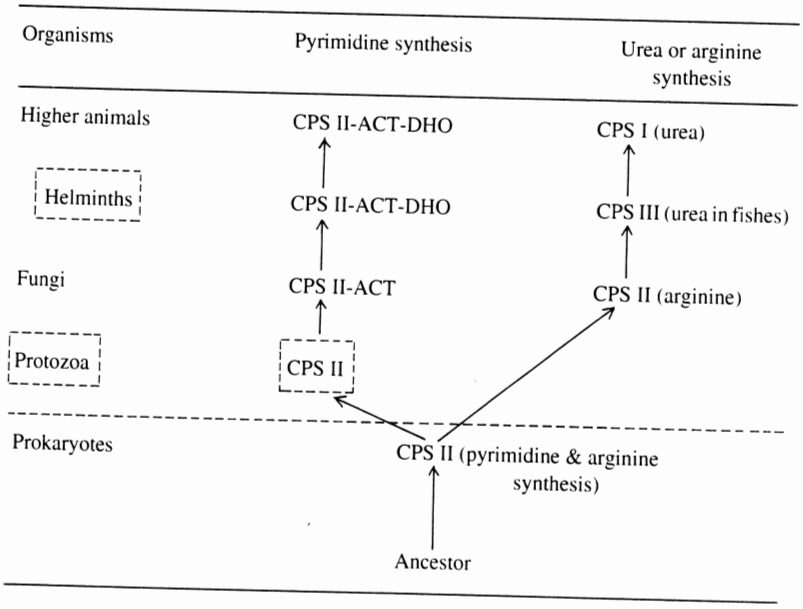


Fig. 5 Possible evolution of CPSs and initial steps of *de novo* pyrimidine biosynthesis. An evolutionary position, which is equivalent to higher animals, of pyrimidine-specific enzymes in helminth parasites is highlighted in this figure. There are three different types of carbamoyl-phosphate synthetases in nature, CPS I, CPS II, and CPS III, which are classified on the basis of substrate specificities and cofactor requirement. CPS II is a glutamine-dependent enzyme, and is categorized as a pyrimidine-specific or arginine-specific enzyme according to its physiological role. See the text for evolution of pyrimidine-specific CPS II. *E. coli* CPS II is composed of a light subunit (GAT) and a heavy subunit (CPS) (Fig. 6). Arginine-specific yeast CPS II possesses a homologous subunit structure. CPS I, single polypeptide with a molecular weight similar to that of *E. coli* CPS II (GAT+CPS), utilizes NH_3 as physiological nitrogen donor and requires *N*-acetyl-L-glutamic acid (AGA) as essential activator. The enzyme catalyzes the first and key step of the urea cycle in liver mitochondria in urea-excreting animals. CPS III is an L-glutamine- and AGA-dependent enzyme localized in mitochondria in some fishes, and responsible for the urea-specific carbamoyl-phosphate synthesis; the production of urea is required for osmotic regulation. The evolutionary relationship between CPS III and CPS I is tentative.

edly altered, with different molecular weights ranging from 160 kDa (*E. coli*) to 240 kDa (mammal), but the physiological role as *de novo* pyrimidine synthesis consistently remained unchanged. On the contrary, the evolution of arginine-specific CPS II (Fig. 5) may have proceeded with a slight alteration in protein structures, but its key role changed quite significantly from arginine-specific (anabolic function) to urea-specific (catabolic function) carbamoyl phosphate synthesis.

Figure 6 shows a schematic representation of currently known protein organizations of the initial three enzymes of *de novo* pyrimidine biosynthesis in various organisms, including *C. fasciculata* and *L. mexicana* (this study). *E. coli* CPS II consists of a GAT subunit and an NH_3-dependent CPS subunit, and ACT is composed of a 17 kDa regulatory subunit and a 34 kDa catalytic subunit. In *Dictyostelium discoideum*, *Drosophila melanogaster*, and syrian hamster, the sequence analyses of cDNAs that encode trifunctional protein CADs exhibited domain organizations, from N-terminus, GAT, CPS, DHO, and ACT (Faure *et al.*, 1989; Freund and Jarry, 1987; Simmer *et al.*, 1990). *Saccharomyces cerevisiae* possesses a bifunctional protein, whose domain order is, from N-terminus, GAT, CPS, DHO-like region, and ACT, where the DHO-like region does not seem to be functional (Souciet *et al.*, 1989). Simultaneously, the yeast has a separate, functional DHO (Fig. 6) that is homologous to prokaryotic DHOs, but, in yeast, the level of similarity between the DHO and DHO-like region is too low to assume these two sequences to be derived from the same ancestral structure (Souciet *et al.*, 1989). However, a different model involving the DHO gene duplication and insertion into an ancestral bifunctional locus is also proposed for the origin and evolution of fused and separated DHOs (Simmer *et al.*, 1990).

Although we have not known the presence of such an ancestral bifunctional protein with no insertion of DHO sequence, we are quite aware of the importance of the evolutionary position of protozoan enzymes, the separated CPS II, ACT, and DHO. We have been recently conducting a molecular analysis of the *T. cruzi* CPS II gene. The result indicates that *trans*-splicing took place for the maturation of CPS II mRNA, and that GAT and CPS domains were covalently linked by a short polypeptide (see Fig. 6). No intron has been detected so far in our examinations (Aoki *et al.*, unpublished). Determination of a complete sequence encoded by the protozoan CPS II gene is awaited for further discussion.

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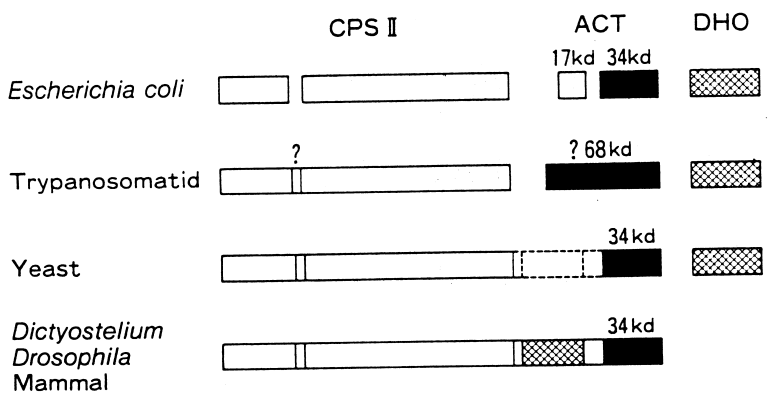

Fig. 6 Subunit or domain organizations of initial three enzymes of *de novo* pyrimidine biosynthesis in representative members in prokaryotes, unicellular eukaryotes, fungi, and multicellular animals.

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