ADVANCES IN ENZYMOLOGY

AND RELATED AREAS OF MOLECULAR BIOLOGY

Founded by E. F. NORD

Edited by ALTON MEISTER

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ENZYMES OF ARGININE AND UREA SYNTHESIS

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CONTENTS

I. Historical Introduction

In the formulation of the ornithine cycle proposed in 1932 by Krebs and Henseleit (1) urea is formed from NH_3 and CO_2 through a cyclical process in which ornithine, citrulline, and arginine are assigned the role of carrier compounds. This novel and far-reaching concept was based on experiments with respiring liver slices (1,2). More than a decade later, coinciding with the introduction of respiring liver homogenates as experimental material, a new burst of activity in the study of urea synthesis occurred in other laboratories. Urea synthesis was, however, still dependent on an adventitious source of ATP and other substrates from respiratory processes. With the demonstrations that the high-energy phosphate bond of ATP is

utilized to form a new C-N bond in the conversion of citrulline to arginine (3) and ornithine to citrulline (4), it was possible to study urea synthesis in soluble systems.

It was shown at the same time that aspartate is the specific nitrogen donor in the conversion of citrulline to arginine (3); thus only one half of the nitrogen of urea originates from free NH_3. The major role of glutamic-aspartic transaminase in the transfer of nitrogen from all amino acids to urea through glutamic dehydrogenase and glutamine synthetase and the interdependence between these nitrogen-transferring reactions and the generation of oxaloacetate and α-ketoglutarate by way of the citric acid cycle became apparent (3,5). These developments and the separation of the enzymes participating in the conversion of citrulline to arginine through the formation and cleavage of a new intermediary compound, argininosuccinate, were the subjects of an earlier review (6).

Experimental advancements of the past fifteen years have reinforced, exponentially, the point of view proposed earlier (6) that the enzymes of arginine biosynthesis are widely distributed in nature to provide arginine for the synthesis of protein, muscle phosphogens, and urea; in ureotelic species the addition of arginase to this pathway represents an exploitation for physiological and excretory purposes. Our knowledge of the mechanism of action of the individual participating enzymes has been greatly advanced in the last ten years, and highly purified preparations of each enzyme have been prepared in sufficient quantity to permit some elucidation of oligomeric structure and catalytic regulation.

With this remarkable progress, and with the availability of the necessary substrates and methods for estimating the individual *in vivo* activities of the ornithine cycle enzymes in tissue homogenates, concurrent studies on the regulation of this pathway, the relation to developmental changes, and the relation to evolutionary development have succeeded in linking molecular behavior to biological processes. A considerable body of comparative literature dealing with the study of enzymes of arginine synthesis from various sources has accumulated.

It is scarcely possible to discuss both the enzymatic and metabolic significance of these developments in deserving detail. Yet all aspects are interrelated, and therefore the catalytic behavior and physical properties of the individual enzymes and their substrates will be

discussed first as background from which to evaluate succeeding sections dealing with adaptive, genetic, developmental, and comparative studies.

II. Carbamyl Phosphate Synthetases

A. INTRODUCTION

The early studies leading to the separation of carbamyl phosphate synthetase from ornithine transcarbamylase and to the recognition of a carbamyl intermediate in the conversion of ornithine to citrulline in mammalian liver have been reviewed (6,7). It will be recalled that the existence of this intermediate was shown by Grisolia and Cohen in 1952 (8). Elucidation of the structure was achieved in 1955 by Jones et al., (9) through chemical synthesis of the compound carbamyl phosphate. This compound is utilized in the first step of two biosynthetic pathways, one leading to arginine and urea and the other to pyrimidines. In microorganisms such as *Escherichia coli* a single enzyme catalyzes the synthesis of carbamyl phosphate for both pathways (10), whereas in ureotelic species (8,11), in yeast (12), and in *Neurospora crassa* (13,14) there are two carbamyl phosphate synthetases, each being pathway specific (Table I). Terrestrial invertebrates have glutamine-requiring and NH_3-requiring enzymes, but both also require *N*-acetylglutamate (15).

It has been a challenge to investigators to obtain requisite proof that the two pools of carbamyl phosphate are separate and do not mix metabolically. In ureotelic livers enzymatic evidence indicates that carbamyl phosphate I, together with ornithine transcarbamylase, is localized in the mitochondia (8). Carbamyl phosphate synthetase II, coupled to aspartic transcarbamylase, is present in the cytosol, as shown by Hager and Jones (11). Intracellular compartmentation and the higher levels of the intramitochondrial enzymes suggest that the two pools do not mix *in vivo*. However, carbamyl phosphate generated within mitochondria under *in vitro* conditions can be trapped outside by supplying an external aspartate acceptor system (16). In *Neurospora crassa,* Davis (13) and Williams and Davis (14) have shown with mutant strains that both carbamyl phosphate-synthesizing enzymes A and P are glutamine dependent. The evidence obtained with ^{14}C labeling by Williams et al. (17) indicates that in *Neurospora* there are two pathway-specific carbamyl phos-

phate pools. Pool separation is linked to the respective transcarbamylases and is probably due to structural compartmentation (cf. discussion in ref. 17a). With mutant yeast strains Lacroute et al. (12) have shown that there are two pathway-specific and glutamine-utilizing carbamyl phosphate synthetases. Pool mixing occurs in yeast since mutants grow on a simple medium. Regulation is such that arginine represses synthetase A and UTP exerts feedback inhibition on synthetase P.

TABLE I
Pathway Specificity of Carbamyl Phosphate Synthetases

Source	Substrate preferred	Pathway	Carbamyl phosphate pools	References
Ureotelic liver			2	8,11,16
Synthetase I	NH_3 (acgl)	Arginine		8
Synthetase II	Glutamine	Pyrimidine		11
Invertebrates			?	
Synthetase I	NH_3 (acglu)	Arginine		15[a]
Synthetase III	Glutamine (acglu)	Pyrimidine		15
Neurospora crassa			2	17
Synthetase A	Glutamine	Arginine		13
Synthetase P	Glutamine	Pyrimidine		14
Sacharomyces			1	12
Synthetase A	Glutamine	Arginine		12
Synthetase P	Glutamine	Pyrimidine		12
Mushrooms	Glutamine			18
Escherichia coli	Glutamine	Both		10,19

[a] Cf. Section XII.B.

In 1962 Levenberg (18) found that the carbamyl phosphate synthetase from mushrooms utilizes the amide nitrogen of glutamine as the preferred nitrogen source. A similar observation was later reported by Piérard et al. (10) for the enzyme in *E. coli*; Anderson and Meister (19) and Kalman et al. (20) found glutamine to be the specific substrate for the purified *E. coli* enzyme. Except for the invertebrate enzymes (15) all glutamine-utilizing enzymes can also utilize NH_3, although a higher concentration is necessary. Two carbamyl phosphate synthetases listed in Table I cannot utilize glutamine; these require acetylglutamate as a cofactor.

B. CARBAMATE KINASE

The carbamate kinase of bacteria was originally described by Jones et al. (9,21). The enzyme catalyzes the synthesis of carbamyl phosphate from carbamic acid and 1 mole of ATP. Carbamate is formed

$$NH_2COO^- + ATP \xrightleftharpoons{Mg^{2+}} NH_2\overset{\overset{O}{\|}}{C}OPO_3^{2-} + ADP \qquad (1)$$

nonenzymatically from NH_4^+ and HCO_3^-. The carbamate kinase reaction is of interest because it reflects the structure and chemistry of carbamyl phosphate and because reversal occurs with ease (22). The equilibrium for reaction 1 favors the generation of ATP from carbamyl phosphate. At pH 9.5 and 10°,

$$K_{eq} = \frac{(\text{carb P})\ (\text{ADP})}{(\text{ATP})\ (\text{carbamate})} = 0.04$$

Carbamate kinase is of interest here only indirectly since much evidence now indicates that this enzyme does not play a significant metabolic role in the synthesis of carbamyl phosphate. A number of bacteria are capable of degrading arginine to citrulline through the action of the hydrolytic enzyme, arginine deiminase. Such species can then form ATP by successive reversal of the ornithine transcarbamylase reaction and reaction 1. Several accounts of the discovery and elucidation of the metabolic function of arginine degradation in bacteria have appeared (6,7). Certain anaerobic strains of *Mycoplasma* are also especially rich in the enzymes involved in the arginine dihydrolase pathway and derive a major supply of ATP from the breakdown of arginine (23,24).

Studies have been carried out with partially purified preparations of carbamate kinase obtained from *Streptococcus faecalis* (25,26) and with homogeneous preparations obtained from *S. faecalis* and *S. lactis* (27–30). The enzyme from *S. lactis* has a molecular weight of 66,000 (30) and an $s_{20,w}$ value of 4.1 S (27). In molecular weight it is probably the same as, or closely similar to, the enzyme from *S. faecalis*. The latter has an S value of 4.1 and a minimum weight of 33,000 (per mole of ADP bound) and a specific activity of 2260 μmoles/min/mg in the reverse direction at 37° (29). Kinetic analyses with respect to binding of substrates by Marshall and Cohen

(29) suggest that with the enzyme from *S. faecalis* a nucleotide is the first to add to the enzyme and the last to dissociate, and that probably a ternary enzyme complex is formed.

C. LIVER CARBAMYL PHOSPHATE SYNTHETASES

1. Carbamyl Phosphate Synthetases from Frog and Rat Liver

Carbamyl phosphate synthetase from frog liver is more stable than the rat liver enzyme; preparations were first obtained about 90% pure from liver mitochondria of the bullfrog (*Rana catesbeiana*), purified 2½-fold over the mitochondrial extract, with a specific activity of about 160 μmoles/hr/mg protein at 37°, a molecular weight of 315,000, and an $s_{20,w}$ value of 11.2 S (31,32).

More recently, Guthöhrlein and Knappe (33) have prepared homogeneous rat liver synthetase with a molecular weight of 250,000 and $s_{20,w}$ and D_{20} values of 10.9 S and 4.02, respectively, and a specific activity of 100 μmoles/hr/mg. Instability was overcome by storing suspensions of the enzyme at −20° in buffered ammonium sulfate solution containing 20% glycerol.

The synthetases from vertebrate liver catalyze the overall reaction in which 2 moles of ATP is utilized to form carbamyl phosphate:

$$NH_4^+ + HCO_3^- + 2ATP^{4-} \xrightarrow{Mg^{2+},\ Acgl} NH_2CO_2PO_3^{2-} + 2ADP^{3-} + P_i^{2-} + 2H^+ \quad (2)$$

2. Role of N-Acetylglutamate

The presence of *N*-acetylglutamate in catalytic amounts is essential for synthetase activity, and this compound appears to be the natural cofactor for the enzyme (7). Preincubation with *N*-acetylglutamate enhances activity and protects against inhibition by —SH reagents (32,34). The facts that analogs such as acetoxyglutarate are effective (35), and that possible functioning of the cofactor as a carrier of active CO_2 has been excluded (36), lend support to the hypothesis of Caravaca and Grisolia (34) that *N*-acetylglutamate is required for conformational reasons.

Guthöhrlein and Knappe (33) have elucidated the mechanism by which acetylglutamate functions as an allosteric effector. By following enzymatic activity at 10° with the use of a kinetic, optical method of assay, an activation phase can be detected that is acetylglutamate dependent. The half-time of the transition from the cata-

lytically inactive conformation, I_1, to the active conformation, A, is about 1 min at 10° with 10 m*M* cofactor. A third transition state, I_2, was detected on longer incubation at low temperatures. Such exposure alters the activation kinetics because of dissociation of the active form of the enzyme into catalytically inactive subunits, I_2. These are presumably 125.000 in molecular weight, as suggested by velocity sedimentation data. Kinetic analysis supports the following sequence of changes:

$$I_1 \underset{\text{acetylglutamate}}{\rightleftharpoons} A \underset{\text{low temperature}}{\rightleftharpoons} I_2$$

Kinetic analysis indicates further that acetylglutamate acts by labilizing I_1, rather than by stabilizing A. The presence of ATP and Mg^{2+} suppresses dissociation by stabilizing the active conformation. The allosteric changes proposed are consistent with much earlier observations that two ATP-binding sites can be distinguished; binding to the second site requires *N*-acetylglutamate (37). The dissociation of the active form, A, into subunits at low temperature may be due to disruption of hydrophobic bonds (33).

3. Mechanism of Action

Reaction 2 has been shown by Metzenberg et al. (38) to consist of at least two steps; the first utilizes ATP to activate CO_2, and the second, which is reversible, utilizes ATP to form the C-N bond (38). Studies by Jones and Spector (39) showed that ^{18}O in bicarbonate is transferred during carbamyl phosphate formation to the orthophosphate formed in reaction 2. It was later proposed, therefore, that the anhydride, carboxy phosphate, is an intermediate in the reaction (40). The contributions bearing on reaction mechanism were reviewed in 1962 (7) and in 1965 (40,40a). The following sequence has been proposed (19,40,40a):

$$E + HCO_3^- + ATP \rightarrow E\text{–carboxy phosphate} + ADP \quad (2a)$$

$$E\text{–carboxy phosphate} + NH_4^+ \rightarrow E\text{–carbamate} + P_i \quad (2b)$$

$$E\text{–carbamate} + ATP \rightleftharpoons E + \text{carbamyl phosphate} + ADP \quad (2c)$$

Steps 2a and 2c are supported, respectively, by a bicarbonate-dependent ATPase activity and by evidence of reversibility (38,41), and step 2a by ^{18}O evidence (39). Step 2c may resemble reaction 1 except that in this case the substrate to enzyme-bound carbamate.

The existence of an enzyme-bound activated form of CO_2 has been tested further by McKinley et al. (42) with the frog liver enzyme by pulse-labeling experiments; the results were negative. In testing substitutes for NH_3, the same investigators found that hydrazine, but not hydroxylamine, can replace NH_3. In the reaction a product is formed which appears to be *N*-aminocarbamyl phosphate. Glutamine when tested in the presence of acetylglutamate could not replace NH_4^+ as substrate (42). The studies (38–42) were carried out with the frog liver enzyme.

Guthöhrlein and Knappe (43) have found that the rat liver enzyme catalyzes an ATP–$^{32}P_i$ exchange which is dependent on HCO_3^-, NH_4^+, and N-acetylglutamate steps (2a and 2b). Since all the substrates necessary for carbamyl phosphate formation were present, exchange due to carbamyl phosphate decomposition had to be excluded. The possibility that exchange might occur by overall reaction reversal seems highly unlikely.

With new K_m values for NH_4^+ (1.1m*M*) and HCO_3^- (5.3 m*M*), Guthöhrlein and Knappe (43) have undertaken a kinetic analysis to distinguish between the possibilities (*a*) that steps 2a and 2b occur simultaneously as a concerted reaction and (*b*) that "active CO_2" is formed first in a rapid, reversible step, and that ADP and P_i are released from the enzyme only on reaction with NH_4^+. The authors favor the second possibility and suggest several structures for possible enzyme-bound intermediates. Estimates based on the second assumption indicate that only a small fraction of enzyme molecules would ever be charged with "active CO_2." The half-life, calculated from the ATPase activity, is estimated to be about 2 sec at 37°. This estimate and ^{14}C dilution through reversal of step 2a may explain(43) the negative results obtained in the pulse-labeling experiments mentioned above (42).

D. MICROBIAL CARBAMYL PHOSPHATE SYNTHETASES

1. Carbamyl Phosphate Synthetase from E. coli

The stoichiometry of the overall reaction catalyzed by the purified enzyme from *E. coli* (19) requires 2 moles of ATP, whether glutamine or NH_3 serves as

$$2ATP + \text{glutamine} + HCO_3^- + H_2O \xrightarrow[Mg^{2+}]{K^+} 2ADP + NH_2\overset{\overset{O}{\|}}{C}OPO_3^{2-} + P_i + \text{glutamate} \quad (3)$$

substrate. The K_m values for HCO_3^-, glutamine, and NH_4^+ are 1.2×10^{-3}, 0.38×10^{-3}, and 93×10^{-3} M, respectively (44).

a. Reaction Mechanism. Anderson and Meister (19,44) originally proposed that the reaction mechanism involves three successive steps. More recent observations by Meister and his collaborators (45) lend support to partial reactions comprising a sequence of five steps:

$$E + ATP + HCO_3^- \rightleftarrows E\text{-}(CO_3PO_3^{3-}) + ADP \quad (3a)$$

$$E\text{-}(CO_3PO_3^{3-}) \rightleftarrows E\text{-}(CO_2) + P_i \quad (3b)$$

$$E\text{-}(CO_2) + \text{L-glutamine} \rightleftarrows E\text{-}(CO_2)\text{-}(\text{L-glutamine}) \quad (3c)$$

$$E\text{-}(CO_2)\text{-}(\text{L-glutamine}) \rightarrow E\text{-}(NH_2CO_2^-) + \text{L-glutamate} \quad (3d)$$

$$E\text{-}(NH_2CO_2^-) + ATP \rightleftarrows E + NH_2\overset{\overset{O}{\|}}{C}OPO_3^{2-} + ADP \quad (3e)$$

Step 3a, the ATP-utilizing activation of CO_2, is supported by the results of pulse-labeling experiments (19) and by the presence of an intrinsic HCO_3^--dependent ATPase activity (44). "Active CO_2" is formulated as an enzyme-bound carboxy phosphate by analogy with the mechanism proposed for the liver enzyme. The bacterial enzyme also catalyzes the reversal of step 3e, that is, the formation of ATP, NH_4^+, and HCO^{3-} from carbamyl phosphate and ADP (44). This activity, which requires Mg^{2+} and K^+, suggests a relationship between the intermediate shown in step 3e and carbamate. The enzyme catalyzes the hydrolysis of γ-glutamyl hydroxamate, for which catalytic amounts of Mg^{2+}, ATP, and HCO^{3-} are required, and the slow hydrolysis of glutamine to glutamate and NH_3 (44,45).

b. Molecular Weight and Subunit Composition. Enzyme preparations thought to be homogeneous (specific activity 280 μmoles of carbamyl phosphate formed/mg/hr at 37° (45) give a range of S values on velocity sedimentation because of self-association (46,47); equilibration between the several species, particularly in the presence of phosphate buffer, explains the variations in S values (48,49). In Tris or veronal buffers, pH 7.8, a single, stable species is obtained having an $s_{20,w}$ value of 7.3 S and a molecular weight, estimated by

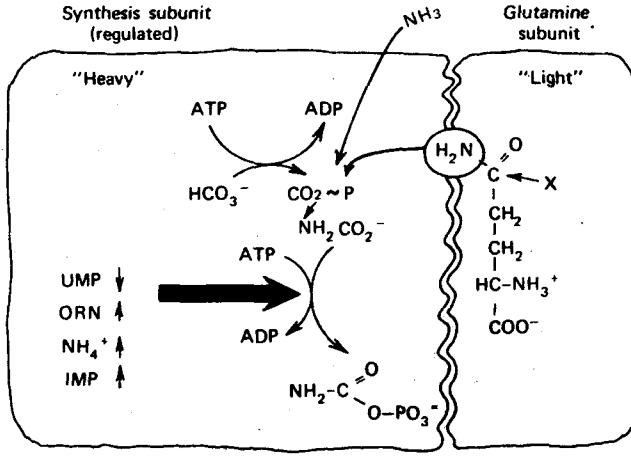

Fig. 1 Schematic representation of the functional role of the two subunits of carbamyl phosphate synthetase. X represents the postulated nucleophile. From Trotta et al. (49).

gel electrophoresis, of 170,000 (49). In confirmation of the report by Anderson et al. (50) that the subunits are not identical, the enzyme dissociates in the presence of denaturing agents (SDS, 8 *M* urea) into two subunits with molecular weights of about 130,000 and 43,000, respectively, for the heavy and light polypeptide chains (49,50).

c. **Role of Glutamine.** After separation in KSCN the heavy subunit catalyzes carbamyl phosphate formation from NH_3 but not from glutamine. Including the activity just mentioned, it retains three of the six activities catalyzed by the intact enzyme; the light subunit retains the sixth activity, that is, the capacity to hydrolyze glutamine (Fig. 1). Reconstruction of the enzyme by mixing the light and heavy chains results in the restoration of activity not shown by either, that is, the ability to synthesize carbamyl phosphate from glutamine and to hydrolyze γ-glutamylhydroxamate (49).

The binding of glutamine to the light subunit is supported by the observations that (*a*) glutamine binds in the absence of other substrates; (*b*) the glutamine analog, 2-amino-4-oxo-chloropentanoic acid, inhibits synthetase activity with glutamine but not with NH_3, suggesting two separate binding sites (51); (*c*) this analog binds

covalently at an —SH group on the light subunit; and (*d*) sulfhydryl reagents greatly increase the glutaminase activity of the intact enzyme (52,52a).

Trotta et al. (49,52) propose that a nucleophilic site for hydrolysis of the glutamine amide group is located on the light chain and that this subunit plays a critical role in the orientation of glutamine and in the orientation of the NH_3 formed in relation to the active site on the heavy subunit.

d. Allosteric Regulation. The synthetase is subject to feedback inhibition by uracil, by arginine, and by pyrimidine nucleotides, whereas purine nucleotides and ornithine stimulate activity. Thus biosynthesis along two metabolic pathways is controlled by regulation of the enzyme that is located at the point of pathway branching. The nucleotide effectors exert their allosteric influence by altering the affinity of the enzyme for ATP (53). The effector sites are located on the heavy subunit (Fig. 1).

The new role assigned to glutamine explains why the stoichiometry of ATP utilization is the same for reaction 2 as for reaction 3, and why it is not changed by substitution of NH_3 for glutamine in reaction 3.

Meister and his associates suggest that bacterial glutamine-dependent carbamyl phosphate synthetases may have evolved by the acquisition of a second polypeptide chain which conferred the ability to replace NH_3 with glutamine as the nitrogen source (49). It is tempting to speculate further that the occurrence of glutamine synthetase in bacterial metabolism and the ability to utilize the amide nitrogen of glutamine may represent nitrogen-conserving measures evolved to combat the loss of free NH_3. In this connection it is of interest to mention the bacterial enzyme, glutamate synthetase, recently described by Tempest and Miers (54), which catalyzes the reductive amination of α-ketoglutarate by TPNH and utilizes the amide group of glutamine as the nitrogen source, in contrast to glutamic dehydrogenase.

Glutamine and the light subunit appear to have the same role in determining the active conformation of bacterial carbamyl phosphate synthetase that is filled by acetylglutamate for the liver enzyme. In lower organisms, such as bacteria, yeast, and *Neurospora*, acetylglutamate is a metabolic intermediate in ornithine biosyn-