ON THE AVAILABILITY OF INTRAMITOCHONDRIAL CARBAMOYLPHOSPHATE FOR THE EXTRAMITOCHONDRIAL BIOSYNTHESIS OF PYRIMIDINES*

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SUMMARY:

Carbamoylphosphate synthesized inside the mitochondria is not compartmentally isolated but, rather, is readily available for extramitochondrial reactions and may constitute a major source of carbamoylphosphate for the biosynthesis of pyrimidines.

INTRODUCTION:

It is generally accepted that the orotate pathway is the major source of the pyrimidine components of DNA and RNA. The sequence of reactions leading to the formation of pyrimidines by this pathway is initiated by the enzyme carbamoylphosphate synthetase (CPS). This enzyme has been shown to exist in two different forms in mammalian tissues: (a) CPS-I which is localized in the mitochondrial fraction of the cell, activated by N-acetyl-L-glutamate, and utilizes bicarbonate, ATP, and ammonia for the synthesis of carbamoylphosphate (CP), and (b) CPS-II which is localized in the soluble fraction of the cell and preferentially utilizes glutamine as the source of nitrogen for the synthesis of CP. Assuming compartmentation within the cell, it has been proposed that CPS-I is the source of CP for the production of urea, the first two reactions of the urea cycle being localized in the mitochondria, while CPS-II is the source of CP for the biosynthesis of pyrimidines, the enzymes of the orotate pathway being localized in the cytosol (Hager and Jones, 1967; Kerson and Appel, 1968). However, the extent to which the CP produced by CPS-I is confined to the mito-

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chondria has not been examined and the ability of the cytoplasmic CPS-II to provide CP for the biosynthesis of pyrimidines appears to be limited since the enzyme has been found only in fetal rat liver (Hager and Jones, 1967), ascites cells (Hager and Jones, 1967), and hematopoietic spleen (Tatibana and Ito, 1967), and only at low levels of activity in these tissues. The purpose of this communication is to assess the extent of compartmentation of the CP synthesized in the mitochondria by measuring the potential of the mitochondrial CPS-I to contribute CP to the soluble portion of the cell for the biosynthesis of pyrimidines.

METHODS:

Mitochondria were isolated in 0. 25 M sucrose from the livers of 200 g male rats of the Charles River Colony according to the method of Hogeboom (1955) with the centrifugal speeds recommended by Myers and Slater (1957). Analysis of the activity of CPS-I in intact mitochondria was accomplished by using ^{14}C-bicarbonate as one of the substrates and coupling the reaction with the ornithine carbamoyltransferase (OCT) endogenous to the mitochondria, thus forming ^{14}C-citrulline. The capacity of CPS-I of intact mitochondria to provide CP for extramitochondrial reactions was assessed by determining the amount of CP converted to carbamoylaspartate when aspartate and aspartate carbamoyltransferase (ACT), extracted from Escherichia coli, were added to the reaction mixture. In all experiments the reaction was stopped by the addition of hydrochloric acid and the contents of each tube boiled 5 minutes to expel the unreacted ^{14}C-bicarbonate. The amount of acid-stable radioactivity, representing citrulline or carbamoylaspartate, was determined in a liquid scintillation spectrometer. The identity of the acid-stable radioactivity has been confirmed using the chromatographic system of Ciardi and Anderson (1968).

RESULTS:

The data in TABLE I show that mitochondrial preparations incorporate ^{14}C-bicarbonate into acid-stable product by a system having the characteristics of CPS-I coupled with OCT (Reactions 1-5) or ACT (Reactions 6-9). Omission of NH_4Cl, ATP, or the activator N-acetyl-L-glutamate, all required for CPS-I activity, markedly reduced the production of acid-stable radioactivity (Reactions 1-4). Omission of ornithine provides evidence that the acid-stable radioactivity in the complete system represents citrulline production through

the coupling of CPS-I with OCT endogenous to the mitochondria (Reactions 1 and 5). Substitution of aspartate and ACT for ornithine shows that the amount of acid-stable radioactivity obtained by coupling CPS-I with extramitochondrial ACT is comparable to that seen when CPS-I is coupled with intramitochondrial OCT (Reactions 1 and 8). The omission of either aspartate or ACT markedly reduces the formation of acid-stable radioactivity providing evidence that the product is carbamoylaspartate (Reactions 6-8). Virtually no carbamoyl-aspartate is produced in the absence of mitochondria (Reaction 9).

TABLE I

SYNTHESIS OF CP BY ISOLATED MITOCHONDRIA AND ITS UTILIZATION FOR INTRAMITOCHONDRIAL AND EXTRAMITOCHONDRIAL REACTIONS

Reaction Number	Description	mμ moles product minute · mg protein
1.	Complete	9.41
2.	-N-acetyl-L-glutamate	1.30
3.	-NH_4Cl	1.50
4.	-ATP	0.15
5.	-Ornithine	0.18
6.	-Ornithine + Aspartate	1.64
7.	-Ornithine + E. coli ACT, 22 units	1.92
8.	-Ornithine + Aspartate + E. coli ACT	7.22
9.	-Ornithine - Mitochondria +Aspartate + E. coli ACT	0.35

Reactions were conducted at 37°C for 15 minutes with each complete incubation mixture (1 ml) containing: Tris-HCl, pH 8.0, 30 mM; $NaH^{14}CO_3$, 34 μC/m mole, 25 mM; mitochondrial protein, 3 mg; NH_4Cl, 10 mM; N-acetyl-L-glutamate, 10 mM; ATP, 7 mM; L-ornithine (or L-aspartate), 20 mM; and sucrose to make the incubation mixture isoosmotic. The reaction was stopped by the addition of 0.25 ml 1N HCl, the acidified reaction mixture placed in a boiling water bath for 5 minutes, and the solution neutralized before counting. Separate analysis of ACT and OCT under these conditions showed the activity of each to be approximately equal. The preparation of ACT is the product of the heat-denaturation step of the purification procedure of Shepherdson and Pardee (1960); 1 unit of ACT produces 1 μmole carbamoylaspartate per hr. at 37°C.

The experiments summarized in TABLE I suggest that the CP synthesized intramitochondrially by the action of CPS-I is readily available for extramitochondrial reactions and may serve as a source of CP for the biosynthesis of pyrimidines. This interpretation of the data is contingent upon the structural integrity of the mitochondria. Therefore, the possibility that lysed mitochondria supplied the CP utilized by ACT was examined.

TABLE II

THE SYNTHESIS OF THE CP BY ISOLATED MITOCHONDRIA AT THE EXPENSE OF EXOGENOUS ATP OR ATP GENERATED ENDOGENOUSLY AND THE UTILIZATION OF CP FOR INTRAMITOCHONDRIAL AND EXTRAMITOCHONDRIAL REACTIONS

SERIES A: ATP generated endogenously	mμ moles product / minute · mg protein	
Addition	No Inhibitor	DNP, 50 μM
1. None (Control)	2.05	1.08
2. + Aspartate (CPS-I coupled with ACT)	7.74	1.95
3. + Ornithine (CPS-I coupled with OCT)	12.00	2.84

SERIES B: ATP added exogenously (7 mM)		Atractyloside, 60 μM
1. None (Control)	1.75	0.88
2. + Aspartate (CPS-I coupled with ACT)	6.91	3.35
3. + Ornithine (CPS-I coupled with OCT)	12.50	6.62

SERIES C: Without ATP	
1. None (Control)	0.36
2. + Aspartate (CPS-I coupled with ACT)	0.43
3. + Ornithine (CPS-I coupled with OCT)	0.36

The components of each incubation mixture and the conditions for assay were as described in the legend of TABLE I except that: (a) ACT of E. coli was included in all reaction mixtures and ornithine or aspartate were added only where indicated and (b) ATP was omitted in SERIES C and the following substrates for the endogenous generation of ATP were substituted for ATP in SERIES A: β-hydroxybutyric acid 10 mM; ADP, 5 mM; inorganic phosphate, 5 mM.

One of the properties of intact mitochondria which may be used to assess their structural integrity is respiratory control (i.e. the coupling of oxidation with phosphorylation), the loss of which allows oxidation to proceed independent of phosphorylation. Our preparations of mitochondria were found to be under respiratory control as judged by measurements of oxygen consumption in the presence of succinate plus or minus ADP, inorganic phosphate, and inhibitors of oxidative phosphorylation. A more direct evaluation of the status of the mitochondria contributing CP to the synthesis of carbamoyl-

aspartate was made by replacing exogenous ATP as a substrate for CPS-I with the ATP derived from oxidative phosphorylation. Under these conditions only intact coupled mitochondria should be capable of synthesizing ATP and, consequently, CP. β-Hydroxybutyric acid was employed as a substrate for oxidation and added ADP and inorganic phosphate as substrates for coupling oxidation with phosphorylation to generate ATP. The ability of this system to contribute CP for the biosynthesis of citrulline or carbamoylaspartate was tested with and without 2, 4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation. The results, given in TABLE II, SERIES A, indicate that the CP produced from endogenously generated ATP is readily available for the extramitochondrial synthesis of carbamoylaspartate (Reaction 2) or the intramitochondrial synthesis of citrulline (Reaction 3). The dependency of this system upon the endogenous generation of ATP is illustrated by the inhibitory action of DNP. The possibility that the ATP generated by intact mitochondria was exported and utilized by CPS-I of lysed mitochondria was excluded by the addition of glucose and hexokinase to deplete the system of extramitochondrial ATP.

These additions did not inhibit the production of carbamoylaspartate. The amount of glucose and hexokinase added was adaquate to block the production of carbamoylaspartate in the presence of 7 mM ATP. These experiments show that the CP synthesized by intact mitochondria is available for extramitochondrial reactions. In order to estimate the fraction of mitochondria which is intact a similar set of reactions was conducted replacing the substrates for the endogenous production of ATP with exogenously added ATP. The results are given in TABLE II, SERIES B. When SERIES A is compared to SERIES B it can be seen that the amount of carbamoylaspartate or citrulline formed through the coupling of CPS-I with extramitochondrial ACT or intramitochondrial OCT is comparable whether the source of ATP is exogenous or endogenous. Thus the CP used for the extramitochondrial synthesis of carbamoylaspartate in both SERIES A and B appears to be derived entirely from intact mitochondria in which oxidation is coupled with phosphorylation. Inhibition of the formation of either carbamoylaspartate or citrulline in SERIES B by the addition of atractyloside, an inhibitor of the entry of ATP into the mitochondria (Klingenberg and Pfaff, 1966), provides additional evidence that the mitochondria are intact. Neither DNP nor atractyloside inhibit the catalytic

activity of CPS-I of lysed mitochondria when ATP is added to the assay system. SERIES C is included as a control series to assess the amount of acid-stable radioactivity, calculated in terms of amount of carbamoylaspartate or citrulline, formed in the absence of exogenous ATP or the substrates for the endogenous generation of ATP.

In conclusion, the carbamoylphosphate synthesized intramitochondrially through the action of CPS-I is not compartmentally isolated in that organelle but, rather, is readily available for extramitochondrial reactions and may constitute a major source of CP for the biosynthesis of pyrimidines.

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