PYRIMIDINE BIOSYNTHESIS IN RAT LIVER: STUDIES ON THE SOURCE OF CARBAMOYLPHOSPHATE*

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Received September 14, 1971

SUMMARY:

The incorporation of labeled bicarbonate into RNA in tissue slices of rat liver exhibits many of the same properties found for the synthesis and export of carbamoylphosphate by isolated intact mitochondria prepared from the same tissue. Thus the intramitochondrial carbamoylphosphate synthetase is shown to contribute carbamoylphosphate toward the biosynthesis of pyrimidines in the cytoplasm.

INTRODUCTION:

The biosynthesis of carbamoylphosphate (CP) serves as the initial step in two separate metabolic pathways: (a) the pathway for the biosynthesis of arginine which, in mammals, provides for the detoxication of ammonia through the urea cycle, and (b) the orotate pathway leading to the production of the pyrimidine components of DNA and RNA. Although there are two distinct enzymes in mammalian tissues which catalyze the biosynthesis of CP, it is generally believed that the product of each enzyme is compartmentally isolated and that this compartmental isolation leads to the exclusive use of each pool of CP by a single metabolic pathway. Thus, in mammalian species, the N-acetyl-L-glutamate-activated carbamoylphosphate synthetase (CPS-I) is thought to be the sole source of CP consumed by the urea cycle, while the glutamine-dependent carbamoylphosphate synthetase (CPS-II) is assumed to be the exclusive source of the CP incorporated into pyrimidine nucleotides (1, 2). This hypothesis is primarily based upon the fact that CPS-I and ornithine carbamoyltransferase (OCT), the second enzyme of the urea cycle, are localized within the mitochondrial fraction of the cell, while CPS-II and the enzymes

*This work was supported by grant numbers HD-03168 and AM-15186 from the National Institutes of Health.

**The work presented herein is in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences.

of the orotate pathway are localized in the cytosol.

We recently reported that the CP synthesized by isolated intact mitochondria is readily available for extramitochondrial utilization, thereby challenging the hypothesis that intramitochondrial CP is compartmentally isolated, an hypothesis for which no other direct experimental evidence is available, and raising the possibility that intramitochondrial CPS-I provides CP for pyrimidine biosynthesis (3). In the present communication the incorporation of labeled bicarbonate into CP by isolated intact mitochondria and the incorporation of labeled bicarbonate into RNA by liver slices exhibit many of the same properties, from which it is inferred that intramitochondrial CPS-I does indeed contribute CP toward the cytoplasmic biosynthesis of hepatic pyrimidines.

RESULTS:

Synthesis of CP from labeled bicarbonate by isolated intact mitochondria is measured by coupling CPS-I with endogenous OCT and the export of CP is assessed by coupling CPS-I with extramitochondrial ACT, prepared from Escherichia coli and added in excess. In addition to the requirements reported earlier (3) for ammonium ion and N-acetyl-L-glutamate for the synthesis of CP by isolated intact mitochondria, we find that the availability of ATP, one of the substrates for CPS-I, is rate-limiting, presumably because ATP does not readily enter mitochondria rich in high energy intermediates of oxidative phosphorylation (4). The addition of 2, 4-dinitrophenol (DNP) and oligomycin, which blocks the generation of high energy intermediates of oxidative phosphorylation from both the oxidation of endogenous substrate (DNP) and the hydrolysis of ATP (oligomycin), causes and 11 fold increase in the synthesis of CP (compare A and B for CPS-I--OCT in TABLE I). Although CP is equally available for intramitochondrial and extramitochondrial utilization (compare A for CPS-I--OCT with A for CPS-I--ACT), apparently not all the CP synthesized under these conditions is exported since export is accelerated only half as much as synthesis (compare A and B for CPS-I--OCT with A and B for CPS-I-ACT). When the mitochondria are made completely dependent upon an exogenous source of ATP for CP synthesis by the addition of DNP and oligomycin, the coupled action of CPS-I and OCT is inhibited 92% by the addition of atractyloside, an inhibitor of the transport of adenine nucleotides across the mitochondrial membrane (5) which has no

TABLE I

PROPERTIES OF THE SYNTHESIS AND EXPORT OF CARBAMOYL-PHOSPHATE (CP) BY ISOLATED INTACT MITOCHONDRIA

ADDITIONS	CPS-I--OCT	CPS-I--ACT
	citrulline	carbamoylaspartate
	(nanomoles/minute · mg mitochondrial protein at 37.5°)	
A. None (control)	5.5 ± 1.0	7.2 ± 0.8
B. 2,4-Dinitrophenol (50 μM) + Oligomycin (3 μg)	61.9 ± 4.0	37.7 ± 6.7
C. 2,4-Dinitrophenol (50 μM) + Oligomycin (3 μg) + Atractyloside (60 μM)	5.1 ± 1.3	8.6 ± 0.5

Mitochondria were isolated in 0.25 M sucrose from the livers of 250 g male rats from the Charles River Colony and the activity of CPS-I was determined by measuring the rate of incorporation of ^{14}C-bicarbonate into citrulline through the coupled action of CPS-I and endogenous OCT as described previously (3). Each incubation mixture contained, unless stated otherwise, the following components in a final volume of 1 ml: Tris-HCl, pH 7.5, 30 mM; $NaH^{14}CO_3$, 34 μCi/m mole, 25 mM; NH_4Cl, 10 mM; ATP, 7 mM; $MgCl_2$, 10 mM; N-acetyl-L-glutamate, 10 mM; L-ornithine, 10 mM; 3 mg mitochondrial protein. The export of CP was measured by omitting L-ornithine from the reaction mixture and converting extramitochondrial CP to carbamoylaspartate through the addition of aspartate, 20 mM, and aspartate carbamoyltransferase (ACT), free from CPS activity and added in excess, purified from Escherichia coli according to the procedure of Shepherdson and Pardee through the ammonium sulfate fractionation step (7). Each value represents the mean of 3 separate experiments ± the standard deviation.

effect on CPS-I--OCT of lysed mitochondria (6), (compare B with C for CPS-I--OCT). The coupled action of CPS-I and ACT is also inhibited by atractylo side, but to a lesser extent (77%) (compare B with C for CPS-I--ACT). The sensitivity of these reactions to atractyloside also attests to the structural integrity of our preparations of mitochondria.

Having established some of the properties characteristic for the synthesis of CP by isolated mitochondria, we proceeded to determine whether there was evidence for the participation of CPS-I in pyrimidine biosynthesis in tissue slices of the rat liver. The incorporation of bicarbonate into RNA

TABLE II

PROPERTIES OF THE INCORPORATION OF BICARBONATE INTO RNA BY TISSUE SLICES OF RAT LIVER

Expt. No.	Additions	(N)	Nanomoles bicarbonate incorporated into RNA per g liver	per mg RNA
1.	None	(11)	6.47 ± 0.39	1.08 ± 0.06
2.	NH_4Cl, 10 mM	(3)	7.64 ± 0.26	1.23 ± 0.02
3.	Glutamine, 10 mM	(3)	6.29 ± 0.35	1.03 ± 0.02
4.	N-acetyl-L-glutamate (NAG, 10 mM)	(3)	12.50 ± 0.45	2.08 ± 0.07
5.	NH_4Cl + NAG	(7)	13.09 ± 0.98	2.25 ± 0.20
6.	2, 4-Dinitrophenol (DNP), 50 μM + Oligomycin (OLIGO), 200 μg	(5)	2.73 ± 0.14	0.45 ± 0.03
7.	DNP + OLIGO + ATP, 5 mM	(3)	21.33 ± 0.75	3.51 ± 0.11
8.	DNP + OLIGO + ATP + NH_4Cl	(3)	37.89 ± 1.94	6.23 ± 0.05
9.	DNP + OLIGO + ATP + glutamine	(3)	20.94 ± 1.03	3.38 ± 0.23
10.	DNP + OLIGO + ATP + NH_4Cl + NAG	(10)	63.03 ± 7.35	11.30 ± 2.70
11.	DNP + OLIGO + ATP + NH_4Cl + NAG + Atractyloside 120 μM	(9)	26.25 ± 2.13	4.68 ± 0.39

Tissue slices, prepared from the liver of 250 g male rats of the Charles River Colony, were incubated for 1 hour at 37°C in Krebs Improved Ringer II Solution (10) containing 68 μCi of ^{14}C-$NaHCO_3$ at a final concentration of 4.67 mM. The incorporation of labeled bicarbonate into RNA was determined by measuring the release of acid soluble labeled nucleotides by mild alkaline hydrolysis of the RNA contained in the acid-insoluble lipid-free fraction of the reaction mixture. The procedures for the preparation, hydrolysis, and colorimetric analysis of RNA were essentially the same as those previously employed (11). Each value represents the mean ± the standard deviation; the number of separate observations represented by each mean is given in the column labeled "(N)".

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is stimulated slightly by the addition of ammonium chloride (TABLE II, Expts 1 and 2) but unaffected by the addition of glutamine, the preferred substrate of CPS-II (Expt 3). The addition of N-acetyl-L-glutamate, and activator of CPS-I which is without effect on CPS-II, doubles the rate of incorporation of bicarbonate into RNA (Expts 4 and 5). These findings are consistent with the interpretation that the incorporation of bicarbonate into RNA is mediated by CPS-I. Additional evidence supporting this interpretation is given in Expts 6, 7 and 10. The addition of DNP and oligomycin affects the incorporation of bicarbonate into RNA and the activity of CPS-I of isolated intact mitochondria in the same manner; a dependency upon an exogenous source of ATP is created (Expt 6), but when ATP is supplied, the incorporation of bicar-

bonate into RNA (and the activity of CPS-I of isolated intact mitochondria) is greatly increased (compare Expts 1, 7 and 10 of TABLE II with A and B for CPS-I--OCT in TABLE I, respectively). The dependency of the incorporation of bicarbonate into RNA on ammonium ion is more apparent in the presence of DNP and oligomycin (Expts 7 and 8) whereas glutamine is still without effect (Expts 7 and 9). The addition of atractyloside to the system dependent upon an exogenous source of ATP has the same effect on the incorporation of bicarbonate into RNA in tissue slices as it does on the synthesis and export of CP by isolated mitochondria; both processes are significantly inhibited (compare Expts 10 and 11 of TABLE II with B and C for CPS-I--OCT and CPS-I--ACT in TABLE I, respectively). Since atractyloside only slightly inhibited the incorporation of ^{14}C-ATP into RNA and did not inhibit the incorporation of ^{14}C-carbamoylaspartate into RNA (8), it can be stated with reasonable certainty that the drug is acting by blocking the intramitochondrial synthesis of CP and not the transport of ATP across the cell membrane or any reaction beyond ACT involved in the incorporation of bicarbonate into RNA. Evidence that bicarbonate is being incorporated into RNA via the orotate pathway includes observations that the incorporation is inhibited more than 80% by the addition of unlabeled intermediates of the pathway or by the addition of 6-azauridine, an inhibitor of the conversion of orotic acid to UMP (9).

The above comparison of the properties for the incorporation of bicarbonate into RNA in slices of rat liver with the properties for the incorporation of bicarbonate into CP in isolated mitochondria, provides strong evidence that the intramitochondrial CPS-I supplies CP for the biosynthesis of pyrimidines in rat liver. A parallel set of experiments employing rat kidney yielded essentially the same results; the incorporation of bicarbonate into RNA of kidney slices exhibits properties characteristic of CPS-I (8). We are currently attempting to assess the contribution of the cytoplasmic CPS-II toward hepatic pyrimidine biosynthesis; preliminary experiments indicate that the addition of azaserine, in amounts reported to significantly inhibit CPS-II, is without effect on the incorporation of bicarbonate into pyrimidines. These studies, with those described above, indicate that the major source of CP incorporated into hepatic pyrimidines is CPS-I.

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