THE UTILIZATION OF INTRAMITOCHONDRIALLY GENERATED CARBAMYL PHOSPHATE FOR MICROSOMAL GLUCOSE 6-PHOSPHATE BIOSYNTHESIS

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1. Introduction

The multifunctional nature of the classical glucose-6-phosphatase (EC 3.1.3.9) of microsomes has been established over the last 7 yr (see [1–4]). Glucose-6-phosphatase catalyzes the hydrolysis of glucose 6-phosphate (eq. 1) as well as several hydrolytic and synthetic reactions involving nucleoside di- or triphosphates, pyrophosphate, and sugar (or polyol) phosphates. Recently we [5–7] described a new synthetic activity of the enzyme, carbamyl phosphate: glucose phosphotransferase (eq. 2), which under certain conditions considerably exceeds the glucose-6-P phosphohydrolase activity of this catalyst.

Carbamyl-P is produced intramitochondrially for urea synthesis by an *N*-acetylglutamate (AG) dependent carbamyl-P synthetase [8–10], and by another carbamyl-P synthetase located extramitochondrially for pyrimidine biosynthesis [11–13]. Natale and Tremblay [14] have recently shown that it is physiologically feasible to utilize carbamyl-P synthesized intramitochondrially for extramitochondrial pyrimidine biosynthesis. We describe here similar studies demonstrating the feasibility of utilizing intramitochondrially synthesized carbamyl-P for microsomal glucose-6-P synthesis (eq. 3).

$$\text{Glucose-6-P} + H_2O \rightarrow \text{glucose} + P_i \quad (1)$$

$$\text{Carbamyl-P} + \text{glucose} \rightarrow \text{glucose-6-P} + \text{carbamate} \quad (2)$$

$$HCO_3^- + NH_4^+ \xrightarrow[\text{acetylglutamate}]{2\ ATP \quad 2ADP + P_i} \text{carbamyl-P}$$

carbamyl-P synthetase (mitochondria)

$$\text{carbamyl-P} + \text{glucose} \xrightarrow{\text{carbamyl-P: glucose phosphotransferase (microsomal)}} \text{glucose-6-P} + \text{carbamate} \quad (3)$$

2. Materials and methods

ATP, *N*-acetylglutamate (AG), β-hydroxybutyrate AMP, and glucose were purchased from Sigma Chemical Co. *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) was obtained from Calbiochem. Deoxycholic acid ("special enzyme grade") was obtained from Mann. All other chemicals were ACS grade or better. Oligomycin was a gift of Dr. H.A. Lardy.

Livers from adult male albino rats weighing between 200–250 g, obtained from Sprague-Dawley, Inc., Madison, Wisc., USA, were employed in all studies. Mitochondria were isolated by the method of Johnson and Lardy [15] and suspended in 0.25 M sucrose to a volume equal to the original liver weight. Microsomes were prepared as previously described [16].

Reactions were carried out for 40 min at 30°, stopped with perchloric acid, neutralized, centrifuged and glucose-6-P determined in the resulting supernat-

Table 1
Glucose-6-P synthesis in systems with exogenous ATP.

Reaction mixture number	Composition	Glucose-6-P formed (nmoles)
	Intact mitochondria	
1	Complete	81.6
2	Minus AG	49.2
3	Minus ATP	3.9
4	Minus ATP, minus AG	3.9
5	Complete + 2 μg oligomycin	101.8
6	Complete + 5 mM L-ornithine	66.1
7	Minus mitochondria	4.6
	Deoxycholate-lysed mitochondria	
8	Complete	114.1
9	Minus AG	7.2
	Sonicated mitochondria	
10	Complete	99.6
11	Minus AG	10.7

Complete reaction mixtures, pH 7.5, contained in 1.5 ml, 30 mM $NaHCO_3$, 10 mM NH_4Cl, 10 mM $MgCl_2$, 180 mM glucose, 10 mM acetylglutamate (AG), 10 mM HEPES buffer, 6.2 mg mitochondrial protein, and 1.2 mg microsomal protein. The deoxycholate-lysed mitochondria, utilized where indicated, were prepared by suspending the mitochondria in 0.25 M sucrose containing 0.2% deoxycholate (w/v). Sonicated mitochondria were prepared according to the method of Henning and Seubert [17].

ant solution [16]. The composition of the reaction mixtures is given in tables 1 and 2.

3. Results and discussion

3.1. *General*

Results of studies in which glucose-6-P synthesis was measured in a reconstituted system which, in its entirety, consisted of a mitochondrial carbamyl-P generating system [8–10] plus added glucose and microsomal preparations, are described in tables 1 and 2. Table 1 depicts results of studies in which ATP was supplied exogenously, while table 2 presents the results of experiments in which ATP for carbamyl-P synthetase activity was generated endogenously via mitochondrial oxidative phosphorylation. The rat liver mitochondria utilized, as specifically indicated in the tables, were either intact, freshly prepared suspensions, or such preparations which had been lysed with deoxycholate or by sonic disintegration. Mitochondria and microsomal fractions were recombined in all systems studied in the same proportions that obtain in liver homogenates; such preparations from 0.26 g wet liver were included per assay mixture.

Table 2
Glucose-6-P synthesis in systems with endogenously generated ATP.

Reaction mixture number	Composition	Glucose-6-P formed (nmoles)
	Intact mitochondria	
1	Complete	34.3
2	Minus AG	16.8
3	Complete + 2 μg oligomycin	9.0
	Deoxycholate-lysed mitochondria	
4	Complete	16.2
5	Minus AG	5.2

Complete reaction mixtures, pH 7.5 contained in 1.5 ml, 30 mM $NaHCO_3$, 10 mM NH_4Cl, 10 mM $MgCl_2$, 180 mM glucose, 10 mM acetylglutamate (AG), 10 mM AMP, 10 mM β-hydroxybutyrate, 5 mM Na_2HPO_4, 40 mM HEPES buffer, 5.0 mg mitochondrial protein, and 1.0 mg microsomal protein. The lysed mitochondria were prepared as in table 1.

3.2 *Synthesis with exogenous ATP*

The data in table 1 indicate that carbamyl-P synthesized intramitochondrially utilizing exogenously supplied ATP is readily available for extramitochondrial glucose-6-P synthesis. As the system is constructed (see legend to table 1), it shows an almost absolute dependence on ATP (compare lines 3 and 1) and mitochondria (lines 7 and 1), and a partial dependence on added AG (lines 2 and 1). Oligomycin, an effective inhibitor of mitochondrial ATPase as well as oxidative phosphorylation, slightly enhances activity under these conditions (line 5). Ornithine, which reacts with carbamyl-P in the presence of mitochondrial ornithine transcarbamylase to produce citrulline [18], inhibits (see line 6, table 1), as would be expected if ornithine competes with glucose for the available supply of carbamyl-P.

Under these conditions, with exogenously supplied ATP, highly significant amounts of glucose-6-P formation were also observed with the complete system in which either deoxycholate-lysed (line 8) or sonically disrupted mitochondria (line 10) were substituted for their intact counterpart (line 1). In these instances, a nearly absolute dependence of glucose-6-P synthesis on the presence of AG were noted (compare lines 9 and 11 with lines 8 and 10, respectively). These observations and the noted lesser extent of dependence on added AG seen with intact mitochondria (see lines 2 and 1) are consistent with the earlier observations of Cohen and Grisolia [19, 20] that carbamyl-P synthesis in intact mitochondria is less highly dependent on added acylglutamate than is the more highly purified carbamyl-P synthetase [8, 10].

Based on the observed dependence of the system on AG, the noted inhibitory effects of ornithine on glucose-6-P formation, and the fact that it has been thoroughly established in this laboratory [6] that ATP, ADP, or AMP will not transfer phosphoryl groups directly to glucose in the presence of microsomal glucose-6-phosphatase at pH values above 7, it is concluded that the observed glucose-6-P synthesis is primarily or entirely a result of carbamyl-P synthesis coupled with carbamyl-P: glucose phosphotransferase as given in eq. 3. The latter activity of microsomal glucose-6-phosphatase, in marked contrast with corresponding activity with nucleotides, is quite potent even at and above pH 7 [5–7].

3.3. *Synthesis with endogenously generated ATP*

The potential physiological significance of the considerations described here is contingent upon the structural integrity of mitochondria in the experiments presented. A direct evaluation of mitochondrial structural integrity was made by replacing added ATP with endogenously generated ATP, utilizing AMP, P_i, and β-hydroxybutyrate as the ATP-generating system (table 2). AMP was chosen rather than ADP as the phosphoryl acceptor for oxidative phosphorylation to avoid possible complications due to the mitochondrial synthesis of ATP by non-oxidative processes, e.g. by adenylate kinase [21–23]. Significant synthesis of glucose-6-P was observed under these conditions (line 1, table 2), and was considerably diminished in the absence of AG (line 2, table 2). In the presence of oligomycin, a potent inhibitor of oxidative phosphorylation linked with mitochondrial electron transport, glucose-6-P formation was reduced very considerably (see line 3, table 2), in contrast with the effects of this compound noted with exogenously supplied ATP (see lines 5 and 1, table 1). Lysis of mitochondria led to a marked decrease in glucose-6-P generation under these conditions (see lines 4 and 1, table 2), in marked contrast with the response noted with added ATP (see lines 8 and 1, table 1), and an increased sensitivity to AG (compare lines 5 and 4, table 2).

3.4. *Studies with kidney preparations*

Supplementary studies similar to those above also were conducted with a rat kidney renal reconstituted particulate system. Glucose-6-P production attributable to prior carbamyl-P synthesis could not be demonstrated. It has been established [24] that the level of renal carbamyl-P synthetase is only approx. 3% that of hepatic tissue; hence it remains to be established whether carbamyl-P: glucose phosphotransferase has any physiological significance in the kidney.

3.5. *Conclusions*

As mentioned above, liver contains, in addition to mitochondrial carbamyl-P synthetase, a cytosolic carbamyl-P synthetase which is thought to provide carbamyl-P as a precursor for pyrimidine biosynthesis [11–13]. The presence of such extramitochondrially synthesized carbamyl-P, together with the extramitochondrial availability of intramitochondrially synthesized carbamyl-P as demonstrated here and elsewhere [14], suggests that glucose-6-P generation via phosphotransferase activity of microsomal glucose-6-phosphatase-phosphotransferase (eq. 2) may be physiologically feasible. These observations thus add further support to our earlier arguments [7], based on kinetic considerations, in favor of a biologically important phosphorylative role in liver for this key multifunctional catalyst.

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