Review Letter

Rapid conversion of newly-synthesized orotate to uridine-5-monophosphate by rat liver cytosolic enzymes

Kottarappat N. Dileepan and James Kennedy

Enzymology Research Laboratory, Veterans Administration Medical Center, 4801 Linwood Boulevard, Kansas City, MI 64128, USA

Received 15 December 1982

It had been noted previously that the activity of mitochondrial dihydroorotate dehydrogenase was lower in crude tissue preparations containing cytosol than in isolated mitochondria. Closer examination reveals that the apparent lower enzyme activity is due to rapid conversion of newly-synthesized orotate to uridine-5-monophosphate by the cytosolic enzymes, orotate phosphoribosyltransferase and orotidylate decarboxylase.

Dihydroorotate dehydrogenase *Effect of cytosol* *Apparent inhibition*
Orotate *Pyrimidine* *Mitochondria*

1. INTRODUCTION

In eukaryotic cells, 5 of the 6 enzymes involved in the biosynthesis of pyrimidines reside in the cytosol as two distinct complexes [1,2]. The first complex contains 3 enzymes which catalyze the initial steps and the second complex contains the last 2 enzymes of the pathway. Dihydroorotate dehydrogenase (DHO-DHase), the fourth enzyme of the sequence, catalyzes the oxidation of dihydroorotate to orotate. In eukaryotes this enzyme is located in mitochondria [3,4] and is linked to the electron transport chain [5–7].

Artificial electron acceptors are generally employed for dihydroorotate dehydrogenase assays but, because of competing reactions, they are unsatisfactory for use with crude tissue preparations. In the latter setting, methods based on direct quantitation of the amount of orotate formed have been used. These include spectrophotometric quantitation of orotate [3] or radiometric assays based on the conversion of [4-^{14}C]dihydroorotic acid to [4-^{14}C]orotic acid and subsequent isolation of the product by electrophoretic [8] or chromatographic [1,9] procedures.

Previously, it had been reported that total dehydrogenase activity in isolated, intact mitochondria exceeded that in whole homogenates [3]. It was suggested that cytosol might contain an inhibitor of the mitochondrial enzyme. Subsequently, the same observation was repeated and ascribed to conversion of dihydroorotate to carbamylaspartate by the enzyme, dihydroorotase, thereby making the concentration of dihydroorotate rate-limiting for DHO-DHase [10]. In both cases the assays were dependent on measurement of orotate accumulation. Here, we report that the lower activity of dihydroorotate dehydrogenase in the presence of cytosol appears to be due to rapid conversion of orotate to uridylic acid by two cytosolic enzymes, orotate phosphoribosyltransferase (OPRTase) and orotidylate decarboxylase (ODCase).

Abbreviations: DHO-DHase, dihydroorotate dehydrogenase (EC 1.3.3.1); ODCase, orotidylate decarboxylase (EC 4.1.1.23); OPRTase, orotate phosphoribosyltransferase (EC 2.4.2.10); PRPP, 5-phosphorylribose-1-pyrophosphate; UMP, uridine-5-monophosphate

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

2. MATERIALS AND METHODS

2.1. *Materials*

L-dihydroorotate, phosphoribosylpyrophosphate, orotic acid, yeast OPRTase, yeast ODCase, Tris, bacterial DHO-DHase (partially purified from *Z. oroticum*), crystalline yeast alcohol dehydrogenase and other chemicals were obtained from Sigma. [carboxyl-^{14}C]Orotic acid, Bray's scintillation solution and Aquasol 2 were purchased from New England Nuclear. L-5,6-[carboxyl-^{14}C]DHO was enzymatically prepared and purified by the method in [11].

2.2. *Preparation of rat liver mitochondrial particles and cytosol*

Livers from 150–200 g male Sprague–Dawley rats were homogenized in 9 volumes of 0.25 M sucrose, and mitochondria were isolated by centrifugation of the 600 × *g* supernatant at 8000 × *g*. The mitochondrial pellet was washed twice in 0.15 M KCl and suspended in 0.15 M Tris–HCl (pH 7.5) to 1/3 of the original homogenate. The mitochondrial preparation was used immediately for experiments with intact mitochondria or frozen at −20°C. The thawed mitochondrial particles were washed with 0.15 M Tris–HCl (pH 7.5), collected by centrifuging at 10000 × *g* for 15 min and resuspended in 0.15 M Tris–HCl (pH 7.5) to 1/3 of the volume of the original homogenate.

For preparation of the cytosolic fraction, the post mitochondrial supernatant of the liver homogenate prepared in 0.15 M Tris–HCl (pH 7.5) was centrifuged at 100000 × *g* for 60 min and the supernatant fraction was collected.

2.3. *Assays of DHO-DHase*

Reaction mixture for the direct measurement of orotate formation contained: 100 mM Tris–HCl (pH 8.0), 1 mM L-dihydroorotate, mitochondrial preparation equivalent to 200–500 μg protein and, when present, the specified amount of supernatant fraction in a final volume of 2 ml. The reaction was carried out at 37°C for 30 min (unless otherwise specified) with shaking and terminated by addition of 2.0 ml of 2 N $HClO_4$. The concentration of orotate in the supernatant was measured by its absorbance at 280 nm [3] or at 290 nm as modified recently to avoid the absorbance due to UMP. Blanks without substrate were run concurrently with each experimental condition.

The radiometric assay of DHO-DHase was carried out as in [11] with minor modifications. The usual incubation system contained: 100 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 0.5 mM phosphoribosylpyrophosphate (PRPP), 0.25 mM [carboxyl-^{14}C]DHO (~250000 dpm/assay), 5 units orotate phosphoribosyltransferase, 5 units orotidylate decarboxylase and the mitochondrial preparation equivalent to 20–50 g protein in a final volume of 1.0 ml. The reactions were carried out in tightly closed Ehrlenmeyer flasks (25 ml) carrying 0.3 ml hyamine hydroxide in the suspended center wells. The flasks were incubated at 30°C for 30 min in a metabolic shaker and the reaction was terminated by injecting 0.5 ml 1 N $HClO_4$. The flasks were incubated for an additional 1 h to ensure complete absorption of $^{14}CO_2$ by hyamine hydroxide. The center wells were transferred directly to the scintillation vials and the radioactivity determined in the presence of 10 ml scintillation solution. When cytosolic fractions (prepared in 0.15 M Tris) were present, the final concentration of Tris in the assay mixture was carefully adjusted so as not to exceed 100 mM because higher concentrations of the salt inhibited the coupling enzymes. One unit of enzyme activity is defined as 1 nmol of orotate or 1 nmol of CO_2 formed in 1 min. Protein was estimated as in [12].

3. RESULTS AND DISCUSSION

Addition of 100000 × *g* supernatant from liver or brain caused an apparent dose-dependent decrease in DHO-DHase activity of mitochondrial particles when assayed spectrophotometrically (fig.1). This finding confirms earlier reports [3,10] with intact mitochondria. Substitution of partially purified dihydroorotase [13] for cytosol did not result in inhibition of dihydroorotate oxidation by isolated mitochondria (not shown), indicating that competition for DHO by DHO-DHase and the cytosolic dihydroorotase is not the cause of the apparent inhibition. Therefore, it was assumed that the decrease in orotate concentration in the presence of cytosol is due either to inhibition of DHO-DHase by some factor or factors present in the cytosol or due to partial loss of orotate as it is being produced. As indicated in table 1, the effect of cytosol on DHO-DHase could be abolished if

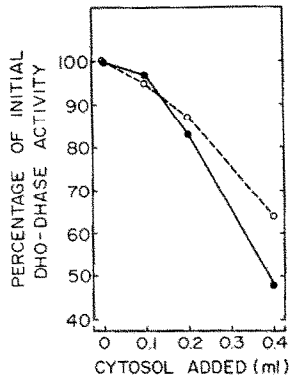

Fig.1. Effect of 100000 × g supernatant fractions from liver or brain on the activity of dihydroorotate dehydrogenase in liver (●—●) and brain (○---○) mitochondria, respectively. The enzyme was assayed spectrophotometrically as described in section 2. Protein concentrations of liver and brain supernatant fractions were 19.3 mg/ml and 5.4 mg/ml, respectively. The indicated amounts of cytosol were added at the beginning and the reaction was initiated by addition of substrate after 5 min pre-incubation. The activities of liver and brain enzymes in the absence of cytosol were 5.32 and 1.83 units/mg protein, respectively. Each value given is the average of duplicate measurements.

the supernatant fraction was either dialyzed or heated. This suggests that the cytosolic factor may be of small M_r which itself is heat labile or, a small compound acting in concert with other heat labile factors.

In order to examine the nature of the apparent inhibition of DHO-DHase by cytosol, the enzyme was assayed as in [11]. This method measures the $^{14}CO_2$ release when [carboxyl-^{14}C]DHO serves as substrate for the DHO-DHase reaction and the latter is enzymatically coupled with large excess of purified OPRTase and ODCase. If the cytosol contained any inhibitors of DHO-DHase there would be less $^{14}CO_2$ produced. On the other hand, if the cytosol did not inhibit at the level of DHO-DHase, there would not be any decrease in $^{14}CO_2$ production because the coupling enzymes will quantitatively convert all orotate into UMP and $^{14}CO_2$. As evident from table 2 addition of cytosol did not bring about any change in the $^{14}CO_2$ production whereas the same amount of cytosol brought about marked decrease in DHO-DHase activity expressed in terms of orotate formed as measured spectrophotometrically.

Cells contain orotate phosphoribosyltransferase and orotidylate decarboxylase existing as a complex (complex U) in the cytosol [1,14–17]. These two enzymes catalyze the conversion of orotate to UMP and CO_2 in the presence of phosphoribosylpyrophosphate and Mg^{2+}. Even though the complex contains two catalytic centers (orotate phosphoribosyltransferase and orotidylate decarboxylase), the decarboxylase of the complex is sufficiently active so that stoichiometric production of orotidylic acid and PP_i by the phosphoribosyltransferase can only be observed if the decarboxylation is completely inhibited [18]. Therefore, if there are sufficient concentrations of PRPP and Mg^{2+} present in the cytosol, orotate formed by DHO-DHase of the mitochondria could get converted to UMP and CO_2 by complex U. In order to examine this possibility, we studied the effect of

Table 1

Effect of heat-treated or dialyzed 100000 × g supernatant fraction on dihydroorotate dehydrogenase activity in mitochondrial particles

Addition to the assay system	DHO-DHase activity (units/mg protein)	% Decrease of activity
None (control)	4.15	–
Supernatant fraction	2.93	29
Heat-treated supernatant fraction	4.20	0
Dialyzed supernatant fraction	4.10	0

DHO-DHase was assayed by the direct observation of the appearance of orotate by measuring its absorbance at 280 nm. Heat inactivation was carried out by heating the cytosol at 50°C for 15 min. Dialysis was carried out for 20 h at 4°C with 3 changes of buffer. Supernatant fraction present in each incubation was equivalent to 6 mg cytosolic protein. Details of the assay system are given in section 2. Each value given is the mean of duplicate assays

Table 2

Comparison of the effect of 100000 × g supernatant fraction on dihydroorotate dehydrogenase activity determined by two different assay methods

Method of assay	DHO-DHase activity (unit/mg protein)		% Decrease in activity
	− Supernatant	+ Supernatant	
Spectrophotometric determination of orotate	4.11	2.38	42
Radiometric determination of CO_2 release	3.88	3.93	0

Details of the assay methods are given in section 2. Supernatant fraction present in each assay system was equivalent to 7–8 mg protein

Table 3

PRPP-dependent removal of orotate from the DHO-DHase assay system and its subsequent decarboxylation by liver cytosol

Addition to the assay system	Orotate recovered (nmol)	$^{14}CO_2$ recovered (nmol)	% Recovery of of products (orotate + CO_2)
None	28.49	0	100
Cytosol	24.11	3.72	98
Cytosol + PRPP, 0.05 mM	19.18	7.44	93
Cytosol + PRPP, 0.1 mM	14.25	15.68	105
Cytosol + PRPP, 0.5 mM	4.38	22.14	93
Cytosol + PRPP, 1.0 mM	0	30.35	106

The control assay mixture contained 100 mM Tris–HCl (pH 8.0), [carboxyl-^{14}C], 0.5 mM DHO (257 dpm/nmol), 5 mM $MgCl_2$, intact liver mitochondria equivalent to 0.31 mg protein and, when present, cytosol equivalent to 5.31 mg protein and the indicated concentrations of PRPP in a final volume of 2 ml. Incubation was carried out in tightly sealed Erlenmeyer flasks at 37°C for 20 min. $^{14}CO_2$ released was trapped in 0.3 ml hyamine hydroxide. Incubation was terminated by addition of 2 ml 2 N $HClO_4$ and the concentration of orotate was determined by the UV absorbance at 290 nm. Each value given is the mean of duplicate observations

liver cytosol on the DHO-DHase assay in the presence of added PRPP and without the addition of exogenous complex U enzymes. In this experiment we used [carboxyl-^{14}C]DHO as substrate and simultaneously measured orotate formation and $^{14}CO_2$ release. As shown in table 3, the amount of orotate recovered was inversely related to the PRPP concentration. The disappearance of orotate resulted in an equivalent increase in the appearance of $^{14}CO_2$ indicating that orotate can be rapidly converted to UMP and CO_2 in this system provided sufficient PRPP and Mg^{2+} are present.

In table 3 the decrement in absorbance with cytosol alone was somewhat less than is usually seen. In this regard, we have noted considerable variability from one cytosol preparation to another. This may reflect different concentrations of labile substrate, PRPP, in different cytosol preparations.

Based on the foregoing, several points bear emphasis:

(1) When orotate formation is measured directly, the 'apparent' reduction in mitochondrial orotate synthesis in the presence of cytosol is due to conversion of orotate to UMP by cytosolic enzymes. Cytosol does not contain an inhibitor of DHO-DHase, nor does cytosolic dihydroorotase reduce dihydroorotate levels to the point that they become rate-limiting for DHO-DHase.

(2) When assaying DHO-DHase in crude tissue preparations containing cytosol, the method of assay must be selected with care. The assay system in [11] appears to be best suited for this purpose.

(3) Orotate produced by mitochondrial DHO-DHase is converted to UMP by cytosolic enzymes in a highly efficient manner. As shown in table 3, addition of a sufficient amount of PRPP results in stoichiometric conversion of newly synthesized orotate to UMP as measured by CO_2 production.

ACKNOWLEDGEMENT

This work was supported by the Medical Research Service of the Veterans Administration.

REFERENCES

[1] Shoaf, W.T. and Jones, M.E. (1973) Biochemistry 12, 4039–4051.

[2] Jones, M.E. (1972) in: Current Topics in Cellular Regulation (Horecker, B.L. and Stadtman, E.R. eds) vol.6, pp.227–265, Academic Press, New York.

[3] Kennedy, J. (1973) Arch. Biochem. Biophys. 157, 369–373.

[4] Chen, J.-J. and Jones, M.E. (1976) Arch. Biochem. Biophys. 176, 82–90.

[5] Miller, R.W., Kerr, C.T. and Curry, J.R. (1968) Canad. J. Biochem. 46, 1099–1106.

[6] Forman, H.J. and Kennedy, J. (1975) J. Biol. Chem. 250, 4322–4326.

[7] Forman, H.J. and Kennedy, J. (1976) Arch. Biochem. Biophys. 173, 119–224.

[8] Westwick, W.J., Allsop, J. and Watts, R.W.E. (1972) Biochem. Pharmacol. 21, 1955–1966.

[9] Herrmann, E.C., Dunn, J.H. and Schmidt, R.R. (1973) Anal. Biochem. 53, 478–483.

[10] Matsuura, T. and Jones, M.E. (1974) in: Energy, Regulation and Biosynthesis (Richter, E.D. ed) pp.422–434.

[11] Smithers, G.W., Gero, A.M. and O'Sullivan, W.J. (1978) Anal. Biochem. 88, 93–103.

[12] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, A.J. (1951) J. Biol. Chem. 193, 265–275.

[13] Kennedy, J. (1974) Arch. Biochem. Biophys. 160, 358–365.

[14] Silva, R.F. and Hatfield, D. (1978) Methods Enzymol. 51, 143–154.

[15] Kasbekar, D.K., Nagabhushanam, A. and Greenberg, D.N. (1964) J. Biol. Chem. 239, 4245–4249.

[16] Kavipurapu, P.R. and Jones, M.E. (1976) J. Biol. Chem. 251, 5589–5599.

[17] Reyes, P. and Guganig, M.E. (1975) J. Biol. Chem. 250, 5097–5108.

[18] Traut, T.W. and Jones, M.E. (1977) Biochem. Pharmacol. 26, 2291–2296.