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2′,3′-CYCLIC NUCLEOTIDE 3′-PHOSPHOHYDROLASE IN RAT LIVER MITOCHONDRIAL MEMBRANES

CHARLES E. DREILING, ROGER J. SCHILLING and RONALD C. REITZ

Division of Biochemistry, School of Medicine, University of Nevada, Reno, NV 89557 (U.S.A.)

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Summary

2′,3′-Cyclic nucleotide 3′-phosphohydrolase (nucleoside-2′:3′-cyclic-phosphate 2′-nucleotidohydrolase, EC 3.1.4.37) activity has been demonstrated in rat liver mitochondria. The enzyme was localized in both the outer and inner mitochondrial membranes but was absent from the intermembrane space and matrix. The mitochondrial (cyclic nucleotide) phosphohydrolase was activated by freezing and thawing and by treatment with digitonin or detergents. It is suggested that (cyclic nucleotide) phosphohydrolase is an integral membrane protein which is buried to a significant degree within the membrane. Atractyloside was found to be a noncompetitive inhibitor of the enzyme both in intact mitochondria and in preparations of the mitochondrial membranes. The enzyme substrate, 2′,3′-cyclic adenosine monophosphate, had no effect on the oxidation of exogenous β-hydroxybutyrate or succinate by intact mitochondria. These findings suggest that 2′,3′-cyclic nucleotide 3′phosphohydrolase is more widely distributed than was previously thought and that the enzyme may play a fundamental role in membranes, independent of their specialized structure or functions.

Introduction

The enzyme, 2′,3′-cyclic nucleotide 3′-phosphohydrolase (nucleoside-2′:3′-cyclic-phosphate 2′-nucleotidohydrolase, EC 3.1.4.37), catalyzes the hydrolysis of 2′,3′-cyclic nucleotides to form the corresponding 2′-monophosphates [1]. High concentrations of (cyclic nucleotide) phosphohydrolase activity were first described in central nervous system white matter [2], and the enzyme has

since been shown to be an integral protein of myelin [3,4]. Although (cyclic nucleotide) phosphohydrolase has been exploited as a myelin marker enzyme, significant levels of activity have also been reported in non-myelin membranes including those derived from the spleen, adrenal gland, kidney, heart and skeletal muscle [1,5,6]; however, it should be emphasized that myelin and myelin-related tissues have an extremely high specific activity compared to these tissues. Enzyme activity also has been demonstrated in the membranes of human erythrocytes, where it was similar to that found in myelin with respect to pH optimum, substrate specificity and effect of metal ions [7]. In view of the apparent ubiquity of (cyclic nucleotide) phosphohydrolase in biomembranes, it has been suggested that the enzyme may be generally present in plasma membranes and their differentiated forms [5,8]. The natural substrate for (cyclic nucleotide) phosphohydrolase and the physiological significance of the enzyme in membranes are not known.

To date, (cyclic nucleotide) phosphohydrolase activity has not been clearly demonstrated in the membranes of intracellular organelles. But, enzyme activity has been reported in the microsomes and mitochondria of nervous tissue [9]; however, this activity was attributed to myelin contamination [2]. Although more recent investigations also have shown (cyclic nucleotide) phosphohydrolase to be present in crude mitochondria and other subcellular membrane preparations obtained from the mouse spleen [6], the location of the enzyme within the mitochondrion was not determined. In the present paper, we have extended these observations and report the presence of (cyclic nucleotide) phosphohydrolase activity in purified liver mitochondria. We also provide evidence that the enzyme is confined to and equally distributed between the inner and outer mitochondrial membranes.

Methods and Materials

Mitochondrial preparations. Rat liver mitochondria were isolated from Long-Evans rats (Simonsen Laboratories) maintained on a liquid diet for 30—40 days prior to killing [10]. The rats were decapitated and their livers were removed, weighed, and homogenized in ice-cold sucrose (0.25 M) containing EGTA (1 mM), Tris-HCl (3.4 mM, pH 7.4) and 1% defatted bovine serum [11]. Mitochondria were isolated by the procedure of Chappell and Hansford [12] as modified by Thompson and Reitz [13] and the final pellet was suspended in 0.25 M sucrose and 3.4 mM Tris-HCl buffer. Aliquots of the whole mitochondria were saved (0—4°C) for subsequent analysis and the remainder was fractionated to yield four subfractions; outer membrane, intermembrane space, inner membrane and matrix [14]. The purity of each subfraction was determined by the enzyme markers; monoamine oxidase (outer membranes) [15]; adenylate kinase (intermembrane space) [16]; cytochrome oxidase (inner membranes) [17] and glutamate dehydrogenase (matrix) [18].

Enzyme determinations. (Cyclic nucleotide) phosphohydrolase was assayed by the phenol red method previously described [19]. The specific activity (U/mg) of (cyclic nucleotide) phosphohydrolase was expressed as the amount of enzyme yielding 1 μmol 2′-AMP/min per mg protein. Protein was determined according to the method of Lowry et al. [20].

To demonstrate the formation of 2′-AMP by the enzyme preparations being studied, the (cyclic nucleotide) phosphohydrolase assay was coupled with excess 3′-nucleotidase. Up to 1.0 mg mitochondrial protein was incubated with 2′,3′-cyclic AMP for 30 min at 37°C. Aliquots of this reaction mixture were diluted with equal volumes of Tris buffer (5 mM, pH 7.5) and incubated for an additional 30 min (37°C) with 40 mU of 3′-nucleotidase (Sigma; less than 1% 2′-nucleotidase contamination). The resultant mixture was assayed for liberated inorganic phosphate by the method of Eibl and Lands [21]. No inorganic phosphate was detected indicating that only the 2′-AMP was formed. For comparison, the 3′-nucleotidase assay was performed on solutions containing known concentrations of 3′-AMP.

Oxygen consumption by mitochondria. Mitochondrial substrate oxidations were determined polarographically as previously described [13]. The state 3, state 4 and respiratory control ratios were determined as described by Estabrook [22].

Results

Preliminary experiments revealed that fresh preparations of whole rat liver mitochondria hydrolyzed 0.133 ± 0.005 μmol 2′,3′-cyclic AMP/min per mg protein. Control studies with heat-treated (boiled for 2 min) mitochondria and assays conducted in the absence of substrate demonstrated the reaction to be enzyme and substrate dependent. The reaction velocity was linear up to 225 μg protein and between 2 and 8 μmol substrate.

The presence of (cyclic nucleotide) phosphohydrolase activity in intact mitochondria prompted investigations as to the location of the enzyme within the organelle. Four major mitochondrial subfractions (outer membrane, intermembrane space, inner membrane and matrix) were isolated, assessed for purity via enzyme markers and assayed for (cyclic nucleotide) phosphohydrolase activity. When each fraction was assayed for the enzyme (Table I), activity was found only in the outer and inner membranes. Marker enzymes showed the outer membrane to contain no cross contamination from the other three fractions. They further showed the inner membrane to contain 14.5 ± 2.6% inner mem-

TABLE I

DISTRIBUTION OF (CYCLIC NUCLEOTIDE) PHOSPHOHYDROLASE ACTIVITY IN FRESHLY ISOLATED MITOCHONDRIAL SUBFRACTIONS

Enzyme determinations were performed on freshly prepared mitochondrial fractions. (U = μmol 2′-AMP formed per min ± S.E.; $n = 4$).

Mitochondrial subfraction	Activity (U/ml)	Volume (ml)	Total activity (U)	Protein (mg)	Specific activity (U/mg protein
Outer membrane	2.61 ± 0.35	2.7 ± 0.2	6.51 ± 0.64	9.3 ± 2.3	0.28 ± 0.03
Intermembrane space	N.D. *	20.1	—	1.8	—
Inner membrane	3.49 ± 0.54	1.3 ± 0.1	4.54 ± 0.33	14.6 ± 2.1	0.24 ± 0.01
Matrix	N.D. *	10.1	—	6.8	—

* N.D., Not detectable.

TABLE II

ACTIVATION OF MITOCHONDRIAL (CYCLIC NUCLEOTIDE) PHOSPHOHYDROLASE BY FREEZING-THAWING AND DETERGENTS

Inner and outer membranes were treated as shown and assayed for (cyclic nucleotide) phosphohydrolase activity [19]. For the detergent treatment, equal volumes of membranes and detergent were incubated at 0—4°C for 30 min. The final concentrations of Lubrol WX and digitonin used were identical to those employed during the isolation of the outer and inner membranes, respectively. For the Triton X-100 treatment, the final concentration of Triton was 0.5%. Each value represents the mean ± S.D. from three to four determinations.

Treatment	Phosphohydrolase activity (U/mg protein)	
	Inner membrane	Outer membrane
Control	0.24 ± 0.03	0.28 ± 0.01
1st freeze-thaw	0.32 ± 0.04	0.41 ± 0.01
2nd freeze-thaw	0.52 ± 0.01	0.61 ± 0.01
3rd freeze-thaw	0.51 ± 0.01	0.60 ± 0.01
Triton X-100	0.49 ± 0.06	0.56 ± 0.05
Lubrol WX	0.55 ± 0.02	0.65 ± 0.03
Digitonin	0.55 ± 0.03	0.58 ± 0.07

brane and 1.5 ± 1.5% matrix contamination. The specific activity of the enzyme in these membranes was approx. twice that found in the whole mitochondria. The specific enzyme activities in the inner and outer membranes (Table I) were essentially identical, i.e., 0.24 and 0.28 μmol/min per mg protein, respectively. However the total (cyclic nucleotide) phosphohydrolase activity recovered in the outer membranes (6.51 μmol/min) was approx. 50% greater than that in the inner membranes (4.54 μmol/min). No activity was detected in either the intermembrane space or the matrix fractions even after prolonged (60 min) incubations with the substrate.

(Cyclic nucleotide) phosphohydrolase, obtained from central nervous system white matter, has been shown to be activated by freezing and thawing and by exposure to certain detergents (i.e., Triton X-100 and deoxycholate) [1,7,23]. Table II shows that (cyclic nucleotide) phosphohydrolase in both the outer and inner membranes was similarly affected by such treatments. The enzyme was

TABLE III

EFFECT OF 2′,3′-CYCLIC AMP ON MITOCHONDRIAL SUBSTRATE OXIDATION

Oxygen consumption was followed polarographically as previously described [13], and respiratory control ratios were calculated from the ratio of state 3 to state 4 respiration [22]. All values except those for 2′,3′-cyclic AMP are measured in nmol O_2/min per mg.

Substrate	2′,3′-cyclic AMP	State 3	State 4	Respiratory control ratio
		nmol O_2/min per mg		
β-Hydroxybutyrate	0	62.9	11.9	5.3
β-Hydroxybutyrate	0.4 mM	61.7	14.7	4.2
β-Hydroxybutyrate	2.0 mM	59.9	13.1	4.6
Succinate	0	83.4	17.6	4.7
Succinate	3.0 mM	72.4	15.1	4.8

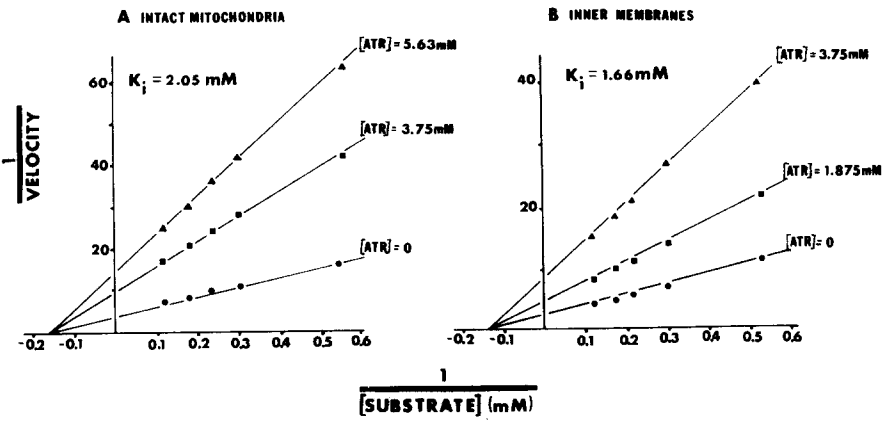

Fig. 1. Effect of atractyloside (ATR) on (cyclic nucleotide) phosphohydrolase activity in (A) intact mitochondria and (B) inner mitochondrial membranes. (Cyclic nucleotide) phosphohydrolase determinations were made via the phenol-red assay method [19]. Neutralized atractyloside (0.1 ml; pH 7.7; Sigma) was added to the (cyclic nucleotide) phosphohydrolase assay mixture (final vol. 0.8 ml). 20 μl freshly prepared intact mitochondria or previously freeze-thawed inner membranes were used to initiate the reaction. The data were analyzed and plotted by the method of linear regression.

maximally stimulated by two overnight freezings and thawings, resulting in a doubling of the specific activity. Lubrol WX, digitonin and Triton X-100 had a similar effect only on freshly prepared membranes.

Although the function of (cyclic nucleotide) phosphohydrolase is unknown, the enzyme's affinity for adenine nucleotides suggests that it may participate in the binding or transporting of adenine nucleotide(s) across biological membranes. If (cyclic nucleotide) phosphohydrolase is involved in adenine nucleotide(s) transport, the enzyme should be competitively inhibited by atractyloside, a known competitive inhibitor of adenine nucleotide(s) transport [24]. Thus, freshly prepared intact mitochondria and their inner membranes were assayed for (cyclic nucleotide) phosphohydrolase activity in the presence of varying concentrations of substrate (2′,3′-cyclic AMP) and inhibitor (atractyloside). Fig. 1 shows that atractyloside acted as a non-competitive inhibitor of the mitochondrial (cyclic nucleotide) phosphohydrolase. The apparent K_i of atractyloside for (cyclic nucleotide) phosphohydrolase in the intact mitochondria (2.05 mM) was similar to that for the inner membrane enzyme (1.66 mM).

The foregoing experiments provide considerable evidence that (cyclic nucleotide) phosphohydrolase is associated with both membranes of mitochondria. Therefore, it was of interest to determine if the artificial (cyclic nucleotide) phosphohydrolase substrate (i.e., 2′,3′-cyclic AMP) had an effect on mitochondrial function. Polarographic tracings of mitochondrial oxygen consumption, during the oxidation of exogenous β-hydroxybutyrate and succinate, showed (Table III) the respiration rates and the respiratory control ratios were essentially unaffected by the added 2′,3′-cyclic AMP. Further, 2′,3′-cyclic AMP had no effect on oxygen consumption whether added before or after the addition of ADP.

Discussion

The results of this study demonstrate (cyclic nucleotide) phosphohydrolase activity in rat liver mitochondria. They also show the enzyme to be confined to both the inner and outer membrane subfractions of the mitochondria. Although (cyclic nucleotide) phosphohydrolase activity has been described in several cellular membrane fractions [2,6,7], this is the first definitive report of the enzyme in mitochondrial membranes. The levels of (cyclic nucleotide) phosphohydrolase reported in this paper (0.10—0.30 μmol/min per mg) are in good agreement with those found in spleen mitochondria and microsomes (0.20—0.40 μmol/min per mg [6]) and in the membranes of human erythrocytes (0.30 μmol/min per mg [7]). Although these levels are only 2—5% of that present in myelin, these data indicate a minimal amount of (cyclic nucleotide) phosphohydrolase activity is present in many, if not all, membrane systems. These data show that very small amounts (1—5%) of myelin contamination could significantly alter (cyclic nucleotide) phosphohydrolase activity indigenous to any subcellular fraction of nervous tissue; therefore, this would account for high levels of (cyclic nucleotide) phosphohydrolase observed in brain subcellular fractions [2].

Several additional properties of the mitochondrial (cyclic nucleotide) phosphohydrolase are noteworthy. The enzyme was activated by freezing and thawing, treatment with non-ionic detergents and was not solubilized after repeated washing of the mitochondrial membranes. These observations, plus the fact that the apparent K_m of the mitochondrial enzyme (6.0—7.5 mM) was similar to the enzyme in myelin (6.3—10.0 mM [25]) and in myelin-free glial cells (5.25 mM [26]), suggest the (cyclic nucleotide) phosphohydrolase in mitochondria is similar to that found in myelin; however, much less (cyclic nucleotide) phosphohydrolase is present in other membranes. The fact that the enzyme is activated by freezing and thawing by detergent treatment without being solubilized (Table II) strongly suggests that (cyclic nucleotide) phosphohydrolase is an 'integral' membrane protein which is buried to a significant degree within the membrane. Further, the doubling of activity in each mitochondrial membrane compared to whole mitochondria could be accounted for by the disruption of the initial membrane structures during the isolation of each individual membrane.

(Cyclic nucleotide) phosphohydrolase activity was not observed in the matrix or intermembrane space of mitochondria. However, approx. equal concentrations of the enzyme were found in the inner and outer membrane fractions. This finding is interesting because these membranes are known to differ widely both with respect to their solute permeabilities and their roles in metabolism. Thus, it appears that the mitochondrial (cyclic nucleotide) phosphohydrolase is not responsible for the specialized functions of the inner and outer mitochondrial membranes. Furthermore, the fact that atractyloside acted as a non-competitive rather than competitive inhibitor of the mitochondrial (cyclic nucleotide) phosphohydrolase, implies that the inhibitor binds to (cyclic nucleotide) phosphohydrolase without interfering with the enzyme's affinity for 2′,3′-cyclic AMP. The Lineweaver-Burk plots (Fig. 1), showing the intersecting lines on the abscissa, also suggest the binding of the inhibitor with

either the 'E' or 'ES' forms of the enzyme are identical. Although the data are not shown, atractyloside was also seen to act as a non-competitive inhibitor of the (cyclic nucleotide) phosphohydrolase isolated from bovine brain white matter. Because atractyloside is a known competitive inhibitor of the adenine nucleotide(s) translocator [24], these results rule against (cyclic nucleotide) phosphohydrolase playing a role in the translocation of adenine nucleotide(s) across the mitochondrial membranes. Finally, the fact that the artificial (cyclic nucleotide) phosphohydrolase substrate (2′,3′-cyclic AMP) had no effect on actively respiring mitochondria suggests the enzyme (or its natural substrate) is not involved with energy production.

In summary, the finding of (cyclic nucleotide) phosphohydrolase in the membranes of rat liver mitochondria suggests the enzyme is widely distributed among biological membranes. Studies on (cyclic nucleotide) phosphohydrolase in defective membranes, and in membranes altered during disease or modified by drugs, are currently underway. The following paper reports on the effects of chronic ethanol ingestion on mitochondrial (cyclic nucleotide) phosphohydrolase. Investigations of this sort should provide information on the importance of the enzyme to the structural and functional integrity of membranes.

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References

1 Drummond, G.I., Iyer, N.T. and Keith, J. (1962) J. Biol. Chem. 237, 3535—3539
2 Olafson, R.Q., Drummond, G.I. and Lee, J.F. (1969) Can. J. Biochem. 47, 961—966
3 Kurihara, T. and Tsukada, Y. (1967) J. Neurochem. 14, 1167—1174
4 Drummond, R.J., Hamill, E.B. and Guha, A. (1978) J. Neurochem. 31, 871—878
5 Kurihara, T., Nussbaum, J.L. and Mandel, P. (1971) Life Sci. 10, 421—429
6 Konings, A.W.T. and Pierce, D.A. (1974) Life Sci. 15, 491—499
7 Sudo, T., Kikuno, M. and Kurihara, T. (1972) Biochim. Biophys. Acta 255, 640—646
8 Matthieu, J.-M. and Waehneldt, T.V. (1978) Brain Res. 150, 307—318
9 Banik, N.L. and Davison, A.N. (1969) Biochem. J. 115, 1051—1061
10 Thompson, J.A. and Reitz, R.C. (1978) Lipids 13, 540—550
11 Goodman, D.S. (1957) Science 125, 1296—1297
12 Chappell, J.B. and Hansford, R.G. (1972) Subcellular Components: Preparation and Purification (G. Birnie, Ed.), pp. 77—92, University Park Press, Baltimore
13 Thompson, J.A. and Reitz, R.C. (1976) Ann. N.Y. Acad. Sci. 273, 194—204
14 Greenawalt, J.W. (1974) Methods Enzymol. 31, 310—323
15 Tabor, C.W., Tabor, H. and Rosenthal, S.M. (1954) J. Biol. Chem. 208, 645—661
16 Schnaitman, C. and Greenawalt, J.W. (1968) J. Cell Biol. 38, 158—175
17 Smith, L. (1955) Methods Enzymol. 2, 732—740
18 Raijam, J. (1974) Biochem. J. 138, 225—232
19 Dreiling, C.E. and Mattson, C. (1980) Anal. Biochem. 102, 304—309
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
21 Eibl, H. and Lands, W.E.M. (1969) Anal. Biochem. 30, 51—57
22 Estabrook, R.W. (1967) Methods Enzymol. 10, 41—47
23 Drummond, G.I., Eng, D.Y. and McIntosh, C.A. (1971) Brain Res. 28, 153—163
24 Vignais, P.V. (1976) Biochim. Biophys. Acta 456, 1—38
25 Lo, K.W. and Tsou, K.C. (1975) J. Neurochem. 25, 181—183
26 Zanetta, J.P., Benda, P., Gombos, G. and Morgan, I.G. (1972) J. Neurochem. 19, 881—883