LOCATION OF A VERY ACTIVE BICARBONATE-DEPENDENT ATPase IN THE OUTER MEMBRANE OF RAT AND FROG LIVER MITOCHONDRIA

S. Grisolia and J. Mendelson

K. U. Med. Ctr., 39th & Rainbow Blvd., Kansas City, Kan. 66103

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SUMMARY: This paper documents the existence of a very active bicarbonate-dependent ATPase which is located in the outer membrane of rat and frog liver mitochondria. The enzyme, which does not require Na^+, is therefore not a Na^+-K^+ type of ATPase and is inhibited by Ca^{++}; in contrast to others the rat liver enzyme is only slightly cold labile and the frog enzyme is not. Since in animals the bulk of bicarbonate is made during metabolism in mitochondria the bicarbonate-stimulated ATPase may have much biological significance.

During early studies of citrulline synthesis, bicarbonate-dependent ATPase activity with mitochondrial preparations from rat liver was noted (1,2). Since carbamyl glutamate, believed at that time to be the natural cofactor for citrulline synthesis, increased the ATPase activity, these findings were interpreted as activation of the synthetase by ATP followed by CO_2 fixation into an unstable intermediate (3). These findings were extended with the more stable carbamyl phosphate synthetase[1] from frog liver and with AG (4). Recently Guthöhrlein and Knappe (5) by extending observations showing stabilization of the rat liver enzyme with glycerol and cyanide (6) obtained preparations which are stable but which also display considerable AG-dependent ATPase activity (7). Although our understanding of carbamyl phosphate synthesis is fairly good, the AG-induced stimulation of ATPase remains unclear.

While attempting to clarify the AG-stimulation of ATPase and to check the characteristics of an enzyme-phosphate intermediate, we found that CPS I preparations from rat and frog liver (8) displayed considerable bicarbonate-dependent ATPase activity in the absence of AG.

MATERIALS AND METHODS

Frogs (Rana Catesbeiana) and Sprague-Dawley rats were used. Pyruvate kinase

[1]Abbreviations used: carbamyl phosphate synthetase E.C. 2.7.2.5., (CPS I), N-acetyl-L-glutamate (AG), cetyltrimethylammonium bromide (CTAB).

and lactic dehydrogenase were obtained from Sigma as salt-free powder. Frog liver CPS I was prepared as described previously (8), except that the final concentration of acetone was 25% by volume.

Rat liver mitochondria were prepared as previously described (9). CPS I was prepared by the method of Guthöhrlein and Knappe (5) except that the mitochondria pellets were extracted in a Sorvall Omni Mixer for two 15 second periods at a speed setting of 4 with (3 volumes of the original weight of liver) 20% glycerol-0.01 M Dithiothreitol-0.05 M Tris-Cl^-, pH 7.4, containing 0.1% CTAB. The extracts were then centrifuged at 15,000 x g for 10 min. Mitochondrial membranes were separated according to Chan, et al. (10).

CPS I was measured according to Chabas, et al. (8). This assay is similar to that used by others for ATPase (10). Therefore, by excluding NH_3 and ornithine, ATPase and bicarbonate-dependent ATPase as well as the effect of AG on ATPase were routinely tested in this system. Occasionally phosphate liberation was measured (11) in a system containing 2.5 mM ATP, 25 mM glycylglycine, pH 7.4, 15 mM Mg^{++} and either 100 mM KCl or $KHCO_3$.

RESULTS AND DISCUSSION

The ATPase and the bicarbonate-dependent ATPase activity of CPS I preparations of frog and rat liver is illustrated in Fig. 1, and also the fact that there is some stimulation by AG on either preparation. Only potassium salts were used except for the sodium ATP; however, replacement of sodium ATP by potassium ATP did not affect activity. Replacing the bicarbonate by KCl or NaCl abolished the stimulation of the ATPase activity. Interestingly, 5 mM Ca^{++} completely inhibited the bicarbonate-dependent ATPase.

The bicarbonate-dependent ATPase activity could be detected in crude CTAB extracts of mitochondrial preparations; however, the activity varied considerably from preparation to preparation. Stable and active preparations were obtained by the use of the digitonin technique (10) which clearly demonstrated location of the activity entirely in the outer membrane. This is illustrated in Table 1. The Table presents data from some 8 preparations. For comparative purposes the activity for CPS I is also included.

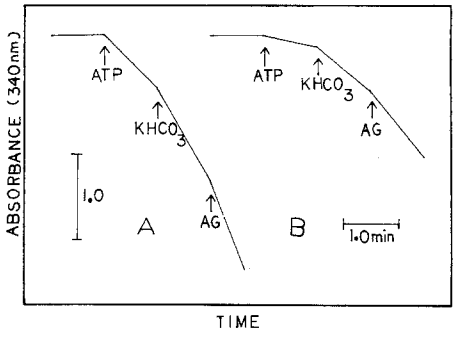

Fig. 1. Bicarbonate-stimulated ATPase in frog and rat liver preparations. 1 mg of frog liver CPS acetone fraction or 0.22 mg rat liver CTAB mitochondrial extract were used. The enzyme was assayed at 25° in 1.0 ml containing: 0.5 µmoles NADH, 50 µmoles glycylglycine, pH 7.4, 15 µmoles $MgSO_4$, 2.5 µmoles phosphoenolpyruvate, 5 µmoles mercaptoethanol, 0.04 mg pyruvate kinase and 0.015 mg lactic dehydrogenase. When indicated saturating quantities of ATP (5 µmoles in 50 µl), $KHCO_3$ (50 µmoles in 50 µl), or AG (10 µmoles in 20 µl) were added. A - frog liver enzyme; B - rat liver enzyme.

Preparations of frog liver also revealed distribution similar to that of rat liver as illustrated in Table 2.

CPS I and ornithine transcarbamylase are located in the mitochondrial matrix (12). While the bicarbonate-dependent ATPase activity here described could reflect a second activity of CPS I, the clear cut location of activity in the outer membrane together with differential stability and Q_{10} indicate that this is not the case. For example, when the rat liver CTAB extracts were stored for about 18 h at 4° and then warmed to 25° for 45 min they showed about 50% of the original CPS I activity. However, these preparations retained ~90% and 70% respectively of the ATPase and the bicarbonate-dependent ATPase. With frog liver digitonin preparations the CPS I activity decreased ~14% after 18 h in the cold, while the ATPase and bicarbonate-dependent ATPase activities increased 14% and 33% respectively. The Q_{10} for CPS I is approximately 3 when measured at 35° and at 25° (13), while the Q_{10}'s for ATPase and the bicarbonate-dependent ATPase in this temperature range are 2 and 1.5.

It has been found recently that stomach and brain (14,15) possess bicarbonate-activated ATPases. It seems that, as illustrated in Table 3, the activity of our preparations is much higher than thus far described for any other tissue.

Table 1. ATPase, Bicarbonate-dependent ATPase and CPS activities in rat liver mitochondria and fractions thereof. One portion of rat liver mitochondria was extracted with CTAB as described in Methods and another treated with digitonin (10). The inner membrane and matrix were extracted with CTAB. The figures given are the mean and S.D. for 8 preparations normalized to 1 g liver. Units are μmoles ATP used or citrulline formed per min. Protein is given in mg.

Fraction		ATPase				CPS	
		without HCO_3^-		with HCO_3^-			
	Protein	Units	S.A.	Units	S.A.	Units	S.A.
Mitochondria	13.8	0.29	0.02 ±0.01	0.43	0.03 ±0.02	3.25	0.24 ±0.13
Outer Membrane	1.4	2.09	1.49 ±0.14	4.68	3.34 ±0.2	0.0	0.0
Inter-membrane Space	3.3	0.37	0.11 ±0.17	0.55	0.17 ±0.23	0.0	0.0
Inner membrane and matrix	9.5	0.0	0.0	0.23	0.02 ±0.03	0.30	0.03 ±0.02

Table 2. ATPase, Bicarbonate-dependent ATPase and CPS activities in frog liver mitochondria and fractions thereof. One portion of frog liver mitochondria was extracted with CTAB as described in Methods and another treated with digitonin (10). The inner membrane and matrix were extracted with CTAB. The figures given are the average of two preparations normalized to 1 g liver. Units are μmoles ATP used or citrulline formed per min. Protein is given in mg.

Fraction		ATPase				CPS	
		without HCO_3^-		with HCO_3^-			
	Protein	Units	S.A.	Units	S.A.	Units	S.A.
Mitochondria	5.0	0.23	0.05	0.0	0.0	1.96	0.39
Outer Membrane	0.4	1.23	3.08	2.22	5.55	0.0	0.0
Inter-membrane Space	4.4	0.60	0.14	0.0	0.0	2.65	0.60
Inner membrane and matrix	2.6	0.45	0.17	0.0	0.0	1.47	0.57

Table 3. ATPase and Bicarbonate-dependent ATPase activities in preparations from stomach, brain and liver. Values are given as μmoles ATP/mg protein/min.

Preparation	Reference	ATPase without HCO_3^-	ATPase with HCO_3^-
Dog gastric mucosa, homogenate	J. G. Spenney, et al. (18)	0.83	1.31
Dog gastric mucosa, mitochondria	A. L. Blum, et al. (14)	0.10	0.12
" " " , microsome	" "	0.29	0.42
Rat brain, cerebral cortex mitochondria	H. K. Kimelberg and R. S. Bourke (15)	0.11	0.145
Rat liver, mitochondrial outer membrane	Present work	1.49	3.34
Frog liver, mitochondrial outer membrane	Present work	3.08	5.55

Although many schemes have been proposed for ATPases, particularly in transport (14,15), their physiological role remains unclear. In this regard the present findings are of interest since the bulk of CO_2 in animals arises in the mitochondria during metabolism, and therefore there must be a gradient which may involve energy. Possibly in most tissues CO_2 is in excess, but it may not be true for liver. The CO_2 produced by liver can be calculated as follows: the liver of man uses ∿15% of the O_2 needed for basal metabolism or for the 70 Kg average man 1.7 mmoles O_2 per min, a figure which remains essentially unchanged with exercise, etc. (16). Then, assuming an R.Q. of 1 (pure carbohydrate diet), 1.7 mmoles of CO_2 would be formed. For a fat diet only 1.2 mmoles would be available. Since man produces from ∿0.3-1 mmole urea per min, it is self-evident that CO_2 may become limiting, or nearly so, if there is also extensive CO_2 utilization for anaplerosis. It is known that in addition to nucleotide pumps liver mitochondria have an ornithine

pump which may largely control citrulline synthesis (12). Whether there are interrelations between these pumps, including the possibility of a bicarbonate-stimulated ATPase pump, as postulated for the stomach enzyme (14), remains to be clarified. The bicarbonate-stimulated ATPase may be related to the cold-labile beef heart mitochondrial ATPase (17), and therefore additional knowledge of the bicarbonate stimulation may also be of interest in relation to oxidative phosphorylation.

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