PYRUVATE OXIDASE (CoA ACETYLATING) IN ENTAMOEBA HISTOLYTICA

Tsutomu Takeuchi, Eugene C. Weinbach and Louis S. Diamond

Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

Received May 30,1975

SUMMARY

A novel type of pyruvate oxidase, which catalyzes acetylation of CoA in the presence of pyruvate, oxygen and FAD, was detected in Entamoeba histolytica. The enzyme was partially purified by centrifugation, ammonium sulfate precipitation and hydroxyapatite chromatography. Enzymatic activity was found in two fractions. The ratio of oxygen consumed to acetyl CoA produced was 1 with the purified enzyme(s). In the presence of excess catalase the velocity of oxygen uptake was one-half. Therefore, the postulated reaction is: pyruvate + CoA + O_2 = acetyl CoA + CO_2 + H_2O_2. For this new enzyme, we propose the trivial name of pyruvate oxidase (CoA acetylating).

Although Entamoeba histolytica, the causative agent of human amebiasis, lacks mitochondria, cytochromes and a functional tricarboxylic acid cycle (1, 2), it actively consumes oxygen when provided (3). Of a large number of substrates tested such as carbohydrates, carboxylic, fatty and amino acids, only glucose and L-serine were found to stimulate the oxygen uptake of this protozoan (3). We have been interested particularly in elucidating the mechanism of L-serine oxidation, and found that the amino acid was not oxidized directly. L-Serine was first converted to pyruvate by L-serine dehydratase, and subsequently, pyruvate was metabolized by a pyruvate oxidase system. Two types of pyruvate oxidases were detected. One is Pi dependent; i.e., phosphorylating pyruvate oxidase. The other is a CoA dependent pyruvate oxidase. This communication deals with the partial purification and characterization of the CoA dependent pyruvate oxidase, which appears to have unique catalytic and molecular properties.

MATERIALS AND METHODS

E. histolytica, HK-9 strain, was grown axenically as described by Diamond (4). After stationary cultivation at 37° for 72 hours, amebae were collected,

washed by centrifugation at 650xg for 5 minutes, and finally suspended in Tris-Cl buffer 50 mM, pH 7.4 containing 0.25 M sucrose.

Enzyme activity was determined either by a polarographic method using the Clark oxygen electrode, or by enzymatic assay of the reaction product (acetyl CoA) (5). Protein was determined either by a modified biuret procedure (6), or by Folin-phenol procedure (7).

Crystalline malate dehydrogenase and citrate synthetase were obtained from Boehringer Mannheim Biochemicals. Catalase was purchased from Worthington Biochemicals. All reagents were of the highest purity commercialiy available.

PARTIAL PURIFICATION OF CoA DEPENDENT PYRUVATE OXIDASE

The amebae collected as above (about 5 g wet weight/10 ml suspension), were disrupted by homogenization for 1 minute using a glass homogenizer with a teflon pestle. The homogenate was centrifuged at 110,000xg for 120 minutes. The volume of the resulting supernatant fraction was adjusted to 10 ml with Tris-Cl buffer 50 mM, pH 7.4, and solid ammonium sulfate was added gradually to 40% saturation during stirring for 60 minutes. The suspension was centrifuged at 23,000xg for 15 minutes, and the sediment discarded. Solid ammonium sulfate was added to the supernatant solution to make 65% saturation in the same way as above, and the precipitate isolated by centrifugation. After the supernatant fluid was discarded, the precipitate was dissolved in 3.0 ml of potassium phosphate buffer 5 mM, pH 6.8, containing 1 mM 2-mercaptoethanol, and dialyzed against 2.5 liters of this buffer overnight. The dialyzed fraction was applied to a column of hydroxyapatite (0.9 cm x 23 cm Hypatite C, Clarkson Chemical Co., Williamsport, Pa.) equilibrated with the same buffer as above. Elution was carried out with a linear gradient of potassium phosphate from 5 mM to 0.2 M, pH 6.8, using the LKB Ultrograd system. Elution speed was about 24 ml/hour, and 4 ml eluent was collected in each tube. All procedures were done at 4°.

RESULTS AND DISCUSSION

The elution profile of hydroxyapatite chromatography is illustrated in Figure 1. The enzymatically active fraction was found in two peaks, designated as El and Ell respectively. Neither fraction exhibited phosphorylating pyruvate

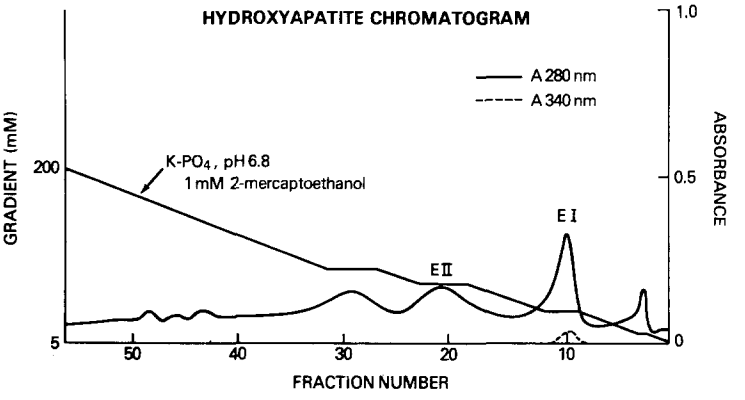

Fig. 1. Hydroxyapatite chromatography of pyruvate oxidase (CoA acetylating). Absorbance at 280 and 340 nm was monitered by the LKB Uvicord 2089.

---

oxidase or phosphotransacetylase activity. Addition of 5 mM sodium arsenate had no effect on phosphorylating pyruvate oxidase. The enzyme purification procedures are summarized in Table 1. Specific activities of oxygen uptake in the E1 and E11 fractions were increased 10 times and 70 times respectively. Although both E1 and E11 lack phosphorylating pyruvate oxidase or phosphotransacetylase as mentioned above, they contained an enzyme that catalyzed the formation of acetyl CoA from pyruvate and CoA in the presence of oxygen. FAD was required as the coenzyme. Addition of 5 mM sodium arsenite had no effect. The ratio of oxygen uptake to acetyl CoA formation was found to be 1 (Table 2). Thus, we postulate that this enzyme catalyzes transacetylation from pyruvate to CoA by an oxidase reaction in which H_2O_2 is produced. Therefore, in the presence of excess catalase, the velocity of oxygen uptake should be diminished. Addition of 300 units catalase reduced the velocity of oxygen uptake by exactly one-half (Table 3). These data suggest that the reaction proceeds as follows: Pyruvate + CoA + O_2 = acetyl CoA + CO_2 + H_2O_2. This enzyme appears to be a new type of pyruvate oxidase.

Phosphorylating pyruvate oxidase, and acetate-producing pyruvate oxidase have been characterized in bacterial cells (8, 9). For example, the

Table 1 Purification of Pyruvate Oxidase (CoA acetylating)

Procedure	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg protein)	Recovery (%)
1. Crude extract	12	432	13.0	0.03	
2. 110,000g x 120 min. supernatant fraction	8	182	12.6	0.07	97
3. 40-65% Ammonium sulfate saturation	4	69	9.0	0.21	69
4. Dialyze	6	58	8.7	0.15	67
5. Hydroxyapatite E1	12	9	2.7	0.3	21
chromatography E11	12	2.4	5.0	2.1	38

One unit catalyzes the oxygen uptake of 1 micromole per minute under the assay conditions. The assay system contained 0.1 mM FAD, 0.5 mM TPP, 5 mM $MgSO_4$, 5mM pyruvate and 0.2 mM CoA. Total volume of the assay mixtures was adjusted to 1.0 ml with Tris-Cl buffer 50 mM, pH 7.4. Before adding pyruvate and CoA, the enzyme was pre-incubated with cofactors (FAD, TPP, and Mg^{++}) for 2.5 minutes at 37°.

acetate-producing pyruvate oxidase from Escherichia coli has been crystallized (9). The pyruvate oxidase from E. coli required phospholipid and an electron acceptor such as ubiquinone-6 or cytochrome b_1 (10-12). A nonphysiological electron acceptor (i.e., ferricyanide) also was functional (13). In contrast, the activity of pyruvate oxidase purified from E. histolytica was not enhanced by any of the phospholipids tested (phosphatidyl-choline, -inositol, and -ethanolamine) or by artificial electron acceptors (dichlorophenolindophenol, ferricyanide, 1,4-naphthoquinone, methylbenzoquinone, and naphthobenzoquinone).

Oxygen has been considered to be toxic to E. histolytica. Results of the present study, however, provide cogent evidence that oxygen is of physiological significance in the metabolism of this putative anaerobe.

Table 2. Stoichiometry between acetyl CoA formation and oxygen uptake in pyruvate oxidase (CoA acetylating)

Conditions	E1		E11	
	Acetyl CoA	O_2	Acetyl CoA	O_2
	μmoles/mg protein/minute			
Pyruvate 5 mM and CoA 0.2 mM	Nil	Nil	Nil	0.06
+ FAD 0.1 mM	0.3	0.3	1.8	2.0
+ FAD 0.1 mM and TPP 0.5 mM	0.3	0.3	1.9	2.0
+ TPP 0.5 mM and Mg^{++} 5 mM	Nil	Nil	Nil	0.1
+ FAD 0.1 mM, TPP 0.5 mM and Mg^{++} 5 mM	0.25	0.3	1.9	1.9

Both experiments were done exactly in the same manner. The enzyme was pre-incubated with cofactors for 2.5 minutes at 37°, and the substrates were added. The reaction was initiated by adding the final substrate (CoA). Incubation was carried out for 2 minutes, and the amount of acetyl CoA produced and oxygen uptake were compared. In the case of acetyl CoA determination, the reaction was stopped by adding 0.2 ml of 25% perchloric acid (PCA). The PCA was neutralized with 3.0 M $KHCO_3$, and the insoluble product removed by centrifugation.

Table 3. Effect of Catalase on Pyruvate Oxidase (CoA acetylating)

Conditions	Oxygen Uptake	
	E1	E11
	μmoles/mg protein/minute	
FAD 0.1 mM and pyruvate 5 mM	0.03	0.1
+ CoA 0.2 mM	0.4	2.0
+ Catalase 300 units and CoA 0.2 mM	0.2	1.1

Catalase was present before the reaction was initiated by addition of CoA.

Our studies of pyruvate oxidase are compatible with the observation of Montalvo *et al.*, (14) that the main product of aerobic metabolism of the parasite was acetate. The fact that anaerobiosis alters the amounts of

the final products formed by glycolysis also can be explained by the requirement for oxygen by the pyruvate oxidase system.

Further studies are in progress in our laboratory, and detailed properties of the catalytic and molecular aspects of this novel pyruvate oxidase will be presented elsewhere. For this new type of pyruvate oxidase, we propose the trivial name of pyruvate oxidase (CoA acetylating).

ACKNOWLEDGEMENT

Technical assistance of C. Elwood Claggett is gratefully acknowledged.

REFERENCES

1. Rosenbaum, R.M. and Wittner, M. (1970) J. Cell Biol. 45, 369.
2. von Brand; T. (1973) Biochemistry of Parasites 2nd. ed. 394, Academic Press, New York;
3. Weinbach, E.C. and Diamond, L.S. (1974) Exp. Parasitol. 39, 249.
4. Diamond, L.S. (1968) J. Parasitol. 54, 1047.
5. Tubbs, P.K. and Garland, P.G. (1969) Methods in Enzymology, vol. XIII, 535, Academic Press, New York.
6. Szarkowska, L. and Klingenberg, M. (1963) Biochem. Z. 338, 674.
7. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randal, R.J., (1951) J. Biol. Chem. 193, 265.
8. Hager, L.P., Geller, D.M. and Lipmann, F. (1954) Fed. Proc. 13, 734.
9. Williams, F.R. and Hager, L.P. (1966) Arch. Biochem. Biophys. 116, 168.
10. Cunningham, C.C. and Hager, L.P. (1971) J. Biol. Chem. 246, 1575.
11. Cunningham, C.C. and Hager, L.P. (1968) Bacteriol. Proc. 133.
12. Williams, F.R. and Hager, L.P. (1960) Biochim. Biophys. Acta 38, 566.
13. Hager, L.P. (1957) J. Biol. Chem. 229, 251.
14. Montalvo, F.R., Reeves, R.E. and Warren, L.G. (1971) Exp. Parasitol. 30, 249.