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NADP$^+$-Dependent α-Glycerophosphate Dehydrogenase Activity in *Entamoeba histolytica*

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Although a number of enzymes involved in glycolysis have been detected in *Entamoeba histolytica*, a human pathogen causing amoebiasis, α-glycerophosphate dehydrogenase has not been identified yet. During the course of investigation on glycolysis of this parasite, however, we have found an activity of α-glycerophosphate dehydrogenase which is primarily dependent on NADP$^+$. Although NAD$^+$ was also functional in the amoebal enzyme, the velocity of NADH oxidation elicited by dihydroxyacetone phosphate was much slower than that of NADPH oxidation. Sometimes, NADH oxidation was almost negligible.

The present communication deals with isolation, identification and partial characterization of this NADP$^+$-dependent α-glycerophosphate dehydrogenase in *E. histolytica*.

Materials and Methods

Parasite: *E. histolytica* (HM-1 strain) was axenically grown in the TYI-S-33 medium as described by Diamond (1968). The procedure of harvesting after 72 hour-cultivation at 35.5 C has also been described in the previous report (Takeuchi *et al.*, 1977). Amoebae were finally suspended in 50 mM tris-HCl, pH 7.4 containing 0.25 M sucrose so that the protein content was approximately 20 mg/ml.

Reagents: NAD$^+$ and NADP$^+$ were supplied by the Oriental Yeast (Tokyo, Japan). NADH, NADPH, α-glycerophosphate and dihydroxyacetone phosphate (lithium salt) were obtained from the Sigma (St. Louis, Mo.). Other chemicals were of the highest purity commercially available.

Assay procedures: The activity of α-glycerophosphate dehydrogenase was assayed by determining the initial velocity of NAD(P)$^+$ reduction or NAD(P)H oxidation spectrophotometrically at 20 C.

The assay mixture for NAD(P)$^+$ reduction contained 1 mM NAD(P)$^+$, 20 mM α-glycerophosphate, the enzyme preparation (2–4 mg protein) and 0.1 M HEPES, pH 7.4 in a final volume of 1 ml. After the endogenous reduction of NAD(P)$^+$, which was practically zero, was recorded for a short period, the enzyme reaction was initiated by adding α-glycerophosphate. Absorbance change was continuously monitored with the Hitachi 556 recording spectrophotometer (Hitachi Co., Tokyo) at 340 nm.

The assay mixture for NAD(P)H oxidation consisted of 0.25 mM NAD(P)H, 20 mM dihydroxyacetone phosphate, the enzyme preparation (2–4 mg protein) and 0.1 M HEPES, pH 7.4 in a final volume of 1 ml. The enzyme reaction was also started by adding the substrate after the endogenous oxidation of NAD(P)H was recorded. Absorbance change was also monitored in the same manner as above.

Protein was determined by Lowry's folin-phenol procedure (Lowry *et al.*, 1951). Bovine serum albumin was used as a standard.

Enzyme preparation: Immediately after harvested, amoebae were disrupted by homogenization using a glass homogenizer with a loosely fitted teflon pestle for 2 min. The crude homogenate was centrifuged at 15,000 g for 20 min and the supernatant fluid isolated. Ammonium sulfate was added to the isolated supernatant fluid to make 40% saturation during stirring for 60 min. The sample was centrifuged at 13,000 g for 20 min and the sediment discarded. The supernatant fluid was brought to 80% saturation of ammonium sulfate and the resulting pellet isolated in the same manner as above. The pellet was dissolved in 3 ml of 50 mM tris-HCl, pH 7.4 and dialyzed against 1 liter of the same buffer overnight with changing the buffer once. The dialyzed sample was used as the enzyme preparation. All of these procedures were done at 4 C.

Results

Table 1 demonstrates $NAD(P)^+$ reduction by α-glycerophosphate as well as NAD(P)H oxidation elicited by dihydroxyacetone phosphate at various concentrations of nucleotides and substrates. It is apparent that addition of α-glycerophsphate up to 20 mM does not elicit reduction of either NAD^+ or $NADP^+$, whereas dihydroxyacetone phosphate readily stimulated NAD(P)H oxidation, particulary NADPH oxidation. The velocity of NADH oxidation elicited by dihydroxyacetone phosphate was always lower than NADPH oxidation irrespective of concentrations of nucleotides and the substrate tested under the present assay conditions. NADH oxidation was normally 1/30–1/40 of NADPH oxidation and sometimes negligible. Thus, it is likely that the amoebal α-glycerophosphate dehydrogenase is primarily $NADP^+$-dependent. It is also probable that the reaction favors reduction of dihydroxyacetone phosphate. It is not known, however, if NADH oxidation elicited by dihydroxyacetone phosphate is also catalyzed by the same enzyme as NADPH oxidation.

Table 2 demonstrates inhibition of dihydroxyacetone phosphate-elicited NADPH oxidation by palmitoyl CoA as well as by various transition metal-chelators. Addition of 0.2 mM palmitoyl CoA almost abolished the activity of $NADP^+$-dependent α-glycerophosphate dehydrogenase. Fifty precent inhibition was accomplished by 40 μM of this compound. Potent inhibitory effects exhibited by bathophenanthroline, salicylaldoxime and o-phenanthroline suggest involvement of a transition metal in the catalysis of this enzyme. In particular, the inhibitory effect

Table 1 α-Glycerophosphate dehydrogenase activity in *Entamoeba histolytica*

Assay mixture	$NAD(P)^+$ reduced or NAD(P)H oxidized
	μmoles/mg protein/min
NAD^+ 1 mM, α-glycerophosphate 20 mM	0
$NADP^+$ 1 mM, α-glycerophosphats 20 mM	0
NADPH 0.25 mM, Dihydroxyacetone phosphate 20 mM	0.045
NDPH 0.25 mM, Dihydroxyacetone phosphate 20 mM	0.001
NADPH 0.25 mM, Dihydroxyacetone phosphate 5 mM	0.028
NADH 0.25 mM, Dihydroxyacetone phosphate 5 mM	0
NADPH 0.1 mM, Dihydroxyacetone phosphate 20 mM	0.030
NADH 0.1 mM, Dihydroxyacetone phosphate 20 mM	0.001
NADPH 0.1 mM, Dihydroxyacetone phosphate 5 mM	0.022
NADPH 0.1 mM, Dihydroxyacetone phosphate 5 mM	0

Values are averages of at least two independent determinations. Other details as in the text.

Table 2 Effect of transition metal-chelators and palmitoyl CoA on α-glycerophosphate dehydrogenase in *Entamoeba histolytica*

Assay mixture	NADPH oxidized
	μmoles/mg protein/min
NADPH 0.25 mM, Dihydroxyacetone phosphate 20 mM	0.045
+Bathophenanthroline 2 mM	0.007
+Salicylaldoxime 10 mM	0.012
+o-Phenanthroline 5 mM	0.007
+Palmitoyl CoA 0.2 mM	0.009

Inhibitors were present in the assay mixture before the reaction was started by adding dihydroxyacetone phosphate. Other details as in the text and the legend to Table 1.

Table 3 Effect of isopropanol on α-glycerophosphate dehydrogenase in *Entamoeba histolytica*

Assay mixture	NADPH oxidized	Inhibition (%)
	μmole/mg protein/min	
NADPH 0.25 mM, Dihydroxyacetone phosphate 5 mM	0.03	..
+Isopropanol 1 mM	0.024	20
+Isopropanol 2 mM	0.015	50
+Isopropanol 3 mM	0.006	80
+Isopropanol 4 mM	0.006	80
NADPH 0.25 mM, Dihydroxyacetone phosphate 20 mM	0.045	..
+Isopropanol 1 mM	0.035	22
+Isopropanol 2 mM	0.027	40
+Isopropanol 3 mM	0.019	58
+Isopropanol 4 mM	0.010	78

Isopropanol was present in the assay mixtures before the reaction was initiated by adding dihydroxyacetone phosphate.

of bathophenanthroline, a highly specific chelating agent for iron, may indicate that the amoebal α-glycerophosphate dehydrogenase contains a non-heme iron.

Table 3 shows a marked inhibition of $NADP^+$-α-glycerophosphate dehydrogenase by isopropanol. Addition of 4 mM isopropanol almost abolished the enzyme activity. Fifty percent inhibition was accomplished by 2–2.5 mM of this alcohol. Other alcohols tested, i. e., methanol ethanol and n-propanol, however, had no effect on the enzyme activity.

Kinetic analysis on the isolated enzyme revealed that Km for dihydroxyacetone phosphate and for NADPH were 10 mM and 0.02 mM respectively. Vmax was 0.045 μmole NADPH oxidized/mg protein/min.

Divalent cations, i. e., 5 mM $MgSO_4$, 2 mM $CaCl_2$ and 1 mM $MnSO_4$, had no effect on the enzyme activity.

Discussion

The present communication showed cogent evidences on functioning of $NADP^+$-dependent α-glycerophosphate dehydrogenase in *E. histolytica*. It has been reported that α-glycerophosphate dehydrogenase from other cell types were primarily NAD^+-dependent (Baranowski, 1963), although requirement of pyridine nucleotides depends on concentrations of dihydroxyacetone phosphate and reduced pyridine nucleotides in some instance. In the case of a chicken liver enzyme, NADPH has 140% activity of that exhibited by NADH at high concentrations of the substrate and pyridine nucleotides (Barman, 1969). At low concentrations of these substances, however, NADPH brought only 5% activity of that by NADH. The amoebal enzyme, however, always showed a higher activity with NADPH than with NADH irrespective of concentrations of dihydroxyacetone phosphate and reduced pyridine nucleotides. Thus, it is likely that the amoebal α-glycerophosphate dehydrogenase is $NADP^+$-dependent. This finding may be compatible with the observation that *E. histolytica* has a high activity of NADPH oxidase (Weinbach *et al.*, 1977). NADH-oxidizing activity has been found to be much lower than NADPH oxidase.

Inhibition of the amoebal α-glycerophosphate dehydrogenase by various transition metal-chelators suggests the similar property of this enzyme to other oxido-reductases of amoeba. In other words, a transition metal,

possibly a non-heme iron, is involved in the catalysis of these enzymes.

Effect of palmitoyl CoA was first studied in detail on yeast glucose-6-phosphate dehydrogenase (Kawaguchi and Bloch, 1974). The mechanism of palmitoyl CoA inhibition of this enzyme appeared to involve dissociation of the enzyme into inactive subunits. Although an endogenous concentration of palmitoyl CoA is not known in *E. histolytica*, it is interesting that a considerable number of enzymes of *E. histolytica* are affected by this compound. These include glyceraldehyde-3-phosphate dehydrogenase and $NADP^+$-dependent ethanol dehydrogenase (Takeuchi *et al.*, 1979). Since all oligomeric enzymes are not susceptible to palmitoyl CoA (Kawaguchi and Bloch 1974), inhibition of the amoebal α-glycerophosphate dehydrogenase by this compound might be of physiological significance.

Inhibition of the enzyme activity by isopropanol at considerably low concentrations seems interesting, since isopropanol elicits alcohol dehydrogenase activity (Reeves *et al.*, 1971; Takeuchi *et al.*, 1979), which participates in the termial step for the endproduct formation of glycolysis mainly under the anaerobic condition (Montalvo *et al.*, 1971; Lo and Reeves, 1978). However, previous studies indicated that a major endproduct of the anaerobic glycolysis was ethanol but not isopropanol (Montalvo *et al.*, 1971). On the other hand, we have showed that $NADP^+$-ethanol dehydrogenase, which is responsible for acetaldehyde reduction, is different from $NADP^+$-isopropanol dehydrogenase in *E. histolytica* (Takeuchi *et al.*, 1979). Thus, it is possibe that isopropanol is produced from acetyl CoA by way of acetoacetyl CoA, acetoacetate and acetone. If this metabolic pathway is functional in amoeba, the inhibitory effect of isopropanol on α-glycerophosphate dehydrogenase may be a kind of a feedback inhibition of the amoebal glycolysis. Further investigations are still in progress in our laboratory in order to identify the matabolic pathway of α-glycerophosphate and to clarify the mechanism of the inhibitory effect of isopropanol. Details will be presented soon.

Summary

The activity of α-glycerophosphate dehydrogenase in *Entamoeba histolytica* was detected and concentrated in 40–80% ammonium sulfate saturated fraction prepared from the supernatant fraction of 15,000 g×20 min centrifugation. Addition of α-glycerophosphate, however, did not elicit NAD^+ or $NADP^+$ reduction, whereas dihydroxyacetone phosphate readily stimulated NADPH oxidation. NADH oxidation was much less than NADPH oxidation irrespective of concentrations of the substrate and reduced pyridine nucleotides. The activity as determined by NADPH oxidation was potently inhibited by various transition metal-chelators, palmitoyl CoA and isopropanol. Among the chelators tested, bathophenanthroline was most active. Additon of 0.2 mM palmitoyl CoA or 4 mM isopropanol almost abolished the enzyme activity. Km for dihydroxyacetone phosphate and for NADPH were 10 mM and 0.02 mM respectively. Vmax was 0.045 μmole NADPH oxidized/mg protein/min.

All of these findings suggest that the amoebal α-glycerophosphate dehydrogenase is primarily $NADP^+$-dependent and probably contains a non-heme iron as a functional catalytic group. Inhibition of the enzyme by isopropanol may represent a kind of a feedback inhibition of the amoebal glycolysis.

References

1) Baranowski, T. (1963): α-Glycerophosphate dehydrogenase. The Enzymes, 2nd ed., (Boyer, P. D. *et al.* eds.), Academic Press, New York, 85–96.
2) Barman, T. E. (1969): Enzyme Handbook, Springer-Verlag, Berlin, 1, 31–32.
3) Diamond, L. S. (1968): Techniques of axenic cultivation of *Entamoeba histolytica*, Schaudinn 1903 and *E. histolytica*-like amoebae. J. Parasit., 54, 1047–1052.
4) Kawaguchi, A. and Bloch, K. (1974): Inhi-

bition of glucose-6-phosphate dehydrogenase by palmitoyl CoA. J. Biol. Chem., 249, 5793–5800.
5) Lo, H. and Reeves, R. E. (1978): Pyruvate-to-ethanol pathway in *Entamoeba histolytica*. Biochem. J., 171, 225–230.
6) Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951): Protein measurement with the Folin-phenol reagent. J. Biol. Chem., 193, 265–275.
7) Montalvo, F. E., Reeves, R. E. and Warren, L. G. (1971): Aerobic and anaerobic metabolism in *Entamoeba histolytica*. Exp. Parasit., 30, 249–256.
8) Reeves, R. E., Montalvo, F. E. and Lushbaugh, T. S. (1971): Nicotinamide-adenine dinucleotide phosphate-dependent alcohol dehydrogenase: The enzyme from *Entamoeba histolytica* and some enzyme inhibitors. Internat. J. Biochem., 2, 55–64.
9) Takeuchi, T., Kobayashi, S., Tanabe, M. and Kaneda: Y. (1979): Submitted for publication.
10) Takeuchi, T., Weinbach, E. C. and Diamond, L. S. (1977): *Entamoeba histolytica*: Localization and characterization of phosphorylase and particulate glycogen. Exp. Parasit., 43, 107–114.
11) Weinbach, E. C., Harlow, D. R., Claggett, C. E. and Diamond, L. S. (1977): *Entamoeba histolytica*: Diaphorase activities. Exp. Parasit., 41, 186–197.

赤痢アメーバの NADP$^+$-依存性 α-グリセロリン酸脱水素酵素について

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無菌培養された赤痢アメーバ（HM—1株）を用い，α-グリセロリン酸脱水素酵素の活性を調べた．酵素活性は 15,000 g×20 min の上清から硫酸アンモニウム分画（40–80 %）を行い，酵素を分離した．α-グリセロリン酸は NAD$^+$，及び NADP$^+$ を還元せず，ディハイドロキシアセトンリン酸が NADPH 酸化を促進した．NADH の酸化は NADPH の $^1/_{30}$～$^1/_{40}$ であり，しばしば検出されぬこともあつた．ディハイドロキシアセトンリン酸による NADPH の酸化は調査した全ゆる基質，及びヌクレオチド濃度で NADH 酸化より速かつた．又，この NADPH 酸化は程々の transition metal-chelator，パルミチル CoA，又はイソプロパノールによつて阻害された．用いた chelator 中ではバソフェナンスロリンが最も強力であり，パルミチル CoA，及びイソプロパノールは各々 0.2 mM，4 mM で酵素活性を約 80 %阻害した．ディハイドロキシアセトンリン酸，及び NADPH に対する Km は各々 10 mM，0.02 mM であり，Vmax は 0.045 μmole NADPH oxidized/mg protein/min であつた．

これらのデータは赤痢アメーバの α-グリセロリン酸脱水素酵素は NADP$^+$-依存性であり，遷移金属（恐らく非ヘム鉄）を含むことを示唆している．イソプロパノールによる阻害は一種のフィードバック阻害に類似のものとして説明されるかもしれない．