THE LACTIC DEHYDROGENASE OF PENICILLIUM CHRYSOGENUM

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Received for publication August 7, 1953

A soluble, cytochrome linked flavoprotein which oxidizes lactic acid to pyruvic acid has been isolated from Penicillium chrysogenum, strain NRRL 1951-B25. The reduction of cytochrome c by a flavin enzyme has precedence in the work of Horecker and Heppel (1949) and Morell (1952) who have shown that xanthine oxidase can reduce cytochrome c. Morell reported the enzyme to be inhibited strongly by phosphate buffers; this agrees with a phosphate inhibition of the lactic dehydrogenase of P. chrysogenum when flavin-adenine dinucleotide is the coenzyme.

A cytochrome linked lactic dehydrogenase from Delft Baker's yeast has been reported by Bernheim (1928); this enzyme was solubilized by Ogston and Green (1935) and purified to a dry stable preparation by Gurchot and Lowman (1936). Ogston and Green compared various electron carriers and concluded that methylene blue and cytochrome c allowed the best oxidation of lactic acid by the enzyme. Later reports by Bach et al. (1942, 1946) show that even with highly purified preparations cytochrome c or methylene blue was the only addition needed for maximum activity although there was some indication that an additional factor was necessary for the system to react with cytochrome c.

This paper describes a soluble cytochrome linked lactic dehydrogenase from P. chrysogenum, strain NRRL 1951-B25.

EXPERIMENTAL METHODS

Penicillium chrysogenum, strain NRRL 1951-B25, was used throughout these studies and was grown in a medium of two per cent lactose and two per cent corn-steep liquor. One hundred ml of medium in 500 ml Erlenmeyer flasks were inoculated with 0.5 ml of a spore suspension and incubated on a reciprocating shaker at 25 C for 48 hours. The cells were harvested on cheese cloth, washed with one to two liters of cold distilled water, and then pressed relatively free of water by hand. All preparations from this point were made in a cold room at 4 C because of instability of the lactic dehydrogenase.

About 20 grams of quartz sand were mixed with 10 grams of damp mycelia, and the mass was ground for 10 minutes in a chilled mortar without the addition of liquid. The resulting paste then was suspended in 50 to 100 ml of cold distilled water (resulting in approximately pH 7.0) or 0.065 M phosphate buffer at pH 7.3 and centrifuged for 30 minutes at approximately 2,000 G. The cell-free supernatant was used directly for enzyme studies or was purified further by the process described later.

Oxygen uptake was measured at 30 C by standard manometric techniques on a Warburg apparatus. After equilibration, 0.5 ml aliquots of substrate were added from the side arm into the main compartment which contained an enzyme preparation of 3 to 5 mg nitrogen in 2.5 ml. Unless otherwise stated the substrate per vessel was 250 \( \mu \)l lactic acid in distilled water, 0.065 M phosphate buffer, or 0.1 M tris (hydroxymethyl) aminomethane buffer, all adjusted to pH 7.1. Other factors in final molar concentration were: Methylene blue, 3.3 \( \times 10^{-4} \) M; cytochrome c, 2.17 \( \times 10^{-5} \) M; sodium riboflavin phosphate, 1.7 \( \times 10^{-7} \) M; flavin-adenine dinucleotide, 8.3 \( \times 10^{-4} \) M. The latter was a preparation of 15 per cent purity from the Sigma Chemical Company.

Dialysis through a cellophane membrane was at 4 C against running distilled water for either 10 or 24 hours. Dialysis against dilute acid inactivated the enzyme.

Nitrogen determinations were by the method of Johnson (1949a). Organic solids were deter-
mined by the method of Johnson (1949) and pyruvate by the method of Green et al. (1945).

RESULTS

Preliminary purification. Many of the experiments were with crude preparations because the mold cytochrome oxidase system was removed during the first purification steps. A simple purification procedure has been developed, however.

A large amount of organic solids in the cell-free enzyme preparation was removed by freezing and thawing. The preparation was frozen at -10 °C and held for 24 hours to allow formation of large ice crystals. Thawing and centrifuging gave a water-clear, orange-brown preparation which had approximately 59 per cent of the original activity, 82 per cent of original organic solids, and 81 per cent of the original nitrogen.

This step removed the cytochrome oxidase activity; the procedure could be repeated, but losses in activity did not compensate for the increased purity. The enzyme could be stored for several months without inactivation at -20 °C although care had to be taken to prevent the enzyme preparation from becoming too warm during thawing.

After purification by freezing and thawing, the enzyme was precipitated in the cold with either alcohol or acetone; the latter gave better results. No lactic dehydrogenase activity was lost with 30 per cent acetone while approximately 53 per cent of the activity was removed by 35 to 40 per cent acetone, and 88 per cent of the activity was removed by 45 to 50 per cent acetone. These fractions were made at pH 6.5; and riboflavin phosphate, methylene blue, and phosphate (1.7 × 10^{-4} M, 2.2 × 10^{-4} M, and 2.2 × 10^{-4} M, respectively) were added for lactic acid oxidation. The ratio of organic solids to activity precipitated over this range of solvent concentrations remained constant.

Optimum pH. The pH optimum for lactic oxidation by the crude cell-free preparation in 0.065 M phosphate buffer was between 6.5 and 7.5. Preparations purified by freezing and thawing had a pH optimum of 6.8 to 7.0 in 1.67 × 10^{-2} M tris (hydroxymethyl) aminomethane buffer. Activity in this buffer dropped sharply at pH 7.5 and above because of the presence of an alkaline phosphatase which attacked the coenzyme; this could be counteracted partially by the addition of 2.2 × 10^{-4} M phosphate.

Michaelis constant. The lactic dehydrogenase of P. chrysogenum had a low affinity for substrate. For crude preparations maximum activity was at approximately 25 µM L-lactate per vessel without methylene blue and 125 µM with methylene blue. Frozen and thawed preparations which had been dialyzed for 10 hours had the same substrate requirement for peak activity (riboflavin phosphate added), and Michaelis constant measurements for both preparations were approximately 1.25 × 10^{-2} M.

Electron acceptors for nondialyzed preparations. In the absence of added electron acceptors the crude dehydrogenase had a maximum QO2 (N) of 16.2; with methylene blue (1.6 × 10^{-2} M) or cytochrome c (4.6 × 10^{-2} M) QO2 (N) values were 111.3 or 132.2, respectively. All preparations contained catalase, and the additions of purified catalase from animal sources had no effect on the enzyme methylene blue system.

Cyanide at 10^{-2} M inhibited lactate oxidation 43.4 per cent; when added to preparations using cytochrome c or methylene blue as electron acceptors, inhibitions of 79.2 per cent and 61.9 per cent, respectively, were obtained. Cyanide at 10^{-3} M had little effect. Additions of 75 µM oxalate per vessel inhibited oxygen uptake 38.5 per cent with no added carrier and 37.4 per cent with methylene blue, indicating inhibition of the enzyme and not the carrier. Similarly saturation with quinine sulfate gave a constant inhibition of 17 to 20 per cent with additions of cytochrome c, methylene blue or no addition.

Comparison with D- and L-amino acid oxidase. The cell-free lactic dehydrogenase also contained D- and L-amino acid oxidase of which neither was able to reduce methylene blue. The lactic dehydrogenase under varying conditions was less stable than either amino acid oxidase.

Rapid heating of a crude preparation to 45 C and immediate cooling had no effect on oxidation of D- or L-methionine but left only 61 per cent of the lactic dehydrogenase activity. The dehydrogenase was inactivated more easily during the freezing and thawing step also.

Coezyme requirement. After dialysis of either the crude or the frozen and thawed preparation, the lactic dehydrogenase showed a definite requirement for riboflavin phosphate or flavin-adenine dinucleotide in addition to the requirement for an electron acceptor. Figure 1 illustrates lactic acid oxidation by an enzyme preparation dialyzed 9 hours against distilled water and with
either riboflavin phosphate or flavin-adenine dinucleotide added.

Flavin-adenine dinucleotide served as a coenzyme, but to obtain comparable activity it was required in concentrations about 3 times greater than riboflavin phosphate. The preparation, however, contained an enzyme which split flavin-adenine dinucleotide yielding riboflavin phosphate; this was followed by observing the increase in fluorescence on a Coleman photofluorometer when flavin-adenine dinucleotide was substrate.

When flavin-adenine dinucleotide or riboflavin phosphate was the coenzyme and when the pH was above pH 7, it was necessary to add phosphate to the dialyzed preparations (figure 2). The enzyme preparation also contained an alkaline phosphatase which split phosphate from riboflavin phosphate and which could be inhibited partially by adding phosphate. Levels of phosphate greater than 0.0055 M inhibited the hydrolysis of flavin-adenine dinucleotide as shown by the decreased activity of the lactic dehydrogenase in figure 2. This has been shown for the crude preparation with methylene blue or cytochrome c as electron acceptor and for the frozen and thawed preparation with methylene blue.

Acetone precipitation of the enzyme also removed the coenzyme. The requirements for phosphate, riboflavin phosphate, and electron acceptor were the same.

Adding diphosphopyridine nucleotide or triphosphopyridine nucleotide to dialyzed preparations gave no increase in activity over that obtained with riboflavin phosphate. The nondialyzed preparations of lactic dehydrogenase contained neither diphosphopyridine nucleotide nor triphosphopyridine nucleotide as evidenced by the complete inactivity of the malic and isocitric dehydrogenases, also present, unless the respective coenzymes were added.

**Figure 1.** The effect of cytochrome c, methylene blue, and flavin-adenine dinucleotide on lactate oxidation by dialyzed cell-free preparation. Vessels contained 2 µM magnesium, 250 µM DL-lactate, 30 µM pH 7.3 phosphate, 2 ml cell-free enzyme dialyzed 9 hours (2.01 mg nitrogen per vessel), and 6.51 × 10⁻⁸ µM cytochrome c, 3 µM methylene blue or 0.025 µM flavin-adenine dinucleotide.

**Figure 2.** The effect of phosphate on oxidation of lactate by a dialyzed cell-free preparation with and without flavin-adenine dinucleotide. Vessels contained pH 7.3 phosphate, 250 µM DL-lactate, 0.025 µM flavin-adenine dinucleotide, 6.51 × 10⁻⁸ µM cytochrome c, and 1.6 ml cell-free enzyme dialyzed 28 hours.
Reaction product. The cell-free preparation oxidized lactic acid one step to pyruvic acid with 101 per cent of the theoretical uptake of O₂ when methylene blue was the electron acceptor and 100 per cent of the theoretical uptake of O₂ when cytochrome c was the electron acceptor. These are based on the amount of pyruvate which theoretically should have been formed according to the oxygen uptake on lactate.

DISCUSSION

The lactic dehydrogenase of P. chrysogenum appears to be different from other dehydrogenases in that it is a flavin enzyme which directly uses the cytochrome system to oxygen. It resembles the soluble cytochrome linked yeast dehydrogenase except that the yeast enzyme is not reported to be a flavoprotein and it has a greater affinity for substrate than does the mold enzyme. It also resembles somewhat the glycolic oxidase of Zelitch and Ochoa (1953) which is a flavoprotein (riboflavin phosphate) and which oxidizes lactic acid.

Meher et al. (1947) stated that the animal lactic dehydrogenase is diphosphopyridine nucleotide linked but can use triphosphopyridine nucleotide to a lesser degree. No requirement could be found in the mold dehydrogenase for diphosphopyridine nucleotide, nor is it probable that any diphosphopyridine nucleotide was present in the preparation since it is decomposed at a rapid rate.

The possibility exists in crude preparations that more than one enzyme is attacking lactic acid since the preparation contains both d- and L-amino acid oxidases, and Blanchard et al. (1945) reported a riboflavin phosphate linked L-amino acid oxidase to oxidize L-alpha hydroxy acids, including lactic acid. The mold amino acid oxidases, however, cannot utilize methylene blue, nor is there a correlation between activity on amino acids and lactic acid in respect to enzyme stability on heating or purification.

The presence of a single enzyme for lactic acid also is implied from results with the flavin enzyme inhibitor, quinine. Hellerman et al. (1946), in studying d-amino acid oxidase, concluded that quinine competes with the flavin for position on the enzyme surface. With the mold dehydrogenase, quinine gives the same per cent inhibition with or without cytochrome c or methylene blue, indicating that in the nondialyzed preparation a flavin is the intermediate for the 3 oxidations.

The yeast lactic dehydrogenase was inhibited strongly by oxalic acid (Bernheim, 1928). Oxalate was also a specific inhibitor for the mold dehydrogenase since the per cent inhibition of lactate oxidation was the same with methylene blue or the mold cytochrome system.

It should be noted that the experiments reported in this paper are in vivo. There is no evidence as yet that the intact mold actually uses cytochrome with this lactic dehydrogenase. The approach to this problem probably will have to be made by a combined use of in vivo inhibitors for both the cytochrome and flavin systems.

SUMMARY

A cell-free lactic dehydrogenase of Penicillium chrysogenum, strain NRRL 1951-B25, is described. This enzyme is soluble and for maximum activity requires riboflavin phosphate and cytochrome c. The presence of only one enzyme attacking lactic acid was shown by use of inhibitors. The enzyme has low substrate affinity as evidenced by a Michaelis constant of approximately 1.4 x 10⁻² M.

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