ON THE PROPERTIES OF A D(-)LACTIC OXIDASE SYSTEM IN RESPIRATORY PARTICLES OF YEAST

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D(-)lactic cytochrome c reductase, the enzyme responsible for D(-)lactate oxidation in aerobic yeast, has been studied using phenazine methosulfate and cytochrome c as artificial electron acceptors and characterized as a flavoprotein containing zinc (Gregolin and Singer, 1962 and 1963). In spite of the appreciable autooxidizability of the enzyme, the velocity of the direct reoxidation by O_2 was insignificant in the catalytic test.

On the other hand, it had been observed (Gregolin and Singer, 1961) that respiratory particles of yeast, which are the best source of the reductase, were incapable of oxidizing D(-)lactate with O_2 uptake. They did, however, exhibit a strong oxidation of succinate, which was sensitive to inhibition by antimycin A.

The observation led to the assumption that D(-)lactic dehydrogenase of aerobic yeast is not functionally linked to the respiratory chain. This view was supported by the observed difference in activation induced by external cytochrome c on the respiration of succinate and D(-)lactate. With succinate, the activity was completely abolished by antimycin A. With D(-)lactate, the activation was unaffected by the addition of inhibitor. Thus there appears to be a "by pass", which connects the dehydrogenase to cytochrome oxidase, with cytochrome c acting as an artificial electron acceptor.

Further investigation of the problem was undertaken using a preparation of particles partially different from that previously employed. This entailed mainly substitution of hypertonic sucrose for 1% NaCl for breaking the cells, with the consequent stabilization of labile cofactors (or structures) involved in the oxidation of D(-)lactate. Particles, isolated essentially according to Linnane and Still (1955), exhibited a clear ability to oxidize D(-)lactate, when tested under suitable conditions. Versene, normally omitted to avoid interferences with the zinc moiety of the enzyme, was of major importance in preserving activity. The sole departure from the procedure of Linnane and Still was the use of a different kind of shaker ("Vibrogen", Groschopp u. Co., Viersen, West-Germany). The scale of the preparation was adjusted to process 20 gm. of pressed baker's yeast at a time. The shaker was operated at 2,800 cycles per min. for 75 seconds. From each 20 gm. of yeast (Distillerie Italiane), 2.5 ml of particles suspension were obtained, which contained usually about 25 mg protein (nitrogen basis) per ml.

The particles oxidize D(-)lactate vigorously with O_2 as ultimate electron acceptor. In contrast to the preparation made in 1% NaCl, the present preparation oxidizes D(-)lactate with phenazine methosulfate, but not with cytochrome c. Also cytochrome c does not stimulate D(-)lactate oxidation.

The most convenient assay conditions are in 0.05 M phosphate, pH 6, in the presence of 0.01 M D(-)lactate and of 0.035 M Mg^{++}. These conditions deserve comment, because they are partially different from those necessary for full activity of the isolated primary dehydrogenase (as described by Gregolin and Singer, 1963). The activity of the particles varies slightly from pH 5.5 to 6.5, then falls rather sharply as the pH rises to 7.5. Substituting other buffers for phosphate does not change this behavior. The optimal concentration of D(-) lactate is the same as for the dehydrogenase. Mg^{++} is added

to the reaction mixture because it causes a four- to five-fold stimulation of the reaction rate. An analogous effect is obtained with similar concentrations of Ca^{++}, Mn^{++}, Fe^{++}, Co^{++}.

For tests with these metals, Tris-acetate buffer was used to avoid precipitation of cations. The ratio of the activation is of the same order of magnitude as obtained with Mg^{++} in phosphate (without addition of Mg^{++}, Tris-acetate buffer gave 80% of the activity in phosphate). When velocity in the presence of Mg^{++} minus velocity in the absence of Mg^{++} is plotted versus Mg^{++} concentration in the double reciprocal plot, a rather steep curve is obtained (Fig. 1). For this reason the activity of the preparation was normally calculated by adding to the activity in the absence of Mg^{++} the activation extrapolated at infinite concentration of metal. The K_m for the substrate in the presence of 0.035 M Mg^{++} is 3.8×10^{-4} M, at pH 6, 22° C, i.e., slightly higher than that for the isolated dehydrogenase as measured in the spectrophotometric assay (2.9×10^{-4} M, at pH 7.5, 22° C); the difference in the optimal conditions of the two assays accounts for the divergence.

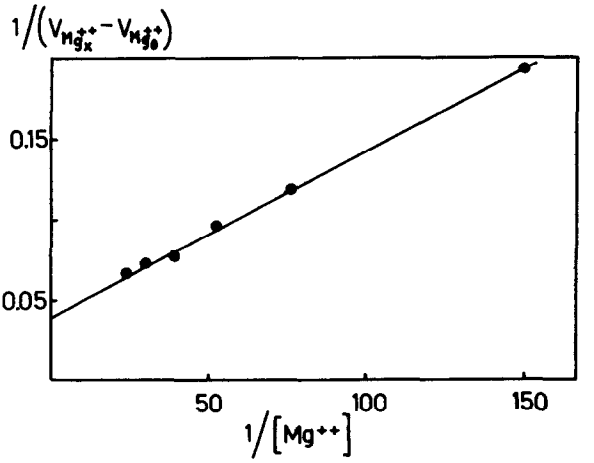

Fig. 1. Dependence of D(-)lactic oxidase activity on Mg^{++} concentration. O_2 uptake was measured for 1 min. with a Gilson Medical Electronics vibrating platinum electrode, at 22° C. In 1.5 ml total volume were present: 3.3 mg protein, 0.01 M D(-)lactate, 0.05 M phosphate buffer pH 6, Mg^{++} as indicated in the abscissa. Ordinate: reciprocals of velocities in the presence of Mg^{++} minus velocity in the absence of Mg^{++}.

The best preparation oxidized 0.12 μM D(-)lactate/min/mg. protein, at 22° C, as measured by the O_2 uptake. The activity shown by the preparation in the spectrophotometric test at infinite concentration of phenazine methosulfate is 0.65 μM D(-)lactate oxidized/min./mg., at the same temperature. The oxidase activity is equal, therefore, to 18.5% of the dehydrogenase activity. However, this is greater than that obtained with cytochrome c used as artificial electron acceptor with the preparation previously employed (Gregolin et al., 1961). When calculated in terms of Q_{O_2}, the activity was 83.5. This value is much larger than that obtained by Utter et al. (1958) using DL-lactate as substrate, and, moreover, larger than that exhibited by yeast cells employed as the starting material in this preparation (Q_{O_2} for D(-)lactate = 70).

Typical activities of this preparation with other substrates (assayed under optimal conditions) are as follows: L(+)lactate (plus Mg^{++}), 0.13 μM; L(+)lactate (without addition), 0.03 μM; succinate 0.1 μM; pyruvate (plus malate as a "sparker"), 0.08 μM; pyruvate (without additions), 0.005 μM oxidized/min./mg. protein, at 22° C. The most remarkable aspect of this list is that L(+)lactate oxidation is stimulated by Mg^{++} as is D(-)lactate.

The stimulation by divalent cations on the D(-) and L(+) lactic oxidase activity is a very interesting feature of this preparation, which casts some light on the path of the electron flow between dehydrogenases and O_2. Since Versene is used in preparation of the particles, a possible inhibitory effect on the primary D(-)lactic dehydrogenase containing zinc was investigated. It was found that Mg^{++} does not stimulate the dehydrogenase activity as measured spectrophotometrically with phenazine methosulfate. It must be concluded therefore that the concentration of Versene used in the preparation of the particles, 0.002 M, does not adversely affect the dehydrogenase. On the other hand, it is known that cyto

chrome b_2 does not contain non-heme metal and is not inactivated by chelating agents. The characteristics of these reactions suggest that Versene inactivates an intermediary component of the lactic oxidase system and that Versene is removed by divalent cations. Such a component (metal? metal containing protein?) would be an obligatory step common to the oxidation of D(-) and L(+)lactate. Unfortunately, attempts to identify such an intermediate by direct addition of metals to the particles prepared in the absence of Versene (and exhibiting only negligible activity) have been unsuccessful. When o-phenantroline was substituted for Versene, the lactic oxidase system was irreversibly inactivated.

Whether the cytochrome chain acts in the oxidase system cannot be decided on the basis of the present information. Antimycin A does not inhibit D(-) and L(+)lactate oxidation. Sodium azide and cyanide exert a powerful and immediate inhibitory effect on D(-) and L(+)lactate oxidation at concentrations which are characteristic of cytochrome oxidase inhibition (5×10^{-3} M and 5×10^{-4} M, respectively); but an action on other factors of the system cannot be excluded.

Experiments on the nature of the intermediate and on the involvement of the respiratory chain in the reactions are at present in progress.

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