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THE LACTIC DEHYDROGENASE OF YEAST

III. D(−)LACTIC CYTOCHROME *c* REDUCTASE, A ZINC-FLAVOPROTEIN FROM AEROBIC YEAST

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SUMMARY

The D-lactic cytochrome reductase of the respiratory particles of aerobic yeast has been solubilized and isolated in apparently homogeneous form. The enzyme possesses high selectivity both toward substrates and electron carriers: only D-lactate and D-α-hydroxybutyrate are oxidized at appreciable rates; phenazine methosulfate and cytochrome *c* are the only electron carriers which have been found to be active. The action of the enzyme appears to be irreversible.

The reductase contains 1 mole FAD per 50 000 ± 5000 g protein and 1 g atom Zn^{2+} per 22 000–27 000 g protein. Partial reversible resolution with respect to the flavin has been accomplished. The Zn^{2+} moiety is very tightly bound. This appears to be the first direct demonstration of the existence of Zn–flavoproteins.

INTRODUCTION

In the course of a study[1,2] of the possible interrelations of the L(+)lactic dehydrogenase (cytochrome b_2) and of the D-α-hydroxy acid dehydrogenase (DHAD, "anaerobic lactic dehydrogenase") of yeast, evidence was uncovered[1] indicating the existence of a third lactic dehydrogenase in yeast cells, which has been named D-lactic cytochrome reductase[1,3]. The enzyme differed from cytochrome b_2 in its inability to oxidize the L-configuration of substrates and to react with ferricyanide, methylene blue and other electron carriers which are satisfactory electron acceptors for cytochrome b_2, as well as in its sensitivity to metal chelating agents and other inhibitors to which cytochrome b_2 is inert. The reductase was readily differentiated from the DHAD of anaerobic yeast, on the other hand, by virtue of the exclusive presence of the reductase in respiratory-chain preparations from aerobic cells, as well as by notable differences in substrate and electron acceptor specificity and response to inhibitors[1,3,4].

In preliminary communications we have reported the solubilization, assay, and general properties of the enzyme[3], its isolation and the identification of its prosthetic groups as Zn–FAD[5], the mechanism of its reversible inactivation by chelating agents[6],

Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; DHAD, D-α-hydroxy acid dehydrogenase.

and its application to the measurement of species differences and preparative modifications in cytochrome *c* preparations[7]. The question of its supposed role in the biosynthesis of cytochrome b_2, suggested in the literature[8,9], has been considered elsewhere[3]. The present paper is a detailed account of the isolation and characterization of the enzyme.

The enzyme was independently discovered by NYGAARD[8], who has reported its partial purification[10] and some aspects of its kinetics[11], without noting, however, that Zn^{2+} is a functional component of the reductase.

MATERIALS AND METHODS

Fresh baker's yeast was a gift of the Red Star Yeast Products Company. Ca D(—)- and L(+)-lactate were purchased from the California Corporation for Biochemical Research and were converted to the Na salt by treatment with Na_2CO_3 before use. Horse-heart cytochrome *c*, Type III, was purchased from the Sigma Chemical Corporation. The lots employed (numbers 31B650 and 61B705) had been shown to be free from inactive or inhibitory materials as far as this enzyme is concerned[7]. The sources of other materials were as follows: DL-*α*-hydroxy acids, Fluka AG Chemische Fabrik, Buchs, (Switzerland); phenazine methosulfate, Aldrich Chemical Company, Inc.; DCPIP, General Biochemicals, Inc.; crystalline bacterial proteinase, Nagase and Company; *Naja naja* venom, Ross Allen's Reptile Institute, and Triton X-100, Rohm and Haas Company. Triton X-100 was evacuated for 6 h at 50° before use in order to remove contaminating acetaldehyde and ethanol.

DEAE-cellulose "Selectacel" and CM-cellulose (both the products of Brown Company) were sized, washed, and equilibrated by standard procedures. Calcium phosphate gel (1–3 months old) was prepared according to SINGER AND KEARNEY[12]. Reagent grade $(NH_4)_2SO_4$ was recrystallized first from EDTA, then from water.

Spectrophotometric assays were performed at 30° with a recording spectrophotometer, and spectra were recorded at 25° with a Cary Model 11 spectrophotometer equipped with a thermostated microcell holder. Protein was determined by the biuret method[13], with the use of the coefficients 0.093 per mg protein in 3 ml through the ammonium sulfate step and 0.111 for the succeeding steps. The latter coefficient was determined on thoroughly dialyzed preparations of known dry weight. In particulate preparations and acetone powders 0.3% desoxycholate was added and in Triton extract protein was first precipitated with 5% trichloroacetic acid.

Flavin was determined by differential fluorometry, according to BURCH[14], following extraction with cold 5% trichloroacetic acid or 2% perchloric acid, with a Farrand Model A photoelectric fluorometer. FAD was also determined by the D-amino acid oxidase test and by ascending paper chromatography with 5% Na_2HPO_4 as solvent.

Zn^{2+} analyses were performed with a modification of MALMSTRÖM's procedure[15], adapted to measure 0.2–0.8 μg of Zn^{2+} in enzyme samples. Iron was determined according to PETERSON[16]. Sedimentation velocity was measured in a Spinco analytical Model L ultracentrifuge. For electrophoretic analyses the Perkin-Elmer Model 38A apparatus was employed.

RESULTS

Assay

Although the existence of D-lactic cytochrome reductase was first recognized by virtue of its ability to reduce cytochrome *c*, phenazine methosulfate reoxidizes the enzyme eight times faster than the most active cytochrome sample tested (Fig. 1). Further, while the reactivity of the enzyme with cytochrome *c* is a function of the species from which the cytochrome is obtained and of the care employed in its isolation[7], its reaction with phenazine methosulfate is free from these complications. Since the reductase does not reduce DCPIP directly, a rapid and convenient assay based on the use of this dye as terminal acceptor and phenazine methosulfate as a mediator was routinely employed. Unless otherwise mentioned assays were conducted in 0.05 M imidazole buffer (pH 7.5) in the presence of 0.01 M D(—)lactate,

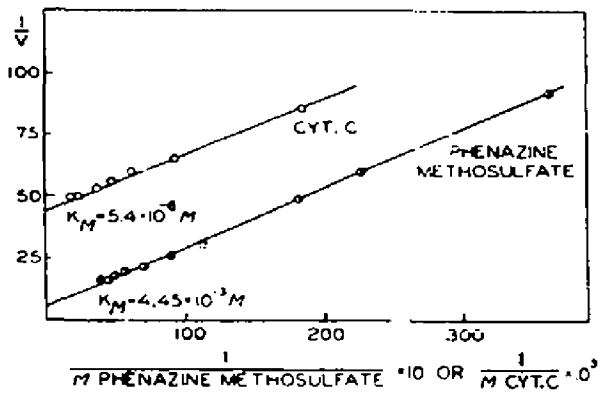

Fig. 1. Assay of the reductase with cytochrome *c* and phenazine methosulfate as electron acceptors. Abscissa, reciprocal concentration of acceptor; ordinate, reciprocal activity in arbitrary units. Test conditions as described in the text. 2 μg of purified enzyme was employed and the reaction period was 15 sec.

$7 \cdot 10^{-6}$ M DCPIP, and $2.2 \cdot 10^{-3}$ M phenazine methosulfate in a total volume of 3 ml at 30°. Reaction rates were calculated from the bleaching at 600 mμ occurring within the first 30–60 sec after addition of the substrate. As has been documented elsewhere[17], the observed rate is independent of the presence of dissolved O_2 and of the DCPIP concentration within a wide range. The rate is a function of the phenazine methosulfate concentration, however (Fig. 1). Nevertheless, owing to the relative constancy of the apparent K_m for phenazine methosulfate during purification, recourse to the determination of $V_{max.}$ with respect to the dye is not required except when precise values are essential. At $V_{max.}$ the rate is 3.1 times that observed at the fixed concentration recommended. Activity was expressed as μmoles of D-lactate oxidized per min at $V_{max.}$ with respect to phenazine methosulfate. Specific activity is the same per mg protein.

In the measurement of cytochrome reductase activity the test was modified by substituting $5 \cdot 10^{-5}$ M horse-heart cytochrome *c* of a suitable batch (*cf.* below) for the two dyes, and measurements were conducted at 550 mμ.

Cytochrome *c* may also be substituted for DCPIP in the phenazine assay, since it

can react directly with leucophenazine methosulfate[18]. With both cytochrome *c* and phenazine methosulfate present the observed rate is, of course, much higher than with cytochrome *c* alone. This technique, however, introduces the unnecessary complication of dual reaction sites for cytochrome *c* with the attendant risk of kinetic artifacts.

The apparent K_m for a given cytochrome *c* sample remains constant throughout the purification of the enzyme. With the cytochrome preparations employed in this study, multiplication by 1.14 converts the rate observed at $5 \cdot 10^{-5}$ M horse-heart cytochrome *c* to $V_{max.}$.

In particulate preparations of the enzyme and in acetone powder suspensions interference by cytochrome oxidase in both types of assay could be eliminated by the inclusion of $5 \cdot 10^{-4}$ M cyanide, which is insufficient to inhibit the reductase under the assay conditions. The inclusion of EDTA in the assay, as employed by NYGAARD[10], is not recommended owing to its ability to chelate with the Zn^{2+} component of the enzyme and thereby inhibit it.

Localization of the enzyme in subcellular fractions

The presence of the reductase in aerobic yeast was first noted in extracts obtained by autolysis of dried yeast[4], but the activity solubilized in this manner was rather low. In view of the tendency of the enzyme to resist extraction by conventional methods, a study was made of its intracellular localization. Breakage in the Nossal shaker and differential centrifugation, using a modification of the method of HEBB *et al.*[19], indicated that some 13% of the activity was in the supernatant solution obtained on 15 min centrifugation at 144 000 × *g*. The rest of the activity was recovered in the particulate fraction sedimented at 144 000 × *g* but not in 10 min at 600 × *g*. Since the particles collected under these conditions appear to consist mainly of membrane fragments (also called mitochondria in the literature), wherein the respiratory chain is localized, it was of interest to examine whether the enzyme is functionally linked to the terminal electron transport system of yeast. As noted elsewhere[3,4], thus far no evidence has been obtained for the existence of a direct linkage of the enzyme to the cytochrome system, at least in respiratory-particle preparations.

Solubilization of the enzyme

Since the particles contained 87% of the reductase activity and were devoid of DHAD activity, they represented an ideal starting material for the isolation of the enzyme. Extraction of the enzyme was first attempted by mild enzymic procedures, which often liberate particle-bound enzymes in soluble form. Digestion with phospholipase A, or a purified bacterial lipase of broad specificity[20], or a concentrate of the autolyzing enzymes of yeast failed to solubilize the reductase. Treatment of acetone powders of the particles with mildly alkaline buffers failed to extract the enzyme at pH values where it was stable. At more alkaline pH values both the extent of extraction and of inactivation increased[4]. Particles desiccated with *n*-butanol behaved in an analogous manner to acetone powders; a maximum of 18% of the activity could be extracted by this procedure[4]. Extraction with aqueous butanol also gave essentially negative results.

Since no means was found to [illegible] particles, an alternative [illegible] enzyme in a highly [illegible] Triton X-100, partial [illegible] by solubilization of [illegible] proteinase. Thus a method of solubilization which was ineffective when applied to the particulate source material, proved highly effective when applied to a purified

TABLE I

PURIFICATION OF D(—)LACTIC CYTOCHROME *c* REDUCTASE

Step	*Total activity (μmoles of D(—)lactate oxidized/min)*	*Specific activity (μmoles D(—)lactate oxidized/min/mg)*
Yeast particles (from 9000 *g*)	115 000	0.4
Triton extract of acetone powder	97 200	7
Eluate from Ca phosphate gel	84 000	32
After digestion with *Naja naja* phospholipase A and bacterial proteinase	69 600	75
Eluate from DEAE-cellulose	62 400	300
53–68% $(NH_4)_2SO_4$ fraction	40 000	440
Eluate from CM-cellulose	24 000	940
After preparative ultracentrifugation	8000*	1670*

* Corrected for inactivation during ultracentrifugation.

preparation. The high yield obtained by this method of extraction (Table I) may be contrasted with the rather poor yield (7.5%) reported by NYGAARD[10] for his solubilization method, which entails precipitation with wet acetone and heating at 55°.

Isolation of the enzyme

Large scale preparation of yeast particles

480 g of pressed yeast were diluted to 1.2 l with 1% NaCl (adjusted to pH 7.5), and 1 l of pre-cooled Ballotini beads (No. 12) were added. Mechanical breakage of the yeast suspension was achieved in the overhead blendor described earlier[21], operated at 18 000 rev./min for 15 min in an 8 l stainless steel beaker surrounded by an ice-bath; the temperature rose to 25° at the end of the breakage. The beads were allowed to settle and the supernatant suspension was decanted and made to 4 l with 0.05 M phosphate (pH 7.5) using part of this buffer to wash the beads two or three times. The suspension was centrifuged for 4 min at 1300 × *g* in an International Model SR-3 centrifuge; the turbid supernatant was collected and the precipitate containing residual cells and cell walls was discarded.

The supernatant solutions obtained from four 480-g batches were united and centrifuged in a refrigerated Sharples centrifuge at 50 000 rev./min at a flow rate of 30 ml/min; the effluent appeared only slightly turbid and was discarded.

With smaller quantities of material other blendors may be substituted but the time and speed of homogenization must be redetermined. Thus, with the same ratio of pressed yeast : NaCl solution : Ballotini beads as described above, satisfactory

results are obtained with 60 g yeast and the VirTis "45" blendor (250-ml bowl), using a rheostat setting of 61 and 30 min blending. With the Lourdes homogenizer 120 g yeast may be worked up in 20 min at a rheostat setting of 62.

Preparation of acetone powder

The thick paste obtained from 1920 g of yeast was homogenized and stirred for 5 min with 4 l of acetone, pre-cooled to −10°, in an 8 l stainless steel beaker, using the overhead blendor operated at 8000 rev./min. The suspension was then centrifuged for 5 min at 1400 × *g* at −10°. The precipitate was homogenized and stirred with acetone as before, rapidly filtered by suction through a Buchner funnel, and the moist cake was washed with 500 ml of ether (−10°). Residual solvent was removed by spreading the cake on heavy paper in the cold room in front of a fan and then was dried in a high vacuum at room temperature. The resulting powder weighed 40–60 g/1920 g of pressed yeast. The enzyme in this form is very stable and the powder can be stored at least for several weeks without appreciable loss of activity.

Extraction with Triton X-100

Treatment of the acetone powder suspension with a buffer of moderately high ionic strength extracts a certain amount of inert protein and thereby simplifies the subsequent purification of the reductase. 40 g of acetone powder suspended by brief homogenization in 1 l 0.1 M Na phosphate (pH 6.5) were stirred vigorously for 15 min at 0° and then centrifuged for 30 min at 18 000 rev./min in the Batch Rotor of the Spinco Model L ultracentrifuge (43 400 × *g* max.). The yellowish supernatant, containing essentially no enzyme, was discarded and the precipitate was resuspended in 800 ml 0.0125 M Na phosphate (pH 6.5). To this 200 ml of a 20% solution (v/v) of Triton X-100 in water were added and the suspension was slowly stirred for 15 min with particular care taken to avoid excessive foaming. The suspension was then centrifuged 30 min at 20 000 rev./min in a Spinco Model L ultracentrifuge (Rotor No. 21) and the resulting clear, intensely brown-yellow supernatant (about 1000 ml) contained 90–100% of the enzyme present in the suspended powder.

Purification with calcium phosphate gel and lyophilization

The protein dispersed by Triton X-100, although not suitable for salt fractionation, can be partially purified by means of calcium phosphate gel. For best yield and purity it is advisable to determine for a given extract the amount of gel which gives 90–95% adsorption of the enzyme, the usual range being 0.4–0.6 mg gel/mg protein.

In practice, the Triton extract was treated with 70 ml of a gel suspension containing 27 mg gel/ml (on a dry weight basis) and, after 10 min of slow stirring, it was centrifuged for 5 min at 1400 × *g*. The supernatant was discarded and the gel was washed with 1 l of 0.05 M phosphate buffer (pH 6.5). The wash was discarded and the enzyme was eluted from the gel with 400 ml of 0.25 M K phosphate buffer (pH 6.5) to which 4 ml of 20% Triton X-100 had been added. The clear, yellow-brown supernatant contained the enzyme in a yield of 75–85%. The salt concentration in the eluate was lowered by passing it through a column of Sephadex G-25

(V_0 = 500 ml), equilibrated with 5 mM phosphate (pH 6.5). The solution was then lyophilized. The resulting powder (350–450 mg protein) could be preserved for several weeks at −20° without appreciable loss of activity.

Treatment with phospholipase A and bacterial proteinase

Digestion of the preparation at this stage with phospholipase A and bacterial proteinase converts it to a soluble form, as evidenced by solubility in $(NH_4)_2SO_4$ solution and behavior on ion exchange columns. Solubilization is principally accomplished by the former enzyme, while the latter serves mainly to remove interfering proteins, such as cytochrome *c*.

Five batches of lyophilized powder were pooled and dissolved in cold distilled water to a concentration of 25 mg protein/ml. The dark brown, clear solution was dialyzed for 8–10 h against 5 mM phosphate buffer (pH 6.5) to reduce the salt concentration. Following 5 min temperature equilibration the preparation was incubated for 45 min at 30° in the presence of *Naja naja* venom (0.04 mg/mg of protein) and crystalline bacterial proteinase (0.005 mg/mg protein). Turbidity developed during incubation, but no inactivation of the enzyme occurred. The mixture was then rapidly cooled to 0° in a salt–ice mixture and centrifuged for 10 min at 14,000 × *g*. The yellow-brown supernatant solution contained 85–90% of the enzyme.

Chromatography on DEAE-cellulose

This step yields four-fold purification and further serves to remove residual Triton and bacterial proteinase.

The soluble enzyme was adsorbed on a DEAE-cellulose column (2 × 15 cm), equilibrated with 5 mM K phosphate (pH 6.5). The column was washed with 0.065 M K phosphate (pH 6.5) until the A_{280} of the effluent was almost zero and the enzyme was then eluted with 0.2 M NaCl–0.1 M phosphate (pH 6.0). The yellow enzyme band was collected batchwise with a yield of 90–95%. From this point particular care was taken to protect the enzyme from light.

The enzyme was immediately concentrated by the addition of solid $(NH_4)_2SO_4$ to 0.75 saturation while the pH was maintained at 6 by the addition of 2 N NH_4OH. After 15 min stirring the precipitate was collected by 15 min centrifugation at 24 000 × *g*.

Salt fractionation

The precipitate was dissolved in 0.1 M phosphate (pH 6.5) to a concentration of 15 mg protein/ml, and the resulting $(NH_4)_2SO_4$ concentration was calculated from the volume of the precipitate in the preceding step. Saturated $(NH_4)_2SO_4$ solution was added to 0.53 saturation and the pH was maintained at 6.5. The resulting precipitate was centrifuged and discarded and the enzyme was precipitated from the supernatant solution by the further addition of saturated $(NH_4)_2SO_4$ solution to 0.68 saturation. The precipitate dissolved in a minimal volume of 0.02 M phosphate (pH 6.5) containing 65% of the enzyme, was dialyzed for 6–8 h against the same buffer in order to remove low-molecular-weight protein impurities resulting,

presumably, from the digestion with bacterial proteinase which had not been removed in the preceding steps.

CM-cellulose chromatography

The dialyzed enzyme was equilibrated with 0.02 M Na acetate, (pH 5.2) on a column of Sephadex G-25; turbidity was removed by 5 min centrifugation, and the enzyme was then adsorbed on a CM-cellulose column (1 × 10 cm), equilibrated with the same acetate buffer. The column was washed with the equilibrating solution and a first inactive fraction was removed. The fraction containing the enzyme was then eluted with a pH gradient obtained as follows: 75 ml of 0.01 M Na succinate buffer (pH 5.2) in the mixer, 0.025 M Na phosphate (pH 7) in the reservoir. Fractions of 2 ml were collected at a flow rate of about 0.5 ml/min. The enzyme was distributed in 12 fractions, and the six central fractions containing about 60% of enzyme of highest specific activity were pooled, adjusted to pH 6.5 with 2 N NH_4OH, and concentrated by precipitation with solid $(NH_4)_2SO_4$ at 0.70 saturation.

At this stage the enzyme is 55–60% pure, as judged by sedimentation analysis. The remaining contaminant is a high-molecular-weight protein, which sediments almost 3.5 times as fast as the reductase. While this contaminant may be removed by differential ultracentrifugation and thus an apparently homogeneous preparation is obtained, there is always an attendant inactivation, since from this point on the enzyme is relatively unstable and fairly readily loses its flavin component.

Ultracentrifugation

The enzyme obtained as described above was dissolved to a concentration of about 10 mg/ml and equilibrated with 0.1 M NaCl–0.01 M K phosphate (pH 6.5) by passage through Sephadex G-25. It was ultracentrifuged in the SW 39L swinging bucket rotor of the Spinco Model L ultracentrifuge (4-ml tubes, 173 000 × $g_{max.}$). After 6 h centrifugation the content of the upper half of the tube was carefully removed. In this fraction the enzyme was present in homogeneous form. Partial inactivation occurred during ultracentrifugation, since about 20% of the activity could not be accounted for; the activity observed in the final product was, therefore, corrected for the inactivation factor (total protein recovery)/(total activity recovered in ultracentrifugation).

The outline of a typical preparation is summarized in Table I.

Properties

Stability: The enzyme is very stable in the cold throughout the purification up to the CM-cellulose step. For example, it may be preserved as a frozen $(NH_4)_2SO_4$ precipitate at the end of the salt fractionation for prolonged periods without inactivation. This behavior is in contrast to the pronounced lability of NYGAARD's preparation[10], even at early stages of the procedure. This difference might well be the consequence of the harsh methods applied by NYGAARD in the solubilization of the enzyme, which may result in progressive denaturation.

As already mentioned, the present preparation is also somewhat labile following

the removal of impurities on CM-cellulose, which may protect the enzyme. Inactivation at this stage is largely, if not entirely, the consequence of dissociation of the FAD moiety.

The pH of optimum stability is about 6.5 at moderate ionic strength (0.02–0.1 M phosphate). While the enzyme may be kept for a short period at low pH (*e.g.*, 30 sec at pH 3.0) without inactivation, even brief exposure to pH values above 7.5 results in major inactivation.

Variation of activity with pH: The pH curve of the enzyme depends both on the nature of the buffer and of the electron acceptor employed (Fig. 2). In the phena-

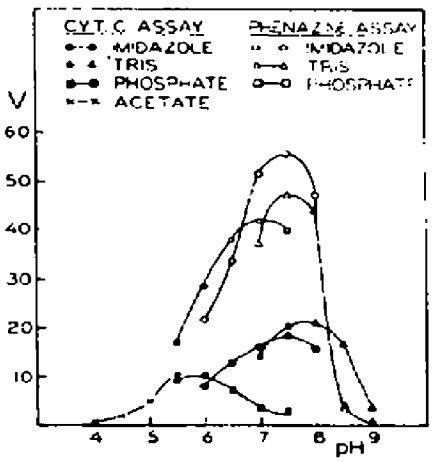

Fig. 2. Variation of activity with pH. Assays were conducted with 0.05 M buffers and the pH values noted are those prevailing in the reaction mixture at 30°. Activity is expressed as μmoles $\times 10^3$ lactate oxidized per 15 sec in the presence of 5 μg purified enzyme (DEAE-cellulose eluate).

zine-DCPIP assay, which is less influenced by ionic strength than the cytochrome assay, highest activity is obtained in imidazole buffer at pH 7.5 (at 30°). The pH optimum in Tris buffer is the same and the alkaline legs of the pH curves in Tris and imidazole are coincident, but below pH 8 the activity in Tris is somewhat lower. In phosphate the pH optimum is 7.0 and the activity at and above this pH is lower in phosphate than in imidazole.

In the cytochrome assay the pH optima at 30° are: 8.0 in Tris, 7.5 in imidazole, and 5.5–6.0 in phosphate–acetate. At the buffer concentration employed (0.05 M) the activity at the pH optimum is much lower in phosphate than in Tris or imidazole. One reason for this is that the reoxidation of the flavoprotein by cytochrome *c* is competitively inhibited at elevated ionic strengths (competition with the electron acceptor) and is much greater in polyvalent than in monovalent buffers, as has also been noted by NYGAARD[11]. It should be mentioned in this connection that the assays in these experiments were conducted at fixed cytochrome concentrations.

Substrates: The D lactic cytochrome *c* reductase, unlike DHAD[22], possesses a high degree of substrate specificity (Table II), since it is active only on D-lactate and

TABLE II

SUBSTRATE SPECIFICITY OF D(−)LACTIC CYTOCHROME *c* REDUCTASE

Substrate	*Activity relative to D(−)lactate at infinite substrate concentration*	*Km at 30° (M)*
D-Lactate	1	$2.85 \cdot 10^{-4}$
D-α-Hydroxybutyrate	0.41	$1.4 \cdot 10^{-3}$
D-Malate	0	
DL-α-OH-isobutyrate	0	
DL-α-OH-caproate	0	
DL-α-OH-isocaproate	0	
DL-α-OH-isovalerate	0	

D-α-OH-butyrate. The following Michaelis constants were obtained at pH 7.5, 30°: K_m for D-lactate = $2.85 \cdot 10^{-4}$ M, for D-α-OH-butyrate = $1 \cdot 10^{-3}$ M. The activity with α-OH-butyrate was measured with a racemic substrate and the K_m was calculated on the assumption that the L-antipode does not inhibit the enzyme competitively.

Electron acceptors: Although the direct reoxidation of the enzyme by O_2 is insignificant in catalytic tests, the reductase is nevertheless appreciably autooxidizable, as may be seen when substrate quantities of the enzyme are bleached by hydrosulfite: upon the readmission of O_2 there is a relatively fast reappearance of the typical yellow color of the oxidized form.

TABLE III

SPECIFICITY OF D(−)LACTIC CYTOCHROME *c* REDUCTASE FOR ELECTRON ACCEPTORS

Oxidant	*Relative activity at Vmax*	*Km at 30° (M)*
Phenazine methosulfate	100	$4.45 \cdot 10^{-3}$
Cytochrome *c* (horse heart)	12.5	$5.4 \cdot 10^{-6}$
Menadione*	0	
Ferricyanide	0	
2,6-DCPIP	0	

* Measured spectrophotometrically with ferricyanide or DCPIP as terminal acceptor.

The specificity of the enzyme toward electron acceptors is very restricted (Table III). Among the oxidants tested only cytochrome *c* and phenazine methosulfate were active. Elsewhere[7,23] we have shown that both the activity of this enzyme at $V_{max.}$ and the K_m for cytochrome are highly dependent on the species from which the hemoprotein is isolated and on the presence of spectrophotometrically undetectable impurities (presumably modified cytochrome *c*) present in varying amounts in different lots of all commercially available preparations. Thus while the

ability of this enzyme to detect structural modifications in the cytochrome molecule is convenient in appraising the quality of cytochrome preparations[7], it necessitates extreme caution in the choice of the cytochrome employed for kinetic studies. Unfortunately, this circumstance has not been generally recognized and, as a result, much of the kinetic information on this enzyme, which has been published from other laboratories, might have to be reevaluated. As to the data in this paper and preceding ones from this Institute, they were obtained with the best horse-heart preparations available, which were devoid of modified cytochrome in all the catalytic tests applied[7]. Nevertheless, the possibility that even these did not represent completely-unmodified cytochrome *c* remains open.

It is of interest in this connection that crystalline horse-, beef-, and tuna-heart cytochromes *c* are considerably more active with the reductase than is yeast cytochrome *c*.

Reversibility: In view of the ease with which the reversibility of the closely related enzyme, yeast DHAD, may be demonstrated[22,23], it was unexpected that attempts to reverse the action of the reductase invariably yielded negative results. Such attempts included the use of leucomethyl viologen, leucobenzyl viologen, and $FMNH_2$ as electron donors and pyruvate as electron acceptor at various pH values in the hydrogenase-coupled assay described elsewhere[24]. Since the possibility that none of the electron donors tested could reduce the enzyme remained open, particularly in view of the restricted specificity of the reductase for dyes, direct proof of the irreversibility of the catalytic reaction was sought by another method. A substrate amount of the purified enzyme (5 mg protein, specific activity = 270; 3 ml volume) was titrated in an anaerobic spectrophotometer cell, under N_2, with hydrosulfite. Bleaching was followed at 450 mμ in a rapid recording spectrophotometer. Addition of hydrosulfite was stopped before complete bleaching occurred so as to avoid the presence of unreacted hydrosulfite. At that time 5 mg solid Na pyruvate was tipped in and no reoxidation of the 450-mμ band was noted. Readmission of O_2, on the other hand, elicited a rapid return of the yellow color. It would appear from these experiments that the action of the enzyme is irreversible and that the reason for this may be in its inability to bind pyruvate. In accord with this interpretation pyruvate is not a competitive inhibitor of D-lactate oxidation.

Inhibitors: In contrast to the DHAD of yeast[22], the enzyme is relatively insensitive to inhibition by SH reagents and substrate analogs. Thus oxidizing agents, such as H_2O_2, do not inhibit the enzyme and it is only partially sensitive to mercurials (Table IV). Remarkably, the same partial (60%) inhibition was noted at $5 \cdot 10^{-7}$ M and $5 \cdot 10^{-3}$ M *p*-chloromercuriphenylsulfonate (5.5 μg protein/3 ml, specific activity = 94). In view of the fact that these data were determined at fixed concentrations of both substrate and electron acceptor, the possibility is open that the incomplete inhibition by the mercurial suggests a steric interference either to the approach of the substrate or of the oxidant, in the sense discussed for wheat-germ lipase[25], rather than the direct participation of a sulfhydryl group in the catalytic cycle.

With regard to substrate analogs, pyruvate, L-lactate and L-malate are not inhibitory even at moderately high concentrations and oxalate is much less inhibitory than in the case of DHAD[22]. Oxalate inhibition may, in any event, reflect its metal-

binding tendency rather than its somewhat remote structural resemblance to the substrate.

The effect of metal chelators on the reductase has been extensively investigated in this laboratory and the results have been summarized elsewhere[6,23,26,27]. The phenomenon is too complex to be discussed here and the inclusion of data on metal chelators in Table IV is only intended to emphasize what is perhaps the most interesting known fact about this enzyme: the complete dependence of its activity on the state of its metal component.

TABLE IV

INHIBITORS OF D(−)LACTIC CYTOCHROME *c* REDUCTASE

The enzyme preparation employed was an $(NH_4)_2SO_4$-precipitated enzyme at the end of the DEAE-cellulose step. Unless otherwise indicated, the enzyme was incubated for 3 min at 30° in the assay mixture with inhibitor and substrate both present and the reaction was started by addition of the electron acceptor (cytochrome *c*).

Inhibitor	*Concentration (M)*	*Inhibition (per cent)*
p-Chloromercuriphenylsulfonate	$5 \cdot 10^{-7}$	60
p-Chloromercuriphenylsulfonate	$5 \cdot 10^{-6}$	60
H_2O_2	$1 \cdot 10^{-3}$	0
Oxalate	$5 \cdot 10^{-6}$	22
Oxalate	$1 \cdot 10^{-4}$	50
Oxalate	$5 \cdot 10^{-3}$	92
EDTA	$4 \cdot 10^{-3}$	25
EDTA	$1 \cdot 10^{-2}$	51
o-Phenanthroline*	$3.5 \cdot 10^{-6}$	95
o-Phenanthroline**	$3.5 \cdot 10^{-3}$	90

* Overnight dialysis at pH 6.5 against the indicated concentration of inhibitor.
** Incubated for 15 min at 30°.

Metal chelators seem to fall into two categories as far as their effect on this enzyme is concerned: those which react rapidly with its Zn^{2+} component and whose inhibitory effect is also rapidly, perhaps instantaneously, reversible on dilution, and those which react and dissociate relatively slowly. In the first category are EDTA and oxalate. The inhibitions by these compounds listed in Table IV obtain only when they are present in the cuvettes at the temperatures and concentrations indicated. Preincubation at these concentrations, followed by dilution of an aliquot for activity determination, results in partial or complete reversal of the inhibition, depending on the extent of dilution and the temperature of the assay.

o-Phenanthroline is representative of the second category of chelators. Its reaction with the enzyme is a moderately slow, first order process, and results in the formation of an enzymically inactive chelate containing 2 moles of *o*-phenanthroline per atom of enzyme-bound Zn^{2+}. The chelator may be dissociated and the activity completely regenerated by a variety of means, including dialysis or displacement with divalent metals. Under no condition studied does the chelator inhibit by dissociating the metal component (*i.e.*, resolving the holoenzyme[8,22,27]).

Constitution

Electrophoresis and ultracentrifugation: The reductase shows almost no mobility in the electrophoretic field in 0.1 M NaCl 0.005 M phosphate solution within the pH range of its stability.

The sedimentation coefficient ($s_{20,w}$) was found to be 6.8 and to be independent of protein concentration in the range of 0.25–1.5% protein.

Flavin content: The association of D-lactic cytochrome reductase activity with a flavoprotein is recognizable at relatively early stages of purification owing to the ability of D(−)lactate to bleach the enzyme in the flavin region. The absorption spectrum of the most purified enzyme is shown in Fig. 3. Since at this stage of purity

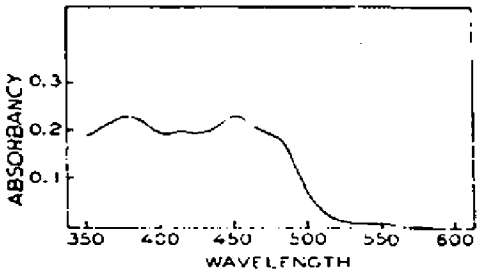

Fig. 3. Absorption spectrum of the reductase as recorded with a Cary Model 11 spectrophotometer equipped with a microcell holder. There were present 13 mg purified enzyme (specific activity = 384) in 1 ml total volume.

flavin tends to dissociate from the enzyme quite readily, this experiment was performed by passing the differentially ultracentrifuged enzyme through Sephadex G-25 immediately before the spectrum was determined, in order to remove free flavin. While consequently the flavin content cannot be calculated from this experiment, the spectrum thus obtained is identical with that found at earlier stages (*e.g.*, before the CM-cellulose step) where the enzyme is stable.

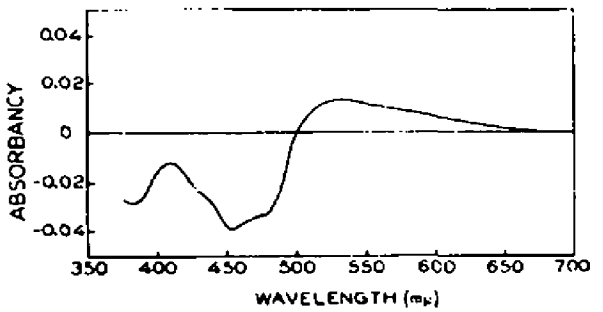

Fig. 4. Difference spectrum of the reductase. 2.8 mg enzyme at the end of the CM-cellulose step were dissolved in 3 ml 0.1 M phosphate (pH 6.5) and placed in an anaerobic cuvette equipped with 2 side arms. After all traces of O_2 were removed, the spectrum was recorded with a Cary Model 11 spectrophotometer (0.1 absorbancy full scale); then the enzyme was titrated with D-lactate and the spectrum immediately redetermined. The curve shown is the difference spectrum elicited by the addition of 1 mole lactate/mole enzyme-bound FAD. A downward deflection denotes bleaching.

On addition of D-lactate under aerobic or anaerobic conditions the difference spectrum reproduced in Fig. 4 is obtained. The notable features of this spectrum are the usual flavoprotein peaks at 450 and 387 mμ in addition to a broad band centering around 530 mμ. Titration of the oxidized enzyme with D-lactate under anaerobic conditions in a recording spectrophotometer showed that (a) addition of as little as 0.5 mole substrate/mole enzyme-bound FAD appears to elicit maximum formation of the 450 mμ trough and the 530 mμ peak within a few seconds (b) addition of 10 moles D-lactate/mole FAD causes no further spectral change (c) on the admission of O_2 there is a gradual disappearance first of the 530 peak then of the 450 mμ trough until the spectrum of the original enzyme is reestablished*. The 530 mμ band may indicate that an appreciable fraction of the enzyme-bound flavin is in the free-radical state, even with excess substrate present. If this interpretation proves to be correct, the semi-quinone would have to be unusually stable since under anaerobic conditions it is preserved for prolonged periods, while even aerobically (with excess substrate) its disappearance requires several minutes.

The flavin present in the enzyme has been unambiguously identified as FAD by differential fluorometry, D-amino acid oxidase test, and paper chromatography. The flavin moiety is readily released from the protein by treatment with 5% trichloroacetic acid or 2% perchloric acid. Prior to the CM-cellulose step milder procedures, such as prolonged dialysis, fail to dissociate the flavin. Treatment with acid $(NH_4)_2SO_4$ at and below pH 3 partially splits the flavin. Despite the use of protective agents (thioglycollate, D-lactate), however, irreversible denaturation always accompanied the resolution of the holoenzyme, and, as the pH was lowered in the range of 3.0 to 2.7, denaturation outstripped true resolution. For these reasons fully resolved and fully reactivable apoenzyme preparations have not been obtained, but the fact that FAD, but not FMN, reactivates the apoenzyme could be nevertheless readily demonstrated (Fig. 5).

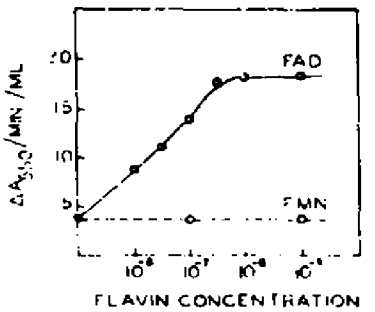

Fig. 5. Reversible dissociation of FAD from D-lactic cytochrome reductase. 1.65 ml enzyme at end of DEAE-cellulose step (6.2 mg protein) in 0.05 M phosphate (pH 6.5) were treated at 0° with 14.9 ml saturated $(NH_4)_2SO_4$ solution, containing 1 mM thioglycollate and previously adjusted to pH 3.0. After 30 sec stirring, the precipitated enzyme was collected by 5 min centrifugation, redissolved in 3.0 ml 0.2 M phosphate –1 mM thioglycollate buffer (pH 6.5) and passed through a Sephadex G-25 column which had been equilibrated with 0.1 M phosphate –1 mM thioglycollate (pH 6.5). It was ascertained that this treatment removed 98% of the activity. For reactivation 10-μl aliquots were incubated for 3 min at 30° in the dark with the complete reaction mixture plus FAD or FMN but without cytochrome *c*. Assays were started by the addition of cytochrome *c* and were conducted at fixed concentration of the acceptor.

* These experiments were kindly performed by Dr. T. CREMONA.

Since in the homogeneous form the flavin is rapidly dissociated, the flavin content of the enzyme had to be determined at less purified stages (just after the CM-cellulose step) and corrections applied for the presence of impurities from analytical ultracentrifuge patterns. Analysis of the five best preparations gave 1 mole FAD/45 000–66 000 g protein. A value of 50 000 ± 5000 is considered probable, since after the CM-cellulose step the only flavoprotein present is the enzyme and thus artifactually high values are unlikely, and since even during the column step some loss of flavin may have occurred occasionally, as judged by the occasional appearance of fluorescence. In the native enzyme the flavin is non-fluorescent.

Since homogeneous preparations have not been obtained in stable form, the determination of the diffusion constant has not been feasible. Consequently, the molecular weight is not known accurately. Taking 45 000 to 55 000 as the minimum molecular weight, the observed sedimentation constant would suggest a molecular weight of the order of 100 000 and, hence, the possible presence of 2 moles flavin/enzyme molecule.

Zinc content: From the DEAE-eluate stage on, the ratio of activity to Zn^{2+} content is rather constant. In homogeneous preparations no metal other than Zn has been detected. Until the differential ultracentrifugation step there is a small amount of Fe present, which is, however, associated with the high molecular weight impurity ($s_{20,w} = 15.3$), which is removed in the last step of the purification procedure.

The Zn^{2+} content* of various samples, determined before and after the CM-cellulose step, as well as at the end of the purification, ranged from 22 000 to 27 000 g protein/g atom of Zn^{2+}, after suitable correction for the homogeneity of the preparation from ultracentrifuge patterns. The uncertainty of the exact flavin content does not permit a definite decision between the extreme values found for the Zn^{2+}/flavin ratio (2–3). While the lability of the flavin might suggest that the value of 2 is the correct one, it should be mentioned that a 3 : 1 ratio has been occasionally observed in fully active samples taken immediately after the CM-cellulose step, before any known loss of flavin occurred, as well as prior to this chromatographic procedure, when the flavin is completely stable.

The linkage of zinc to the protein appears to be considerably more stable than that of the FAD moiety, since neither 12 h dialysis at pH 6.5, dialysis against *o*-phenanthroline[6], nor 4.5 h dialysis at pH 5.2 or passage through CM-cellulose at this pH lower the Zn^{2+} content. The stability of the protein–Zn^{2+} bond to treatment at pH 5.2 was ascertained in order to eliminate the possibility that Zn^{2+} might be lost in the chromatographic step, particularly since the Zn^{2+} moiety of carboxypeptidase is dissociated under these conditions[20].

Turnover number

The specific activity of the most purified preparations averaged 1670 μmoles D-lactate oxidized/min/mg protein in the phenazine methosulfate assay. On the basis

* We are indebted to Dr. B. VALLEE for the quantitative spectrographic analysis of an early preparation.

of an average flavin content of 1 mole per 56 000 g protein, the turnover number corresponds to $9 \cdot 10^4$ moles substrate oxidized/min/mole flavin. Occasional samples possessed a turnover number as high as $12 \cdot 10^4$.

DISCUSSION

The identification[5] of Zn^{2+} and FAD as the prosthetic groups of D-lactic cytochrome reductase provided the first unambiguous demonstration of the existence of zinc–flavoproteins. Zn^{2+} has also been postulated[29,30] to be present in the DHAD of anaerobic yeast, an enzyme whose flavoprotein nature has been clearly demonstrated[32] but one which has not been isolated in sufficiently pure form to permit analytical proof of the identity either of its flavin or of its metal constituents. The conclusion of LABEYRIE *et al.*[31] that FAD is the flavin component of DHAD was based on the prevention of atebrin inhibition by FAD, a tenuous basis in view of the established circumstances[32,33] that atebrin is by no means a specific inhibitor of flavoenzymes and that it does not act by displacing the flavin from flavoproteins. Their conclusion[29] that Zn^{2+} is present in DHAD was based in part on the assumption that EDTA resolves the holoenzyme and Zn^{2+} specifically reconstitutes it, in part on the assumption that, following treatment with EDTA only the Zn^{2+}-reactivated DHAD possesses kinetic constants identical with the untreated enzyme. Both of these assumptions have been shown to be fallacious[6,23,27,34]. The possibility that DHAD, like the reductase described here, is a Zn^{2+}–FAD enzyme is nevertheless open but remains to be proven.

The discovery of this new class of metal-flavoproteins reopens the much debated question of the function(s) of the metal components in these enzymes. An oxidoreductive role for zinc has been suggested[29] but is clearly unlikely and available evidence suggests that zinc functions as a substrate-binding site rather than a participant in electron transfer. This possible function of zinc has been repeatedly voiced for the reductase as well as the DHAD of yeast and of animal tissues[4,28,32,35]. The evidence is in all cases indirect and incomplete. As far as the reductase is concerned, the substrate-binding function is suggested by the facts that (a) lactate and metal chelating agents compete for the same binding site on the enzyme; thus the substrate prevents combination of *o*-phenanthroline with the Zn^{2+} moiety (b) the *o*-phenanthroline-chelated, inactive form of the reductase is not bleached in the visible region of the spectrum by the substrate[23].

Another aspect of this enzyme which remains to be further explored is its biological role. As is also true of the flavohemoprotein, cytochrome b_2 (L(+)lactic dehydrogenase), and of the DHAD of anaerobic yeast, almost nothing is known of the role they play in the metabolism of yeast cells.

Lastly, it might be of some interest to compare the properties of the enzyme described here with the preparation simultaneously and independently purified by NYGAARD[10]. Since no physical criteria of homogeneity have been published by that author, comparison must be based on specific activities. In comparing activities reported by NYGAARD with those found in this study, considering the differences in assay conditions and units employed, the former should be divided by 35.1 for conversion to μmoles lactate/min at fixed phenazine concentration, by 11.2 to convert to μmoles lactate/min at infinite phenazine concentration, and by 102 to convert to

μmoles lactate/min in the cytochrome *c* assay at the fixed cytochrome concentration employed in the present study.

These conversion factors take into consideration the different pH and temperature employed in the assays in the two laboratories as well as the inclusion of inhibitory EDTA in NYGAARD's assay medium. On the basis of these considerations, the best preparations obtained by NYGAARD (5200 in NYGAARD's units[10]/mg ≙ specific activity = 462 at infinite phenazine concentration in our units) appears to be about 28% of that attained in this study. This lower activity may either be due to the presence of impurities in NYGAARD's best fractions or to inactivation caused by loss of flavin, since per mole of enzymically reducible flavin NYGAARD obtained a turnover of $0.75 \cdot 10^4$ moles D-lactate oxidized/min, which is only slightly lower than the value $1 \cdot 10^4$ moles D-lactate oxidized/min/mole of total flavin reported in this paper. This minor difference may well be due to the somewhat less favorable assay conditions employed in Oslo. Besides this difference, the major divergence of the two preparations is in stability. While both are unstable at the terminal stage of purification, NYGAARD's samples are said to be highly labile throughout the purification procedure, while ours are gratifyingly stable at earlier stages. The reason for this difference might well be the relatively harsh treatments employed in NYGAARD's solubilization step. As is often the case, damage to a protein at the beginning of the isolation leads in part to an immediate loss of activity (*i.e.*, low yield on extraction), in part to a progressive denaturation of the extracted enzyme, manifest from its tendency to undergo inactivation under conditions to which it is stable if prepared by milder procedures.

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