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FLAVIN-LINKED MITOCHONDRIAL α-GLYCEROPHOSPHATE DEHYDROGENASE OF *CANDIDA UTILIS*

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The 150-fold purification of the L-α-glycerophosphate dehydrogenase of *Candida utilis* electron-transport particles by very mild procedures is described. The active enzyme contains FAD, iron and copper. The function of the metals, if any, is not clear. Its molecular weight is about $5 \cdot 10^5$. The subunit composition is complex and remains unresolved because the enzyme is contaminated with protease(s). The activity of this enzyme is very low in *Saccharomyces cerevisiae* unless the cells are grown in glycerol. The NAD-dependent cytoplasmic α-glycerophosphate dehydrogenase is present in *C. utilis* but could not be demonstrated in glucose-grown *S. cerevisiae*.

Introduction

The flavin-dependent L-α-glycerophosphate (EC 1.1.99.5) dehydrogenases of a variety of cells have been extensively purified [1–4]. This activity is found associated with membranes and, in particular, with mitochondria in cells which have these organelles. In mammalian systems this enzyme allows the entry into mitochondria of reducing equivalents from cytoplasmically generated NADH via a shuttle involving a cytoplasmic NAD-dependent L-α-glycerophosphate dehydrogenase poised to reduce dihydroxyacetone phosphate [5]. The physiological importance of the flavin-dependent enzyme is indicated by the response of the enzyme to thyroid hormone and to cold stress [6]. In mammals the enzyme has been shown to be affected by iron deficiency and to recovery rapidly on refeeding [7]. The enzyme from yeast mitochondria has not previously been purified. Some properties of the enzyme in an insoluble, solvent-treated preparation from *Saccharomyces cerevisiae* [8] and in extracts from alkane-grown *Candida tropicalis* [9] have been reported.

This communication reports the purification of flavin-dependent L-α-glycerophosphate dehydrogenase from the electron-transport particles of *Candida utilis*. The enzyme has been purified about 150-fold and in 20% yield. We have found it necessary to use very mild procedures in agreement with indications that the enzyme from *S. cerevisiae* is not as stable as the mammalian enzyme [10]. We also report some experiments on the level of this activity in *S. cerevisiae* and on the NAD-dependent L-α-glycerophosphate dehydrogenase in both yeasts. Our results suggest that *C. utilis* resembles mammalian systems in its α-glycerophosphate dehydrogenases.

Materials and Methods

The culture of *C. utilis* and its growth were as previously described [11]. The medium for growth

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; CTAB, cetyltrimethylammonium bromide

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of yeast for 'minimal medium' electron-transport particles contained 0.1% sodium acetate, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.3% yeast extract and 1% dextrose. A triploid strain (5148B) of *S. cerevisiae* was obtained from Dr. Donald C. Hawthorn and was grown as described previously [12].

Electron-transport particles were prepared as described previously [13]. Glass-distilled water was used except for metal determinations where the water was additionally deionized. Buffer pH values were measured at 25°C. In the case of concentrated buffers they were diluted to 0.1 M first. All enzyme assays were performed at 25°C.

α-Glycerophosphate dehydrogenase activity was determined routinely by following the decrease in absorption of DCIP at 600 nm in a Beckman DU Spectrophotometer modified to record rates [14]. The reaction mixture contained 20 mM sodium phosphate buffer, 10 mM NaN_3, 25 mM DL-α-glycerophosphate (sodium salt), 0.033% Brij 58, 0.033% CTAB, 0.025 mM Cr(III) in the form of a glycine complex, 0.035 mM DCIP and 0.5 mM menadione. The final pH in the reaction mixture was 7.1–7.2. The stock Cr(III) complex solution was made by mixing a 10 mM Cr(III) salt with 1 M glycine at pH 7.4 in 1 : 1 proportion. This mixture was allowed to stand at room temperature for 24 h before use, by which time the color was magenta. Menadione was added in the form of a 50 mM solution in methanol. This amount of methanol does not affect the assay. The extinction coefficient of DCIP in the reaction mixture was determined to be 27.4 $mM^{-1} \cdot cm^{-1}$ at 600 mm in the presence of CTAB or lauryldimethylamine *N*-oxide (rather than 21 $mM^{-1} \cdot cm^{-1}$ [15]). The reaction rate was essentially linear in the standard assay for the first 3 min.

NAD-dependent α-glycerophosphate dehydrogenase in the cytoplasm was assayed at 340 nm in a cuvette containing 1 ml water, 0.1 ml of 5 mg/ml of the lithium salt of dihydroxyacetone phosphate, 0.05 ml of 0.5 M sodium phosphate, pH 7.5, and 0.01 ml of 0.1% NADH. The reaction was started with addition of dihydroxyacetone phosphate after determining the blank rate with NADH alone. The 'S_1' fraction used for this assay was prepared as described in Ref. 13.

Oxidase assays with a Clark oxygen electrode were performed as previously described [14] using 20 mM phosphate, pH 7.2, and 25 mM sodium D-α-glycerophosphate.

Flavins were determined by high-performance liquid chromatography [11]. The amounts of metals in the purified enzyme were determined by atomic absorption spectrophotometry as previously described [14].

SDS gel electrophoresis was performed according to Ref. 16 and 20°C and 10 mA per gel. The low molecular weight standards from Pharmacia Fine Chemicals, Piscataway, NJ, were used (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin). To perform dissociation we added the protein solution to 2–4 vol. of dissociating solution at 100°C and heated the mixture for 1–2 min. The dissociating solution contained 0.46 M SDS, 0.28 M dithiothreitol, 5.3 M urea and 1.2 mM sodium phosphate, pH 7.2. Between 1 and 10 μg of protein were applied to each gel usually in a total volume of 3–40 μl after addition of bromcresol blue and glycerol. Subunits were also determined with dissociation by CTAB following the published procedure [17].

Electrophoresis of native protein was carried out by using a low phosphate concentration in the gel (30 mM sodium phosphate, pH 7.25) and running the gel at 1–2 mA per tube. The electrode buffer was 0.1 M sodium phosphate, pH 7.15. Gels were either stained for protein in the usual way with Coomassie blue or for activity [18] by using a solution like the standard assay mixture except that it had double the substrate concentration and containing tetranitroblue tetrazolium instead of DCIP. The position of the bands at several gel concentrations was compared with those of bovine thyroglobulin, ferritin and bovine serum albumin and its multimers (our sample of Sigma bovine serum albumin had a significant amount of dimer and trimer) by plotting relatively mobility against log molecular weight. We also used the Pharmacia high molecular weight standards (bovine thyroglobulin, ferritin, catalase, lactate dehydrogenase and bovine serum albumin).

Isoelectric focusing of the native protein was done at 4°C in 4.4% gels contained 0.2 ml per 10 ml gel solution of Bio-Lyte 3/10, 5% glycerol and 0.05% Brij 58. The gels were run with the anode in

0.01 M phosphoric acid and the cathode (top) in 0.4% ethylenediamine. They were run for about 20 h with the first hour at a constant current of 1 mA (per 6 mm internal diameter gel) and then at a constant voltage of 204 V. The gels were either directly stained for activity (as above) or soaked in 5% sulfosalicyclic acid in 11% methanol and then stained for protein with Coomassie blue.

Chromatography on A1.5 m agarose (0.6 × 16 cm column) was carried out at 4°C in a buffer made by mixing 70 ml water, 20 ml of 0.5 M sodium citrate, 10 ml of 0.5 M NaH_2PO_4, 7.5 ml of 1 M K_2HPO_4 and 0.05 ml of 10% Brij 58. This buffer was also used to dissolve and store enzyme. Blue Dextran, bovine thyroglobulin and bovine serum albumin were used to establish the molecular weight versus effluent position. About 20 mg protein was the maximum amount applied on this column.

The inorganic chemicals used were reagent grade and used without further purification. All the proteins, detergents and organic chemicals were obtained from Sigma with the exception of the molecular weight standards (mentioned above) from Pharmacia, ferritin from Boehringer, 1,10-phenanthroline from Eastman, bathocuproine from G. Frederick Smith Chemical Co., Columbus, OH, CoQ_1 analog (2,3-dimethoxy-*t*-methyl-6-pentyl-1,4-benzoquinone). a generous gift from Dr. T.P. Singer, lauryldimethylamine *N*-oxide a generous gift from Onyx Chemical Co., Jersey City, NJ, and agaroses and Bio-Lyte from Bio-Rad Labs.

Purification

All operations were carried out at 0–5°C. The enzyme was kept in the dark as much as possible and precipitates were kept on ice before centrifugation. Frozen electron-transport particles at a concentration of about 20 mg protein/ml were thawed with the addition of 50 mg phenylmethylsulfonyl fluoride (in 1 ml methanol) per 140 ml of electron-transport particles. The electron-transport particles were added to a mixture of 0.006 vol. of 1 M acetic acid, 0.005 vol. of 2 M sodium acetate and 0.11 vol. of 10% (w/v) Brij 58. After 20 min, they were centrifuged at 160000 × *g* for 35 min. The clear supernatant (called Brij 58 supernatant) was mixed with 0.06 vol. of 0.5 M sodium phosphate buffer, pH 7.5, 0.06 vol. 2 M sodium acetate and enough streptomycin sulfate to give a final concentration of 10 mg/ml. This solution was centrifuged in the same way after 10 min. The clear supernatant was saved. Enough finely ground succinic anhydride, suspended in 0.05 vol. of water, to give a final concentration of 2.5 mg/ml was added to this supernatant and allowed to sit with gentle stirring for 40 min on ice. 0.19 gm/ml of solid $(NH_4)_2SO_4$ was added and after 20 min the mixture was centrifuged at 36000 × *g*. The floating precipitate was discarded. An additional 0.21 gm/ml of $(NH_4)_2SO_4$ was added and the precipitate collected by centrifugation. This precipitate often floats and may be fragile and difficult to collect. The precipitate was dissolved in the agarose column buffer and concentrated on an Amicon CF25 filter cone to a volume of the 0.1–0.2 ml before chromatography on A1.5 m agarose as described above. The enzyme appears almost directly after the excluded volume and the first three or four fractions containing protein (0.2 ml each) have the highest specific activity.

Results and Discussion

The enzyme has 70% less activity if DCIP is replaced by ferricyanide in the assay. Phenylmethazine sulfonate will not replace menadione. The activity with the Q_1 analog is 63% of that with menadione. The use of the Cr(III)-glycine complex (or Ni(II)-glycine) in the assay increases the activity by 25–50%. The preparation was essentially free of lactate, lipoate, NADH and succinic dehydrogenases. The K_m of the enzyme was found to be 5.17 mM DL-α-glycerophosphate in the standard assay. A simple pH vs. activity curve was seen with a maximum between 7.0 and 7.4. The enzyme has essentially the same activity in imidazolium buffers as in phosphate. No activation with dithiothreitol was seen using either DCIP or ferricyanide.

Table I presents data for a typical preparation from *C. utilis* electron-transport particles. The final purification ranged from 134- to 240-fold and the final yields from 15 to 25%. The mild procedures used in the preparation allow the total activity to be accounted for at every step. Although the presence of sodium α-glycerophosphate tends to stabilize the enzyme it cannot be used during

TABLE I

PURIFICATION AND YIELD

The material was assayed as described in Material and Methods. Units are mmol DCIP produced/min.

Purification method	Fraction	Total units	Units mg protein	% recovered	Times purified
Procedure using low pH extraction	Electron-transport particles	800	0.69	100	1.0
	Brij 58 supernatant	660	5.9	82	8.5
	0.19–0.40 g/ml ammonium sulfate fraction	350	25.0	44	36.0
	Best fraction off A1.5 m agarose column	150	98.0	18	142.0

extraction of the electron-transport particles because it decreases the amount of enzyme extracted. It was found necessary to extract as few proteins as possible initially because we found few subsequent purification procedures to be effective. We were not able to get any better purification with Sepharose coupled with DL-α-glycerophosphate, DCIP or FMN than by sieving on A1.5 m agarose.

TABLE II

ANALYTICAL DATA

Preparation	Activity (mM DCIP/min per mg protein)	Molecular weight ($\pm$S.D.) ($\times 10^{-3}$)	Flavin content (mol/520000 g protein)		Metals (mol/520000 g protein)	
			FAD	FMN	Fe	Cu
(1) Standard	90	520 ± 45 [a] 516 ± 84	2.26	0.45	–	–
(2) Same as 1, but deionized on Bio-Gel P-60	30	–	–	–	1.07	0.91
(3) Same as 1, but dialyzed 48 h against 0.05 M Tris	0	150 [a]	–	–	0.98	0.30
(4) $NaClO_4$ and Tween 20 preextracted preparation [b]	62	530 ± 121	1.1	0.85	2.8	4.0
(5) Same as 4, but dialyzed 48 h against 0.05 M Tris	0	–	–	–	1.65	1.35
(6) Extracted at neutral pH from electron-transport particles of yeast grown in minimal medium	73	558 [a]	1.55	0.31	–	–
(7) Same as 6, after treatment with 1% each lauryldimethylamine *N*-oxide and Triton X-100	30	120	–	–	–	–

[a] From gel electrophoresis. The other molecular weight figures are from chromatography on A1.5 m agarose. Where no S.D. is given, the figure is from a single determination.

[b] Electron-transport particles were preextracted with (final concentrations) 0.3 M $NaClO_4$, 0.15% Tween 20, and 10 mg/ml streptomycin sulfate. The electron-transport particles were then extracted with Brij 58, fractionated with $(NH_4)_2SO_4$ and chromatographed on A1.5 m agarose.

We have found that the enzyme is not stable to repeated precipitations with $(NH_4)_2SO_4$ once its purity reaches 20 U/mg protein. For this reason it is advantageous to fractionate the Brij 58 supernatant with $(NH_4)_2SO_4$ in such a way as to obviate the necessity for refractionation.

When our strain of *C. utilis* is grown in the minimal medium (see Materials and Methods) it is found that, although the dehydrogenases are normal and cytochrome oxidase is normal, there is a deficiency in between such that overall oxidation of NADH or succinate by O_2 is not carried out (Mackler, B., unpublished observations). The isolation of α-glycerophosphate dehydrogenase can be carried out from yeast grown in minimal medium. These yeast contain as much activity as yeast grown in enriched media. However, the enzyme is less stable to the isolation procedure and the initial extraction must be at neutral pH (no acetic acid).

The molecular weight from gel electrophoresis agrees with the determination on A1.5 m agarose satisfactorily (Table II). Since on gels the enzyme sometimes appears larger than a molecular weight of 520000, both in pure preparations and in the Brij 58 supernatant, it is probable that the enzyme aggregates. In some preparations (not treated with any detergent except Brij 58) we have also seen active bands on gel electrophoresis that corresponded to a molecular weight of about 250000. The major part of the material extensively dialyzed for metal determinations was about 150000 molecular weight but was totally inactive. No method of deionizing the enzyme was found that did not result in concomitant losses of activity. Menadione and CTAB (or lauryldimethylamine *N*-oxide) are essential in the assay of enzyme that has been either chromatographed or dialyzed.

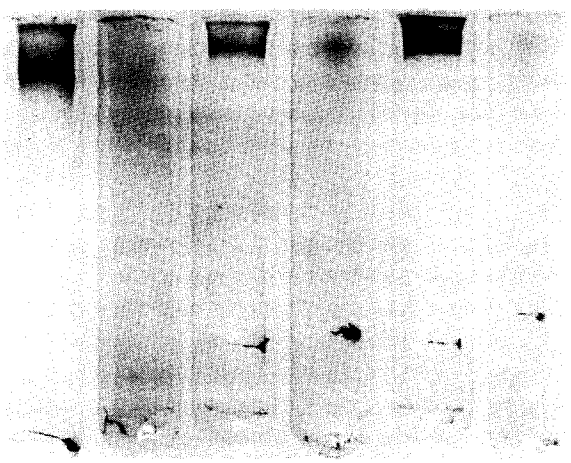

Fig. 1. Gels after electrophoresis of pure enzyme at 4°C in phosphate buffer as described in Materials and Methods. From the left: The first two gels contained no Brij 58, first gel stained for activity the second for protein; the third and fourth gels contained Brij 58, the third was stained for activity and the fourth for protein; the fifth and sixth gels contained Brij 58 and the buffer on top of the tube contained α-glycerophosphate, the fifth was stained for activity and the sixth for protein. 5 μg protein were applied to gels stained for activity and 2.5 μg to those stained for protein.

In both gel electrophoresis (Fig. 1) and isoelectric focusing of the native protein it is usual to see more than one band. The main band appears at about pH 5.2 in isoelectric focusing. The presence of proteolytic impurities in the preparation (see below), the indications of aggregation and disaggregation of the enzyme, the probability of different forms from variations in the amount of bound detergent [1,2], and the possibility that the enzyme is by its nature not very stable can all contribute to the appearance of more than one band. It is possible to make a Brij 58 extract of the enzyme in the presence of the three proteolytic inhibitors used by Ryrie [19] (these probably also inhibit lipases [20]) but we have not yet made preparations from these extracts. The figures in Table II for FAD and FMN indicate that the FMN is probably a contaminant.

EPR measurements at 12 K of both resting enzyme and glycerophosphate reduced enzyme showed only small signals at *g* 4.3 and *g* 2.0 which do not appear to be of significance. No signals were seen in the dithionate-reduced enzyme. Absence of the usual EPR signals indicate that metals (Table II) are probably not involved in the enzyme reaction. The fact that dialysis against metal-free solutions removes both almost all of the copper and all of the activity suggests that disaggregation to the inactive 150000 molecular weight form may be associated with loss of copper. In the atomic absorption spectrophotometry no significant amounts of Mo, Mn or Zn were found.

Because the α-glycerophosphate dehydrogenase is a membrane protein some doubt must attach to the apparent [1,23] subunit sizes found with SDS

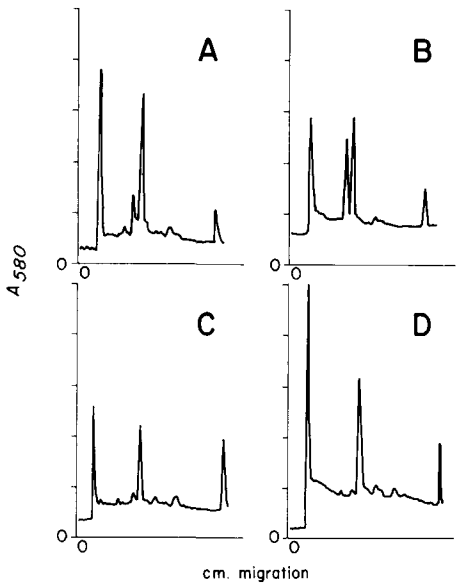

Fig. 2. Gels scans after SDS electrophoresis. (A) Standard preparation (ratio 71000/67000 units = 1/4), activity = 90 U/mg. (B) Preparation made from electron-transport particle preextracted with $NaClO_4$ and Tween 20 (see Table II for details), (ratio 71000/64000 units = 1/1.3), activity = 62 U/mg. (C) Preparation made from electron-transport particles grown in minimal medium with Brij 58 extraction at neutral pH (ratio 71000/67000 units = 1/6), activity = 73 U/mg. (D) Same as C except pure enzyme treated with 1% lauryldimethylamine *N*-oxide and 1% Triton X-100 (ratio 71000/67000 units = 1/10), activity = 30 U/mg.

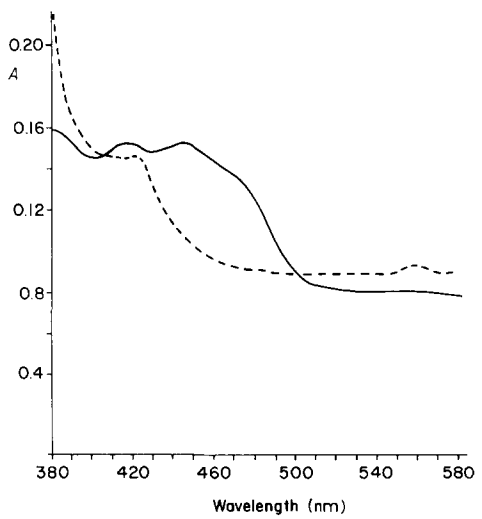

Fig. 3. Spectra of standard preparation (activity = 98 U/mg) in 0.1 M phosphate, pH 7.1, with phosphate buffer only in the blank cuvette. Protein concentration 0.8 mg/ml. The solid line is the oxidized (resting) enzyme. The dashed line is after reduction by 1–2 mg hydrosulfite.

(Fig. 2). CTAB dissociation gave essentially the same subunits as SDS, indicating that lipid binding is probably not reflected in the subunit sizes found [17]. The *C. utilis* enzyme resembles the mammalian and *Escherichia coli* enzymes, since they all have two or more protein subunits per native molecular weight and per flavin. All three enzymes contain FAD as the flavin. Some or all of the material at and below 45000 molecular weight

TABLE III

INITIAL REACTION RATE IN THE PRESENCE OF VARIOUS COMPOUNDS

The reaction mixture was as described in Materials and Methods except those in the final four rows, which were assayed in the absence of Cr(III). Compounds marked with one asterisk were added in methanol (antimycin A only) or ethanol, and in these cases the relative activity is that compared to reaction mixture containing the same amount of solvent. The concentration shown is that in the reaction mixture.

Compound added	Relative activity
None	1.0
0.15 deoxycholate	0.18
0.2% Triton X-100	0.61
0.1% cetylpyridinium bromide	0.60
1% (v/v) chloroform	1.26
1% (v/v) methanol	1.00
2% (v/v) methanol	0.75
1% (v/v) ethanol	0.91
2% (v/v) ethanol	0.95
0.015 mg/ml antimycin A*	0.97
0.03 mg/ml antimycin A*	0.84
10^{-5} M rotenone*	1.00
$2 \cdot 10^{-5}$ M rotenone*	0.96
10^{-3} M seconal	0.76
$2 \cdot 10^{-3}$ M seconal	0.74
$1 \cdot 10^{-3}$ M *p*-chloromercurisulfonate	0.70
$2 \cdot 10^{-3}$ M *p*-chloromercurisulfonate	0.62
10^{-3} M ethacrynic acid*	0.77
$5 \cdot 10^{-2}$ M lithium chloride	0.61
10^{-1} M lithium chloride	0.48
10^{-1} M sodium chloride	0.70
$2 \cdot 10^{-4}$ M bathocuproine*	1.05
5 M urea	0.09
5 M acetamide	0.43
(No Cr(III) in the assay)	
10^{-4} M *o*-phenanthroline*	0.84 (0.76) [a]
10^{-4} M bathocuproine*	1.17
$2 \cdot 10^{-4}$ M aluminum sulfate	0.62
$2 \cdot 10^{-4}$ M ammonium molybdate	0.62

[a] Assayed at half the enzyme concentration.

may result weight from proteolysis. The preparations show no chains above this weight if the dissociation is carried out at 40°C instead of 100°C. Proteolytic contamination of yeast enzymes is not uncommon [21,22]. 71000 and 67000 molecular weight chains are found in all the preparations. The data do not allow any conclusions to be made about whether one or both chains are essential for activity. Subunit complexity may be because extraction of the enzyme by Brij 58 might carry along neighboring proteins, perhaps those involved in the site I phosphorylation which occurs in *C. utilis* [6].

The spectrum of the pure enzyme (Fig. 3) yields a ratio of 3.5 flavins (using ϵ_{451} (oxidized minus reduced) $\mu M = 10.3\ cm^{-1}$) per 520000 molecular weight. This agrees quite well with 2.7 from Table II considering the possibility of loss on isolation of the flavins. The flavin content indicates a minimum molecular weight of 230000 or less. We have seen electrophoresis bands indicating this size in Brij 58 extracts treated with lauryldimethylamine *N*-oxide and deoxycholic acid. Table III shows the activation or inhibition of the pure preparation found with a variety of compounds added to the assay. No effect was found with sodium arsenate, sodium arsenite, sodium cacodylate, magnesium chloride, calcium chloride or EDTA. CTAB inhibits the enzyme completely in the absence of added menadione. It is possible that the residual quinone left on the enzyme after purification is displaced by this detergent. The effect of chloroform in apparently accelerating the reaction has been seen consistently. The effect of ethanol on the enzyme is minimal in the standard assay but in imidazolium buffers 2% ethanol inhibits the enzyme by 80%. Effects seen with compounds which must be added in organic solvents are difficult to evaluate because the enzyme is rather sensitive to added solvents (Table III).

The data in Table IV show that the α-glycerophosphate oxidase activity of *S. cerevisiae* electron-transport particles is low unless the yeast is grown on glycerol. In *C. utilis* the S_1 supernatant (crude cell extract minus electron-transport particles) [13] had an NAD-dependent α-glycerophosphate dehydrogenase activity of 0.39 mM NADH/min per mg protein. The corresponding S_1 fraction from *S. cerevisiae* inhibited the reaction with the *C. utilis* extract. There may be some of this activity present in the cytoplasm of glycerol-grown *S. cerevisiae*, although the actual change in NADH concentration was very small when corrected for the dihydroxyacetone phosphate-independent change. The activities of this enzyme found here are only a small fraction of that found in *C. tropicalis* [9]. The bulk of their activity was, however, in nonmitochondrial par-

TABLE IV

OXIDASE ACTIVITY (SEE MATERIALS AND METHODS) OF MITOCHONDRIAL α-GLYCEROPHOSPHATE DEHYDROGENASE IN *C. UTILIS* and *S. CEREVISIAE*

ETP, electron-transport particles.

	μl O_2/min per mg protein				Ratio of maximum oxidase activity to activity in standard assay (μl O_2/mM DCIP)
	Substrate and buffer only	+0.05 mg/ml cytochrome *c*	$+5 \cdot 10^{-4}$ M menadione	Standard assay, except no DCIP	
C. utilis ETP	0.10	0.13	0.57	0.57	1.5
Brij 58 supernatant	0	0	0.42	2.38	0.75
Second $(NH_4)_2SO_4$ precipitate	0	0	–	10.7	0.64
S. cerevisiae ETP	0.012	0.014	–	0.08	2.0
S. cerevisiae ETP (glycerol-grown)	0.10	0.10	0.28	0.02	5.4

ticulates and their assay conditions were different, particularly as we used the lithium salt of dihydroxyacetone phosphate.

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