An Electron Transport Particle from Yeast: Purification and Properties*

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Previously, Crane, Glenn, and Green (1) have described the purification and properties of an electron transport particle (ETP)[1] from beef heart. The ETP was similar in composition and properties to the reduced diphosphopyridine nucleotide oxidase described earlier by Mackler and Green (2, 3) and, in addition, carried out the oxidation of succinate by molecular oxygen. The ETP preparation contained copper, nonheme iron, lipid, coenzyme Q, flavin, and cytochromes b, c_1, c, and a.[2] Succinate and DPNH oxidase activities were inhibited by antimycin A, but only the DPNH oxidase was inhibited by Amytal. Other electron transport particles, isolated from *Azotobacter vinelandii* (4) and from cauliflower buds (5), differ from the beef heart ETP in their cytochrome composition. The ETP from *A. vinelandii* contained copper, nonheme iron, flavin, and lipid in amounts similar to the beef heart system but was antimycin-insensitive and did not catalyze the reduction of mammalian cytochrome c.

The present paper describes the purification and properties of an ETP prepared from *Saccharomyces cerevisiae*. Differences in composition, structure, and kinetic properties between this enzyme system and the previously reported preparations of ETP from other sources (1, 4, 5) will be discussed.

EXPERIMENTAL PROCEDURE

Materials and Methods

The oxidation of DPNH by oxygen was measured at 38° spectrophotometrically (2) or by means of an oxygen polarograph (6) as described previously. The complete system contained 0.1 μmole of DPNH (0.5 μmole in the polarographic assay), 0.2 ml of 0.2 M phosphate buffer of pH 7.5, 0.02 ml of 1% cytochrome c solution, and water to a final volume of 1 ml. Reactions were started by addition of an appropriate amount of enzyme protein: 4 μg for the spectrophotometric assay and 0.2 mg for the polarographic assay. The oxidation of succinate by oxygen was measured by means of the oxygen polarograph as described above, with the exceptions that 0.05 ml of a 1 M solution of succinate was substituted for DPNH and approximately

1 mg of enzyme was added to start the reaction. In some determinations, where indicated, cytochrome c was not included in the assay mixtures.

The oxidations of DPNH and succinate by cytochrome c were measured spectrophotometrically at 38° by the increase in absorbancy at 550 mμ. The assay systems were the same as those described above, except for the inclusion of 0.1 ml of a 0.1 M solution of sodium azide and 0.1 ml of a 1% cytochrome c solution in addition to the other components; in assays for succinic cytochrome c reductase activity, 0.01 ml of 1 M succinate was used.

Cytochrome c oxidase activity was assayed spectrophotometrically at 38° as described previously (7).

Assays for the following activities were performed by methods described elsewhere: TPNH-cytochrome c reductase (8), isocitric dehydrogenases (9), lactic dehydrogenase (10), alcohol dehydrogenase (11), fumarase (12), malic dehydrogenase (13), butyryl-CoA dehydrogenase (14), ATPase (15), lipoic dehydrogenase (16), and oxidative phosphorylation (17).

Flavin was released from the enzyme preparation by two methods. In the first method, the enzyme solution was heated at 100° for 6 minutes, cooled to 5°, acidified with perchloric acid to a final concentration of 10%, and centrifuged to remove the denatured protein. The supernatant solution was then adjusted to pH 8 with 6 N potassium hydroxide, and the potassium perchlorate was removed by centrifugation. The second method was identical, except that the enzyme solution was incubated with trypsin (0.5 mg per mg of protein) for 90 minutes at 38° before the heat and acid-deproteinization steps.

In the above extracts, total flavin was measured by the decrease in absorbancy at 450 mμ upon treatment with hydrosulfite (18). FAD was determined by assay with D-amino acid apo-oxidase,[3] and riboflavin 5'-phosphate, by assay with TPNH-cytochrome c aporeductase from yeast (8). Flavins were also identified by paper chromatography (19) in two solvent systems: *n*-butanol-acetic acid-water (4:1:5, organic phase) or 5% Na_2HPO$_4$.

Phosphorus was determined by a modification of the method of King (20), protein, by the method of Lowry *et al.* (21), ribonucleic acid, by the method of Littlefield *et al.* (22), and copper, by the method of Bouchardt and Butler (23). Analyses for lipid, iron, coenzyme Q, and the cytochrome components were performed as described previously (24, 25). Spectra of the turbid enzyme preparations were recorded at room temperature and at −195° (precooled in liquid nitrogen) by means of a sensitive wave length-scanning spectrophotometer (26).

[1] The abbreviations used are: ETP, electron transport particle; FMN, riboflavin 5'-phosphate (flavin mononucleotide).

[2] In this paper, the cytochrome (or cytochromes) in the cytochrome oxidase portion of the electron transport chain will be designated as cytochrome "*a*."

[3] N. A. Rao, S. P. Felton, F. M. Huennekens, and B. Mackler, to be published.

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Chemicals were obtained from the following sources: Fleischmann's yeast, from Standard Brands, Inc.; TPNH, DPNH, DPN, TPN, cytochrome *c*, coenzyme A, ATP, hexokinase, catalase, lactic dehydrogenase, FAD, and FMN, from the Sigma Chemical Company; antimycin A, from the Wisconsin Alumni Research Foundation; seconal and Amytal, from the Eli Lilly Company; and trypsin (1:300), from Nutritional Biochemicals Corporation. FAD and FMN were purified by chromatography on diethylaminoethyl cellulose as described elsewhere.[3] The Superbrite glass beads, size 071, used in the preparation of the enzyme were obtained from the Minnesota Mining and Manufacturing Company.

Preparation of Enzyme

Step I. Bakers' yeast (2 kg) was washed twice with distilled water by centrifugation and then diluted to a final volume of 2 liters by addition of a solution containing 0.01 M K_2HPO_4, 0.001 M Versene (the disodium salt of ethylenediaminetetraacetic acid), and 8.5% sucrose. The suspension of yeast was added to approximately 4 liters of moist glass beads in a 6-liter stainless steel beaker immersed in an ice bath. All subsequent steps in the purification of the enzyme were performed at 0–5°. The suspension of yeast and glass beads was homogenized for 30 minutes at top speed with a large overhead blender equipped with a circular glass blade and then centrifuged at 1370 × *g* in the refrigerated International centrifuge; the packed residue was discarded.

Step II. The turbid supernatant fluid from Step I was centrifuged at 3100 × *g* for 30 minutes, and the residue was discarded. The supernatant fluid was now centrifuged at high speed, *viz.* 30,000 r.p.m. for 30 minutes in the No. 30 rotor of the Spinco model L ultracentrifuge, and the clear supernatant fraction was discarded. The residue was suspended in 250 ml of 8.5% sucrose solution and then homogenized at top speed for 10 minutes in a VirTis high speed homogenizer.

Step III. The material obtained after homogenization in the VirTis blender was diluted with 8.5% sucrose solution to a protein concentration of 20 mg per ml, and the pH was adjusted to 10 with 6 N KOH. The preparation was then centrifuged in the No. 30 rotor of the Spinco ultracentrifuge at 30,000 r.p.m. for 30 minutes. After centrifugation, three layers were observed: a clear supernatant layer, which was discarded; a reddish brown middle layer, which was saved; and the packed residue, which was discarded. The middle layer was diluted with 8.5% sucrose solution to a protein concentration of 15 mg per ml, and the pH was lowered to 8.5 with 0.1 N HCl.

Step IV. The suspension obtained from Step III was centrifuged in the No. 40 rotor of the Spinco ultracentrifuge at 15,000 r.p.m. for 20 minutes, and the turbid supernatant suspension was then recentrifuged at 40,000 r.p.m. for 90 minutes. The final residue, which represented the purified ETP, was suspended in sufficient 8.5% sucrose solution to give a protein concentration of 20 mg per ml.

RESULTS

Table I summarizes data showing the purification and recovery of protein for the various steps in the preparation of ETP from yeast. Preparations of the highest purity catalyzed the oxidation of 3 to 4.5 μmoles of DPNH and 1 μmole of succinate per minute per mg of protein with oxygen as the acceptor. The enzyme was specific for DPNH and did not utilize TPNH as

TABLE I
Purification of ETP from yeast

Fraction	Total protein	DPNH oxidase		Succinoxidase	
		Specific activity*	Units of activity†	Specific activity*	Units of activity†
	mg				
Step I.....	80,000	0.3	24,000	0.05	4000
Step II....	19,000	0.8	15,200	0.10	1900
Step III...	2,500	2.4	6,000	0.50	1250
Step IV....	1,500	3.3	4,950	0.70	1050

* Specific activity: micromoles of substrate oxidized per minute per mg of protein.

† Units of activity: Specific activity × total protein.

TABLE II
Rates of enzymatic activity of yeast ETP with various substrates and acceptors

Preparation	Activity*						
	DPNH			Succinate			Reduced cytochrome c oxidase
	Oxidase		Cytochrome c reductase	Oxidase		Cytochrome c reductase	
	With added cytochrome c	Without cytochrome c		With added cytochrome c	Without cytochrome c		
1	3.3	1.1	1.1	0.86	0.59	0.23	3.1
2	3.2	0.9	1.4	0.89	0.65	0.18	4.8
3	2.7	1.0	1.1	1.0	0.63	0.26	†
4	4.2	1.7	1.3	0.70	0.43	0.21	†

* Activity: micromoles of substrate oxidized per minute per mg of protein.

† Assays were not performed.

substrate. The enzymatic oxidation of DPNH or of succinate was not accompanied by phosphorylation of ADP to ATP under the conditions of assay (17). Preparations of yeast ETP contained negligible amounts of malic, isocitric, lactic, alcohol, lipoic, and butyryl-CoA dehydrogenases, TPNH-cytochrome *c* reductase, adenosine triphosphatase, and glucose 6-phosphatase. Preparations of ETP at pH 8.5 did not lose activity when stored at −20° for periods of up to 2 weeks.

The Michaelis constants (K_m) for DPNH and succinate were determined by the method of Lineweaver and Burk (27) to be 7.7 × 10^{-5} M and 6.0 × 10^{-4} M, respectively.

Table II shows the rates of enzymatic activity for several preparations of ETP when DPNH, succinate, and reduced cytochrome *c* were used as substrates, and when oxygen and cytochrome *c* were employed as acceptors. As seen from the data, addition of cytochrome *c* stimulated DPNH oxidase activity 2- to 4-fold but did not appreciably affect succinoxidase activity. In addition, succinate cytochrome *c* reductase activity was found to be considerably slower than either succinoxidase or DPNH-cytochrome *c* reductase activities. Preparations of enzyme also catalyzed: (*a*) the reduction of ferricyanide and 2,6-dichloroindophenol with DPNH as substrate and (*b*) the reduction of phenazine methosulfate with succinate as substrate. The rates of these reactions, however, were much slower than rates of oxidation of DPNH or succinate with oxygen.
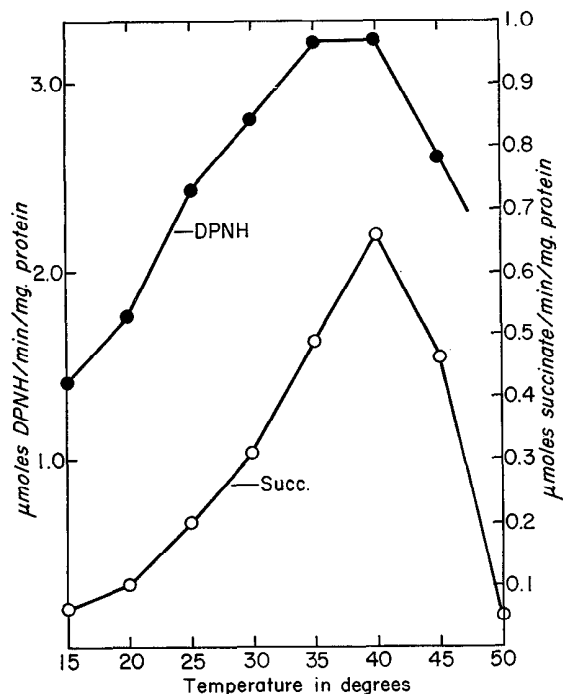
Fig. 1. Effects of temperature on the succinic and DPNH oxidase activities of yeast ETP. The assays were performed polarographically as described in "Experimental Procedure" by using a vessel in which the temperature was controlled.

The effect of temperature on DPNH and succinic oxidase activities is shown in Fig. 1. Succinoxidase activity was increased to a much greater degree by an increase in temperature than was DPNH oxidase activity, suggesting different rate-limiting reactions for the two activities.
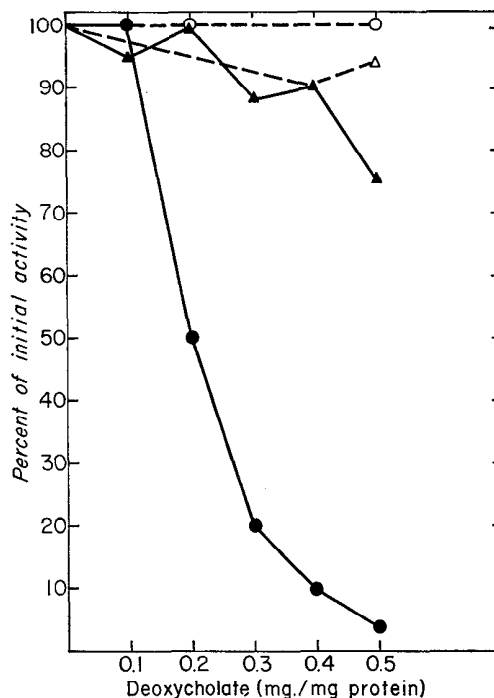

Fig. 3. Effects of preincubation with deoxycholate on DPNH-cytochrome c reductase and succinic cytochrome c reductase activities of ETP assayed in the presence of cyanide or azide. DPNH-cytochrome c reductase was assayed in the presence of 0.001 M cyanide (●——●) or 0.01 M azide (▲——▲). Succinic cytochrome c reductase activity was assayed in the presence of 0.001 M cyanide (○- - -○) or 0.01 M azide (△- - -△). ETP suspended in 8.5% sucrose was preincubated with varying amounts of deoxycholate, as indicated, for 3 minutes at 0° and then assayed for cytochrome c reductase activities in the presence of 0.001 M cyanide or 0.01 M azide.
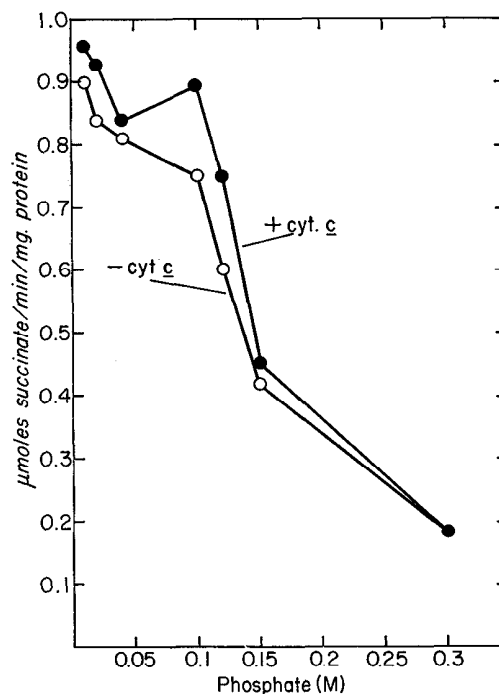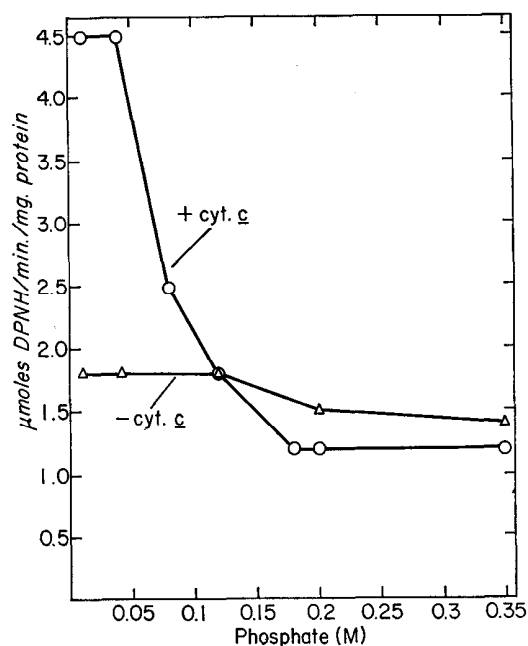

Fig. 2. Effects of phosphate concentration on DPNH oxidase activity (*left*) and succinoxidase activity (*right*). Assays were performed spectrophotometrically with and without added cytochrome c as described in "Experimental Procedure."

Enzymatic activities in 0.04 M phosphate or Tris buffer with succinate or DPNH as substrates showed broad maxima between pH 7 and 8 with peaks at approximately 7.5. As shown in Fig. 2 (left), increasing amounts of phosphate markedly interfered with the ability of cytochrome c to stimulate DPNH oxidase activity but had virtually no effect on the activity without added cytochrome c. Succinoxidase activity, on the other hand, was progressively inhibited by increasing phosphate concentration in the absence or presence of external cytochrome c (Fig. 2 (right)). The inhibition of enzymatic activity by high concentrations of phosphate may be explained by the recent work of Machinist et al. (28) showing that phosphate interferes with the binding of cytochrome c to phospholipid.

Antimycin A (1 μg per ml), cyanide (0.001 M), azide (0.01 M), and p-chloromercuribenzoate (0.0001 M) inhibited both DPNH and succinic oxidase activities approximately 90 to 100%. Antimycin A (1 μg per ml) also completely inhibited DPNH and succinic cytochrome c reductase activities. Seconal (0.002 M) and Amytal (0.002 M) were without effect on any of the above activities. Cyanide (0.001 M) had no effect on rates of reaction of the succinic and DPNH-cytochrome c reductases, but when the concentration was increased to 0.004 M, the reactions were inhibited 50 and 90%, respectively.

When the enzyme was preincubated with increasing concentrations of deoxycholate and assayed in the presence of 0.001 M cyanide, progressive inhibition of DPNH-cytochrome c reductase
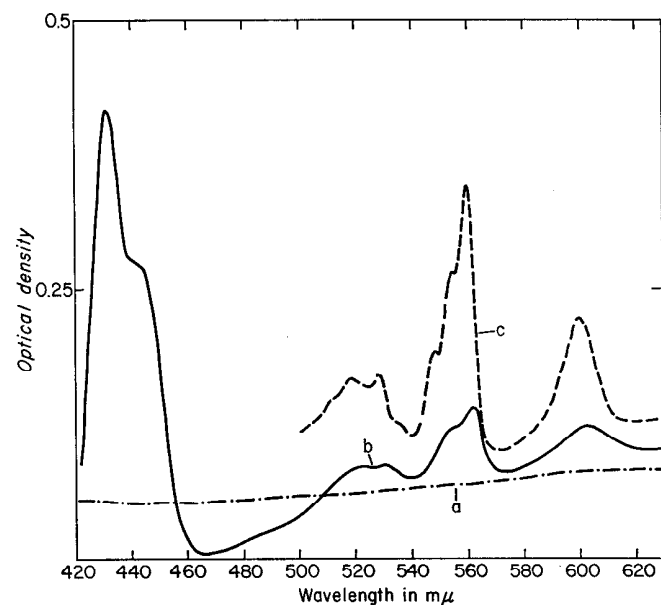

Fig. 4. Difference spectra (reduced minus oxidized) of yeast ETP recorded at b 25° and c −195°. Curve a represents the baseline for the spectrum recorded at 25° before reductant was added. The sample and control cuvettes used for the room temperature spectrum had light paths of 10 mm, and each contained 8 mg of enzyme protein in 3 ml of 0.02 M phosphate buffer, pH 7.5. The cuvettes used for the low temperature spectra had light paths of 2 mm, and each contained 2.5 mg of enzyme protein in 1 ml of phosphate buffer, pH 7.5. Preparations were reduced by the addition of a small amount of hydrosulfite to the sample cuvettes. For the low temperature spectrum, the sample was first reduced with hydrosulfite, the sample and control cuvettes were next cooled to −195° by immersion in liquid nitrogen for 2 to 3 minutes and then withdrawn and quickly placed in the light path of the spectrophotometer, and the spectrum was recorded.

TABLE III
Composition of yeast ETP

All values represent the average of determinations performed on at least four different preparations of enzyme and are expressed as millimicromoles per milligram of protein, with the exception of lipid, which is expressed as per cent of dry weight.

Component	Concentration
Flavin:	
Acid-extractable	0.41
Trypsin- and acid-extractable	0.73
Coenzyme Q	0.30
Cytochromes:	
a	0.90
b	1.2
$c + c_1$	0.85
Total	3.0
Nonheme iron	0.80
Copper	1.3
Lipid	21.0

TABLE IV
Composition of flavin component of yeast ETP

All values except for percentage of recovery are expressed as millimicromoles of flavin per mg of protein. Total flavin was measured by the decrease in absorbancy at 450 mμ upon reduction with hydrosulfite. FAD and FMN were determined by specific enzymatic assays.

Preparation	Trypsin- and acid-extractable flavin				Acid-extractable flavin			
	Total	FAD	FMN	Recovered as FAD and FMN	Total	FAD	FMN	Recovered as FAD and FMN
				%				%
1	0.92	0.72	0.02	81	0.36	0.26	0.01	75
2	0.50	0.50	0.05	110	0.28	0.29	0.01	107
3	0.58	0.50	0.01	88	0.35	0.23	0.01	69
4	0.59	0.60	0.02	105	0.34	0.31	0.01	94
Average	0.65	0.58	0.02	96	0.33	0.27	0.01	86

activity was found, as shown in Fig. 3. There was only a slight inhibition of DPNH-cytochrome c reductase when 0.01 M azide replaced cyanide in the reaction. Succinic cytochrome c reductase activity was not affected by treatment with deoxycholate and 0.001 M cyanide. Preincubation of the enzyme with deoxycholate (0.4 mg per mg of protein) in 0.03 M histidine buffer, pH 7.5, resulted in an 80% loss of DPNH oxidase activity accompanied by similar decreases in both DPNH and succinic cytochrome c reductase activities; these results are contrary to previous findings with beef heart preparations (12), in which there were marked increases in the cytochrome c reductase activities concomitant with the fall in oxidase activity.

Difference spectra (reduced with hydrosulfite minus oxidized) of a preparation of yeast ETP recorded at room temperature and at −195° are shown in Fig. 4. Absorption maxima (room temperature spectrum) at 604, 562, 553, and 445 mμ demonstrate the presence of cytochromes a, b, and $c_1 + c$ in the preparation. The spectrum recorded at low temperature has an additional absorption maximum at 548 mμ, indicating the presence of a small amount of cytochrome c.
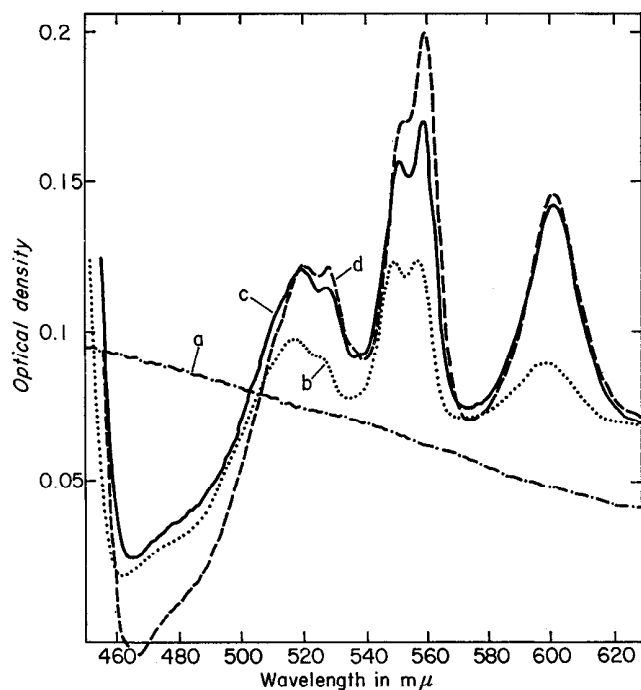
FIG. 5. Difference spectra (reduced minus oxidized) of yeast ETP recorded in steady state (*b*) as quickly as possible after addition of DPNH, (*c*) fully reduced by DPNH, and (*d*) after addition of hydrosulfite. The base-line, shown as *Curve a*, was obtained as described in the legend for Fig. 4. Sample and control cuvettes had 10-mm light paths and contained 15 mg of enzyme protein in 3 ml of 0.02 M phosphate buffer, pH 7.5. Solid DPNH (1 mg) was added to the sample cuvette and the spectra were recorded in three stages: immediately, after anaerobiosis was obtained, and finally, after addition of a small amount of hydrosulfite to the sample cuvette.
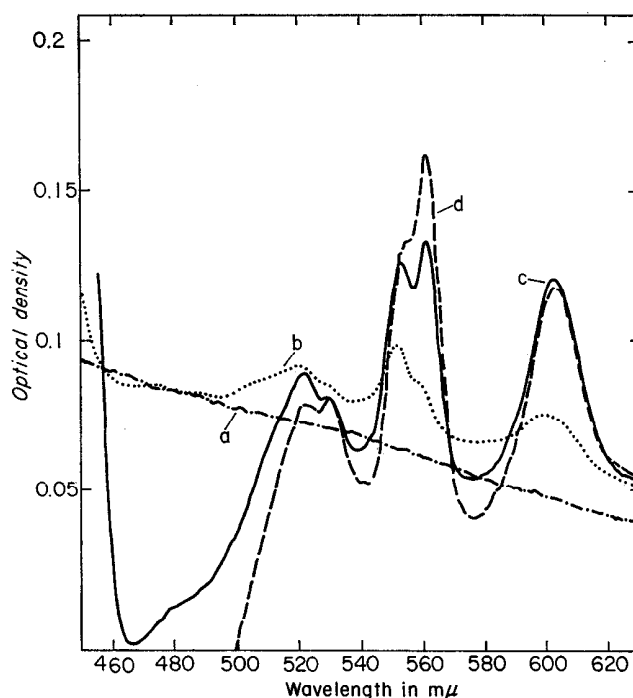

FIG. 6. Difference spectra (reduced minus oxidized) of yeast ETP recorded in steady state (*b*) after addition of succinate, (*c*) fully reduced by succinate, and (*d*) after addition of hydrosulfite. The base-line, shown by *Curve a*, was obtained as described in the legend for Fig. 4. Conditions were the same as given for Fig. 5, except that 0.02 ml of 1 M succinate was added to the sample cuvette in place of DPNH.

The composition of yeast ETP is shown in Table III. The general composition and the concentrations of total flavin and the cytochromes are similar to values reported previously for preparations of beef heart ETP (29), but concentrations of non-heme iron, copper, coenzyme Q, and lipid are considerably lower in the yeast ETP. Data for the individual flavin components of yeast ETP are shown in Table IV. Assays performed with sensitive, specific apoenzyme systems (*cf.* "Experimental Procedure") revealed that approximately 90% of the flavin is FAD, whereas only trace amounts of FMN could be detected. These results were confirmed by subjecting the total flavin extract to a paper chromatographic analysis.

Difference spectra (reduced minus oxidized) of a preparation of yeast ETP recorded at room temperature immediately after the addition of DPNH or succinate (steady state), and again after anaerobiosis was reached, are shown in Figs. 5 and 6, respectively. Spectra of the preparations after reduction with hydrosulfite are also included in these figures. It is apparent that DPNH and succinate each reduce the flavin and cytochrome components of the preparation to a similar degree during anaerobiosis, suggesting that there are functional links or common components between the two chains. However, the spectrum obtained during enzymatic catalysis (steady state) induced by DPNH differs markedly from that obtained with succinate. Values for percentage reduction of the flavin and cytochrome components during the steady states induced by DPNH and succinate are shown in Table V. With DPNH, the degree of

TABLE V

Percentage reduction of components of yeast ETP during steady states induced by DPNH and succinate

Substrate	Component			
	Cytochrome			Flavin
	a	*c + c₁*	*b*	
DPNH	22	75	60	90–100
Succinate	25	35	18	25
DPNH + succinate	24	77	62	90–100

reduction of the components decreased in the following order: flavin $> b > c + c_1 > a$, in accord with the previous results of Estabrook and Mackler with beef heart DPNH oxidase (30). When steady states were induced with succinate, however, the components remained mostly oxidized and to approximately the same degree, suggesting that a relatively slow rate-limiting step is located in the succinate chain at a point preceding the flavin component. The possibility that the succinic chain was damaged during preparation of the enzyme is suggested by the results reported above. Indeed, Chance and Williams (31) have demonstrated in rat liver mitochondria that cytochrome reduction during succinate-induced steady states is similar to that described with DPNH (30).

DISCUSSION

Preparations of yeast ETP are similar in many respects to the DPNH oxidase (2, 3) and ETP (1, 29) previously isolated from

beef heart. Each of these preparations contains flavin (of which approximately 50% is peptide-bound), coenzyme Q, cytochromes b, c_1, c, and a, nonheme iron, copper, and lipid. The amount of nonheme iron in yeast ETP is approximately equivalent to the amount of flavin and is much lower than the nonheme iron in heart preparations. This result is in accord with the recent work of Beinert and Lee (32), which suggests that only 1 atom of nonheme iron per mole of flavin is active during enzymatic catalysis in the heart preparations. The finding that copper is present in significant amounts in preparations of ETP from such different sources as beef heart, yeast, and *A. vinelandii* strongly supports the results of electron spin resonance studies recently reported by Sands and Beinert (33), demonstrating that copper is an active component of beef heart cytochrome oxidase.

Preparations of ETP from yeast and heart muscle differ significantly in the nature of their flavin components. The present finding that all of the flavin of yeast ETP is FAD may be contrasted with our previous studies (34), which demonstrated that FMN is the flavin coenzyme of beef heart DPNH oxidase. Furthermore, the flavin component in yeast ETP, which requires tryptic digestion for its removal from the enzyme, is nevertheless active in the D-amino acid apo-oxidase system, and it thus appears to be identical enzymatically and chromatographically with FAD. In contrast, the trypsin-extracted flavin from heart preparations, is inactive in the enzymatic assay for FAD (35), although it appears to be FAD linked to a peptide fragment. Finally, the finding that the DPNH oxidase activity of yeast ETP is unaffected by seconal or Amytal under conditions in which heart ETP is completely inhibited may be related to the nature of the flavin, or its binding, in the yeast ETP chains.

The results in this paper demonstrate that external cytochrome c interacts with the yeast ETP in a different manner when DPNH and succinate, respectively, are used as electron donors. For example, the succinic cytochrome c reductase activity of the enzyme preparations is much slower in rate than either the succinoxidase activity or the DPNH-cytochrome c reductase activity, and the DPNH oxidase activity, but not the succinic oxidase activity, was greatly stimulated by addition of cytochrome c. It is possible that the pool of cytochrome c associated with the DPNH oxidase system is depleted during preparation of the enzyme, whereas the cytochrome c linked to the succinoxidase system remains intact. Thus, added cytochrome c is able to interact readily with the DPNH oxidase system but cannot interact with, or stimulate, the intact succinoxidase system. The demonstration that the amount of cytochrome c present in yeast ETP is much less than that of the other cytochrome components (see Fig. 4) supports the above hypothesis.

SUMMARY

An electron transport particle, isolated from bakers' yeast, catalyzes the oxidation of reduced diphosphopyridine nucleotide and succinate by molecular oxygen. DPNH oxidase activity, but not succinoxidase activity, is stimulated several fold by the addition of cytochrome c. Antimycin A inhibits both DPNH and succinic oxidase activities, and Amytal and seconal have no effect on either activity. Preparations of yeast electron transport particle contain flavin, coenzyme Q, nonheme iron, copper, lipid, and cytochromes a, c, c_1, and b. The flavin component, identified as flavin adenine dinucleotide, is present in two fractions, one removable by acid treatment and the other requiring

tryptic digestion for removal from the enzyme. Kinetic studies suggest that the rate-limiting reaction in the succinoxidase system is located at a point in the enzymatic sequence before the flavin component and that external cytochrome c interacts in a different manner with the DPNH and succinic oxidase systems.

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