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Electron-Transport Systems of Yeast. II. Purification and Properties of a Soluble Reduced Diphosphopyridine Nucleotide Dehydrogenase*

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ABSTRACT: A soluble highly purified reduced diphosphopyridine nucleotide (DPNH) dehydrogenase has been prepared from yeast. The enzyme catalyzed the oxidation of DPNH by ferricyanide, 2,6-dichlorophenolindophenol (indophenol), and cytochrome *c*, and contained 0.6 mole of flavin–adenine dinucleotide, (FAD) and 0.4 g.-atom of iron per mole of enzyme. Both FAD and iron were removed from the enzyme by acid treatment, but complete reactivation of the apoenzyme was effected by addition of FAD alone.

Two *p*-mercuriphenylsulfonic acid (PCMS) sensitive sulfhydryl groups, active in enzymatic catalysis, appear to be located after the flavin in the sequence of electron transport and in close spatial approximation to the site of binding of DPNH by the enzyme.

Previously, we have described the properties of an electron-transport particle (ETP)[1] isolated from *Saccaromyces cerevisiae* (Mackler *et al.*, 1962). The ETP differs from similar preparations from beef heart (Mackler and Green, 1956; Crane *et al.*, 1956) in several properties, although the enzymes catalyze the same reactions. Preparations of ETP from yeast contain only flavin–adenine dinucleotide (FAD), in contrast to heart ETP which contains both flavin mononucleotide (FMN) and FAD and much higher amounts of nonheme iron and coenzyme Q (ubiquinine) than does the yeast ETP. In addition, amytal and seconal inhibit the reduced diphosphopyridine nucleotide (DPNH) oxidase activity of the heart enzyme, but do not affect the activity of the yeast preparation.

The present report describes the preparation and properties of a soluble DPNH dehydrogenase from preparations of yeast ETP. Differences in structure, composition, and kinetic properties between this enzyme and the previously reported DPNH dehydrogenase (Mackler, 1961) prepared from beef heart will be discussed.

Materials and Methods

Assays of enzymatic activity were performed spectrophotometrically as described previously (Mackler, 1961; Rao *et al.*, 1963) at 38° with the exception that the assays contained either 0.2 ml of 0.2 M potassium phosphate buffer, pH 7.5, or 0.2 ml of 0.2 M sodium

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[1] Abbreviations: ETP, electron-transport particle; DPNH, reduced diphosphopyridine nucleotide; FAD, flavin–adenine dinucleotide; FMN, flavin mononucleotide; indophenol, 2,6-dichlorophenolindophenol; PCMS, *p*-mercuriphenylsulfonic acid.

TABLE I: Purification of Yeast DPNH Dehydrogenase.

Fraction	Total Protein (mg)	Specific Activity[a]	Total Activity	% Recovery
Yeast ETP	15,500	1.0	15,500	...
Crude extract after 1st lyophilization	1,019	3.0	3,057	20
DEAE-cellulose eluates	11.4	52	593	4
After ammonium sulfate fractionation	3.0	182	546	4

[a] Specific activity is defined as μmoles of DPNH oxidized per min per mg of enzyme protein. Ferricyanide was used as acceptor. In the case of yeast ETP the assay included 1 μg of antimycin A/ml. Assays were performed at pH 5.5.

acetate buffer, pH 5.5. From 1 to 4 μg of enzyme protein was used in the assays, depending on enzymatic activity.

Total flavin, FAD, FMN, iron, and protein were determined as described previously (Mackler *et al.*, 1962; Rao *et al.*, 1963). Chemicals were obtained from sources described elsewhere (Mackler *et al.*, 1962; Rao *et al.*, 1963). Determination of sulfhydryl groups was performed by the method of Ellman (1959).

Preparation of Enzyme. ETP was prepared from Fleischmann's yeast as described previously (Mackler *et al.*, 1962). All procedures were carried out at 0–5°. The ETP (approximately 15 g of protein) was suspended in cold water (30 mg of protein/ml) and the pH was lowered to 5.5 by slow addition of 1 M KH_2PO_4. Absolute ethyl alcohol was added to a final concentration of 9%, the suspension was incubated at 35° for 15 min and then cooled, and the pH was adjusted to 7.0 with 6 N KOH. The suspension was centrifuged in the No. 30 rotor of the Spinco Model L ultracentrifuge for 15 min at 30,000 rpm, and the clear supernatant was evaporated to dryness by lyophilization. The lyophilized residue was dissolved in a minimum amount of water and dialyzed overnight against 8 l. of 0.005 M phosphate buffer, pH 7.0. A column measuring 2.5 cm in diameter was packed to a height of 25 cm with DEAE-cellulose which had been repeatedly washed with water and then equilibrated with the dialysis solution diluted with an equal volume of cold water. The dialyzed enzyme solution (0.5–1 g of protein) was then placed on the column, the column was washed with 500 ml of diluted dialysis fluid, and gradient elution was applied. The mixing chamber contained 400 ml of the diluted dialysis fluid, and the reservoir contained 200 ml of 0.05 M phosphate buffer, pH 7.0. The rate of flow of the column was such that 12 tubes, 9.0 ml each, were obtained over a 1-hr period. Fractions of enzyme having the highest activities were found in tubes 40–60 and these fractions were combined and lyophilized. The lyophilized residue was dissolved in a minimum amount of water and dialyzed overnight against 8 l. of 0.01 M phosphate buffer of pH 7.0. A saturated solution of ammonium sulfate (pH 7.0, saturated at 0°) was added slowly with stirring to the enzyme solution to a final saturation of 70%. The suspension was centrifuged in the No. 40 rotor of the Spinco ultracentrifuge at 40,000 rpm for 15 min and the residue was discarded. Sufficient solid ammonium sulfate was added to the supernatant solution to make it 100% saturated, and the suspension was allowed to stand with gentle stirring at 5° for 10 min. The precipitate, which was the purified enzyme, was collected by centrifugation and dissolved in 0.02 M phosphate buffer, pH 7.5. Preparations of purified dehydrogenase were very stable and retained full activity on storage at −20° for several months.

Results

The over-all purification of the enzyme prepared according to the above procedure is shown in Table I. The specific activity of the enzyme increased approximately 180-fold during fractionation with recovery of 4% of the initial activity. However, it should be stated that it is difficult to estimate the final yield of enzyme or over-all purification, since ferricyanide may react at more than one site with antimycin-inhibited ETP from yeast, thus giving values for activity of the ETP which may be unduly high and cannot be compared directly with the activity of the dehydrogenase. The most active preparation of enzyme had specific activities of 190 (μmoles of DPNH oxidized per min per mg of enzyme protein) with ferricyanide as acceptor at pH 5.5 and with FAD added to the assay vessels. When such preparations were studied in the Spinco analytical ultracentrifuge[2] by velocity techniques, the protein moved as a single symmetrical peak as shown in Figure 1 and was estimated to be over 90% homogeneous by calculation of the relative area distribution. The enzyme was calculated to have a molecular weight

[2] We are indebted to Mr. Roger Wade, Department of Biochemistry, University of Washington, for carrying out the ultracentrifuge measurements.

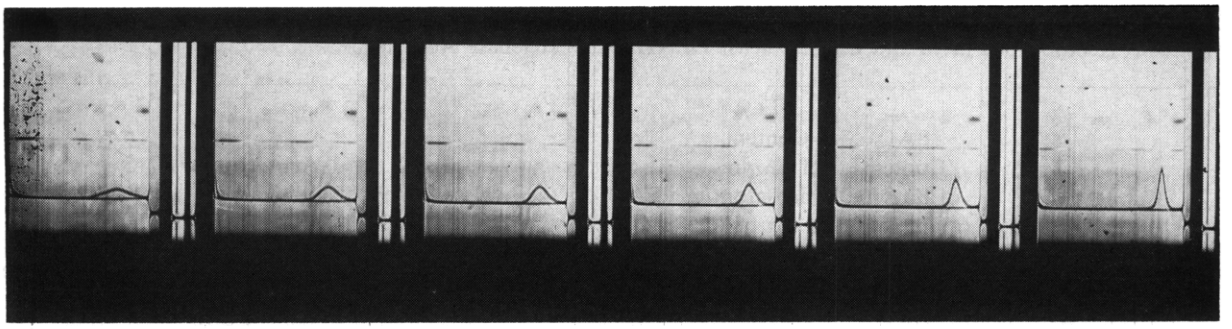

FIGURE 1: Analytical ultracentrifuge pattern of velocity studies of yeast DPNH dehydrogenase. Sedimentation is from right to left at 0, 8, 16, 24, 32, and 56 min.

TABLE II: Activity of DPNH Dehydrogenase with Various Acceptors.

	Acceptor[a]					
	Ferricyanide		Indophenol		Cytochrome *c*	
pH	−FAD	+FAD	−FAD	+FAD	−FAD	+FAD
5.5	194	192	...	...	16.2	18.1
7.5	55	88	16.9	30.0	4.6	5.8

[a] Activity expressed as μmoles of DPNH oxidized per min per mg of enzyme protein. FAD (2 mμmoles) was added to assays where indicated. Assays contained 1–2 μg of enzyme protein as described in Methods.

of 51,000–58,000 (average 55,000, $s_{20,w}$ 2.4) in other studies in the ultracentrifuge performed by equilibrium sedimentation techniques (Richards and Schachman, 1959).

Preparations of DPNH dehydrogenase from yeast catalyzed the oxidation of DPNH when ferricyanide, 2,6-dichlorophenolindophenol (indophenol), and cytochrome *c* were used as electron acceptors, as shown in Table II. Enzymatic reactions with all acceptors tested had a pH maximum between 5 and 6 and proceeded as well at pH 7.5 in Tris buffer as in phosphate buffer. The enzyme did not catalyze the reduction of added free FAD or FMN, or molecular oxygen as does the DPNH dehydrogenase prepared from heart muscle (Mackler, 1961), but required added flavin for full activity.

Preparations of enzyme of the highest purity contained 10.6, 6.3, and 20 mμmoles of flavin, iron, and sulfhydryl groups, respectively, per mg of enzyme protein, or 0.6, 0.35, and 1.1 moles of flavin, iron, and sulfhydryl groups, respectively, per mole of enzyme (based upon an average molecular weight of 55,000 as determined from experiments in the ultracentrifuge). The flavin was completely released from the enzyme by first heating the preparation at 100° for 6 min and then by addition of trichloracetic acid to a final concentration of 5%. Analysis of the flavin by assay with D-amino acid apooxidase and by chromatographic techniques as described previously (Rao *et al.*, 1963) demonstrated that it consisted entirely of FAD. The yeast preparations appear to have lost flavin during purification since all activities at pH 7.5 were stimulated markedly by the addition of free FAD (see Table II). This stimulation is greater at pH 7.5 than at pH 5.5 since the flavin is dissociated from the enzyme at acid pH. When preparations of the enzyme were precipitated two to three times with ammonium sulfate at pH 2 and then dialyzed overnight at 5° against 400 volumes of 0.01 M phosphate buffer, pH 8, containing 0.001 M Versene, the flavin and iron contents of the enzyme were reduced considerably, as shown in Table III (the enzymes used in this study were partially purified preparations). Accompanying the loss of flavin and iron was a loss of all the enzymatic activities, which were completely restored (at pH 7.5) by assay of the apoenzyme with FAD or FMN added to the assay tubes. However, in other studies, as shown in Figure 2, FAD reactivated the apoenzyme at much lower concentrations than did FMN (the preparation used in this experiment was of the highest purity). In control studies the apoenzyme did not reduce added FAD or FMN with DPNH as substrate. Since the activities at pH 7.5 of preparations of purified enzyme were stimulated approximately 60% by addition of FAD to the assay (see Table II), it would appear that flavin was lost during purification and that the true flavin content of the enzyme was 60% higher than the value determined, or approximately 17 mμmoles/mg of enzyme protein. The minimum molecular weight of the enzyme calculated from the

TABLE III: Relation of Flavin and Iron to Yeast DPNH Dehydrogenase Activities.[a]

Preparation	FAD Content of Enzyme (mμmole/mg of protein)	Fe Content of Enzyme (mμg-atom/mg of protein)	FAD Added (mμmole)	FMN Added (mμmole)	Activity at pH 7.5 (μmole of DPNH/mg of protein per min) Ferricyanide	Indophenol	Cytochrome *c*
I before acid treatment	2.4	...	...	...	16	7.6	0.39
			2	...	22	8.0	0.34
			...	4	22	7.8	0.34
I after acid treatment	0.44	...	...	...	1.0	0.2	0.02
			2	...	23	7.5	0.24
			...	4	20	7.2	0.28
II before acid treatment	7.5	5.0	...	...	44	16	4.6
			2	...	74	26	5.8
II after acid treatment	1.8	1.3	...	...	23	5	1.8
			2	...	78	24	4.8

[a] Flavin was added to the assays for enzymatic activity as noted in the table. The assays contained 1–4 μg of enzyme protein as described in Methods.

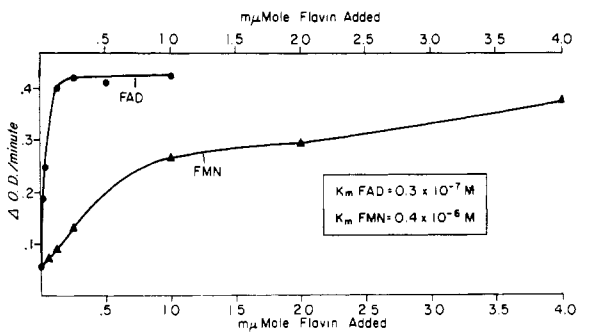

FIGURE 2: Reactivation of the apoenzyme of DPNH dehydrogenase by FAD and FMN. Assays were performed at pH 7.5 with ferricyanide as acceptor, as described in the section on Methods. FAD or FMN was added to the assay mixtures in the amounts indicated. Four micrograms of enzyme protein was used in the experiments.

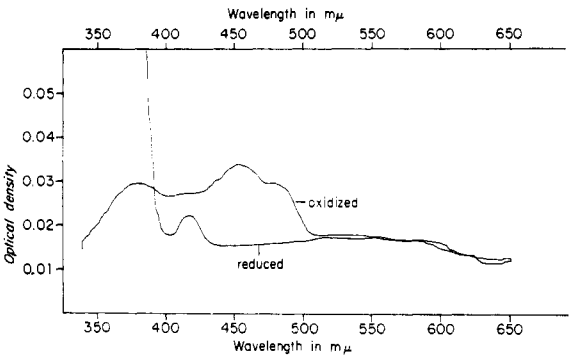

FIGURE 3: Spectra of an oxidized and reduced preparation of purified DPNH dehydrogenase. The sample cuvet (10-mm optical pathway) contained 0.16 mg of enzyme protein in 1 ml of 0.02 M phosphate buffer, pH 7.5. The control cuvet contained H_2O. The enzyme was reduced by addition of approximately 1 mg of hydrosulfite to the sample cuvet.

corrected flavin content is 59,000, and this value is in good agreement with that obtained from studies in the analytical ultracentrifuge.

Figure 3 shows the spectra of a preparation of purified enzyme in the oxidized state and after reduction by hydrosulfite. In the oxidized form there are absorption maxima at 452 and 380 mμ which represent the flavin component of the enzyme, and another maximum at 485 mμ. Upon reduction with either DPNH or hydrosulfite the three absorption maxima disappear (reduction of the peak at 380 mμ is obscured in the figure by the absorption due to dithionite). When the flavin and iron components were removed from the enzyme by treatment with acid ammonium sulfate as described earlier and a spectrum was obtained of the apoenzyme, it was found that the three absorption peaks present in the intact enzyme at 485, 452, and 380 mμ were absent. As shown in Figure 3 there is a small amount of absorption in the 450–650-mμ region which remains after reduction of the enzyme; the significance of this is not known at present.

p-Mercuriphenylsulfonic acid (PCMS) completely inhibited enzymatic activity with all electron acceptors at a concentration of 10^{-4} M without preincubation with the enzyme. Amytal (10^{-3} M), arsenite (10^{-3} M), Versene (10^{-3} M), quinacrine (10^{-3} M), and antimycin A (1 μg/ml) had no effect on enzymatic activity. Cadmium

(10^{-3} M) inhibited activity approximately 50%. As shown in Table IV, the enzyme after preincubation with PCMS for 10 min at 0° showed an increased sensitivity to the inhibitor and was completely inhibited by 10^{-6} M PCMS. Preincubation with DPNH in addition to PCMS markedly protected the enzyme from inhibition by PCMS (see Table IV), suggesting that the PCMS-sensitive sulfhydryl groups are located on the enzyme either at or close to the site of binding of DPNH. However, when spectra were obtained of a preparation of DPNH dehydrogenase in the oxidized state and after reduction with DPNH in the presence of 5×10^{-3} M PCMS, the flavin component was fully reduced by DPNH despite the fact that PCMS was present in a great excess (10^3 moles of PCMS/mole of enzyme), thus suggesting that the sensitive sulfhydryl groups are located after the flavin in the chain of electron transport, and that DPNH probably protects the enzyme from inhibition by PCMS by sterically hindering the binding of the PCMS or by causing conformational changes in the enzyme rather than by competing directly with PCMS for the active sulfhydryl groups. When the preparations of DPNH dehydrogenase were assayed in the presence of 1 M urea, enzymatic activity was inhibited approximately 50%, but the degree of sensitivity of the enzyme to inhibition by PCMS was unchanged and preincubation with DPNH protected the preparation against PCMS inhibition to the same degree as described above. The spectrum (oxidized) of the dehydrogenase dissolved in a solution of 1 M urea was similar to the spectrum (oxidized) shown in Figure 2 having absorption maxima at 485 and 452 mμ. Analysis of the enzyme for the presence of "labile sulfide" by a modification of the method of Fogo and Popowsky (1949) showed that no "labile sulfide" groups were present, and this is in agreement with the finding that there is no evolution of hydrogen sulfide when the enzyme is precipitated with strong acid. Figure 4 shows the curve calculated from the average of data obtained in four different experiments in which preparations of dehydrogenase of the highest purity were preincubated with varying amounts of PCMS at 0° for 10 min and were then assayed at pH 7.5 with ferricyanide as acceptor. As shown in the figure, preincubation of PCMS with enzyme in a ratio of 1 mole of PCMS/mole of enzyme resulted in 50% inhibition of activity, suggesting strongly that there are two sulfhydryl groups/mole of enzyme active in the catalysis. Similar experiments performed with indophenol as acceptor at pH 7.5 and with ferricyanide as acceptor at pH 5.5 yielded identical results. When the enzyme was preincubated with DPNH in addition to PCMS, approximately 10 times as much PCMS was required to produce a 50% inhibition of activity than when the preincubation mixture contained only enzyme and PCMS.

TABLE IV: Effects of Preincubation with PCMS and DPNH on Yeast DPNH Dehydrogenase Activity.[a]

Additions to Preincubation Mixture[b]			Further Additions at Time of Assay[c]		% Inhibition of Enzyme Activity
Enzyme	PCMS (M)	DPNH	PCMS (M)	DPNH	
+	−	−	−	+	0
+	−	−	10^{-6}	+	47
+	−	+	10^{-6}	−	36
+	10^{-6}	−	−	+	97
+	10^{-6}	+	−	−	65
+	−	−	10^{-7}	+	26
+	−	+	10^{-7}	−	0
+	10^{-7}	−	−	+	71
+	10^{-7}	+	−	−	17

[a] Amounts of DPNH and enzyme were similar to those given in section on Methods for assay of enzymatic activity with ferricyanide as acceptor; 0.5 ml of 1% DPNH and 2 μg of enzyme protein were used in all experiments. [b] The preincubation mixture contained 0.2 ml of 0.2 M phosphate buffer, pH 7.5, and 0.7 ml of water. [c] Enzymatic reactions were begun by addition of 0.1 ml of 8.1 mM ferricyanide solution. The assays were performed as described in the section on Methods.

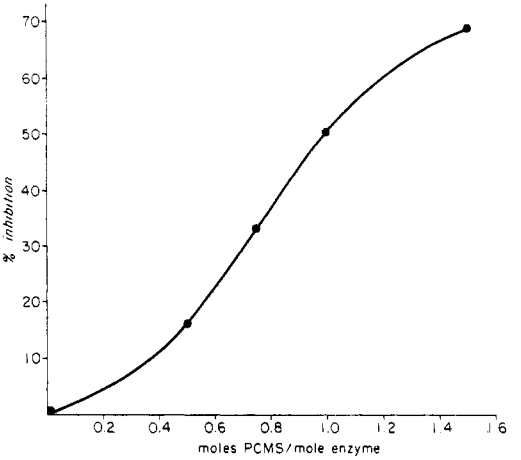

FIGURE 4: Inhibition of dehydrogenase activity by PCMS. The curve represents the average of data obtained in four different experiments. In a typical experiment, several 0.1-ml aliquots of 0.02 M phosphate buffer, pH 7.5, containing 0.12 mg of enzyme protein were incubated with varying amounts of PCMS as indicated in the figure for 10 min at 0°. The preincubated enzyme samples were then assayed at 38° and pH 7.5 with ferricyanide as acceptor, as described in the section on Methods. Assays were begun by addition of 0.02 ml of the preincubation mixtures to the assay cuvets.

Discussion

DPNH dehydrogenase prepared from yeast ETP differs in several respects from the DPNH dehydrogenase prepared similarly from beef heart (Mackler, 1961). In contrast with the heart enzyme which contains FMN as prosthetic group (Rao *et al.*, 1963), the yeast DPNH dehydrogenase contains FAD. In addition, the heart preparation can catalyze the reduction of added FAD, FMN, and molecular oxygen, but yeast preparations are unable to do so. The DPNH dehydrogenase of yeast has an average molecular weight of 55,000, approximately two-thirds that of the heart preparation which has a molecular weight of 70,000–90,000.[2] Preparations of DPNH dehydrogenase from beef heart contain 2 g-atoms of iron/mole of flavin or enzyme, whereas the yeast enzyme has 0.4 g-atom of iron/mole of enzyme. Furthermore, most of the iron in the yeast DPNH dehydrogenase can be removed by acid treatment and is not essential for enzymatic catalysis, since complete reactivation of dehydrogenase activity is obtained by addition of FAD alone to the apoenzyme. It is of interest that although both enzymes catalyze the reduction of ferricyanide by DPNH, the pathways of electron transport differ in that enzyme-bound flavin is required for the enzymatic reduction of ferricyanide by the yeast dehydrogenase, but not by the heart enzyme (Rao *et al.*, 1963). Both dehydrogenases require bound flavin for the enzymatic reduction of indophenol and cytochrome *c*, although the yeast apoenzyme is more completely reactivated by flavin than is the heart apoenzyme. Finally, the spectrum (oxidized) of the yeast dehydrogenase resembles the spectra of flavoprotein enzymes, such as the microsomal cytochrome b_5 reductase (Strittmatter and Velick, 1957) and the electron-transferring flavoprotein (Beinert, 1963), in that there are two absorption maxima present in the 450–500-mμ region. The spectrum of the DPNH dehydrogenase from heart muscle differs in that only a single broad maximum is present in this spectral region. Since the electron-transferring flavoprotein contains no metal component, and the iron content of the yeast DPNH dehydrogenase is much lower than 1 g-atom/mole of enzyme, it would appear that the absorption maxima at 485 and 452 mμ found in the yeast DPNH dehydrogenase are both related to the flavin prosthetic group and its mode of binding to the enzyme.

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