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Short Communication

Partial Purification of the NADH-Nitrate Reductase Complex from *Chlorella pyrenoidosa*[1]

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ABSTRACT

The nitrate reductase complex from *Chlorella pyrenoidosa* has been purified by a procedure which includes as main steps, ammonium sulfate fractionation, polyethylene glycol treatment, and DEAE-cellulose chromatography. The Michaelis constants for NADH, FAD, and NO_3^- in the NADH-nitrate reductase assay are 10 μM, 2.6 μM, and 0.23 mM, respectively. Heat treatment exerts varying effects on the enzymatic activities associated with the nitrate reductase complex.

The enzymatic reduction of nitrate to nitrite has been characterized in fungi and in higher plants as a sequential transfer of electrons from NAD(P)H to NO_3^- via FAD and molybdenum (1, 2, 4, 6, 8, 9). Cytochromes may be involved (5, 11). This electron transfer sequence can be interpreted as two enzymatic activities which operate sequentially (6, 7, 13). The first is an NADH diaphorase and the second is the nitrate-reducing activity which can use reduced flavin nucleotides as the electron donor ($FADH_2$-nitrate reductase). Reduced viologen dyes can replace $FADH_2$ in this latter activity. Although the diaphorase and the nitrate-reducing activities are not physically separable by conventional enzyme purification techniques, they can be assayed independently of each other. Functioning together, they comprise the physiological nitrate-reducing unit, NAD(P)H-nitrate reductase (6). Aparicio *et al.* (1) have reported a 98-fold purification of the $FADH_2$-nitrate reductase from *Chlorella pyrenoidosa,* but no data were given in this report concerning the relative nitrate-reducing activity using either NADH or reduced viologen dyes as electron donors. This report describes a partial purification and characterization of the nitrate reductase complex from *C. pyrenoidosa* in which the over-all NADH-nitrate reductase activity was followed, in addition to the associated $FADH_2$- and reduced viologen-nitrate reductase activities.

MATERIALS AND METHODS

Cultures of *C. pyrenoidosa* were grown on an inorganic salt media (3). For experimental purposes, *C. pyrenoidosa* was first subcultured at 26 C in 2-liter flasks containing 1400 ml of media, with light provided by eight "Gro-lux" bulbs (Sylvania). The cultures were vigorously aerated with a 95% air-5% carbon dioxide gas mixture. After 4 to 5 days of growth, the contents of the 2-liter flask were used to inoculate 60 liters of media. The cells were collected during the late log phase of growth and frozen until use.

In the course of purifying the nitrate reductase complex, NADH-nitrate reductase, $FADH_2$-nitrate reductase, and reduced methyl viologen-nitrate reductase activities were assayed as previously described (6) with the following exceptions: 0.05 ml of 2 mM NADH and 0.1 M phosphate buffer, pH 7.8, were used.

One unit of activity is defined as the formation of 1 nmole of nitrite in 1 min. Specific activity is expressed as units of activity per mg protein.

RESULTS AND DISCUSSION

The requirements for maximal extraction of enzymatic activity were determined to be a high ionic strength buffer (0.1 M), phosphate ions, and a sulfhydryl-protecting agent, such as 2-mercaptoethanol. All buffers contained 0.5 mM EDTA and 1 mM 2-mercaptoethanol.

In a typical purification, 265 g of cells, 1060 ml of 0.1 M phosphate buffer, pH 7.5, and 1060 g of acid-washed glass beads were homogenized in a Waring Blendor for 10 min at 0 C. The resulting homogenate was centrifuged at 16,300*g* for 30 min. The nitrate reductase was then precipitated between 20 to 45% ammonium sulfate saturation and the precipitate was dissolved in 0.1 M glycylglycine-NaOH buffer, pH 7.5. Removal of nucleic acids was achieved by the addition of 30% w/w polyethylene glycol 6000 (0.3 ml/ml enzyme) (6, 12). The mixture was stirred for 15 min and then centrifuged. The resulting supernatant solution was dialyzed in a Dow Chemical Dialyzer (b/HFD-1) against 5 mM phosphate buffer, pH 7.5, for 1.5 hr, and clarified by centrifugation. This dialyzed enzyme was then chromatographed on a diethylaminoethyl cellulose column (3 × 6 cm), the column was eluted with a 0.5-liter linear gradient solution between 5 mM phosphate buffer, pH 7.5, and 1.0 M phosphate buffer, pH 7.5. The fractions containing the greatest enzymatic activity (the portion of the linear gradient between 0.2 and 0.3 M phosphate) were pooled. The enzyme was then precipitated by the addition of ammonium sulfate (40% saturation). The precipitate was dissolved in 0.1 M glycylglycine-NaOH buffer, pH 7.5, containing 20% glycerol, and dialyzed for 3 hr against a 0.1 M phosphate buffer, pH 7.5, solution containing 20% glycerol and sufficient ammonium sulfate to give a final concentra-

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Table I. *Summary of the Purification of NADH-Nitrate Reductase and Associated Activities*

Fraction	Volume	Total Protein	NADH-Nitrate Reductase				FADH₂-Nitrate Reductase				MVH-Nitrate Reductase[1]			
			Total activity	Specific activity	Recovery	Purification	Total activity	Specific activity	Recovery	Purification	Total activity	Specific activity	Recovery	Purification
	ml	*mg*	*units*		%	*fold*	*units*		%	*fold*	*units*		%	*fold*
1. Crude extract	987	6500	290,200	44.5	100	1	39,700	6.1	100	1	232,200	35.6	100	1
2. 20–45% $(NH_4)_2SO_4$ ppt.	80	1060	320,000	303	110	6.8	54,900	52	138	8.5	342,000	323	147	9
3. PEG supernatant	96	720	227,000	315	79	7.1	43,300	60.1	110	9.9	272,900	379	119	10.6
4. Dialyzed PEG supernatant	105	525	128,000	243	44	5.5	33,800	64.4	86	10.6	202,800	386	89	10.8
5. DEAE-eluates	61	126	70,600	551	24	12.4	24,800	194	63	31.8	131,600	1027	57	28.8
6. 0–40% $(NH_4)_2SO_4$ ppt.	9	74.5	87,000	1164	29	26.2	21,000	281	54	46	158,850	2126	70	59.7
7. 25–35% $(NH_4)_2SO_4$ pt.	2	11	19,200	1747	7	39.3	10,270	933	30	153	90,200	8200	44	230.3

[1] MVH: reduced methyl viologen; PEG: polyethylene glycol.

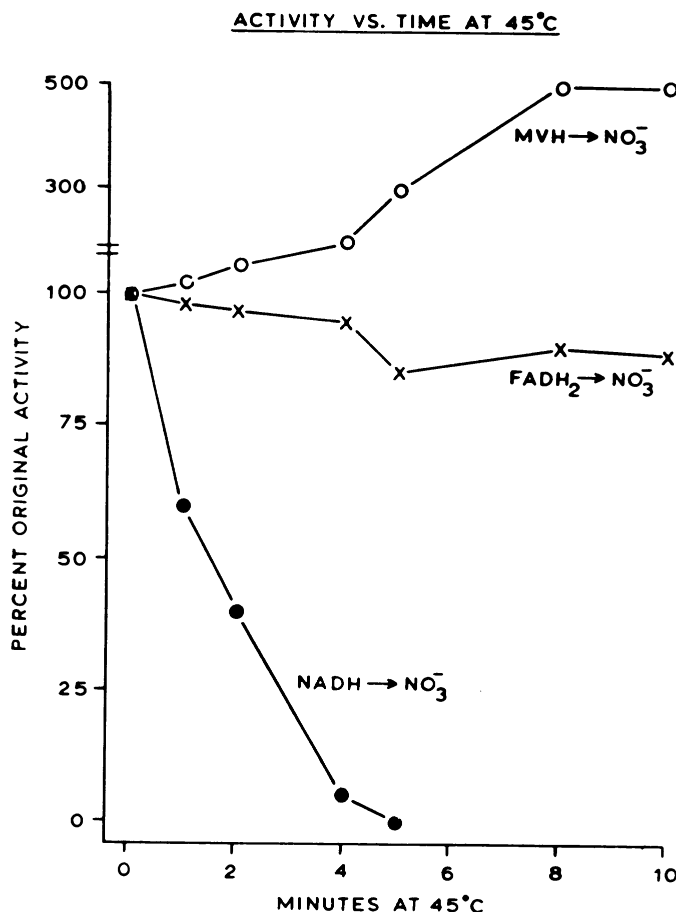

FIG. 1. Effect of temperature treatment on the NADH-nitrate reductase and associated activities. One-milliliter aliquots of fraction 2 (specific activity, 303 units/mg, 13.2 mg/ml) were placed in water bath at 45 C for the indicated time intervals. After cooling in ice, each heat-treated aliquot was assayed for the various activities. The results are compared with those of an unheated sample. MVH: reduced methyl viologen.

tion of 25% saturation. This dialyzed enzyme fraction was centrifuged and the resultant supernatant fraction was dialyzed again for 4 hr against 0.1 M phosphate buffer, pH 7.5, solution containing 20% glycerol and ammonium sulfate at final concentration of 35% saturation. The ammonium sulfate precipitate which formed was collected by centrifugation and dissolved in 0.1 M glycylglycine-NaOH buffer, pH 7.5.

The partially purified NADH-nitrate reductase (fraction 7) exhibited a 50% loss in activity in 24 hr at −15 C. The addition of glycerol (20% v/v final concentration) stabilized the enzyme somewhat, so that only 25% of the activity was lost in 48 hr. The $FADH_2$- and reduced methyl viologen-nitrate reductase activities showed no loss in activity in 48 hr at −15 C, in 20% glycerol. The data of the purification procedure is summarized in Table I.

Other enzymatic activities associated with the NADH-nitrate reductase, namely the $FADH_2$-nitrate reductase and the reduced methyl viologen-nitrate reductase, were followed during the purification (Table I). The enzymatic activities were retained in more or less the same proportions in the steps which do not entail dialysis either against a low ionic strength buffer (fraction 4) or for prolonged periods (fraction 7). The $FADH_2$-nitrate reductase exhibited a normal decline in recovery of total enzyme activity in the purification, whereas the NADH-nitrate reductase, besides showing this normal decline in total enzyme units, possessed an unusual degree of lability as reflected by the low recovery. It is of interest to note that both the fold purification and recovery of the NADH-nitrate reductase are 25% of that of the $FADH_2$-nitrate reductase. The reduced methyl viologen-nitrate reductase activity increased slightly relative to that of the $FADH_2$-nitrate reductase.

As shown in Fig. 1, a 45 C heat treatment of the preparation in the absence of added FAD results in a selective loss in the ability of the enzyme complex to use NADH as an electron donor, as reported earlier (13). Reduced FAD-nitrate reductase activity remains unaffected whereas the reduced methyl viologen-nitrate reductase activity is increased 5-fold. Similar results were found earlier with the *Neurospora* nitrate reductase (6).

The results of the heat treatment and of the purification procedure are consistent with the concept of one enzyme complex catalyzing the three enzymatic activities measured (6). The initial enzymatic function of the complex, the NADH diaphorase, is both susceptible to heat treatment and refractory to purification, indicating a high degree of lability for this portion of the nitrate-reducing system. Consequently, in those instances where the activity of the purified nitrate reductase preparations is assayed using reduced FAD as the electron donor, the activity may *not* be a valid measurement of the "physiological" nitrate reductase, since the physiological elec-

tron donor is probably NADH, not $FADH_2$. On the other hand, $FADH_2$-nitrate reductase activity is probably the best indication of total nitrate reductase concentration in extracts. The concomitant activation of reduced methyl viologen-nitrate reductase which occurs on inactivation of the NADH diaphorase moiety renders the viologen activity inadequate as a quantitative measure of relative nitrate reductase complex concentrations under normal circumstances.

The Michaelis-Menten constant for nitrate of the NADH-nitrate reductase was determined to be 0.23 mM which is quite similar to that reported in other organisms (6, 10). Also exogenously added FAD (Km = 2.6 μM) stimulated the NADH-nitrate reductase activity of the purified enzyme more than 15-fold and was therefore routinely included in those assays. NADH (Km = 10 μM) is the specific electron donor for this enzyme preparation; NADPH at similar concentrations was ineffective.

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