Purification and Properties of NADH-Cytochrome o Reductase from Vitreoscilla*

Victoria Gonzales-Prevatt‡ and Dale A. Webster

From the Department of Biology, Illinois Institute of Technology, Chicago, Illinois 60616

A component of the NADH-cytochrome o reductase system of Vitreoscilla purified to near homogeneity as judged by disc electrophoresis had a molecular weight of 61,000 when estimated by gel filtration. Electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate showed that the enzyme consists of two 35,000-dalton subunits. The enzyme contains one FAD and two cytochromes per molecule all noncovalently bound. Acid-labile sulfide was not detectable.

The flavoprotein can catalyze the reduction by NADH of partially purified cytochrome o and a number of artificial electron acceptors such as ferricyanide, cytochrome c, dicyclohexylidene-phenol, and p-iiodonitro-tetrazolium violet, but not pure cytochrome c. The reduction of partially purified cytochrome o was assayed aerobically by following formation of the oxygenated cytochrome; the kinetics of this reduction were hyperbolic with respect to NADH. The anaerobic reduction of cytochrome c catalyzed by the reductase in the presence of excess NADH occurred in two phases: an initial rapid decrease in absorption of the oxidized cytochrome at 405 and 540 nm followed by a slow shift of these maxima to 423 and 555 nm, respectively. This result is evidence for a sequential reduction of the high and low potential hemes of cytochrome o.

The reductase can accept 4 electrons as determined by anaerobic titration of the fully reduced enzyme with ferricyanide and photochemical titration in the presence of sodium dithionite. Midpoint potentials of the four oxidation-reduction centers estimated from the latter titration were in the range of -257 to -318 mV. The flavosemiquinone was present during intermediate stages of the titrations. Titration of the oxidized protein with NADH reduced only two of the four electron centers. The inability of NADH to fully reduce the purified reductase and the unreactivity of the enzyme with pure cytochrome c suggest that other components are required for the reduction of the cytochrome.

Cytochrome c is a common terminal oxidase in bacteria. The respiratory chain of Vitreoscilla, a filamentous, strictly aerobic prokaryote, is unique in that it does not contain c-, a-, or d-type cytochrome and employs cytochrome c as its only terminal oxidase (1). This cytochrome has been purified to homogeneity and its properties have been studied in detail (2–4). It was reported previously that a NADH-cytochrome c reductase co-purified with the cytochrome and that oxygen uptake with NADH as substrate was primarily associated with those preparations containing high NADH-cytochrome c reductase activity (5). Subsequently, evidence for the involvement of a flavoprotein in the NADH-cytochrome c oxidase reaction was obtained (6). This paper reports the purification of NADH-cytochrome o reductase, some of its electron-accepting and -transferring properties, and discusses some implications of these properties with regard to the mechanism of reduction of cytochrome c.

METHODS

Purification of NADH-Cytochrome c Oxidoreductase—Culture of cells, preparation of cell-free extracts, removal of nucleic acids with protamine sulfate, and ammonium sulfate fractionation were performed as previously described (7). Subsequent steps, herein described, were carried out at 0–4°C. The fraction precipitated at 45 to 65% ammonium sulfate saturation was dissolved in and dialyzed against 0.02 M sodium phosphate buffer, pH 7.2, and adsorbed onto a DEAE-cellulose column (4.7 × 55 cm) equilibrated with the same buffer. The column was eluted with 3 liters of a linear gradient of 0 to 0.5 M sodium chloride in equilibration buffer. The reductase eluted at 0.3 to 0.4 M sodium chloride and was separated from the cytochrome which eluted at 0.2 to 0.3 M (Fig. 1). The pooled cytochrome o fractions were concentrated and chromatographed on Sephadex G-75 according to Tyree and Webster (2). The resulting cytochrome preparation was used for NADH-cytochrome c reductase assays. It had an A450/A550 of approximately 1.8, indicating a purity of about 75%. Fractions containing NADH-cytochrome c reductase activity were pooled, concentrated by ultrafiltration using an Amicon PM10 ultrafilter, and chromatographed on Sephadex G-200 (5 × 84 cm) equilibrated with 0.02 M sodium phosphate buffer, pH 7.5, containing 0.1 M sodium chloride. Gel filtration was repeated on a Sephadex G-100 column (4.7 × 50 cm) under the same conditions. The pooled reductase fractions were then concentrated and further purified by preparative electrophoresis on 10% polyacrylamide gel slabs (3 × 140 × 170 mm) according to Davis (8). Gel slabs were made in a Desaga polymerizing chamber and consisted of 50 ml of separation gel, 6 ml of stacking gel, and 4 ml of sample gel containing 40 to 50 mg of protein. Electrophoresis was run for 6 to 20 h at a constant current of 30 mA in a Desaga TLC chamber with a Brinkmann-regulated power supply. Temperature was maintained at 0–4°C with a Haake circulating pump. The bright yellow band that migrated toward the anode stained for NADH-cytochrome c oxidase activity using p-iodonitrotetrazolium violet (9). This band was sliced off the gel slab and extracted with three 20-ml washes of 0.02 M sodium phosphate buffer, pH 7.5, containing 1.0 M sodium chloride, each wash being allowed to equilibrate at 4°C for 12 to 16 h. The combined washes were dialyzed against 0.02 M sodium phosphate buffer, pH 7.5, concentrated with Aquacide II A (Calbiochem) and stored frozen as 0.5-ml aliquots. Purity of the preparation was determined by disc electrophoresis (8).

Determination of Molecular Weight—Molecular weight was estimated by gel filtration on Sephadex G-100 (1.9 × 150 cm) equilibrated with 0.02 M sodium phosphate buffer, pH 7.5, containing 0.1 M sodium chloride at 4°C (10). The void volume was determined with blue dextran 2000 and the column calibrated with the following proteins: equine cytochrome c (Mr = 13,400), soybean trypsin inhibitor (Mr = 20,126), and bovine serum albumin (Mr = 68,000). The molecular size of the purified reductase was calculated to be 51,000, as compared to the value of 60,000 reported by others (7). This suggests that the reductase is a dimer, consistent with the molecular weight of 61,000 determined by gel filtration. The molecular weight calculated for this preparation is 88,000, which indicates that the reductase is a tetramer.
NADH-Cytochrome o Reductase

dithionite and were excluded from the calculations. Extent of reduction of the flavoprotein was calculated from the change in absorption at 455 nm. Total flavin content of the oxidized enzyme was determined fluorometrically (12).

**Determination of Oxidation-Reduction Potentials**—Oxidation-reduction potentials were estimated by the dye equilibrium method (15) using safranin O (Fisher Scientific Co.). All concentrations in 2.0 ml of 0.1 M sodium phosphate buffer, pH 7.0, were reduced, 34.7 µM safranin O, 2 units of glucose oxidase, 2 units of catalase, 3.5 units of superoxide dismutase, 0.5 µM FNM, 0.2 µM glucose, and 10 µM EDTA. Glucose and EDTA were added from the sidearm in the dark following evacuation and flushing of the cuvette with argon (Matheson, ultra high pure) 10 times. Reduction was achieved by illuminating the cuvette in a 23°C water bath with a 30-watt tungsten bulb at a distance of about 5 cm. Illumination for 30 min under these conditions had no significant effect on the NADH-INT reductase activity of the enzyme. Extent of reduction of dye and protein was determined spectrophotometrically after each illumination. Corrections were made for the contribution of the dye to the protein’s absorption maxima and vice versa. Safranin O was recrystallized from ethanol prior to use.

Assays—Protein was estimated by the method of Lowry et al. (18). Cytochrome o concentration was based on heme content determined by the pyridine hemochromogen method (19). All spectra were recorded with a Zeiss DMR 21 spectrophotometer.

**RESULTS**

The yield and purification achieved could not be accurately estimated because of the lack of a specific and simple assay of enzyme activity. The NADH-cytochrome o reductase reaction may be specific, but it is complex and probably involves other components, whereas the use of artificial electron acceptors such as INT suffers from lack of specificity. However, polyacrylamide gel electrophoresis of the purified reductase showed only one major protein band and two minor bands, and only the major band stained for NADH-INT reductase activity. The purification is summarized in Table I. Electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate showed one major band of molecular weight 35,000 and three minor contaminants. Since the molecular weight of the native enzyme estimated by gel filtration on Sephadex G-100 was 61,000, the enzyme most likely consists of two subunits.

The visible absorption spectrum of the oxidized enzyme is characteristic of that of flavoproteins (Fig. 4b, spectrum 9), and it has a fluorescence emission maximum at 525 nm when excited at 450 nm. Prosthetic group analysis revealed that the purified enzyme contained 0.89 ± 0.08 molecule of FAD (Table II) and 2.1 ± 0.7 non-heme iron/molecule (average of three determinations), but no acid-labile sulfide. Both prosthetic groups are noncovalently bound and can be removed under...

---

**Table I**

| Purification of NADH-cytochrome o reductase from Vitreoscilla (per 100 g of cells) |
|---------------------------------|-------------------|-----------------|-----------------|
| Step                           | Total protein       | NADH-cytochrome o reductase activity (ΔA/min/mg) | NADH-INT reductase activity (ΔA/min/mg) | No. of INT-staining bands on disc electrophoresis |
| Crude extract                    | 4700 mg             | 0.072           | 0.117           | 5-6               |
| Potassium sulfate supernatant   | 4550 mg             | 0.080           | 0.155           | 5-6               |
| 45 to 65% Ammonium sulfate precipitate | 2330 mg          | 0.082           | 0.791           | 3                 |
| DEAE-cellulose pool             | 902 mg              | 0.045           | 0.743           | 2-3               |
| Sephadex G-200 pool             | 265 mg              | 0.140           | 1.340           | 2                 |
| Sephadex G-100 pool             | 190 mg              | 0.126           | 2.140           | 2                 |
| Preparative polyacrylamide gel electrophoresis | 2 mg               | 1.260           | 11.29           | 1                 |

---

1 The abbreviations used are: INT, p-iodonitrotetrazolium violet; n, the number of electron equivalents transferred in an oxidation-reduction reaction according to the Nernst equation.
TABLE II

| Procedure as described by Faeder and Siegel (12) |
| Sample | Concentration | Relative fluorescence at 525 nm excited at 450 nm |
| Flavin | Protein pH 7.7 pH 2.6 pH 2.6/ pH 7.7 |
| FMN | 0.25 | 0.87 | 0.57 | 0.7 |
| FAD | 0.41 | 0.32 | 0.66 | 2.1 |
| Reductase (boiled) | 0.21 | 0.25 | 0.11 | 0.30 | 2.7 |

* Based on M, = 61,000.

TABLE III

| Acceptor | Concentration | Apparent turnover number |
| Ferricyanide | 2.0,000. | 246 |
| INT | 406 | 162 |
| Dichlorophenolindophenol | 100 | 135 |
| Cytochrome c | 50 | 122 |
| Cytochrome o | 1.0 | 2 |

The purified enzyme can catalyze the reduction by NADH, at this concentration under aerobic conditions. The deflavoenzyme, prepared by overnight dialysis against 3 mM potassium iodide (20) which removed FAD but not the non-heme iron, followed by removal of potassium iodide, was totally inactive. Simple addition of either flavin or 2.0 mM AMP. No additions; – – –, 2.5 mM NAD. Similar results were obtained with 2.0 mM AMP.

The purified enzyme can catalyze the reduction by NADH of partially purified cytochrome o, a number of artificial electron acceptors, but not the pure cytochrome (Table III). The rate of reduction of impure cytochrome o under aerobic conditions showed hyperbolic kinetics with NADH and this reaction was inhibited by 2.5 mM NAD and 2.0 mM AMP (Fig. 2). Lineweaver-Burk plots of these data were biphasic with apparent K values of 10 and 50 μM, suggesting the presence of two different NADH binding sites, but the need to use impure cytochrome o preparations to observe even low rates of reduction makes interpretation of these data premature.

Moreover, using the formation of the oxygennated compound as a direct measure of the rate of reduction of the cytochrome may not be entirely valid (see "Discussion"). The low turnover number observed for cytochrome o (Table III) is at least partially attributable to the apparent high K values of 10 and 50 μM, suggesting the presence of two different NADH binding sites, but the need to use impure cytochrome o preparations to observe even low rates of reduction makes interpretation of these data premature.

Fig. 2. The NADH-cytochrome o reductase activity of purified enzyme as a function of NADH concentration under aerobic conditions and the effect of NAD. Procedure as described in the text. The reaction mixture contained 1.06 μM cytochrome o, 0.25 μM reductase, and indicated concentrations of NADH in 0.2 ml of 0.02 M sodium phosphate buffer, pH 7.5, at room temperature. No additions; – – –, 2.5 mM NAD. Similar results were obtained with 2.0 mM AMP.

Fig. 3. Anaerobic reduction of high and low potential hemes of cytochrome o catalyzed by purified reductase. The reaction mixture contained 4.5 nmol of cytochrome o, 3.5 nmol of reductase, 2 units of glucose oxidase, 2 units of catalase, 3.5 units of superoxide dismutase, and 0.3 nmol of gluconate in 1.5 ml of 0.05 M sodium phosphate buffer, pH 7.5 under an argon atmosphere. – – –, Spectrum of oxidized cytochrome o; – – –, 7 min after addition of 1 μmol of NADH; – – –, 55 min after addition of NADH.

Fig. 4. Anaerobic ferricyanide titration of reductase reduced with dithionite. Reductase (containing 94.6 nmol of flavin), 3 units of glucose oxidase, 3 units of catalase, 5.2 units of superoxide dismutase, and 2.9 nmol of glucose were mixed in 1.5 ml of 0.1 M sodium phosphate buffer, pH 7.0, under an argon atmosphere. Excess dithionite was added to fully reduce the enzyme (Spectrum 1). Spectra 2 to 6 (a) and 7 to 9 (b) after addition of 38, 75, 112, 150, 188, 262, 338, and 375 nmol of ferricyanide, respectively. a and b are parts of the same titration that were separated for clarity.
FIG. 5. Anaerobic titration of oxidized reductase with NADH. The reaction mixture contained 11.8 nmol of reductase, 3 units of glucose oxidase, 3 units of catalase, 5.2 units of superoxide dismutase, and 2.9 nmol of glucose in 1.5 ml of 0.1 M sodium phosphate buffer, pH 7.0, under an argon atmosphere. Spectrum 1, and 14.2 nmol of NADH, respectively.

FIG. 6. Photochemical titration of reductase in the presence of safranin O. Procedure as described under "Methods." Absorbance changes of reductase at 450 nm (○—○) and 630 nm (■—■) are plotted versus illumination time.

for cytochrome o, which was estimated to be approximately 5 μM. When pure cytochrome o was used, no reaction was observed, so it is likely that an additional protein component(s) still present in small amounts in the impure preparations is needed in higher concentrations for full activity. This is supported by the observation that the rate of INT reduction catalyzed by the reductase was stimulated when impure cytochrome o was added but not when boiled impure cytochrome o was added.

Under anaerobic conditions, the reduction of the two hemes of cytochrome o in the presence of purified reductase and excess NADH occurred in two phases (Fig. 3). The initial rapid decrease in absorption of the oxidized cytochrome at 405 and 540 nm was followed by a slow shift of these maxima to 423 and 555 nm, respectively. Anaerobic reductive titration of cytochrome o chemically with dithionite or photochemically showed that the decrease in absorption at 405 nm and 540 nm was associated with reduction of the high potential heme \((E'_{m1} = 118 \text{ mV})\) and the shift of these maxima with the reduction of the low potential heme \((E'_{m2} = -122 \text{ mV})\) (3).

The reductase reduced with dithionite was titrated anaerobically with ferricyanide. The first 2 mol of ferricyanide/mol of flavin produced an increased absorption in the visible region characteristic of the flavosemiquinone (Fig. 4a). Further additions of ferricyanide resulted in continued increase of absorption at 380, 430, 455, and 485 nm but decreased absorption at 520 to 680 nm (Fig. 4b), indicating conversion of the flavosemiquinone to the oxidized form. This transition occurred isosbestically at 505 and 353 nm. Addition of ferri cyanide in excess of 4 mol/mol of flavin did not elicit further changes in the absorption spectrum of the reductase. A plot of moles of enzymes reoxidized versus moles of ferricyanide added showed that 4.3 electrons were required to oxidize 1 molecule of reduced enzyme.

Anaerobic titration of the oxidized enzyme with the first 1.2 mol of NADH/mol of flavin produced a decrease in absorption at 380, 430, 455, and 485 nm but an increase at 520 to 680 nm with isosbestic points at 503 and 358 nm (Fig. 5). Additional NADH did not produce further spectral changes. Thus, the end point of the titration was the formation of the flavosemiquinone (Fig. 5) with the consumption of approximately 2 electrons.

Photochemical titration of reductase in the presence of safranin O (Fig. 6) enabled estimates of the oxidation-reduction potentials of its electron centers. The Nernst plot calculated from absorbance changes at 455 nm was sigmoidal and was resolved into two linear plots corresponding to titration of a high \((E'_{m1} = -269 \text{ mV}, n = 3)\) and low \((E'_{m2} = -318 \text{ mV}, n = 1)\) potential center (Fig. 7a). In a similar manner, a Nernst plot was made based on absorbance changes at 630 nm in phase 1 of Fig. 6 and the assumption that the maximum increase in absorption at this wavelength was equivalent to complete conversion of the reductase to the semiquinone. This portion of the titration consumed 2 electrons with a \(E'_{m} = -257 \text{ mV}\) (Fig. 7b).

**DISCUSSION**

The reduction of cytochrome o catalyzed by the reductase...
under aerobic conditions was assayed assuming that the rate of formation of the oxygenated compound was a direct measure of the rate of cytochrome o reduction. This assumption may not be true under all conditions. The reaction of reduced cytochrome o with oxygen involves the formation of at least two identifiable intermediates, Compound D (Fe(III)-O2) and oxygenated cytochrome o (Fe(III)-O2). While the oxygenated compound is stable under certain conditions and remains at a constant level during the steady state, the concentration of oxygenated compound may not always be an accurate measure of the concentration of reduced cytochrome o. For example, three species of the ferrous enzyme could be present under the conditions of the reductase assay if 1 molecule of oxygenated compound reacts with 1 molecule of reduced cytochrome o to reform Compound D as part of the catalytic cycle proposed by Tyree and Webster (4).

The reductase can accept 4 electrons as determined by both anaerobic titration of the reduced enzyme with ferricyanide (Fig. 4) and photochemical reductive titration in the presence of safranin O (Fig. 7). The Nernst plot of the photochemical titration calculated from the absorbance changes of the reductase at 455 nm was sigmoidal, but was resolved into two linear plots corresponding to reduction of high and low potential hemes (Fig. 3). The enzyme-catalyzed thermodynamically favorable rate of reduction of this heme is apparently due to a kinetic limitation imposed by the structure of the one electron-reducible reduction of this heme thus behaves similarly to the chemical reduction with dithionite and the photochemical reduction of the flavosemiquinone as the endpoint. The midpoint potential of the flavosemiquinone was estimated to be between -257 and -259 mv (Fig. 7a). Thus, the first three electron centers have similar potentials and titrated simultaneously. A Nernst plot based on the assumption that the maximum absorbance change at 630 nm (end of phase 1, Fig. 6) corresponded to 100% formation of the flavosemiquinone had a n = 2 and a midpoint potential of 257 mv (Fig. 7b). Since maximum formation of the flavosemiquinone required 2 electrons, 1 electron must be in the non-heme iron. Full reduction of the flavosemiquinone by the third electron did not occur and the spectrum of the semiquinone persisted until the end of the titration (Fig. 6). This persistence of the flavosemiquinone during all intermediate stages of both the photochemical reduction (Fig. 6) and the oxidative titration of the dithionite-reduced enzyme (Fig. 4) is evidence that the flavin and non-heme irons titrate simultaneously or are coupled such that electron exchange between them occurs in partially reduced forms of the enzyme. The scheme presented in Fig. 8 is consistent with the titrometric data.

Anaerobic titration of the oxidized enzyme with NADH reduced only the first two electron centers with formation of the flavosemiquinone as the endpoint. The midpoint potentials of the third and fourth centers were estimated to be higher (between -269 and -318 mv) than the NAD/NADH oxidation-reduction couple (2.3 mV) and, therefore, reducible by NADH. The inability of NADH to fully reduce the purified reductase and the unreactivity of the enzyme with pure cytochrome o suggest that the NADH-cytochrome o oxidase reaction requires other components which could be involved in regulation of the catalytic properties of the reductase.

Acknowledgments—We are grateful to Dr. Francis C. G. Hoskins of the Department of Biology, Illinois Institute of Technology for the use of his spectrophotofluorometer and to Dr. William D. Prett of Beatrice Foods Inc., Chicago, Illinois for drawing the figures.

REFERENCES


Fig. 8. Proposed oxidation-reduction transitions occurring during photochemical reduction (top to bottom) or oxidative titration (bottom to top) of purified reductase in vitro. FI, oxidized flavin; FIH-, half-reduced flavin; FIH2, fully reduced flavin.