Purification and Comparison of NADH-Cytochrome b_5 Reductase from Mitochondrial Outer Membrane of Bovine Heart and Turnip

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The NADH-cytochrome b_5 reductase (NCBR), a mitochondrial external electron carrier, was purified from bovine heart and turnip and their properties were examined. The mitochondrial outer membranes separated were subjected to NCBR isolation through DEAE-Cellulose ion exchange, DEAE-Sephadex gel chromatography, and hydroxyapatite adsorption chromatography. These processes yielded the purification folds of 88 and 42 and the recovery percentages of 0.2%, 5.67% for turnip and bovine heart, respectively. The molecular weight of the NCBR from the two sources was estimated to be 35,000 using SDS polyacrylamide gel electrophoresis. The Michaelis constant K_m and maximum velocity V_{max} were determined by measuring the NADH-ferricyanide redox system as well as the NADPH-ferricyanide redox system. The kinetics showed that both NCBRs had higher affinities for NADH than artificial electron-acceptor substrate ferricyanide. Although NADPH had a lower affinity for the enzymes than NADH, this study showed the 2'-phosphate dinucleotide could be used as a substrate.

Introduction

The electron transport event in a mitochondrial inner membrane, the internal pathway of which is an amytal/rotenone sensitive route coupling to ATP synthesis, has been widely studied in view of the examination of the biological energy production mechanism. It was reported that heat production through a nonphophorylation process; *i.e.*, through no ATP synthesis, was performed using ATP/ADP antiporter, thermogenin and the external pathway of the outer membrane in the mitochondria from skeletal muscles, brown fat tissue and rat liver, respectively.[1-4] Lehninger[5] first proposed that exogenous NADH oxidation was performed through cytochrome b_5 and NADH-cytochrome b_5 reductase flavoprotein (fp_5:[6] abbreviated to NCBR in this report) as an electron carrier and a catalyst, respectively. He described that this external pathway did not relate to the coupling of the electron transport with ADP phosphorylation. He also stated that exogenous NADH oxidation driven slowly by rat liver mitochondria was stimulated through the external electron transport pathway by virtue of the addition of cytochrome *c*. However, the external-transport problem has remained a mitochrondrial enigma for a long time.[4] Related to the activation of the external electron transport system, it was proposed that cytochrome *c* operates as a mobile electron carrier between the inner and outer membranes.[6-8] Bernardi and Azzone[9] also reported as to mitochondrion that the electrons produced from exogenous NADH oxidation are first transfered to cytochrome b_5 *via* NCBR in the outer membrane and then reach oxygen molecules as the final electron acceptor of the respiratory chain through cytochrome *c* oxidase in the inner membrane. In this process, cytochrome *c* was thought to function as an electron shuttle between the outer and inner mitochondrial membranes. NCBR and cytochrome b_5 are also well known to possess catalyst and electron carrier capacities, respectively, for the electron transfer reactions occurring within microsome organelle; *i.e.*, fatty acid desaturation, cholesterol biosynthesis, and certain substrate oxidation by cytochrome P-450.[10-16] Day and Wiskich[17] confirmed the activity of antimycin-insensitive NADH-cytochrome *c* reductase in the outer membrane compartment prepared by treating plant mitochondria isolated from a turnip with digitonin. However, the studies on NCBR and cytochrome b_5 within the external pathway in the outer mitochrondrial membrane are not common.

On the basis of the experimental data mentioned above, the NCBRs catalyzing the external pathway were isolated from bovine and turnip tissues, respectively, and then their reactivities for substrates were examined in this study.

Experimental

Preparation of outer mitochondrial membrane. According to the method of Day and Wiskich,[17] mitochondria and their outer membrane were isolated from a white turnip.

One kg of turnip was divided into small pieces and homogenized in a blender with 0.3 M sucrose containing 8 mM EDTA, 0.1 M *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) buffer (pH 7.2), 0.03 M Tris, 0.5%(w/v) bovine serum albumin, and 4% cysteine. The homogenate was filtered to centrifuge the filtrate for 5 minutes at 15,000 g. The supernatant obtained was recentrifuged for 15 minutes at 12,000 g to separate the turnip mitochondria pellet. The mitochondrial pellet obtained was suspended in 0.3 M sucrose to make a mitochondrial concentration of 10 mg protein/mL. One fifth digitonin by weight on the basis of the mitochondrial protein was added to the mitochondrial suspension to extract the outer membrane, by stirring, at 0 °C for half an hour. This surfactant-containing mitochondrial suspension was diluted twice by volume adding 0.3 M sucrose to centrifuge the diluted suspension for 30 minutes at 30,000 g. The supernatant obtained which was seemingly clear was recentrifuged at 144,000 g for 5 hours. The pelleted outer-membrane component from this re-

centrifugation was stored at −80 °C before NCBR purification.

The mitochondria from bovine heart tissue were prepared using a differential centrifugation after Ragan[18] to separate the mitochondrial outer membrane fraction according to the method of Comte and Gautheron.[19]

Purification of NADH-cytochrome b_5 reductase. According to the method of Cooper,[20] DEAE-Cellulose ion exchange resin and DEAE-Sephadex gel resin were prepared and packed in chromatographic columns. The hydroxyapatite prepared by the method of Levin[21] was stored in 0.001 M sodium phosphate (pH 6.8), and eliminating air bubbles was followed by packing in the columns.

DEAE-Cellulose resin was packed into two columns (2.5×50 cm) and equilibrated with 0.1 M Tris-HCl, pH 8.0 containing 0.001 M EDTA. The sample containing the mitochondrial outer membrane isolated from the turnip tissue was solubilized in a small volumn of 0.3 M sucrose and it was made to develop in one column. By the method of Lee,[22] the mitochondria from bovine heart tissue were treated with *n*-dodecyl β-D-maltoside (DMS) and then centrifuged to obtain the supernatant. This supernatant was developed in the other column. The development of the samples in the two columns was performed with a continous concentration gradient method of NaCl (from 0 to 0.4 M) in 0.1 M Tris-HCl, pH 8.0 containing 0.001 M EDTA to collect 5 mL eluates. For the eluate fractions showing NCBR activity and absorbances at 280 nm, another development was carried out in the column of DEAE-Sephadex gel resin. Packing in the two columns (2.5×20 cm) and equilibrating of DEAE-Sephadex resin were done with 0.02 M Tris-HCl, pH 8.0, 0.001 M EDTA and 0.1% Triton X-100. The elution was performed with the equilibrating buffer containing 0.05 M KCl. The elution and identification of NCBR activity were carried out as in the method described above and the active fraction was then subjected to adsorption chromatography. The hydroxyapatite prepared was packed in two columns (1.5×30 cm) and equilibrated in a 0.01 M potassium phosphate buffer (pH 6.5). The developing was performed with the step-by-step gradient method using 0.01, 0.04 and 0.09 M potassium phosphate (pH 6.5). The elution and determination of NCBR activity were performed by the same method employed above.

All the steps in the chromatographic purification were carried out in a cold room at 4 °C.

NCBR assay. An activity measurement of NCBR in the NADH-ferricyanide redox system was performed by the method of Mihara and Sato.[23] The redox reaction system consisted of 0.1 M potassium phosphate buffer (pH 7.5), 1 mM potassium ferricyanide and NCBR purified/NCBR sources from the turnip and bovine heart tissues. This reaction system was made to be 25 °C and activity measurement started by adding 1×10^{-4} M NADH. A 420 nm absorption change due to ferricyanide reduction was recorded by using a Cary spectrophotometer with a temperature controller. The reactivity measurements of purified NCBRs for NADH, NADPH and ferricyande were done by identical monitoring techniques to determine the Michaelis constants (K_m) and maximum velocities (V_{max}).

Electrophoresis and Protein determination. Electrophoresis was performed by employing the SDS-discontinuous polyacrylamide gel system of Laemmli[24] and Hames.[25]

The Bradford method[26,27] was used for protein determination with bovine serum albumin as a standard.

Results and Discussion

It is well known that NCBR catalyzes in microsomal electron transport. The purification of NCBR, therefore, has been mostly performed from liver microsomes. After the initial isolation according to the method of solubilization incubating liver microsomes with cobra venom by Strittmetter and Velick,[14,15] the various methods of NCBR purification from microsomes were reported by many researchers.[16,28] However, the characterization and purification of NCBR and the other membrane proteins existing in the mitochondrial outer membrane have not been investigated intensively.

The attempts to purify the NCBR from bovine heart tissue and the NCBR from white turnip were designed individually. NCBR purification was initiated with the treatment of detergents for isolated mitochondria and mitochondiral outer membrane preparations from bovine heart and turnip tissues followed by the development through column chromatographies using ion-exchanger resin, gel-filtration resin and hydroxyapatite. On developing the turnip mitochondrial outer membrane on DEAE-Cellulose resin to obtain the eluates of 5 mL/fraction, proteins were detected for fractions of numbers 25-46 within which fractions 31, 35 and 37 had NCBR activity (Figure 1). In the case of bovine heart tissue, DMS was added to the mitochondrial outer membrane to make a mixture and then extracted overnight. This mixture was spun to obtain the supernatant for the NCBR purification through the DEAE-Cellulose resin column. This chromatographic development was performed under the same conditions used for the turnip mitochondrial outer membrane. Protein elution and maximum specific activity of NCBR were detected for the fractions of 32-40, and 32-45 respectively (Figure 4). The NCBR fractions obtained through the DEAE-Cellulose resin column were developed secondly on DEAE-Sephadex resin. Protein elution and NCBR activity were certified at 5 mL fractions 3-26

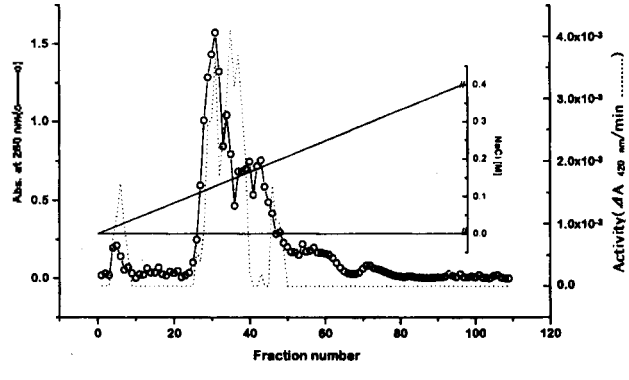

Figure 1. DEAE-Cellulose purification chromatogram for NCBR from turnip tissue. The mitochondrial outer membrane treated with digitonin was developed on a DEAE-Cellulose anion exchanger. The elution was carried out by the continuous concentration gradient method of NaCl from 0 M to 0.4 M with 0.1 M Tris-HCl, pH 8.0 containing 1 mM EDTA.

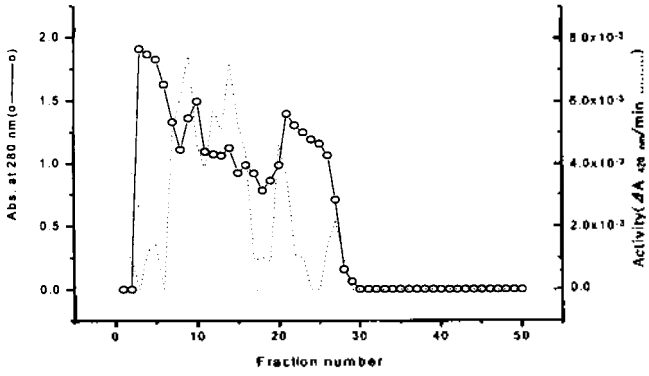

Figure 2. DEAE-Sephadex purification chromatogram for NCBR from turnip tissue. DEAE-Sephadex gel resin was packed and equilibrated with 0.02 M Tris-HCl, pH 8.0, 1 mM EDTA containing 0.1% Triton X-100 in the column of 2.5×20 cm. The fractions including NCBR eluted from the ion exchanger were developed with the identical equilibrium buffer containing KCl.

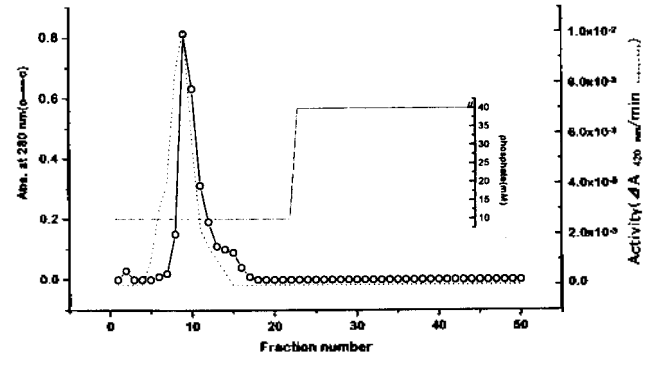

Figure 3. Hydroxyapatite purification chromatogram for NCBR from turnip tissue. This is an adsorption chromatography profile for the fractions having NCBR activity from the DEAE-Sephadex gel column. A column of 1.5×30 cm containing hydroxyapatite adsorbent was used. Developing was conducted increasing the phosphate concentration by means of a step-by-step gradient method.

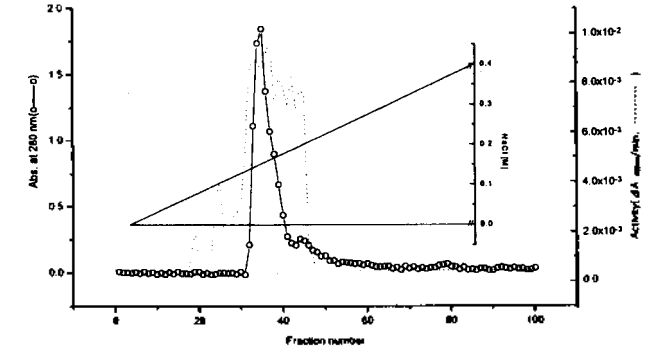

Figure 4. DEAE-Cellulose purification chromatogram for NCBR from bovine heart tissue. This chromatogram expresses the ion exchanger chromatography profile for the spun bovine heart mitochondrial outer membrane treated with DMS. The development and elution from column were carried out by means of the identical method applied in the Figure 1 experiment.

and 9, 14, 20/6, 16, 21 and 15 in turnip/bovine tissue, respectively (Figure 2, 5). Hydroxyapatite was used for a stationary phase in adsorption chromatography, thirdly. The

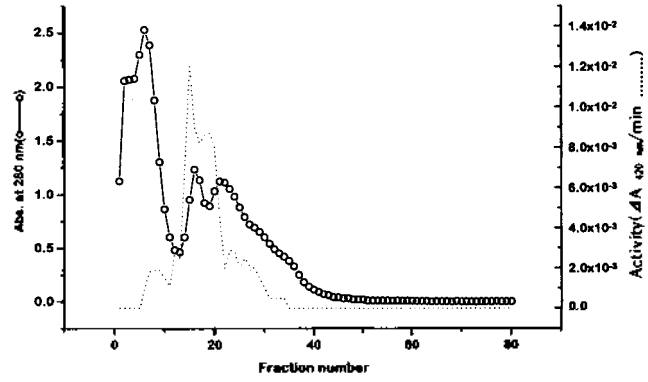

Figure 5. DEAE-Sephadex purification chromatogram for NCBR from bovine heart tissue. The chromatography was carried out by means of the identical method used for Figure 2.

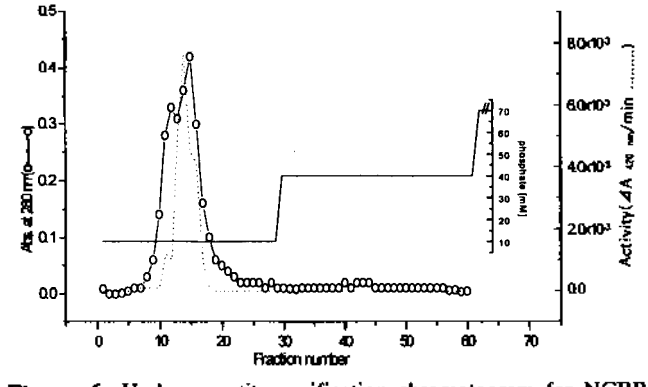

Figure 6. Hydroxyapatite purification chromatogram for NCBR from bovine heart tissue. This chromatography was performed using the method for Figure 3.

specific activity of the NCBR reaction was identified in the elutes released with 0.01 M phosphate buffer in both turnip and bovine heart (Figure 3, 6).

For NCBRs purified through the procedures described above from the plant tissue and the animal tissue, the purification degrees and recoveries were 88.3 fold and 0.2%, and 42.1 fold and 5.67%, respectively (Table 1). Considering the difference in purification degrees shown in this experiment, NCBR purification from mitochondria must be designed taking into account the characteristic properties of animal and plant tissues individually. In the method for NCBR purification from animal tissue of the bovine heart purification steps and options of packing materials used in column development must be reconsidered. The purification degree calculated for plant tissue taken from a turnip, on the other hand, means that the method employed in this work is successful.

Electrophoresis was performed for purified and crude NCBRs from each tissue with standard proteins (Figure 7). The bands on A, B lanes and D, E lanes represent samples which are released from a DEAE-Cellulose ion exchanger and DEAE-Sephadex gel filter for turnip and bovine heart tissues. Purified NCBRs eluted from the adsorption column for the two tissues appeared as a single band at an identical position on the C and F lanes. Comparing the positions of these two bands with those of standard protein markers, both of the purified NCBRs were estimated to have a molecular weight of about 35 kDa. Mihara and Sato[23] reported

Table 1. Purification of turnip NADH-cytochrome b_5 reductase/bovine NADH-cytochrome b_5 reductase

	Total protein (mg)	Total activity (U)	Specific activity (u/mg)	Purification fold	Recovery (%)
Mitochondrial outer membrane preparation	210/132.1	98.7/89.8	0.47/0.68	1/1	100/100
DEAE-Cellulose	172.5/120.1	293.3/245.18	1.7/2.1	3.6/3.01	82.1/90.5
DEAE-Sephadex	6.36/80	43.9/188	6.9/2.4	14.7/3.53	3.0/60.6
Hydroxyapatite	0.34/7.5	14.1/216.98	41.5/28.6	88.3/42.1	0.2/5.67

The activity of NADH-cytochrome b_5 reductase in the purification processes was determined by measuring its NADH-ferricyanide reductase activity. The one unit, u, is defined as the amount of enzyme changing 0.001 of $\Delta A_{420\ nm}$/min.

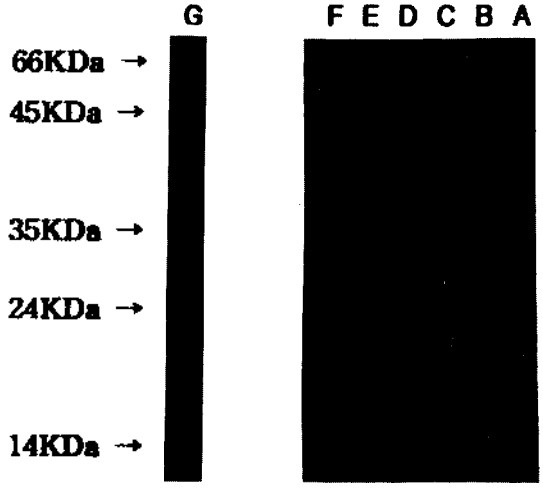

Figure 7. The purification degree and molecular weight of purified NCBR were estimated by means of SDS-PAGE. The A, B and C lane/D, E and F lane are the eluates from ion exchanger, gel filter and hydroxyapatite columns for the tissues of turnip and bovine heart, respectively.

the molecular weight of NCBR from rabbit liver microsomes to be 33,000 by the methods of SDS-polyacrylamide gel electrophoresis, Sepharose 6B gel chromatography and Sephadex G-100 gel chromatography. Spatz and Strittmatter[29] also supposed the molecular weight of NCBR from calf liver microsomes to be 43,000 based on the sedimentation data and partial specific volume data obtained from centrifugation. The reason for the difference in their molecular weight is not clear. According to the findings suggested by this study, the molecular weights of NCBRs from mitochondrion and microsomes may be equal ranging from 30,000 to 40,000.

NCBRs purified through fractionations on columns for isolated mitochondria outer membranes from turnip and bovine heart tissues were given for enzyme activity measurements (Table 2). The velocity of the NCBR reaction was examined using the NADH-ferricyanide system and NADPH-ferricyanide system on the basis of Michaelis-Menten kinetics to calculate the Michaelis costant (K_m) and maximum velocity (V_{max}) for each substrate. Two substrates, an electron donor and an acceptor, were necessary for NCBR activity. NADH and cytochrome b_5 are regular substrates as an electron donor and an acceptor, respectively. The potassium ferricyanide instead of cytochrome b_5 was used for the determination of reactivity and substrate specificity of NCBR in this study taking the following reasons into account. There is no commercially available cytochrome b_5

Table 2. Determination of the kinetic parameters K_m and V_{max} of turnip NCBR/bovine NCBR for substrates

Substrate	K_m (mM)	V_{max} ($\Delta A_{420\ nm}$/min)
NADH	3.32/2.17	0.03/0.12
NADPH	7.69/2.34	0.56/0.03
Ferricyanide	4.35/3.85	0.025/0.027

These reactivities of the reductases were estimated using NADH-ferricyanide and NADPH-ferricyanide redox reaction systems. The values are calculated using the least-square method.

and trypsin-solubilized cytochrome b_5 has a high K_m value for reductase.[23]

$$NADH + 2\ Fe(CN)_6^{3-} \rightleftarrows NAD^+ + 2\ Fe(CN)_6^{4-} + H^+$$

The K_m values of NADH and ferricyanide are 3.32 mM, 4.35 mM and 2.17 mM, 3.85 mM for the NCBRs purified from turnip and bovine heart tissues, respectively. There is a slight difference in the assay methods for NCBR activities from plant and animal tissues. Both reductases purified from plant and animal tissues have more affinities for NADH as a regular substrate than ferricyanide as an artificial substrate. The NADPH instead of NADH could serve as the electron donor for NCBR catalysis. Stryer stated that NADPH is the reductant in biosynthesis.[30] The information shown in the present experiment suggests that it would be worthwhile to scrupulously reexamine this elusive problem of the NADPH role in the future. The K_m values of plant NCBR and animal NCBR for NADPH were estimated to be 7.69 mM and 2.34 mM, respectively. Although NADPH had a lower affinity for NCBR than NADH, it is known through present assays that NADPH is able to work equally well as an electron donor.

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Direct Analysis of Tackifying Resins in Vulcanized Rubber by Simultaneous Pyrolysis Methylation-Gas Chromatography/Mass Spectrometry

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Vulcanized rubber containing three kinds of oligomeric resins such as cashew resin, t-octylphenol formaldehyde resin and terpene modified wood rosin has been characterized by simultaneous pyrolysis methylation-gas chromatography/mass spectrometry (SPM-GC/MS). After methylation by the SPM method using tetramethylammonium hydroxide, the methylated pyrolyzates of the corresponding resins were detected with higher sensitivity than underivatized pyrolyzates without any interferences from other ingredients of vulcanized rubber.

Introduction

Pyrolysis gas chromatography/mass spectrometry (Py-GC/MS), a direct analysis method, is a powerful analytical technique for macromolecules and oligomers that are insoluble or not extractable from matrix by organic solvents. Simultaneous pyrolysis methylation (SPM) with tetramethylammonium hydroxide (TMAH) is frequently used as a derivatization method of macromolecules in analytical pyrolysis. The method improves the gas chromatographic analysis of polar pyrolysis products of natural, synthetic, and biological polymers with carboxyl, aromatic amino, and hydroxyl groups. There are many reports on structural analysis of resin and resinite since the method was first introduced by Challiner.[1-4] The latest works on analytical and applied pyrolysis of polymers, copolymers and blend, were reviewed by Blazso.[5] Challinor has applied the SPM method for several types of rosin-based commercial resins[6] and alkyd resins[7] that are polyesters prepared by reaction of polyhydric alcohols with polybasic acids. According to the Challinor's report, the composition of *t*-butylphenol, *p*-nonylphenol formaldehyde condensates, and their rosin ester derivatives were successfully determined by the SPM method for the purpose of forensic uses. According to their reports, however, the SPM method gave more complex chromatographic profiles under the experimental condition, that is, somewhat high pyrolysis temperature of 770 ℃.

Most studies concerning the SPM method were done for the structural examination of oligomers and macromolecules themselves. Commercial rubbers are quite complex materials. In addition to various polymers, rubber formulations contain a number of compounding additives that are plas-