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DETECTION OF IRON-SULFUR CENTER-CONTAINING SUBUNITS OF MITOCHONDRIAL NADH DEHYDROGENASE BY SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS AND BY HIGH-PERFORMANCE GEL PERMEATION CHROMATOGRAPHY

Morimitsu Nishikimi, Yoshiharu Shimomura,
Hiroshi Yamada, and Takayuki Ozawa

Department of Biomedical Chemistry, Faculty of Medicine,
University of Nagoya, 466 Japan

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Summary: Soluble NADH dehydrogenase resolved from Complex I of the mitochondrial electron-transfer chain was subjected to gel electrophoresis in the presence of sodium dodecyl sulfate at 4°C, and then the gel was stained for iron with bathophenanthroline disulfonate and thioglycolic acid. The 23,000-dalton subunit was markedly stained, and the 51,000-dalton subunit was also stained, but only slightly. High-performance gel permeation chromatography using an eluant containing 0.1% sodium dodecyl sulfate also demonstrated that these subunits contain an iron-sulfur center: the elution pattern recorded by light absorption at 400 nm gave two peaks corresponding to the positions of the subunits.

Complex I of the mitochondrial respiratory chain, which catalyzes electron transport from NADH to ubiquinone, contains several iron-sulfur centers, as demonstrated by electron spin resonance spectroscopy (1). Hatefi and his coworkers (2,3) isolated soluble NADH dehydrogenase from this complex, and showed that the enzyme was composed of three subunits. Recently, Ragan *et al.* (4) have further fragmented this enzyme into the largest subunit and the mixture of the other two. Their data indicated that the former possesses an iron-sulfur center, and that another iron-sulfur center is present in the latter. Since the amino acid analysis data indicate that the smallest subunit does not contain enough cysteine residues to form an iron-sulfur center, they reasoned that the center is present in the subunit of middle size (4).

Since proteins possessing a prosthetic group such as chlorophyll-protein complexes (5) and hemoprotein (6) can be separated with retention of the prosthetic groups by gel electrophoresis in the presence of dodecyl sulfate at low temperature, we have attempted to identify the iron-sulfur center-containing subunits in the dehydrogenase by a similar gel electrophoresis procedure.

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As another approach, we have also used high-performance gel permeation chromatography using an eluant containing SDS[1]. The results obtained by both techniques indicate that the subunit of middle size in soluble NADH dehydrogenase does possess an iron-sulfur center.

Materials and Methods

Materials. Complex I was isolated from beef heart mitochondria by the method of Hatefi (7), and soluble NADH dehydrogenase was prepared from this complex as described by Galante and Hatefi (8).
Polyacrylamide gel electrophoresis. Polyacrylamide disc gels (10%, 0.5 x 6 cm) were formed in 25 mM Tris/190 mM glycine (pH 8.3)/0.1% SDS/20% glycerol. Samples were prepared by treatment of soluble NADH dehydrogenase (1.4 mg/ml) in 5 mM Tris-Cl (pH 7.8)/0.1% SDS/50 mM dithiothreitol/10% glycerol/0.003% bromophenol blue at 0°C, and placed on the tops of gels. Electrophoresis was carried out at 4°C at 1.5 mA per tube using 25 mM Tris/190 mM glycine (pH 8.3)/0.1% SDS as an electrode buffer. After electrophoresis, gels were stained for iron with bathophenanthroline disulfonate in the presence of thioglycolic acid (9), and for protein with amidoblack 10B. In the experiment of two-dimensional gel electrophoresis, the first dimensional electrophoresis was carried out on the same gel as above (0.2 x 6 cm) at 0.12 mA per tube; and, after the gel was equilibrated in 125 mM Tris-Cl (pH 6.8)/5% 2-mercaptoethanol/2% SDS, it was electrophoresed on a slab gel which had been made as described previously (10) except that the gel concentration was 10%. The gel was stained with Coomassie blue. The molecular-weight marker proteins used were bovine serum albumin, ovalbumin, α-chymotrypsinogen A, and cytochrome c.
Gel permeation chromatography. High-performance liquid chromatography was carried out using a Jasco model TRI ROTAR III equipped with a column of silica-based aqueous gel (Finepack SIL AF-102, 0.72 x 50 cm) and a Jasco UVIDEC-100-III UV spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo). The solvent system used was 10 mM sodium phosphate (pH 6.8)/0.1% SDS/200 mM sodium sulfate. The flow rate was 0.7 ml/min, and the column temperature was 30°C.

Results and Discussion

To resolve soluble NADH dehydrogenase into subunits with limited destruction of its iron-sulfur centers, SDS-polyacrylamide gel electrophoresis under mild conditions was developed. Since the structure of the centers may be stabilized at low temperature, samples were prepared at 0°C in a solution containing a relatively low concentration of SDS (0.1%)[2] and subjected to electrophoresis at 4°C. When a gel was stained for iron with bathophenanthroline disulfonate in the presence of thioglycolic acid (9), a pink-colored band was clearly visible (Fig.1A). This band corresponded to the middle band of the three major protein bands which appeared upon staining the same gel

[1] Abbreviation: SDS, sodium dodecyl sulfate

[2] The usage of Tris-glycine buffer (pH 8.3) for preparation of both gels and electrode buffer prevents precipitation of SDS at 0-4°C.

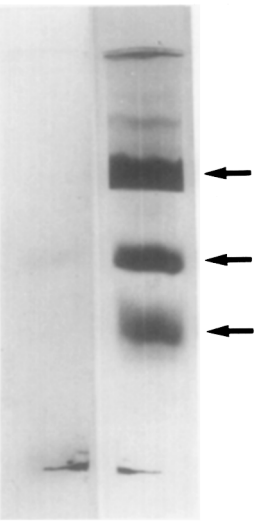
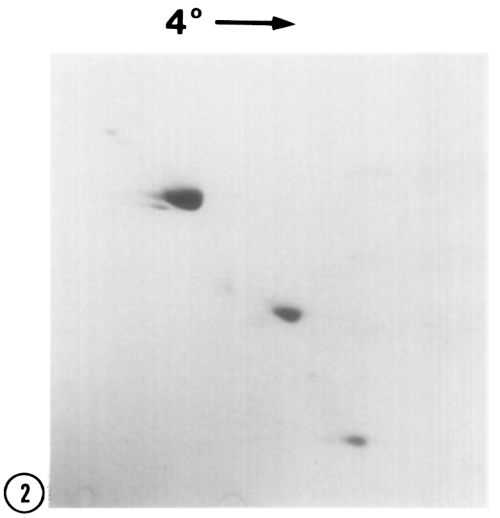


Fig. 1. SDS-polyacrylamide disc gel electrophoresis of soluble NADH dehydrogenase under mild conditions. The dehydrogenase (35 μg) was subjected to electrophoresis at 4°C as specified in "Materials and Methods". After electrophoresis, the gel was stained for iron with bathophenanthroline disulfonate and thioglycolic acid (A), and for protein with amidoblack 10B (B). The India Ink marks at the bottom indicate the position of bromophenol blue, and the three arrows, three subunits of the dehydrogenase.

with amidoblack 10B (Fig.1B). A faint band was also observed at the position of the uppermost band, when a gel was stained for iron after shorter period of electrophoresis (data not shown). It may be that the iron-sulfur center associated with this band may be prone to destruction under the electrophoresis conditions.

To examine whether the three subunits of soluble NADH dehydrogenase were separated according to their molecular weights, two-dimensional gel electrophoresis was performed in which the first dimensional separation of the enzyme proteins was done on the same gel as described above, and then SDS-polyacrylamide gel electrophoresis was carried out at room temperature (20°C) in the second dimension. The results indicated that three major spots were located nearly on the diagonal line, indicating that the electrophoresis procedure used in the first dimension can separate the subunits of soluble NADH dehydrogenase according to their size (Fig.2). The molecular weights of the subunits



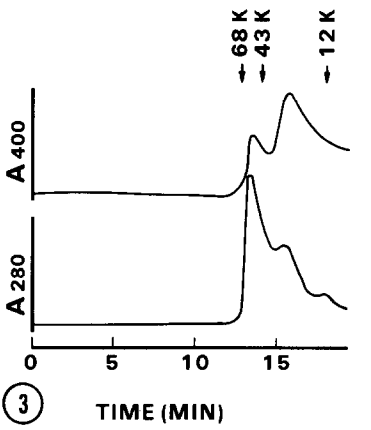


Fig. 2. Two-dimensional electrophoresis of soluble NADH dehydrogenase. The enzyme (7 μg) was subjected to electrophoresis under the same conditions as in Fig. 1 in the first dimension, then to SDS-slab gel electrophoresis at room temperature (20°C). The gel was stained with Coomassie blue.

Fig. 3. High-performance gel permeation chromatography of soluble NADH dehydrogenase. The enzyme was subjected to chromatography as specified in "Materials and Methods". Elution of protein was recorded spectrophotometrically at 280 nm and 400 nm. The amounts of the enzyme injected were 35 μg for the trace at 280 nm and 70 μg for the trace at 400 nm. The sensitivity of detection at 400 nm was 4 times that of detection at 280 nm. The arrows indicate the elution positions of bovine serum albumin (68K), ovalbumin (43K), and cytochrome c (12K).

were estimated to be 51,000, 23,000 and 11,000. Thus, it follows that the subunit of middle size possesses an iron-sulfur center.

In another approach to the separation of subunits of soluble NADH dehydrogenase with retention of its iron-sulfur centers, we utilized high-performance gel permeation chromatography using an eluant containing 0.1% SDS. When elution of protein was detected spectrophotometrically at 280 nm, the subunits of the enzyme were found to be separated in three peaks (Fig.3), and their molecular weights were determined to be 48,000, 24,000, and 13,000. Corresponding to the first and second peaks, two peaks appeared in the elution pattern recorded at 400 nm. Since iron-sulfur centers absorb the light at this wavelength, these findings indicate again that both the largest and middle subunits have an iron-sulfur center. It is noted that the first peak is fairly small compared to the second one. It seems that the iron-sulfur center of the largest subunit is more labile than that of the subunit

of middle size; therefore, the former is more easily destructed than the latter during the course of chromatography. Although this chromatography was performed at 30°C because of the better resolution of the peaks than at lower temperature, the iron-sulfur centers appeared to be retained on the polypeptides to appreciable extents during the course of chromatography. Irrespective of the relatively high temperature, the rapidness of the chromatography may allow us to detect the iron-sulfur center-containing subunits.

The two techniques used in the present study could provide direct evidence that the subunit of middle size possesses an iron-sulfur center. The methods are simple in that each of the subunits does not need to be purified to homogeneity by time-consuming procedures. Moreover, they require only less than 100 μg of protein for analysis. They may be useful in analyzing other iron-sulfur protein in enzymes that are composed of multiple subunits.

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