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ORGANELLE SENSOR: AMPEROMETRIC DETERMINATION OF NADH BY IMMOBILIZED MITOCHONDRIAL ELECTRON TRANSPORT PARTICLES

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A new sensor for NADH was developed by making use of an immobilized subcellular organelle. Mitochondria was used as a model system for assembling an organelle sensor. Mitochondrial electron transport particles (ETP) were prepared from beef heart muscle and entrapped in the membrane formed of agar gel. The membrane-bound ETP was found capable of NADH oxidation:

$$NADH+\tfrac{1}{2}O_2+H^+ \xrightarrow{ETP} NAD^-+H_2O$$

The membrane was tightly attached to the surface of an oxygen electrode capable of amperometric detection of O_2. The sensor responded to NADH in solution with a resulting electric output. The response was enhanced by the addition of 2,4-dinitrophenol (DNP). NADH was determined in the concentration range 1–300 μM. NADH was alternatively determined for 2 weeks without replacing the ETP-bound membrane.

INTRODUCTION

Many biocatalysts have been successfully employed as analytical reagents. Enzymatic assays and microbioassays are typical examples of processes that depend for their selectivity and sensitivity on the function of biocatalysts. In the past 10 years various types of biosensors capable of detecting a specific substance in a simple manner have been developed. These sensors use the unique combination of biocatalysts and electrochemical devices. The biosensors provide promising applications in clinical and environmental analysis and process control (1–10). However, there have been few biosensors for cofactors such as NAD(P), NAD(P)H, and ATP. This investigation was undertaken to develop a new biosensor for NADH, a cofactor of clinical importance.

Most essential features of mitochondrial function, such as electron transfer, are retained by submitochondrial particulate preparations. The mitochondrial electron transport particle (ETP) can be included in such a

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particulate preparation. The ETP is the particular subunit carrying out the special function that transfers electrons from NADH and succinate to molecular oxygen. The phosphorylation function of ETP is inactivated by such decoupling agents as 2,4-dinitrophenol (DNP). In addition, the oxidation of succinate is inhibited by malonate. Therefore NADH is considered to be a specific electron donor to ETP in the presence of malonate and DNP:

$$NADH + \tfrac{1}{2}O_2 + H^+ \xrightarrow{ETP} NAD^+ + H_2O$$

This suggests that ETP can be used as a specific analytical agent for NADH. The concentration of NADH can be determined by following the consumption of O_2.

In order to stabilize ETP function and to assemble a biosensor for NADH, ETP was immobilized in a membrane matrix with retention of its function. Agar was chosen as the matrix from the preliminary investigation (11). The membrane-bound ETP was tightly attached to a membrane-type oxygen probe, so as to detect the change of O_2 concentration in its vicinity. The assembled biosensor responded quantitatively to NADH. Since the biocatalyst is utilized in the subcellular organelle form, the new biosensor may be termed an organelle sensor in a similar manner to the enzyme sensor, in which immobilized enzyme is combined with an electrochemical device.

There have been many investigations on the electrochemical characterization of NAD(P)H and NAD(P) (12–20). Coughlin and his coworkers (21) developed the electrochemical determination of NADH. Because of the direct electrochemical oxidation of NADH at an anode, the method suffers from the presence of electroactive contaminants and nonspecific adsorbents on electrodes. The newly developed biosensor overcomes the above problems. NADH is oxidized selectively by the biocatalyst. The electrochemical device is completely separated from the biochemical reaction by an oxygen permeable Teflon membrane. Only small gaseous molecules such as O_2 can penetrate into the electrochemical device. Therefore, the electrodes are completely protected from contamination. This paper describes the characteristics of the organelle sensor for NADH.

EXPERIMENTAL

Preparation of ETP

Beef hearts were collected at the slaughterhouse and packed in ice immediately after the animals were killed. The hearts were first trimmed of excess fat and connective tissue and then minced. The minced muscle was

FIG. 2. Schematic profile of the organelle sensor.

washed four times with 5 vol of 0.25 M sucrose containing 10^{-3} M Versene. The pH was maintained at 7.0 by the addition of 1.0 N KOH whenever necessary. Two hundred g (wet weight) of the washed mince was suspended in 500 ml of 0.25 M sucrose and homogenized in a Waring blender at top speed for 20 s. The homogenate was centrifuged at $1000 \times g$ for 10 min. The turbid supernatant was carefully decanted from the loosely packed cell debris and centrifuged at $15{,}000 \times g$ for 10 min. The pellet was thoroughly homogenized with a Potter-Elvejhem homogenizer (glass-Teflon) in 10 vol of 0.25 M sucrose containing at 5 mM Tris buffer of pH 7.4 $1000 \times g$ for 10 min. The supernatant was then centrifuged at $10{,}000 \times g$ for 10 min. The pellet was carefully suspended in 100 ml of 0.25 M sucrose containing 5 mM Tris buffer of pH 7.4, and the suspension was centrifuged at $10{,}000 \times g$ for 10 min. The protein concentration of the precipitated mitochondrial fraction was adjusted at 10 mg/ml with 0.25 M sucrose containing 5 mM Tris buffer of pH 7.4. The suspension was sonicated for 5 min in a 20 kHz sonic oscillator and centrifuged at $105{,}000 \times g$ for 60 min. Six g of ETP (wet weight) was precipitated. The preparations were preserved by freezing.

Preparation of the Organelle Sensor

The ETP was immobilized in a thin layer of agar gel. Forty mg of agar was dissolved in 1 ml of 0.25 M sucrose containing 5 mM Tris-HCl buffer of pH 7.4 and 270 μM 2,4-dinitrophenol (DNP) at about 80°C. The solution

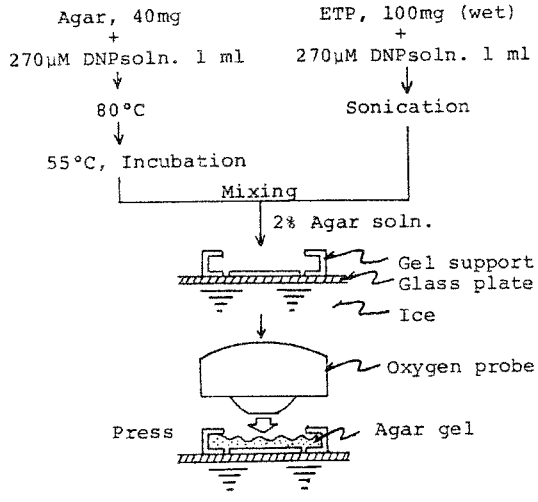


FIG. 1. Procedure for the preparation of an organelle sensor.

was cooled at 55°C. The frozen ETP (100 mg wet weight, 4 mg protein) was thawed and dispersed in 1 ml of 0.25 M sucrose containing 5 mM Tris-HCl buffer of pH 7.4 and 270 μM DNP by sonication at 20 kHz for 10–15 sec. The ETP suspension was vigorously mixed with the agar solution.

A plastic support was placed on a glass plate cooled by ice as illustrated in Fig. 1. The agar-ETP solution was immediately added to the plastic support. An oxygen probe, consisting of an oxygen-permeable Teflon membrane, a lead anode, and a platinum cathode were put in the plastic support and kept in press until the solution was solidified. The immobilized ETP membrane was tightly fixed to the Teflon membrane. The thickness of the membrane was about 400 μm in the wet state.

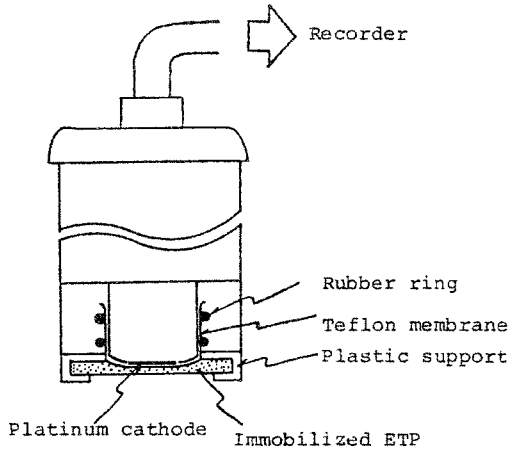
Measurements of NADH with the Organelle Sensor

The organelle sensor was connected with a recorder through a standard register. A schematic diagram of the measurement apparatus is shown in Fig. 2. Each sample was injected into an oxygen saturated buffer contained in a reaction vessel. The buffer was kept stirred magnetically and thermostated at 25 ± 0.1°C.

RESULTS

Response of the Organelle Sensor to NADH

The organelle sensor was immersed in 5 mM Tris-HCl buffer of pH 7.4 containing 270 μM DNP. The solution was initially saturated with O_2 by air



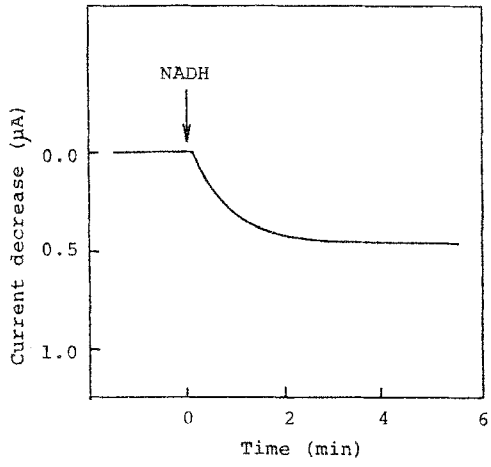


FIG. 3. Typical response of the organelle sensor to NADH. Into a solution containing 270 μM DNP, pH 7.4, 25°C, 100 μl of 16 mM NADH was injected with magnetic stirring.

bubbling. Due to dissolved oxygen, a steady current was obtained when the solution was gently stirred. An NADH solution was injected into the oxygen-saturated solution. The final concentration of NADH was 80 μM. The output current of the sensor was recorded as the difference from the initial current. The addition of NADH resulted in a sharp decrease in the current, as presented in Fig. 3. The output current reached a steady state within 2 min.

The organelle sensor was then washed with Tris-HCl buffer of pH 7.4. The washing caused the sensor to show the initial steady current due to dissolved oxygen. Furthermore, the organelle sensor responded repeatedly to NADH in a similar way to the above. The response of the sensor was reproducible.

Effects of Temperature and pH on the Response

The response of the organelle sensor was determined at various values of pH and temperature, while maintaining the concentration of NADH at 160 μM. Glycine-HCl buffer at pH 3.0, sodium acetate–acetic acid buffer at pH 4.5, phosphate buffer at pH 6.0, and Tris-HCl buffers at pH 7.0, 8.0, and 9.0, with an ionic strength of 0.1, were used to control the pH. The pH profile is presented for the response of the organelle sensor to NADH in Fig. 4. The maximal response was obtained in the pH range 5.5–6.0. Above a pH

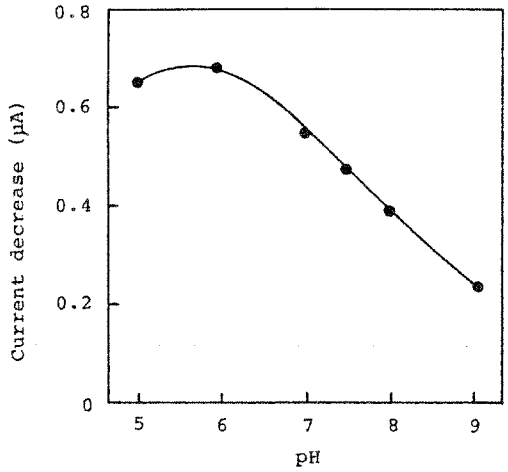


FIG. 4. Responses of the organelle sensor to NADH at various pH. 160 μM NADH, 270 μM DNP, 25°C.

of 6, the response decreased with increasing pH. After measurement at pH 9.0, the sensor was contacted with the buffer of pH 7.0. The sensor responded reversibly to NADH. It indicated that the immobilized ETP retained its activity up to a pH of 9. Under acidic conditions, the response was considerably suppressed. In contrast to alkaline conditions, an irreversible change occurred below a pH of 3; the immobilized ETP was completely inactivated.

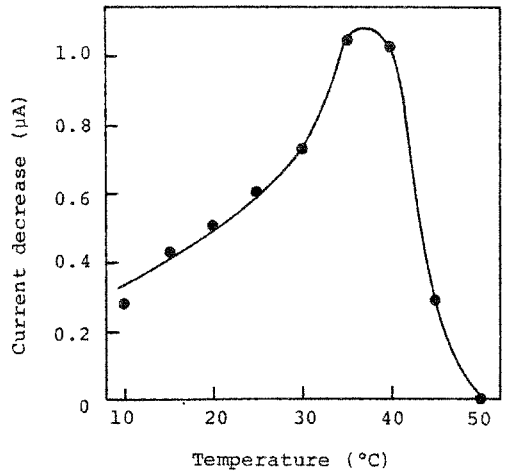


FIG. 5. Temperature dependence of the response to NADH. 160 μM NADH, 270 μM DNP, 25°C.

Figure 5 shows the temperature dependence of the response of the organelle sensor to NADH. The measurements were conducted with 160 μM NADH. The response of the organelle sensor was enhanced with increasing temperature, reaching a maximum around 35–40°C. Above 40°C, the response was sharply depressed. The organelle sensor gave no appreciable response to NADH at 50°C. The inactivation of the immobilized ETP occurred irreversibly. The lost activity was not recovered even when the temperature was lowered at 25°C.

These results indicate that the response of the organelle sensor is considerably influenced by temperature and pH. The measurements should be made under a controlled temperature and pH as in most biochemical analyses.

Calibration Curve for the Determination of NADH

The organelle sensor responded with such sensitivity to NADH that a calibration curve was prepared by following either the initial rate of current or the steady state current of the sensor. Figure 6 contains two calibration curves obtained by the initial rate and the end-point methods. A linear correlation was obtained in the concentration ranges 1–300 and 1–250 μM NADH by the initial rate and the end-point methods, respectively.

The interference due to any other electron donors such as ascorbate was carefully checked. No appreciable effects on the response were developed

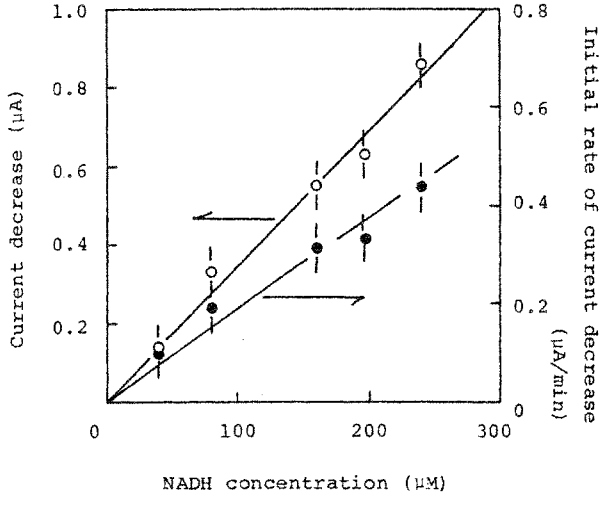


FIG. 6. Calibration curves for the determination of NADH. ○, initial rate method; ●, end-point method.

by these electron donors except succinate. Only succinate gave considerable effects, although the organelle sensor required a little longer response time as compared with NADH. However, the response to succinate was specifically and sufficiently inhibited by the presence of malonate, which was a competitive inhibitor of the succinate oxidase of the ETP. Moreover, malonate gave no harmful effects on the NADH oxidase activity of the ETP. In the case where NADH is contained together with succinate, NADH is specifically determined in the presence of malonate.

Reuse of the Organelle Sensor

The immobilization made it possible to reuse the ETP. The organelle sensor was repeatedly used for the determination of NADH without replacing the immobilized ETP gel. NADH was measured several times a day using the sensor. The sensor was then stored in a buffer of pH 7.4 at 4°C. The responses that are averaged for each day are plotted in Fig. 7. Reproducible response was obtained for at least 2 weeks. The response gradually decreased after 2 weeks. In prominent contrast to the native ETP, the immobilized one showed a considerably long lifetime. This may be an advantage of the immobilized ETP, particularly for analytical purposes.

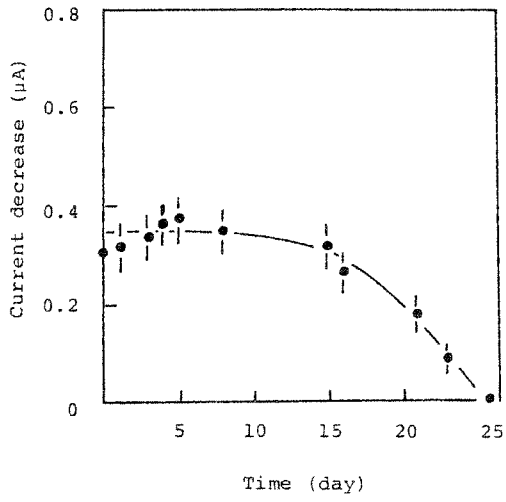


FIG. 7. Reusability of the organelle sensor. NADH (100 μM) was determined every day. After measurement, the sensor was washed and stored in Tris-HCl buffer of pH 7.4, containing 0.25 M sucrose around 4°C.

DISCUSSION

Subcellular organelles are commonly so unstable *in vitro* that they are not reusable. A reusable subcellular organelle has been prepared by immobilizing the mitochondrial ETP in agar gel. The ETP consists of membraneous vesicles resulting from the resealing of the mitochondrial inner membrane fragments (22, 23). Multienzyme systems catalyzing electron transport might be localized on the surface of vesicles, which causes the enzyme activity to be unstable. In our work, these enzymes were entrapped by the agar gel matrix with resulting stabilization of their activity. Therefore, the immobilized ETP can claim two advantages. One is an enhanced accessibility of NADH, because the electron transport enzymes contact directly with NADH in solution. The other is prolonged lifetime and reusability.

NADH has been sensitively determined photometrically in clinical and biochemical analyses. The turbidity of a sample, however, seriously interferes with the photometric assay. The present method overcomes the disadvantage of photometry. The organelle sensor may be used with a turbid sample. As compared with the conventional electrochemical determination of NADH (21), the metal electrodes involved in an oxygen probe have no chance to contact with a sample, because an oxygen permeable Teflon membrane separates the electrochemical device from the sample solution. Thus the metal electrodes are free from contamination.

The 100% response time of the organelle sensor was 2 min. The 2-min duration might account for the slight interference of the diffusion of NADH through the gel layer. The agar gel matrix caused the ETP to have a longer lifetime on the one hand and to respond to NADH with a little longer duration on the other. A possible improvement of the response time might be accomplished with a porous membrane matrix.

CONCLUSIONS

NADH was determined in the concentration range 1–300 μM with an organelle sensor composed of an immobilized mitochondrial electron transport particle, an oxygen permeable Teflon membrane, a lead anode, a platinum cathode, and an alkaline electrolyte. To our knowledge, the present system is the first application of a subcellular organelle to the construction of a biosensor.

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