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The Catalytic Activity of Glass Beads, Silica Gels and Anion-Exchange Resins Modified with Metal-Porphines in the Oxidative Reaction of NADH

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Silica gel and glass beads were modified using the acid chloride of metal-tetrakis(4-carboxyphenyl)porphine (M-TCPP) through a peptide bond, and an anion-exchange resin was modified with M-TCPP by electrostatic interaction and physical adsorption. The carriers modified with Co^{3+}-TCPP proved to accelerate the following redox reaction:

$$\mathrm{NADH+O_2} \xrightarrow{\text{carriers modified with Co-TCPP}} \mathrm{NAD^+ + H_2O_2}.$$

The formation of hydrogen peroxide was confirmed by means of a colored reaction. The glass beads modified with Co^{3+}-TCPP exhibited the strongest activity among the tested carriers, and were expected to be useful practically as a solid catalyst for the formation of hydrogen peroxide from NADH.

Key words NADH; oxidative catalysis; modified carrier; metal–porphine; hydrogen peroxide; determination

NADH (a reduced form of 1,4-dehydronicotinamide adenine dinucleotide) is one of the important coenzymes widely used in clinical analyses. For the sensitive determination of NADH, various methods using coupling colored reactions have been developed to date.[1—4] For this purpose, another idea is that NADH can be determined indirectly by determining the hydrogen peroxide generated from NADH in the oxidative reaction, since extra-sensitive methods for the determination of hydrogen peroxide have already been developed.[5,6] Previously, we studied the glutathione oxidase (GSHPx)-like activity of the carriers modified with metal-porphines (M-P) and found that a carrier modified with Co^{3+}-porphine catalyzes the oxidative reaction of glutathione to generate hydrogen peroxide.[7] In the present paper, we report the oxidative catalytic activity of glass beads and silica gel modified with Co^{3+}-tetrakis(4-carboxyphenyl)porphine (Co-TCPP), as well as anion-exchange resin modified with Co-TCPP. Consequently, it was revealed that the carriers modified with Co-TCPP catalyzed the following redox reaction (Eq. 1).

$$\mathrm{NADH+O_2} \xrightarrow{\text{carriers modified with Co-P}} \mathrm{NAD^+ + H_2O_2} \qquad (1)$$

In addition, the amount of hydrogen peroxide in the reaction mixture was revealed to be related to a fixed rate of NADH. This result suggested that NADH can be determined spectrophotometrically based on the amount of hydrogen peroxide produced in the reaction (Eq. 1) catalyzed by glass beads modified with Co-TCPP.

Experimental

Materials and Reagents Reagent-grade tetrakis(4-carboxyphenyl)porphine (H_2-TCPP) and tetrakis(4-sulfophenyl)porphine (H_2-TSPP) were purchased from Wako Junyaku Co., Ltd., and used without further purification. Amberlite IRA 900 was used as a mother anion-exchange. The resins were washed with 1 mol/l HCl and NaOH solutions, and with water and acetone, successively, and dried up. Aminopropyl-controlled-pore glass beads (AMP00500C: particle size, 200—400 mesh) and aminopropyl-silica gel (Licrosorb-NH_2: particle size, 10 mm) were purchased from CPG Inc., and Merck AG, respectively, and used as mother carriers. Aqueous solutions of NADH were prepared before use from NADH (reduced form, Sigma & Aldrich Co., Ltd.). Peroxidase (type I, from horseradish) for the determination of hydrogen peroxide was purchased from Sigma & Aldrich Co., Ltd. Other reagents and chemicals were of analytical or of reagent grade.

Preparation of Ion-exchange Resins Modified with M-P H_2-Porphines were converted into metal-complexes (M-TCPP and M-TSPP, M= Co^{3+}, Fe^{3+}, Mn^{3+} and Cu^{2+}) by the methods described in the literatures.[8,9] Modified resins (M-P_r, M-P 25 μmol/g dry resin) were prepared from aqueous solutions of the metal-porphines and ion-exchange resins, Amberlite IRA 900, according to the method reported previously.[10—14]

Preparation of Glass Beads and Silica Gels Modified with Co-TCPP Co^{3+}-tetrakis(4-chloroformylphenyl)porphine (Co-TCPPCl, see Fig. 1), the acid chloride of Co-TCPP, was prepared by treating Co-TCPP with thionyl chloride in dioxane at 70 °C in a conventional manner. After the excess thionyl chloride and dioxane were removed under a vacuum, the solid Co-TCPPCl was washed with dioxane and dried under a vacuum. Then, a mixture of Co-TCPPCl and silica gels or glass beads in dioxane was refluxed until the mixture became clear. The resultant silica gel or glass beads modified with Co^{3+}-TCPP (Co-$TCPP_s$ and Co-$TCPP_g$, respectively, Co-TCPP 25 μmol/g dry carrier) were filtered, washed with water and acetone, and dried under a vacuum.

Apparatus Absorption spectra and absorbances were measured on Shimadzu UV-180 and UV-160 spectrophotometers, respectively, with a 10 mm fused-silica cell.

Evaluation of the Oxidative Catalytic Activity A modified carrier (50 mg) was added to a mixture of NADH (1 mmol/l, 1.0 ml), 0.1 mol/l acetate buffer (pH 5.0, 2.0 ml) and 2.0 mol/l NaCl (1.0 ml) solutions, and the mixture was incubated at 35 °C for 30 min. For the determination of unchanged NADH, the absorbance of the supernatant at 340 nm was measured against the control. As the control, the supernatant obtained in the same manner using IRA 900 was used in the place of the M-$TCPP_r$, or M-$TSPP_r$ was used for the modified resins, and the reagent blank was used for Co-$TCPP_g$ and -$TCPP_s$. The activity of the modified carrier was evaluated based on the decrease in the absorbance against the control.

Determinations of Hydrogen Peroxide Produced by Oxidation of NADH A sample solution (0.2 ml, 4.0 μmol of NADH) was mixed with 0.1 mol/l acetate buffer (pH 5.0, 2.0 ml) solution. After the addition of a carrier modified with M-P (25 mg), the mixture was incubated at 35 °C for 30 min to oxidize NADH. A supernatant (0.5 ml) of the mixture was added to 4.0 ml of a 4 : 1 : 1 : 1 mixture of 0.05 mol/l borate buffer (pH 7.5), 7.0 mg/ml phenol, 0.5 mg/ml 4-aminoantipyrine and 10 unit/ml peroxidase (from horseradish) solutions, and the absorbance of the resulting quinoid dye in the mixture was measured at 505 nm against the reagent blank.

Procedure of Determination of NADH The procedure was essentially

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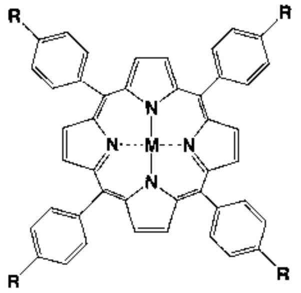

R = COO$^-$ Metal-tetrakis(4-carboxyphenyl)porphine
(M-TCPP ; M=Fe^{3+}, Mn^{3+}, Co^{3+} and Cu^{2+})

R = SO$_3^-$ Metal-tetrakis(sulfophenyl)porphine
(M-TSPP ; M=Fe^{3+}, Mn^{3+}, Co^{3+} and Cu^{2+})

R = COCl Metal-tetrakis(4-chloroformylphenyl)porphine
(M-TCPPCl ; M=Co^{3+})

Fig. 1. Structures of Metal-Porphines

the same as that used to determine hydrogen peroxide using peroxidase described in the above section. The absorbance of quinoid dye formed from hydrogen peroxide, which was formed from NADH by the oxidative catalytic activity of carriers modified with metal-porphine, was estimated as the absorbance of NADH. A calibration curve of NADH against the absorbance was drawn, to which NADH can be indirectly referred.

Results and Discussion

Metalloporphyrins The metal-porphines used in this study are shown in Fig. 1. All except for Co-TCPPCl are water-soluble. They were immobilized easily and firmly on the ion-exchange resins, as described previously,[10—14] by electrostatic and physical interactions. Co-TCPPCl, which was an acid chloride of Co-TCPP and was easily prepared from Co-TCPP, was firmly linked to aminopropyl–silica gels and aminoacyl–glass beads through peptide bonds. All of the modified carriers were stable under variation in temperature, in moisture and storage, and the M-TCPP and -TSPP on the carriers were hardly eluted with water and organic solvents.

Reactions The progress of the reaction (Eq. 1) was confirmed through the detection of NAD$^+$ and hydrogen peroxide in a reaction mixture containing Co-TCPP$_r$. The formation of hydrogen peroxide was confirmed using the following color reaction (Eq. 2).

$$\text{Hydrogen peroxide}+\text{4-aminoantipyrine (AAP)}+\text{phenol} \xrightarrow{\text{peroxidase}} \text{quinoid dye}+H_2O \qquad (2)$$

As a result, a considerable amount of hydrogen peroxide was detected in the supernatant of the reaction mixture, as discussed in the following section.

Since the higher the activity of the modified carrier, the greater the decrease in NADH and the more hydrogen peroxide generated, quantities of unchanged NADH and generated hydrogen peroxide should be a reliable indication of the activity.

Table 1. Unchanged NADH (%)

Modified carrier	Unchanged NADH (%)
Co-TSPP$_r$	27.1
Cu-TSPP$_r$	93.0
Fe-TSPP$_r$	98.3
Mn-TSPP$_r$	—[a]
Co-TCPP$_r$	30.6
Cu-TCPP$_r$	87.4
Fe-TCPP$_r$	91.9
Mn-TCPP$_r$	—[a]
Co-TCPP$_g$	2.6
Co-TCPP$_s$	2.8

a) Can not be examined due to elution of Mn-P.

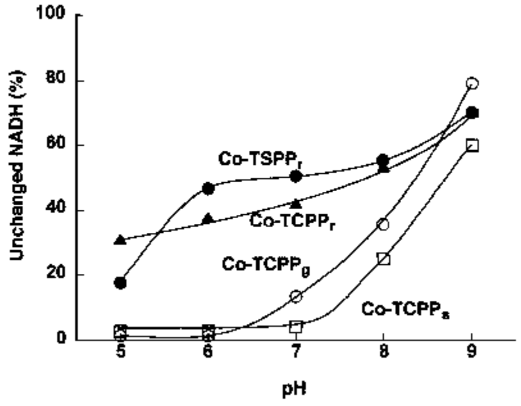

Fig. 2. Effect of pH

Co-TCPP$_g$ (—▲—), Co-TCPP$_s$ (—⊟—), Co-TCPP$_r$ (—⊖—), Co-TSPP$_r$ (—●—). Modified carrier, 50 mg; incubated for 30 min at 35 °C.

Reaction Conditions for Examination of the Activity The anion-exchange resin (IRA 900), which serves as the mother resin of M-P$_r$, adsorbs a considerable amount of NADH. To prevent the adsorption of NADH on IRA 900, the ionic strength of the solution was elevated with the NaCl solution. As a result, it was revealed that the addition of 2.0 mol/l NaCl solution minimized the adsorption of NADH on IRA 900. Accordingly, each examination was conducted using a reagent solution containing 2.0 mol/l NaCl solution referring to the result obtained by the use of IRA 900 as a control. However, since glass beads or silica gels do not absorb NADH, the reagent blank was used as a reference in the cases of Co-TCPP$_g$ and -TCPP$_s$.

The Oxidative Catalytic Activity The (%) unchanged NADH after treatment with M-P$_r$ at pH 5.0 is summarized in Table 1. As seen in Table 1, NADH was almost zero after treatment with the carriers modified with Co-P, while most of the NADH remained unchanged in the case of Cu- and Fe-P$_r$. Furthermore, a small elution of Mn-TCPP and -TSPP from Mn-TCPP$_r$ and -TSPP$_r$, respectively, was observed. These results indicate that carriers modified with Co-P can exhibit strong oxidative catalytic activity. We then carried out the following examinations in detail to obtain optimum conditions for carriers modified with Co-P.

Effect of pH on the Activity: The (%) unchanged NADH against pH is presented in Fig. 2. Comparing the unchanged NADH at pH 5.0, NADH was nearly zero after the treatment with Co-TCPP$_g$ and Co-TCPP$_s$, but about one-third of the NADH still remained after treatment with Co-TCPP$_r$ and -TSPP$_r$, as seen in Fig. 2. This result indicates that Co-TCPP$_g$ and -TCPP$_s$ can exhibit stronger oxidative catalytic activity than Co-TCPP$_r$ and -TSPP$_r$. In addition, Co-TCPP$_g$ seems to

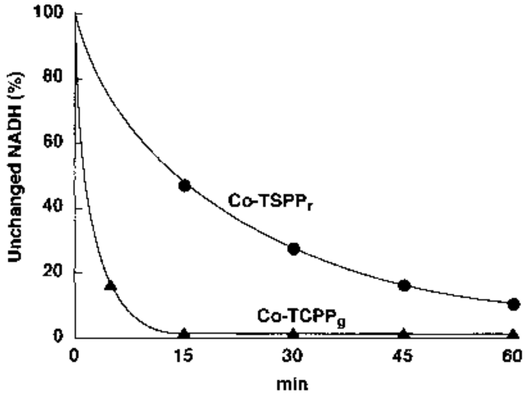

Fig. 3. Time Courses

Co-TCPP$_g$ (–▲–), Co-TSPP$_r$ (–●–). Modified carrier, 50 mg; pH 5.0; incubated at 35 °C.

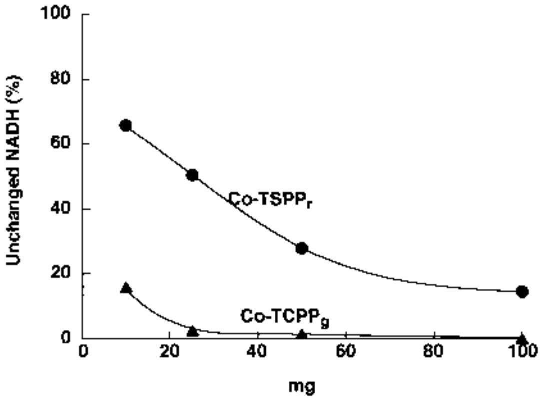

Fig. 5. Effect of Amount of the Modified Carriers

Co-TCPP$_g$ (–▲–), Co-TSPP$_r$ (–●–). pH 5.0; incubated at 35 °C for 30 min.

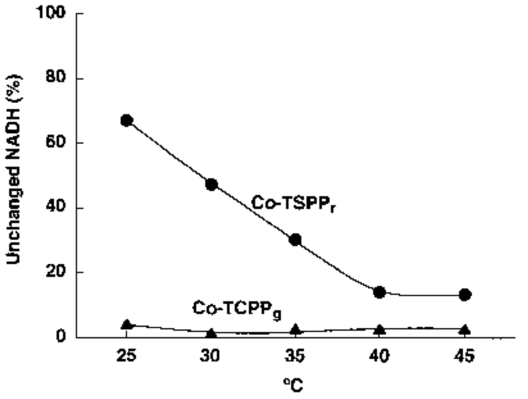

Fig. 4. Effect of Incubating Temperature

Co-TCPP$_g$ (–▲–), Co-TSPP$_r$ (–●–). Modified carrier, 50 mg; pH 5.0; incubated for 30 min.

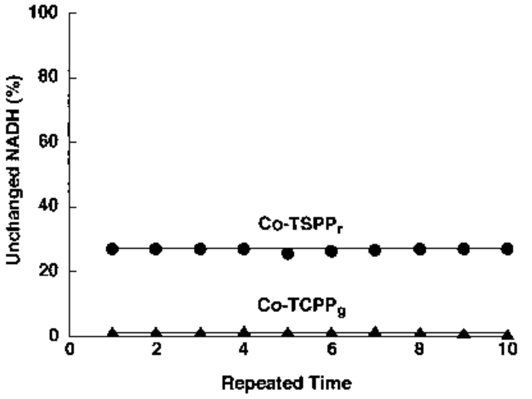

Fig. 6. Repeated Uses

Co-TCPP$_g$ (–▲–), Co-TSPP$_r$ (–●–). Modified carrier, 50 mg; pH 5.0; incubated at 35 °C for 30 min.

be slightly more active than Co-TCPP$_s$. We thus decided mainly to examine Co-TCPP$_g$ at pH 5.0 and to use Co-TSPP$_r$ as reference, because Co-TSPP$_r$ showed the strongest oxidative catalytic activity of NADH among the modified resins examined.

Time Course: Figure 3 shows the time course of the reaction (Eq. 1). Initially, we presumed that Co-TSPP$_r$ would give more rapid equilibrium of the reaction (Eq. 1) than Co-TCPP$_g$, since a considerable amount of NADH could be decreased due to adsorption on Co-TSPP$_r$. Contrary to our presumption, Co-TCPP$_g$ allowed the reaction (Eq. 1) to reach equilibrium after a 15-min incubation, but Co-TSPP$_r$ required at least a 100-min incubation before equilibrium was reached, as seen in Fig 3. Co-TCPP$_g$ accelerated the reaction (Eq. 1) to a much greater extent than Co-TSPP$_r$, and was suggested to be superior to Co-TSPP$_r$ as a catalysis. For the present examination, we determined that a 30-min incubation should be sufficient for the evolution of the activity, taking into consideration time to spare.

Effect of Incubation Temperature: The effect of the incubating temperature on the (%) unchanged NADH was examined between 25 and 45 °C by using Co-TCPP$_g$ and -TSPP$_r$. Most of the NADH disappeared in the tested region (see Fig. 4), while the activity of Co-TSPP$_r$ increased as the temperature rose. The standard incubating temperature was settled at around 35 °C, where most of the enzyme reactions were carried out in general. It is an advantageous features of Co-TCPP$_g$ that its activity was not affected seriously by the incubating temperature in contrast to most enzymes.

Effect of the Amount of Co-TCPP$_g$ and -TSPP$_r$: To examine the effect of the amount of catalysis on the rate of reactions, we checked the relationships between the amount of the modified carriers and unchanged NADH. As a result, the reaction (Eq. 1) reached equilibrium when 50 mg or more Co-TCPP$_g$ was added (see Fig. 5). The unchanged NADH was only about 2.6% when 25 mg of Co-TCPP$_g$ was added, but was 50% when the same amount of Co-TSPP$_r$ was added. Thus Co-TCPP$_g$ exhibited much stronger activity than Co-TSPP$_r$ in small amounts, which indicates that Co-TCPP$_g$ is more effective than Co-TSPP$_r$. Thus, in the present study, we concluded that Co-TCPP$_g$ was the best catalyzing agent among the modified carriers tested, and that the addition of 50 mg of Co-TCPP$_g$ was significant in examining the activity in more detail.

Repeated Uses To confirm the capacity of Co-TCPP$_g$ as a catalyst in the oxidative reaction of NADH, repeated uses of Co-TCPP$_g$ were checked. During 10 trials, both Co-TCPP$_g$ and Co-TSPP$_r$ maintained their activity, as seen in Fig. 6. Thus, we concluded that the carriers modified with Co-TCPP serve as catalysts in the oxidative reaction (Eq. 1).

Quantity of the Resulting Hydrogen Peroxide According to the reaction (Eq. 1), one mole of hydrogen peroxide

Table 2. Resulting Hydrogen Peroxide When NADH Was Oxidized

Solid catalysis	Hydrogen peroxide determined[a] (μmol)
Co-TCPP$_s$	1.8
Co-TCPP$_g$	1.8
Co-TCPP$_r$	0.7
Co-TSPP$_r$	0.7

a) 4.0 μmol of NADH were used.

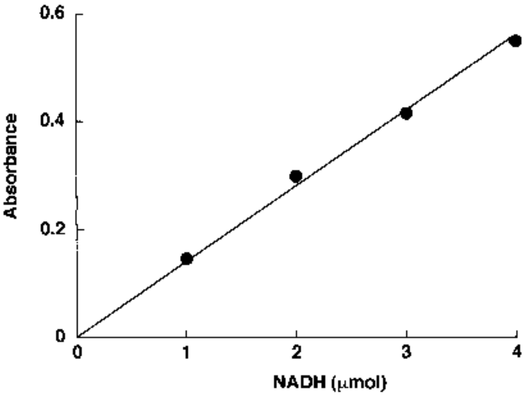

Fig. 7. Plot of NADH Amount against Absorbance of Hydrogen Peroxide

Co-TCPP$_g$, 50 mg; pH 5.0; incubated at 35 °C for 30 min.

should be formed from one mole of NADH. To check this point, the quantity of hydrogen peroxide was determined by following the procedures described above. The determination was carried out at pH 7.5, which is the optimum pH of peroxidase for determining hydrogen peroxide. As a result, the reaction catalyzed by Co-TCPP$_g$ and -TCPP$_s$ gave only 1.8 μmol of hydrogen peroxide from 4.0 μmol of NADH, as shown in Table 2. The lower-than-expected yield of hydrogen peroxide was attributed to the fact that hydrogen peroxide was partially decomposed by Co-TCPP on the carrier, which had been previously reported to exhibit weak catalase-[13] and/or GSHPx-like[7] activities. We therefore concluded that this result supported the above scheme.

In the case of Co-TSPP$_r$, the resulting hydrogen peroxide was 0.7 μmol less than that observed in the case of Co-TCPP$_g$. Since an anion-exchange resin adsorbs a considerable quantity of NADH and the adsorbed NADH is expected not to take part in the reaction (Eq. 1), this result again would not contradict reaction (Eq. 1). To confirm that the amount of hydrogen peroxide is formed at a fixed rate of the NADH, the resulting hydrogen peroxide from a reaction mixture containing Co-TCPP$_g$ and NADH at different concentrations of 1 to 4 μmol was determined. When the amount of NADH was plotted against absorbance, which corresponds to the quantity of hydrogen peroxide, a straight line sufficient for use as a calibration curve of NADH was obtained (Fig. 7). This line indicated that hydrogen peroxide was produced in proportion to NADH at a fixed rate.

Analytical Application As mentioned above, since the plot of NADH against absorbance was regarded as a calibration curve of NADH, where Beer's law was applicable between 1.0 and 4.0 μmol, we could be able to determine NADH using the curve based on the determination of hydrogen peroxide. From this point of view, Co-TCPP$_g$, having the strongest activity among the tested carriers, is a promising solid catalysis agent in the determination of NADH.

Furthermore, the sensitivity of the measurement of enzyme activity using the determination of NADH[4] might be improved by the use of a combination of this Co-TCPP$_g$ method and the highly sensitive determination methods of hydrogen peroxide, such as fluorometry[5] or the chemiluminescence method.[6]

In conclusion, carriers modified with Co-TCPP exhibited oxidative catalytic activity for NADH. Particularly, Co-TCPP$_g$ was expected to be practically useful in the determination of NADH as a solid catalysis agent.

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