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POLYETHYLENE GLYCOL-MODIFIED HEMIN HAVING PEROXIDASE ACTIVITY IN ORGANIC SOLVENTS

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Summary: Hemin, having two carboxyl groups, was coupled with monomethoxy-polyethylene glycol, PEG, through the ester bond formed with carbodiimide. The PEG-modified hemin was readily soluble not only in neutral aqueous solution but also in organic solvents. Its absorption spectrum in 1,1,1-trichloroethane showed a sharp Soret band at 398 nm. The modified hemin catalyzed the peroxidase-reaction in organic solvent and in aqueous solution using hydrogen peroxide or peroxidized linolenic acid as hydrogen acceptor and o-phenylene diamine as hydrogen donor . The activity of PEG-hemin in 1,1,1-trichloroethane was greater than that in an aqueous solution; k_1 values in 1,1,1-trichloroethane were 2.3×10^3 $M^{-1}sec^{-1}$ with hydrogen peroxide and 7.0×10^2 $M^{-1}sec^{-1}$ with peroxidized linolenic acid, and the value in an aqueous solution was 3.0×10 $M^{-1}sec^{-1}$ with hydrogen peroxide.

Chemical modification of proteins and enzymes with synthetic polymers such as polyethylene glycol and polyaspartic acid derivative were performed to cure various diseases including leukemia(1-4) and to alleviate allergenic reactions(5,6), or to make enzymes soluble and active in organic solvents in the field of new bioreactor(7-19). In the series of study on alteration and improvement of original function and property of a biological material, the investigation in a wider variety of the material to be modified is intriguing and important.

Hemin, a prosthetic group of heme-proteins and enzymes, plays an important role by itself in oxido-reductions such as oxidatoin of a compound with peroxide. Hemin liberated from apoprotein is not soluble in neutral aqueous solution and several organic solvents such as benzene and chloroform(20). This paper deals with chemical modification of hemin with polyethylene glycol, an amphipathic polymer. The modified hemin became

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amphipathic and exerted peroxidase activity in organic solvents as well as aqueous solution.

Materials and Methods

Hemin crystal and monomethoxypolyethylene glycol(MW=5000) were purchased from Wako Pure Chemical Industry Ltd.(Osaka, Japan) and Polyscience Inc.(Warrington, PA, USA), respectively. Dicyclohexyl carbodiimide(DCC) and o-phenylene diamine were obtained from Tokyo Kasei Co. Ltd.(Tokyo, Japan). Other reagens were of analytical grade.

Preparation of PEG-modified hemin. Sixty mg of crystal hemin chloride, ferriprotoporphyrin IX, were dissolved to 10 ml of pyridine. To the pyridine-hemichrome solution were added 1 g of monomethoxypolyethylene glycol(MW=5000) and 0.5 g of Molecular Sieves 3A(Nikka Seiko K.K., Tokyo, Japan), followed by stirring for 3 hr to remove water. To the mixture were added 250 mg of DCC, and the reaction mixture was kept at 25°C for 2 days under stirring to complete the reaction. After mixing with 200 ml of chloroform, the mixture was filtrated to remove insoluble materials, N,N'-dicyclohexyl urea(DCU) or unmodified hemin, and the filtrate was completely evaporated. The residue was dissolved in 100 ml of water, in which unreacted DCC was hydrolyzed, and this was centrifugated to remove insoluble materials containing unmodified hemin. After the lyophilization, the residue was re-dissolved in 100 ml of chloroform. The PEG-hemin free from pyridine was obtained by centrifugation and evaporation, and was subjected to the experiments. The PEG-hemin preparation was found to have 0.8 carboxyl group per the molecule by titration. The milli-molar extinction coefficient of PEG-hemin was spectrophotometrically determined by measuring the absorbance at 408 nm after adding pyridine to a solution of hydrolyzed PEG-hemin. The milli-molar extinction coefficient of pyridine-hemichrome is 110(21).

Measurement of peroxidase activity. The peroxidase activity of PEG-hemin was determined in 1,1,1-trichloroethane. In the present study, we used o-phenylene diamine as the hydrogen donor, and hydrogen peroxide or peroxidized linolenic acid as the hydrogen acceptor. Peroxidized linolenic acid was obtained by autooxidation of linolenic acid, and its peroxide concentration was determined with potassium iodide and sodium thiosulfate by the method of titration(22). The concentration of hydrogen peroxide was also determined with titanium sulfate by the method of Pobinor(23). The concentration of o-phenylene diamine in oxidized form was spectrophotometrically determined by measuring the absorbance at 470 nm, assuming the molar extinction coefficient at 470 nm to be 2.9×10^3 $M^{-1}cm^{-1}$. To 2 ml of 34 mM o-phenylene diamine were added 0.15 ml of hydrogen peroxide (or peroxidized linolenic acid) and 0.03 ml of PEG-hemin solution, and the initial velocity was spectrophotometrically measured at 25°C. Each reagents were all dissolved in 1,1,1-trichloroethane, and the concentration of o-phenylene diamine was in excess. The rate constant, k_1, for the reaction between PEG-hemin and hydrogen acceptor was calculated by assuming that the reaction is first-order(24,25).

Results and Discussion

Chlorohemin, ferriprotoporphyrin IX, is practically insoluble in water and in organic solvents except for strong organic base such as trimethylamine and dimethylaniline. The polyethylene glycol-modified hemin, PEG-hemin, was readily soluble not only in aqueous solution but also

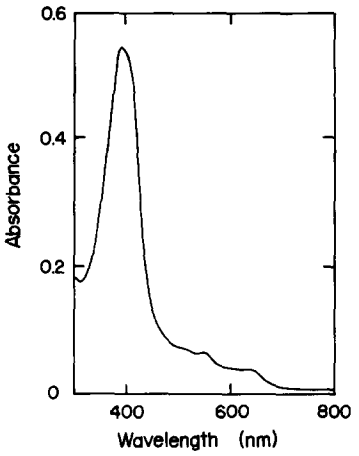

Fig. 1. Absorption spectrum of PEG-hemin in 1,1,1-trichloroethane. The concentration of PEG-hemin was 0.25 mg/ml.

in organic solvents, such as benzene, chloroform and 1,1,1-trichloroethane. This is due to the attachment of amphipathic polymer of monomethoxypolyethylene glycol to carboxyl groups in the hemin molecule. By the titration of the PEG-hemin solution, it was revealed that average one out of two carboxyl groups in the hemin molecule was coupled with the hydroxyl group of monomethoxypolyethylene glycol to form ester-bond with the carbodiimide. Fig. 1 represents the absorption spectrum of the PEG-hemin in 1,1,1-trichloroethane, and it shows a sharp band at 398 nm with ε_{mM}(milli-molar extinction coefficient) of 73. The similar sharp bands of PEG-hemin were also observed at 398 nm in chloroform and in aqueous solution, and the ε_{mM} values were 81 in chloroform and 51 in water. These indicate that PEG-hemin is soluble in many solvents without aggregation.

PEG-hemin catalyzed peroxidase-reactions shown below not only with hydrogen peroxide(Eq.1) but also with peroxidized linolenic acid, ROOH, (Eq. 2) as hydrogen acceptor in organic solvents such as 1,1,1-trichloroethane:

$$H_2O_2 + 2AH \longrightarrow 2H_2O + 2A \qquad \text{(Eq. 1)}$$

$$ROOH + 2AH \longrightarrow ROH + H_2O + 2A \qquad \text{(Eq. 2)}$$

Fig. 2 represents the initial velocity of peroxidase-reaction of o-phenylene diamine(AH, hydrogen donor) with hydrogen peroxide(hydrogen

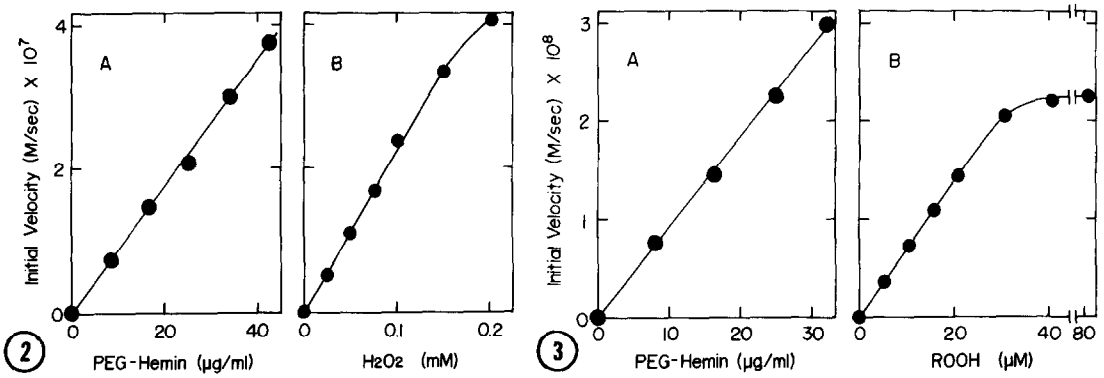

Fig. 2. The initial velocity of peroxidase-reaction with hydrogen peroxide catalyzed by PEG-hemin in 1,1,1-trichloroethane with varied concentrations of PEG-hemin (curve A) and hydrogen peroxide (curve B). Curve A; 34 mM o-phenylene diamine and 340 μM hydrogen peroxide were used. Curve B: 34 mM o-phenylene diamine and 25 μg/ml of PEG-hemin.

Fig. 3. The initial velocity of peroxidase-reaction with peroxidized linolenic acid catalyzed by PEG-hemin in 1,1,1-trichloroethane with varied concentrations of PEG-hemin (curve A) and peroxidized linolenic acid, ROOH(curve B). Curve A; 34 mM o-phenylene diamine and 190 μM peroxidized linolenic acid were used. B: 34 mM o-phenylene diamine and 25 μg/ml of PEG-hemin.

acceptor)(Eq. 1) catalyzed by PEG-hemin in 1,1,1-trichloroethane when the concentrations of PEG-hemin(curve A) and hydrogen peroxide(curve B) were changed. As seen in curve A, plotting of the velocity and the amount of PEG-hemin gave a straight line, indicating that the reaction proceeded catalytically with PEG-hemin in 1,1,1-trichloroethane. The velocity was enhanced gradually with increasing the concentration of hydrogen peroxide(curve B) up to 0.2 mM.

Using peroxidized linolenic acid instead of hydrogen peroxide as the hydrogen acceptor and o-phenylene diamine as the hydrogen donor, the peroxidase-reaction by PEG-hemin(Eq. 2) was investigated in 1,1,1-trichloroethane. The results were shown in Fig. 3. The initial velocity was increased linearly, accompanied by increasing the amount of PEG-hemin(curve A). This indicates that PEG-hemin also catalyzed the reaction with peroxidized linolenic acid, a hydrophobic peroxide, as much as hydrogen peroxide. As seen in curve B, the velocity enhanced with increasing the peroxidized linolenic acid and tended to reach a constant level up to 40 μM peroxidized linolenic acid in 1,1,1-trichloroethane.

Table 1. Rate Constant(k_1) with PEG-Hemin

Hydrogen Acceptor	Solvent	k_1 Value $M^{-1}sec^{-1}$
H_2O_2	TCE	2.3×10^3
ROOH	TCE	7.0×10^2
H_2O_2	PBS	3.0×10

ROOH is peroxidized linolenic acid. TCE and PBS are 1,1,1-trichloroethane and phosphate buffered saline(pH 7.0), respectively. o-Phenylene diamine(34 mM) was used as hydrogen donor.

Rate constants with hydrogen acceptors(k_1) were determined for the peroxidase-reaction by PEG-hemin in 1,1,1-trichloroethane and aqueous system(phosphate buffered saline, pH 7.0). The results were shown in Table 1. The k_1 values with hydrogen peroxide and peroxidized linolenic acid as the hydrogen acceptor in 1,1,1-trichloroethane were 2.3×10^3 M^{-1} sec^{-1} and 7.0×10^2 $M^{-1}sec^{-1}$, respectively, which were over ten folds greater than the k_1 value with hydrogen peroxide in aqueous system(3.0×10 $M^{-1}sec^{-1}$). It is intriguing that peroxidase-reaction catalyzed by PEG-hemin proceeds more efficiently in an organic solvent than in aqueous system.

This PEG-hemin exerts peroxidase-like activity also in other organic solvents such as benzene and chloroform, also using other hydrogen donor such as o-aminophenol, leucomalachite green and leucocrystal violet in the presence of peroxidized linolenic acid as the hydrogen acceptor. Therefore, the PEG-hemin is sure to be effective for quantitative microanalysis of peroxide which is hardly soluble in aqueous system.

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