Immunoassay Using a Metal-complex Compound as a Chemiluminescent Catalyst. IV. The Investigation of a Metal Porphine Complex as a Labeling Reagent

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Synopsis. Among the metal porphine and phthalocyanine complexes synthesized by the present authors, a iron(III) complex of 5,10,15,20-tetrakis(4-carboxyphenyl)-porphine was found to be most sensitive for the chemiluminescence reaction of luminol-H_2O_2; it was successfully used in the determination of albumin as a protein model, with a detection limit of about 130 pg.

A chemiluminescence complex catalyst immunoassay (CLCCIA), in which a synthesized iron(III) phthalocyanine complex compound, acting as a catalyst in the chemiluminescence(CL) reaction of 5-amino-2,3-dihydro-1,4-phthalazinedione(luminol) hydrogen peroxide(H_2O_2), was used as a labeling reagent, was previously described for practical use by the present authors.[1-3] This method has the advantages of being sensitive and of enabling one to select the desired chemical structure of the labeling reagent[2] since it is based on the use of the catalytic reaction of a synthesized metal-complex compound. In order to make this method very useful as an immunoassay, the preparation of a more sensitive complex compound than a iron(III) phthalocyanine complex compound as a labeling reagent is necessary.

It is well known that the natural substances having a porphine skeleton in their active centers, that is, peroxidase, microperoxidase, hemin, and hematin, act as sensitive catalysts for the CL reaction of luminol-H_2O_2. Several metal porphine complexes with a skeleton similar to those of the above natural substances were, therefore, synthesized by the present authors, and their catalytic activities were compared with each other; our studies included hematin, which had been previously reported[4] to show the highest catalytic activity among the above natural substances. Here, the catalytic activity was compared for a liberated catalyst and a catalyst-albumin conjugate obtained by the carbodiimide method.

Experimental

A metal 5,10,15,20-tetrakis(4-carboxyphenyl)porphine (TCPP) complex was prepared by synthesizing TCPP by Adler's procedure[5] and by reacting it with the desired metal salt under refluxing in an acetic-acid medium.[6] The labeling of human serum albumin(HSA) as a protein model with a metal-complex compound except for iron(III) 1,8,15,22-tetracarboxyphthalocyanine (TCP-Fe(III)) was carried out by the carbodiimide method used by Karube *et al.*[7] Here, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) or *N*-cyclohexyl-*N'*-(2-morpholinoethyl)carbodiimide-methyl *p*-toluenesulfonate(CMC) as a water-soluble carbodiimide was used and the column charged with TOYOPEARL HW-50 was used for the gel filtration. The labeling of TCP-Fe(III) was carried out as follows: A 5-cm^3 phosphate buffer solution(pH 7.2) containing 25 mg of HSA was added to the solution immediately after 5 mg of iron(III) 1,8,15,22-tetrakis (chlorocarbonyl)phthalocyanine had been dissolved in 1 cm^3 of dimethyl sulfoxide. The contents were made to react at room temperature for 1 h and then at 4 °C for 18 h. Thereafter, the contents were treated much as in the method used by Karube *et al.*

The catalytic activity was measured by diluting a sample solution with a borate buffer solution(pH 10.2) and by subjecting the diluted solution to the flow-injection analysis apparatus shown in Fig. 1. By the use of the peristaltic pumps (d) in Fig. 1, a 1.0×10^{-3} mol/dm^3 luminol solution (pH 10.2) (a), a 7.5×10^{-3} mol/dm^3 H_2O_2 solution(b), and a borate buffer solution (pH 10.2) (c) were delivered at the rate of 2.5 cm^3/min. A definite volume of the sample solution was taken by means of the sampling loop (200 mm^3) (f) and injected into the flow line by operating a six-way cock (e). Then the CL emitted was measured with a photon counter (Hamamatsu TV, C-1230, R-464) (i).

Results and Discussion

It has been established that the salts of Fe^{III}, Co^{II}, and Os^{VIII}, belonging to the eighth-group of the periodic table, and natural substances with a iron porphine skeleton, such as peroxidase, microperoxidase, hemoglobin, hematin, and hemin, show high catalytic activities for the CL reaction of luminol-H_2O_2. Several water-soluble porphine complexes of the eighth-group elements were synthesized, and their catalytic activities were examined. A useful labeling reagent for CLCCIA was required to satisfy the following conditions: 1) the labeling reagent should be soluble in an alkaline solution when the CL of luminol-H_2O_2 gave a high quantum yield, and 2) the labeling reagent should contain a functional group by which the labeling reagent could be covalently bonded to the desired constituent, such as protein, under mild conditions. From these points of view, the TCPP complex was selected as the labeling reagent in the pres-

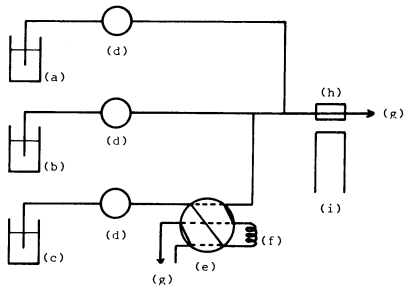

Fig. 1. A flow diagram of the flow-injection system for the measurement of catalytic activity.
a: Luminol, b: H_2O_2, c: buffer solution, d: pump, e: six-way cock, f: sampling loop, g: waste, h: flow-cell, and i: photon counter.

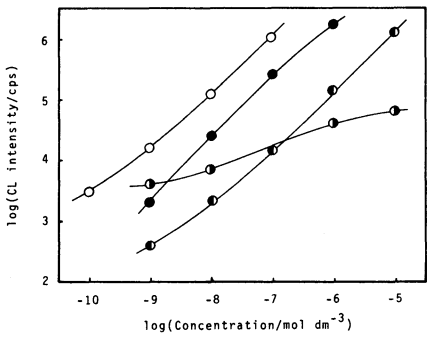

Fig. 2. The relationship between catalyst concentration and CL intensity.
○: TCPP-Fe(III), ◑: TCPP-Pd(II), ◐: TCPP-Pt(II), and ●: TCP-Fe(II).

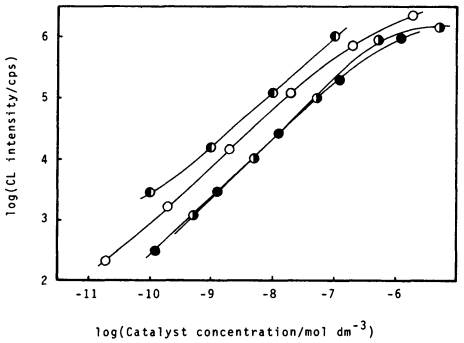

Fig. 3. The catalytic activities of labeled HSA on the basis of catalyst concentration.
Liberated TCPP-Fe(III) (◐), and HSA labeled with TCPP-Fe(III) (○), TCP-Fe(III) (●), and hematin (◑), respectively, by the use of CMC as a carbodiimide.

ent study. Though Co^{II} belonged to the eighth-group, its complexes[1] did not show very high catalytic activity. The catalytic activities of the TCPP complexes of Fe^{III}, Pt^{II}, and Pd^{II}, all belonging to the eighth-group, were examined (Fig. 2). The TCPP-Fe(III) was found to be most sensitive among them; its catalytic activity was higher than that of TCP-Fe(III),[1] which had been previously reported to be most sensitive, and that of hematin,[4] which had been reported to be the most sensitive among the natural-substance catalysts. The TCPP complexes of Mn^{III} and Sn^{IV} other than the eighth-group elements were also synthesized in order to examine their catalytic activities. The catalytic activity of TCPP-Mn(III) was about one-three hundredth that of TCPP-Fe(III), and TCPP-Sn(IV) showed a slight catalytic activity.

HSA as a model protein, was labeled with TCPP-Fe(III), TCP-Fe(III), and hematin, and the catalytic activities of the labeled HSA were examined. Each concentration of the HSA and the catalyst in the labeled HSA was determined by the use of the calibration curves of TCPP-Fe(III) at 414 nm and 280 nm, of TCP-Fe(III) at 637 nm and 280 nm, of hematin at 384 nm and 280 nm, and of HSA at 280 nm, on the assumption that the absorption of each constituent remained unchanged before and after the labeling. The catalytic activities of the labeled HSA are shown in relation to

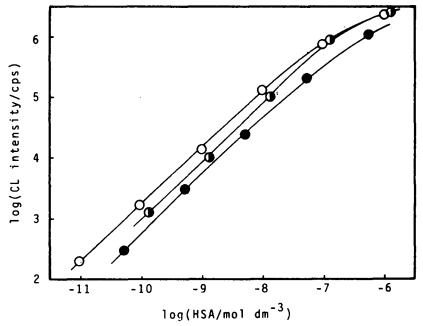

Fig. 4. The catalytic activities of labeled HSA.
HSA labeled with TCPP-Fe(III) (○), TCP-Fe(III) (●), and hematin (◑), respectively, by the use of CMC as a carbodiimide.

the catalyst concentration in Fig. 3. It was concluded from the absorbance measurement that the mole ratio of HSA to TCPP-Fe(III) was 1:3 or 1:2 for the HSA labeled with TCPP-Fe(III) by the use of EDC or CMC as a carbodiimide. The HSA labeled with TCPP-Fe(III) was not so sensitive as the value to be expected from the above mole ratio. As can be seen from Fig. 3, the catalytic activities of a liberated TCPP-Fe(III) and the TCPP-Fe(III) labeled onto HSA were not as similar as TCP-Fe(III) and hematin.

The catalytic activities of TCPP-Fe(III), TCP-Fe(III), and hematin, based on the HSA concentration, were also compared with each other (Fig. 4). The detection limit of the HSA labeled with TCPP-Fe(III) gave the lowest value among them, and a linear calibration curve was obtained at the concentration range of 9.4×10^{-12}—9.4×10^{-8} mol/dm^3-labeled HSA, with a detection limit of about 130 pg HSA. The HSA labeled with TCPP-Fe(III) was about 5.6 times as sensitive as the HSA labeled with TCP-Fe(III), since the detection limit of the latter was about 730 pg; it was also about twice as sensitive as the HSA labeled with hemin reported by Ikariyama *et al.*[8]

It was concluded from the above results that TCPP-Fe(III) was a more sensitive and more excellent labeling reagent for the determination of protein than those reported so far, though the catalytic activity of TCPP-Fe(III) was lowered by the labeling, unlike the case of the other chemiluminescence complex catalysts.

References

1) T. Hara, M. Toriyama, and K. Tsukagoshi, *Bull. Chem. Soc. Jpn.*, **56**, 2267 (1983).
2) T. Hara, M. Toriyama, and K. Tsukagoshi, *Bull. Chem. Soc. Jpn.*, **56**, 2965 (1983).
3) T. Hara, M. Toriyama, and K. Tsukagoshi, *Bull. Chem. Soc. Jpn.*, **57**, 587 (1984).
4) H. A. Neufeld, C. J. Conklin, and R. D. Towner, *Anal. Biochem.*, **12**, 303 (1965).
5) A. D. Adler, *J. Org. Chem.*, **32**, 476 (1967).
6) T. Osa, "Porphyrin no Kagaku," Kyoritsu Shuppan, Tokyo (1982), pp. 57—58.
7) I. Karube, T. Matsunaga, T. Satoh, and S. Suzuki, *Anal. Chim. Acta*, **156**, 283 (1984).
8) Y. Ikariyama, S. Suzuki, and M. Aizawa, *Anal. Chim. Acta*, **156**, 245 (1984).