Determination of a Small Amount of a Biological Constituent by Use of Chemiluminescence. IX. Effect of a Ligand on a Peak Shape

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Synopsis. The chemiluminescence intensity of a 1,10-phenanthroline-hydrogen peroxide system was systematically examined in the presence of various types of copper(II) chelate-forming ligands. There was a good relation between the peak shape on a recorder chart and the types of ligands; they could be classified into four groups as follows: A) Both bidentate and tridentate ligands gave positive and negative peaks. B) The ligand having more than four donor atoms, as in a quadridentate ligand or protein, gave a single negative peak. C) A liberated porphine ring gave a single sharp negative peak. D) Some metal organic compounds such as vitamine B-12 and ferritin gave broader positive and negative peaks in comparison with those in A). These unique peak shapes were applicable to the estimation of the type of ligand. α-Fetoprotein as a tumor marker could be also easily detected with a detection limit of 250 pg and with a typical coefficient of variation of 3.8%.

In order to establish a new analytical method by which several proteins can be sensitively and rapidly determined without labeling, the flow-injection method using the fact that the catalytic activity of copper(II) for the chemiluminescence (CL) reaction between 1,10-phenanthroline (phen) and hydrogen peroxide (H_2O_2) decreased in the presence of protein has been reported by the authors.[1)]

As pointed out in the previous paper, the peak shape of the CL intensity changed, depending upon the type of ligand. The relationship between the type of ligand (e.g. uni-, bi-, tri-, and quadri-dentate ligands) and the peak shape has been systematically investigated in the present paper.

Furthermore, such ligands as α-fetoprotein (AFP), ferritin, and polyamines which are well-known as cancer markers were also investigated.

Experimental

All chemicals used were reagent grade. A phen solution, a H_2O_2 solution, and a copper(II) catalyst solution were prepared as in the previous paper. The sample solution of AFP (human placental) from The Green Cross Co., and ferritin (TYPE IV, human liver) from Sigma Chemical Co., and polyamines (sperminetetrahydrochloride, spermidine, and putrescine), and all other samples were prepared by diluting them in a buffer solution (pH 10.2) consisting of 0.1 mol dm^{-3} boric acid and 0.1 mol dm^{-3} potassium hydroxide.

The experiment was carried out using the same apparatus as in the previous paper.

Results and Discussion

According to the previous paper, the injection of an α-amino acid solution into the copper(II)–tartrate catalyst solution flow showed a first positive peak and then a second negative peak based on the formation of a copper(II)–amino acid complex compound. Since this result appeared to have a close relation with the composition of a copper(II) complex compound, an investigation was systematically carried out for various ligands, and the results obtained are shown in Table 1.

The effect of an unidentate ligand such as ammonia and pyridine on a peak shape was small. Neither positive nor negative peaks were observed until the mole ratio of ligand to copper(II) ([ligand]/[copper(II)]=r) exceeded 10^5.

Both positive and negative peaks were observed for a bidentate or a tridentate ligand such as in Group A. This was explained on the assumption that the positive peak corresponding to the positive catalytic activity was obtained first due to the formation of a

Table 1. Observed Peak Shape for Various Ligands

Group	Ligand	Donor atoms	Positive peak	Negative peak
A	α-Amiao acid[a)]	2-(5)	○	○
	2,2′-Bipyridine	2	○	○
	Ethylenediamine	2	○[d)]	○
	Diethylenetriamine	3	○[e)]	○
	Peptide[b)]	3-(6)	○[f)]	○
	Spermine	4	○	○
	Spermidine	3	○	○
B	Triethylenetetramine	4	×	○
	Tetraethylenepentamine	5	×	○
	EDTA	6	×	○
	Diethylenetriamine-pentaacetic acid	8	×	○
	Protein[c)]		×	○
	α-Fetoprotein		×	○
C	TCPP	4	×	○
	TPPS	4	×	○
	Hematoporphyrin	4	×	○
D	Ferritin		○	○
	Vitamine B-12		○	○

a) L-Aspartic acid, L-glutamic acid, L-glycine, L-alanine, L-proline, L-lysine hydrochloride, L-arginine hydrochloride, and L-histisine hydrochloride. b) glycylglycine, glycyl-L-phenylalanine, glycyl-L-proline, and glycylglycylglycine. c) bovine serum albumin, bovine serum γ-globulin, human serum albumin, human serum γ-globulin, and ovalbumin. d) $r<10^6$. e) $r<10^3$. f) $r<10^6$.

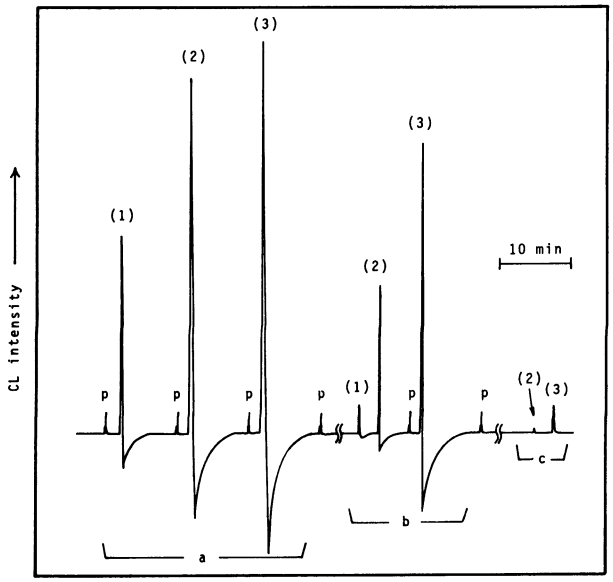

Fig. 1. Observed signals of polyamines.
a: Spermine, b: spermidine, c: putrescine, p: periodical shock peak accompanying with a reciprocation of the pump piston, (1): 1.0×10^{-6} mol dm^{-3}, (2): 1.0×10^{-5} mol dm^{-3}, and (3): 1.0×10^{-4} mol dm^{-3}.
Conditions: 5.0×10^{-5} mol dm^{-3} phen(4.0×10^{-3} mol dm^{-3} ethylhexadecyldimethylammonium bromide (EHDAB), 2.0×10^{-7} mol dm^{-3} tetraethylenepentamine (TEPA), 1.0×10^{-1} mol dm^{-3} sodium hydroxide(NaOH)), 5% H_2O_2, and 1.0×10^{-7} mol dm^{-3} copper(II) (5.0×10^{-5} mol dm^{-3} L-arginine hydrochloride(Arg)).

1:1 copper(II)-ligand complex compound, followed by the negative peak corresponding to the inactivation of the catalyst due to the complete occupation of the four coordination sites of copper(II) by the ligand. These results are consistent with the fact that the negative peak area increased with an increase of the *r* value.

A ligand which had more than four donor atoms, as in a quadridentate ligand, strongly linked with all four coordination sites in a planar square by way of a chelate effect, in which case a negative peak alone was obtained (Group B).

In addition, such liberated porphine ring as 5,10,15,20-tetrakis(4-carboxyphenyl)porphine (TCPP), 5,10,15,20-tetraphenylporphinesulfonic acid (TPPS) (Dojindo Laboratories.), and hematoporphyrin (Porphyrin Products.) (Group C) showed sharp negative peaks which were different from the peak shape of Group B and proteins. These results suggest that the center of the porphin molecule was rapidly occupied by copper(II), and that the catalytic activity of copper(II) was lowered rapidly.

The present method was further applied to other compounds which were of importance in clinical diagnostics as a tumor marker. The peaks obtained for polyamines such as sperminetetrahydrochloride, spermidine, and putrescine are shown in Fig. 1. Considerably large positive and negative peaks were obtained for both spermine and spermidine while a small positive peak alone was obtained for putrescine.

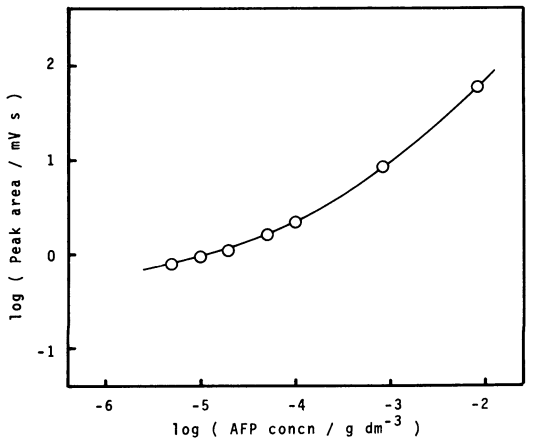

Fig. 2. Calibration curve of AFP.
Conditions: 5.0×10^{-5} mol dm^{-3} phen(4.0×10^{-3} mol dm^{-3} EHDAB, 2.0×10^{-7} mol dm^{-3} TEPA, 1.0×10^{-1} mol dm^{-3} NaOH), 5% H_2O_2, and 1.0×10^{-7} mol dm^{-3} copper(II) (5.0×10^{-5} mol dm^{-3} Arg).

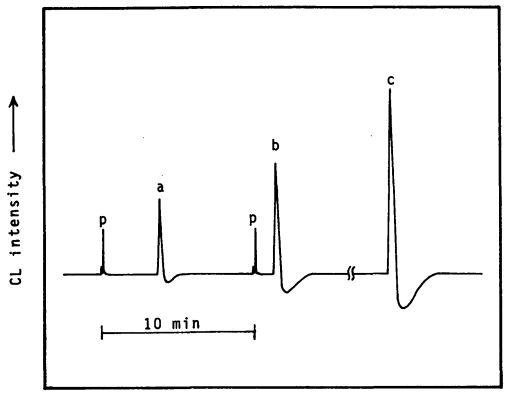

Fig. 3. Observed signals of ferritin.
a: 2×10^{-5} g dm^{-3}, b: 1×10^{-4} g dm^{-3}, c: 4×10^{-4} g dm^{-3}, and p: periodical shock peak accompanying with a reciprocation of the pump piston.
Conditions: 5.0×10^{-5} mol dm^{-3} phen(4.0×10^{-3} mol dm^{-3} EHDAB, 2.0×10^{-7} mol dm^{-3} TEPA, 1.0×10^{-1} mol dm^{-3} NaOH), 5% H_2O_2, and 1.0×10^{-7} mol dm^{-3} copper(II) (5.0×10^{-5} mol dm^{-3} Arg).

This was reasonably understood by considering the following complex formations: two six-membered chelate rings for spermine; one six-membered chelate ring for spermidine; and one single seven-membered chelate ring for putrescine.

The calibration curve of AFP which is well-known as a tumor marker is shown in Fig. 2. The AFP as a glycoprotein also formed a stable complex compound with copper(II) and showed a single negative peak. Using the present detector, the detection limit of AFP was 250 pg with the coefficient of variation of 3.8% for five measurements of 1×10^{-4} g dm^{-3} AFP. This sensitivity is satisfactory for practical use from the standpoint that the criterion of hepatoma is approximately 20 ng.

The peaks of ferritin which are also utilized as a marker for lung cancer and another malignant tumor including a blood disease are also shown in Fig. 3.

Judging from the molecular structure containing iron(III) at the central position, the positive peak was assumed to be due to the existence of iron(III) as a CL catalyst, while the negative peak to be due to the existence of protein. Vitamine B-12 containing a cobalt(II)–porphine complex structure was experimentally confirmed to act as a catalyst similarly to ferritin. The detection limit of ferritin corresponding to the negative peak was 200 pg, while the diagnostic significance of ferritin is regarded as "abnormaly large value" for more than 25 ng.

To sum up, there were good relations between the peak shape and the type of ligands that formed stable complex compounds with copper(II). The peak shapes were available for the estimation of the type of a ligand. According to the present study, several cancer markers are also sensitively determinable though their peak shapes differ from one another.

Reference

1) T. Hara, T. Ebuchi, A. Arai, and M. Imaki, *Bull. Chem. Soc. Jpn.*, **59**, 1833 (1986).