The Determination of a Small Amount of a Biological Constituent by the Use of Chemiluminescence. X. The Determination of Protein Using a 1,10-Phenanthroline-Hydrogen Peroxide-Ruthenium(III) System

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Synopsis. The catalytic activity of ruthenium(III) chloride in a chemiluminescence reaction with 1,10-phenanthroline-hydrogen peroxide has been found to decrease in the presence of protein. On the basis of this phenomenon, bovine serum albumin as a model protein was determined within the concentration range of 5×10^{-5}—1×10^{-1} g dm^{-3} and a detection limit of about 1 ng.

Several new methods for the determination of protein by the use of a chemiluminescence(CL) reaction between 5-amino-2,3-dihydro-1,4-phthalazinedione or 1,10-phenanthroline(phen) and hydrogen peroxide(H_2O_2) have been reported by the present authors in previous papers.[1-4] The methods are based on the measurement of the decreasing catalytic activity of copper(II), the iron(III) complex, or the cobalt(III) complex for a CL reaction in the presence of protein. According to Suzuki et al.,[5,6] CL emission from phen in the presence of H_2O_2 was markedly enhanced by a copper(II) catalyst, but not another catalyst, such as Cd(II), Co(II), Hg(II), Zn(II), Pb(II), or Ni(II). They used this phenomenon in determining a small amount of copper(II). It has now been found by the present authors that the ruthenium(III) chloride acts as a catalyst for a CL reaction between phen and H_2O_2 and that, moreover, its catalytic activity decreases in the presence of protein. As bovine serum albumin(BSA), human serum albumin(HSA), bovine serum α-globulin, and human serum γ-globulin show similar CL responses, BSA was selected as the model protein for their determination. A new procedure for the determination of protein has been established on the basis of the above-mentioned phenomenon. The present method is 10^2—10^3 times as sensitive as the conventional method[7] for the determination of protein and is no less sensitive than the most sensitive other method[4] in the present series of reports by the present authors.

Experimental

All the reagents were of a commercially available special grade. The ion-exchange water was distilled for use. A phen solution, a H_2O_2 solution, and a buffer solution were prepared as in the previous paper.[8] A 1.0×10^{-3} mol dm^{-3} ruthenium(III) catalyst solution was prepared as a stock solution by dissolving ruthenium(III) chloride hydrate(NAKARAI CHEMICAL, LTD.) in 0.36 N HCl; it was then diluted to the desired concentration with a buffer solution. BSA and HSA from NAKARAI CHEMICAL, LTD., bovine serum α-globulin from ICN Pharmaceuticals, Inc., and human serum γ-globulin from the SIGMA CHEMICAL COMPANY were prepared by diluting them with the above-mentioned buffer solution.

The experiment was carried out using the apparatus described in a previous paper.[8] The optimum flow rates and the optimum distance between the point where a phen-H_2O_2 mixture solution and a buffer solution were mixed and a certain cell were decided by the experiment. A definite volume of the ruthenium(III) solution was added to a sample solution containing protein, and the resulting solution was heated for a definite period. After the solution had then cooled to room temperature, an aliquot of the solution (50 mm^3) was injected into the flow-through system and the CL intensity was measured.

Results and Discussion

The CL intensity of the 1.0×10^{-6} mol dm^{-3} ruthenium(III) catalyst solution was measured by the use of 3-, 90-, 180-, and 270-cm portions of the tube, portions which corresponded to the distance between the point where a phen-H_2O_2 mixture solution and a buffer solution were mixed and a certain cell. The maximum CL intensity was observed by the use of a 90-cm tube. Both the flow rate of a phen solution and that of a H_2O_2 solution were chosen simultaneously at the following rates: 0.5, 0.7, 1.0, and 1.3 ml min^{-1}; the CL intensity of a 1.0×10^{-6} mol dm^{-3} ruthenium(III) catalyst solution was also measured. The maximum CL intensity was observed at 0.7 ml min^{-1}. The flow rate of a buffer solution was always set at 2.3 ml min^{-1}, the maximum capacity of the pump, so as to deal with as many samples as possible in a definite time. On the basis of the experimental results, the optimum conditions were established as follows: tube length: 90 cm; flow rates of a phen, a H_2O_2, and a buffer solution: 0.7, 0.7, and 2.3 ml min^{-1} respectively.

The calibration curve for the ruthenium(III) catalyst solution is shown in Fig. 1; it was almost linear for 1.0×10^{-7}—1.0×10^{-5} mol dm^{-3} ruthenium(III).

The relationships between the CL intensity and the reaction time at 20, 60, and 95 °C are shown in Fig. 2. On the basis of the results pertaining to the decrease in the CL intensity and the reaction time, the CL intensity of a protein sample solution was examined after it had been treated for 45 min at 95 °C in all the following experiments.

The CL responses of a sample solution containing 3×10^{-3} g dm^{-3} each of BSA, HSA, bovine serum α-globulin, and human serum γ-globulin were examined (Table 1). As they showed similar responses, BSA was selected as the model protein in the following experiment.

Figure 3 shows the calibration curve for BSA. As

Table 1. The CL Responses of Various Proteins

Protein	Experimental CL intensity/kcps	Relative CL intensity
(Protein-free)	3.44	1.00
BSA	1.68	0.49
HSA	1.80	0.52
Bovine serum α-globulin	1.84	0.53
Human serum γ-globulin	1.74	0.51

a) Conditions: 1.2×10^{-4} mol dm^{-3} phen, 5.0 wt% H_2O_2, 3.0×10^{-6} mol dm^{-3} Ru(III), and 3.0×10^{-3} g dm^{-3} protein.

Table 2. The Apparent Coupling Ratio and Coupling Constant When a Ru(III) or Cu(II) Catalyst is Used

Catalyst	Detection limit of BSA/ng	Catalyst concn used for BSA determination/mol dm^{-3}	Coupling ratio (Metal:Protein)	Coupling constant
Ru(III)	1	3.0×10^{-6}	6.0 : 1	3.5×10^{35} (mol^{-1} dm^3)$^{6.0}$
Cu(II)	5[a)]	5.0×10^{-8}	2.3 : 1	1.9×10^{18} (mol^{-1} dm^3)$^{2.3}$

Conditions: 6.1×10^{-7} mol dm^{-3} BSA against 2.0×10^{-6}—5.0×10^{-6} mol dm^{-3} Ru(III), 6.1×10^{-8} mol dm^{-3} BSA against 7.5×10^{-8}—1.5×10^{-7} mol dm^{-3} Cu(II), ionic strength $\fallingdotseq$0.1 mol dm^{-3}, and reaction temperature=95 °C. The molecular weight of BSA was regarded as being 66000 for purposes of calculation. a) Estimated using the apparatus described in a previous paper.[8)]

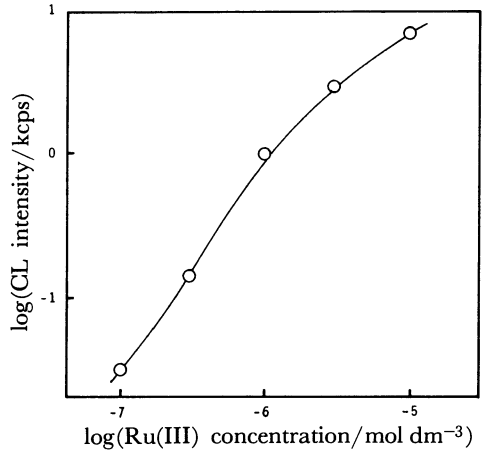

Fig. 1. Calibration curve of Ru(III) catalyst solution. Conditions: 1.2×10^{-4} mol dm^{-3} phen and 5.0 wt% H_2O_2.

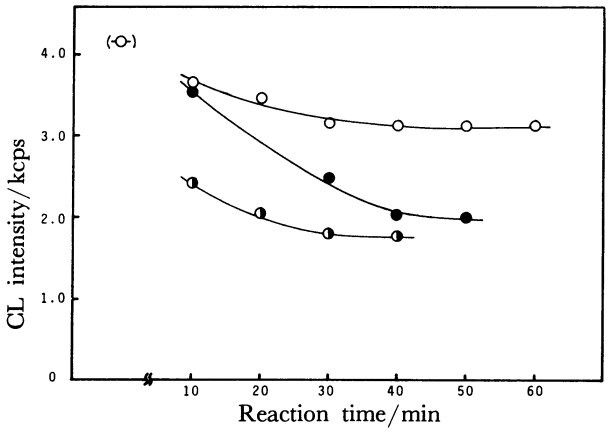

Fig. 2. Relationship between CL intensity and reaction time. Reaction temperature O: 20 °C, ●: 60 °C, and ◑: 95 °C. (-O-): BSA free. Conditions: 1.2×10^{-4} mol dm^{-3} phen, 5.0 wt% H_2O_2, 3.0×10^{-6} mol dm^{-3} Ru(III), and 5.0×10^{-3} mol dm^{-3} BSA.

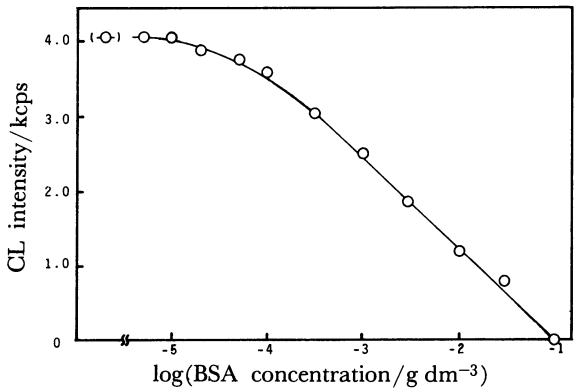

Fig. 3. Calibration curve of BSA. (-O-): BSA free. Conditions: 1.2×10^{-4} mol dm^{-3} phen, 5.0 wt% H_2O_2, and 3.0×10^{-6} mol dm^{-3} Ru(III).

can be seen from Fig. 3, BSA in the concentration range of 5×10^{-5}—1×10^{-1} g dm^{-3} could be determined within a detection limit of about 1 ng. The coefficients of variation for 10 analyses of 1×10^{-3} g dm^{-3} BSA and protein-free sample solutions by the present method were 1.27 and 2.03% respectively. The lowering of the CL activity of the ruthenium(III) catalyst solution in the presence of 5.0×10^{-3} g dm^{-3} glycylglycine and 5.0×10^{-3} g dm^{-3} glycylglycylglycine was examined; their relative CL intensities were 0.88 and 0.79 respectively. The lowering of the CL activity of the ruthenium(III) catalyst solution in the presence of protein seems to be caused by the formation of a ruthenium-polypeptide linkage. The results suggest that the present method can be applied to the determination of biological constituents containing a peptide linkage, such as albumin and globulin as well as an enzyme.

The apparent ratios of ruthenium(III) to a protein and the coupling constant of a ruthenium(III)-protein complex were estimated in a way similar to that de-

scribed in a previous paper.[8] These value were then compared with those of a copper(II)-protein complex in the previous paper;[8] they are shown in Table 2, together with the detection limit of BSA. The apparent coupling ratio and coupling constant of a ruthenium(III) complex were larger than those of a copper(II) complex. It was difficult to compare these values exactly because they were obtained under quite different conditions. However, the data suggest that BSA can be estimated with a detection limit of about 1 ng even though a ruthenium(III) catalyst with a concentration 60 times as high as that of a copper(II) catalyst is used. The reaction mechanism between the metal and the protein still remains unknown.

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