The Determination of a Small Amount of a Biological Constituent by the Use of Chemiluminescence. XVII. Experimental Consideration on the Chemiluminescence Detection of Protein Using the 1,10-Phenanthroline-Hydrogen Peroxide-Copper(II) System

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Synopsis. With an objective to clarify the relationship between chemiluminescence intensity and the reaction time in the flow injection analysis of a small amount of protein using a 1, 10-phenanthroline-hydrogen peroxide-copper(II) chemiluminescence system, an apparatus was newly set up for continuously measuring the chemiluminescence intensity. With this apparatus a lowering of the catalytic activity of copper(II) was observed in the presence of α-amino acids, polyamines, polypeptides, and proteins. In addition, the effect of heating on the reaction between copper(II) and protein was examined, and the reaction rate was found to markedly increase. Heating was therefore indispensable for the rapid and sensitive detection of proteins.

The method for protein determination established by the authors[1-3] is based on lowering the catalytic activity of a metal ion or metal complex in the presence of some protein. According to flow injection analysis (FIA) using a 1,10-phenanthroline(phen)-hydrogen peroxide (H_2O_2)-copper(II) (Cu(II)) system, various proteins could be detected with a detection limit of about 250 pg; its sensitivity was about 10^2—10^6 times those of conventional methods, such as colorimetry and fluorometry.[4-6] Though these satisfactory results were experimentally obtained by an optimization of the FIA conditions, the relationship between the chemiluminescence (CL) intensity and the reaction time were not clear. With an objective to clarify it, an apparatus was newly set up in the present study for a continuous measurement of the CL intensity against the reaction time.

According to the obtained results, the catalytic activity of Cu(II) was inhibited more or less in the presence of α-amino acids, polyamines, polypeptides, and proteins. The reaction rate between Cu(II) and protein were also found to markedly increase by heating a sample solution. Moreover, the results obtained in a previous study[7] showed that the apparent coupling ratio between Cu(II) and protein increased and the apparent coupling constant of a Cu(II)-protein complex increased drastically upon heating a protein solution. On the basis of these results, heating was found to be indispensable for a rapid and sensitive detection of protein.

Experimental

Reagents. All of the reagents used were of a commercially available special grade. Ion-exchanged water was distilled for use. A 5×10^{-5} mol dm^{-3} phen solution containing ethylhexadecyldimethylammonium bromide (EHDAB) in 4×10^{-3} mol dm^{-3}, tetraethylenepentamine (TEPA) in 5×10^{-7} mol dm^{-3} and sodium hydroxide (NaOH) in 1×10^{-1} mol dm^{-3} were prepared. A 5 wt% H_2O_2 solution was prepared by diluting a 30 wt% H_2O_2 solution with distilled water. A 1×10^{-7} mol dm^{-3} Cu(II) solution was prepared by diluting a 1×10^{-2} mol dm^{-3} Cu(II) stock solution with a mixed solution containing NaOH in 1×10^{-1} mol dm^{-3} and sodium chloride in 1×10^{-1} mol dm^{-3}. All α-amino acids from Kyowa Hakko Co., Ltd., glycylglycine (Gly-Gly), and glycylglycylglycine (Gly-Gly-Gly) from Peptide Institute, Inc., polyamines (spermine-tetrahydrochloride, spermidine-trihydrochloride, and putrescine-dihydrochloride), and bovine serum albumin (BSA) from Nakarai Tesque, Inc., human serum γ-globulin (HγG), and ovalbumin (Ova) from Sigma Chemical Co., and human serum albumin (HSA) from Miles Labolatories, Inc. were dissolved in water and used.

Apparatus and Procedure. All measurements were carried out in accordance with the following two operations:

1) Ordinary Operation. A schematic diagram of the apparatus used for the present study is shown in Fig. 1. All of the tubes and connectors used were made of Teflon. A Cu(II) solution (a) was delivered at the flow rate of 1.6 cm^3 min^{-1} with the pump P_1 (PERISTAMINI PUMP SJ-1211), and a H_2O_2 solution(b) and a phen solution(c) were delivered at each flow rate of 0.8 cm^3 min^{-1} with the pump P_2 (Tokyo Rikakikai Co., Ltd., MICRO TUBE PUMP). Both solutions fed with P_1 and P_2 were completely mixed through the mixer (h). A poly(vinyl chloride) tube (0.8 mm i.d. ×15 cm) was spirally wound and then used as the cell (i). The CL intensity of the solution passing through the cell was measured by a photon counter (j) (Hamamatsu TV Co. Ltd., C1230) and recorded on a recorder (k) (Yokogawa Electric Works, Ltd., 3056). For ordinary operation, each definite volume of an aqueous sample solution of an α-amino acid, a polyamine, a polypeptide, and an intact or preheated protein

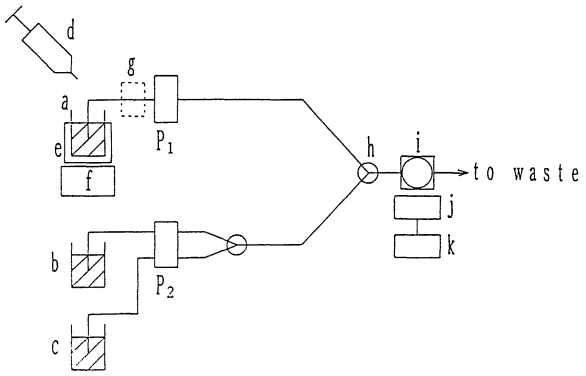

Fig. 1. Schematic diagram of the CL detector system. a: copper(II) solution, b: H_2O_2 solution, c: phen solution, d: syringe, e: heating bath, f: stirrer, g: cooling bath, h: mixer, i: flow cell, j: photon counter, k: recorder, and P_1, P_2: pump.

was injected into (a) with a syringe (d) immediately after the blank CL intensity had stabilized; the resulting solution was sufficiently stirred with a stirrer (f) (Tokyo seikakusha, Model AS-2) during the feed, followed by a measurement of the CL intensity.

2) The Operation Accompanying with Heating. The copper(II) solution (a) was heated at the desired temperature in a heating bath (e) (Yamato WATER BATH MODEL BM-41) and a definite amount of sample solution was added immediately after the blank CL intensity had stabilized. The resulting solution was sufficiently stirred with a stirrer during the feed, followed by a measurement of the CL intensity. Here, the Teflon tube (1.0 mm i.d. ×39.5 cm) leading to P_1 was cooled at room temperature in a cooling bath (g).

Results and Discussion

Generally speaking, the measurement involving the CL reaction is not always satisfactory regarding reproducibility. Therefore, the blank values obtained by different measurements in the present experiment were corrected to become the same value.

Various concentrations of Cu(II) could be determined with certainty using the present apparatus, and there was a reasonable correlation between the Cu(II) concentration and the CL intensity. A 1×10^{-7} mol dm^{-3} of Cu(II) solution was chosen as the optimum concentration and was used in the following experiment.

Then, the effect of various ligands, such as α-amino acids, polyamines, polypeptides, and proteins, on the CL intensity was investigated. First of all, the relationship between the logarithmic stability constant of Cu(II)-α-amino acid complex compounds and the relative CL intensity was examined; the relative CL intensity was found to decrease with an increasing value of the stability constant. This result is well understood by the fact that the catalytic activity of Cu(II) is more or less lowered by occupying all of the coordination sites of Cu(II) with ligands.[8]

The effect of polyamines on the CL intensity was as follows: putrescine did not influence the CL intensity, since it formed no stable chelate ring with Cu(II); spermidine and spermine, however, showed a lowering of the CL intensity, since spermidine formed a six-membered chelate ring and spermine formed two six-membered chelate rings with Cu(II). This lowering of the CL intensity could easily be understood by taking the chelate effect into consideration.

The effect of Gly-Gly and Gly-Gly-Gly containing a peptide linkage on the CL intensity is shown in Table 1, together with that of glycine (Gly), on the basis of the relative CL intensity (1.00) in the absence of a peptide. As can be seen from Table 1, the relative CL intensity decreased with an increasing number of peptide linkages. These values of the relative CL intensity were found to be reasonable by considering the

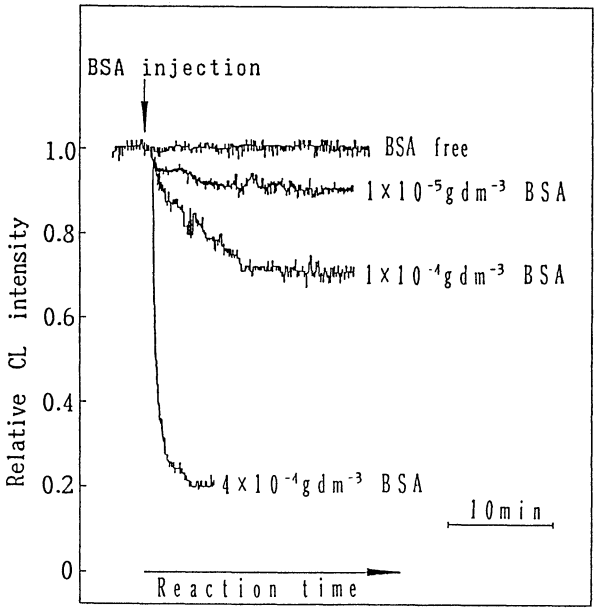

Fig. 2. The effect of BSA concentration on the CL intensity at 16 °C.

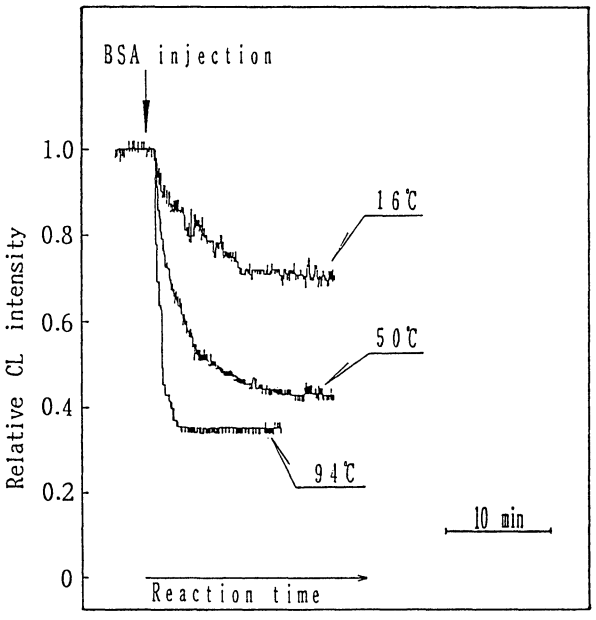

Fig. 3. The effect of heating on the reaction rate between Cu(II) and BSA. BSA concn=1×10^{-4} g dm^{-3}.

Table 1. The Effect of Peptides on the CL Intensity

Sample	Chemical formula	Relative CL intensity
Peptide free	—	1.00
Gly	H_2NCH_2COOH	0.95
Gly-Gly	$H_2NCH_2CONHCH_2COOH$	0.83
Gly-Gly-Gly	$H_2NCH_2CONHCH_2CONHCH_2COOH$	0.39

Concn of peptides and Gly=1.0×10^{-4} mol dm^{-3}.

possibility that Gly-Gly gives two five-membered chelate rings and Gly-Gly-Gly gives three five-membered chelate rings.

The effect of BSA concentration on the CL intensity is further shown in Fig. 2. As can be seen from Fig. 2, the CL intensity decreased with an increasing concentration of BSA, but the mechanism for the bond formation between Cu(II) and BSA still remained undissolved.

The effect of several proteins (such as BSA, HSA, HγG, and Ova) on the CL intensity showed no marked difference among the above-mentioned proteins.

The effect of heating on the detection of proteins was investigated using intact or preheated BSA. The effect of heating on the reaction rate between Cu(II) and intact protein(BSA) is shown in Fig. 3. As can be seen, both the apparent reaction rate presented by the slope at an arbitrary point on each curve and the lowering of the relative CL intensity from the blank CL intensity increased with an increase in the reaction temperature. The final relative CL intensity at 94 °C, which is parallel to the abscissa, showed good agreement with that obtained from BSA, which had been left standing for 18 hr at pH 13 and at about 16 °C. Moreover the authors reported in a previous paper that the apparent coupling ratio between Cu(II) and protein increased and that the apparent coupling constant of a Cu(II)-protein complex also increased drastically upon heating a protein solution.

These results show that the denaturation of protein proceeds by heating much more rapidly than the rise of pH, and that denatured protein rapidly combines with Cu(II). The effect of heating on the reaction rate between Cu(II) and α-amino acids, such as L-aspartic acids, glycine, and L-glutamine, was examined; no phenomenon similar to that mentioned above was observed. The relationship between the relative CL intensity and the reaction time for preheated BSA, which was obtained by heating a BSA solution alone, was further examined at 16 °C. Since the reaction rate between Cu(II) and denatured protein was larger in the presence of Cu(II) than in the absence of Cu(II), the heating of intact BSA at 94 °C in the presence of Cu(II) was more preferable to the heating of BSA alone in the absence of Cu(II) at 94 °C for a rapid and sensitive detection of BSA. Though the reaction rate, the apparent coupling constant, and the apparent coupling ratio between Cu(II) and protein should be sufficiently large for a sensitive detection of protein by FIA, these requirements could be satisfied with a heat treatment of protein. However, the reaction mechanism between Cu(II) and protein still remains unknown.

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