Determination of a Small Amount of a Biological Constituent by Use of Chemiluminescence. VIII. Effect of Heating on the Determination of Protein

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Synopsis. The effect of heating on the determination of protein by chemiluminescence was examined by estimating the apparent coupling constant of a copper(II)-protein complex after treatment of a solution at 25 °C or 95 °C and by comparing the values with each other. The values obtained for two proteins were as follows: 1.3×10^{8} mol^{-1} dm^3—2.1×10^{11} (mol^{-1} dm^3)$^{1.5}$ at 25 °C and 4.3×10^{17} (mol^{-1} dm^3)$^{2.3}$—1.9×10^{18} (mol^{-1} dm^3)$^{2.2}$ at 95 °C. These values suggest that heating makes a copper(II)-protein complex more stable and improves the concentration range for the determination of protein.

New methods for the determination of protein by chemiluminescence (CL) reaction between 5-amino-2,3-dihydro-1,4-phthalazinedione(luminol) or 1,10-phenanthroline(phen) and hydrogen peroxide (H_2O_2) were reported by the authors in the previous papers.[1,2] The determination is based on the measurement of the decreasing catalytic activity of copper(II) (Cu(II)) for CL reaction in the presence of protein. Both the concentration range of determinable protein and the detection limit were improved by heating a Cu(II)-protein complex sample solution.[1] This seemed to be attributed to the formation of a more stable Cu(II)-protein complex by heating. However, no attempt to confirm this has been carried out in detail. The present study deals with the estimation of an apparent ratio of Cu(II) to protein and a coupling constant of a Cu(II)-protein complex after treatment of a sample solution at 25 °C or 95 °C. The values obtained were compared and discussed.

Experimental

A phen solution, a H_2O_2 solution, a Cu(II) catalyst solution, and a buffer solution were prepared as in the previous paper.[2] The 1.2×10^{-4} mol dm^{-3} phen solution contained 4.0×10^{-3} mol dm^{-3} ethylhexadecyldimethylammonium bromide, 2.0×10^{-7} mol dm^{-3} tetraethylenepentamine, and 1.0×10^{-1} mol dm^{-3} sodium hydroxide. A 5.0 wt% H_2O_2 solution was prepared by diluting 30 wt% H_2O_2 with water. A Cu(II) catalyst solution was prepared by diluting a 2.0×10^{-2} mol dm^{-3} Cu(II) stock solution with a buffer solution (pH 10.2) consisting of 1.0×10^{-1} mol dm^{-3} boric acid and 1.0×10^{-1} mol dm^{-3} potassium hydroxide. This solution also contained L-aspartic acid (its concentration is 1.2×10^{3} times of Cu(II)) instead of tartrate in a biuret reagent.

All reagents were of commercially available special grade. Ion-exchange water was distilled before use. Bovine serum albumin (BSA) and human serum albumin (HSA) from NAKARAI CHEMICAL, LTD were prepared by diluting them with a buffer solution. A definite volume of Cu(II) solution was added to a sample solution containing protein and the resulting solution was heated for a definite period.

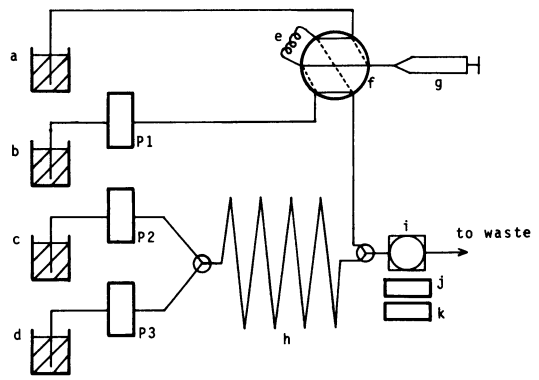

Fig. 1. Flow-injection system.
a: Sample solution, b: buffer solution, c: hydrogen peroxide solution, d: 1,10-phenanthroline solution, e: sampling loop, f: six-way cock, g: sylinge, h: mixing coil, i: flow cell, j: photon counter, k: recorder, and P1, P2, P3: pump.

After the solution was cooled to room temperature, an aliquot of the solution was injected into the flow-through system.

The schematic diagram of the apparatus which was set up for flow-injection analysis is shown in Fig. 1. All the tubes and connectors used were made of Teflon. A buffer solution(b), a H_2O_2 solution(c), and a phen solution (d) were delivered at the flow rate of 1.6 cm^3 min^{-1} with the corresponding pumps: (p_1), (p_2), (p_3)-(PERISTA MINI PUMP SJ-1211). A phen solution and a H_2O_2 solution were first mixed using a mixing coil(h) (346 cm, 0.5 mm i.d.) and the resulting mixture was combined with a buffer solution. A definite amount (50 mm^3) of the sample solution was injected into the line of a buffer solution. A poly(vinyl chloride) tube (45 cm, 0.8 mm i.d.) was spirally wound and was then used as the cell(i). The CL intensity of the solution passing through the cell was measured by a photon counter-(j) (HAMAMATSU, C1230) and recorded on a recorder(k) (National. PEN RECORDER). The net CL intensity of a sample solution was obtained by subtracting the background (the CL intensity of the solution containing no sample) from the intensity obtained.

Results and Discussion

After a Cu(II) catalyst solution was held at 25 °C or 95 °C for a definite period and then cooled at room temperature, the catalytic activity of Cu(II) for CL was measured. The calibration curve thus obtained was linear for 1×10^{-9}—1×10^{-7} mol dm^{-3} Cu(II) (Fig. 2).

The relationship between reaction time and the CL intensity in the presence of 1.0×10^{-7} mol dm^{-3} Cu(II) and BSA as a model protein was examined at 25 °C and 95 °C, respectively. The reaction between Cu(II)

Table 1. The Concentration of Species at Equilibrium for BSA at 25 °C

Initial Cu(II) concd mol dm^{-3}	CL intensity kcps	Free Cu(II) concn[a] mol dm^{-3}	Bound Cu(II) concd mol dm^{-3}
7.50×10^{-8}	4.83	2.70×10^{-8}	4.80×10^{-8}
8.00×10^{-8}	5.55	3.10×10^{-8}	4.90×10^{-8}
1.00×10^{-7}	8.70	4.80×10^{-8}	5.20×10^{-8}
1.25×10^{-7}	12.40	6.90×10^{-8}	5.60×10^{-8}

Initial BSA concd $=6.1\times10^{-8}$ mol dm^{-3}. a) Estimated from Fig. 2.

Table 2. An Apparent Coupling Ratio and Coupling Constant at 25 and 95 °C

Protein	25 °C		95 °C	
	Coupling ratio (Cu(II) : Protein)	Coupling constant	Coupling ratio (Cu(II) : Protein)	Coupling constant
BSA	1 : 1	1.3×10^{8} mol^{-1} dm^{3}	2.3 : 1	1.9×10^{18} (mol^{-1} dm^{3})$^{2.3}$
HSA	1.5 : 1	2.1×10^{11} (mol^{-1} dm^{3})$^{1.5}$	2.2 : 1	4.3×10^{17} (mol^{-1} dm^{3})$^{2.2}$

Protein concd $=6.1\times10^{-8}$ mol dm^{-3}. Cu(II) concd $=7.50\times10^{-8}$—1.50×10^{-7} mol dm^{-3}. Ionic strength $\fallingdotseq 0.1$ mol dm^{-3}.

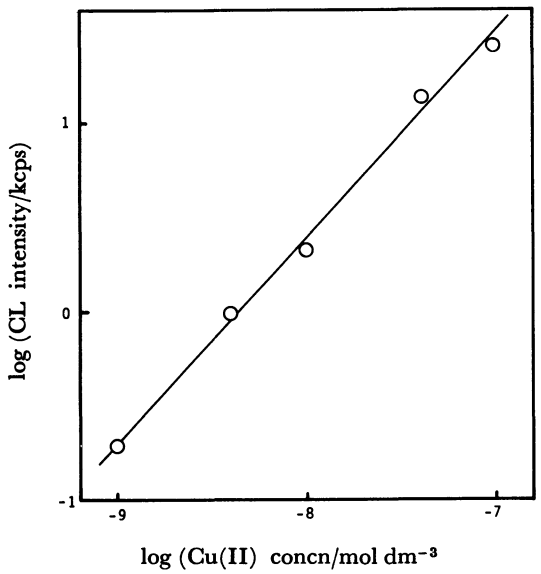

Fig. 2. Calibration curve of Cu(II) catalyst solution.

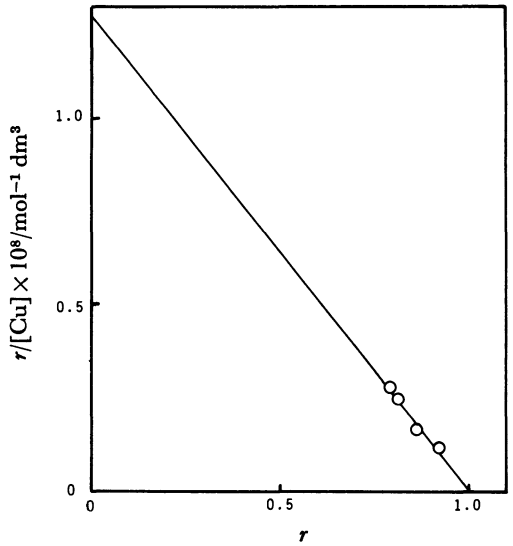

Fig. 3. Relationship between r and $r/[Cu]$ at 25 °C for BSA.

catalyst and BSA went to completion in 30 min at 25 °C and in 45 min at 95 °C. These reaction times contain the times required for a sample solution to reach the individual temperature from room temperature. In the following experiment, the CL intensity of a protein solution was examined in 45 min at 25 °C and 60 min at 95 °C.

The reaction between Cu(II) and protein is assumed to proceed as follows (Eq. 1).

$$x[Cu] + [Prot] \rightleftharpoons [Cu\,x\cdot Prot] \quad (1)$$

where [Cu], [Prot], [Cu x·Prot], and x are the concentration of free Cu(II), free protein, and associated complex, and an apparent coupling ratio, respectively. An apparent coupling constant K is defined by Eq. 2.

$$K=\frac{[Cu\,x\cdot Prot]}{[Cu]^{x}[Prot]} \quad (2)$$

From Eq. 2, Eq. 3 is derivered.

$$r/[Cu]^{x} = xK-rK \quad (3)$$

where r means the mole ratio of bound Cu(II) to total protein. The free Cu(II) concentration is estimated from the CL intensity and the bound Cu(II) concentration is obtained by subtracting the free Cu(II) concentration from the initial Cu(II) concentration. Thus r is found from the bound Cu(II) and total protein concentration.

The value of x was determined so as to satisfy Eq. 3 by the use of the experimental data, and the plot of $r/[Cu]^{x}$ to r was obtained. The value of K was found from the slope of a straight line in the plot. Table 1

and Fig. 3 show the data and the plot for BSA at 25 °C. An apparent coupling ratio and coupling constant were Cu(II):protein=1:1 and 1.3×10^{8} mol^{-1} dm^{3} respectively under the following conditions: initial Cu(II) concentration 7.50×10^{-8}—1.25×10^{-7} mol dm^{-3} and ionic strength about 0.1 mol dm^{-3}. An apparent coupling ratio and coupling constant for two proteins (BSA and HSA) were estimated similarly as above at 25 °C and 95 °C, respectively (Table 2). A molecular weight of 66000 was assumed for these proteins for the calculation.

On the basis of these data the effect of heating on the determination of protein by the use of CL can be understood. Though heating made a Cu(II)–protein complex more stable and improved the concentration range for the determination of protein, the reaction mechanism between Cu(II) and protein still remains unknown.

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References

1) T. Hara, M. Toriyama, and K. Tsukagoshi, *Bull. Chem. Soc. Jpn.*, **57**, 1551 (1984).
2) T. Hara, T. Ebuchi, A. Arai, and M. Imaki, *Bull. Chem. Soc. Jpn.*, **59**, 1833 (1986).