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Competitive Binding to Cuprous Ions of Protein and BCA in the Bicinchoninic Acid Protein Assay

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Abstract: Although Bicinchoninic acid (BCA) has been widely used to determine protein concentration, the mechanism of interaction between protein, copper ion and BCA in this assay is still not well known. Using the Micro BCA protein assay kit (Pierce Company), we measured the absorbance at 562 nm of BSA solutions with different concentrations of protein, and also varied the BCA concentration. When the concentration of protein was increased, the absorbance exhibited the known linear and nonlinear increase, and then reached an unexpected plateau followed by a gradual decrease. We introduced a model in which peptide chains competed with BCA for binding to cuprous ions. Formation of the well-known chromogenic complex of BCA-Cu^{1+}-BCA was competed with the binding of two peptide bonds (NTPB) to cuprous ion, and there is the possibility of the existence of two new complexes. A simple equilibrium equation was established to describe the correlations between the substances in solution at equilibrium, and an empirical exponential function was introduced to describe the reduction reaction. Theoretical predictions of absorbance from the model were in good agreement with the measurements, which not only validated the competitive binding model, but also predicted a new complex of BCA-Cu^{1+}-NTPB that might exist in the final solution. This work provides a new insight into understanding the chemical bases of the BCA protein assay and might extend the assay to higher protein concentration.

Keywords: Bicinchoninic acid, Protein measurement, Nearby two peptide bonds, Cupric ion, Cuprous ion, Equilibrium equation, Competitive binding.

INTRODUCTION

Since the original work of Smith *et al.* (1985) [1], bicinchoninic acid (BCA) has been widely used to determine protein concentration because of its ease of use, high sensitivity and tolerance of interfering species [2, 3]. This assay is based on two chemical reactions. The first is the reduction of cupric ions (Cu^{2+}) to cuprous ions (Cu^{1+}) by the peptide bonds, known as the biuret reaction, and by some specific residues in an alkaline environment [1]. The second step is the chelation of one Cu^{1+} with two BCA molecules (Fig. **1a**), forming an intense purple complex, which has a peak absorbance at 562nm. The protein concentration in a solution is determined by comparing this absorbance with a standard curve of absorbance from varying bovine serum albumin (BSA) [1]. For the Micro BCA™ Assay Kit of Pierce (23235#), the standard curve appears nonlinear above the working range of 0.5 to 20 μg/mL protein. The reason for this nonlinearity has not been investigated. When we increased the concentration of BSA up to 6000 μg/mL, an unexpected absorbance maximum was observed. The molar concentrations of cupric ions, BCA molecules and peptide bonds were calculated from the given contents of the kit. It was found that the absorbance maximum appeared where the peptide bonds were about six times more concentrated than the cupric ions. It is reasonable to assume that the excess peptide bonds compete with the BCA for binding to cuprous ion, thereby reducing production of the chromogenic group. We describe a study of this competition by experiments and theoretical modeling.

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EXPERIMENTAL SECTION

Materials and Methods

Following the standard protocol for the Micro BCA Protein Assay Kit (23235#, Pierce Company), the working reagent (WR) was prepared from 25 parts of MA (sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH), 24 parts of MB (4% BCA in water) and 1 part of MC (4% cupric sulfate, pentahydrate in water). A series of WR with increasing BCA was prepared with varying MB present in 24, 36.5, 49, 74, and 124 parts. The volumes of all WR solutions were kept constant by adding pure water so that only the BCA concentration varied. The pH value of all WR solutions was 11.16 ± 0.06 measured with an Orion 310 pH meter. Twelve BSA solutions with concentrations ranging from 0.02 to 40 mg/mL were prepared by dissolving BSA powder (purity $\geq$ 99.9%, purchased from Bailingke Company, Beijing) in pure water. After mixing one part BSA solution with seven parts WR, a total of sixty solutions with varying concentrations of protein or BCA were obtained. The concentration given in the experimental results is that in the final mixture, and only the concentration of copper ions was invariant, 0.4 mM in all sample solutions. Each measurement was performed in duplicate.

The samples were incubated at 60 ˚C for one hour before cooling to room temperature in accordance with the standard procedures. All the absorbances were corrected by the corresponding blank replicate. The absorbance of the blank

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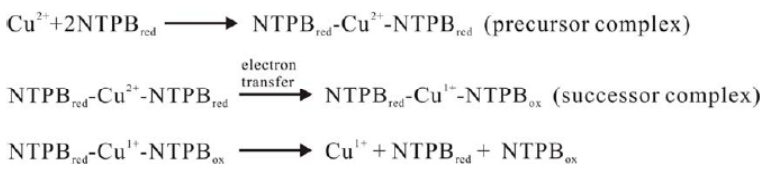

Fig. (8). Proposed three-step reduction reaction of Cu^{2+} to Cu^{1+} by peptide bonds: 1) the cupric ion forms a precursor complex NTPB-Cu^{2+}-NTPB with groups on the peptide; 2) Electron transfer occurs inside the precursor complex, reducing Cu^{2+} to Cu^{1+}; 3) The successor complex dissociated.

forms a precursor complex NTPB-Cu^{2+}-NTPB with some groups on the peptide backbone. Second, electron transfer occurs inside the precursor complex and Cu^{2+} is reduced to Cu^{1+}, then a successor complex NTPB-Cu^{1+}-NTPB forms. Finally, the successor complex dissociates and the cuprous ion can be detected by BCA or other reagent. But we know that there is still a long way to clarify the reductive process.

It should be noted that in the simplified model, $\rho_B \approx \rho_B^0$ is a basic hypothesis, so this model is restricted to the case of $\rho_B^0 >> \rho_{C^2}^0$ (in our experiments, $\rho_B^0 > 15\rho_{C^2}^0$). The error caused by this approximation can be estimated. In most cases, the relative error of $\rho_{B_2C^1} / \rho_{C^2}^0$ calculated by Eq. (9) and Eq. (10) is less than 5%, which is acceptable in experiment. If the mechanism of the reduction reaction and the contribution of peptide bonds to the production of cuprous ions are clarified, the accurate model can be deduced from a series of equilibrium equations and conservation equations.

Based on the above discussion, adding more cupric ions would increase the linear working range, in which $\rho_P^0 / \rho_{C^2}^0 << 1$. When applying the model established in this paper to measure the concentration of a protein in the nonlinear working range, the parameter b must be determined, as it is dependent on the amino acid composition and thus is variable from protein to protein.

CONCLUSIONS

Using the micro BCA protein assay kit of the Pierce Company and varying the concentration of BCA, we measured the absorbance at 562 nm with varying concentrations of BSA. To explain the nonlinearity of the color formation with increasing protein concentration and the plateau value at high protein concentrations, we considered the competition of peptide bonds with BCA for binding to cuprous ions, assuming that there might exist two new complexes besides the chromogenic complex. For the limiting condition of $\rho_B^0 / \rho_{C^2}^0 >> 1$, that is when [BCA] was in excess of copper ions, a simplified model was presented to describe the concentrations of, protein, copper ions, BCA and their complexes at equilibrium. The parameters in the equation were fitted with experimental data, and the curves predicted by the equilibrium equation coincided well with experimental results. The experimental results and theoretical model indicated that the peptide bonds that are not involved in the reduction reaction compete with BCA for binding to cuprous ions. In addition to the chromogenic complex of BCA-Cu^{1+}-BCA, there was evidence for the formation of a new complex of BCA-Cu^{1+}-NTPB. Another possible complex of NTPB-Cu^{1+}-NTPB was shown to be negligible based on the fitting results. The mechanism of the reduction reaction is still unknown but an empirical exponential function with three parameters could describe the production process of cuprous ions quantitatively. The parameters may be dependent on the amino acid composition of a protein and further work should be done to clarify quantitatively the reduction reaction. This work partially clarified the mechanisms of interaction between protein, copper ions and BCA in the BCA protein assay and explained the nonlinearity of absorbance at high protein concentration. The affinity of protein for cuprous ions may be the basis of further investigation of redox reactions in protein-copper systems and the design of new protein assay methods.

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GLOSSARY

A	=	absorbance at 562 nm
b	=	equilibrium constant of reductive reaction of Cu^{2+} to Cu^{1+}
b_1	=	upper limit of b
b_0	=	lower limit of b
c	=	parameter describing the effect of concentration of BCA on b
K_1	=	equilibrium constant of BCA-Cu^{1+}-BCA complex
K_2	=	equilibrium constant of BCA-Cu^{1+}-NTPB complex
K_3	=	equilibrium constant of NTPB-Cu^{1+}-NTPB complex
l	=	optical length path of cuvette
R^2	=	coefficient of determination
ε	=	molar absorption coefficient
ρ_x	=	molar concentration of substance x at the equilibrium state

ρ_x^0 = initial molar concentration of substance x

ρ_x^t = total molar concentration of substance x

χ^2 = chi squares statistics

χ_v^2 = reduced chi squares statistics (= χ^2 / v)

v = number of degrees of fitting freedom

ABBREVIATION FOR THE SUBSTANCE IN EXPRESSION OF CONCENTRATION

B = bicinchoninic acid (BCA)

C^1 = cuprous ion (Cu^{1+})

C^2 = cupric ion (Cu^{2+})

P = peptide bond

B_2C^1 = BCA-Cu^{1+}-BCA complex

BC^1P_2 = BCA -Cu^{1+}-NTPB complex

P_4C^1 = NTPB-Cu^{1+}-NTPB complex

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