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A TECHNIQUE FOR THE DETERMINATION OF PROTEIN CONCENTRATION BY NEUTRON ACTIVATION ANALYSIS OF SILVER BINDING

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A method for the quantitative determination of small amounts of protein samples was developed employing neutron activation analysis. Current methods of protein concentration determination are severely limited as a result of differences in the specific characteristics of each protein. Silver binding has been used as a sensitive colorimetric method to indicate the presence of protein. However, silver-protein complexes can have a variety of absorbance spectra unique to each protein, which complicate the analysis. Various amounts of specific proteins were equilibrated in an excess of silver nitrate prior to the reduction of the silver by the addition of $NaBH_4$, HCHO, and NaOH. The protein-silver complex was rapidly separated from the unbound silver by centrifugation chromatography and the amount of bound silver was determined by INAA. The amount of silver was proportional to the amount of protein present in each sample. When the silver was not reduced prior to removal of the unbound silver by chromatography, only negligible amounts of silver remained bound to the protein. The stoichiometry of bound silver to protein on a molar basis showed relatively small differences for the proteins that were examined. This ratio was found to depend on the conditions of the binding and reduction of the silver. The results suggest that the binding of silver is not specific to any charged or polar groups on these proteins and may, therefore, provide a means of determination of the concentration of protein that has general application for all proteins.

Introduction

Polyacrylamide gel electrophoresis can separate on the order of 2000 proteins from one sample due to the differences in the size and the charge of these molecules. Although this procedure is limited primarily to the separation of very small amounts of protein, color-sensitive staining procedures which employ silver have been developed that are capable of detecting picomole quantities of protein in these gels/1,2/. The mechanism of silver staining is not well understood, yet the intensity of the stain has been used as a measure of the abundance of proteins because it is the only procedure available that can detect such small amounts of protein.

In this study, we used INAA to examine the binding of silver to protein that results when the silver-staining procedure of Sammons et al./1/ is used. Treatment with formaldehyde was found to be required for the binding of the silver to the proteins and not simply for color development. When the amount of silver that was associated with the proteins was compared to the optical absorbance spectra of the silver-protein complex, the absorbance maxima and extinction coefficients were found to be unique to each protein and to depend on the concentration of the protein during silver binding.

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Materials and methods

Proteins purchased from Sigma include bovine pancreatic ribonuclease A, bovine carbonic anhydrase, bovine serum albumin, lactoperoxidase and RuBP carboxylase. Coupling factor one was isolated from spinach as described by Frasch and Selman/3/.

The protocol for the binding of silver to protein was similar to the silver-staining procedure of Sammons et, al./1/. The proteins were incubated for one hour at room temperature in 1.4 mM $AgNO_3$ as an aqueous solution in Tricine buffer, pH 8. The incubation was terminated by the addition of an equal volume of the reducing solution which consisted of 0.75 M NaOH, 7.5mL/L of formaldehyde (37% v/v) and 2.5 mM $NaBH_4$. The protein was removed from the silver free in solution by the centrifuge column chromatography method of Penefsky/4/. Subsequent protein concentration determination was made by the method of Bradford/5/ where a standard curve for each protein was individually determined. Optical absorbance spectra were determined with a Perkin Elmer model Lambda-9 spectrophotometer.

Instrumental neutron activation analysis (INAA) was performed on all samples at the FNR reactor facilities of the Phoenix Memorial Laboratory, University of Michigan. Each sample, 0.5 mL in polyethylene, was irradiated in the pneumatic irradiation system under automatic control of a Nuclear Data 6700 computer analyzer. Each sample was exposed to a neutron flux of 1.5×10^{13} $n/cm^2/s$ for a period of 120 seconds followed by a delay of 60 seconds before start of counting. The 2.4 min ^{108}Ag 0.633 MeV, was used for the determination of the silver. Comparator standards of $AgNO_3$ were used to determine the unknown silver quantities. Gamma spectral analysis was performed using an Ortec Gamma-x intrinsic germanium detector arranged so that each sample was positioned exactly at the same distance from the detector.

Results

Neutron activation analysis was used to quantitate the amount of silver bound to proteins of various molecular weights as shown in Table I. The data show an average ratio of silver to protein when all the proteins were analyzed in duplicate in a single experiment (experiments 1 and 2) as well as when each protein was analyzed separately with eight replications (experiment 3). The uncertainty in any one experiment was about 10% and resulted primarily from the variability of the conditions of silver binding rather than in the quantitation of the silver. Although the proteins that were examined represent a range of molecular weights that span more than an order of magnitude, the mole ratio of bound silver to protein was about 20 for most of these proteins. An apparent exception is RuBP carboxylase which was found to bind an average of 227 moles of silver per mol of protein. However, in experiment 2, RuBP carboxylase bound only 8 moles of silver which is more consistent with the values of the other proteins.

Table I
The stoichiometry of silver bound to protein

	Molecular Weight	Mole Ag bound/mole protein Experiment 1	Experiment 2	Experiment 3	Mean
carbonic anhydrase	30000	43±4.3	6± 1.4		24±17.8
bovine serum albumin	66000	29±2.9	39±17.8	53±16	40± 8.2
lactoperoxidase	100000	18	22± 1.4		20± 1.9
coupling factor one	400000	18±4.3		9± 3.2	13± 4.3
RuBP carboxylase	550000	162±4.8	8± 0.7	512±56.8	227± 176

The optical density of silver-stained proteins that has been used extensively as a measure of protein concentration is polyacrylamide electrophoresis gels. To examine the accuracy of this procedure, silver was bound to proteins under conditions in which the concentration of the protein was varied during the binding. The optical absorbance spectra of the silver-protein complexes that were obtained are shown in Figures 1-4. Ribonuclease (Fig. 1) and coupling factor one (Fig. 2) were found to have a single absorbance maximum at about 400nm when silver was bound. This maximum did change slightly with protein concentration. The absorbance spectra of bovine serum albumin

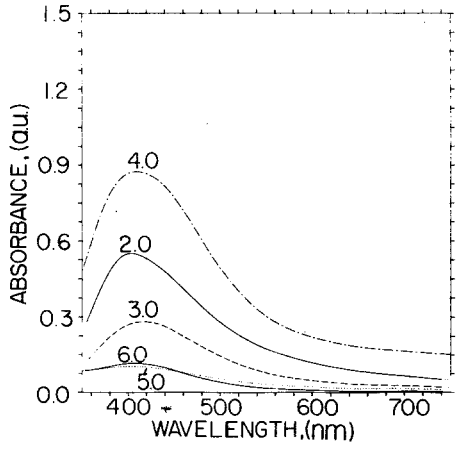

Fig. 1. Optical absorbance spectra of ribonuclease bound to silver. In samples 2–6 the protein concentration during the binding of silver was 100, 300, 500, 700 and 900 μg/ml, respectively

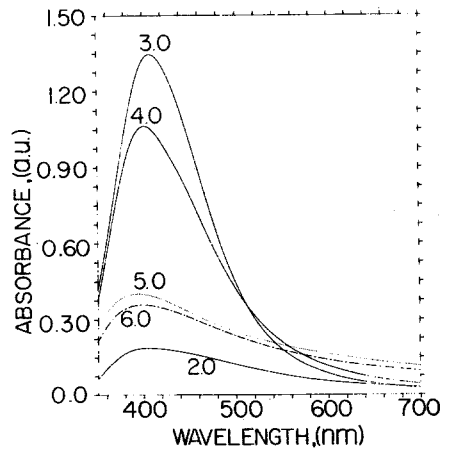

Fig. 2. Optical absorbance spectra of coupling factor one bound to silver. The protein was incubated in silver at the concentrations described in Fig. 1

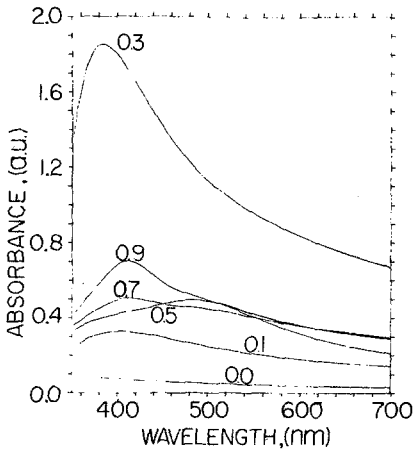

Fig. 3. Optical absorbance spectra of bovine serum albumin bound to silver. The concentration of the protein during the binding of silver is as indicated in μg/ml in each spectrum

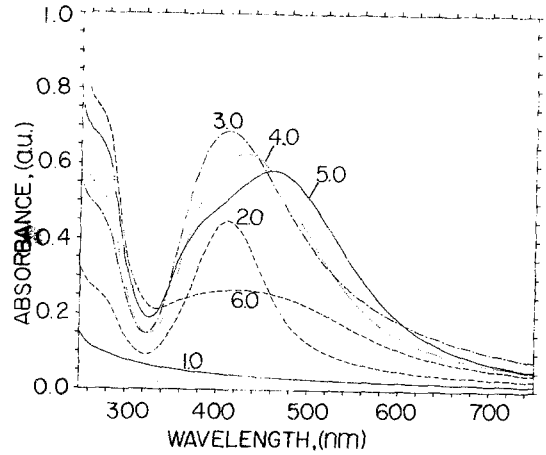

Fig. 4. Optical absorbance spectra of RuBP carboxylase bound to silver. No protein was present in sample 1. In samples 2–6 the protein concentration during the binding of silver was 200, 400, 600, 800 and 1000 μg/ml, respectively

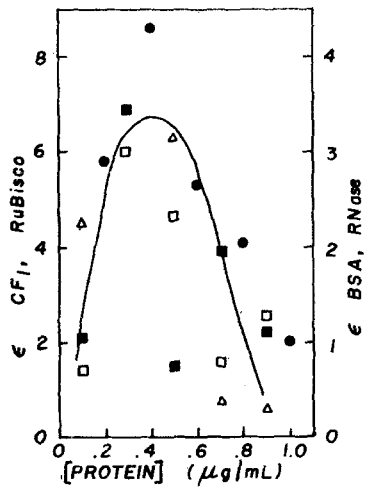

Fig. 5. The optical extinction coefficients at 400 nm of the protein-silver complexes from Fig. 1–4. as a function of protein concentration during the binding of silver. The values are expressed as A_{400} (μg protein/ml)$^{-1}$. Open squares; BSA, closed squares; RNase, tri-angles; RuBisCo, circles

(Fig. 3) and RuBP carboxylase (Fig. 4) showed a single maximum at 400 nm at low protein concentrations and maxima at 460 nm at higher concentrations.

If the optical density of the silver-protein complexes is to be an accurate measure of protein concentration, the optical extinction coefficient of these complexes must be constant with concentration. The extinction coefficients determined at 400nm from Figures 1-4 are shown in Figure 5 as a function of protein

concentration. The extinction coefficient was not constant but exhibited the same maximum at 300-400 ng protein/mL for all of the proteins that were studied.

Figure 6 shows the amount of bound silver as a function of the amount of bovine serum albumin or coupling factor one in the assay. In both cases there was a linear relationship between the silver bound and the amount of protein at low protein con-

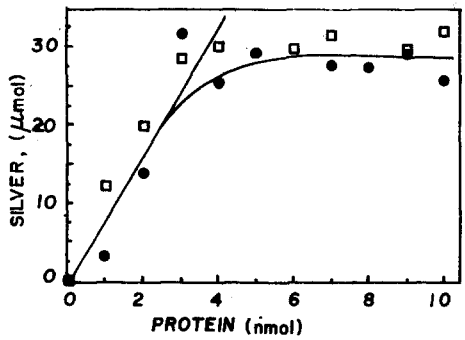

Fig. 6. The amount of silver associated with RuBP carboxylase (squares) and with coupling factor one (circles) as a function of the amount of protein present during the binding

centrations. The lack of increase of bound silver at higher protein concentrations results because the amount of silver available for binding has been exhausted. The conditions for silver binding used in the experiments of Figure 6 resulted in a stoichiometry of bound silver to protein on a molar basis of the order of 1×10^4. It is of note that when the silver was not reduced prior to centrifuge chromatography only negligible amounts of silver remain bound to the protein.

Discussion

The data presented here indicate that although the optical density of silver-protein complexes may be an extremely sensitive indication of the presence of protein, the quantitation of the amount of protein requires the direct measurement of the amount of silver bound. If the conditions of the binding of silver are held constant (i.e. there must be excess amounts of silver and reducing reagents and the duration of the incubation in the silver as well as in the reducing solution must be held constant) the mole ratio of silver to protein will remain constant.

Although the mechanism of the association of the silver with the protein is not known, it has been hypothesized to result from a Tollen's type of reaction/6/. In such a reaction Ag(I) is reduced to silver metal and, thus, would not be able to coordinate to the protein. If this hypothesis is correct, then

the protein is possibly acting as a catalyst for the reaction. The terminal amino groups of the protein may be forming the silver ammonia ion that can be reduced to silver by the oxidation of formaldehyde. Several observations reported here indirectly support this hypothesis including; (i) the requirement of the addition of the reducing solution for silver binding; (ii) the observation that several different proteins will have approximately the same amount of silver bound when the conditions of binding are held constant; (iii) that the amount of silver ultimately associated with the proteins can be altered by changing the conditions of silver binding and reduction and can be several orders of magnitude greater than the amount of protein present. These results suggest that the binding of silver is not specific to any charged or polar groups on the proteins and may, therefore, provide a means of determination of the concentration of protein that has general application for all proteins.

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