Binding of Zn(II) by the 11S Fraction of Soybean Proteins

A. G. Appu Rao and M. S. Narasinga Rao*

Binding of Zn(II) by the 11S fraction of soybean proteins in 0.2 M acetate buffer of pH 6.5 was determined by equilibrium dialysis. The Scatchard plot of binding data indicated heterogeneity of binding sites. Binding occurs possibly at the imidazole groups of histidine residues. Addition of 0.5 M NaCl to the buffer or prior treatment of the protein with EDTA had no effect on binding. Zn(II) binding did not change the sedimentation velocity pattern of the protein or its $s_{20,w}$ value. It did not affect the fluorescence spectrum and ORD in the visible range. However, it increased heat coagulation of the protein. At 4 $\times 10^{-3}$ M and higher Zn(II) concentrations the protein was quantitatively precipitated. Precipitation at low concentrations of Zn(II) was suppressed by NaCl but was not at higher concentrations.

Soybean seeds contain various minerals (Cartler and Hopper, 1942). The availability of minerals from soybean is quite low (Harmon et al., 1969). Constituents from soybean interfere with the availability and utilization of minerals. Allred et al. (1964) have shown that Zn(II) is bound by soybean proteins. Binding of Zn(II) by unfractionated soybean proteins has been reported by Malik and Singh (1969). However, there has been no study of the binding of Zn(II) by the isolated protein fractions of soybeans. The 11S and 7S proteins have been fractionated to homogeneity and considerable information is available on their chemical and physico-chemical properties (Wolf, 1970). The binding of Ca(II) and Mg(II) by 11S protein and the effect of such binding on its physico-chemical properties have been reported (Appu Rao and Narasinga Rao, 1975a,b). In this paper we report the results of a study of the binding of Zn(II) by 11S protein and the effect on its physico-chemical properties. The results have been compared with those from Ca(II) and Mg(II) binding studies.

EXPERIMENTAL SECTION

Materials. Improved pelican variety soybeans were used in this investigation. Zn-65 in the form of $ZnCl_2$ was purchased from the Bhabha Atomic Research Center, Bombay, India. All reagents used were of analytical reagent grade. Deionized water was used for the preparation of solutions.

11S Protein. This was prepared by the method of Wolf et al. (1962). The preparation contained 2–3% of 7S protein impurity as judged by sedimentation velocity experiments in 0.05 M phosphate buffer of pH 7.8 containing 0.35 M NaCl and 0.1% β-mercaptoethanol, but was free of 15S protein.

EDTA-Treated 11S Protein. The protein was dissolved in acetate buffer containing 5×10^{-3} M EDTA, left for 60 min at room temperature, and dialyzed extensively against the same buffer which did not contain EDTA.

Protein Concentration. This was determined by measuring the absorbance of the solution at 280 nm and calculated using a value of $E_{1\ \mathrm{cm}}^{1\%} = 9.2$ (Wolf and Briggs, 1959).

Equilibrium Dialysis. An aliquot (2.5 ml) of 0.5–1.0% protein solution in appropriate buffer was dialyzed at 30 °C against 5.0 ml of buffer solution containing varying amounts of Zn(II) (in the form of $ZnSO_4$) for 48 h, an interval sufficient for equilibration. Corresponding "blanks" containing only buffer solution were also run. At the end of the period, the concentration of Zn(II) of the solutions outside the dialysis bag in the experimental and the "blank" was estimated. From the difference in the Zn(II) concentration, the number of Zn(II) ions bound by 100000 g of protein was calculated.

Estimation of Zn(II). A cold $ZnSO_4$ solution was mixed with Zn-65 in the form of $ZnCl_2$. The Zn(II) concentration was determined by titration with standard EDTA solution using Eriochrome Black T as indicator. Using this stock solution, a series of solutions of known concentration was prepared by dilution. Radioactivity was measured on a Beckman liquid scintillation counter, LS 100. The composition of the scintillation liquid was 200 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in 100 ml of absolute methanol, 20 ml of ethylene glycol, 4 g of 2,5-diphenyloxazole (PPO), and 60 g of naphthalene made to 1 l. with dioxane. No decay corrections were applied since a calibration curve of counts per minute vs. concentration was prepared for each set of experiments. The measurements were made at 25 °C.

Ultracentrifugation. The experiments were made with 1% protein solution at 25 °C using a Spinco Model E analytical ultracentrifuge fitted with phase-plate schlieren optics and an RTIC unit, and $s_{20,w}$ values were calculated (Schachman, 1959).

Optical Rotatory Dispersion. Measurements were made at 25 °C in the visible range with a JASCO-J-20 automatic spectropolarimeter fitted with xenon lamp photomultiplier, using a quartz cell of 10-mm optical path and 1% protein solutions. The data were analyzed by the Moffitt–Yang equation (Urnes and Doty, 1961). Values of 113 for mean residue weight (Fukushima, 1968) and 212 for λ_0 were used.

Fluorescence Measurements. The measurements were made at room temperature (~25 °C) with a Perkin-Elmer fluorescence spectrophotometer Model 203. A protein solution having an absorbance of 0.1 at 280 nm was used.

Heat Coagulation. Five milliliters of 0.5% protein solution in 0.2 M acetate buffer of pH 6.5 containing 0.5 M NaCl with and without added metal ion was heated for 15 min at various temperatures from 30 to 97 °C. For each temperature a separate aliquot was used. At the end of 15 min the solution was cooled under tap water to room temperature. Centrifugation was at 6000 rpm for 20 min and the absorbance of the supernatant was read at 280 nm from which the extent of precipitation was calculated.

Precipitation of the Protein with Zn(II). Two milliliters of 1% protein solution in 0.2 M acetate buffer of pH 6.5 containing either 0.1 or 0.5 M NaCl was mixed with 2.0 ml of metal ion solution in the buffer. The mixture was shaken at 30 °C for 6 h. The resulting precipitate was removed by centrifugation at 6000 rpm for 30 min. The absorbance of the supernatant was read at

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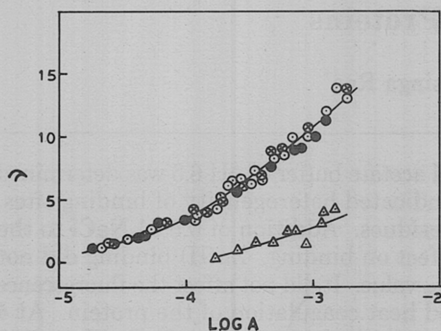

Figure 1. Binding of Zn(II) by the 11S protein: (⊙) 11S protein in 0.2 M acetate buffer (pH 6.5) containing 0.1 M NaCl; (⊗) EDTA treated 11S protein in 0.2 M acetate buffer (pH 6.5) containing 0.1 M NaCl; (●) 11S protein in 0.2 M acetate buffer (pH 6.5) containing 0.5 M NaCl; (△) 11S protein in 0.2 M acetate buffer (pH 5.0) containing 0.5 M NaCl.

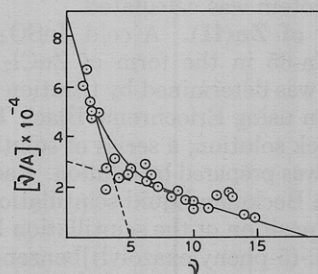

Figure 2. Scatchard plot of binding data in 0.2 M acetate buffer (pH 6.5) containing 0.1 M NaCl.

280 nm after proper dilution, if necessary. From absorbance measurements the percent of protein precipitated was calculated.

Other Measurements. The absorbance measurements were made at room temperature with a Carl-Zeiss spectrophotometer. The pH measurements were made with a Radiometer pH meter TTT2.

RESULTS AND DISCUSSION

Binding measurements were made at pH 6.5 in 0.2 M acetate buffer since Zn(II) precipitated at higher pH values. Also, 0.1 M NaCl was added to the buffer as the protein did not dissolve easily in NaCl-free buffer solution. The binding of Zn(II) by the 11S protein is shown in Figure 1; ν, the number of Zn(II) ions bound by 100000 g of protein is plotted as a function of the logarithm of free Zn(II) concentration, A. The binding data were analyzed with the Scatchard equation (Scatchard, 1949) to evaluate the binding constant, k, and the maximum number of ions bound, n. The equation is $\nu/A = k(n - \nu)$. For a set of equivalent binding sites the plot would be a straight line with the intercept on the abscissa giving n and the slope giving k. If the binding involves more than one type of binding sites, the plot would be nonlinear. The Scatchard plot could be fitted to two straight lines (Figure 2). The initial linear portion gave on extrapolation a value of $n \simeq 5$ and the second linear portion gave $n \simeq 17$–18. Thus, the binding sites on the protein molecule appear to be nonidentical. In the case of nonlinear Scatchard plots, the intercepts on the y axis cannot be equated with different binding constants. These are complex functions of the binding constants (Klotz and Hunston, 1971). Therefore, from the intercepts k_1, k_2, n_1, and n_2 were obtained by successive approximation which gave a calculated curve that fitted the experimental data. Using values of k_1, k_2, n_1, and n_2 of 1×10^4, 1×10^3, 5, and 12, respectively, ν was calculated as a function of A with eq 1. The calculated

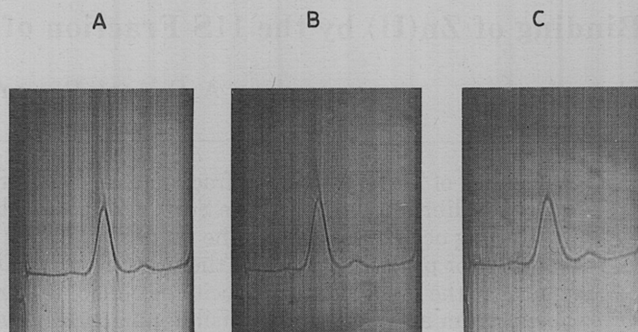

Figure 3. Sedimentation velocity pattern of 11S protein in 0.2 M acetate buffer of pH 6.5 containing 0.1 M NaCl. Sedimentation proceeds from left to right: (A) 11S protein (untreated); (B) 11S protein in the presence of 5.0×10^{-5} M Zn(II); (C) 11S protein EDTA treated.

$$\nu = \frac{k_1 n_1 A}{1 + k_1 A} + \frac{k_2 n_2 A}{1 + k_2 A} \quad (1)$$

curve is shown as a solid line in Figure 1. The agreement between the experimental data and calculated curve was good. This would suggest that binding occurred at two sets of nonequivalent binding sites. The total number of sites ($n_1 + n_2$) was 17–18 which agreed with the histidine content of the 11S protein (Shvarts and Vaintraub, 1967). Therefore the probable binding sites were the imidazole groups of the histidine residues. The binding of Ca(II) and Mg(II) also appears to occur at the imidazole groups (Appu Rao and Narasinga Rao, 1975a,b).

The binding of Zn(II) at pH 5.0 (0.2 M acetate buffer containing 0.5 M NaCl) was negligible (Figure 1). This is compatible with the conclusion that binding occurs at the imidazole groups.

Metal ions are found in association with soybean proteins (Wolf and Briggs, 1958). To remove any contaminating metal ions the 11S protein was treated with EDTA and the binding of Zn(II) by EDTA-treated protein was measured at pH 6.5. The binding data before and after EDTA treatment fitted the same calculated curve, suggesting that prior treatment of 11S protein with EDTA did not affect Zn(II) binding (Figure 1). In the case of Mg(II) binding at pH 7.8, EDTA treatment decreased the affinity of the protein for the metal ion and rendered the binding sites equivalent (Appu Rao and Narasinga Rao, 1975b).

In Figure 1 the binding of Zn(II) by the 11S protein at pH 6.5 in the presence of 0.5 M NaCl is shown. An increase in NaCl concentration from 0.1 to 0.5 M had no effect on the binding of Zn(II). This contrasts with Ca(II) or Mg(II) binding where addition of NaCl reduced the binding (Appu Rao and Narasinga Rao, 1975a,b).

The sedimentation velocity pattern of 11S protein in 0.2 M acetate buffer containing 0.1 M NaCl consisted of a major peak of 11S and a minor peak of 15S (Figure 3A); the latter was perhaps an aggregation product due to the absence of mercaptoethanol in the buffer. No lower molecular weight fractions were observed. The addition of Zn(II) did not bring about any change either in the pattern or the $s_{20,w}$ value (Figure 3B). A similar measurement in buffer solution containing 0.5 M NaCl also did not reveal any change in the $s_{20,w}$ value. The sedimentation velocity pattern of the EDTA-treated protein in 0.2 M acetate buffer containing 0.1 M NaCl did not show any difference from the untreated protein (Figure 3C). In buffer solutions of low ionic strength the EDTA-treated protein shows lower molecular weight fractions (Wolf and Briggs, 1958; Appu Rao and Narasinga Rao, 1975a,b). Probably high salt concentration reverses

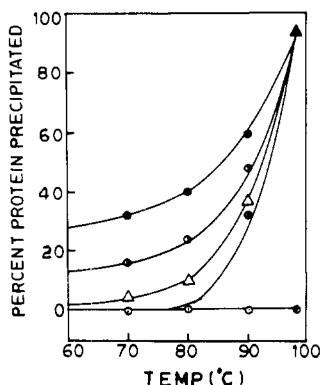

Figure 4. Heat coagulation of 11S protein (untreated) in 0.2 M acetate buffer containing 0.5 M NaCl in the presence of Zn(II): (◎) 11S protein alone; (⊗) 11S protein in the presence of 2.0×10^{-3} M Zn(II); (△) 11S protein in the presence of 3.0×10^{-3} M Zn(II); (◐) 11S protein in the presence of 4.0×10^{-3} M Zn(II); (●) 11S protein in the presence of 5×10^{-3} M Zn(II).

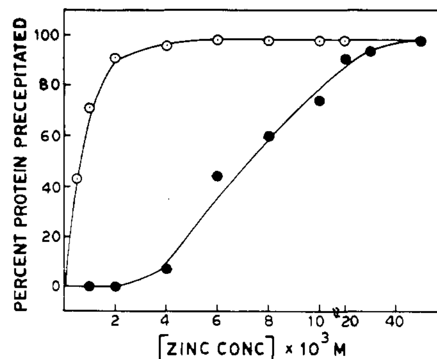

Figure 5. Precipitation of 11S protein by Zn(II) (0.2 M acetate buffer of pH 6.5; temperature, 30 °C): (◎) 11S protein in buffer containing 0.1 M NaCl; (●) 11S protein in buffer containing 0.5 M NaCl.

the dissociation caused by EDTA treatment. The addition of Zn(II) did not cause any aggregation of EDTA-treated protein. These results suggest that ultracentrifugally detectable conformational changes had not occurred due to Zn(II) binding.

At pH 7.8 the 11S protein has an emission maximum at 330 nm and an excitation maximum at 280 nm (Appu Rao and Narasinga Rao, 1975b). The spectrum in 0.2 M acetate buffer of pH 6.5 containing 0.5 M NaCl had the same emission and excitation maxima. The addition of Zn(II) had no effect on the fluorescence spectrum. Since the fluorescence spectrum is due to tryptophan and tyrosine residues (Chen et al., 1969) conformational changes involving these residues had not occurred.

The effect of Zn(II) binding on the conformation of 11S protein was studied by optical rotatory dispersion in the visible region. The measurements were made in unbuffered KCl solution whose pH was about 6.5. The data were analyzed with the Moffitt–Yang equation (Moffitt and Yang, 1956). The value of b_0 was essentially zero and that of a_0 was −360°. These agree with the reported values (Fukushima, 1968; Catsimpoolas et al., 1970; Koshiyama, 1972). The addition of Zn(II) at 5×10^{-4} M had no effect on the value of either a_0 or b_0. At the highest Zn(II) concentration used (1×10^{-3} M) there was no change in the value of b_0 although a slight change was found in a_0. These results again suggest that Zn(II) binding did not cause any conformational changes in the protein.

Heat destroys the quaternary structure of 11S protein at temperatures above 70 °C; the protein dissociates into subunits (Catsimpoolas et al., 1970; Wolf and Tamura, 1969). The protein does not precipitate when heated at 97 °C for 15 min (Figure 4). However, at all the concentrations of Zn(II), the 11S protein was quantitatively precipitated at 97 °C. At lower temperatures, the protein was not quantitatively precipitated, although there was some precipitation. Increase in Zn(II) concentration also did not bring about quantitative precipitation at lower temperatures. However, at any temperature, except at 97 °C, an increase in Zn(II) concentration increased the extent of heat coagulation of the protein. This contrasts with the behavior of Ca(II) or Mg(II) which did not enhance heat coagulation significantly (Appu Rao and Narasinga Rao, 1975a,b).

The precipitation of 11S protein by different concentrations of Zn(II) at 30 °C in 0.1 M acetate buffer of pH 6.5 containing 0.1 M NaCl is shown in Figure 5. At 4×10^{-3} M Zn(II) there was almost complete precipitation of 11S protein in buffer containing 0.1 M NaCl. However, at the same Zn(II) concentration an increase in NaCl concentration to 0.5 M decreased the precipitation to 10%. Up to 5×10^{-2} M Zn(II) precipitation in buffer containing 0.5 M NaCl was less than that in buffer containing 0.1 M NaCl. Thus, whereas NaCl concentration had no effect on Zn(II) binding (Figure 1) it had a marked effect on the precipitation of the protein.

A Comparison of Binding of Ca(II), Mg(II), or Zn(II) by 11S Protein. Binding of Ca(II) or Mg(II) by the 11S protein at pH 7.8 appears to occur at the imidazole groups of the histidine residues of the protein molecule (Appu Rao and Narasinga Rao, 1975a,b). Zn(II) binding at pH 6.5 also appears to occur at imidazole groups. The 11S protein showed heterogeneity of sites for binding Mg(II) and Zn(II) whereas no such effect was observed in the binding of Ca(II). Addition of NaCl at 0.5 M concentration to the buffer had no effect on Zn(II) binding, decreased Ca(II) binding, and completely suppressed Mg(II) binding. EDTA treatment of the protein decreased its affinity for Ca(II) and Mg(II) but not for Zn(II). Also, this treatment abolishes the heterogeneity of sites for Mg(II) binding but not for Zn(II) binding. The addition of Ca(II), Mg(II), or Zn(II) to the protein did not alter its sedimentation velocity pattern or $s_{20,w}$ value. The fluorescence spectrum was not altered by the addition of these metal ions. However, they increased heat coagulation of the protein. Ca(II) or Mg(II) caused increased turbidity whereas Zn(II) caused precipitation. The protein was quantitatively precipitated by 5×10^{-3} M Ca(II), 8×10^{-3} M Mg(II), or 4×10^{-3} M Zn(II). NaCl (0.5 M) suppressed precipitation by Ca(II) or Mg(II) at all the concentrations of the metal ion. Although precipitation by low concentrations of Zn(II) ion was suppressed by 0.5 M NaCl it was not affected at higher concentrations of the metal ion.

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Binding of Ca(II), Mg(II), and Zn(II) by 7S Fraction of Soybean Proteins

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Binding of Ca(II) or Mg(II) by 7S protein at pH 7.8 appears to occur at the imidazole groups of the histidine residues of the protein molecule. Zn(II) binding at pH 6.5 also occurs at imidazole groups. The 7S protein binds more Ca(II) or Mg(II) in borate buffer than in Tris-HCl buffer of the same pH. Rate of proteolysis, fluorescence, optical rotatory dispersion, and circular dichroism measurements do not indicate any conformational change in the protein due to metal ion binding. Ca(II), Mg(II), or Zn(II) increases the heat coagulation of 7S protein. At room temperature the protein is precipitated to an extent of 40% by Ca(II), 10% by Mg(II), and 90% by Zn(II). NaCl (0.5 M) suppresses precipitation by Ca(II) or Mg(II) and decreases only slightly precipitation by Zn(II).

Precipitation of soybean proteins by Ca(II) or Mg(II) is used in the isolation of 7S fraction (Koshiyama, 1965; Appu Rao and Narasinga Rao, 1976a). These cations precipitate the 11S protein, the other major protein in soybeans; they are bound by the protein also (Appu Rao and Narasinga Rao, 1975a,b). Of the various soybean proteins, 11S and 7S proteins have been isolated in homogeneous form and considerable information is available on their chemical and physico-chemical properties (Wolf, 1970). The interaction of Ca(II), Mg(II), or Zn(II) with 11S protein has been reported (Appu Rao and Narasinga Rao, 1975a,b, 1976b). In this paper we describe the interaction of Ca(II), Mg(II), and Zn(II) with 7S protein and compare it with that of 11S protein.

EXPERIMENTAL SECTION

Improved Pelican variety soybean was used in this investigation. Ca-45 and Zn-65 were purchased from Bhabha Atomic Research Center, Bombay, India, and α-chymotrypsin from Worthington Biochemicals. All the reagents were of analytical or equivalent grade. Solutions were prepared with deionized water.

Preparation of 7S Fraction. From the water extract of soybean proteins, 15S and 11S proteins were precipitated with Ca(II) (Koshiyama, 1965). The 7S and 2S proteins which remained in the supernatant were precipitated by full $(NH_4)_2SO_4$ saturation. The precipitate was dissolved in 0.05 M phosphate buffer of pH 7.8 containing 0.35 M NaCl and passed through a column of Sephadex G-100 which had been equilibrated with the same buffer. Fractions corresponding to the peak emerging immediately after the void volume of the column were collected. To the pooled fraction EDTA was added to a concentration of 5×10^{-3} M to remove any bound Ca(II); the solution was then dialyzed extensively against the buffer. The homogeneity of the preparation was determined by ultracentrifugation and gel electrophoresis at pH 7.8. It contained 5–10% of 11S protein.

Protein Concentration. This was determined by absorbance measurements at 280 nm. A value of $E_{1\,cm}^{1\%} = 6.0$ was used for converting absorbance to concentration. This value was determined by making absorbance measurements with a series of solutions of known protein concentration which had been determined by Kjeldahl nitrogen estimation. A value of 6.25 was used to convert nitrogen content to protein content.

Equilibrium Dialysis. The details of the method are the same as described earlier (Appu Rao and Narasinga Rao, 1975a,b, 1976b).

Estimation of Metal Ions. Ca(II), Mg(II), and Zn(II) were estimated by the methods described earlier (Appu Rao and Narasinga Rao, 1975a,b, 1976b).

Rate of Proteolysis by α-Chymotrypsin. Ten milliliters of 2% protein solution in 0.05 M Tris-HCl buffer of pH 7.8 containing varying amounts of metal ions was incubated at 37 °C. After preincubation, 10 ml of α-chymotrypsin (20 μg/ml) was added and the solutions were mixed well. At the end of each time interval, 2 ml of the reaction mixture was withdrawn and the reaction was "stopped" by the addition of 2 ml of 20% Cl_3CCOOH. After centrifugation, absorbance of the supernatant was read at 280 nm. Time intervals from 2 to 30 min were used.

Optical Rotatory Dispersion (ORD) and Circular Dichroism (CD). A JASCO-J-20 automatic spectropolarimeter fitted with xenon lamp photomultiplier was used for ORD and CD measurements using a quartz cell of 5-mm optical path. Protein solutions (0.04%) were used. The optical rotation was converted to mean residue ro-

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