Kinetics and Thermodynamics of the Mechanism of Interaction of Sodium Phytate with α-Globulin†

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ABSTRACT: The precipitation mechanism of α-globulin in the presence of *myo*-inositol hexaphosphate (sodium phytate) was studied in detail. The maximum interaction was found at pH 2.3 where the protein was in a dissociated state having an 8.3S aggregate and a 1.5S monomer. This interaction was predominantly dependent upon the sodium phytate to protein ratio. Velocity sedimentation studies indicated polymer formation due to preferential progressive binding of ligand to polymer, whose size and concentration increased with an increase in sodium phytate concentration. The polymer formation was shown to be ligand mediated and exists independently in solution along with the monomer. The binding isotherm by equilibrium dialysis confirmed differential binding of sodium phytate to the polymer and the monomer as indicated by two sets of binding sites, one having 7 ± 2 sites of a K value 1.3×10^{-4} mol^{-1} and the other having 56 ± 3 sites with a K value of 2.8×10^{-3} mol^{-1}. Binding resulted in perturbation of chromophores of protein due to charge effects. The kinetics of the polymer formation was shown to be a pseudo-first-order reaction having two steps. The initial fast reaction involving conformational changes has rate constants of $k_1 = 52.4 \times 10^{-3}$ s^{-1} and $k' = 67.5 \times 10^{-3}$ s^{-1}, followed by a slow reaction step of rate constants $k_2 = 4.3 \times 10^{-3}$ s^{-1} and $k'_2 = 2.9 \times 10^{-3}$ s^{-1} at sodium phytate concentrations of 1×10^{-4} M and 5×10^{-4} M, respectively.

α-Globulin, the major protein fraction of sesame seed total proteins, forms nearly 65–70% of the protein. It is the highest source of phytic acid in nature (nearly 6%; Cheryan, 1980). The protein was isolated to homogeneity and its biophysical properties were well-established (Prakash & Nandi, 1978; Prakash et al., 1980; Prakash, 1985; Plietz et al., 1986; Prakash & Narasinga Rao, 1986).

Among inositol phosphates, phytic acid (inositol hexaphosphate, $InsP_6$) and Ins[1,3,4,5,6]P_5 are present in cells at higher concentration than others (Shears, 1991). Inositol triphosphate (Ins[1,4,5]P_3) arising out of phytase action on phytic acid is shown to be a second messenger which regulates intracellular calcium both by mobilizing calcium from internal stores and by stimulating external calcium entry (Berridge & Irvine, 1989).

Since the major protein of sesame seed is well-characterized and phytic acid and its derivatives are well-recognized for their physiological significance, the interaction between phytic acid and α-globulin is followed in order to understand the mechanism, kinetics, and thermodynamics of the reaction.

MATERIALS AND METHODS

Materials

An authentic variety of sesame seeds (*Sesamum indicum L.*) was obtained from a local market, and the defatted flour was prepared as described by Prakash and Nandi (1978). The chemicals and reagents used were as follows: phytic acid (sodium salt), bisacrylamide, *d*-10-camphorsulfonic acid, *N,N,N',N'*-tetramethylethylenediamine (Sigma Chemical Co.), and the other chemicals used were of analytical grade.

Methods

α-Globulin from sesame seed was isolated by the method of Prakash and Nandi (1978). The final precipitate was dissolved in 0.15 M glycine–hydrochloric acid buffer of pH 2.3 (GH buffer) and used for all the experiments.

Protein Concentration. Protein concentration was determined spectrophotometrically by using an absorption coefficient ($E^{1\%,1cm}_{280nm}$) of 10.8 (Prakash & Nandi, 1978).

Sodium Phytate Determination. Sodium phytate was hydrolyzed by using 10 N H_2SO_4, and the inorganic phosphorus liberated was estimated by the method of Taussky and Shorr (1953).

Precipitation Experiments. The percentage of protein precipitated was determined over a wide pH range and at various sodium phytate to protein concentrations (in GH buffer) by absorbance measurements at 280 nm (A_{280}) of the control protein solution (without sodium phytate) and of the supernatant after centrifuging the precipitate.

In all other experiments, the precipitate formed upon addition of sodium phytate was removed by centrifugation and the clear supernatant was used for further experiments. The protein concentration was measured spectrophotometrically.

Analytical Ultracentrifugation. Sedimentation velocity experiments were carried out at 27 ± 1 °C in a Spinco Model E of analytical ultracentrifuge at various concentrations of sodium phytate in GH buffer at 59 780 rpm in a Kel F cell. Sedimentation coefficient values ($s_{20,w}$) were calculated as described by Schachman (1959).

Fast Kinetics. The fast kinetics of sodium phytate interaction was followed by using a stopped flow spectrophotometer (Union Giken Model No. RA 401) attached with a monochromator and a PMT tube interfaced to a computer at 27 ± 1 °C. Protein solutions of 5.1×10^{-6} M were used. The final data were analyzed by established methods (Gutfreund, 1972; Hiromi, 1979; Rajendran & Prakash, 1988). The slow kinetics was followed in a Shimadzu UV-150-02 double-beam spectrophotometer at 600 nm and 27 ± 1 °C using a protein concentration of 3.7×10^{-7} M.

Fluorescence. Fluorescence measurements were made in an Aminco Bowman spectrofluorometer at 27 ± 1 °C in the

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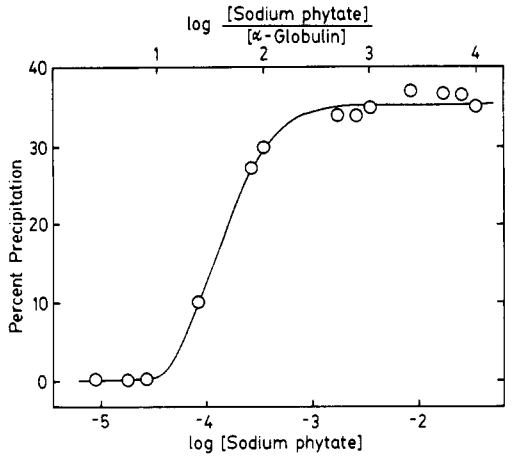

FIGURE 1: Precipitation isotherm of α-globulin with sodium phytate in GH buffer at 27 ± 1 °C. An α-globulin concentration of 3.6 × 10^{-6} M was used.

range of 300–400 nm with a protein solution of 3.4 × 10^{-7} M, keeping the fluorescence excitation at 279 nm. The fluorescence emission was measured after 10 s when fluorescence intensity attained constancy.

Circular Dichroism. Circular dichroic (CD) measurements were made in a Jasco J20C spectropolarimeter in the wavelength region of 200–250 nm at 27 ± 1 °C. The instrument was calibrated with *d*-10-camphorsulfonic acid. The data were expressed as mean residue ellipticities, $[\theta]_{MRW}$, using a value of 115.5 for the mean residue weight of α-globulin. A protein concentration of 2 × 10^{-6} M was used in a 0.1-cm-path-length cell. The dichroic absorbance differences were averaged from two recordings, and the mean ellipticity was calculated from the averaged spectrum.

Equilibrium Dialysis. Spectrapor membrane tubing of 16 mm diameter was boiled in distilled water followed by 0.2% sodium carbonate and 0.2% EDTA solutions to remove metal contaminants. The tubing was finally boiled in glass double-distilled water twice and stored in double-distilled water. All the equilibrium dialysis experiments were performed using a 12-mL volume of outer solution at 40 °C in a uniform shaking Queue orbital shaker at 140 rpm. One milliliter of protein solution of 1.8 × 10^{-5} M concentration was taken in the dialysis bag. The range of concentration of sodium phytate in the outer solution was 1.6 × 10^{-6} M to 1 × 10^{-2} M. After a 24-h interval, the bags were removed and the amount of sodium phytate present in the outer solution was estimated. The binding isotherm, calculated from these data, was analyzed as described by Steinhardt and Reynolds (1969) and Scatchard (1949).

RESULTS

The turbidimetric titration of α-globulin with sodium phytate shows that turbidity increases with an increase in sodium phytate and α-globulin concentrations (Figure 1). The precipitation isotherm indicates that up to a sodium phytate concentration of 3.6 × 10^{-5} M no precipitation is observed (Figure 1). This corresponds to a molar concentration ratio of 10 between sodium phytate and α-globulin. As the sodium phytate concentration is increased further, the percent precipitation increases and reaches a plateau region at 1 × 10^{-3} M sodium phytate concentration (which corresponds to a sodium phytate to α-globulin molar concentration ratio of 300). Above this concentration, there is no change in the percent precipitation value indicating the saturation of α-globulin with sodium phytate.

α-Globulin at neutral pH sediments as a single peak with a sedimentation coefficient of 12.8S at neutral pH (Figure 2a). As the pH is reduced, the protein is dissociated and the pattern shows one fast moving component with an $s_{20,w}$ value 8.3 ± 0.2 S and a slow moving component with an $s_{20,w}$ value of 1.5 ± 0.15 S at pH 2.3 (Figure 2b, upper). Upon addition of sodium phytate, initially the concentration of the fast moving component decreases with a concomitant increase in the slow moving component concentration (Figure 2b (lower) and 2c). At a 1 × 10^{-3} M sodium phytate concentration, only the slow moving component is present in the velocity sedimentation pattern (Figure 2c).

The $s_{20,w}$ value of the fast moving component in GH buffer is 6.0 S, at a protein concentration of 7.3 × 10^{-5} M (20 mg/mL) (Figure 3). At the same protein concentration, in the presence of sodium phytate (free ligand concentration, 1 × 10^{-4} M), the $s_{20,w}$ value increases to 8 S. The $s_{20,w}$ value of this component (polymer) increases as a function of sodium phytate concentration and reaches a value of 15.0 s at 1 × 10^{-3} M sodium phytate (Figure 3, inset). This value is nearly 3-fold higher than the $s_{20,w}$ of the polymer found in the absence of sodium phytate (6 S at 7.3 × 10^{-5} M protein concentration). The reaction may be represented as

$$6S \xrightarrow[\text{sodium phytate}]{\text{upto } 1 \times 10^{-3}\text{ M}} \underset{\substack{\text{precursor of}\\ \text{precipitate}\\ \text{(polymer)}}}{15S} \longrightarrow \text{precipitate}$$

In Figure 3 the $s^0_{20,w}$ value of the fast moving component in the presence of 1 × 10^{-4} M sodium phytate concentration is shown. It has a value of 15.3 S (Figure 3). This is nearly twice the $s^0_{20,w}$ value of the fast moving component (8.3 s) in the absence of sodium phytate. The values of *g* (nonideality coefficient) for the aggregate in the absence and presence of sodium phytate are 0.1 mL/mg and 0.3 mL/mg, respectively. The evaluation of $s^0_{20,w}$ of the 1.5S component in both the absence and presence of sodium phytate shows very marginal change in the value of $s^0_{20,w}$. Thus, the aggregate found in the absence of sodium phytate, i.e., acid-induced product, is different from the polymer generated in the presence of sodium phytate.

Velocity sedimentation profiles of α-globulin at different protein concentrations at a 1 × 10^{-4} M sodium phytate concentration indicate that at low concentrations of α-globulin the polymer is not seen. Above a 5 mg/mL protein concentration, the fast moving component (polymer) forms a Schlieren peak and increases with increase in protein concentration. This is quantitated in Figure 4. These data demonstrate that there exists a critical concentration of the protein which is necessary for the formation or recognition of the polymer at a defined free concentration of sodium phytate.

The binding of sodium phytate to α-globulin, investigated by equilibrium dialysis, indicates that initially the binding is very rapid, reaching a value of 11 binding sites and increasing further to 20 binding sites (C_{Eq} = 1.65 × 10^{-3} M) as shown in the Figure 5 inset. The data indicate that there is more than one interacting site on α-globulin for sodium phytate. The value increases progressively above 1 × 10^{-4} M and ascends very rapidly above 1 × 10^{-3} M ligand concentration. Most of the changes are seen in the region between 1 × 10^{-4} M and 1 × 10^{-3} M ligand concentration. At a 1.65 × 10^{-3} M ligand concentration, a value of 20 is obtained from the above data. The Scatchard plot of the data shows that the binding profile can be fit into two sets: one below 7 ± 1 binding sites and the other above it (Figure 5). The low-affinity binding

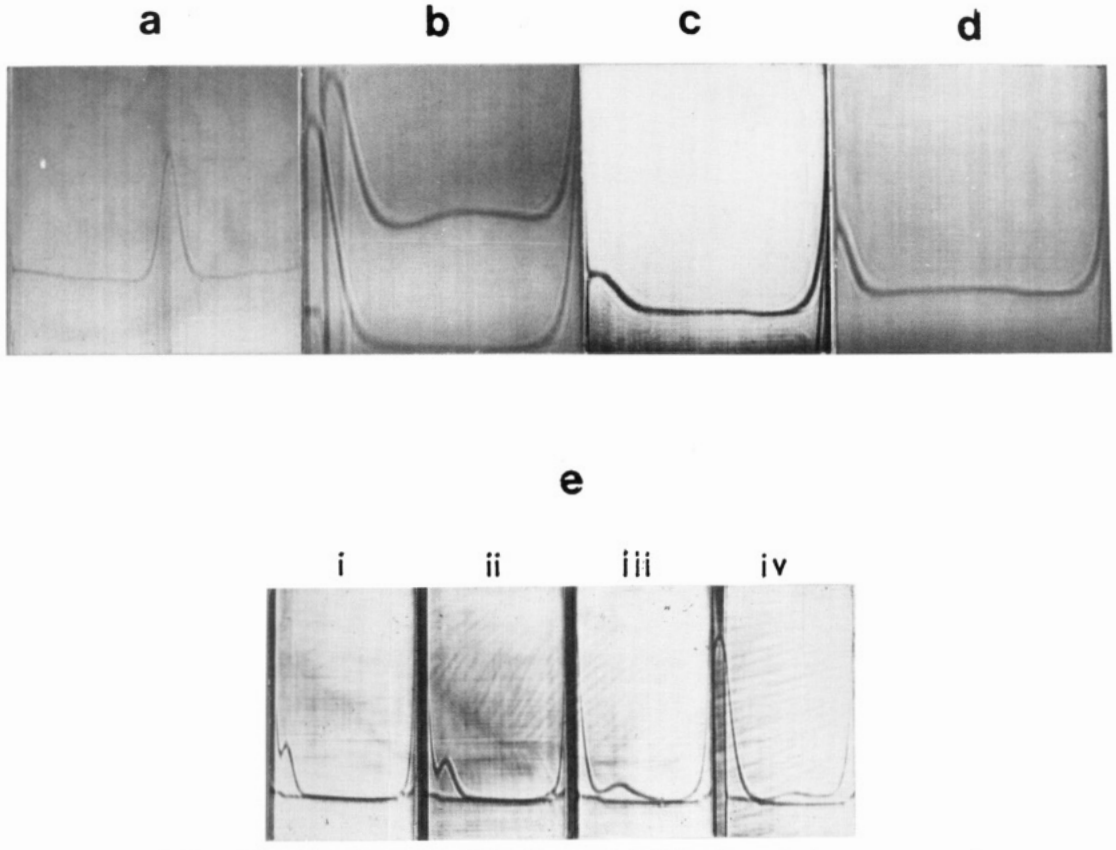

FIGURE 2: Sedimentation velocity patterns of α-globulin in GH buffer at 27 ± 1 °C using a single sector centerpiece with quartz windows. The photographs were taken at specified bar angles and times after reaching two-thirds maximum speed which is indicated in parentheses along with concentrations of sodium phytate (M) and α-globulin (mg/mL): (a) pH 7.5, 13.5 mg/mL (60° 35 min); (b) 5×10^{-5} M, 15.7 mg/mL (upper), 5×10^{-4} M, 15.7 mg/mL (lower) (60°, 45 min); (c) 1×10^{-3} M, 16 mg/mL (60°, 61 min); (d) 1.0×10^{-3} M, 16.08 mg/mL (60°, 29 min); (e) formation of polymer peak of α-globulin in GH buffer containing 1×10^{-4} M free concentration of sodium phytate at 27 ± 1 °C. A standard 12-mm Kel-F double-sector centerpiece having protein in one sector and GH buffer containing 1×10^{-4} M sodium phytate in another sector was used. All photographs were taken at a bar angle of 60° unless otherwise mentioned. A protein concentration of 22 mg/mL was used. The times of photographs after attaining two-thirds maximum speed are (i) 13 min (70°), (ii) 17 min, (iii) 33 min, and (iv) 48 min.

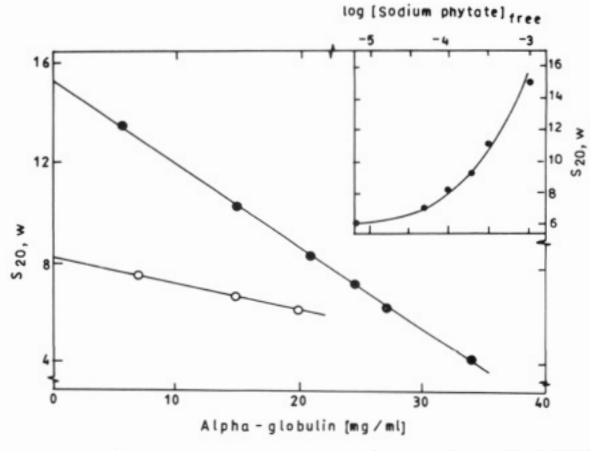

FIGURE 3: Effect of protein concentration on the sedimentation coefficient of (i) acid-induced aggregate of α-globulin (○) and (ii) sodium phytate induced polymer at 1×10^{-4} M sodium phytate concentration (●). (Inset) Effect of sodium phytate concentration on the sedimentation coefficient of the polymer of α-globulin in GH buffer as monitored by sedimentation velocity in an analytical ultracentrifuge at 27 ± 1 °C. A constant α-globulin concentration of 20 mg/mL was used in all the experiments. The concentration values of the slow moving species are the apparent concentrations through which this species sediments.

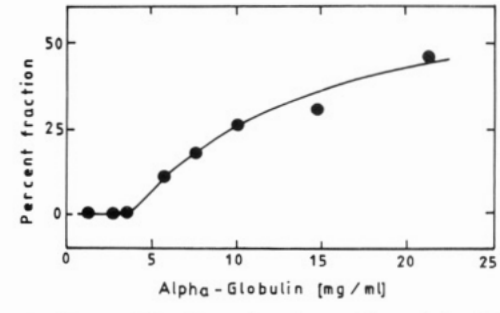

FIGURE 4: Percent fraction of polymer formed in GH buffer containing 1×10^{-4} M sodium phytate as a function of α-globulin concentration as monitored by velocity sedimentation patterns in analytical ultracentrifuge at 27 ± 1 °C.

sites (7 ± 1) have a K value of 1.29×10^{-4} mol^{-1} and the K value of high-affinity binding sites (56 ± 3) is 2.8×10^{-3} mol^{-1}.

The effects of increasing concentration of sodium phytate on the fluorescence emission spectra of the protein show both quenching and shift in the fluorescence emission maximum. The quenching of fluorescence is seen in the range of 1×10^{-4} M to 1×10^{-2} M. Up to 1×10^{-5} M sodium phytate concentration, no change is observed in the fluorescence emission maximum indicating that the tryptophan residues have not undergone any change as compared to the control protein. Upon an increase in the sodium phytate concentration, the emission maximum shifts to 340 nm from 332 nm of the control protein at pH 3.2 (5×10^{-3} M), where it remains constant even with a further increase in ligand concentration.

α-Globulin is rich in β and aperiodic structures as reported in earlier publications from this laboratory by Prakash et al. (1980). At acidic pH, the protein shows higher rotation (Figure 6a). In presence of sodium phytate there is a reduction in ellipticity with a further shift in minimum which indicates

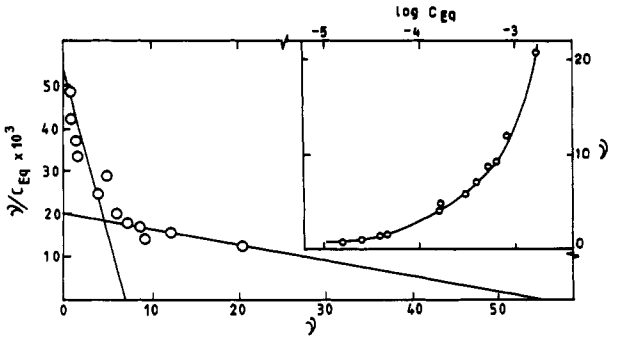

FIGURE 5: Scatchard plot of binding isotherm. (Inset) Relationship of a number of moles of sodium phytate bound per mole of α-globulin (γ) to the logarithm of equilibrium concentration of sodium phytate (log C_{Eq}) at 40 °C. The concentration of α-globulin was 1.8×10^{-5} M.

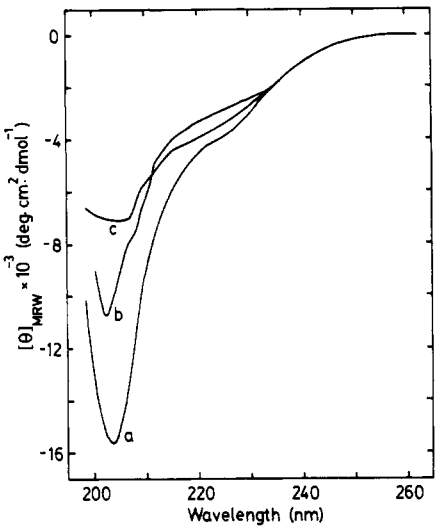

FIGURE 6: Far-ultraviolet circular dichroic spectra of α-globulin in phosphoric acid at pH 2.3, as a function of the following sodium phytate concentrations: (a) without sodium phytate and (b) 1×10^{-5} M and (c) 5×10^{-5} M at 27 ± 1 °C.

a gross conformational change upon sodium phytate binding to the protein (Figure 6b,c).

The fast kinetics of α-globulin sodium phytate interaction indicates that the increase in absorbance at 600 nm against time (up to 100 s) follows a hyperbolic curve. The analysis of the data by a plot of ln ($\Delta A/\Delta A_\infty$) versus time up to 30 s fits into straight line. The calculated rate constants are 52.4×10^{-3} s^{-1} and 67.5×10^{-3} s^{-1} for sodium phytate concentrations of 1×10^{-4} M and 5×10^{-4} M, respectively. The slow kinetics also shows pseudo-first-order kinetics. The rate constants are 4.3×10^{-3} s^{-1} and 2.9×10^{-3} s^{-1} for equilibrium sodium phytate concentrations of 1×10^{-4} M and 5×10^{-4} M, respectively. These data show that the reaction follows pseudo-first-order kinetics.

DISCUSSION

The turbidimetric titration of α-globulin with sodium phytate indicates that (i) the protein–sodium phytate complex precipitates, (ii) the extent of precipitation is protein concentration dependent, and (iii) the precipitation starts at higher sodium phytate concentration as the protein concentration is increased (Figure 1, inset). The sigmoidal curve of precipitation isotherm plateaus at 1×10^{-3} M sodium phytate concentration, indicating the saturation of α-globulin molecule at this concentration of sodium phytate. This binding does not appear to be cooperative as the process is spread over a nearly 100-fold concentration range (Figure 1).

Table I: Percent Fraction and Sedimentation Coefficient of Different Components as a Function of Sodium Phytate Concentrations

		12.8S		8.3S		1.5S	
		%	s	%	s	%	s
1	pH 7.4[a]	100	12.8[b]	–	–	–	–
2	pH 2.3[c]	–	–	35	8.3[d,g]	65	1.5[e]
3	1.0×10^{-5} M[f]	–	–	30		70	1.5[e]
4	3.2×10^{-5} M[f]	–	–	26		74	1.5[e]
5	5.0×10^{-5} M[f]	–	–	23	7.1[g]	77	1.5[e]
6	1.0×10^{-4} M[f]	–	–	20	8.2[g]	80	1.5[e]
7	2.5×10^{-4} M[f]	–	–	13	9.3[g,h]	87	1.5[e]
8	4.0×10^{-4} M[f]	–	–	5	11.1[g,i]	95	1.5[e]
9	5.0×10^{-4} M[f]	–	–	4		96	1.5[e]
10	1.0×10^{-3} M[f]	–	–	3	15.0[g]	97	1.5[e]
11	5.0×10^{-3} M[f]	–	–	–	j	100	1.5[e]
12	1.0×10^{-2} M[f]	–	–	–	j	100	1.5[e]

[a] Native α-globulin in 0.05 M sodium phosphate buffer. [b] The value is $s^0_{20,w}$ of native hexamer. [c] In GH buffer. [d] $s^0_{20,w}$ of aggregate component in the absence of sodium phytate. [e] $s^0_{20,w}$ of low molecular weight component. [f] Free concentration of sodium phytate in GH buffer. [g] Values are $s_{20,w}$ of the polymer formed at 20 mg/mL α-globulin concentration in the presence of respective sodium phytate free concentration. [h] The free sodium phytate concentration is 2×10^{-4} M. [i] The free sodium phytate concentration is 3.2×10^{-4} M. [j] The polymer precipitates out.

What is the mechanism of precipitation of α-globulin upon sodium phytate binding? The analysis of velocity sedimentation data partly answers this question. α-Globulin at pH 7.4 has a $s^0_{20,w}$ value of 12.8S (Figure 2a). At pH 2.3, α-globulin forms a 8.3S aggregate and a 1.5S component (Figure 2b, upper). The 8.3S component disappears with a concomitant increase in the 1.5S component with an increasing concentration of free sodium phytate. This is accompanied by self-association of aggregate components into soluble polymers with defined $s_{20,w}$ values depending upon the concentration of free sodium phytate (Figure 2b, lower, and 2c). The shape of the polymer peak is skewed because of the presence of polymers of different sedimentation rates, moving differently at various sodium phytate concentrations. These are all in equilibrium with the monomer. These results are tabulated in Table I, and they show that sodium phytate interacts preferentially with the aggregates leading to further polymerization. This type of bimodality in a sedimentation velocity pattern of a homogeneous protein can be due to several possibilities as explained by Cann (1970), Gilbert and Gilbert (1973), and Prakash and Timasheff (1986). According to the Cann and Goad and Gilbert and Gilbert systems, the minima never descend to baseline even in the later stages of the experiment. In the case of α-globulin–sodium phytate interaction, the descent of the minima to the baseline suggests that the system is comprised of two independent components: (i) a stable 1.5S component and (ii) the polymer (Figure 2e). This is further confirmed by the $s_{20,w}$ value and the fact that the concentration of polymer formed increases with an increase in sodium phytate concentration (Figure 3, inset). This is in accordance with the Wyman theory according to which the ligand binding should favor polymer formation as well as an increase in the size of the polymer formed (Wyman, 1964). Such a drastic change in the size of the molecule can occur only by preferential binding of ligand to polymer, favoring polymer formation.

At what stage does the sodium phytate binding induce association of the protein? Above 5 mg/mL α-globulin concentration, the polymer is seen at a constant free sodium phytate concentration of 1×10^{-4} M, and the polymer concentration increases, with an increase in protein concentration (Figure 4). This shows that there is a critical concentration for the appearance of a rapid peak. This

progressive reaction in the presence of ligand favored by protein concentration can be explained by the following scheme:

$$2P \xrightarrow[\text{phytic acid}]{K_2} P_2$$

$$P_2 + P \xrightarrow[\text{phytic acid}]{K_3} P_3$$

$$P_{n-1} + P \xrightarrow[\text{phytic acid}]{K_n} P_n$$

where P is the monomer protein molecule and K's are the association constants and n is the induced polymer.

At the P_n formation point, the reaction stops; i.e., the final step is not identical with all the other steps as it could be the terminal step. Similar results are observed in the case of tubulin interaction with various ligands (Prakash & Timasheff, 1986).

At what stage does the soluble aggregate formed precipitate out? As seen earlier, sodium phytate binding results in soluble aggregates of different sizes and concentrations. The average size of the polymer increases with an increase in ligand concentration. This chain reaction of higher polymer formation increases the size of the polymer to a size which ultimately precipitates out of solution.

The binding isotherm indicates that there are multiple interacting sites on α-globulin for sodium phytate (Figure 5). Also, it supports the hypothesis that there are two classes of binding sites (Figure 5). The analysis of more points at higher sodium phytate concentrations was hindered by precipitation of α-globulin.

Several earlier reports indicate that the interaction of phytic acid with globular proteins induces conformational changes in them (Prakash & Narasinga Rao, 1986; Mothes et al., 1990). The expected large changes in conformation of α-globulin upon sodium phytate binding are in fact supported by far-ultraviolet circular dichroic spectra (Figure 6). Deshpande and Damodaran (1989) reported that sodium phytate binding resulted in conformational changes in the secondary structure of both trypsin and chymotrypsin which influenced their activity. McConnell et al. (1991) reported that the Epstein–Barr virus transformed B lymphocytes contain substantial amounts of multiple isomers of $InsP_5$, which is supposed to be the precursor of $InsP_6$. Recently, it has been shown that activation of appropriate cell surface receptors stimulates the hydrolysis of a membrane lipid, phosphatidylinositol 4,5-bisphosphate generating, Ins[1,4,5]P_3, which is recognized as an authentic second messenger in intracellular communication. This has been shown to act by controlling the mobility of calcium ions (Nishizuka, 1984; Streb et al., 1983; Carpenter et al., 1989). In the case of α-globulin, polymer formation follows the initial conformational change that occurs even at 1×10^{-5} M ligand concentration. These initial conformational changes in the protein molecule may possibly be the rate limiting step at such low concentrations of sodium phytate ion in most of the proteins.

The investigation of reaction kinetics show that the interaction has an initial fast reaction component which may be the rate-limiting conformational step and is dependent on ligand concentration. This fast reaction could be the first step in the formation of precipitate. This is followed by the slow reaction component, the protein–protein interaction. This protein–protein interaction leading to polymer formation is favored by the large excess of ligand in the solution. These two steps of formation of precipitate on interaction of sodium phytate with α-globulin thus have different kinetic rates.

α-Globulin, a hexamer having a trimer–trimer arrangement of its six subunits, is predominantly held by hydrophobic interactions (Prakash, 1985). The protein dissociates by destabilization of this subunit assembly to an 8.3S and a 1.5S component at pH 2.3. Sodium phytate interacts maximally with α-globulin in this state. The aggregate and 1.5S protein components interact separately with sodium phytate leading to two classes of binding sites. Sodium phytate binding initially results in a conformational change of the protein. This is the first fast step of the two-step interaction which is complete by 30 s. This is followed by the second slow step of the progressive association of protein components to form polymers of definite sizes. The ligand preferentially binds to the polymers, favoring the polymer formation as well as increasing their hydrodynamic size. As the ligand concentration increases further, the polymer size exceeds a certain critical size, resulting ultimately in the precipitation of the protein.

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