Interaction of Myoinositoltrisphosphate−Phytase Complex with the Receptor for Intracellular Ca^{2+} Mobilization in Plants

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ABSTRACT: One of the myoinositol trisphosphates produced by the phytase−myoinositol hexakisphosphate ($InsP_6$) reaction is Ins(2,4,5)P_3. That Ins(2,4,5)P_3 can elicit Ca^{2+} mobilization from intracellular stores in plants [Samanta, S., Dalal, B., Biswas, S., & Biswas, B. B.(1993) *Biochem. Biophys. Res. Commun. 191*, 427] prompted us to elucidate the mechanism. The $InsP_3$ [Ins(1,4,5)P_3/Ins(2,4,5)P_3]−phytase complex has been found to interact with the receptor for $InsP_3$ *in vitro* forming a ternary complex, and a nanomolar concentration of $InsP_3$ is required. For enzymatic cleavage of $InsP_3$ by phytase, micromolar concentrations are needed, and the affinities of the phytase for different myoinositol phosphates have been found to depend upon the number of phosphate groups present in the substrate. Fraction accessibility of tryptophan residues to a neutral fluorescence quencher, acrylamide in free and myoinositol phosphate bound phytase, as determined by Stern−Volmer plot, records a progressive decrease starting from $InsP_6$ to InsP with the notable exceptions of both Ins(1,4,5)P_3 and Ins(2,4,5)P_3. This deviation from the trend of change in the accessibility of tryptophan residues in myoinositol phosphate bound phytase is recorded from the fact that there is a high affinity (dissociation constant of the nanomolar order) and noncatalytic binding site in phytase for the two isomers of $InsP_3$. In the nanomolar range of concentrations, both isomers of $InsP_3$ bind to a second site of phytase having about 40-fold higher affinity than the normal substrate binding site. $InsP_3$, when bound to noncatalytic site in phytase is not hydrolyzed but induces a significant change in the conformation of phytase as assayed from the relative accessibility of tryptophan residues. This conformational change in phytase is recognized by the receptor for $InsP_3$, because in absence of $InsP_3$ no interaction between the receptor and phytase is detected. However, $InsP_3$−phytase complex is a better elicitor of Ca^{2+} efflux from microsomal/vacuolar fractions than free $InsP_3$. This is further confirmed by the fact that when Ins(1,3,4)P_3−phytase complex can elicit Ca^{2+} efflux from the intracellular stores, Ins(1,3,4)P_3 *per se* is minimally effective.

Phytase or myoinositol hexakisphosphate phosphohydrolase, which hydrolyzes myoinositol hexakisphosphate ($InsP_6$)[1] to myoinositol and inorganic phosphate, is an enzyme that has varied characteristics from different sources (Loewus & Loewus, 1983; Biswas et al., 1984; Drobak, 1992). It has not been detected in the cotyledons of ungerminated seeds, but it appears upon germination (Biswas et al., 1984). $InsP_6$ accumulates in the seeds and other storage tissues in very significant amounts (Biswas et al., 1984; Loewus et al., 1990). Earlier studies from this laboratory (Maiti et al., 1974) showed the following pathway of degradation for $InsP_6$: $InsP_6 \rightarrow InsP_5 \rightarrow InsP_4 \rightarrow InsP_3 \rightarrow InsP_2 \rightarrow InsP_1 \rightarrow$ Ins. The nature of the different isomers in terms of the position of phosphate residues formed as intermediates and their relative distribution are not yet well established (Maitra et al., 1988). The reported myoinositol trisphosphate intermediates are Ins(2,4,5)P_3 and Ins(1,2,3)P_3 in the stepwise dephosphorylation by phytase in germinating mung bean seeds and in pollen, respectively (Maitra et al. 1988; Loewus et al., 1990). Ins(1,4,5)P_3 is now established as an intracellular second messenger in plants (Trewavas & Gilroy, 1991; Drobak, 1992). Ins(2,4,5)P_3, one of the intermediary products of phytase and $InsP_6$, has also been found to be effective in stimulating release of Ca^{2+}, though to a lesser extent than Ins(1,4,5)P_3 (Samanta et al., 1993). The presence of low amounts of ptd$InsP_2$ along with large amounts of $InsP_6$ suggests an alternative pathway for generation of $InsP_3$. In animal systems, other homologs of $InsP_3$ are formed as a sequence of specific phosphorylation and dephosphorylation of Ins(1,4,5)P_3, though their physiological role is not known (Shears et al., 1987). $InsP_3$, the transient product of the reaction involving phytase and $InsP_6$, could lead to mobilization of Ca^{2+}. Preliminary observations reported from our laboratory suggest the possibility of an alternative pathway for $InsP_3$ generation, particularly Ins(2,4,5)P_3, that might play an important role in Ca^{2+} release from intracellular stores (Samanta et al., 1993).

Mobilization of Ca^{2+} from microsomal/vacuolar fractions was detected when a mixture of $InsP_6$ and phytase was added after a definite time of hydrolysis that coincides with the time of production of $InsP_3$ (Samanta et al., 1993). Another

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[1] Abbreviations: $InsP_n$ ($n = 1-6$), D-myoinositol phosphate where n denotes the number of phosphate groups; ptd$InsP_2$, phosphatidylinositol-4,5-bisphosphate; PVP, polyvinylpyrrolidone; Quin-2, (2-[2-bis-[carboxymethyl]amino-5-methylphenoxy)methyl]-6-methoxy-8-bis-[carboxymethyl]aminoquinoline; βME, β-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; ELISA, enzyme-linked immunosorbent assay.

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notable observation from ligand competition experiment was that a mixture of Ins(1,4,5)P_3 or Ins(2,4,5)P_3 and phytase was more effective than Ins(1,4,5)P_3 or Ins(2,4,5)P_3 alone in displacing [^{3}H]Ins(1,4,5)P_3 bound to microsomal/vacuolar fractions containing the putative receptor for InsP_3. It was also noticed that addition of a mixture of Ins(1,4,5)P_3 or Ins-(2,4,5)P_3 and phytase leads to enhanced Ca^{2+} release relative to that observed with free InsP_3 (Samanta et al., 1993). Both Ins(1,4,5)P_3 and Ins(2,4,5)P_3 showed similar trends in competition and Ca^{2+} release from the microsomes/vacuoles. Initially these observations gave an indication that those InsP_3−phytase complexes *per se* might interact with the putative receptor for InsP_3 associated with the microsomal/vacuolar membrane. Though Ins(2,4,5)P_3 is the phytase product, Ins(1,4,5)P_3 was used for the receptor binding and other complex formation for comparison. The present studies aim to provide experimental data to verify the above proposition for the role of phytase in intracellular Ca^{2+} mobilization in plants.

MATERIALS AND METHODS

InsP_6, Ins(1,3,4)P_3, and Ins(1,3,4,5)P_4 were purchased from Sigma Chemical Co. Ins(1,4,5)P_3 was obtained from Calbiochem. [^{3}H]Ins(1,4,5)P_3 was obtained from NEN, DuPont. Ins(2,4,5)P_3 and Ins(4,5)P_2 were from Boehringer Manheim. All other reagents were of analytical grade and purchased from local markets. Mung bean (*Vigna radiata* B_1) seeds were obtained from Seed Multiplication Farm, Behrampur, West Bengal, India.

Isolation of Phytase. Phytase was isolated from cotyledons of 72 h old germinating seeds as described earlier (Mandal et al., 1972). About 200 g of cotyledons was homogenized with 50 mM Tris-HCl buffer, pH 7.0. The homogenate was filtered through cheese cloth, and the filtrate was centrifuged at 10000*g* for 25 min. The supernatant was subjected to heat treatment at 57 °C for 6 min, cooled, and then centrifuged at 10 000*g* for 20 min. The supernatant was 50% saturated with ammonium sulfate and then centrifuged for 20 min at 10 000*g*. The supernatant fraction was made 70% saturated with ammonium sulfate and centrifuged at 10 000*g* for 20 min. The pellet was dissolved in 50 mM Tris-HCl buffer, pH 7.0, and dialyzed overnight. An equal volume of cold (4 °C) acetone was added to the dialyzed fraction and centrifuged after being kept on ice for 5 min. The supernatant was made to 80% (v/v) with respect to acetone, and the mixture was kept on ice for 30 min. It was centrifuged and the resulting pellet was dissolved in the same buffer and dialyzed. The fraction was subjected to 5% native PAGE. The protein from the major band out of three corresponding to phytase was eluted, dialyzed, and tested for activity from estimation of liberated phosphate from the substrate, InsP_6, at 37 °C (Chen et al., 1956). Purity of the protein was confirmed by means of SDS−PAGE from the presence of a single band of 160 kDa. Contamination of nonspecific phosphatase in the purified enzyme was not detected because the preparation does not hydrolyze glucose 6-phosphate and *p*-nitrophenyl phosphate, substrates for phosphatase. For fluorescence spectroscopic studies, protein was eluted from a 5% nondenaturing gel, and then it was passed through Sephadex G-75 (Superfine) to remove possible contaminants from the gel. The ratio of fluorescence intensities at 340 and 400 nm ($\lambda_{ex} = 295$ nm) of the enzyme was monitored as an index of removal.

Preparation of Antibody for Phytase. Polyclonal antibody was raised in rabbits by injecting 100 μg of purified phytase in Freund's complete adjuvant subcutaneously. Booster injections of 100 μg of protein were given at intervals of 3 weeks in Freund's incomplete adjuvant. The titers of sera were tested by immunoprecipitation. The immunoglobulin fraction was isolated by ammonium sulfate precipitation and purified by DEAE-Cellulose (Pal et al., 1990).

Fluorescence Spectroscopic Studies. All fluorescence spectra were recorded with either a Hitachi F-4010 in Computer Average Time scan mode for each spectrum or a Shimadzu F-540 spectrofluorometer. For evaluation of the dissociation constants between phytase and its substrates, small aliquots of myoinositol phosphate were added to a fixed concentration of phytase. That the interaction between the enzyme and respective substrate had attained equilibrium was indicated from the absence of any change in the emission spectrum of the enzyme with time. Excitation wavelengths were 278 or 295 nm (selective excitation of the tryptophan residues). All studies were carried out in 50 mM Tris-HCl buffer, pH 7.0, at 14 °C. Appropriate subtraction of the contribution from the buffer was done. Correction due to the inner filter effect was not made because the absorbance of the samples did not exceed 0.03. Any dilution effect was considered for the estimation of the substrate induced quenching in fluorescence of the enzyme. The dissociation constant for enzyme−substrate interaction was calculated from the following equation:

$$1/\Delta F = 1/\Delta F_{max} + K_d/(\Delta F_{max}[S]) \quad (1)$$

where [S] denotes the concentration of the substrate. ΔF is the extent of quenching of the enzyme fluorescence at 340 nm as a function of the input concentration of the substrate. The ratio of the slope and intercept of the straight line from the plot of $1/\Delta F$ against 1/[S] gives the value of K_d (Wang & Edelman, 1971).

The affinity of Ins(1,4,5)P_3/Ins(2,4,5)P_3 for its receptor was evaluated from the ligand-induced quenching of the fluorescence of the receptor at $\lambda_{ex} = 295$ nm. The emission intensity of the receptor at 340 nm was plotted as a function of input concentration of the ligand to obtain the binding isotherm. The affinity of Ins(1,4,5)P_3 for the receptor was also determined by the filter binding assay using [^{3}H]Ins-(1,4,5)P_3.

A neutral fluorescence quencher, such as acrylamide (2× crystallized, Sigma Chemical Co.) was used as a probe to evaluate the accessibility of the tryptophan residues in free and inositol phosphate bound enzyme. Fluorescence quenching data were analyzed according to the Stern−Volmer equation: $F_0/F = 1 + K_{sv}[Q]$, where F_0 and F are initial and final fluorescence intensities of the protein, respectively. K_{sv} is the quenching constant, and [Q] denotes the input concentration of acrylamide. A modified Stern−Volmer plot (Lehrer & Laevis, 1978) was also done with a view to estimating the fraction of accessible (f_e) tryptophan residues that was evaluated from the following equation:

$$F_0/(F_0 - F) = 1/(K_{sv}f_e[Q]) + 1/f_e \quad (2)$$

Preparation of Microsomes/Vacuoles. Hypocotyl from the 48 h germinating seeds were homogenized in buffer A containing 25 mM Tris-HCl buffer, pH 8.0, 0.25 M sucrose,

3 mM EDTA, 10 mM βME, 1 mM PMSF, 1 mM benzamidine, and 10 g/L PVP. The homogenate was passed through two layers of cheese cloth. It was then centrifuged at 10000*g* for 40 min at 4 °C. The supernatant was centrifuged at 80000*g* for 55 min at 4 °C. The microsomal/vacuolar pellet after centrifugation was suspended in buffer B, 2.5 mM Tris-MOPS, pH 7.2, containing 0.25 M sorbitol and layered on a discontinuous gradient of 7% and 12% dextran in buffer B, respectively. This was centrifuged in a SW55 rotor at 80000*g* for 2 h at 4 °C. The layer between 7% and 12% was collected and mixed with 4 volumes of buffer C (4 mM Tris-HCl buffer, pH 7.2, 100 mM KCl, 0.25 M sucrose, and 2 mM $MgCl_2$) and pelleted at 80000*g* for 45 min at 4 °C. This pellet was resuspended in buffer C for Ca^{2+} uptake and release experiments.

Assay of Ca^{2+} Release. The concentration of free Ca^{2+} in the incubation medium was determined using the Ca^{2+} indicator fluorescent dye Quin-2 in a Hitachi F-3010 spectrofluorometer. The medium consisted of a suspension of microsomal/vacuolar fraction in buffer C (124 μg/mL of protein), 3 mM sodium azide, 100 μM Quin-2, and 100 μM $CaCl_2$. This was incubated at 25 °C, and ATP was added to a final concentration of 2 mM to initiate the uptake of Ca^{2+} by the microsomes/vacuoles. The uptake was monitored from the decrease in fluorescence of Quin-2 (λ_{ex} = 339 nm, λ_{em} = 492 nm). After the process of uptake had reached equilibrium as indicated from the absence of any further significant change in the fluorescence, $InsP_3$ or $InsP_3$−phytase complex was added to stimulate the Ca^{2+} release. $InsP_3$−phytase complex was made separately by incubating appropriate concentrations of the two components at 25 °C. Concentration of Ca^{2+} was calculated from the following relation (Mazorow & Millar, 1990):

$$[Ca^{2+}] = K_d(F - F_{min})/(F_{max} - F) \quad (3)$$

where K_d, F_{max}, and F_{min} denote the dissociation constant (115 nM) for Quin2−Ca^{2+} interaction, maximum fluorescence in the presence of 2 mM $CaCl_2$, and minimum fluorescence in the presence of 5 mM EGTA, respectively. F is the observed fluorescence upon addition of ATP and the Ca^{2+} mobilizing agents. The release of Ca^{2+} was monitored after a time period of 20 s. The concentration of Ca^{2+} released was expressed as nanomolar (nM)/mg of protein to make it independent of the volume of the assay medium.

Purification of Putative Ins(1,4,5)P_3 receptor (InsPR). The receptor was isolated from the microsomal/vacuolar fraction of mungbean hypocotyl and purified as described (Biswas et al., 1995). The receptor activity was monitored from [^{3}H]-Ins(1,4,5)P_3 binding to the receptor (Miyanaki et al., 1991).

Evaluation of Dissociation Constants. Different concentrations of [^{3}H]Ins(1,4,5)P_3 (specific activity, 0.677 μCi/nmol) were incubated with a fixed concentration of phytase (100 nM) in 50 mM Tris-HCl buffer, pH 8.0, plus 25 mM NaCl at 5 °C. After 15 min, the incubation mixture was passed through Whatman GF/C filter paper to separate the free ligand from enzyme-bound ligand. The filter paper was washed with the same buffer and dried for determination of radioactivity. Nonspecific binding was checked in the presence of an excess of unlabeled $InsP_3$, and the appropriate value was subtracted from the total counts to construct the binding isotherm.

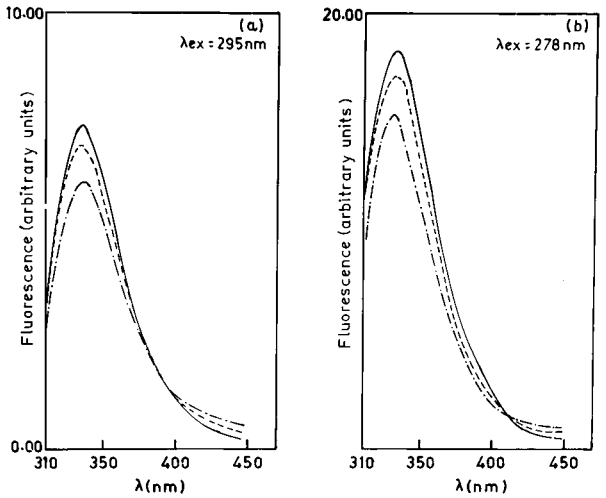

FIGURE 1: Fluorescence emission spectrum of phytase (0.3 μM): alone (—) and in the presence of $InsP_6$ (2 μM, - - - and 8.7 μM, —·—) corresponding to excitation wavelengths of (a) 295 nm and (b) 278 nm in 50 mM Tris-HCl buffer, pH 7.0, at 14 °C.

The binding of $InsP_3$ to purified receptor isolated from microsomes /vacuoles was estimated as follows. Different concentrations of [^{3}H]Ins(1,4,5)P_3 (specific activity, 0.677 μCi/nmol) were added to the receptor (250 nM) in a total volume of 100 μl in 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM βME, 1 mM EDTA, and 1 mM PMSF. It was incubated at 5 °C for 15 min. Extent of binding was monitored by filter binding assay as described above.

In Vitro Formation of a Ternary Complex between Receptor and $InsP_3$−Phytase Complex. This was determined as follows. The mixture of three components, [^{3}H]Ins(1,4,5)-P_3 (0.3 μM; specific activity, 1.54 μCi/nmol), putative receptor (0.3 μM), and phytase (0.3 μM), was incubated in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM βME, 3 mM EDTA, and 25 mM NaCl for 15 min at 5 °C. It was passed through Sephadex G-200 (column size: 1 cm × 20 cm; fractionation range: 5−600 kDa) in order to detect the formation of the ternary complex. Formation of ternary complex was also checked by fluorescence detection of the peaks at an emission wavelength of 340 nm. This was particularly useful to detect the formation of ternary complex in the case of nonradioactive Ins(2,4,5)P_3 and Ins(1,3,4)P_3.

Formation of the ternary complex was also determined by fluorescence spectroscopy, e.g., phytase (100 nM) was added to an equilibrium mixture of the receptor (20 nM) and Ins(1,4,5)P_3 or Ins(2,4,5)P_3 (400 nM) because the above concentration of the ligand is necessary to bind the receptor. Fluorescence of the mixture was compared with that for the free phytase at the same concentration in 50 mM Tris-HCl buffer, pH 8.0, containing 25 mM NaCl at 5 °C after appropriate subtraction of any contribution from $InsP_3$−InsPR complex. As a control experiment, the same concentration of the receptor was added to the phytase to check its effect upon the fluorescence spectrum of phytase.

RESULTS AND DISCUSSION

Interaction of Myoinositol Phosphates with Phytase. Phytase has fluorescence with an emission peak at 335 nm for excitation wavelengths of 278 and 295 nm. Progressive quenching of the fluorescence upon addition of myoinositol phosphate(s), as shown for the representative example with $InsP_6$ (Figure 1a,b), indicates binding of the substrate with

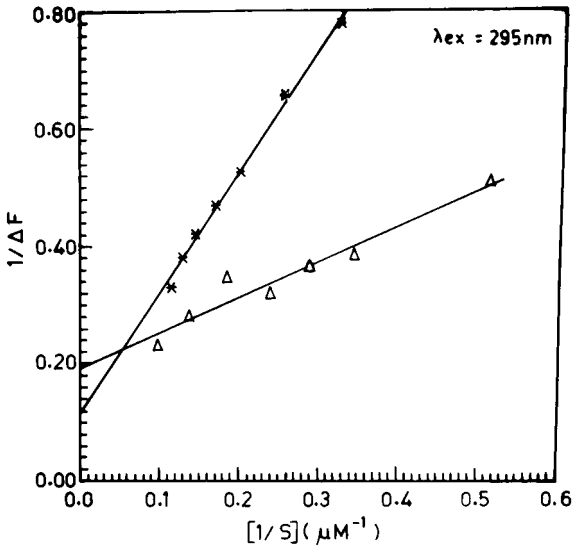

FIGURE 2: Plot of $1/\Delta F$ against 1/[S] for the interactions of phytase with different ligands: $InsP_6$ (∗) and Ins(1,4,5)P_3 (△) corresponding to an excitation wavelength of 295 nm in 50 mM Tris-HCl buffer, pH 7.0, at 14 °C.

Table 1: Dissociation Constants (K_d) for Interactions of Phytase with Different Myoinositol Phosphates[a]

ligand	dissociation constant (μM)
Ins(1,2,3,4,5,6)P_6	17.7 (22.1)[b]
Ins(1,3,4,5,6)P_5	8.8
Ins(1,3,4,5)P_4	8.0
Ins(1,4,5)P_3	3.1 (3.1)
Ins(2,4,5)P_3	5.3
Ins(4,5)P_2	2.1

[a] Values in 50 mM Tris-HCl buffer, pH 7.0, at 14 °C as calculated from fluorescence titration at $\lambda_{ex} = 295$ nm. Mean of three sets of estimation with standard deviation of 20% are mentioned above. [b] Fluorescence titration was done at $\lambda_{ex} = 278$ nm.

the enzyme. The extent of quenching depends upon the nature of myoinositol phosphates (data not shown). A single crossover point characterizes the set of spectra (Figure 1) and indicates the formation of a single type of complex between the enzyme and myoinositol phosphates over the range of ligand concentrations used in this study. Quenching of fluorescence corresponding to an excitation wavelength of 295 nm originates from either a change in the conformation of the enzyme induced by myoinositol phosphates and/or the presence of electronegative phosphate groups in the vicinity of the tryptophan residues. The release of inorganic phosphate from the substrates due to interaction with phytase is insignificant over the time in which the fluorescence change has been recorded at 14 °C .

The dissociation constants, K_d, for the association of phytase with myoinositol phosphates were evaluated from the quenching of fluorescence of the enzyme. Representative plots of $1/\Delta F$ against 1/[S] are plotted according to eq 1 for $InsP_6$ and Ins(1,4,5)P_3 (Figure 2). Results are summarized in Table 1. Values of the dissociation constants for a particular set of phytase−inositol phosphate interactions are independent of the excitation wavelengths. On the other hand, these values are dependent on the nature of the myoinositol phosphates. The dissociation constant decreases with the decrease in number of phosphate residues.

Conformational Change of Phytase. We compared the accessibilities of tryptophan residues in free and myoinositol

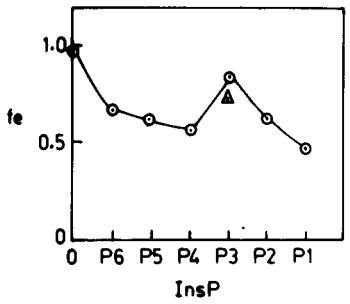

FIGURE 3: Plot of tryptophan accessibility of phytase against different myoinositol phosphates: The symbol (△) denotes the value for Ins(2,4,5)P_3. In all cases acrylamide was added to an equilibrium mixture of phytase (0.3 μM) and myoinositol phosphate (25 μM) in 50 mM Tris-HCl buffer, pH 7.0, at 14 °C. The initial point on the ordinate refers to the value for free phytase. The accessibility value is the mean of three sets of experiments with a standard deviation of 10%.

phosphate bound phytase with a view to checking whether there is any ligand-induced change in the conformation of the enzyme. Stern−Volmer plots were constructed from progressive quenching of fluorescence of enzyme−ligand complex as a function of the concentration of acrylamide (data not shown). Knowledge of the dissociation constants for different phytase−$InsP_n$ interactions (Table 1) helped us to fix the concentration of $InsP_n$ such that there was a homogeneous population of enzyme−ligand complex to start with. The slopes of the resultant plots were different; therefore, modified forms of the Stern−Volmer plots, as stated under Materials and Methods, were drawn with the same set of data. A graphical representation of accessibility as a function of the number of phosphate groups in the myoinositol phosphate is presented in Figure 3. The notable feature is that there is a progressive decrease in accessibility starting from $InsP_6$ to InsP with the exceptions of both Ins(1,4,5)P_3 and Ins(2,4,5)P_3 (other $InsP_3$ not tested). The change in accessibility could originate from a conformational change in the enzyme.

Detection of a High Affinity Binding Site in Phytase Specific for $InsP_3$. Having established that the conformational change of phytase by $InsP_3$ does not follow the general trend shown for other $InsP_n$ ($n \neq 3$), we attempted to check the possibility that there was a second high affinity noncatalytic binding site in phytase for Ins(1,4,5)P_3. In fact, a second high affinity site for Ins(1,4,5)P_3 in the enzyme was detected. Figure 4 shows the relevant binding isotherm for the high affinity binding between the enzyme and Ins(1,4,5)P_3, as monitored from the filter binding assay using [^{3}H]-Ins(1,4,5)P_3. The dissociation constant evaluated by this method was 75 ± 10 nM. The noncatalytic nature of the site was tested from the absence of any phosphate release under the above conditions at 37 °C. The binding of Ins(1,4,5)P_3 to this site did not perturb the fluorescence spectrum of phytase to a major degree; hence the association could not be detected by the fluorescence assay. The specificity of Ins(1,4,5)P_3 for this site is demonstrated from the observation that a 50-fold excess of $InsP_6$, Ins(1,3,4,5)P_4, Ins(4,5)P_2, and Ins-2-P did not displace Ins(1,4,5)P_3 from phytase (Table 2). The presence of such a high affinity site in phytase for Ins(2,4,5)P_3/Ins(1,3,4)P_3 was detected from similar competition experiments in which displacement of prebound [^{3}H]Ins(1,4,5)P_3 from phytase upon addition of unlabeled Ins(2,4,5)P_3/Ins(1,3,4)P_3 was monitored. The

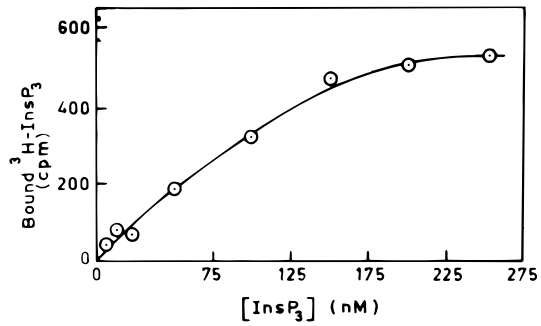

FIGURE 4: High affinity and noncatalytic binding of myoinositol trisphosphate to phytase: Representative binding isotherm for the interaction of phytase (0.1 μM) and Ins(1,4,5)P_3 in 50 mM Tris-HCl buffer, pH 8.0, plus 25 mM NaCl at 5 °C. The dissociation constant was calculated from the concentration of Ins(1,4,5)P_3 corresponding to half of the bound cpm.

Table 2: Competition of Other Myoinositol Phosphates for Ins(1,4,5)P_3 Binding to Phytase[a]

ligand (μM)	cpm of [^{3}H]Ins(1,4,5)P_3 displaced by the ligand
none	408 ± 51[b]
Ins(1,2,3,4,5,6)P_6 (15)	423 ± 17
Ins(1,3,4,5)P_4 (0.3)	413 ± 37
Ins(1,4,5)P_3 (0.3)	278 ± 20
Ins(2,4,5)P_3 (0.3)	233 ± 13
Ins(1,3,4)P_3 (0.5)	215 ± 29
Ins(4,5)P_2 (15)	469 ± 40
Ins-2-P (15)	420 ± 19

[a] [^{3}H]Ins(1,4,5)P_3−phytase complex was obtained by mixing phytase (0.1 μM) with the radiolabeled Ins(1,4,5)P_3 (0.3 μM; specific activity, 0.677 μCi/nmol) in 50 mM Tris-HCl buffer, pH 8.0, containing 25 mM NaCl at 5 °C for 15 min. It was then competed with various myoinositol phosphates at the concentrations mentioned above. After 15 min of incubation, the samples were spotted on GF/C filter paper, dried, and counted for radioactivity. Nonspecific binding was determined by using 100-fold excess of cold Ins(1,4,5)P_3, this value was subtracted from total binding to obtain the specific binding. [b] Mean value obtained from three sets of experiments with different batches of phytase.

results (Table 2) show that Ins(2,4,5)P_3/Ins(1,3,4)P_3 could displace Ins(1,4,5)P_3 from its high affinity binding site in phytase. It further indicates a comparable affinity constant of Ins(2,4,5)P_3 for the high affinity binding site in phytase and a relatively lower value for Ins(1,3,4)P_3.

The binding of either of the two InsP_3 isomers to the noncatalytic site leads to a significant change in the conformation of phytase. This was demonstrated from the change in the accessibility of tryptophan residues of the enzyme upon binding of Ins(2,4,5)P_3 to its high affinity binding site in phytase (Figure 5). The accessibility of tryptophan residues to acrylamide for free phytase ($f_e = 0.98$) changed in the presence of Ins(2,4,5)P_3 ($f_e = 0.55$) and Ins-(1,3,4)P_3 ($f_e = 0.65$). A similar change in the accessibility took place when Ins(1,4,5)P_3 bound to the high affinity site in phytase ($f_e = 0.62$). The accessibility (f_e) values were different when low affinity (Figure 3) and high affinity (Figure 5) binding sites of InsP_3 in phytase are saturated. The high affinity binding and its effect upon the conformation of phytase may account for the exceptional influence of InsP_3 in terms of the substrate induced conformational change in phytase (Figure 3).

Formation of a Ternary Complex Involving Phytase, InsP$_3$, and InsPR. Having established the presence of a high affinity binding site in phytase for Ins(2,4,5)P_3 and Ins(1,4,5)-P_3, we checked the possibility of the formation of a ternary complex. The gel elution profile of the mixture in the case of Ins(1,4,5)P_3 when the three components were incubated is shown in Figure 6a,b. Peak positions were detected by both fluorescence and radioactivity. The elution profile shows the presence of four peaks (Figure 6b). The three peaks (II, III, and IV) correspond to the InsP_3−InsPR complex, the InsP_3−phytase complex, and InsP_3, respectively, as evidenced from the elution profile coinciding with that when InsPR, phytase, and InsP_3 were run separately through the column. The presence of a peak I to the left of peak II suggests the elution of a component with higher molecular mass than that of the InsP_3−InsPR complex (molecular mass 400 kDa, peak II). The same elution profile was detected from the fluorescence at 340 nm of the protein. The absence of peak IV is obvious because InsP_3 is not fluorescent. For Ins(2,4,5)P_3/Ins(1,3,4)P_3, the detection of the peaks was done only by fluorescence, because radio labeled isomers were not available. The number and position of the peaks were the same as obtained in the case of fluorescence detection of the peaks for Ins-(1,4,5)P_3.

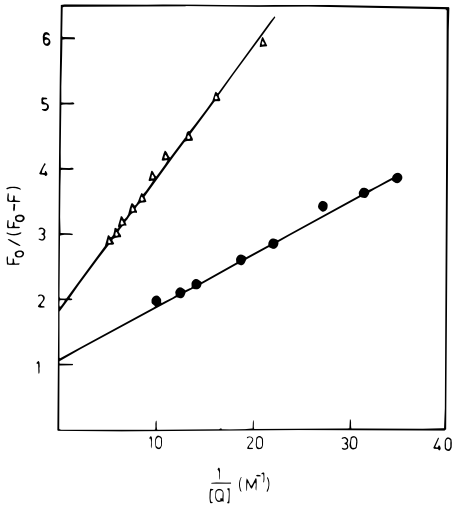

FIGURE 5: Comparison of the accessibilities of tryptophan residues in free and ligand bound (at high affinity site) phytase: Modified Stern−Volmer plots of $F_0/(F_0 - F)$ against 1/[Q] for free phytase (0.15 μM, ●) and Ins(2,4,5)P_3 (0.3 μM)−phytase (0.15 μM) complex (△) in 50 mM Tris-HCl buffer, pH 8.0, plus 25 mM NaCl at 5 °C. The concentration of Ins(1,3,4)P_3 is 1 μM for the detection of change in the accessibility of tryptophan residues in Ins(1,3,4)-P_3 bound phytase (data not shown).

The position of elution for peak I indicates that it corresponds to the ternary complex (mass = mass of phytase, 160 kDa + mass of InsPR, 400 kDa, = 560 kDa). This is also indicated from a standard calibration curve for molecular weight determination from elution volume (Figure 6c). It was further confirmed from the following properties of peak I: (i) the nature of its fluorescence spectrum ($\lambda_{ex} = 295$ nm) characteristic of a protein, (ii) its phytase activity devoid of any nonspecific phosphatase activity measured from standard assays, (iii) the presence of phytase as detected by means of ELISA ($A_{450nm} = 0.27$ for antibody blank and 0.35 for peak I) using phytase antibody, (iv) the presence of [^{3}H]Ins(1,4,5)-P_3, and (v) the receptor activity as indicated from its ability to bind Ins(1,4,5)P_3. The InsPR activity was measured by [^{3}H]Ins(1,4,5)P_3 binding assay after subjecting the peak to

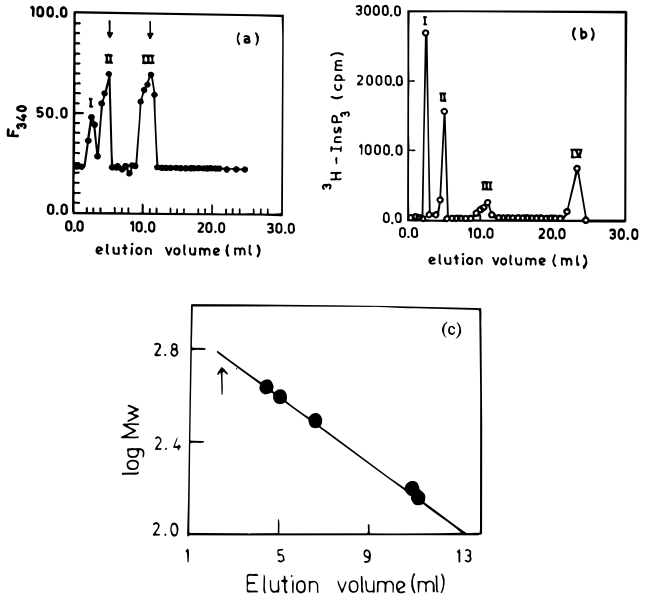

FIGURE 6: Formation of ternary complex $InsP_3$−InsPR−phytase: Gel filtration (Sephadex G-200) of a mixture of InsPR (0.3 μM), phytase (0.3 μM), and [^{3}H]Ins(1,4,5)P_3 (0.3 μM) incubated in 50 mM Tris-HCl, pH 8.0, containing 10 mM βME, 3 mM EDTA, and 25 mM NaCl, for 15 min at 5 °C. Peaks I, II, III, and IV refer to Ins(1,4,5)P_3−InsPR−phytase complex, Ins(1,4,5)P_3−InsPR complex, Ins(1,4,5)P_3−phytase complex, and Ins(1,4,5)P_3, respectively. (a) Plot of fluorescence (at 340 nm corresponding to λ_{ex} = 295 nm) for each fraction against elution volume. Arrows indicate the positions of the markers, 400 and 160 kDa. (b) Plot of radioactivity for each fraction against elution volume. (c) Molecular mass determination of ternary complex by the size exclusion chromatography on Sephadex G-200 (fractionation range: 5−600 kDa). Standard proteins were apoferritin (440 kDa), InsPR (400 kDa), pod coat protein from *Impatiens balsamina* (300 kDa), phytase (160 kDa), and aldolase (154 kDa). The arrow indicates the position of elution for the ternary complex corresponding to a mass of 560 kDa.

SDS−PAGE and elution of the band corresponding to 100 kDa (because the receptor is a homotetramer of 100 kDa). In a control experiment, a mixture of phytase and the receptor, after incubation for 15 min at 5 °C, was passed through the same column and monitored by fluorescence for the presence of a third peak corresponding to a binary complex of higher molecular mass, but no such complex formation was found. This indicates that in the absence of $InsP_3$ there is no interaction between InsPR and phytase (data not shown).

Figure 7 shows the quenching in the fluorescence of phytase upon addition of the equilibrium mixture of Ins(1,4,5)P_3 and InsPR. The absence of a significant change in the fluorescence spectrum of phytase under the same conditions in the presence of InsPR alone also shows the absence of any nonspecific association (data not shown). Similar results were obtained when Ins (2,4,5)P_3 was used instead of Ins(1,4,5)P_3 under identical conditions.

Phytase binds to InsPR in the presence of Ins(1,4,5)P_3 leading to the formation of a ternary complex. Ins(2,4,5)P_3 can substitute for Ins(1,4,5)P_3. An essential prerequisite for the formation of such a ternary complex is that both isomers of $InsP_3$ should have comparable affinities for the phytase and InsPR. Therefore, the affinity of $InsP_3$ for the purified receptor was estimated. The binding isotherm for its association with Ins(2,4,5)P_3 at 5 °C is shown in Figure 8, and the dissociation constant calculated from the binding

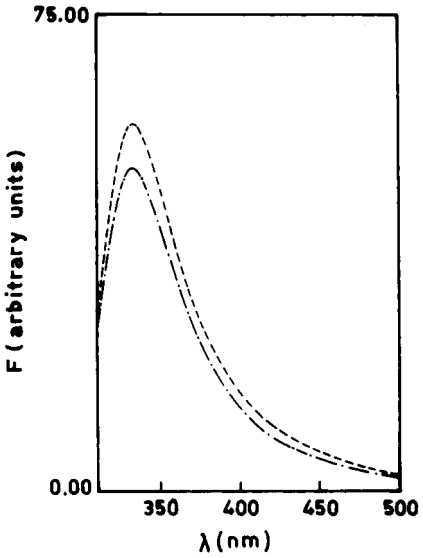

FIGURE 7: Fluorescence change of phytase due to formation of ternary complex: Fluorescence emission spectrum (λ_{ex} = 295 nm) of phytase (0.1 μM, - - -) alone and in the presence of an equilibrium mixture of InsPR (0.02 μM) and Ins(1,4,5)P_3 (0.4 μM) (−·−) in 50 mM Tris-HCl buffer, pH 8.0, plus 25 mM NaCl at 5 °C. Spectrum of phytase in the presence of receptor alone overlaps with that for free phytase, hence it is not shown here. Contribution to the spectrum from InsPR or Ins(1,4,5)P_3−InsPR complex have been subtracted.

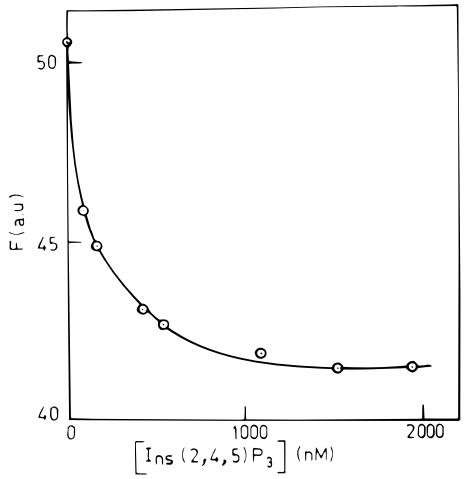

FIGURE 8: Binding of myoinositol trisphosphate to InsPR: Binding isotherm in 50 mM Tris-HCl buffer, pH 8.0, plus 25 mM NaCl at 5 °C for the interaction of Ins(2,4,5)P_3 with InsPR (42 nM) purified from the microsomes/vacuoles. The emission intensity of the receptor at 340 nm (λ_{ex} = 295 nm) is plotted against the input concentration of Ins(2,4,5)P_3. Apparent dissociation constant was calculated as follows: $K_d = (c - 0.5a)$, where c and a denote the concentrations of Ins(2,4,5)P_3 and receptor corresponding to half of the total change in fluorescence.

isotherm was 110 ± 5 nM. The corresponding value for Ins(1,4,5)P_3 was 90 ± 10 nM. The filter binding assay also gave a comparable dissociation constant, 100 nM, for the same system. The above values for the dissociation constant are comparable to those characteristic of the high affinity, noncatalytic binding between Ins(1,4,5)/(2,4,5)P_3 and phytase (75 ± 10 nM). The possibility of transfer of free $InsP_3$ from the transient $InsP_3$−phytase complex to InsPR appears to be minimal. The results thus far obtained support the idea that a ternary complex could, in principle, be formed at least *in vitro*. The binding of Ins[(1,4,5)/(2,4,5)/(1,3,4)]-P_3 to the high affinity site leads to a significant change in

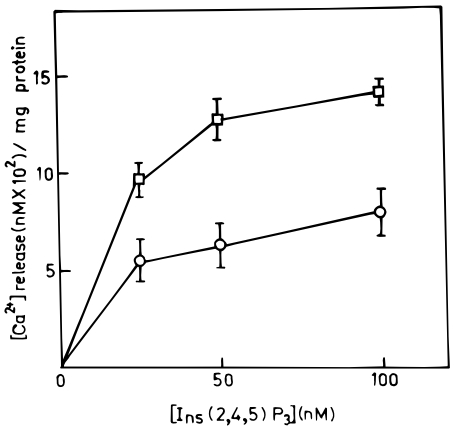

FIGURE 9: Intracellular Ca^{2+} release: Ca^{2+} efflux from microsomes/vacuoles by Ins(2,4,5)P_3 (○) and Ins(2,4,5)P_3−phytase complex (□) was monitored as described under Materials and Methods. Each point shown with standard error was obtained from three independent experiments with different batches of microsomes/vacuoles.

Table 3: Effect of $InsP_3$−Phytase Complex on the Release of Ca^{2+} from Microsomes/Vacuoles at 25 °C[a]

ligand (μM)	Ca^{2+} release (nM/mg of protein)
Ins(1,4,5)P_3 (0.1)	1080 ± 120
phytase−Ins(1,4,5)P_3 complex (0.1)	2580 ± 180
Ins(2,4,5)P_3 (0.1)	785 ± 115
phytase−Ins(2,4,5)P_3 complex (0.1)	1415 ± 68
Ins(1,3,4)P_3 (0.1)	120 ± 35
phytase−Ins(1,3,4)P_3 complex (0.1)	648 ± 108

[a] Release of Ca^{2+} from microsomes/vacuoles was followed from increase in fluorescence of Quin-2 (as described under Materials and Methods) after a time period of 20 s since the addition of $InsP_3$ or the $InsP_3$−phytase complex. The complex was made by incubating 1 μM each $InsP_3$ and phytase for 2 min at 25 °C. Fifty microliters of the complex was then added to a final volume of 500 μL of microsomes/vacuoles suspension containing the remaining components. Mean value of Ca^{2+} release from three different batches of microsomes/vacuoles was calculated.

the conformation of phytase, which probably favors its binding to InsPR, because phytase without $InsP_3$ does not bind with the receptor.

Physiological Significance of the Ternary Complex $InsP_3$−Phytase−InsPR. Since the $InsP_3$−phytase complex binds to InsPR and leads to the formation of a ternary complex, the question arises whether this has any relevence *in vivo*. Figure 9 illustrates the relative effects of Ins(2,4,5)P_3 and its high affinity complex with phytase upon release of Ca^{2+} from microsomes/vacuoles. The concentration−response curves for free Ins(2,4,5)P_3 and Ins(2,4,5)P_3−phytase complex clearly showed that there was an increase in the level of Ca^{2+} release when the complex is added. A similar trend was noticed for Ins(1,4,5)P_3, though there was a difference in the relative extent of release of Ca^{2+} (Table 3). On the other hand, this could be ascribed to the fact that $InsP_3$−phytase complex binds to the endogenous microsomal/vacuolar receptor and elicits the release of Ca^{2+}. This is further confirmed by the ability of Ins(1,3,4)P_3−phytase complex to release Ca^{2+} when free Ins(1,3,4)P_3 could elicit insignificant amount of Ca^{2+} release (Table 3). This is also in conformity with the observation that Ins(1,3,4)P_3 has a very low affinity for InsPR. Even 2 μM Ins(1,3,4)P_3 did not displace [^{3}H]Ins(1,4,5)P_3 from $InsP_3$−InsPR complex. The question now arises as to what is the possibility of the $InsP_3$−phytase complex coming into proximity with the InsPR *in vivo*? The localization of phytase in the cell has not been studied in detail. However, phytase has been found to be distributed in the cytosol and in membraneous structures of the cell in several plant systems (Loewus et al., 1990). Further, $InsP_6$ synthesis takes place in the cytoplasm prior to its deposition within protein bodies (Greenwood & Bewley, 1984). That the microsomes/vacuoles contain the receptor sites with different affinities and densities has been reported from several laboratories (Biswas et al., 1995). Of the 20 possible $InsP_3$ isomers, the following three have been identified as phytase products: Ins(1,2,3)P_3, Ins(1,2,6)P_3, and Ins(2,4,5)P_3. The first two are formed as intermediates in the acid phytase catalyzed hydrolysis of $InsP_6$ (Cosgrove, 1980). Ins(1,2,3)P_3 has also been shown to be a product of alkaline phytase from lilly pollen (Barrientos et al., 1994). Ins(2,4,5)P_3 as one of the products of phytase has been reported from mung bean (Maitra et al., 1988).

Interest in Ins(1,4,5)P_3 as a second messenger has led to the investigations of its metabolism in a number of tissues (Drobak, 1992). Pattern of degradation indicates that plant cells contain enzymes that selectively remove phosphate group from different positions. The ratios of intermediate $InsP_3$ and $InsP_2$ varied with tissue as did the pH optima and sensitivity to metal ions such as Mg^{2+}, Li^+, and Ca^{2+} suggesting that the enzyme(s) in these systems might contain multiple kinases and phosphatases acting on $InsP_3$. In the mung bean system, the distribution of phytase and the InsPR is not known. The specific additional role the $InsP_3$−InsPR−phytase complex in the cell is perhaps to utilize $InsP_3$ generated either by phytase or different pathways as an elicitor of intracellular Ca^{2+} efflux. Thus in the light of the above observations it is speculated that other inositol trisphosphates which *per se* can not trigger Ca^{2+} release at physiological concentrations may do so when complexed with the phytase.

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