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Analysis of Aluminum and Divalent Cation Binding to Wheat Root Plasma Membrane Proteins Using Terbium Phosphorescence

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ABSTRACT

A phosphorescent trivalent cation, terbium [Tb(III)], has been used to study the binding of different polyvalent cations to the proteins of wheat (*Triticum aestivum* L.) root plasma membranes. The phosphorescence emission intensity of Tb(III) was enhanced after Tb(III) binding to wheat root plasma membranes as a result of nonradiative resonance energy transfer from the membrane protein tyrosine and phenylalanine residues. Complex, saturable Tb(III) binding was observed, suggesting multiple binding sites. Bound Tb(III) could be displaced by divalent cations in the general order: Mn(II) > Ca(II) > Mg(II). Al(III) was very effective in reducing the protein-enhanced Tb(III) phosphorescence at pH values below 5. Al(III) also altered the Tb(III) phosphorescence lifetime, suggesting Al(III)-induced changes in membrane protein conformation. The more Al(III)-sensitive wheat cultivar (Anza) bound Al(III) with higher affinity than the more tolerant cultivar (BH 1146). At pH 5.5 where Al(III) did not displace bound Tb(III), low levels of Al(III) reduced the ability of Mn(II) to decrease Tb(III) phosphorescence. The significance of these results is discussed with respect to the mechanisms of Al(III) tolerance in wheat and the potential beneficial effects of Al(III) in reducing Mn(II) phytotoxicity.

The phytotoxic effects of Al(III) on plant growth under acidic soil conditions has been the subject of numerous investigations (11). *In vivo* experiments have demonstrated the ability of certain cations, primarily Ca(II), to reduce the toxic effects of Al(III) under acidic growth conditions (10, 11, 17). However, only recently (17) has the decrease in Al(III) phytotoxicity by Ca(II) been shown to be the result of specific interactions rather than simply the consequence of alterations in Al(III) activity by changes in ionic strength.

Considering the genetics of Al(III) tolerance in wheat, Moore (22) suggested that Al(III) tolerance involves a specific mechanism. He proposed that the exclusion of Al(III) from the root cells may be responsible for the reduced Al(III) phytotoxicity in some wheat cultivars and that this process involves proteins in the wheat root plasma membrane. Kinraide and Parker (17) recently demonstrated that cation reduction of Al(III) toxicity in wheat may involve an extracellular mechanism. They suggested that Al(III) may either displace Ca(II) from plasma membrane binding sites or bind to some other site from which it can be displaced by Ca(II) and other cations.

Any *in vitro* investigation of Al(III) interactions with biological membranes is complicated by the difficulties in distinguishing between ion binding to lipids and to proteins. Al(III) can associate strongly with negatively charged phospholipids in the absence of proteins, resulting in reduced membrane surface charge, release of bound divalent cations, and changes in lipid structure (7). Al(III) can interact with proteins, either competing with other cations for negatively charged binding sites or inducing changes in conformation which could influence ion-protein interactions (14).

The ability of different lanthanide cations to replace Ca(II) in Ca(II)-binding proteins has been extensively documented (4, 9, 15, 19). The luminescent lanthanide, terbium [Tb(III)], has been used in numerous studies to investigate Ca(II)-binding sites in membranes and isolated proteins (4, 8, 13, 20, 21, 28). Tb(III) is only weakly phosphorescent in aqueous solutions. However, upon binding to proteins, Tb(III) phosphorescence is often greatly enhanced by resonant energy transfer (19). This process can be summarized: (a) absorption by an aromatic amino acid side chain of phenylalanine, tyrosine, or tryptophan in the 250 to 300 nm region; (b) nonradiative energy transfer from an aromatic side chain to a adjacent Tb(III); (c) enhanced Tb(III) phosphorescence in the green, 535 to 555 nm spectral region. Since the optimal excitation wavelengths for this process are the same as those for protein aromatic amino acid absorbance and the efficiency of the energy transfer is very short range with a radius^{-6} dependence on the distance between the donor and acceptor sites (19), only protein-bound Tb(III) should display protein-enhanced phosphorescence (13). The subsequent displacement of protein-bound Tb(III) by other cations reduces the Tb(III) phosphorescence and permits the estimation of apparent cation binding constants (9, 23). Although the association of Tb(III) with sites that do not bind Ca(II) may be possible, this method has been used to investigate *in vitro* the properties of Ca(II)-binding sites of various animal membrane-bound proteins (8, 13, 20, 21, 23).

Considering the apparent interaction of Al(III) and Ca(II) in Al(III) phytotoxicity and the possibility that this results from Al(III) displacement of Ca(II) from membrane proteins, this study was undertaken in order to determine whether protein-enhanced Tb(III) phosphorescence could be used to measure *in vitro* cation association with the proteins of root plasma membranes isolated from wheat cultivars of differing Al(III) tolerance. Since the symptoms of Al(III) phytotoxicity are usually expressed only at pH values below 5, these investigations were performed at pH 5 and below. Furthermore,

since Al(III) has been reported to be beneficial to plants under conditions which should induce Mn(II) toxicity, the interactions between Al(III) and Mn(II) were also considered.

MATERIALS AND METHODS

Plant Material and Membrane Isolation

Wheat (*Triticum aestivum* L. cv Anza or BH1146) seed was germinated and grown over an aerated solution of 0.25 mM $CaSO_2$ (initial pH 5.5) in the dark at 20 ± 1°C. The roots of 5-d-old seedlings were excised, and a plasma membrane-enriched microsomal fraction was isolated by the methods of Sommarin *et al.* (27) with the following modifications. The homogenization solution consisted of 5 mM EDTA, 5 mM EGTA, 2 mM SHAM,[1] 2 mM Na metabisulfite, 0.5 mM PMSF, 1% (w/v) soluble PVP, and 10% (w/v) glycerol in 50 mM Hepes-KOH (pH 7.6). The KCl concentration in the two-phase partition system was increased to 5 mM. The plasma membrane preparation was washed and resuspended (4 mg membrane protein/mL) in 5 mM Mes-Tris buffer (pH 6.5), containing 10% (w/v) glycerol, and used immediately. Membrane protein was measured by the method of Wang and Smith (30).

Phosphorescence Measurements

All phosphorescence and fluorescence measurements were performed using a Perkin-Elmer[2] model LS-5 fluorescence spectrometer. Membrane samples were resuspended in 5 mM Mes-Tris, containing 10% (w/v) glycerol, to give the desired pH and 40 μg membrane protein/mL. Tb(III) and the other cations, as their chloride salts, were added from concentrated solutions so that the total increase in sample volume during the experiment did not exceed 2% of the original volume. The concentrated Al(III) solutions, 10 mM aqueous $AlCl_3$ adjusted to pH 2.5 with dilute HCl, were prepared immediately before use. Since pH below 5.5 is outside the useful buffering range of Mes (2), Mes-Tris solutions below pH 5.5 cannot be considered buffered. Therefore, before the phosphorescence measurements, the pH of all sample solutions was checked and adjusted after each addition. All experiments were performed at 20°C, correcting for background phosphorescence. The sample absorbance at 546 nm was determined at the beginning and end of every phosphorescence experiment to ensure that ion-induced membrane aggregation did not occur. Unless otherwise noted, all phosphorescence measurements were performed with an excitation wavelength of 280 nm and an emission wavelength of 546 nm. The time-averaged phosphorescence measurements were performed with a delay (t_d) of 0.3 ms and a gate (t_g) of 8 ms.

[1] Abbreviations: SHAM, salicylhydroxamic acid; t_d, delay time; t_g, gate time; $t_{1/2}$, half-life; I_{max}, maximal inhibition; P_{max}, maximal phosphorescence; N, Hill coefficient; K_d, apparent binding constant; pK_a, log acid disassociation constant; aluminon, aurintricarboxylic acid ammonium salt.

[2] Mention of trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

The Tb(III) phosphorescence decay constants and maximal phosphorescence intensities (P_{max}) were measured with the LS-5 spectrometer by varying t_d (50 μs increments) at a constant t_g (50 μs), correcting for background phosphorescence (13). The values for the decay constants and extrapolated P_{max} were estimated by standard linear regression analysis of phosphorescence decay curves converted to the semilogarithmic form (13). When appropriate, the various cation binding constants, maximal inhibition values, and Hill coefficients were estimated using a previously described computer program (6).

RESULTS

Preliminary Experiments

Although Good *et al.* (12) reported that Tris and Mes had negligible abilities to bind divalent cations, more recent work has suggested that both Tris and 'Goods' buffers could chelate polyvalent cations (24). Therefore, the ability of both Tris and Mes to chelate Tb(III) at different pHs was examined, using aluminon as a colorimetric indicator of free Tb(III) concentration under conditions essentially the same as those described for 8-hydroxyquinoline (24). Although usually utilized as an Al(III) indicator (25), aluminon (0.5 mM) absorbance at 545 nm and pH 4.5, 5.5, or 6.5 increased linearly as the Tb(III) concentration was raised from 10 to 100 μM. There were indications of low levels of Mes (25 mM) chelation of Tb(III) at pH 6.5. However, neither Mes nor Tris at concentrations up to 25 mM significantly reduced the free Tb(III) levels at pH 5.5 and below. These results suggest that the 5 mM Mes-Tris used in this study had negligible effect on the cation concentrations.

At Tb(III) concentrations from 10 to 100 μM, there was a slight increase in the activity at 20°C of the Mg(II)-dependent ATPase associated with the wheat root plasma membranes. This suggests that either Tb(III) may not significantly impair the biochemical properties of the wheat plasma membranes or Tb(III)-ATP may serve as an alternative substrate for the ATPase.

Characteristics of the Protein-Enhanced Tb(III) Phosphorescence

The phosphorescence spectra of Tb(III) bound to wheat root plasma membranes are shown in Figure 1. There was a significant increase in the time-averaged Tb(III) phosphorescence (546 nm emission) upon the addition of the membrane suspension. In the excitation spectrum (Fig. 1A), the addition of membrane results in increased phosphorescence with excitation below the spectrometer lower limit (230 nm) which may correspond to the absorption maximum of free Tb(III) in aqueous solution (218 nm) (13). The phosphorescence was significantly enhanced relative to free Tb(III) at excitation wavelengths from about 250 to 290 nm with the suggestion of maxima at about 260 and 280 nm. The protein-enhanced Tb(III) phosphorescence emission spectrum (Fig. 1B) shows the four maxima typical of Tb(III) phosphorescence (13). Varying the sample concentration from 0.05 to 0.25 absorbance units at 280 nm did not modify the shapes of the

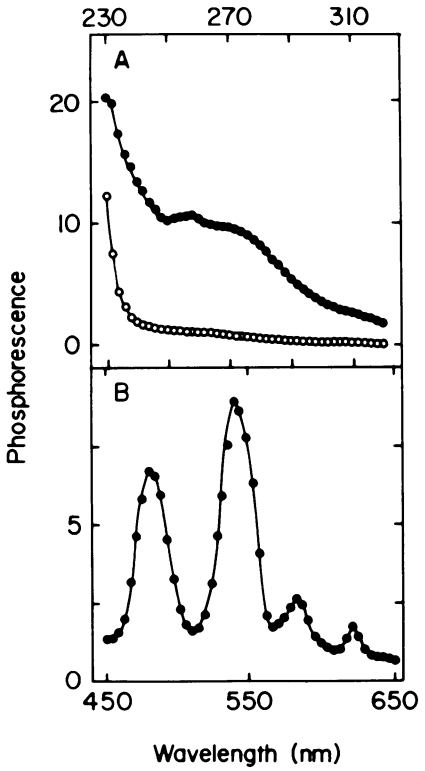

Figure 1. Phosphorescence excitation (A) and emission (B) spectra of bound Tb(III) in wheat (cv Anza) root plasma membranes. The excitation spectra of Tb(III) (25 μM) in the presence (●) and absence (○) of wheat plasma membrane (40 μg/mL) were obtained by measuring luminescence at 546 nm. The emission spectrum was obtained by excitation at 280 nm. Phosphorescence between 0.3 and 8 ms after pulse excitation was measured at pH 6.5 and 20°C. The phosphorescence intensity is given in arbitrary units.

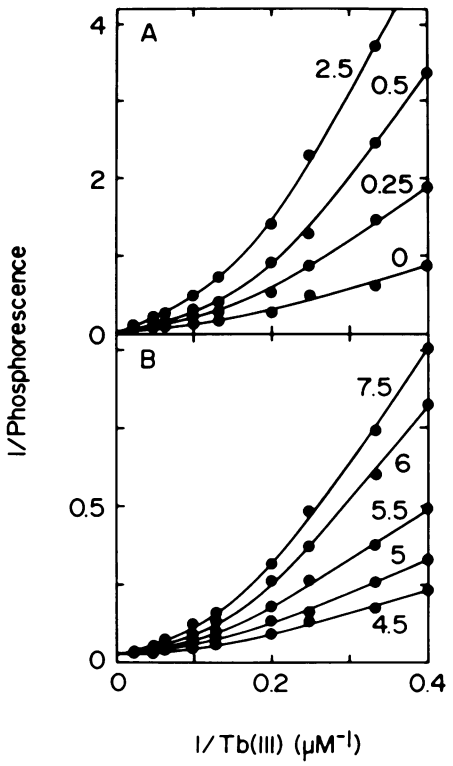

Figure 2. Double reciprocal plots of the phosphorescence produced by the titration of wheat (cv Anza) root plasma membranes with Tb(III) in the presence of Ca(II) (A) and at different pH (B). Phosphorescence between 0.3 and 8 ms after pulse excitation was measured at 20°C with excitation at 280 nm and emission at 546 nm. In part A, the curves are labeled with the millimolar Ca(II) concentration used in the phosphorescence measurements at pH 6.5 and 40 μg/mL membrane protein. In part B, the curves are labeled with the sample pH.

phosphorescence excitation and emission spectra (data not shown), indicating that the multiple phosphorescence excitation maxima probably did not result from distortions produced by inner filter effects.

Titration of Anza plasma membrane suspensions with Tb(III) resulted in concentration-dependent increases in Tb(III) phosphorescence which were sigmoidal and saturable. The complex nature of Tb(III) binding to the membrane proteins is evident from the double reciprocal plots of phosphorescence as a function of Tb(III) concentration (Fig. 2). Similar plots using plasma membranes from BH1146 roots showed the same curvature (data not shown). Although suggestive of positive cooperativity, these plots probably indicate the presence of multiple Tb(III) binding sites of differing affinities. In preliminary experiments, the effect of increasing Tb(III) levels on the intrinsic membrane protein fluorescence was examined, as previously described (4). With excitation at 280 nm, Tb(III) concentrations up to 100 μM did not visibly reduce the intrinsic fluorescence intensity at 340 nm.

Effect of pH, Calcium, and Aluminum on the Time-Averaged, Protein-Enhanced Tb(III) Phosphorescence

Since Tb(III) has been shown to be a useful probe for Ca(II) binding to proteins (19), Ca(II) is often a competitive inhibitor of the Tb(III) luminescence. As shown in Figure 2A, increasing amounts of Ca(II) significantly reduced the time-averaged Tb(III) phosphorescence sensitized by the Anza membranes at pH 6.5. These curves converge to the same point on the y axis. Similar double reciprocal plots have been interpreted as suggestive of the competitive inhibition of Tb(III) fluorescence by Ca(II) (20). Membranes isolated from BH1146 generated a similar double reciprocal plot, although Ca(II) was slightly less effective in reducing the Tb(III) phosphorescence (data not shown).

Since pH is extremely important in determining the phytotoxicity of Al(III), the effect of pH on the protein-enhanced Tb(III) phosphorescence was investigated. Decreasing the sample pH increased the Tb(III) phosphorescence of the Anza membranes (Fig. 2B). Furthermore, the double reciprocal plots became more linear. The pH dependence of Tb(III) phosphorescence obtained with membrane from BH1146 were similar except that the phosphorescence intensity was routinely 23 to 27% lower than the Anza membrane phosphorescence at the same protein concentration (data not shown).

Although the data presented in Figure 2 are more appropriately interpreted as indicating multiple Tb(III)-binding sites in the wheat plasma membrane, the data were analyzed as

though there were a single class of Tb(III)-binding sites, displaying positive cooperativity. This analysis was performed to permit an estimation of the average binding affinity of Tb(III) and to allow comparison between the two wheat cultivars. As the pH was reduced from 7.5 to 4.5, with the Anza membrane the Hill coefficient decreased from 1.89 to 1.45 and the apparent Tb(III)-binding constant (K_d) declined from 119 to 28 μM. For the BH1146 plasma membrane, the same change in pH reduced the Hill coefficient from 1.76 to 1.34 and K_d for Tb(III) from 106 to 25 μM. These results suggest a general increase in the affinity of the Tb(III)-protein interactions as the pH is reduced. Furthermore, the Tb(III) binding to the membrane proteins of the two wheat cultivars appears to be very similar.

The similarity between the Tb(III) phosphorescence of the two wheat cultivars was also evident when sample pH was varied at a constant Tb(III) concentration of 10 μM (Fig. 3). Even though, as mentioned above, the Tb(III) phosphorescence was higher in membranes from Anza, the pH dependencies of the Tb(III) phosphorescence associated with the membranes of both cultivars were essentially the same. The phosphorescence intensity decreased with increasing pH in manner which indicates a pK_a of about 6 for some component involved in the protein-enhanced Tb(III) phosphorescence of the plasma membranes. The difference in phosphorescence intensity for the membranes of the two wheat cultivars suggests that Anza plasma membrane has either more Tb(III)-binding sites or more efficient energy transfer from the protein aromatic amino acids to the bound Tb(III).

As shown in Figure 4, Al(III) diminished the time-averaged Tb(III) phosphorescence of Anza plasma membranes at high Tb(III) levels. However, Al(III) stimulated the Tb(III) phosphorescence at Tb(III) concentrations below 3 μM. Therefore,

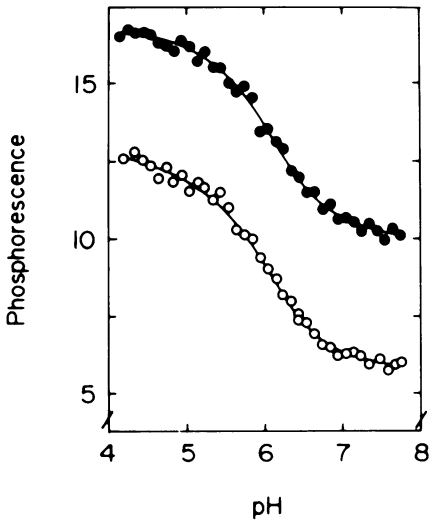

Figure 3. Effect of pH on the Tb(III) phosphorescence sensitized by wheat root plasma membrane proteins. Phosphorescence between 0.3 and 8 ms after pulse excitation was measured at 20°C with excitation at 280 nm and emission at 546 nm, using either wheat cultivar Anza (●) or BH1146 (○) membrane at 40 μg membrane protein/mL and 10 μM Tb(III). The sample pH was increased by the addition of μL amounts of concentrated Tris directly to the sample cuvette.

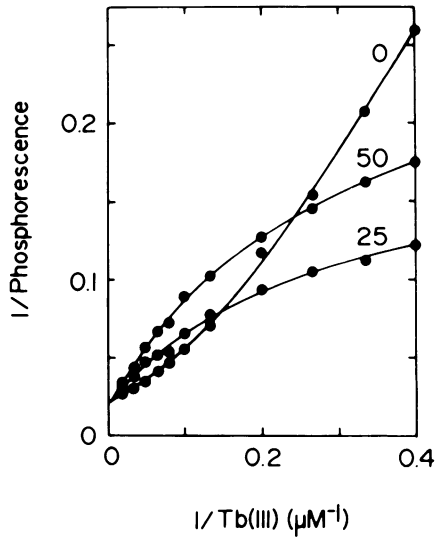

Figure 4. Double reciprocal plots of the phosphorescence produced by the titration of wheat (cv Anza) root plasma membranes with Tb(III) in the presence of Al(III). Phosphorescence between 0.3 and 8 ms after pulse excitation was measured at 20°C and pH 4.5 with excitation at 280 nm and emission at 536 nm, using 40 μg membrane protein/mL. The curves are labeled with the sample Al(III) concentration in μM.

in the presence of Al(III), the results gave the appearance of negative cooperativity ($n < 1$) for Tb(III) binding to the membrane proteins. The enhancement of Tb(III) phosphorescence by Al(III) at low Tb(III) levels might involve either the release of Tb(III) from nonphosphorescent sites on the membrane which can then reassociate at phosphorescent sites or the alteration of the efficiency of resonant energy transfer.

Estimation of Cation Binding to Plasma Membrane Proteins by Time-Resolved Tb(III) Phosphorescence

The lifetime ($t_{1/2}$) of Tb(III) phosphorescence is sensitive to the cation microenvironment (15). Therefore, Tb(III)-binding sites in the wheat plasma membrane which have the same ion-protein coordination characteristics should have phosphorescence decays with similar $t_{1/2}$. Since there are apparently multiple types of Tb(III)-binding sites in the wheat membrane (Figs. 2 and 4), the use of time-resolved phosphorescence may simplify the investigation of cation binding to the membrane proteins by permitting the examination of single classes of Tb(III)-binding sites which have similar properties.

The effect of Al(III) and other divalent cations on the phosphorescence decay kinetics was examined, using the gate-shift method (13). Time-averaged phosphorescence intensity was determined with a constant gate time of 50 μs and increasing the delay times (50 μs increments). Since the Tb(III) phosphorescence lifetime is significantly longer than the 50 μs measurement period (13), the time-dependence of the phosphorescence decay can then be estimated by plotting the time-averaged phosphorescence as a function of delay time. The accuracy of the gate-shift method for phosphorescence decay measurement was confirmed by determining the decay kinetics of free Tb(III) in aqueous solution and comparing

the lifetime ($t_{1/2}$ = 274 μs) with the literature value ($t_{1/2}$ = 280 μs) (13).

A representative semilogarithmic plot of phosphorescence intensity (t_g = 50 μs) as a function of delay time is presented in Figure 5. In this and all other cases, there were at least three phases of phosphorescence decay, indicating three major classes of Tb(III) microenvironment. The rates of decay of the fast and slow phases could be resolved by subtracting the extrapolated values of the intermediate decay phase (0.5–1.8 ms) from that of the total phosphorescence. Although not readily apparent in decay profiles presented in Figure 5, the slow phase (t_d > 1.8 ms) contributed up to 13% of the total time-averaged phosphorescence (t_d = 0.3 ms, t_g = 8 ms).

Considering only the decay between 0.5 and 1.8 ms, the data presented in Figure 5 indicate that increasing levels of Al(III) reduced the P_{max} of the Tb(III) phosphorescence and increased the $t_{1/2}$ of the phosphorescence decay (Fig. 5A). However, Ca(II) concentrations up to 1 mM did not alter appreciably the $t_{1/2}$ of Tb(III) phosphorescence at pH 4.5 (Fig. 5B). Plotting the $t_{1/2}$ of the intermediate phase of the protein-bound Tb(III) phosphorescence decay as a function of cation concentration (Fig. 6) demonstrates the ability of Al(III) to increase $t_{1/2}$. It is apparent that change in the Tb(III) phosphorescence $t_{1/2}$ induced by Al(III) with the Anza membrane

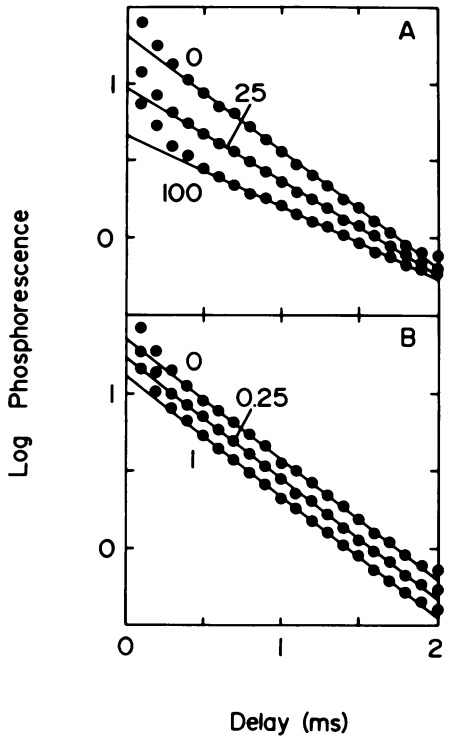

Figure 5. Cation effect on the phosphorescence decay kinetics of wheat root plasma membrane-bound Tb(III). Tb(III) (10 μM) phosphorescence at 546 nm with excitation at 280 nm was measured with a gate time of 0.05 ms and the given delay time after pulse excitation, using wheat (cv Anza) membrane (40 μg protein/mL) at pH 4.5 and 20°C. The lines produced by linear regression analysis of the phosphorescence intensities obtained between 0.5 and 1.8 ms are labeled with either the micromolar concentration of Al(III) (A) or the millimolar concentration of Ca(II) (B) present in the sample.

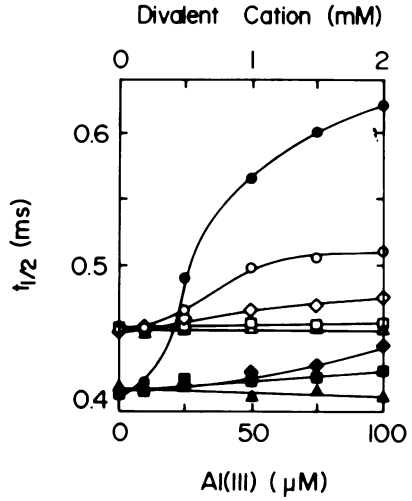

Figure 6. Cation effect on the phosphorescence decay half-life ($T_{1/2}$) of wheat root plasma membrane-bound Tb(III). Tb(III) (10 μM) phosphorescence at 546 nm with excitation at 280 nm was measured with a gate time of 0.5 ms and delay times from 0.5 to 1.8 ms after pulse excitation, using cv Anza (closed symbols) or cv BH1146 (open symbols) membrane (40 μg membrane protein/mL) at pH 4.5 and 20°C. The samples contained increasing concentrations of either Al(III) (●, ○), Mg(II) (▲, △), Ca(II) (■, □), or Mn(II) (◆, ◇). The Tb(III) phosphorescence decay half-life was computed from the best-fit line through the log phosphorescence intensity as a function of delay time.

was greater than that observed with the BH1146 membrane. Furthermore, the 50 μs difference between the Tb(III) phosphorescence $t_{1/2}$ of the membrane from the two cultivars in the absence of other added cations indicates somewhat dissimilar Tb(III) microenvironments. These results suggest that Al(III) has the ability to both displace Tb(III) bound to the membrane proteins and modify the environment around the Tb(III) which remains associated with the membrane proteins. With the possible exception of Mn(II), the results presented in Figure 6 also suggest that the divalent cations tested did not modify the Tb(III) microenvironment in the membrane proteins at this pH.

The ability of Al(III) to displace protein-bound Tb(III) was determined for both wheat cultivars. As shown in Figure 7, the apparent binding constants for Al(III) decreased with decreasing pH. The two wheat cultivars had different responses to Al(III). BH1146 membrane proteins had lower apparent affinity for Al(III) at all pH values with a pK_a of 4.25 for the pH-dependent reduction in K_d. The decrease in Al(III) K_d for the cultivar Anza had a pK_a of 4.6. At pH values above 4.5, these results indicate that Al(III) has limited effect on Tb(III) binding at pHs above 5 and that the more Al(III)-resistant wheat variety (BH1146) was less sensitive to Al(III)-induced changes in Tb(III) phosphorescence at pHs below 5. Figure 8 shows the effects of increasing concentrations of Mg(II), Ca(II), and Mn(II) on the protein-enhanced Tb(III) phosphorescence of the two wheat cultivars at pH 4.5. The Tb(III) phosphorescence of the membrane from both cultivars responded similarly to the divalent cations. The apparent K_d of Mg(II) was 2.53 mM (I_{max} = 35.6%) and 3.21 mM (I_{max} = 38.9%) for the membrane proteins of Anza and BH1146,

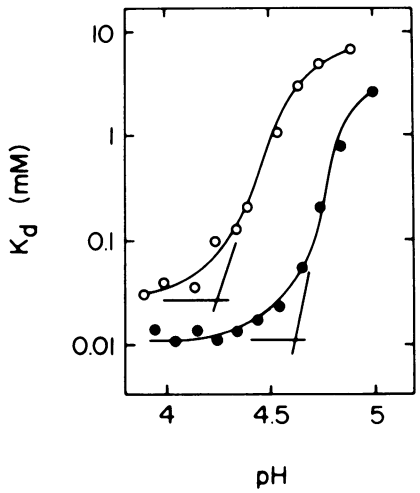

Figure 7. pH effect on the apparent Al(III) binding constants of membrane-bound Tb(III) associated with either wheat cv Anza (●) or cv BH1146 (○) plasma membrane. Maximal phosphorescence intensities in the presence of increasing Al(III) concentrations (5–200 μM) were computed by extrapolation to 0 delay time of the best linear fit of time-resolved phosphorescence intensities obtained at 20°C and 10 μM Tb(III), as described in Figure 5. The apparent Al(III) binding constants (K_d) were computed from the maximal membrane-bound Tb(III) phosphorescence intensities as a function of Al(III) concentration.

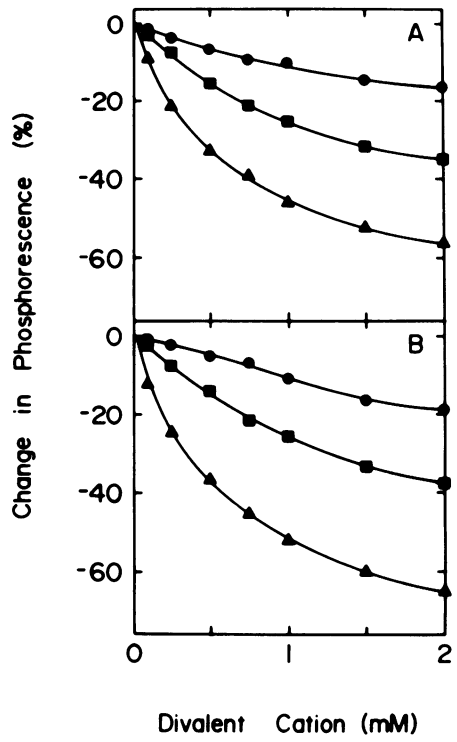

Figure 8. Cation effect on the time-resolved maximal phosphorescence of Tb(III) bound to either cv Anza (A) or cv BH1146 (B) plasma membranes. Maximal Tb(III) (10 μM) phosphorescence intensities in the presence of increasing concentrations of Mg(II) (●), Ca(II) (■), or Mn(II) (▲) at 20°C and pH 4.5 were determined, as described in Figure 6, and converted to percent inhibition relative to the maximal phosphorescence in the absence of added cation.

respectively. Compared to Mg(II), Ca(II) bound to the membrane proteins with higher affinity (Anza: K_d = 1.26 mM, I_{max} = 56.1% and BH1146: K_d = 1.44 mM, I_{max} = 64.4%). Mn(II) significantly reduced the Tb(III) phosphorescence. Using membrane from Anza roots, Mn(II) had an apparent K_d of 0.54 mM (I_{max} = 85.1%). Mn(II) bound to the BH1146 plasma membrane proteins with a K_d of 0.58 mM (I_{max} = 81.5%). These results demonstrate that Ca(II) could displace about 25% more Tb(III) from the membrane binding sites than Mg(II).

It has been reported that Al(III) could reduce the symptoms of Mn(II) phytotoxicity (11). Therefore, the effect of Al(III) on the Mn(II)-induced reduction of Tb(III) phosphorescence was examined under conditions (pH > 5) where low levels of Al(III) did not inhibit Tb(III) phosphorescence (Fig. 7). As shown in Figure 9, increasing levels of Al(III) at pH 5.5 reduced the effects of Mn(II) on the Tb(III) phosphorescence intensity. This phenomenon was more pronounced with membrane from BH1146 (Fig. 9B). Comparison of the Mn(II)-induced reduction in Tb(III) phosphorescence at pH 5.5 without Al(III) (Fig. 9) and at pH 4.5 (Fig. 8) indicates that the binding affinity for Mn(II) increased with increasing pH.

DISCUSSION

The addition of wheat plasma membrane to an aqueous solution of Tb(III) significantly enhanced the Tb(III) phosphorescence. Since the excitation wavelengths for optimal membrane protein-sensitized Tb(III) phosphorescence was between 250 and 280 nM, most of the increased phosphorescence must have resulted from resonant energy transfer from the protein aromatic amino acids to protein-bound Tb(III). This broad peak with indications of maxima at about 260 and 280 nm is typical of protein-enhanced Tb(III) phosphorescence sensitized by energy transfer from phenylalanine (A_{max} = 259 nm) and tyrosine (A_{max} = 280 nM) (4, 13). The decline in phosphorescence intensity at wavelengths above 280 nm suggests that tryptophan (A_{max} = 295 nm) was not involved in the protein-enhanced phosphorescence. Brittain *et al.* (4) examined the Tb(III) emission of 33 soluble proteins, most of which had known Ca(II)-binding abilities. Only one protein, carp parvalbumin, displayed resonant energy transfer from phenylalanine to Tb(III). In contrast, 23 proteins had an excitation maximum indicating tryptophan as the primary energy donor to Tb(III). Therefore, the absence of tryptophan-sensitized Tb(III) phosphorescence in the wheat membranes was surprising. However, the spectrum presented in Figure 1A is very similar to that obtained with intact rat liver mitochondria which also had no evidence of tryptophan-sensitized Tb(III) phosphorescence (13).

In most of the experiments which have utilized Tb(III) fluorescence to investigate membrane protein Ca(II)-binding sites, the addition of Tb(III) reduced the protein intrinsic fluorescence (20, 21, 23). This was not the case with the wheat root plasma membrane. However, the optimal excitation wavelengths for the protein fluorescence were 285 to 295 nm (data not shown) which are the same as those found for barley

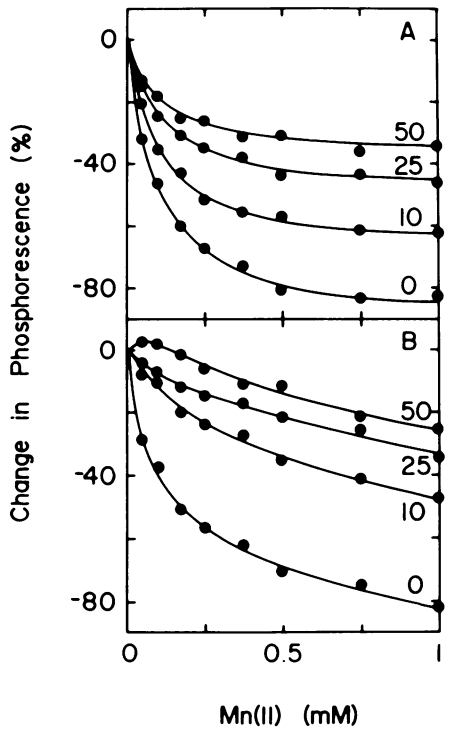

Figure 9. Al(III) effect on the percent Mn(II) inhibition of the time-resolved phosphorescence of Tb(III) bound to either cv Anza (A) or cv BH1146 (B) plasma membranes. Maximal Tb(III) (10 μM) phosphorescence intensities at 20°C and pH 5.5 were determined in the presence of increasing concentrations of Mn(II) and the given fixed concentration of Al(III) in micromoles, as described in Figure 6, and converted to percent inhibition relative to the maximal phosphorescence in the absence of added cation.

root plasma membrane (5). If the intrinsic protein fluorescence primarily results from the excitation of tryptophan, then the absence of any decrease in wheat plasma membrane protein fluorescence by Tb(III) is consistent with phenylalanine and tyrosine being the primary energy donors to the bound Tb (III). Furthermore, these results and the low phosphorescence intensity of Tb(III) bound to the membrane proteins suggests that there are few phosphorescent Tb(III)-binding sites in the wheat plasma membrane proteins and, therefore, the protein-enhanced Tb(III) phosphorescence may result from specific ion-protein interactions.

Considering the literature and the data presented in Figures 2A and 8, Tb(III) is a valid probe for the analysis of Ca(II)-binding sites in wheat root plasma membrane proteins. The site selectivity for Ca(II) over Mg(II) (Fig. 8) suggests that these cation-binding sites are of physiological relevance (8). The Ca(II) to Mg(II) binding ratio of about 2 and magnitudes of the Ca(II) and Mg(II) binding affinities of the wheat membrane proteins are similar to those measured using animal membrane preparations (8, 23). Even though wheat does not absorb significant amounts of Ca(II) under normal conditions (10), these plasma membrane Ca(II)-binding sites may be related to Ca(II) transport.

The reduction in membrane protein-sensitized phosphorescence intensity as the sample pH was increased (Fig. 3) is consistent with the results of Lux *et al.* (18). These researchers demonstrated a significant decrease in protein tyrosine fluorescence quantum yield with increased pH which they attributed to deprotonation of aspartyl or glutamyl carboxylic acid groups (18). Changes in the quantum yield of the protein tyrosine groups would modify the efficiency of any tyrosine to Tb(III) phosphorescence energy transfer. Brittain and Konteatis (3) reported that the pH-dependent change in the luminescence of a Tb(III)-glycine complex was caused by the ionization of the amino acid carboxylic acid groups at pHs below 7.5. This ionization could have a significant effect on the Tb(III) emission quantum yield by altering the number of water ligands in the primary hydration sphere of the glycine-complexed Tb(III) (15, 28). Above pH 7.5, measurements were prevented by reactant precipitation, probably as a result of Tb(III) hydrolysis (26). Although the multiplicity of Tb(III)-binding sites in the wheat plasma membranes precludes detailed analysis, the apparent pK_a of about 6 for the pH dependence of the Tb(III) phosphorescence (Fig. 3) is consistent with the presence of protein glutamic or aspartic acid residues near the membrane protein Tb(III)-binding sites and/or the tyrosyl groups which participate in the resonance energy transfer to the bound Tb(III). Considering that Tb^{3+} has a pK_a of 2.94 (9), it is unlikely that Tb(III) ionization would contribute to a process with a pK_a of 6.

As reviewed by Foy *et al.* (11), increased concentrations of Ca(II) in the growth medium often decreased the uptake and phytotoxicity of Mn(II) *in vivo*. Since Mn(II) is a potent inhibitor of Tb(III) binding to the wheat membrane proteins without significantly modifying the Tb(III) microenvironment (Figs. 6, 8, and 9), it is possible that Mn(II) competes directly with Tb(III) for the Ca(II)-binding sites. Since Mn(II) reduces Ca(II) transport *in vivo* (11), this possibility is consistent with the proposition that these Ca(II)-binding sites are involved in the transport of divalent cations.

Although Al(III) could not displace Tb(III) from the membrane proteins at pHs above 5 (Fig. 7), Al(III) reduced the ability of Mn(II) to inhibit Tb(III) phosphorescence at pH 5.5 (Fig. 9). Therefore, the reported ability of Al(III) to decrease Mn(II) phytotoxicity (11) may involve Al(III) preventing Mn(II) from binding to Ca(II) sites on the root plasma membrane proteins.

If Tb(III) associates with sites related to Ca(II) transport, the ability of Al(III) to displace membrane-bound Tb(III) is consistent with the observed *in vivo* reduction of Ca(II) uptake by Al(III) at pHs below 5 (16). However, the Al(III)-induced increase in phosphorescence $t_{1/2}$ (Fig. 6) suggests that the reduction of Tb(III) phosphorescence by Al(III) is not simply the result of competition for the same binding sites. In order to alter the microenvironment of bound Tb(III), Al(III) probably must interact with separate binding sites, changing the conformation of the Tb(III)-binding sites. This concept was proposed earlier for the effects of Al(III) on Ca(II) binding by calmodulin (14). Since Mg(II), Ca(II), and Mn(II) did not change appreciably the phosphorescence $t_{1/2}$ of bound Tb(III) at pH 4.5 (Fig. 6), these divalent cations probably could not associate with the separate Al(III)-binding sites. Alternatively, if these divalent cations can bind to the proposed Al(III)-binding sites, then they are unable to modify the microenvironment of the bound Tb(III). It should be noted that these

Al(III)-binding sites need not be on the membrane proteins. The known interactions between Al(III) and the membrane phospholipids (7) could result in lipid structural changes which, in turn, could modify protein conformation and cation binding. However, at the concentrations employed in this study, the divalent cations should also have the ability to restructure the membrane lipids. As cited above, no apparent change in bound Tb(III) $t_{1/2}$ was observed at pH 4.5 in the presence of Ca(II) and Mg(II).

Although the nature of this Al(III)-induced change in membrane protein-bound Tb(III) microenvironment is not known, research with purified proteins has indicated that any process which alters the hydration of the bound Tb(III) ions will influence Tb(III) fluorescence quantum yield (15, 28) and luminescence lifetime (15). An Al(III)-induced membrane protein conformational change could result in more protein ligands coordinated with the bound Tb(III), displacing water ligands from the primary hydration sphere of the Tb(III). This situation would result in a longer luminescence $t_{1/2}$ (28), increasing the Tb(III) luminescence quantum yield.

There were definite cultivar differences in the binding of Al(III) to the wheat root plasma membrane proteins. Based upon the data presented in Figure 7 and the estimated upper concentration limit for Al(III) in soil solution of 150 μM (1), it seems unlikely that Al(III) would significantly reduce Ca(II) binding to the membrane proteins until the pH is lowered below about 4.7 for Anza and 4.35 for BH1146. Obviously, the Al(III)-binding constants exceeding 500 μM obtained at higher pH values are of little physiological significance. Furthermore, at the lower pH values, the apparent K_d for Al(III) binding to the BH1146 and Anza membrane proteins began to level out at about 40 and 16 μM, respectively. Therefore, if these proposed Ca(II)-binding sites are involved in the expression of Al(III) phytotoxicity in these two spring wheat varieties, then the cultivar BH1146 should be more Al-tolerant than Anza.

Taylor and Foy (29) examined the Al(III) tolerance of 20 spring wheat cultivars exposed to 74 μM of Al(III) for 14 d. The cultivar Anza ranked 11th and the cultivar BH1146 ranked 6th. Based upon the values of the root tolerance index (29), BH1146 was about 25% more Al(III) tolerant than Anza. Therefore, although BH1146 is more Al(III) tolerant than Anza, the difference based on root growth of all the varieties tested is not particularly large. Therefore, to confirm that differences in Al(III) binding to membrane proteins results in dissimilar Al(III) tolerances, it will be necessary to perform similar analyses of the Al(III) binding to protein Ca(II) sites in membranes isolated from wheat cultivars of higher and lower Al(III) tolerances.

Regardless of whether the obvious differences between the membrane protein Al(III)-binding characteristics of the two wheat cultivars have any relevance to Al(III) tolerance, it is apparent that Al(III) can remove Tb(III) and, by inference, Ca(II) from wheat root plasma membrane protein binding sites. This is apparently the first supportive evidence of the hypothesis that Al(III) displacement of membrane protein-bound Ca(II) may be one of the primary lesions in Al(III) phytotoxicity.

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