Plant Physiol. (1990) 92, 919–923
0032-0889/90/92/0919/05/$01.00/0

Received for publication July 10, 1989
and in revised form November 11, 1989

Biochemical Similarities between Soluble and Membrane-Bound Calcium-Dependent Protein Kinases of Barley[1]

Leszek J. Klimczak* and Geoffrey Hind

Biology Department, Brookhaven National Laboratory, Upton, New York 11973

ABSTRACT

The soluble and membrane-bound forms of the calcium-dependent protein kinase from barley leaves (*Hordeum vulgare* L. cv. Borsoy) have been partially purified and compared. Both forms showed an active polypeptide of 37 kilodaltons on activity gels with incorporated histone as substrate. They eluted from chromatofocusing columns at an identical isoelectric point of pH 4.25 ± 0.2, and also comigrated on several other chromatographic affinity media including Matrex Gel Blue A, histone-agarose, phenyl-Sepharose, and heparin-agarose. Both activities comigrated with chicken ovalbumin during gel filtration through Sephacryl S-200, indicating a native molecular mass of 45 kilodaltons. The activities share a number of enzymatic properties including salt and pH dependence, free calcium stimulation profile, substrate specificity, and *Km* values.The soluble activity was shown to bind to artificial lipid vesicles. These data suggest strongly that the soluble and membrane-bound calcium-dependent protein kinases from barley are very closely related or even identical.

Calcium-dependent protein kinases (CDPKs[2]) are thought to be involved in signal transduction in plants since there is a growing body of evidence that calcium can function in many plant systems as a second messenger (for review, see refs. 5 and 13). Numerous examples from animal, as well as a few plant systems (*e.g.* 12), show that calcium can effect its regulatory action via protein phosphorylation. It is apparent that accumulation of more biochemical data on CDPKs in plants is necessary at this time to develop means to investigate their functional role.

Plant CDPKs are present in the cell in both soluble and membrane-bound form (1, 4) and individual research groups have tended to concentrate on one or other of these. The laboratory of Polya analyzed both the soluble and membrane-bound activities and recently noted certain similarities between them (6, 7). Further elucidation of the question of relatedness of these activities is necessary to gain better insight into the mechanisms of their action. Specifically, the demonstration that there is only one activity would help to focus the research and to address questions of the physiological/biochemical significance of the two-phase distribution. In contrast, evidence for two distinct protein kinase species would indicate the existence of separate signal transduction pathways involving calcium and CDPKs.

The data presented here strongly support the former possibility, since the soluble and membrane-bound activities share many important characteristics including molecular weight of the active polypeptide, isoelectric point, and enzymatic properties.

[1] This research was carried out at Brookhaven National Laboratory under the auspices of the U.S. Department of Energy and with funding from its Office of Basic Energy Sciences, Division of Energy Biosciences.

[2] Abbreviations: CDPK, calcium-dependent protein kinase; PBE, Polybuffer exchanger.

MATERIALS AND METHODS

Plant Material

Seedlings of barley, *Hordeum vulgare* L. cv. Borsoy, were grown in the dark for 7 d at room temperature.

Chemicals

[γ-^{32}P]ATP (specific activity 6000 Ci/mmol) was obtained from New England Nuclear. Acrylamide was purchased from Accurate Chemical, SDS from Bio-Rad; phenyl-Sepharose, Polybuffer Exchanger 94, and Polybuffer 74 from Pharmacia; Matrex Gel Blue A from Amicon; histone IIIS, histone-agarose, heparin-agarose, and PVP (insoluble and mol wt 40,000) from Sigma.

Protein Kinase Assay

This assay was performed in a 40 μL assay mixture containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 200 μg/mL histone IIIS, and 2 μM [γ-^{32}P] ATP (1 μCi). It was not necessary to add exogenous calcium to the assay to obtain optimal activity in routine assays (see "Results"). When absolute levels of activity had to be determined under completely standardized conditions, $CaCl_2$ was added to a final concentration of 1 mM. After adding up to 10 μL of enzyme preparation, the assay was incubated for 30 min at room temperature. The samples were spotted on 2 × 2 cm squares of Whatman 3MM filter paper, which were washed three times for 10 min in 10% TCA, 1% sodium pyrophosphate, rinsed in ethanol, dried, and counted for Cerenkov radiation in a scintillation counter. One unit of activity is defined as the amount of enzyme catalyzing the incorporation of 1 pmol of phosphate into histone per 30 min under these conditions.

Determination of Effect of Free Calcium on Protein Kinase Activity

The concentration of free calcium in the assay medium was controlled by adding a Ca^{2+}/EGTA buffer comprised of a

final concentration of 0.2 mM EGTA and variable concentrations of $CaCl_2$, calculated using a computer program COMICS described in Perrin and Sayce (11) on the basis of stability constants from Martell and Smith (9).

Purification of Soluble CDPK

All operations were performed at 4°C. Aliquots of 150 g of etiolated 7 day old barley leaves were homogenized for 1.5 min in a Waring blender in 3 volumes of 50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 1% insoluble PVP, and 100 μM PMSF. The homogenate was filtered through four layers of cheesecloth and centrifuged for 10 min at 10,000*g*. The supernatant was fractionated with ammonium sulfate to obtain a 25 to 40% saturation cut. The pellet was suspended in a minimal volume of buffer A (50 mM Tris-HCl [pH 7.0], 100 μM PMSF) (*ca.* 150 ml) and the resultant suspension was centrifuged for 10 min at 10,000*g*. The supernatant was loaded on a 2.5 × 8 cm column of phenyl-Sepharose, which was extensively washed with 25 mM Tris-HCl (pH 7.0) until no protein emerged in the eluate. The column was then washed with 5 volumes of a buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, and 15% glycerol; and was finally eluted with 5 volumes of 1 mM EGTA in the same buffer. The active fractions were pooled and loaded on a 0.8 × 5 cm column of PBE94. The column was washed with 25 mM histidine-HCl (pH 6.2), 5 mM 2-mercaptoethanol, and 15% glycerol, and was eluted with 14 volumes of 1:8 diluted Polybuffer 74 containing 5 mM 2-mercaptoethanol and 15% glycerol.

Purification of Membrane-Bound CDPK

All operations were performed at 4°C. Samples of 2 kg of etiolated 7 day old barley leaves were homogenized for 2 min in a Waring blender in 3 volumes of 50 mM Tris-HCl (pH 7.0), 250 mM sucrose, 1 mM EDTA, 1% PVP (mol wt 40,000), and 100 μM PMSF. The homogenate was filtered through four layers of cheesecloth and centrifuged for 10 min at 10,000*g* and for 20 min at 80,000*g*. The pellet of the second centrifugation was suspended in a minimal amount of 20 mM Tris-HCl (pH 8.0) and 100 μM PMSF and added dropwise into a stirred beaker with 20 volumes of ice-cold acetone. After stirring for at least 1 h, the acetone powder was separated by filtration, vacuum-dried, and suspended in a minimal volume of buffer A. A Potter glass homogenizer with Teflon pestle was used to disperse the powder and the suspension was stirred for 30 min and centrifuged for 15 min at 20,000*g*. The supernatant was subjected to ammonium sulfate fractionation to obtain the 25 to 40% saturation fraction. The pellet was suspended in a minimal amount of buffer A and loaded on a 1 × 3.5 cm column of phenyl-Sepharose. The elution of the activity and the subsequent chromatofocusing step were performed as described for the soluble activity.

Detection of Protein Kinase Activity *in Situ* after SDS-PAGE (Activity Gels)

Gel electrophoresis under denaturing conditions was performed in the presence of SDS as described in Laemmli (8). The stacking and separating gels contained 4 and 10% w/v polyacrylamide, respectively (acrylamide/bis-acrylamide, 30:1, w/w). The activity assay *in situ* was a slight modification of the procedure described in Geahlen *et al.* (3); the procedure was previously employed for a plant protein kinase by Harmon *et al.* (4). Histone IIIS was added to the separating gel solution to a final concentration of 100 μg/mL prior to polymerization. Gel thickness was 0.75 mm, the running distance 5 cm. After electrophoresis, the gel was washed four times for 15 min at 4°C in 30 gel volumes of 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol and incubated for 4 and 1 h in two changes of 40 volumes of assay buffer (50 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 10 mM DTT, 200 mg/L BSA, and 20% glycerol). The *in situ* assay was performed for 5 to 8 h at room temperature with 100 μCi [γ-^{32}P]ATP in one gel volume of assay buffer contained in a sealed plastic bag. Afterward, the gel was washed four times for 20 min with 20 gel volumes of distilled water and incubated overnight in the presence of 5 g Dowex 1-X8 wrapped in one layer of Miracloth. Subsequently, the gel was washed twice with 20 gel volumes of 10% TCA and 1% sodium pyrophosphate and equilibrated with distilled water. Finally, the gel was covered with plastic wrap (or dried onto Whatman 3MM paper) and autoradiographed using Cronex 4 X-ray film (DuPont) and Kodak intensifying screens.

Preparation of Liposomes and Reconstitution Assays

The protocol for reconstitution of protein-lipid complexes was based on the procedure described in McDonnel and Staehelin (10), omitting the detergent. Phosphatidylcholine was dissolved in chloroform and vacuum-dried in a Speed-Vac centrifuge. The pellet was suspended by sonication in 50 mM Tris-HCl (pH 8.0), 15% glycerol (suspension buffer) at a final concentration of 10 mg/mL. The reconstitution assay was performed in a 100 μL suspension, which contained variable amounts of enzyme fractions and phospholipid vesicles. The assay suspension was frozen in liquid nitrogen, thawed, and sonicated for 2 min in a water bath. The samples were centrifuged at 100,000*g* for 30 min and the pellet was rinsed with 100 μL of suspension buffer. It was solubilized in 30 μL of suspension buffer containing 1% Triton X-100, and assayed for protein kinase activity.

RESULTS

Abundance and Distribution in Crude Extract

Following homogenization of barley leaves, protein kinase activity can be detected both in the supernatant and pellet fractions after centrifugation at 80,000*g* for 30 min (soluble and membrane-bound activities, respectively). With histone IIIS as a substrate under conditions described in "Materials and Methods," both the soluble and membrane-bound protein kinase are predominantly calcium-dependent, since addition of 5 mM EGTA to the assay mixture inhibits 90 to 95% of the soluble and 80 to 85% of the membrane-bound activity. The EGTA-sensitive activity can be detected at the level of about 10,000 units/g of fresh weight in the soluble and about 400 units/g of fresh weight in the membrane-bound fraction,

which corresponds to *ca.* 4% of CDPK being associated with membranes (data not shown).

Purification and Elution Pattern in Chromatofocusing

Both membrane-bound and soluble activities were isolated using calcium-dependent hydrophobic chromatography on phenyl-Sepharose. They eluted from the column at identical positions in a calcium-dependent manner (data not shown), indicating a comparable effect of calcium on the exposure of hydrophobic domains. This step resulted in major purification of the preparation (*ca.* 300 times for the soluble, and *ca.* 100 times for the membrane-bound activity). Further purification was achieved by chromatofocusing, where again the elution pattern was identical (Fig. 1). Both activities show, accordingly, the same isoelectric point of pH 4.25 ± 0.2. The soluble activity was purified 470-fold with 30% yield and a final specific activity of 19,500 units/mg, while for the membrane-bound activity these values were 320-fold, 31%, and 16,200 units/mg, respectively. The relative elution position of the activities was also determined on several other chromatographic media as follows. The phenyl-Sepharose eluate was supplemented with 2 mM $CaCl_2$ and 5 mM $MgCl_2$ and was loaded on 1 mL columns equilibrated with a buffer containing 50 mM Tris-HCl (pH 8.0), 15% glycerol, and 5 mM 2-mercaptoethanol. The columns were eluted with a gradient of KCl in the equilibration buffer. The activities eluted identically in the following effluent fractions from the columns: heparin-agarose, 150 mM KCl; Matrex Gel Blue A agarose, 500 mM KCl; histone-agarose, 1000 mM KCl. Upon gel filtration through a Sephacryl S-200 column, both activities comigrated with chicken ovalbumin, indicating a native molecular weight of *ca.* 45,000.

Calcium Dependence

Both activities were assayed in the presence of varying concentrations of free calcium. The determined dependence profiles appear to be very similar and show half-maximal activation values of around 20 μM free Ca^{2+} (Fig. 2). Since activation was achieved by low concentrations of calcium, no addition of exogenous calcium to the assay solution was necessary due to sufficient contaminating amounts present in the other reagents, or calcium tightly bound to the enzyme.

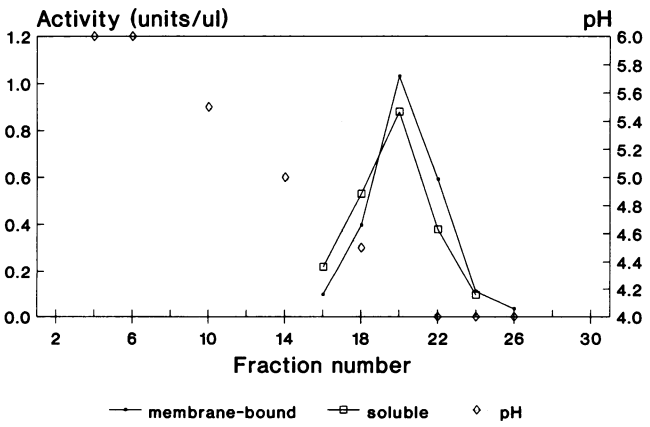

Figure 1. Elution profiles of the soluble and membrane-bound activities from a chromatofocusing column of PBE94. For assay conditions and definition of activity unit see "Materials and Methods."

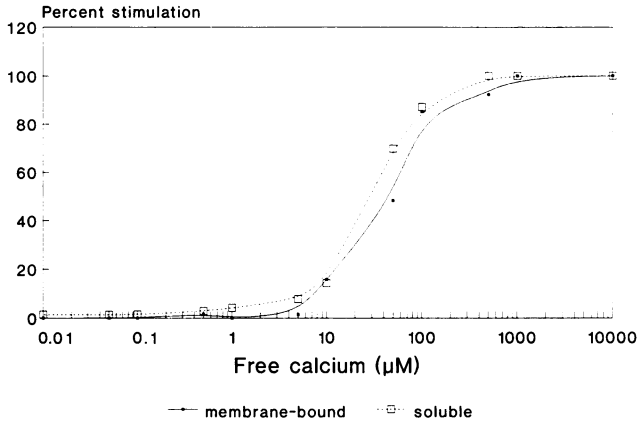

Figure 2. Effect of free calcium in the assay buffer on the protein kinase activities.

Table I. *Comparative Characterization of Assay Conditions for the Membrane-Bound and Soluble Protein Kinase*

Conditions	Activity	
	Membrane-bound	Soluble
	%	
Standard assay	100	100
KCl added (mM)		
100	29	23
200	7	8
400	2	5
N-Ethyl maleimide added (mM)		
2	40	45
10	6	8
pH[a]		
7.0	65	43
7.5	72	55
8.0	100	100
8.5	120	107
9.0	165	140
Protein substrate[b]		
Myelin basic protein	65	61
Dephosphorylated casein	10	12
Myosin light chain	0	0

[a] The assay contained 50 mM Tris-HCl of the corresponding pH. [b] Histone IIIS was replaced by given proteins at a final concentration of 200 μg/mL.

Comparison of Enzymatic Properties

The effect of assay conditions on protein kinase activities is shown in Table I. In each situation studied, both activities were identically affected. Both activities were strongly inhibited by monovalent ions and *N*-ethyl maleimide, were stimulated by alkaline pH values, and preferred basic protein substrates. K_m values for ATP were 6.0± 1.2 μM and 9.3 ± 2.0 μM and for histone IIIS, 150 ± 30 μg/mL and 170 ± 40 μg/mL for the soluble and membrane-bound activities, re-

spectively. Within the limits of experimental error, these values could be ascribed to a single enzyme component.

Detection of Protein Kinases in Denaturing Gels

When the activity assay for protein kinase was performed *in situ* after SDS-PAGE, a single, strong activity band predominated at 37 kD in both the soluble and membrane-bound preparation (Fig. 3). These bands were cut out and subjected to analysis of phosphoamino acids, which revealed that radioactive label was found principally in phosphoserine. An identical result followed when the enzyme fraction used for activity gel was assayed directly in solution and phosphorylation products were analyzed (data not shown). The 37 kD bands correspond to a calcium-dependent activity, since inclusion of EGTA in the gel assay buffer reduced band intensity to *ca.* 5 to 10% (data not shown). Together with the native molecular weight of *ca.* 45,000, this result indicates that both protein kinases are monomeric polypeptides. Weak activity bands of around 20 kD were sometimes observed in the membrane-bound preparation, but these represent a minor activity component and probably result from proteolysis.

Pelletability of Soluble Activity with Liposomes

Since the above data strongly indicated the identity of the enzymes, we sought to determine whether the soluble activity can attach itself to membranes. Artificial liposomes prepared of phosphatidylcholine were used, and mixtures containing different amounts of liposomes and the enzyme were subjected to a treatment developed for reconstitution of membrane proteins into protein-lipid complexes (10) and were assayed for the recovery of protein kinase in the pellet from a 100,000*g* centrifugation. The amount of activity recovered in the supernatant was 150 to 200% of that added, due to a stimulatory effect of low concentrations of phospholipids (20–100 μg/mL). Higher phosphatidylcholine concentrations (above 1 mg/mL) were inhibitory. As shown in Table II, between 3.3 and 10% of the total activity recovered could be found in the pellet, whereas the control values without liposomes were between 0 and 0.5% (values higher than 0% were obtained only with high activity loading). The amounts of pelletable kinase were not affected by the presence of EGTA in the suspension buffer (data not shown). When hemoglobin was used in the assay as a control for entrapment and nonspecific binding, 1.6 ± 1.4% of protein was recovered in the pellet. Since the values obtained with protein kinase are above this range, its binding shows some specificity.

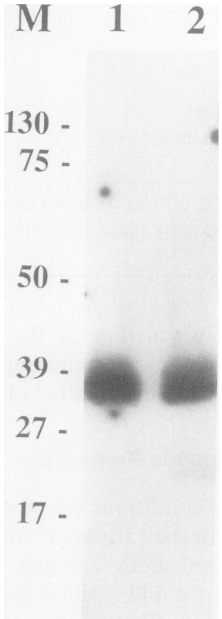

Figure 3. Autoradiogram of a protein kinase activity gel with histone IIIS as incorporated substrate. Lanes: M, protein molecular mass standards given in kD; 1, soluble activity; 2, membrane-bound activity.

DISCUSSION

The paper of Klucis and Polya (7) provided some preliminary data that the soluble and membrane-bound plant CDPKs are similar activities. The authors noted that the enzymes have in common their behavior on DEAE-Sephacel columns, native molecular size, and several enzymatic characteristics. In this work, we further substantiate these indications by comparing key physicochemical properties such as the mol wt of the active subunits, the isoelectric point, and further enzymatic characteristics. The activity gel technique provides a very precise determination of mol wt, since it employs separation under denaturing conditions in polyacrylamide gels. The data obtained with this method show clearly that the active subunits of the kinases have identical mol wts, and constitute most probably the only subunit in a monomeric enzyme. Although identical molecular size may nonetheless be coincidental, the enzymes do have the same isoelectric point, and elution pattern from several chromatographic materials and do share important enzymatic properties.

The data presented in this paper are in good agreement with earlier work on plant CDPKs. Harmon *et al.* (4) showed that the molecular mass of an active subunit of a soluble protein kinase from suspension-cultured soybean cells fell in the 46 to 51 kD range. Klucis and Polya (7) determined the native mol wts for two membrane-bound forms from silver beet as 51,000 and 53,000 and two membrane-bound forms as 56,000 and 57,000. K_m values for ATP and histone IIIS reported in the latter work were higher than those reported here, but still lay within one order of magnitude. However, we did not observe separation of the activities studied here into two different forms on DEAE-cellulose, although overloaded columns resulted in broad elution, nor was separation

Table II. *Adsorption of Soluble CDPK Activity to Phosphatidylcholine Liposomes*

Activity per Assay	Phosphatidylcholine Concentration	Activity Recovered in Pellet
units	*mg/mL*	*units*
0.5	0	0.000 ± 0.003
0.5	5	0.054 ± 0.014
0.5	10	0.043 ± 0.009
5	0	0.032 ± 0.01
5	5	0.165 ± 0.05

obtained on FPLC Mono Q (data not shown). Furthermore, we could not identify any partial independence of Ca^{2+} in the presence of unsaturated fatty acids. The reports from the group of Trewavas (1, 2) are in more contrast to ours and those discussed above, since we detected at most only very weak activity bands around 20 kD. We conclude that the putative 18-kD kinase subunit described therein is either not active in our system, or is a proteolytic fragment.

The soluble activity was shown to become pelletable with artificial liposome vesicles, and the percentage of activity recovered in the pellet in these *in vitro* experiments closely parallelled the amounts of activity found in the membrane fraction upon homogenization (3.3–10% and 4%, respectively). A number of interpretations could be applied to these data. There could be a single species of protein which partitions between the water and lipid phases. The partitioning might occur either *in vivo*, where it may or may not have some physiological significance, or as an artifact produced during cell disintegration. Since the amount of activity associated with membranes both upon isolation and *in vitro* is low, an artificial association cannot be excluded and could arise from unspecific adsorption or entrapment. Alternatively, a small fraction of the enzyme could be subtly modified so as to be indistinguishable by activity gels or chromatofocusing, yet exhibit preferential binding to membranes. The association of the membrane-bound activity with the membrane is not very strong since is can be released by 0.1% w/v Triton X-100. The soluble form clearly does not require detergent to preserve activity, as might be expected for a solubilized integral membrane protein.

Although it has frequently been found that soluble enzymes (and protein kinases in particular) can be associated with different cellular organelles, including membranes, in very many cases neither the biological significance nor the mechanism of association are known, mainly owing to methodological problems in addressing these aspects. Especially difficult for interpretation are situations in which only a small percentage of total activity becomes associated with an organelle and where artificial interaction cannot be fully excluded. Since this is the case in the plant CDPK system, it would be premature to speculate on the nature and mechanism of its membrane association both *in vivo* and *in vitro* solely on the basis of the available data.

The extent of identity and diversity among plant CDPKs can be fully assessed when sequence information becomes available. Since the purification of these enzyme close to homogeneity will be the prerequisite of sequencing and cloning work, information about a significant similarity between the soluble and membrane-bound activities, as described in this paper, is important. The membrane-bound activity is very low in abundance and its purification is difficult. Our attempts involved scaling up to 7 kg of starting plant material and still did not yield a pure protein in spite of *ca.* 1500-fold purification in relation to the acetone powder extract. In view of the extensive similarity between the two activities, a concentration of effort on the more abundant soluble activity may be a more practical and efficient initial approach.

ACKNOWLEDGMENTS

L.J.K. performed preliminary characterization of the membrane-bound activity during his Visiting Fellowship at the Plant Biotechnology Institute, National Research Council Canada, Saskatoon, and thanks Dr. William Crosby for facilities and support, and Dr. Vincenzo DeLuca for discussion and encouragement.

LITERATURE CITED

1. **Blowers DP, Trewavas AJ** (1987) Autophosphorylation of plasma membrane bound calcium calmodulin dependent protein kinase from pea seedlings and modification of catalytic activity by autophosphorylation. Biochem Biophys Res Commun **143:** 691–696
2. **Blowers DP, Trewavas AJ** (1989) Rapid cycling of autophosphorylation of a Ca^{2+}-calmodulin regulated plasma membrane located protein kinase from pea. Plant Physiol **90:** 1279–1285
3. **Geahlen RL, Anostario MA, Low PS, Harrison ML** (1986) Detection of protein kinase activity in SDS-PAGE gels. Anal Biochem **153:** 151–158
4. **Harmon AC, Putnam-Evans C, Cormier MJ** (1987) A calcium-dependent but calmodulin-independent protein kinase from soybean. Plant Physiol **83:** 830–837
5. **Kauss H** (1987) Some aspects of calcium-dependent regulation in plant metabolism. Annu Rev Plant Physiol **38:** 47–72
6. **Klucis E, Polya GM** (1987) Calcium-independent activation of two plant leaf calcium-regulated protein kinases by unsaturated fatty acids. Biochem Biophys Res Commun **147:** 1041–1047
7. **Klucis E, Polya GM** (1988) Localization, solubilization and characterization of plant membrane-associated calcium-dependent protein kinases. Plant Physiol **88:** 164–171
8. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
9. **Martell AE, Smith RM** (1974) Critical Stability Constants. Plenum Press, New York
10. **McDonnel A, Staehelin LA** (1980) Adhesion between liposomes mediated by the chlorophyll *a/b* light-harvesting complex isolated from chloroplast membranes. J Cell Biol **84:** 40–56
11. **Perrin DD, Sayce IG** (1967) Computer calculation of equilibrium concentrations in mixtures of metal ions and complexing species. Talanta **14:** 833–842
12. **Ranjeva R, Refeno G, Boudet AM, Marme D** (1983) Activation of plant quinate:NAD^+ 3-oxidoreductase by Ca^{2+} and calmodulin. Proc Natl Acad Sci USA **80:** 5222–5224
13. **Trewavas AJ** (1986) Molecular and Cellular Aspects of Calcium in Plant Development. Plenum Press, New York