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Diacylglycerol Kinase from Suspension Cultured Plant Cells[1]

Purification and Properties

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ABSTRACT

Diacylglycerol kinase (ATP:1,2-diacylglycerol 3-phosphotransferase, EC 2.7.1.107) from suspension-cultured *Catharanthus roseus* cells was extracted from a membrane fraction with 0.6% Triton X-100 and 150 millimolar NaCl and was purified about 900-fold by DEAE-cellulose, blue Sepharose, gel permeation, and phenyl-Sepharose chromatography. The enzyme is obviously membrane bound as activity in the cytosol could not be detected. In the presence of detergents such as Triton X-100 (3-[3-cholamidopropyl]dimethylamino)-1-propanesulfonate (Chaps), or deoxycholate, a molecular weight of about 250,000 was determined by gel filtration. In glycerol density gradients, the enzyme sedimented slightly more slowly than bovine serum albumin, indicating a molecular weight of less than 68,000. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis enzyme activity could be assigned to a protein of 51,000 daltons. As found previously for bacterial and animal diacylglycerol kinases, the purified enzyme was completely devoid of activity without the addition of phospholipids or deoxycholate. Cardiolipin was found to be most effective, whereas higher amounts of detergent were inhibitory. The enzyme needs divalent cations for activity, with Mg^{2+} ions being the most effective. Apparent K_m values for ATP and diacylglycerol were determined as 100 and 250 micromolar, respectively.

Although one function of DG[2]-kinase in *de novo* phospholipid synthesis is conceivable, that of balancing the action of PA phosphatase (25, 26), its main role is considered to be the recycling of DG generated from different sources. Whereas in *Escherichia coli,* DG is obtained from phospholipids other than PI (6, 29), in animal cells it is mainly PI which is cleaved by phospholipase C. This step is part of the so-called PI cycle which is under regulatory control in a variety of physiological processes (for review, see refs. 1 and 22).

In plant cells, enzyme activities of the PI cycle, *i.e.* the existence of PI and PI-4-phosphate kinases (8, 9, 31, 32) and phospholipase C (4, 5, 10, 11, 18, 23, 24, 27, 28), have been reported recently. We assayed the *in situ* activity of DG-kinase during the growth cycle of suspension-cultured plant cells (9). In the present work the purification of DG-kinase from suspension-cultured *Catharanthus roseus* cells is reported and its properties are compared with enzymes described from *E. coli* (3, 17, 20, 30, 34) and from animal cells (14, 15, 19).

MATERIALS AND METHODS

Enzyme Assay

DG-kinase activity was determined by measuring the label incorporated from [γ-^{32}P]ATP into PA at 25°C. The standard assay mixture contained, in a total volume of 250 μL, 40 mM Bis-Tris (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM spermine, 0.5 mM DTT, 1 mM sodium-deoxycholate, 0.02% Triton X-100, 250 μM cardiolipin, 500 μM dioleoylglycerol, 1 mM ATP (containing about 1 μCi labeled ATP), and 25 μl of the enzyme fraction. The stock solution (8.3-fold concentrated) of DG together with cardiolipin had to be prepared in the presence of deoxycholate. The lipids, dissolved in chloroform/methanol (1:1), were placed in plastic reaction vessels and dried under a stream of nitrogen; Na-deoxycholate dissolved in water was added and the mixture sonified 4 times with a Branson sonifier (50 W for 20 s followed by 2 min cooling in ice).

The assay mixture was preincubated for 5 min at 25°C, the ATP was added, and the reaction was stopped after 30 min by adding 760 μL chloroform/methanol (1:2) containing 1% concentrated HCl. Extraction and separation of phospholipids was performed as described previously (7, 8). An aliquot of the phospholipids dissolved in chloroform was used to determine the total amount of the incorporated labeled phosphate, a second aliquot to separate the components by TLC. The plates were scanned with a Berthold LB 512 thin layer analyzer and the spots were also visualized by autoradiography. After the purification step on DEAE-cellulose, the enzymic product was only assayed in the chloroform/methanol phase used for phospholipid extraction by counting in a liquid scintillation counter.

Purification of the DG-kinase

The following buffers were used: (basic buffer) 40 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 5 mM mercaptoethanol; (A) basic buffer supplemented with 0.25 M sucrose;

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[2] Abbreviations: DG, diacylglycerol; Chaps, (3-[3-cholamidopropyl]dimethylamino)-1-propanesulfonate; PA, phosphatidic acid; PI, phosphatidylinositol.

(B) basic buffer supplemented with 0.6% Triton X-100, 0.06% sodium deoxycholate, 150 mM NaCl, 0.1 mM PMSF, 0.1 mM pepstatin, and 10% glycerol; (C) basic buffer supplemented with 0.2% Triton X-100, 0.018% sodium deoxycholate, 10% glycerol, and 50 mM NaCl; (D) buffer C supplemented to 500 mM NaCl; (E) buffer C supplemented to 1.2 M NaCl; (F) buffer C supplemented to 100 mM NaCl; (G) basic buffer supplemented with 0.1% Triton X-100, 1.7 M NaCl (pH 6.8); (H) basic buffer supplemented with 3 M NaCl (pH 6.8); (I) basic buffer supplemented with 50 mM NaCl and 0.2% Triton X-100.

The *Catharanthus roseus* suspension-cultured cells (7) were harvested on the 6th day of the growth cycle (about the middle of the growth phase) and frozen in liquid nitrogen. All further operations were carried out at 0 to 4°C. The cells were suspended in buffer A (3 mL per g cells) and homogenized with four pulses of 30 s duration with an Ultra Turrax homogenizer (Janke and Kunkel). The suspension was centrifuged for 5 min at 160 *g* (swing out rotor), and the sediment was resuspended in buffer A and again homogenized and centrifuged at 160 *g*. The supernatants were combined and centrifuged at 44,000 g for 1 h. The pellets were resuspended in buffer B (50 mL per 100 g cells) and extracted with 30 strokes (maximum speed) in a Potter Elvehjem homogenizer. After centrifugation at 44,000 *g* for 1 h the supernatant was frozen in liquid nitrogen and stored at −70°C.

For DEAE-cellulose chromatography, the extract from 100 g cells (50 mL) was diluted 3:1 with basic buffer supplemented with 15% glycerol, to obtain the composition of buffer C, applied to a column (2.6 × 10 cm) of DEAE-cellulose (DE-52, Whatman), which had been equilibrated with 300 mL of buffer C, and eluted with a gradient profile (buffer C and D) as indicated in Figure 1A. The enzyme appeared in the step gradient with 30% buffer D as indicated. The activity-containing fractions (15–20 mL) were pooled and frozen in liquid nitrogen for further purification.

For blue Sepharose chromatography, the pooled fractions from four DEAE-cellulose column preparations were desalted on a Sephadex G-25 column (2.6 × 50 cm), which had been equilibrated with buffer C, and applied to a column (2.6 × 3 cm) of blue Sepharose. After a wash with 30 mL of buffer C, the enzyme was eluted by a linear gradient of 0 to 100% of buffer E in buffer C as shown in Figure 1B. The pooled activity containing fractions were desalted as before and frozen in liquid nitrogen.

For size exclusion chromatography, the pooled fractions were first concentrated on a 4 mL column of Q-Sepharose (fast flow) by elution with a small volume (about 6 mL) of 250 mM NaCl (in buffer C). Gel filtration was performed with a Sephacryl S 200 HR and S 300 HR column in series (each 2.6 × 94 cm) equilibrated and eluted with buffer F.

Hydrophobic interaction chromatography was performed with the above activity-containing fractions, which were diluted 1:1 with buffer H, the pH adjusted to 6.8, and applied onto a 2 mL phenyl-Sepharose column that had been equilibrated with buffer G. After application of the enzyme, the column was washed with buffer G and eluted with buffer C. The active fractions were concentrated on a 1 mL "Mono-Q" column from Pharmacia using buffer D for elution.

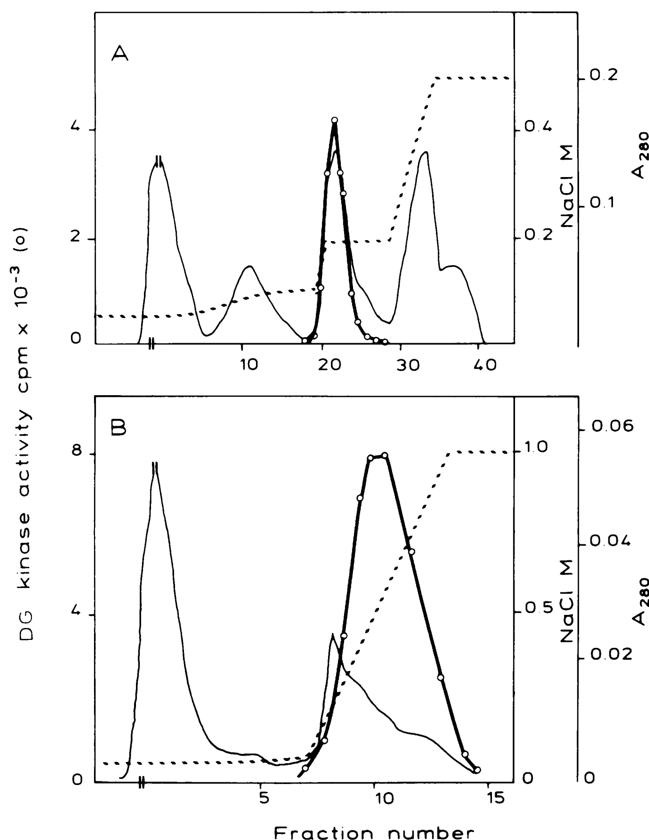

Figure 1. DEAE-cellulose (*top*) and Blue-Sepharose Chromatography (*bottom*) of DG-kinase. The extract of 100 g wet cells was applied to a 53 mL DEAE-cellulose column (A); thereafter the pooled, activity-containing fractions of four DEAE-cellulose runs were desalted by Sephadex G-25 gel filtration and applied to a 16 mL blue Sepharose column (B) (for details, see "Materials and Methods." The indicated interruptions correspond to the end of sample application. Fractions of 5 mL were collected. (O——O) DG kinase activity, - - - - - NaCl gradient, extended line without symbols is the absorption recorded at 280 nm (protein).

Gel Electrophoresis

Electrophoresis was performed with the purified enzyme on a 8% polyacrylamide gel, containing 0.1% SDS, at about 10°C. After electrophoresis the gel was washed twice (5 min) in the buffer used for the enzyme activity assay, containing 0.03% Triton X-100 and 10 mM NaCl. One-third of the gel was cut in 2-mm strips, which were ground in liquid nitrogen in a mortar. The frozen powder was placed in an Eppendorf reaction vessel and 1 mL of the assay buffer was added. After thawing, vortexing, and centrifuging to remove the polyacrylamide, the supernatant was assayed for enzyme activity for 2 h with 2 μCi [γ-^{32}P]ATP. A further third of the gel was used for silver staining and the last part was stored at −20°C.

After assignment of the enzyme activity containing band, this band was cut out from the third part, ground in liquid nitrogen, and resuspended in assay buffer, and the polyacrylamide was removed by centrifugation. The protein was precipitated with 10% TCA and the pellet resuspended in 40 μL buffer C and 10 μL electrophoresis sample buffer. After

heating for 5 min at 80°C, this sample was subjected to SDS gel electrophoresis.

Glycerol Density Gradient Sedimentation

Linear gradients of 10 to 40% (v/v) glycerol in buffer I were prepared by fast protein liquid chromatography equipment from Pharmacia. Linearity was determined by adding 1 mg/mL BSA in the 40% glycerol buffer of a control sample and measuring the absorption at 280 nm. Samples were applied in 100 μL aliquots containing both DG-kinase and standard proteins (catalase, alcohol dehydrogenase, BSA, and cytochrome *c*). Gradients were centrifuged for 15 h at 50,000 rpm in a swinging bucket rotor (SW 60 Beckmann) at 4°C. Fractions of 150 μL were collected and assayed for DG-kinase activity, for marker enzymes, and for UV absorption in the case of BSA and cytochrome *c*.

RESULTS

Substrate Specificity of Phosphorylation

The presence of endogenous lipid substrates and different phospholipid kinases in the crude cell homogenate caused phosphate transfer from labeled ATP to occur not only into PA but into a variety of other phospholipids in spite of the fact that an excess of exogenous DG was used in the enzyme assay. (With crude homogenate an inhibition of phosphate incorporation from ATP was observed when the DG concentration was larger than 250 μM.) Table I shows that the main competing activity was a PI kinase whose activity and available substrate was significant in the crude cell extract and the membrane fraction. Only with DEAE-cellulose chromatography were the endogenous phospholipids removed and hence the PI-kinase activity. Also PA phosphatase activity could not be detected when assayed in the DG-kinase assay buffer replacing ATP by labeled PA (50,000 cpm). After this step the DG-kinase activity could be determined by simply counting the radioactivity in the chloroform/methanol extract.

Enzyme Purification

Purification started with the preparation of a membrane fraction from the crude cell extract by centrifugation at 44,000*g* (Table II). In the supernatant of this centrifugation step, containing 50% of the original protein, DG-kinase activity could not be detected. This shows that the enzyme is not cytosolic but located in an as yet undefined membrane fraction. DG-kinase extraction with a buffer containing both Triton X-100 and deoxycholate was very efficient as the remaining membrane pellet obtained by centrifugation contained only about 1% of the total enzyme activity but more than 50% of the total membrane protein. Total DG-kinase activity increased from the crude cell homogenate (Table II) to the membrane extract by a factor of about 5, probably due to inhibitory substances which were removed by preparation of the membrane extract. The degree of purification was therefore calculated from the protein balance. DG-kinase was unstable in the membrane extract; upon storage overnight at 4°C 90% of the activity was lost. However, the enzyme was stable for at least 6 months when stored at −70°C. Hence between the subsequent purification steps the active fractions were usually stored at −70°C.

DEAE-cellulose chromatography (Fig. 1) obviously removed most of the endogenous lipids, as after this step the DG-kinase activity was completely dependent on the added DG substrate and lipid cofactors such as cardiolipin. Blue Sepharose chromatography proved to be very efficient with an almost seven-fold purification; a steep salt gradient was used for elution as smooth gradients gave very extented activity peaks. This purification step resulted in a rather stable enzyme; furthermore radioactive incorporation was linear with time for at least 2 h in the usual activity assay. As this fraction was used for the enzymatic characterization of the DG-kinase, it was verified that the K_m values for ATP and DG did not change (within the limit of 5% deviation) by further purification to the phenyl-Sepharose step.

Further purification was performed with a combination of Sephacryl S200 and S300 chromatography operated in series

Table I. *Phosphorylation Specificity at Different Stages of Purification*

The fractions were obtained at the different purification steps, as described in "Materials and Methods," and were frozen before the determination of the enzymic activities. Determination was performed with 250 μM (first two fractions) or 500 μM exogenous DG. In the unextracted membrane fraction the activities were determined in situ, *i.e.* before the extraction (*cf.* "Materials and Methods"). The different labeled phospholipids were determined by scanning: the values are the percentage of the total radioactivity determined.

Fraction	Phospholipids Determined by Scanning[a]				
	PA	PI	PIP	PIP_2	Others
	% total radioactivity				
Cell homogenate	45	10	34	2	9
Unextracted membrane fraction	50	8	36	1	5
Membrane extract	75	7	18	0	0
DEAE-cellulose	>98	0	0	0	<2
Blue Sepharose	≤100	0	0	0	≤1

[a] PIP = PI-4-phosphate; PIP_2 = PI-4,5-bisphosphate.

Table II. *Purification of DG-Kinase*

The data are related to 400 g (wet weight) *C. roseus* cells as starting material. DG-kinase activity determination and the purification steps are described in "Materials and Methods." The crude extract was divided into the 44,000*g* supernatant and the pellet (membrane fraction) by centrifugation. Detergent extraction of this pellet fraction and a second centrifugation lead to the membrane extract (supernatant) and the second 44,000*g* pellet.

Fraction	Total Protein	Total Activity	Specific Activity	Degree of Purification
	mg	*nmol min^{-1}*	*nmol min^{-1} mg^{-1}*	*-fold*
Crude extract	2255	107	0.05	1
44,000*g* supernatant	1105	0	0	
Membrane extract	407	581	1.4	5.6
Second 44,000*g* pellet	504	6.9	0.013	
DEAE-cellulose	99	344	3.4	13.6
Blue Sepharose	8.4	192	22.8	91
Sephacryl S200/S300	2.0	177	99	355
Phenyl-Sepharose	0.62	143	231	922

which gave a four-fold purification without an appreciable loss of activity. For hydrophobic interaction chromatography, the enzyme was bound to phenyl-Sepharose at 1.7 M NaCl and 0.1% Triton X-100, and eluted by an immediate reduction of the ionic strength (buffer C) to obtain a sharp activity peak and an efficient separation (gradient elution did not result in further resolution). The overall degree of purification was about 900 with a yield of about 25%. Table II shows a typical purification; the specific activity obtained varied slightly from purification to purification depending on the age and physiological stage of the suspension cultured cells, but the degree of purification from step to step was rather reproducible.

Properties of DG-kinase

Complete removal of salt and/or detergent resulted in an irreversible loss of total enzyme activity; hence in the course of the purification process the ionic strength was not allowed to fall below 50 mM NaCl and 0.1% Triton X-100 and 0.018% deoxycholate were used. Larger amounts of detergents were inhibitory; starting with the detergent composition of the enzyme assay (0.28 mM Triton X-100 and 1 mM deoxycholate) Triton X-100 reduced the activity at slightly lower concentrations (50% inhibition after addition of about 0.5 mM) than deoxycholate (50% inhibition after addition of about 1 mM), but complete inhibition at higher concentrations was less efficient with Triton-100 (10% residual activity at 10 mM) than with deoxycholate (2% residual activity at 10 mM). Furthermore, Triton did not denature the enzyme irreversibly and was most efficient for extraction from the membrane fraction, being more efficient than other detergents tested such as Chaps, octylglucosides, Tween, or deoxycholate.

The enzyme had an activity optimum at pH 7 with a rapid decrease of activity below pH 6.5. It showed Michaelis-Menten kinetics with Mg-ATP as substrate. The apparent K_m value was 100 μM determined in the presence of 5 mM Mg ions. With Mg ions maximum activity was obtained at about 10 mM with only a slow inhibition at higher concentrations. Other divalent cations including Ca ions, however, were mainly inhibitory with only Mn ions showing a slight activation at 5 mM, whereas Co ions showed a strong inactivation.

Attempts to run the enzyme on native PAGE failed; with SDS gel electrophoresis of a protein fraction not heat treated, DG-kinase activity could be recovered from one protein band by washing the gel twice in assay buffer containing 0.03% Triton X-100 and 100 mM NaCl. The extracted protein band showed DG-kinase activity with PA as product as shown by TLC by comparison with authentic PA. Part of the active protein band was again run on SDS gel electrophoresis after extraction and heat denaturation. Silver staining revealed one band with mol wt of about 51,000.

Gel permeation chromatography on an analytical Sephacryl S 300 HR and TSK G 3000 SW column in the presence of detergent showed a mol wt of about 250,000; there were no significant differences for the various detergents used (0.2% Triton, 1% Chaps or 1% deoxycholate). The micells from pure Triton X-100 eluted on the same columns with an apparent mol wt of about 60,000 shortly after BSA. The enzyme fraction purified through the phenyl-Sepharose step was subjected to centrifugation in a glycerol density gradient. DG-kinase activity was found to sediment slightly more slowly than BSA.

DISCUSSION

Activity measurements in cellular fractions of suspension cultured cells and the condition of extraction showed that the *C. roseus* DG-kinase is located in a membranous fraction. The nature of this structure is at present not known. Studies are in progress to define the membrane structures which contain the main activity of DG-kinase. Preliminary studies on the differentiated *C. roseus* plants (leaves and stalks) also showed that DG-kinase is membrane bound.

DG-kinases have been previously described from *E. coli*, (3, 17, 20, 30, 34) *Dictyostelium discoideum* (13) and from mammalian sources (14, 15, 19). Whereas the *E. coli* enzyme is totally membrane bound (3, 17, 35), cytosolic location was reported for different mammalian enzymes (14–16, 19) with part of the activity located in membranes (16, 21, 33) and a

lipid-dependent translocation from the cytosol to membranes (2, 12). With *D. discoideum* a cytosolic and a particulate form were described which showed translocation to the particulate form upon induction of proliferation (13). Translocation could be stimulated by addition of the substrate DG or by action of phospholipase C (2).

The present DG-kinase could not be purified to homogeneity, as several bands were visible after staining the SDS gel. Due to extractable activity, we could assign a band of 51,000 D. The molecular mass of 51,000 D is markedly larger than the *E. coli* enzyme with 14,000 D (20, 30) but smaller than the cytosolic enzymes from mammalian brain with mol wt from 72,000 to 80,000 (15, 16, 19). Determination of the native size is difficult, as the DG-kinase from *C. roseus* was irreversibly inactivated when the detergents were completely removed. An apparent mol wt of 250,000 was determined in the presence of different detergents, such as Triton X-100 and Chaps known to form micells of different size. The high apparent mol wt is indicative of micellular structures containing the enzyme protein. With the *E. coli* enzyme sizes of 240,000 and 120,000 D have been reported upon gel filtration in the presence of Triton X-100 (20). The sedimentation behavior of the enzyme in a glycerol gradient, however, indicated that the enzyme existed as a monomer with a mol wt similar to that determined in SDS-PAGE.

The apparent K_m value for ATP of 100 μM determined for the *C. roseus* DG-kinase is rather low; for other enzymes values of 300 to 600 μM were reported (15, 19, 34). With the *E. coli* DG-kinase (34) the effect of divalent cations were tested which showed a slightly different behavior than with the present enzyme. With both enzymes free Mg is an activator; for the *E. coli* enzyme Mn and Co were also activating, whereas the present enzyme showed only a slight activating effect with Mn and a strong inhibitory effect of Co.

The distribution of the DG-kinase activity in the different membrane systems, the lipid and detergent dependency, as well as the substrate behavior of DG and a mapping of the ATP site, will be described in a subsequent study.

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