Diphosphatase Related to Lipid Metabolism and Gluconeogenesis in Cucumber Cotyledons: Localization in Plasma Membrane and Etioplasts but not in Glyoxysomes

Thomas Kirsch, Barbara Rojahn, and Helmut Kindl

Biochemie, FB Chemie, Philipps-Universität Marburg, D-3550 Marburg, Bundesrepublik Deutschland

Z. Naturforsch. **44c,** 937–945 (1989); received June 7/July 25, 1989

Plastid, Plasma Membrane, Glyoxysome, Diphosphatase (Pyrophosphatase), Cucumber Mobilization

Diphosphatase (inorganic pyrophosphatase) activity was localized within compartments of cotyledons of germinating cucumber seeds during the stage of maximal conversion of fat into carbohydrates. At this stage, almost 2 mol pyrophosphate are produced during the formation of one mole sucrose from 0.28 mol triglyceride. When organelles of the $2000 \times g$ pellet or $10{,}000 \times g$ pellet were separated by density gradient centrifugation and gradient flotation, the diphosphatase activity paralleled the profiles of markers of the plastid stroma but was virtually absent from the glyoxysomes. Within the fraction of small vesicles and membranes, diphosphatase was attributed to the plasma membrane.

The main portion of diphosphatase, contained in the plastids, was partially purified by chromatography on anion exchange resin and molecular sieving, leading to a 75-fold enrichment compared to the stroma fraction. Trace amounts of diphosphatase observed in the glyoxysomal fraction were analyzed in the same way. Comparison of the isoelectric points and the activity profile at different pH values and the inhibitory effect of the various cations indicated that the trace amounts of diphosphatase activity in the glyoxysome fraction represented contaminations originating from the plastids.

The plasma membrane form of diphosphatase is an integral membrane protein which was solubilized with octylglucoside. It was shown to differ from the plastid form in pH optimum and sensitivity towards bivalent cations. All forms of diphosphatase were clearly distinguished from other phosphohydrolytic activities.

Introduction

Cotyledons of germinating cucumber seeds are characterized as tissue that releases carbohydrates and amino acids into the meristematic portion of roots and hypocotyls of the seedling [1]. In the storage tissue, triglycerides are degraded to provide building blocks for the synthesis of carbohydrates [2] to be transported into the area of dividing cells. In the process of fat mobilization and fatty acid β-oxidation [2, 3], glyoxysomes synthesize fatty acyl-CoA from free fatty acid, ATP and CoA [4]. The products of this synthetase reaction are acyl-CoA, AMP and diphosphate (pyrophosphate). In addition to fatty acid degradation, diphosphate is formed in these cells in the cytosol during the synthesis of UDP-glucose and export of sucrose.

One can calculate that the mobilization of one molecule of triglyceride, *e.g.* palmitoyl-linoleoyl-stearoyl glyceride, leads *via* fat degradation and glyoxylate cycle to one molecule glycerol and 13 molecules malate, and eventually to 7 molecules hexose phosphate. Considering sucrose formation as main task, the mobilization of one molecule triglyceride and the concomitant formation of 3.5 molecules sucrose would lead to the production of 6.5 molecules diphosphate. It is important to know at which site this large amount of metabolite and potential energy source is formed and to describe the intracellular location of its removal.

To determine the fate of diphosphate and to get additional indirect evidences as to the intraorganellar localization of the diphosphate-forming step we investigated the metabolism of diphosphate and, thus, the distribution of diphosphatase within the various compartments and subcompartments of fat-degrading cotyledons. Processes as the formation of fruc-

Abbreviations: MES, 2-[N-morpholino]ethanesulfonic acid; MOPS, 3-[N-morpholino]propanesulfonic acid; PIPES, piperazine-N,N″-bis[2-ethanesulfonic acid]; TRICINE, N-tris-[hydroxymethyl]methylglycine.

Reprint requests to H. Kindl.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/89/1100–0937 $ 01.30/0

tose-1,6-bisphosphate or phosphoribosylpyrophosphate [5] were not considered in this particular metabolic situation.

It was assumed that a possible occurrence of diphosphatase within the matrix of glyoxysomes would indicate the release of products of the fatty acyl-CoA synthetase reaction into the interior of these organelles. In contrast to that assumption, we found no diphosphatase in glyoxysomes, but attributed the main portion of diphosphatase activity to plastids and plasma membrane.

Materials and Methods

Sources

Cucumber seeds (*Cucumis sativus* L.) purchased from Küpper, Eschwege, F.R.G., were surface sterilized and germinated on sterilized vermiculite at 26 °C, >90% humidity in the dark. Cotyledons of 4 day old seedlings were harvested and cooled to 4 °C.

Preparation of glyoxysomes and subfractions

The method for glyoxysome purification was a modification of the procedure of Köller and Kindl [6]. The homogenization buffer consisted of 1 mM EDTA, 2 mM $MgCl_2$, 10 mM KCl and 50 mM MOPS-NaOH, pH 6.8, in 25% (w/w) sucrose. The buffer was used at a ratio of 4 $ml \cdot g^{-1}$ fresh weight of cotyledons. The homogenate was filtered through two layers of Miracloth and centrifuged at $2000 \times g$ for 5 min, and the pellet was discarded. The supernatant was centrifuged at $10{,}000 \times g$ for 25 min. The sedimented organelles of the last pellet were carefully suspended in homogenization medium and loaded onto a sucrose gradient ranging from 30–56% (w/w) sucrose. Following centrifugation in a swinging bucket rotor (Kontron TST 28.38) at $70{,}000 \times g$ (av.) for 2 h, the content of the tubes was fractionated using a ISCO gradient fractionator model 640.

Glyoxysomes were subfractionated into matrix, peripheral proteins and residual membranes [7]. Membranes obtained after salt treatment were subjected to selective solubilization with Triton X-100.

Glyoxysomes were further purified by gradient flotation as follows. A glyoxysomal preparation obtained by sucrose density gradient centrifugation was brought to 62% (w/w) by slowly adding 67% (w/w) sucrose. Five ml of this mixture were pipetted into a 36.5 ml centrifuge tube and overlayered with 5 ml 60% (w/w), 13 ml 56%, 6 ml 50%, 4 ml 45% and 2 ml 40% sucrose. The density gradient flotation was at 27,000 rpm (Kontron TST-28.38 rotor) for 35 h. Following fractionation of the resulting gradient, the marker enzymes were determined in the 30 fractions.

Preparation of plastids

Plastids were prepared from etiolated cotyledons by homogenation with a scalpel. The extraction medium contained 70 mM TRICINE-NaOH, pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1% dextran 40, 1% Ficoll, 25% (w/w) sucrose, and 0.1% serum albumin. The homogenate was filtered through two layers of Miracloth and centrifuged at $2000 \times g$ for 5 min in a Sorval GSA rotor. The main portion of plastids were recovered in the pellet.

Preparation of plasma membrane

A fraction highly enriched in plasma membrane was prepared from microsomes according to [8]. The microsomal pellet was obtained by sedimenting the membranes contained in the supernatant after a $7000 \times g$ centrifugation of the crude homogenate. The sedimentation was performed at $100{,}000 \times g$ and 4 °C for 1 h in a Beckman rotor Ti 60. Consecutively, the pellet was suspended in 5 mM phosphate buffer, pH 7.5, containing 0.25 mM sucrose ("partition buffer"). The suspension was layered onto 5 ml cushion of 31% (w/w) sucrose in phosphate buffer and centrifuged for 2 h at 27,000 rpm in a Kontron rotor TH641. With the sediment, this treatment was repeated. The membranes suspended in partition buffer were transferred into a 27 g two-phase partition system which was eventually adjusted to 36 g. The final concentrations were: 6.2% dextran T500, 6.2% polyethylenglycol 4000, 0.25 mM sucrose, and 5 mM phosphate, pH 7.5. Prior to the 1 min period of gentle shaking, the temperature of the whole system was adjusted to 5 °C by standing for 30 min with occasional movement at this temperature. The phases were separated by centrifugation at $3000 \times g$ for 3 min. The upper phase (90%) was removed with a pipette and transferred to a second tube already containing 0.1 volume of fresh upper phase and 1 volume fresh lower phase. Furthermore, fresh upper phase (0.9 volume) was added to the first tube containing the lower phase including 10% of the upper phase. As described before, the two-phase extraction

was repeated and led to the purified upper phase and the purified lower phase. Finally, the separated phases were diluted with 7-fold volume of partition buffer, and the membranes collected by centrifugation for 2 h at 100,000 × *g*. Enriched plasma membranes recovered from the upper phase could be stored in a buffer containing 20% glycerol.

Following treatment of the plasma membrane with 100 mM $MgCl_2$, the integral membrane proteins were solubilized with 0.5% (w/w) octylglucoside. The micellar form of diphosphatase was recovered in the supernatant after a centrifugation in the airfuge at 200,000 × *g* for 40 min. The enzyme activity was not decreased by this procedure and remained unchanged during standing for 2 days at 4 °C.

Assays

ATPase activity was assayed at 30 °C in a 0.5 ml reaction volume containing 1 mM ATP, 30 mM PIPES-KOH, pH 7.0, and 5 mM $MgSO_4$, unless otherwise indicated. Inorganic phosphate was determined after reduction with *p*-methylaminophenol and sodium sulfite according to [9]. Diphosphatase activity was determined with an incubation mixture comprising 30 mM MOPS-KOH, pH 8.0, 10 mM sodium diphosphate, 5 mM $MgCl_2$, and 1 mM ammonium molybdate. For tests in the pH range pH 6.0–7.0, the respective MES-buffer was used. The production of phosphate was determined after incubation at 30 °C for 30 min. Triosephosphate isomerase [10], isocitrate lyase [11], malate synthase [12], acyl-CoA oxidase [13], fumarase [6] were assayed according to established methods. Galactosyl transferase activity was determined analogous to the procedure for glucosyl transferase [8]. Protein was assayed by the method of [14].

The amount of diphosphate was determined as described by [15]. Diphosphate was used by a bacterial diphosphate:fructose 6-phosphate 1-phosphotransferase, and the fructose-bisphosphate was assayed *via* aldolase and glycerol 3-phosphate dehydrogenase.

Enzyme purification

For large-scale preparation, 270 ml containing plastid stroma proteins were prepared from 800 g cotyledons. 270 ml of this fraction were chromatographed on a column of TSK-DEAE (Merck, Darmstadt, F.R.G., v_{total} = 30 ml; 20 cm length) which was previously equilibrated with 10 mM MOPS-NaOH, pH 7.0, and 10% (w/w) glycerol. Following pumping the solution onto the column, the column was washed with 120 ml of the equilibration buffer, and the proteins eluted by applying an anion gradient ranging from 0–330 mM NaCl. The peak fractions containing diphosphatase activity were combined. The solution was transferred into a dialysis bag and concentrated at 4 °C by embedding the bag into dry Sephadex G-100. The protein solution was then applied to a column of Sephacryl S-200 (Pharmacia, Uppsala, Sweden) (v_{total} = 100 ml; length: 58 cm) previously equilibrated with the previously used buffer. Chromatography was at a flow rate of 5 ml·h^{-1}.

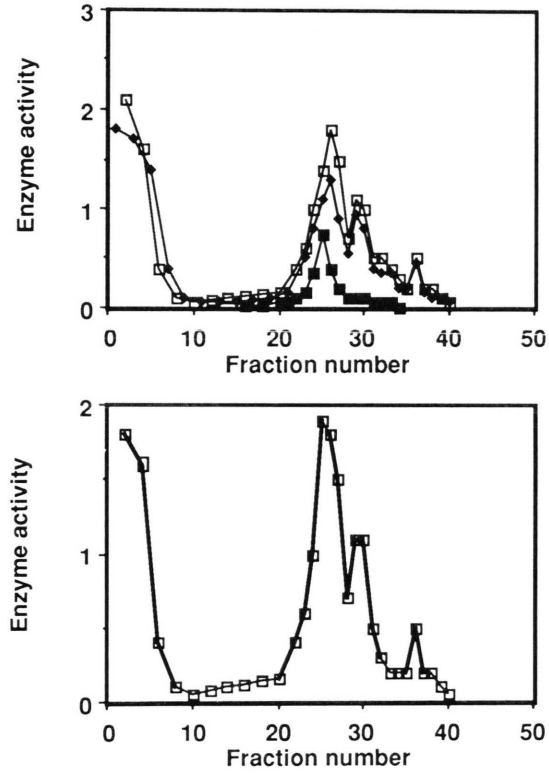

Fig. 1. Profiles of diphosphatase and marker enzymes after separation of etioplasts on a sucrose density gradient under equilibrium density conditions. A crude organelle preparation, *i.e.* a 2000 × *g* pellet obtained from a homogenate, was separated by centrifugation on a sucrose density gradient ranging from 25 to 58% (w) sucrose. Lower part: –□–□–□–, diphosphatase (1 arb. unit = 100 nkat). Upper part: –◆–◆–◆–, aldolase (1 arb. unit = 5 nkat); –□–□–□–, triosephosphate isomerase (1 arb. unit = 500 nkat); –■–■–■–, isocitrate lyase (1 arb. unit = 1 nkat).

Results

Distribution of diphosphatase activity among the organelles in the 2000 × g pellet or the 10,000 × g pellet prepared from etiolated cucumber cotyledons

Diphosphatase activity was assumed to take part in several processes subsequent to the cleavage of diphosphate from a nucleoside triphosphate. An exclusive location of diphosphatase within a single compartment seemed to be highly unlikely. Therefore, more than one way of separating organelles starting from several kinds of pellets was applied. We used a preparation of a 2000 × *g* pellet which is particularly suited for the isolation of plastids and also a 10,000 × *g* pellet which is enriched in glyoxysomes.

A fraction representing the large organelles of the cucumber cotyledons was prepared by a differential centrifugation at 2000 × *g*. The diphosphatase activity in this pellet amounted to 50% of the total extractable enzyme activity. Separation of the organelles contained in the 2000 × *g* pellet by equilibrium density centrifugation led to a profile of diphosphatase activity which was characteristic of plastids. Except the gradient supernatant representing the content of broken organelles, the profile showed one major peak and two minor peaks (Fig. 1). This profile coincided with the profiles of triosephosphate isomerase or aldolase. However, also a small amount of isocitrate lyase activity was sedimenting in the range of plastids. This findings needed to be investigated more thoroughly in the 10,000 × *g* pellet enriched in glyoxysomes. We compared the distribution of marker enzymes in 18 h gradients with the results from 2 h gradients. The peaks of optically dense material shifted to slightly greater densities during the longer centrifugation, though the pattern was similar for 2 h and 18 h.

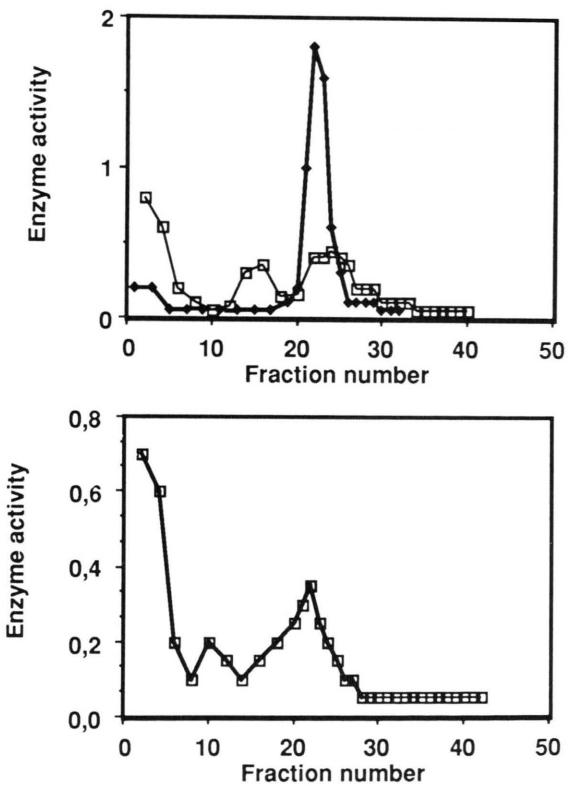

Fig. 2. Separation by sucrose density gradient centrifugation of organelles contained in a 10,000 × *g* pellet. Lower part: −□−□−□−, diphosphatase (1 arb. units = 1 nkat). Upper part: −◆−◆−◆−, isocitrate lyase (1 arb. unit = 100 nkat); −□−□−□−, triosephosphate isomerase (1 arb. unit = 200 nkat).

Starting with crude homogenates, and removing the large organelles by centrifugation at 2000 × *g*, we prepared a 10,000 × *g* pellet which encompassed 15% of the particle-bound diphosphatase activity of the cell extract. Within the 10,000 × *g* pellet, the main portion of diphosphate activity was attributed to plastids, as shown in Fig. 2 as the results of another sucrose density gradient centrifugation. Sucrose density gradient centrifugation afforded a clear separation of glyoxysomes from other organelles but left some activity of plastids in the range of glyoxysomes. Thus, an additional step was required for the purification of glyoxysomes contained in the fraction at density $d = 1.24\ \mathrm{g \cdot ml^{-1}}$ (Fig. 2). Subsequent flotation of the glyoxysome containing fraction in another density gradient (Fig. 3) proved that the profile of diphosphatase activity did not coincide with the activity of isocitrate lyase. In addition, acyl-CoA oxidase and malate synthase activities were assayed to further corroborate the separation of glyoxysomes and etioplasts (data not shown). The main portion of diphosphatase floated up to a density which was previously shown to be indicative of etioplasts.

By three consecutive purification steps, it became evident that by far the main portion of the organellar diphosphate activity was confined to etioplasts. This form of diphosphatase activity behaved during the centrifugation steps similar to triose phosphate isomerase activity which is located within the stroma of plastids. Less than 2% of the particle-bound form of diphosphatase activity was detectable in the purified glyoxysomes.

Partial purification and characterization of plastid diphosphatase

Starting out from a large preparation of plastid stroma proteins we purified the diphosphatase 75-fold by two consecutive steps, chromatography on an anion exchanger (Fig. 4) and molecular sieving. The results of the purification procedure are summarized in Table I. As the specific activity of the diphosphatase increased approximately 50-fold during the preparation of etioplast stroma from crude homogenate *via* 2000 × *g* pellet (data not shown), the actual purification of the plastid form of the enzyme may be close to 350-fold.

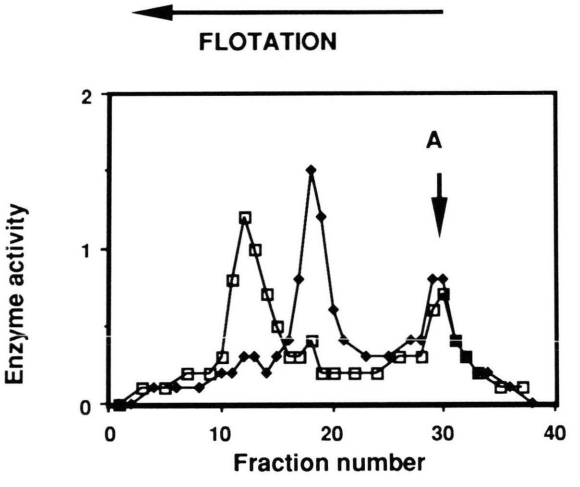

Fig. 3. Equilibrium flotation of glyoxysomes previously isolated by density gradient sedimentation. Glyoxysomes taken from fractions 22, 23 of the former gradient (Fig. 2) were adjusted to 56% (w) sucrose and layered onto a cushion of 58% sucrose. Then, a shallow sucrose gradient was overlaid. Following a 35 h centrifugation at 90,000 × *g*, the gradient was fractionated and assayed. The zone where the glyoxysomes were applied to the gradient prior to the centrifugation is marked as A. −◆−◆−◆−, isocitrate lyase (1 arb. unit = 100 nkat); −□−□−□−, diphosphatase (1 arb. unit = 0.2 nkat).

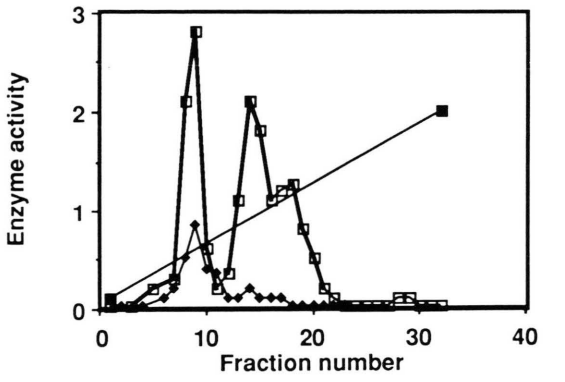

Fig. 4. Separation of diphosphatase on an anion exchange column. Proteins of the plastidic stroma were chromatographed on TSK-DEAE as described under "Materials and Methods". −□−□−□−, diphosphatase from stroma of etioplasts (1 arb. unit = 10 nkat). −◆−◆−◆−, diphosphatase activity from glyoxysomes (1 arb. unit = 0.5 nkat); −■−■−■−, NaCl (1 arb. unit = 100 mM).

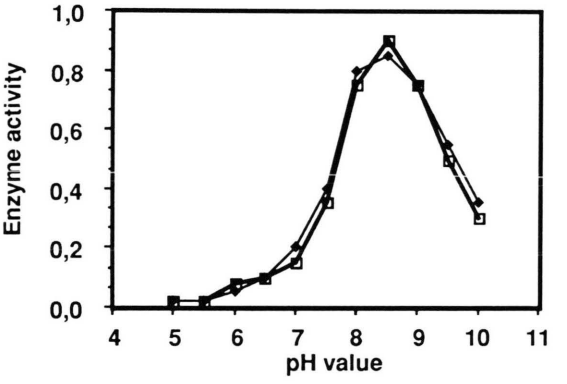

Fig. 5. Comparison of pH optimum of the etioplast diphosphatase and of diphosphatase activity adhering to glyoxysomes. −◆−◆−◆−, diphosphatase activity purified from stroma; −□−□−□−, diphosphatase activity assayed using purified glyoxysomes. Relative enzyme activities are drawn on the ordinate. They were determined with 40 times higher amounts of glyoxysomal protein compared to the amount of etioplast protein.

Table I. Large scale purification of soluble diphosphatase from etioplasts.

Fraction	Total activity [nkat]	Specific activity [nkat/mg]	Purification -fold	Yield [%]
Plastids	49.95	0.23	1	100
Stroma	29.70	0.28	1.2	59
Chromatography on anion exchange resin	11.98	3.07	13	24
Molecular sieving	5.49	17.30	75	11

Several properties of the stroma diphosphatase were determined in order to use them in comparing other diphosphatase preparation with the plastid form. Usually, the diphosphatase activity was tested in the presence of 1 mM molybdate which inhibits, by more than 90%, phosphatase activity possibly present in organellar fractions. The particular phosphatase was assayed with *p*-nitrophenylphosphate and exhibited a pH-optimum between pH 5.0 and 6.0; it was predominantly localized within the fraction of peripheral membrane proteins. In the purified preparation of matrix diphosphatase, however, the potential contamination of diphosphatase by traces of phosphatase was less than 0.5%, comparing the hydrolysis of *p*-nitrophenylphosphate with diphosphate in the absence of molybdate.

The pH optimum of the stroma form of diphosphatase was between pH 8–9 (Fig. 5). An apparent K_M of 60 μM diphosphate was calculated for the range 0.02–2 mM substrate. Concentrations of more than 5 mM diphosphate are strongly inhibitory. The diphosphatase activity was dependent upon Mg^{2+}, with an optimal concentration at 5–10 mM, but was not affected by 1 mM molybdate or 0.1 mM vanadate. 0.1 mM concentrations of dithiothreitol or mercaptoethanol give a 40% increase in activity. Ca^{2+} and Cd^{2+} at concentrations above 0.1 mM were strong inhibitors. 1 mM Ca^{2+} tested in the presence of 5 mM Mg^{2+} inhibited 98% of the diphosphatase activity while 1 mM Mn^{2+} had almost no effect. Chelating agents such as EDTA (0.1 mM), tested in the presence of 5 mM Mg^{2+}, increased the activity by a factor of 1.9. Likewise, 0.1 mM Zn^{2+} functions as activator in the presence of Mg^{2+}.

The enzymatic properties of the stroma enzyme were determined from highly enriched fractions obtained after chromatography on Sephacryl S-200. The enzyme cleaved phosphate from ATP, GTP, IDP, fructose-1,6-bisphosphate with a rate which was less than 1% of the one observed with diphosphate, if tested in the range of 1–10 mM substrate. Cleavage of ADP, however, was significant, namely 4% of the hydrolysis of diphosphate.

Comparison of properties of stroma diphosphatase and diphosphatase activity detected in glyoxysomes

Trace amounts of diphosphatase activity, *i.e.* less than 2% of the amount in the crude particle preparations, were detected in purified glyoxysomal fractions. In order to rule out the occurrence of a glyoxysomal isoenzyme, we compared carefully some characteristic properties of the plastid enzyme and the residual activity detectable in glyoxysomes. The activity adhering to glyoxysomes was separated on a column with anion exchanger exactly as described for the plastid enzyme in Fig. 4. The enzyme profile indicated that the minor amounts of enzyme derived from glyoxysomes had the same isoelectric point as the plastid enzyme. In addition, the activity profiles

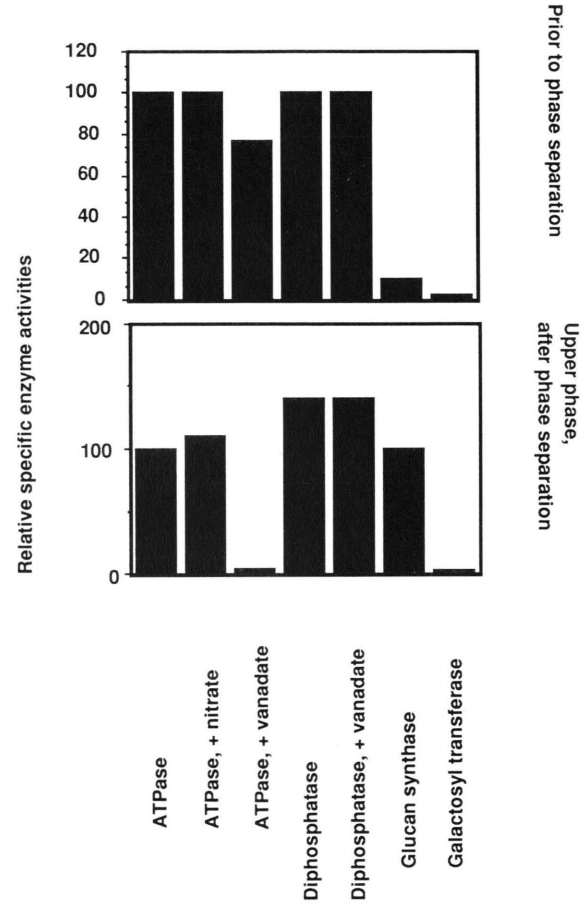

Fig. 6. Comparison of marker enzymes in the fraction of purified microsomal membranes with equilibrium densities higher than 1.10 g/cm^3 (upper part) and in the plasma membrane-enriched fraction (lower part). Nitrate-sensitive ATPase as marker of the tonoplast and galactosyl transferase indicative of the plastid envelope were included. The relative units were as follows: ATPases, 200 μmol/min mg (= 100%); diphosphatase, 100 μmol/min mg (= 100%); glucan synthase, 3 nmol/min mg (= 100%); galactosyl transferase, 3 nmol/min mg (= 100%). Inhibitors used as indicated were added to give the following concentrations: nitrate (50 mM), vanadate (10 μM).

of the plastid enzyme and the enzyme from the glyoxysomes for different pH values matched exactly (Fig. 5). Likewise, a comparison of the inhibition or activation by various effectors proved the close similarity or identity of both enzyme activities.

Diphosphatase localized in plasma membranes

Evidences were provided that a minor but significant and reproducible portion of diphosphatase activity, *i.e.* approximately 10% of the total cellular activity, is bound to plasma membranes. A specific enzyme activity of 120 mU/mg protein (2 nkat/mg protein) was determined for the diphosphatase in highly purified preparations of plasma membrane subsequent to two-phase partition. The preparation was free of enzyme activities originating from plastids, mitochondria or glyoxysomes. Less than 1% of the total NADH:cytochrom *c* oxidoreductase activity was detectable in the upper phase. Fig. 6 compares the enzyme activities in the purified microsomal fraction which has been sedimented through 31% sucrose with the one of the final plasma membrane-enriched fraction obtained by two-phase separation. The figure demonstrates that nitrate-sensitive ATPase and galactosyl transferase were absent. In addition, ATPase activity in the plasma membrane-enriched fraction was virtually completely inhibited by vanadate, and can thus not contain significant amounts of other ATPases, *e.g.* a tonoplast ATPase.

It was excluded that ATPase activity on the plasma membrane could interfere with the diphosphatase assay. Addition of 20 μM vanadate which inhibited more than 90% of the ATPase activity caused a very slight increase in diphosphate cleavage. The diphosphatase, in contrast to the plastidic form, exhibited a more acidic pH-optimum and was less influenced by divalent metal ions. Various attempts to remove the diphosphatase from the membranes by treatment with buffer or high salt failed. However, a high yield of soluble diphosphatase activity was achieved by solubilization with non-ionic detergents. Solubilization experiments revealed that the main portion of plasma membrane diphosphatase activity stems from an integral membrane protein. Octylglucoside at concentrations of 0.3–0.5% led to a 90% recovery of membrane diphosphatase as micel. In this form, diphosphatase activity was rather stabile and could be subjected to gel permeation chromatography.

Estimation of diphosphate content in cells of cucumber cotyledons

The total cellular amount of diphosphate was determined for etiolated cucumber cotyledons at day 4 of germination at 26 °C. A value of 0.21 μmole diphosphate per g fresh weight from which an intracellular concentration of more than 0.25 mM was estimated.

Discussion

The intracellular sites of diphosphate metabolism

Gluconeogenesis from fat is intrinsically intertwined with diphosphate formation. Our data obtained with etiolated cotyledons of germinating cucumber seeds demonstrate that during the intensive fat degradation in storage tissue diphosphatase is mainly located in plastids and plasma membrane, but not in glyoxysomes.

It is known that chloroplasts require diphosphatase activity to get rid of the diphosphate formed in the pyruvate:phosphate dikinase reaction [16] and that amyloplasts degrade the diphosphate produced during formation of ADP-glucose [17]. Plastids, therefore, potentially are the compartment where diphosphatase activity is expected. In the case of cucumber cotyledons in the dark, a role of glyoxysomes as site of diphosphate cleavage was likely since glyoxysomal membranes are known as site of diphosphate formation. A cytosolic site on the outer surface of organelles was the other location where diphosphate cleavage had to be postulated since intensive sucrose formation in the cytosol is paralleled by the production of large amounts of diphosphate.

The methods applied here were not optimal for assaying plastids and glyoxysomes in the same gradient. Thus, a procedure for good recovery of glyoxysomes and the isolation procedure optimized for etioplasts were used independently. The recovery of a large portion of triosephosphate isomerase in the supernatant of the first gradient is due to the fact that the isomerase is located both in the plastids and in the cytosol and emphasizes the fragility of the plastids upon resuspension of a previously pelleted fraction. These findings make it probable that most of the diphosphatase activity found in the supernatant of the first gradient (Fig. 1) and originating from the $2000 \times g$ pellet, actually was housed in the plastids like the triosephosphate isomerase.

It was a major point of this investigation to unravel whether diphosphatase is located within glyoxysomes. If diphosphatase would be a constituent of the glyoxysomal matrix, that would indicate that the product of the acyl-CoA synthetase reaction is released into the matrix of glyoxysomes. In contrast, the lack of diphosphatase in glyoxysomes and the localization of diphosphatase activity at the plasma membrane makes it highly likely that the cleavage of ATP into AMP and diphosphate, during the formation of acyl-CoA, takes place at the outer surface of glyoxysomes. The fact has to be stressed that a removal of diphosphate is absolutely necessary in order to let the fatty acid degradation proceed. The equilibrium constant determined for the formation of acyl-CoA from fatty acid plus ATP and CoA is approximately 1. A concentration of diphosphate higher than 5 mM at the site of acyl-CoA synthesis eventually switches off the fatty acid degradation.

Unlike other organisms where an inorganic diphosphatase keeps the cytosolic concentration of diphosphate below 1 μM [18], plants maintain a considerably higher concentration of diphosphate in the cytosol. A rough estimation for the cucumber cotyledons gave a value of 0.25 mM for the intracellular diphosphate which compares favourably with the values of 0.25 mM diphosphate measured in the cytosol of spinach [19]. Weiner *et al.* [19] argued that diphosphate may present an energy source for the function of ion pumps at the tonoplast or other membranes.

We conclude from our data that the cotyledon cells use a plasma membrane bound diphosphatase to maintain in the cytosol a diphosphate concentration below 1 mM. It is our interest to unravel how this potential of diphosphate at the plasma membrane is used and to investigate whether alterations of this potential and the cytosolic diphosphate concentration are caused during the receiving of hormone like signals from the extracellular space. In cells other than the one which are characterized by a large space of protein bodies being degraded and becoming vacuoles, a highly active diphosphatase is attributed to the tonoplast [20–22]. There are no hints that the altering membranes of protein bodies in cotyledons of 4 day old cucumber seedlings have already resumed all functions of the tonoplast including diphosphatase activity. However, we have analyzed our membrane fraction highly enriched in plasma membrane for nitrate sensitive ATPase as marker of tonoplast. Such an activity was not detectable. This is not surprising as the membrane preparation which was subjected to two-phase partition was previously centrifuged through 31% sucrose, a procedure which removes the main portion of the endoplasmic reticulum (data not shown). The tonoplast is characterized by an equilibrium density lower than the one of the endoplasmic reticulum. If present, small amounts of tonoplast will be separated at this step.

The production of diphosphate in the step of acyl-CoA formation was demonstrated for microbodies other than glyoxysomes. Gerbling and Gerhardt [23] determining latency and resistance to protease came to the conclusion that the acyl-CoA synthetase might be located at the matrix face of peroxisomes. This reaction, together with the more relevant synthesis of diphosphate as product of the synthesis of sucrose to be transported into sinks, produces diphosphate in green leaves. Therefore, the question of how diphosphate concentration is maintained in various kinds of plant cells is of interest under normal and pathogenic situations.

Properties of the diphosphatases in fat mobilizing tissue

The pH-optimum above pH 8 (Fig. 5) is characteristic of diphosphatases in plastids or of the constitutive enzyme in *Escherichia coli* [24]. The relative effectiveness of bivalent metal ions as inhibitors $Cd^{2+} > Ca^{2+} \gg Mn^{2+}$ found in the case of diphosphatase from cucumber etioplasts was similar but not identical to the results reported for the enzyme from mesophyll chloroplasts [25]. Noteworthy is the pronounced inhibitory effect of 1 mM Ca^{2+} in the presence of 5 mM Mg^{2+}. Even 0.1 mM Ca^{2+} and a ratio Ca^{2+}/Mg^{2+} of 0.02 reduced the diphosphatase activity to 1/3.

Most remarkable in terms of diphosphatase interfering with sugar phosphate metabolism is the finding that plastid diphosphatase is highly specific and does not cleave hexose phosphates or phosphates in the C_3 metabolism.

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie for financial support.

[1] R. N. Trelease, Annu. Rev. Plant Physiol. **35,** 321–347 (1984).
[2] H. Beevers, Annu. Rev. Plant Physiol. **30,** 159–193 (1979).
[3] H. Kindl, in: Biochemistry of Plants. A Comprehensive Treatise (P. K. Stumpf and E. E. Conn, eds.), pp. 31–52, Academic Press, London 1987.
[4] T. C. Cooper, J. Biol. Chem. **246,** 3451–3455 (1971).
[5] J. E. Dancer and T. ap Rees, Planta **177,** 261–264 (1989).
[6] W. Köller and H. Kindl, Arch. Biochem. Biophys. **181,** 236–248 (1977).
[7] H. Kindl and C. Kruse, Meth. Enzymol. **96,** 700–715 (1983).
[8] Ch. Larsson, in: Modern Methods of Plant Analysis, Cell Components, **Vol. 1,** 85–104, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo 1985.
[9] D. Lebel, G. G. Poirier, and A. R. Reaudoin, Anal. Biochem. **85,** 86–89 (1978).
[10] P. M. Burton and S. G. Waley, Biochem. J. **100,** 702–710 (1966).
[11] J. Frevert and H. Kindl, Eur. J. Biochem. **92,** 35–43 (1978).
[12] B. Hock and H. Beevers, Z. Pflanzenphysiol. **55,** 405–414 (1966).
[13] T. Kirsch, H.-G. Löffler, and H. Kindl, J. Biol. Chem. **261,** 8570–8575 (1986).
[14] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. **193,** 265–275 (1951).
[15] J. Edwards, T. ap Rees, P. M. Wilson, and S. Morrell, Planta **162,** 188–191 (1984).
[16] M. D. Hatch, Aust. J. Plant Physiol. **9,** 287–296 (1982).
[17] P. Gross and T. ap Rees, Planta **167,** 140–145 (1986).
[18] L. G. Butler, in: The Enzymes (P. D. Boyer, ed.), **Vol. IV,** Academic Press, New York, London 1971.
[19] H. Weiner, M. Stitt, and H. W. Heldt, Biochim. Biophys. Acta **893,** 13–21 (1987).
[20] A. Chanson, J. Fichmann, D. Spear, and L. Taiz, Plant Physiol. **79,** 159–164 (1985).
[21] A. Hager, W. Berthold, W. Biber, H.-G. Edel, Ch. Lanz, and G. Schiebel, Ber. Deutsch. Bot. Ges. **99,** 281–295 (1986).
[22] C. Bremberger, H.-P. Haschke, and U. Lüttge, Planta **175,** 465–470 (1988).
[23] H. Gerbling and B. Gerhardt, Planta **171,** 386–392 (1987).
[24] S. C. K. Wong, D. C. Hall, and J. Josse, J. Biol. Chem. **245,** 4335–4345 (1970).
[25] V. Ananda Krishnan and A. Gnanam, Arch. Biochem. Biophys. **260,** 277–284 (1988).