Plant Physiol. (1992) 98, 927–935
0032-0889/92/98/0927/09/$01.00/0

Received for publication August 12, 1991
Accepted October 14, 1991

Subcellular Distribution of *O*-Acetylserine(thiol)lyase in Cauliflower (*Brassica oleracea* L.) Inflorescence

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ABSTRACT

The subcellular localization of *O*-acetylserine(thiol)lyase (EC 4.2.99.8) in nongreen tissue from higher plants has been studied using purified proplastids, mitochondria, and protoplasts from cauliflower (*Brassica oleracea* L.) buds as a source of subcellular fractions. *O*-Acetylserine(thiol)lyase has been detected in both organelles (proplastids and mitochondria) and a cytosolic extract obtained by protoplast fractionation. We confirmed these observations, demonstrating that a form of the enzyme different in global charge and separated from others by anion-exchange chromatography corresponded to each subcellular location. Our observations are consistent with the need for cysteine biosynthesis in each subcellular compartment where the synthesis of proteins occurs.

The biosynthesis of L-cysteine in microorganisms and higher plants proceeds via a two-step enzymatic pathway wherein L-serine is first acetylated by acetyl-CoA to yield *O*-acetyl-L-serine; this reaction is catalyzed by serine transacetylase (EC 2.3.1.30): L-serine + acetyl-CoA → *O*-acetyl-L-serine + CoA-SH. *O*-Acetyl-L-serine then reacts with sulfide in a reaction catalyzed by *O*-acetylserine (thiol) lyase (EC 4.2.99.8) to form L-cysteine: *O*-acetyl-L-serine + S^{2-} → L-cysteine + acetate.

In leaves from higher plants, assimilatory sulfate reduction is located in chloroplasts (31, 32) and in the cytosol (17). In *Euglena* mutants lacking plastids, the enzymes of assimilatory sulfate reduction were localized in mitochondria (6, 30), and the chloroplasts of wild-type *Euglena* appear to lack the sulfate-activating enzymes (29). *Euglena* is thought to be a special case (2), and therefore chloroplasts are generally considered as the main site for cysteine synthesis in leaves. Recently, in spinach leaves, *O*-acetylserine(thiol)lyase was detected not only in chloroplasts and in the cytosol but also in purified mitochondria, thus demonstrating that cysteine synthesis was possible in all cell compartments in leaves where proteins are synthesized (23).

In nongreen tissues, the localization of enzymes of assimilatory sulfate reduction is not clear: *O*-acetylserine(thiol)lyase was found in a proplastid fraction prepared from spinach roots (16), whereas serine transacetylase was detected in a mitochondrial fraction obtained from *Phaseolus vulgaris* roots (34). ATP-sulfurylase (EC 2.7.7.4), adenosine 5′-phosphosulfate sulfotransferase, and sulfite reductase (EC 1.8.7.1) were almost exclusively located in plastids from pea roots, whereas *O*-acetylserine(thiol)lyase was predominantly detected in the cytosol (8). It is, however, rather difficult to prepare intact and purified organelles from nongreen tissues, and this limitation hampers a proper localization of the enzymes involved in cysteine synthesis within these tissues.

In this investigation, we analyzed purified proplastids and mitochondria from cauliflower buds and also used protoplasts from cauliflower buds as a source of subcellular fractions. We conclude from these experiments that, in cauliflower buds, *O*-acetylserine(thiol)lyase activity can be detected in the cytosol, proplastids, and mitochondria.

MATERIALS AND METHODS

Preparation of Total Homogenate from Cauliflower Buds

Cauliflower (*Brassica oleracea* L.) buds were purchased from the local market. To determine enzymatic activities in the whole cauliflower bud tissue, a total homogenate from the top part of the inflorescence was prepared as follows: 100 g of inflorescence tissue was homogenized at high speed for 1 min at 0°C in a 1-L Waring Blendor in 100 mL of a medium containing: 50 mM KH_2PO_4-K_2HPO_4 buffer (pH 7.5), 2 mM DTT, 1 mM EDTA, 0.1% (w/v) BSA, 1% (w/v) PVP (M_r 25,000; Serva), and 0.05% (w/v) Triton X-100 (Merck). The homogenate was filtered through eight layers of muslin (Ruby; Voiron, France) and centrifuged for 10 min at 10,000*g*. The supernatant fraction, containing soluble and solubilized enzymes released from the cytosol and all organelles, was stored on ice and assayed for enzyme activities within 2 h.

Preparation of Cauliflower Bud Organelles

Cauliflower bud organelles (plastids and mitochondria) were prepared according to a previously published procedure (21). The top parts of the inflorescence (1 kg) were disrupted (three times) with a 4-L Waring Blendor at low speed for 3 s (twice) in 1.5 L of chilled extraction medium containing: 0.3 M mannitol, 20 mM PPi buffer (pH 7.6), 2 mM DTT, 1 mM EDTA, and 0.1% (w/v) BSA (fraction V; Boehringer). The temperature was maintained at 0 to 4°C throughout organelle isolation. The homogenate was filtered through eight layers of muslin (see above) and a 50-μm nylon net (Tripette et Renaud, Sailly-Saillysel; Combles, France) and then centrifuged for 20 min at 3000*g* (JA-10 rotor, J 2–21 M/E centri-

fuge; Beckman) using low acceleration (2 min from 0–50*g*). This first differential centrifugation was common to both plastids (pellet fraction) and mitochondria (supernatant fraction) preparations.

Preparation of Plastids

The pellet obtained from the first differential centrifugation (3000*g*) was resuspended in approximately 500 mL of wash medium A, containing: 0.3 M sucrose, 10 mM KH_2PO_4-K_2HPO_4 buffer (pH 7.5), 2 mM DTT, 1 mM EDTA, and 0.1% (w/v) BSA. The resuspended pellet was centrifuged at 200*g* (JA-10 rotor) for 5 min to pellet starch and a sticky fraction containing a high proportion of nucleic acids. The supernatant fraction was recentrifuged at 3000*g* (JA-10 rotor) for 10 min to yield the crude plastids, still contaminated by mitochondria. The pellet was resuspended in approximately 12 mL of medium A; aliquots (3-mL sample; 20–30 mg protein) were then layered over 36 mL of medium A containing 38% (v/v) Percoll (Pharmacia) and centrifuged for 20 min at 40,000*g* (JA-20 rotor). To avoid the adverse effects of rapid acceleration and deceleration on the gradient, we used low acceleration (2 min from 0–50*g*) and deceleration (no brake). Under these conditions, centrifugation of Percoll resulted in formation of a smooth density gradient (27). The plastids containing starch were found in a broad, greenish band near the bottom of the tube, and mitochondria and lighter plastids (*i.e.* almost devoid of starch) remained near the top of the tube, just below a sticky layer. The mitochondria/lighter plastid fraction were removed by aspiration from the top of the tube. After the heavy plastid fraction was diluted at least fivefold with medium A, it was pelleted by centrifuging at 3500*g* for 10 min. To remove the last contaminating mitochondria, this pellet was resuspended in 4 mL of medium A, layered on top of a new Percoll layer (medium A containing 38% [v/v] Percoll) and centrifuged for 20 min at 25,000*g* in a JS-13 horizontal rotor. Centrifugation in a horizontal rotor does not result in formation of a density gradient because of the long path length and unequal gravity forces along the tube. Plastids were found in a greenish band near the bottom of the tube, and mitochondria stayed at the top of the Percoll bed. Plastids were carefully removed and pelleted as described above in wash medium A without BSA. The purified plastids were resuspended (10–20 $mg \cdot mL^{-1}$) in this medium and stored at 0°C. The intactness of plastid preparations was evaluated according to the method of Journet and Douce (21).

Preparation of Mitochondria

Mitochondria from cauliflower buds were prepared as rapidly as possible by the method of Bonner (4), except that the first step (JA-10 rotor, 10,000*g*, 20 min) was performed on the supernatant fraction of the first differential centrifugation (3000*g*) to remove the bulk of the plastids. Mitochondria thus obtained (washed mitochondria, still heavily contaminated with plastids) were purified by isopyknic banding in Percoll density gradients, as previously described by Neuburger *et al.* (25), except that we controlled acceleration and deceleration as described above for plastids. The purification procedure was repeated once, *i.e.* after pelleting the mitochondria, the resuspended pellet was layered on top of a new Percoll gradient (medium A containing 28% [v/v] Percoll), to remove plastids containing small starch granules. The purified mitochondria were resuspended (10–20 $mg \cdot mL^{-1}$) in the wash medium A, used for plastid conservation, and stored at 0°C.

Preparation of Protoplasts from Cauliflower Inflorescence

For the preparation of protoplasts from cauliflower, tops of the inflorescence (10 g) were cut into thin strips using razor blades and suspended in 100 mL of a preplasmolysis medium, containing: 0.5 M mannitol, 10 mM Mes (pH 5.8), and 10 mM $CaCl_2$. After 1 h, the tissue was incubated in 20 mL of this solution, containing: 2.5% (w/v) cellulase (Onozuka RS; Yakult Pharmaceutical Co., Nishinomiya, Japan), 1% (w/v) pectinase (Pectolyase Y-23; Seishin Pharmaceutical Co., Tokyo, Japan), 1% (w/v) Hemicellulase (Sigma), and 0.1% (w/v) defatted BSA (fraction V powder; Sigma). To facilitate penetration of the cell wall-degrading enzyme mixture, tissue was vacuum infiltrated for 5 min. After a 1-h digestion in the dark, at 25°C with constant shaking (maximum 50 $cycles \cdot min^{-1}$), the enzyme solution containing protoplasts was filtered through a 48-μm nylon net (Zurich Bolting Cloth Manufacturing Co., Ltd.; Zurich, Switzerland) to remove larger portions of undigested tissues and cell clumps. All operations were then carried out at 0 to 4°C. The solution was centrifuged for 10 min at 100*g*, and the protoplast-enriched pellet was resuspended in approximately 5 mL of wash medium B: 0.5 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 5 mM Mops buffer (pH 7.5), 0.1% (w/v) BSA, and 1% (w/v) PVP (M_r 25,000; Serva). Washing was performed by resuspending the protoplasts in this solution and pelleting by centrifugation at 100*g* for 10 min. This washing was repeated two times, and resuspensions were carried out with great care to avoid protoplast rupture. The washed protoplasts (3–4 mL) were then layered on top of a 0.5 M sucrose bed (1 mL) in wash medium B and centrifuged at 100*g* for 10 min. The purified and intact protoplasts stayed at the top of the sucrose bed, and some cells were pelleted to the bottom of the tube. Intact protoplasts were carefully pipetted from the interface. An aliquot of this preparation was used for diameter measurements and protoplast concentration determination in a hemacytometer (Fig. 1). Protoplasts were then collected and placed in a known volume of wash medium B and stored at 0°C.

Note that, using cellulase Caylase 345 L (Cayla Laboratories; Toulouse, France), aggregation and multiple fusions were observed in cauliflower bud protoplast preparations, as also observed in our laboratory during the preparation of maize protoplasts (not shown).

Gentle Rupture of Protoplasts and Separation of the Organelles from the Cytosolic Fraction

The best way to rupture protoplasts without affecting proplastid integrity was to use a combination of two techniques generally used for cell fractionation. The protoplasts were suspended in wash medium containing only 0.1 M mannitol (osmotic shock) and filtered twice (mechanical treatment) through a 20-μm nylon net (Zurich Bolting Cloth) affixed to

the cut end of a 2-mL disposable syringe. This procedure ruptures almost all the protoplasts, leaving the organelles largely intact (Table I). To separate the cytosolic fraction from the cell organelles, we subjected the broken protoplast fraction to centrifugation which yielded a pellet largely free of cytosolic contaminants and a supernatant enriched in cytosolic enzymes. To prevent starch grains from tearing through the plastid envelope, centrifugation was carried out in four separate runs (5 min at 100, 500, 4,500, and 12,000g in a horizontal rotor). Each supernatant was carefully removed and centrifuged in a new tube at the next higher gravity force. The four successive pellets were then combined and resuspended in a volume equivalent to the volume of the supernatant and in the same wash medium B previously described. Marker enzymes (see below) were then assayed on the pellet ("organelles" fraction) and the supernatant ("cytosolic" fraction) and their activities compared to estimate the reciprocal contaminations. A known volume of the initial protoplast suspension was used to prepare a crude extract.

Some subcellular compartment marker enzyme activities (see below), measured on detergent-treated protoplasts (crude extract), were partially affected when 0.1% (w/v) BSA and 1% (w/v) PVP were omitted from the cell wall digestion and storage media. Enzymic activities were normalized to a number of protoplasts (previously evaluated in all preparations) and expressed as nmol product formed·min^{-1} for 10^7 protoplasts, because of the need for BSA in the protoplast storage medium.

Measurement of Marker Enzyme Activities

All assays were first performed on blanks to detect any nonspecific drift or enzyme contamination. They were then optimized with respect to the concentration of each component and to the pH of the reaction mixture. We verified that, under these conditions, activities were linear with respect to time for at least 2 min and were proportional to the amount of protein. For the spectrophotometric assays (Uvikon 860; Kontron), the coupling enzyme systems (Boehringer) were determined not to be rate limiting. When NADPH was present in the assay, the rates were corrected for any NADPH oxidase activity. BSA was omitted from the medium and enzymatic activities were rapidly measured to determine specific activities in organelles or total homogenate. For enzyme activities sensitive to the presence of phosphate, Percoll-purified organelles were resuspended in the wash medium B used for protoplast conservation. The following marker enzyme assays were performed in a 1-mL final reaction volume and references to the procedures are given, together with any features of the reaction mixtures that differed from those previously described.

PPi: PFP[1] (EC 2.7.1.90) (11): 100 mM Hepes-NaOH (pH 7.8), 1 mM $MgCl_2$, 5 mM fructose 6-phosphate, 150 μM NADH, 0.2 unit aldolase, 10 units triose-phosphate isomerase, 0.5 unit glyceraldehyde 3-phosphate dehydrogenase. The samples were preincubated for 2 min with 2 μM fructose 2,6-bisphosphate (to activate PFP) and 5 mM NaF (to inhibit any pyrophosphatase activity without affecting the PFP activity), and the reaction was initiated with 1 mM pyrophosphate.

ADP-glucose pyrophosphorylase (EC 2.7.7.27) (14): 50 mM Tricine-NaOH (pH 8.0), 5 mM $MgCl_2$, 2 mM DTT, 5 mM NaF (pyrophosphatase inhibitor), 1 mM ADP-glucose, 5 mM glycerate 3-phosphate (to activate ADP-glucose pyrophosphorylase), 10 μM glucose 1,6-bisphosphate (to activate phosphoglucomutase), 500 μM NADP, 1 mM PPi, 0.8 unit phosphoglucomutase, and 0.7 unit glucose 6-phosphate dehydrogenase.

Gluconate 6-phosphate dehydrogenase (EC 1.1.1.44) (33): 50 mM Tricine-NaOH (pH 8.2), 5 mM $MgCl_2$, 500 μM NADP, and 2 mM gluconate 6-phosphate.

Cyt *c* oxidase (EC 1.9.3.1) (28): 10 mM KH_2PO_4-K_2HPO_4 buffer (pH 7.2), 5 mM $MgCl_2$, 10 mM KCl, 1 mM ascorbate, and 20 μM Cyt *c*. The enzymatic reaction was inhibited after 2 min by addition of 200 μM KCN to measure the nonenzymatic oxidation of Cyt *c* by O_2.

Hydroxypyruvate reductase (EC 1.1.1.81) (35): 50 mM Mes-NaOH pH (6.4), 200 μM NADH, and 1 mM hydroxypyruvate.

Citrate synthase (EC 4.1.3.7) (12): 50 mM Hepes-NaOH pH (7.6), 250 μM $MgCl_2$, 200 μM acetyl-CoA, 1.5 mM 5,5′-dithiobis(2-nitrobenzoic acid), and 1 mM oxaloacetate. An extinction coefficient of 13,600 $M^{-1}·cm^{-1}$ was used for the thionitrobenzoate.

Measurement of *O*-Acetylserine(thiol)lyase Activity (EC 4 2.99.8)

Activity was measured according to the method of Burnell and Whatley (10): 100 mM KH_2PO_4-K_2HPO_4 buffer (pH 7.5), 5 mM DTT, 50 μM pyridoxal 5′-phosphate, 10 mM *O*-acetyl L-serine, 2 mM Na_2S, and a suitable amount of enzyme in a 100-μL final volume. The reaction was stopped as previously described (23), and L-cysteine was spectrophotometrically measured at A_{560} (extinction coefficient of 25,000 $M^{-1}·cm^{-1}$) according to the method of Gaitonde (18). The omission of pyridoxal 5′-phosphate from the reaction mixture did decrease the enzyme activity significantly (30%). We observed (data not shown) that addition of pyridoxal 5′-phosphate seemed to protect the enzyme, probably by preventing cofactor loss as suggested by Bertagnolli and Wedding (3). For this reason, pyridoxal 5′-phosphate was included in all buffers used for the storage of fractions containing *O*-acetylserine(thiol)lyase activity. *O*-Acetylserine(thiol)lyase activity was also decreased (40%) when assayed in the absence of DTT. However, this compound prevents the oxidation of cysteine to cystine as confirmed by comparing the titration curves for cysteine measurements in the presence or absence of DTT. Because cystine is not detected by the colorimetric assay used for cysteine detection (18), DTT should be added to the incubation mixture for quantitative determinations. Under our experimental conditions, the rate of *O*-acetylserine(thiol)lyase was linear for at least 20 min and up to 0.1 mg protein·mL^{-1}.

Thermolysin Treatment of Purified Intact Plastids

Proteolytic digestion was performed according to a previously published procedure (1). Intact plastids (final concen-

[1] Abbreviation: PFP, fructose 6-phosphate phosphotransferase.

tration, 6 mg·mL^{-1}) were incubated for 1 h at 4°C in the following medium: 0.3 M saccharose, 10 mM tricine-NaOH (pH 7.8), 1 mM $CaCl_2$, and 0.2 mg·mL^{-1} thermolysin from *Bacillus thermoproteolyticus* (Boehringer). Thermolysin is active only in the presence of Ca^{2+}, and its activity can be easily inhibited by addition of 10 mM EGTA. Polypeptides localized in the inner envelope membrane or in the stroma are not hydrolyzed during the incubation because thermolysin is unable to cross the outer envelope membrane. In addition, the integrity of cauliflower proplastids is maintained during incubations under mild conditions (at 4°C, with low thermolysin concentration) (1). Therefore, after incubation in the presence of thermolysin under these conditions, cauliflower proplastids were repurified on a Percoll gradient containing protease inhibitors (1 mM PMSF, 1 mM benzamidine-HCl, and 5 mM ε-amino caproic acid) to remove the protease and the broken plastids. The treated, intact plastids were recovered, stored on ice, and assayed for enzyme activities, and the soluble enzymes were analyzed by chromatography on the Mono Q anion-exchange column (see above).

Mono Q Anion-Exchange Chromatography

All chromatography experiments were performed at 4 °C using a Mono Q HR 5/5 (Pharmacia) coupled to an Fast Protein Liquid Chromatography system (Pharmacia) to obtain precise and repetitive elution patterns. Organelles or protoplasts were ruptured by addition of 0.05% (w/v) Triton X-100 in their wash medium (see above). After vortex mixing, the organelles were left on ice for 10 min to ensure complete lysis, and the soluble protein fractions were separated from the cellular membranes using centrifugation at 40,000g for 10 min and filtration of the supernatant on a 0.2-μm filter (Minisart; Sartorius Filters, Inc.) Under these conditions, we have verified that *O*-acetylserine(thiol)lyase activity, associated with the cell organelles, was entirely recovered in the supernatant (extract). The protein solution was desalted on a G-25 M column (Sephadex PD-10; Pharmacia) in a medium containing 30 mM KH_2PO_4-K_2HPO_4 buffer (pH 7.5) and 1 mM DTT and then loaded (0.5–3 mg) onto the anion-exchange column equilibrated with the same buffer. After the column was washed with one bed volume of this medium, proteins were eluted using a linear gradient from 0 to 350 mM NaCl in the same buffer. Fractions of 1 mL were collected at a flow rate of 0.5 mL·min^{-1}, stored on ice, and assayed for *O*-acetylserine(thiol)lyase activity within 1 h.

Latency Measurements

Corrections were made for extraorganellar activity by comparing the activities in ruptured and intact organelles. The organelles were kept intact by adding an osmoticum to the reaction medium (0.3 M mannitol) or ruptured by adding 0.05% (w/v) Triton X-100. Percentage of latent activity is the ratio of organellar (ruptured minus intact) activity to total (ruptured) activity. We verified that, under this conditions, enzymatic activities were not affected by the presence of the detergent.

Protein Determination

Protein was measured by the method of Bradford (5), using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories), and bovine γ-globulin as the standard. For the Mono Q anion-exchange chromatography elution profiles, proteins were measured by determination of A_{280} (monitor UV-2; Pharmacia).

RESULTS

Intracellular Localization of O-Acetylserine(thiol)lyase Activity in Cauliflower Buds

This was first investigated in protoplasts prepared from cauliflower buds (Fig. 1). The following marker enzymes were used: mitochondria, citrate synthase; plastids, ADP-glucose pyrophosphorylase; cytosol, PPi:PFP. As discussed in "Materials and Methods," it was difficult to rupture the small protoplasts without affecting the proplastid integrity; therefore, about 45% of the total ADP-glucose pyrophosphorylase activity derived from proplastids was recovered in the supernatant (Table I). Most (86.6%) of the mitochondrial marker activity was measured in the pellet, together with 56% of the plastidial marker activity. In contrast, the pellet contains little cytosolic marker activity (<15% of the total activity). Therefore, the data in Table I demonstrate that the supernatant obtained after differential centrifugation of lysed cauliflower bud protoplasts contains cytosolic enzymes but also an appreciable amount of soluble enzymes derived from fragile proplastids that contain large starch grains (21). *O*-acetylserine(thiol)lyase activity was detected in both the pellet and the supernatant, but the distribution of enzyme activity was intermediate between that of cytosolic and plastidial marker enzymes because 60% of the total *O*-acetylserine(thiol)lyase activity was recovered in the supernatant and 40% in the pellet (Table I). This observation is consistent with the location of *O*-acetylserine(thiol)lyase activity in both the cytosol and organelles.

The pellet obtained from protoplasts contained different

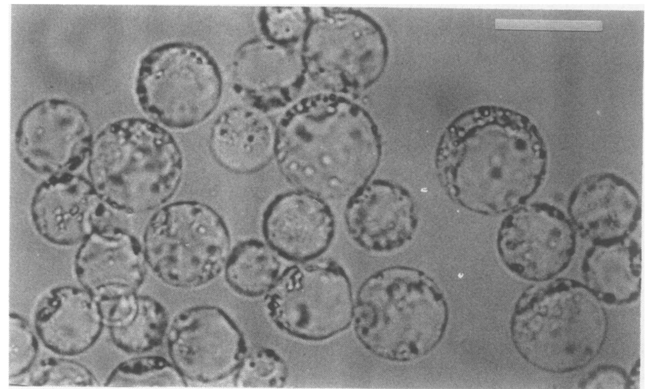

Figure 1. Optical microscopy of a cauliflower bud protoplast preparation. The purification method is described in "Materials and Methods." Note the heterogeneity in protoplast size in the preparation. The protoplasts, thus obtained, were utilized to prepare the cytosolic compartment of the cell, as described in the text. Bar, 25 μm.

Table I. *Subcellular Localization of ADP-Glucose Pyrophosphorylase (Plastidial Marker), Citrate Synthase (Mitochondrial Marker), PPi:PFP (Cytosolic Marker), and O-Acetylserine(thiol)lyase in Cytosolic and Organelle Fractions Obtained from Mechanically Ruptured Cauliflower Bud Protoplasts*

Preparation of intact and broken protoplasts (crude extract), centrifugation of intact organelles, and enzymic activities measurements were carried out as described in "Materials and Methods." Activities in the crude extract are the mean of values obtained from five different experimental determinations ± SEM. The data presented for distribution in supernatant and pellet are from a representative experiment and have been reproduced five times.

Enzymes (Associated Cell Compartment)	Crude Extract	Distribution	
		Supernatant	Pellet
	nmol·min⁻¹·10⁷ protoplasts	*% of total activity*	
O-Acetylserine(thiol)lyase	149 ± 17	60.2	39.8
Citrate synthase (mitochondria)	8 ± 0.6	13.4	86.6
ADP-glucose pyrophosphorylase (plastids)	10.5 ± 1.25	44.0	56.0
PPi:PFP (cytosol)	4.5 ± 0.25	86.9	13.1

types of organelles (proplastids, mitochondria, peroxisomes). Therefore, with the aim to localize further *O*-acetylserine(thiol)lyase activity in the pellet containing cell organelles, intact mitochondria and proplastids were purified from cauliflower buds. Because cauliflower buds contain small proplastids with almost the same size and density as mitochondria (21), the purity of the organelle preparation was carefully monitored. Evidence for intactness of proplastid preparations was confirmed by measuring the latency of gluconate 6-phosphate dehydrogenase activities (21). The latency thus measured on freshly prepared cauliflower bud proplastids was, on average, 96%. Assays of various markers (citrate synthase, Cyt-*c* oxidase, hydroxypyruvate reductase, PPi:PFP; Table II) showed that a double purification through Percoll gradients almost completely eliminated cytosolic, mitochondrial, and peroxisomal contamination. *O*-Acetylserine(thiol)lyase activity was associated with proplastids (Table II). The specific activity of *O*-acetylserine(thiol)lyase in proplastids was two-fold higher when compared to the specific activity of the enzyme in crude extracts from whole tissue, (1240 and 590 $nmol \cdot mg^{-1} protein \cdot min^{-1}$, respectively).

Purification of cauliflower bud mitochondria by Percoll gradient centrifugation almost completely eliminates cytosolic and peroxisomal contamination (Table II), although the mitochondrial extract was found to contain substantial activity of the plastid marker enzymes, ADP-glucose pyrophosphorylase and gluconate 6-P dehydrogenase. On average, the mitochondrial fractions contained between approximately 10 and 18% proplastids. In addition, the rate of KCN-sensitive Cyt *c*-dependent O_2 uptake was only 5% of that recorded for osmotically shocked mitochondria, giving an apparent 95% intactness. A very active *O*-acetylserine(thiol)lyase could be measured in purified mitochondria (Table II). The specific activity thus obtained was very high (about 950 $nmol \cdot mg^{-1} protein \cdot min^{-1}$) and of the same order of magnitude as that measured in purified proplastids. The ratios of *O*-acetylserine(thiol)lyase activity to ADP-glucose pyrophosphorylase activity in extracts of cauliflower bud proplastids and mitochondria were 7.3 and 30.6, respectively. Therefore, *O*-acetylserine(thiol)lyase activity measured in preparations of purified mitochondria could not be due to the contamination by proplastids.

Consequently, these results strongly support the presence of *O*-acetylserine(thiol)lyase activity in at least two plant cell organelles: mitochondria and proplastids. In addition, the experiments performed with protoplasts suggest that some activity could also be present in the cytosol, but because the cytosolic fraction is contaminated by plastid enzymes, further evidence is needed to support this hypothesis.

Separation of O-Acetylserine(thiol)lyase Activities by Mono Q Anion-Exchange Chromatography

To determine whether *O*-acetylserine(thiol)lyase activity from cauliflower buds could be resolved into several forms by anion-exchange chromatography, extracts from cauliflower buds (crude extract, proplastids, mitochondria, and cytosol) were fractionated on a Mono Q column. Organelles or protoplasts were first lysed by addition of 0.05% (w/v) Triton X-100, and the soluble protein fractions were separated from the cellular membranes and desalted as described in "Materials and Methods."

The elution profile obtained with the crude extract (Fig. 2a) demonstrates that *O*-acetylserine(thiol)lyase activity was resolved into four peaks (A–D), eluting in a NaCl concentration range between 70 and 200 mM. The major peak (peak B), eluting at 100 to 110 mM NaCl, represented about 40 to 45% of the total activity. Peaks A (eluting at 80 mM NaCl) and D (eluting at 170 mM NaCl) were of similar amplitude, and each peak usually contained about 20 to 25% of the total activity. Peak C was only a minor peak and represented about 15% of the total activity present in the crude extract.

The mitochondrial pattern (Fig. 2b) contained only one major peak exhibiting *O*-acetylserine(thiol)lyase activity, displaying chromatographic properties similar to that of peak A of the crude extract, but peaks B, C, and D were barely detectable. In all mitochondrial preparations analyzed, peak A was always the major peak, representing about 80% of the total activity. In contrast, peak A was barely detectable in the elution profile obtained with purified proplastids (Fig. 2c).

Table II. *Distribution of Various Markers and O-Acetylserine(thiol)lyase within Percoll-Purified Organelles (Plastids and Mitochondria) and Crude Extract from Cauliflower Bud Tissue*

Preparations of cell organelles, measurement of different enzymatic activities, and latent activity definition are described in "Materials and Methods." Before enzymatic activities were measured, all cellular fractions were first incubated in the same medium containing: 0.3 M mannitol, 5 mM Mops buffer (pH 7.5), 5 mM $MgCl_2$, 10 mM KCl, 1% (w/v) PVP, and 0.05% (w/v) Triton X-100. All activities are the mean of values obtained from six different experimental determinations ± SEM and are expressed as specific activities (nmol of product formed·min^{-1}·mg^{-1} protein). Starting from 1 kg of cauliflower bud tissue, the protein content of the organelle preparations was 40 mg from purified plastids and 5 mg from purified mitochondria.

Enzyme (Associated Cell Compartment)	Crude Extract from Bud Tissue	Percoll-Purified Plastids	Percoll-Purified Mitochondria
O-Acetylserine(thiol)lyase[a]	590 ± 81	1240 ± 81[b]	949 ± 23[c]
Citrate synthase (mitochondria)	31 ± 0.5	8 ± 1.1	417 ± 3
Cyt c oxidase (mitochondria)	ND[d]	5	202
ADP-glucose pyrophosphorylase (plastids)	46 ± 11	170 ± 38	31 ± 6
Gluconate 6-phosphate dehydrogenase (plastids + cytosol)	83 ± 6	268 ± 61	40 ± 14
Hydroxypyruvate reductase (microbodies)	80 ± 2.5	8 ± 1.2	6 ± 6
PPi:PFP (cytosol)	17 ± 1	0	0

[a] Optimum pH = 7.8, apparent K_m value for O-acetylserine about 2 mM in both plastids and mitochondria. [b] Activity in Percoll-purified plastids (96% intact): 80% latent activity. [c] Activity in Percoll-purified mitochondria (95% intact): 90% latent activity. [d] Not determined.

However, in this fraction, three peaks were observed and had a similar profile to B, C, and D. The major peaks were peaks B and C, together representing about 80% of the enzymatic activity in plastids, but peak D, eluting at 170 mM NaCl, was consistently present in the extracts from proplastids. Finally, the elution profile obtained with the cytosolic extract (Fig. 2d) contained the same three peaks B, C, and D but in different proportions: peak D was always the major component, representing about 60% of the total activity measured in the cytosolic extract.

From these results (Fig. 2), we can conclude that *O*-acetylserine(thiol)lyase activity is resolved into several forms by anion-exchange chromatography. These results also indicate that peak A is associated with mitochondria, because it is always a minor component in both proplastid and cytosolic extracts and because the *O*-acetylserine(thiol)lyase activity measured in the mitochondrial fraction exhibits a very high latency (Table II). We can also conclude that peaks B and C are concentrated in plastids. The purest mitochondrial fractions are mostly devoid of peaks B and C. These peaks are present only in mitochondrial preparations containing significant ADP-glucose pyrophosphorylase activity. On the other hand, the cytosolic fraction always contains peaks B and C, but as minor components (Fig. 2d). This observation is consistent with the data presented in Table I which demonstrates that the supernatant obtained after fractionation of cauliflower bud protoplasts always contains soluble enzymes derived from proplastids.

Finally, peak D is the major component of the cytosolic fraction, but it was always found in the proplastid extract, although cytosolic contamination of proplastids was always negligible. Indeed, PPi:PFP was barely detectable in this fraction (Table II). This observation led us to question whether some cytosolic *O*-acetylserine(thiol)lyase was absorbed to the outer surface of the outer membrane of the proplastid envelope and therefore remained in the proplastid fraction during the purification. To probe this hypothesis, we used a mild proteolytic digestion of intact proplastids with thermolysin because this nonpenetrating proteolytic enzyme was demonstrated to be an efficient tool for characterizing those envelope proteins that are accessible from the cytosolic side of the outer membrane (1, 22). The elution pattern of the extract obtained from thermolysin-treated proplastids confirms that *O*-acetylserine(thiol)lyase activity was indeed a genuine constituent of proplastids because peaks B and C were not affected by the treatment (Fig. 2e). In contrast, peak D was no longer detectable in thermolysin-treated proplastids, thus providing clear evidence for an extraplastidial localization of this *O*-acetylserine(thiol)lyase activity. Therefore, from the results presented in Figure 2, d and e, and in Tables I and II, we can conclude that peak D indeed corresponds to *O*-acetylserine(thiol)lyase activity present in the cytosol.

As a control, we used the same proteolysis treatment to confirm that *O*-acetylserine(thiol)lyase activity associated with the mitochondria was clearly inside the organelle. The identical elution profiles before and after thermolysin treatment confirmed the high latency of the activity associated with the mitochondria.

DISCUSSION

Fractionation of cauliflower bud protoplasts and purification of proplastids and mitochondria from this tissue clearly indicate that *O*-acetylserine(thiol)lyase activity is associated with several subcellular compartments. *O*-Acetylserine(thiol)lyase was resolved into four peaks by anion-exchange chromatography, two of them (making up about 50% of the total activity) were located in purified proplastids. The two other peaks were present in similar amounts in the crude extract prepared from cauliflower buds and were located in

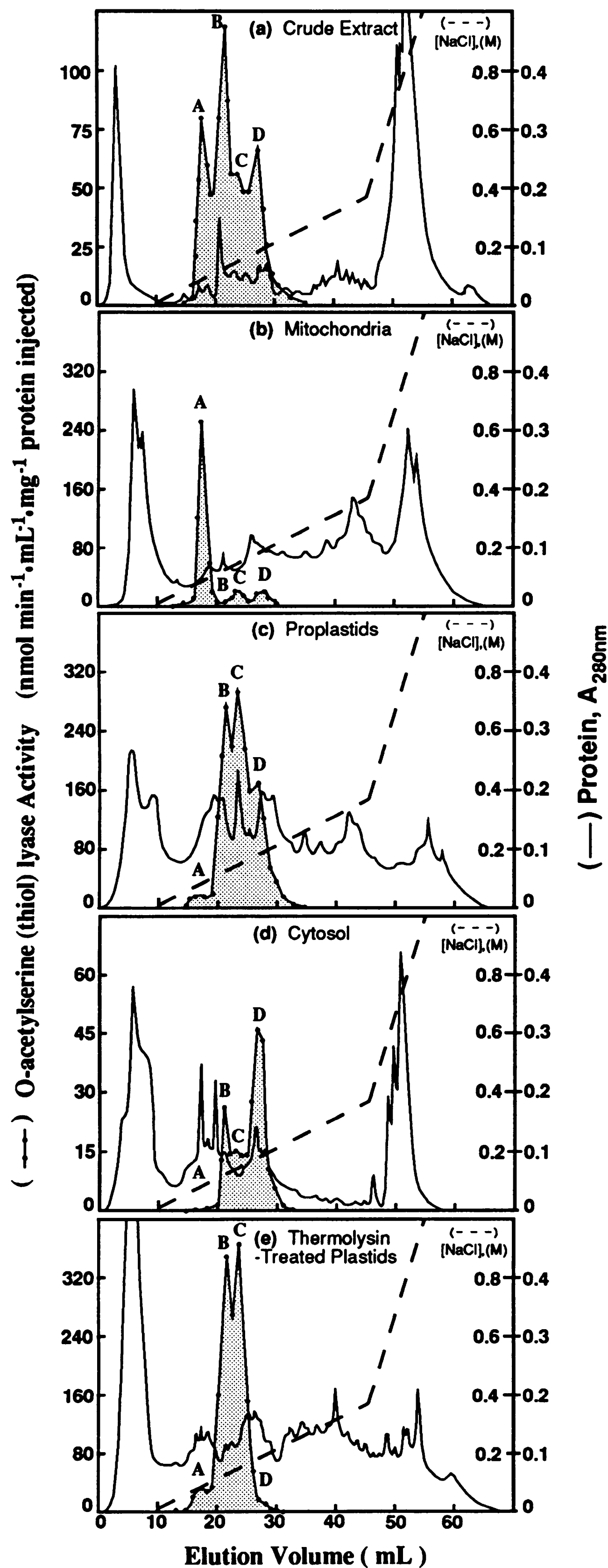

Figure 2. Separation of multiple forms of O-acetylserine(thiol)lyase from cauliflower buds by chromatography on Mono Q. Subcellular fractions (plastid, mitochondria, cytosolic fraction) and crude extracts from cauliflower buds were first solubilized with 0.05% (w/v) Triton X-100 and centrifuged for 10 min at 40,000*g*. Pellets were discarded and supernatants (containing the soluble and solubilized proteins) were filtered through a 0.2-μm filter. Each sample, 0.5 to 3 mg protein first desalted on a G-25 column, was loaded on the anion-exchange (Mono Q HR 5.5) column and eluted (1-mL fraction size) with a linear NaCl (0–350 mM) gradient in a 10 mM KH_2PO_4-K_2HPO_4 buffer (pH 7.5) containing 1 mM DTT. The O-acetylserine(thiol)lyase activity was measured (within 1 h) in an aliquot of each elution fraction and expressed as nmol cysteine formed·min^{-1}·mg^{-1} of protein injected.

two distinct compartments: one in mitochondria and the other in a cytosolic fraction.

Multiple forms of *O*-acetylserine(thiol)lyase have been demonstrated in a number of plant species (for a review, see ref. 20). The existence of two forms in spinach (19) and in leaves of two species of *Phaseolus* (3) have been reported, whereas five isoforms were purified in rape leaves (24). In contrast, few investigators have reported careful analysis of the subcellular localization of the enzyme. Because most of the reactions of reductive sulfate assimilation are present in chloroplasts (2, 20), the presence of *O*-acetylserine(thiol)lyase in chloroplasts was generally assumed and was conclusively demonstrated in some experiments (15, 17, 26). A plausible explanation for the recovery of enzyme in a soluble fraction was that the chloroplasts were disrupted during isolation, releasing the enzyme (20). In addition, the enzyme was never detected in higher plant mitochondria (9; for reviews, see refs. 2 and 20). In mutants of *Euglena gracilis* lacking plastids, however, all of the enzymes of assimilatory sulfate reduction were localized in the mitochondria (6, 30), and in wild-type *Euglena*, the small amounts of sulfate-activating enzymes in chloroplasts were attributed to mitochondrial contamination (29). In nongreen tissues from higher plants, the scarcity of data together with the inherent difficulties in handling characterized organelles from these tissues made the available information even more confusing because the enzyme was only partially located in plastids (8, 16). In contrast, recent observations from our laboratory (23) led to the conclusion that chloroplastic, mitochondrial, and cytosolic extracts from spinach leaves contain *O*-acetylserine(thiol)lyase activity that can be resolved by anion-exchange chromatography. Furthermore, the results presented here provide further evidence to conclude that: (a) *O*-acetylserine(thiol)lyase is present in plastids (chloroplasts as well as proplastids) and (b) the extraplastidial localization is, in fact, rather complex. Only careful analysis of the distribution of *O*-acetylserine(thiol)lyase activity and marker enzymes in the different cell fractions made it possible to conclude that *O*-acetylserine(thiol)lyase activity is present in mitochondria and also in a soluble compartment (cytosol). Therefore, we can conclude that chlorophyllous and nongreen tissues are rather similar with respect to the localization of enzymes involved in cysteine biosynthesis. In addition, serine transacetylase, which provides *O*-acetylserine for *O*-acetylserine(thiol)lyase, was characterized in spinach leaf chloroplasts (7) and mitochondria from *Phaseolus* leaves (34). Preliminary results obtained in our laboratory indicate that serine transacetylase is also present in all cell compartments (cytosol,

mitochondria, and plastids) in which cysteine synthesis occurs.

The presence of three peaks of *O*-acetylserine(thiol)lyase activity in proplastids was rather surprising. Thermolysin experiments demonstrated that peak D was actually due to some cytosolic activity present on the exterior of the outer envelope membrane. Binding of the activity was strong enough to remain during Percoll purification of the cauliflower bud proplastid, but the activity was released in the soluble phase after rupture of the organelle in the presence of Triton X-100. Preliminary experiments suggest that *O*-acetylserine(thiol)lyase is somewhat hydrophobic and may interact with cell membranes and especially the plastid outer envelope membrane, due to its high polar lipid content (13). The value the *O*-acetylserine(thiol)lyase-specific activity measured in Percoll-purified plastids (Table II) was then overestimated. About 15% of this activity was attributable to *O*-acetylserine(thiol)lyase cytosolic contamination (peak D). The two other peaks of *O*-acetylserine(thiol)lyase activity found in proplastids were peaks B and C. Preliminary data obtained in our laboratory strongly suggest that the occurrence of two peaks of *O*-acetylserine(thiol)lyase activity during anion-exchange (Mono Q HR) chromatography was an artifact of the experimental protocol, because peak B can be transformed in an irreversible manner into peak C during protein purification. In addition, we observed that the distribution between the two forms varied from one experiment to another. The mechanism involved in this conversion is currently under investigation in our laboratory.

The physiological significance of three sites of cysteine synthesis in plant cells remains unclear. Indeed, the plastids are believed to be the sole site for the synthesis of almost all amino acids. The need for enzymes to be represented in different cell compartments derives from the impermeability of the membrane to some metabolites, so that duplication of the reactions that generate these metabolites are necessary to make them available where needed. The existence of distinct isoenzymes can potentially provide, in each subcellular compartment, an enzyme form with biochemical characteristics appropriate for the substrate concentration and reaction equilibrium prevailing in that location.

Finally, the results presented here raise the problem of the origin of reduced sulfur for cysteine biosynthesis. It is possible, therefore, that in higher plant cells, mitochondria, plastids, and cytosol possess all the enzymatic machinery incorporating reduced sulfur into cysteine (sulfate assimilation). Unfortunately, the presence of several sources of reduced sulfur production in higher plant cells cannot be assessed at present.

ACKNOWLEDGMENTS

We are grateful to Dr. J. Joyard for helpful discussions during the development of this work and to Dr. R. DeRose and Dr. M.C. Walsh for critical reading of the manuscript.

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