Acyl Carrier Protein Is Conjugated to Glutathione in Spinach Seed

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ABSTRACT

Acyl carrier protein (ACP) contains an essential sulfydryl group in its phosphopantetheine prosthetic group. We have investigated the state of this sulfhydryl in developing and mature spinach seed (Spinacia oleracea). Seed extracts were separated on sodium dodecyl sulfate or native polyacrylamide gels, blotted to nitrocellulose, and probed with antibodies raised against spinach ACP-I. In extracts of mature seeds prepared with reducing agents, ACP-II migrated as a single major band, whereas extracts prepared without reducing agents gave two major bands. The additional band was identified as a conjugate of ACP-II to glutathione (ACP-S-S-G) on the basis of its sensitivity to reducing agents and its comigration with standards in both native and sodium dodecyl sulfate gel electrophoresis. In developing spinach seeds ACP-II exists primarily in its free phosphoryl form or as acyl derivatives, with essentially no ACP-S-S-G present. During later stages of seed development, as seed water content declines, ACP-S-S-G accumulates to approximately 50% of the total ACP. Seed imbibition results in a rapid decline in ACP-S-S-G levels. The ACP-S-S-G:ACP-SH ratio of seeds during storage was found to be a function of seed water content and this could be manipulated by controlling the relative humidity under which the seeds were stored. We speculate that conjugation of ACP to glutathione protects the ACP from sulfhydryl oxidative damage in dry seeds.

The synthesis of fatty acids in plants occurs mainly in plastids and is catalyzed by a series of enzymes together with the protein cofactor, ACP3 (6). In plants, ACP is a small (ca. 9000 D), acidic cofactor which plays a central role in plant lipid metabolism (13). A phosphopantetheine prosthetic group is attached to a serine residue near the middle of the polypeptide and is the site at which acyl chains are esterified during the reactions of fatty acid assembly. In addition to the six enzymatic steps of fatty acid synthesis, ACP also participates in reactions catalyzed by stearoyl-ACP desaturase, two acyl-ACP dependent chloroplast acyltransferases which acylate glycerol-3-phosphate and oleoyl-ACP thioesterase, which releases fatty acids from ACP. In spinach leaves, two major isofoms of ACP are present but in seeds and roots one form predominates (ACP-II) (14). ACP activity requires that the sulfhydryl of the phosphopantethein prosthetic group be in a reduced state. However, sulfhydryl groups are subject to several oxidative reactions which in the case of ACP could lead to its inactivation. In biological systems these oxidation reactions are prevented or reversed through a variety of protective mechanisms including the glutathione/glutathione reductase system. However, in dry seeds where enzyme activities are greatly reduced, these protective mechanisms may not be operative and long term exposure to oxygen could lead to oxidation of essential sulfhydryl groups. Because ACP is essential for fatty acid synthesis, and therefore membrane biogenesis during seed germination, it was of interest to determine the form of ACP-II that is stored in mature seeds. In this study we show that a major portion of the ACP found in mature seeds is conjugated as a mixed disulfide to glutathione.

MATERIALS AND METHODS

Plant Material

Spinacia oleracea L. (Hybrid 624) was grown in potting soil:vermiculite (1:1) at 25°C with 9 h illumination daily for 6 to 8 weeks after which plants were transferred to constant illumination to induce flowering.

Protein Gels and Immunoblot Analysis

Developing and mature spinach seeds collected from plants were used immediately or stored at 5°C. To extract ACP, seeds were crushed to a fine powder in liquid nitrogen and then homogenized in PBS (45 mM KH2PO4, 150 mM NaCl) in the presence of 10 mM NEM (pH 6.0) or 10 mM DTT (pH 7.0). Cellular debris was removed by centrifugation (5 min, 15,000g) and proteins in the supernatant were separated by native (13% polyacrylamide) or SDS-PAGE (15% gel) and electroblotted to nitrocellulose (16). The blots were soaked in 5% (v/v) formamide for 5 min, rinsed three to four times with water, and then prepared for immunoblot analysis (5). Crude rabbit serum containing antibodies raised against purified spinach ACP-I (15) was used as the primary antibody at a dilution of 1:1000 in 5% (w/v) nonfat dried milk in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% (v/v) Tween 20. Immobilized antibody was detected with goat-anti-rabbit IgG-alkaline phosphatase conjugate diluted 1:2000 (5).

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Preparation of Standards

ACP-II was purified from spinach leaves as described by Ohlrogge and Kuo (14). An ACP-II-glutathione adduct was prepared as follows. Spinach ACP-II (2 μL, 2 μg) was mixed with 1 μL of 1.0 M Tris pH 8.5, and 1 μL 100 mM DTT and incubated at 37°C for 15 min. Thereafter, 50 μL of 50 mM oxidized glutathione (dissolved in 100 mM Tris pH 8.5) were added and the solution incubated at 30°C for 3 h. TCA was added to a final concentration of 6% (w/v) and the mixture placed on ice for 30 min. The solution was centrifuged (5 min 15,000g) and the pellet redissolved in 100 mM Tris buffer, pH 8.0. Malonyl-ACP-II was prepared enzymatically with malonyl-CoA transacylase (9).

Seed Storage at Controlled RH

Seeds (4.0 g) were placed in 50 mL beakers and stored in sealed jars at constant RHs such that the seeds achieved various hydration states. Anhydrous CaSO₄ was used to maintain 0% RH, and higher RHs (50–100%) were obtained by using mixtures of water and sulfuric acid (21). The sealed humidity chambers were kept under laboratory temperature (20°C) and lighting conditions. Seed water content was approximated from the loss in weight of a sample heated at 110°C for 24 h. Less than 2% additional weight loss was observed when seeds were heated at 110°C for longer periods. For imbibition studies, seeds were placed in a 250 mL flask containing 100 mL of oxygenated distilled water for 24 h.

RESULTS

ACP-II Exists in Two Forms in Mature Spinach Seeds

The sulphydryl group of ACP is easily oxidized during sample preparation and gel electrophoresis resulting in altered electrophoretic mobility. Although such oxidations can be prevented by inclusion of reducing agents such as DTT, these agents can lead to deacylation of acyl-ACPs (12, 17) and reduction of in vivo disulfide forms of ACP. We have recently shown that analysis of in vivo forms of ACP is simplified by preparing extracts in the presence of NEM (17). NEM reacts irreversibly with the free sulphydryl of spinach ACP to form an adduct which retains the same mobility as ACP-SH during native PAGE.

In mature seeds, ACP-II extracted in the presence of NEM exists in two major forms of approximately equal abundance (Fig. 1, lane 3). The major band closest to the dye front comigrates with a fully reduced spinach ACP-II standard. In the absence of NEM, the reduced ACP-II band disappears, probably as a result of its sulfhydryl oxidation during electrophoresis, but the second, slower migrating form remains (Fig. 1, lane 2). When seed extracts were prepared in the presence of DTT (Fig. 1, lane 1) the more slowly migrating form disappeared, whereas the mobility of the fully reduced form of ACP-II was unaffected by this treatment. These results suggest that the more slowly migrating form of ACP-II may be conjugated to another molecule at its sulfhydryl group. Fully reduced ACP-II and the unknown ACP-II were the major bands detected in dry, mature spinach seeds by immunoblot analysis, but after 24 h of imbibition, additional bands, presumably corresponding to acylated forms of ACP-II appeared (Fig. 1, lanes 4 and 5). The identity of these bands was based on their comparable mobility to acylated ACP-II standards, and their sensitivity to hydrolysis by DTT (Fig. 1, lane 6). The appearance of these acylated ACPs suggests the initiation of fatty acid synthesis in response to hydration and was accompanied by a decline in the amount of the unknown ACP-II in comparison to levels found in dry seeds. There was no evidence (based on the combined intensity of the ACP-II bands), for increased levels of ACP-II during the 24 h imbibition period.

Comigration of Unknown ACP with ACP-Glutathione Standard on Both Native and SDS-Polyacrylamide Gels Suggests that ACP-II Is in Disulfide Linkage to Glutathione

It was hypothesized that the unknown ACP-II was an ACP-glutathione adduct (ACP-S-S-G). To test this hypothesis, an ACP-S-S-G adduct was prepared in vitro and served as a

Figure 1. ACP-II exists in two forms in mature spinach seeds. Aliquots (50 μg total protein) of extracts from imbibed seed or dry seed prepared in 50 mM phosphate buffer alone or in the presence of NEM (10 mM) or DTT (10 mM) were separated on a native gel and ACPS were detected by immunoblot analysis. Lanes 1 to 3, dry seed; lanes 4 to 6, imbibed seed.
A. NATIVE GEL

Figure 2. Comigration of the unknown ACP with an ACP-glutathione standard on both native and SDS-polyacrylamide gels indicate that ACP-II is in disulfide linkage to glutathione. A, Native gel. Lane 1, extract of dry seed prepared with NEM; lane 2, glutathione-ACP-II standard; lane 3, malonyl-ACP-II standard; lane 4, ACP-II standard. B, SDS-gel. Lane 1, glutathione-ACP-II standard; lane 2, extract of seeds stored at 80% RH for 3 weeks.

standard for immunoblot analysis. Antibodies raised against ACP-I recognized the ACP-S-S-G standard (Fig. 2A, lane 2), and upon reduction with DTT the ACP-S-S-G was converted to fully reduced ACP-II. The ACP-S-S-G standard comigrated on a native gel as a single band with the unknown ACP extracted from mature spinach seeds (Fig. 2A, lane 1). Malonyl-ACP-II is also known to migrate slightly above ACP-II in this gel system and therefore to confirm that the unknown was not malonyl-ACP-II, a malonyl-ACP-II standard was prepared and analyzed (Fig. 2A, lane 3). Both malonyl-ACP-II and ACP-S-S-G can be resolved on this native gel system, and clearly the unknown is not malonyl-ACP-II.

As shown in Figure 2B, immunoblot analysis of seed extracts separated on SDS-gels indicated that ACP proteins also comigrated in this gel system with the ACP-S-S-G standard. Under some conditions ACP resolves into two bands during polyacrylamide gel electrophoresis. Two electrophoretic bands have been detected previously from pure preparations of both spinach (1) and Escherichia coli ACP (8) and are perhaps related to two stable conformations of the polypeptide (11). In addition, the ACP-S-S-G standard migrated as two major bands (Fig. 2B, lane 1). Upon reduction with DTT, the ACP-S-S-G standard disappeared coincident with the appearance of a band which comigrated with the ACP-II standard reduced with DTT (data not shown). Similarly, seed extracts analyzed in the presence of DTT resulted in the disappearance of proteins comigrating with the ACP-S-S-G standard and the appearance of a band which comigrates with fully reduced ACP-II. Thus, as with native gels, both the comigration of the unknown band with standards and its susceptibility to reduction by DTT supports its identification as a glutathione adduct.

Levels of ACP-S-S-G Accumulate during Final Stages of Seed Development

ACP-S-S-G was not detected in green immature seeds (Fig. 3, lane 3) but was observed at low levels during the late stages of seed development (Fig. 3, lane 4). Acylated ACPs were detected in seeds of both of these developmental stages, indicative of fatty acid synthesis in these seeds. When seeds were imbibed for 24 h, the ACP-S-S-G adduct disappeared (Fig. 3, lane 5).

Levels of ACP-S-S-G Are Function of Seed Water Content

Mature seeds were harvested and stored at different RHs. The effect of storage for 12 d at different constant RHs on the water content of spinach seeds is shown in Table I. Preliminary investigations were concerned with the effects of various RHs (0, 50, 85%) on levels of ACP-SH and ACP-S-S-G in seeds stored for 4 months (Fig. 4). Seeds stored at 0% RH (Fig. 4, lanes 1–3) showed no change in the levels of ACP-SH and ACP-S-S-G and seed water contents declined from 10.0% (water content of mature seeds stored at ambient conditions) to 8.7%. A similar result was found with seeds stored at 50% RH (Fig. 4, lanes 4–6) although seed water contents increased from 10 to 11.6%. In contrast, seeds stored at 85% RH (Fig. 4, lanes 7–9) showed a dramatic loss of ACP-S-S-G and an increase in water content from 10.0 to 17.5%.

Figure 3. Levels of ACP-S-S-G accumulate during the final stages of seed development. Aliquots (50 μg total protein) of seed extracts from imbibed seed or mature seed prepared in 50 mM phosphate buffer alone or in the presence of NEM (10 mM) or DTT (10 mM) were separated on a native gel and processed for immunoblot analysis. Lanes 1 and 3, immature seeds; lanes 2 and 4, mature seeds; lanes 5 and 6, mature seeds imbibed in water for 24 h before homogenization.
Table I. Effect of Storage for 12 d at Different Constant RHs on Water Content of Spinach Seeds

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<th>RH (%)</th>
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<tr>
<td>0</td>
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<td>50</td>
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<td>90</td>
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Restoration of ACP-S-S-G levels was observed when seeds stored at 85% RH were returned to 0% RH (Fig. 4, lanes 10–12).

The time course of changes in levels of ACP-S-S-G and ACP-SH in mature seeds stored at 80% RH was measured over a period of 12 d (Fig. 5A, lanes 1–5) and after 3 weeks (Fig. 5B, lane 1). During this time, seed water contents increased from 10.0 to 16.3% and concomitantly, the relative proportion of ACP-S-S-G accumulated such that after 3 weeks, little ACP-SH remained (Fig. 5B, lane 1). Upon transfer of these seeds from 80 to 100% RH (Fig. 5A, lanes 6–10) seed water contents increased to 21.3%, whereas levels of ACP-S-S-G declined. After 3 weeks at 100% RH, ACP-SH predominated (Fig. 5B, lane 2).

**DISCUSSION**

The results presented in this paper indicate that approximately half of the ACP in mature spinach seeds is conjugated to glutathione, while the remainder apparently exists in a fully reduced form. Evidence for this conclusion is based upon the following arguments. In mature seeds, ACP extracted in the presence of NEM exists in two forms of approximately equal abundance (Fig. 1). The form closest to the dye front comigrates with a fully reduced spinach ACP-II standard. In the absence of NEM the reduced ACP band disappears, probably as a result of its oxidation during electrophoresis, while the second, slower migrating form remains. This result indicates that the nonreduced form of ACP is not simply an ACP-NEM adduct because it is also present in the absence of NEM. Preparation of seed extracts in the presence of DTT (Fig. 1) results in the disappearance of the slower migrating form, suggesting that this form of ACP is conjugated to another molecule at its sulfhydryl group. Comigration of the ACP adduct with an ACP-S-S-G standard on both native and SDS-polyacrylamide gels (Fig. 2) suggests that ACP-II is in disulfide linkage to glutathione.

Pools of ACP-S-S-G are not found at most stages of seed development but accumulate only during the final stage at a time when the seed is in the process of dehydration. Interestingly, acylated ACP intermediates of fatty acid synthesis, are also detected in seeds during late development. In fully mature and dry seeds, half the total ACP occurs as ACP-S-S-G, and the remainder as ACP-SH, while none is acylated. Seed hydration during imbibition results in a dramatic decrease in ACP-S-S-G levels and the appearance of acylated and reduced ACP (Fig. 1).

**Figure 5.** Time course of changes in levels of ACP-S-S-G and ACP-SH in response to altered seed water content. Seed extracts were prepared by homogenizing seeds in 50 mM phosphate buffer (pH 6.0) in the presence of NEM (10 mM). A, Lanes 1 to 5, seeds stored for 0, 2, 4, 7, 12 d at 80% RH; lanes 6 to 10, seeds stored at 100% RH for 0, 2, 4, 7, 12 d. B, Lane 1, seeds stored for 3 weeks at 80% RH; lane 2, seeds stored for 3 weeks at 100% RH.

**Figure 4.** During seed storage, levels of the ACP-S-S-G in spinach seed are a function of seed water content. Aliquots (5, 15, 50 µg total protein) of extracts from seeds stored at different constant RHs for 4 months. Lanes 1 to 3, 0% RH; lanes 4 to 6, 50% RH; lanes 7 to 9, 85% RH; lanes 10 to 12, seeds stored at 85% RH for 1 month and then transferred to 0% RH for 3 months; lane 13, spinach ACP-II.
The water content of seeds appears to be a major factor in determining the levels of ACP-SH and ACP-S-S-G in spinach seeds. When seeds are stored for three weeks at 80% RH, most ACP becomes conjugated to glutathione (Fig. 5B) while very little occurs as ACP-SH and very little is acylated. It is likely that the coincident accumulation of ACP-S-S-G and the disappearance of ACP-SH results from their interconversion. The reverse process can also be demonstrated: seeds transferred from 80% to 100% RH show a loss of ACP-S-S-G and an accumulation of ACP-SH and acylated ACP (Fig. 5, A and B). Figure 4 indicates that seeds stored at between 0 and 50% RH do not change in the relative levels of ACP-S-S-G and ACP-SH for prolonged periods (more than 4 months). Seeds transferred from 85 to 0% RH lose water and accumulate ACP-S-S-G. However, the interconversion is not complete and consequently about half the ACP remains as ACP-SH (Fig. 4). This is analogous to the drying process during seed maturation on the plant where mature seeds were also found to have only half the ACP conjugated to glutathione (Fig. 1). Only under experimental conditions, where seed water content is maintained at approximately 16% by incubation at 80% RH, is most ACP found as ACP-S-S-G (Fig. 5B).

The pattern of ACP-S-S-G accumulation in seeds during spinach seed maturation and ACP-SH regeneration upon imbibition is similar to the thiol-disulfide interconversions of glutathione found in Neurospora crassa conidia spores (3, 4). Most glutathione in the mycelia of N. crassa is in its thiol form (G-SH), but in conidia G-SH is converted to its disulfide, G-S-S-G. In addition, proteins were found adducted to glutathione, although the identity of these proteins was not determined. During spore imbibition, glutathione disulfide and mixed disulfide levels decreased sharply and G-SH accumulated. The conversion of CoA from thiol to disulfide forms has been reported to accompany spore formation in Bacillus megaterium, and reduction of such disulfides was found to occur early in germination (18). The results of these investigations parallel those presented here with seeds.

Glutathione is the most abundant low mol wt thiol in most biological systems and functions primarily as a reductant to maintain sulphydryl groups in a reduced state. Under normal physiological conditions, the ratio of reduced to oxidized glutathione is high. Maintenance of this state by regeneration of reduced glutathione is catalyzed in many tissues by an NADPH-dependent glutathione reductase. When biological systems dehydrate, the resulting loss in activity of enzymes such as glutathione reductase and NADPH generating pathways leads to an increase in the oxidative environment (20). Under such conditions the formation and accumulation of disulfides and mixed disulfides between glutathione and other thiols such as ACP can be expected to occur. Mixed disulfide formation can occur in vitro spontaneously and would be expected to also occur in vivo under oxidizing conditions (22).

Upon rehydration, glutathione reductase can be expected to be reactivated and reducing conditions restored. Studies with dormant spores of B. megaterium show that within minutes of rehydration, NADPH is generated through the reactivation of the pentose phosphate pathway (19).

Considering the close association between changes in hydration status and thiol-disulfide states in fungal, bacterial and animal systems, the results presented here with spinach seeds suggest that a similar phenomenon is responsible for ACP modifications in seeds. Thus, we interpret these data as indicating that as the seed matures and dries, glutathione reductase activity declines, the ratio of G-S-S-G to G-SH increases and mixed disulfide formation between glutathione and ACP occurs. Free radical activity is known to increase with increasing RH (7). Storing seeds at 80% RH results in the fastest rate of ACP-S-S-G accumulation. Presumably, it is under these conditions that the reduction systems of the cell are inoperative but oxygen radical activity is highest. With further dehydration, molecular diffusion rates decline (2) so that the conversion of ACP-SH to ACP-S-S-G through the interaction of ACP with glutathione or free radicals becomes negligible. This is consistent with the observation that over a period of several months, no change in levels of ACP-SH and ACP-S-S-G could be detected in spinach seeds stored under dry conditions (50 and 0% RH) (Fig. 4). Increasing spinach seed water content by imbibition or by storage at 85% RH-most likely allows the regeneration of NADPH and an increase in the activity of glutathione reductase.

ACP present in spinach seeds is utilized for lipid metabolism during seed germination. During long term exposure of seeds to oxygen in the air, the sulphydryl group which forms the active site of ACP is likely vulnerable to inactivation by its oxidation to a variety of oxidation states (10). Disulfides are the predominant oxidation product of thiols under mild oxidizing conditions and are very unreactive to further oxidation. Furthermore, the formation of disulfides is easily reversed in the presence of excess thiol, thereby regenerating the active reduced sulphydryl. Glutathione in disulfide linkage to ACP may therefore have a protective function in reducing the loss of ACP from irreversible oxidation during seed dormancy.

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LITERATURE CITED