Polyamines in chloroplasts: identification of their glutamyl and acetyl derivatives

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Incubation of chloroplasts of *Helianthus tuberosus* with labelled putrescine and/or spermidine and proteolytic digestion of their trichloroacetate-soluble and -insoluble proteins revealed the presence of *N*-(γ-glutamyl)-putrescine, N^1,N^4-bis-(γ-glutamyl)-putrescine and N^1,N^8-bis-(γ-glutamyl)spermidine. This finding may be regarded as unequivocal proof of the presence of transglutaminase activity in chloroplasts. In addition, the recovery of spermidine or putrescine and acetylspermidine from chloroplasts incubated with [^{3}H]putrescine or [^{3}H]spermidine respectively indicates the existence of biosynthetic and oxidative pathways. These results suggest that polyamines may have an important function in chloroplasts both in their free form and by covalently binding to proteins.

INTRODUCTION

Aliphatic polyamines, putrescine, spermidine and spermine, have been detected in animals and plants. Cellular levels of these small organic cations are highly responsive to stimuli, such as different types of hormones and stress. Their administration to cultured cells can cause growth stimulation and probably differentiation, as well as preventing senescence (Galston and Kaur-Shawney, 1990; Bagni and Torrigiani, 1992).

The correlation between polyamine titre and a physiological response does not necessarily provide information as to their direct connection and the molecular events involved. However, such correlative data are the necessary prelude to further investigation of the mechanism of action of polyamines at the molecular level. In order to move on from the phase of establishing correlations, we at first turned our attention to polyamines bound to nucleic acids (Serafini-Fracassini et al., 1984) and, more recently, to proteins (reviewed by Del Duca and Serafini-Fracassini, 1993).

It has been established that, in animal cells, post-translational covalent linkage of polyamines to proteins is catalysed by a class of enzymes known as transglutaminases (TGase; R-glutaminyl-peptide–amine γ-glutamyltransferase, EC 2.3.2.13), which have been localized both intra- and extra-cellularly (Folk, 1980; Lorand and Conrad, 1984; Beninati et al., 1985). The Ca^{2+}-dependent acyl-transfer reaction catalysed by TGases has a limited specificity in that only the γ-carboxamide groups of peptide-bound glutamine residues serve as acyl donors. Primary amino groups in a relatively wide variety of compounds can act *in vitro* as acyl acceptors with the subsequent formation of monosubstituted γ-amides of peptide-bound glutamic acid. The ε-amino groups of lysine residues of specific proteins can act as acyl acceptors, with the formation of ε-(γ-glutamyl)lysine cross-links. It has been observed that polyamines are acyl-acceptor substrates for TGases. They are covalently bound to proteins through one of their terminal primary amino groups to form mono-(γ-glutamyl)polyamines or by means of two linkages, with both terminal amino groups, to form bis-(γ-glutamyl)polyamines (Folk et al., 1980).

Until recently, studies on the physiological role of TGases focused on animal cells. Although the occurrence of structural proteins as substrates for TGases is frequently related to differentiation (Green, 1980), cell death (Piacentini et al., 1991) and metastatic potential of cancer cells (Beninati et al., 1993), the direct role of intracellular TGase in these biological events is still unknown.

An enzyme with several TGase-like characteristics has only recently been studied in different organs of higher plants. A pioneering paper dealing with the occurrence of light-stimulated 'fixed' polyamines in chloroplasts of *Brassica pekinensis* was published by Cohen et al. (1982). Owing to an apparent Ca^{2+}-independence of this binding activity, these authors assumed that it was not catalysed by a TGase. Later, results suggesting the presence of TGase activity in cycling cells and etiolated sprout apices of *Helianthus tuberosus* tubers (Mossetti et al., 1987; Serafini-Fracassini et al., 1988, 1989) and in *Pisum sativum* (Icekson and Apelbaum, 1987) were reported. A possible TGase isoform was also detected in the particulate fraction of leaves of several species (Signorini et al., 1991; Falcone et al., 1993). It was also found in unopened flower buds of *Medicago sativa*, where ribulose bisphosphate carboxylase/oxygenase (Rubisco) was indicated as its major substrate (Margosiak et al., 1990). In leaves, other proteins of relatively low M_r were also found to be conjugated to polyamines (Falcone et al., 1993) and considerable binding activity was detected in non-photosynthetic medullary explants of *H. tuberosus* induced to greening with respect to their non-green controls (Del Duca et al., 1993). In isolated chloroplasts of *H. tuberosus* leaves, proteins conjugated to putrescine or spermidine have been identified as apoproteins of the chlorophyll *a*/*b* antenna complex, namely light-harvesting complex II

Abbreviations used: acetyl-SD, acetylspermidine; bis-PU, bis-(γ-glutamyl)putrescine; bis-SD, bis-(γ-glutamyl)spermidine; mono-PU, mono-(γ-glutamyl)putrescine; PAO, polyamine oxidase; Rubisco, ribulose bisphosphate carboxylase/oxygenase; TGase, transglutaminase; LHC, light-harvesting complex.

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(LHCII), CP26, CP24 and CP29, as well as the large subunit of Rubisco (Del Duca et al., 1994).

Although the above papers provide much circumstantial evidence indicating that these binding reactions are catalysed by a TGase, the detection of polyamines covalently linked to glutamyl residues of endogenous proteins is the only unequivocal demonstration of the existence of these enzymes in plants. Signorini et al. (1991) showed that only mono-(γ-glutamyl)-putrescine (mono-PU) could be identified on incubation of extracts from *Beta vulgaris* leaves with putrescine and *N,N'*-dimethylcasein exogenously supplied in the assay mixture.

MATERIALS AND METHODS

Materials

Leaves of *H. tuberosus* L. cv OB 1 plants, selected and grown in the Botanical Garden of Bologna University, were collected and, after being left in the dark overnight, were used to extract chloroplasts as previously described (Del Duca et al., 1994). In some experiments, 5% (w/v) non-fat dried milk, 5 mM ε-aminohexanoic acid and 0.2 mM phenylmethanesulphonyl fluoride were added to the extraction buffer as proteinase inhibitors (Gray, 1982).

Protein determination

Protein was determined by the method of Lowry et al. (1951).

TGase assay

Chloroplasts (1500 μg of protein) were incubated for 30 min at 30 °C in the light (3000 lm·m^{-2}). In the buffered (pH 8.5) incubation mixture prepared as previously reported (Del Duca et al., 1994) 0.2 mM putrescine or spermidine and 370 kBq of [1,4-^{3}H(n)]putrescine or [1,4-^{3}H(n)]spermidine (1.5 TBq/mmol) (NEN–Dupont) were added. In some experiments 4 mM instead of 0.2 mM unlabelled putrescine was used. The reaction was stopped with 5% (w/v) (final concentration) trichloroacetic acid also containing 2 mM unlabelled spermidine in order to remove trapped free polyamines; the mixture was stored at 4 °C for 24 h and then centrifuged at 13250 *g* for 10 min. The pellets were solubilized overnight with 0.1 M NaOH at 37 °C and then reprecipitated twice with 5% trichloroacetic acid. The supernatants and final pellet, washed with anhydrous ethyl ether, were proteolytically digested as described by Folk et al. (1980).

Identification of polyamine derivatives and products of TGase catalysis

Ion-exchange chromatographic separation of γ-glutamylpolyamines and other derivatives in the acid-soluble and -insoluble fractions was performed on an LKB Alpha Plus 4151 amino acid analyser equipped with a 4.5 mm × 90 mm column packed with Ultropac 8 resin (Na^+ form), using the five-buffer system previously reported (Folk et al., 1980). The identity of conjugated polyamines (γ-glutamylpolyamines and N^1- and N^8-acetylated spermidine) was determined from the release of free polyamines after acid hydrolysis of the ion-exchange chromatographic fractions corresponding to the predicted retention times (Folk et al., 1980). An attempt was made to recognize the acetylated forms of spermidine by incubating the corresponding ion-exchange chromatographic fractions with rat liver polyamine oxidase (PAO) purified by the procedure of Hölttä (1977).

To verify further the identity of the acetylated polyamines, ion-exchange chromatographic fractions that released spermidine on acid hydrolysis were purified by gas chromatography (Daves et al., 1983) and the spermidine derivative was isolated. A fast-atom bombardment m.s. analysis was also performed on this fraction.

RESULTS

Figures 1 and 2 show the chromatographic profiles of the digested trichloroacetate-soluble and -insoluble fractions respectively of *H. tuberosus* chloroplasts incubated with polyamines. It is evident that active metabolism of polyamines takes place in these chloroplasts. Incubation of chloroplasts with 0.2 mM spermidine or putrescine led to the recovery in both cases of the other free polyamines, bis-(γ-glutamyl)putrescine (bis-PU) and bis-(γ-glutamyl)spermidine (bis-SD) and their mono-(γ-glutamyl) derivatives, as well as two chromatographic peaks with retention times similar to those of N^1- and N^8-acetylspermidine (acetyl-SD). Identification of γ-glutamyl polyamines was performed by acid hydrolysis of the ion-exchange chromatographic fractions corresponding to the predicted retention times for these derivatives (Beninati et al., 1985). Fast-atom bombardment m.s. analysis of the fractions with retention times corresponding to those of N^1- and N^8-acetyl-SD gave a positive ion M^+ 1 of 188.29 (the value calculated for *N*-acetyl-SD = 188.0).

A preliminary evaluation of the identity of the two chromatographic peaks eluted in the region of N^1- and N^8-acetyl-SD was obtained from the following results: (i) acid hydrolysis of

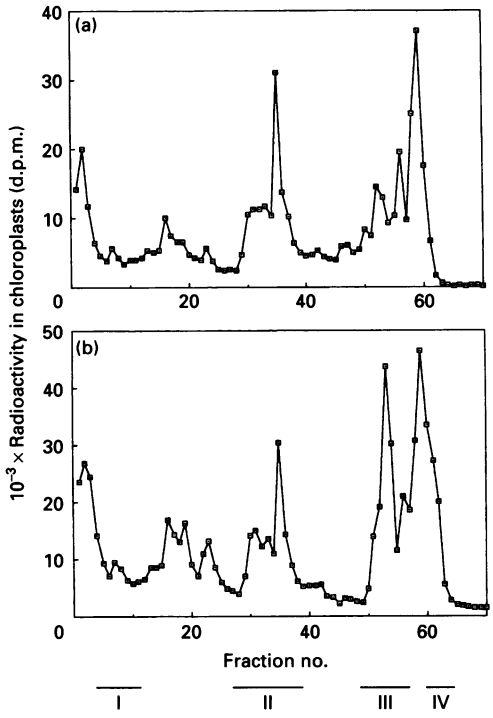

Figure 1 Distribution of radioactivity in chloroplasts incubated in the presence of tritiated polyamines (trichloroacetate-soluble fraction)

Chloroplasts were incubated in the presence of 0.2 mM spermidine (**a**) or 0.2 mM spermidine + 0.2 mM putrescine (**b**). Proteins were precipitated with trichloroacetic acid. Portions of the acid-soluble fraction were proteolytically digested and analysed by automated ion-exchange chromatography. The retention time ranges indicated in the chromatograms represent the expected elution regions: I, N^1,N^4-bis-PU; II, *N*-mono-PU, N^1,N^8-bis-SD; III, N^1N^8-mono-SD and N^1,N^8-acetyl-SD; IV, free polyamines.

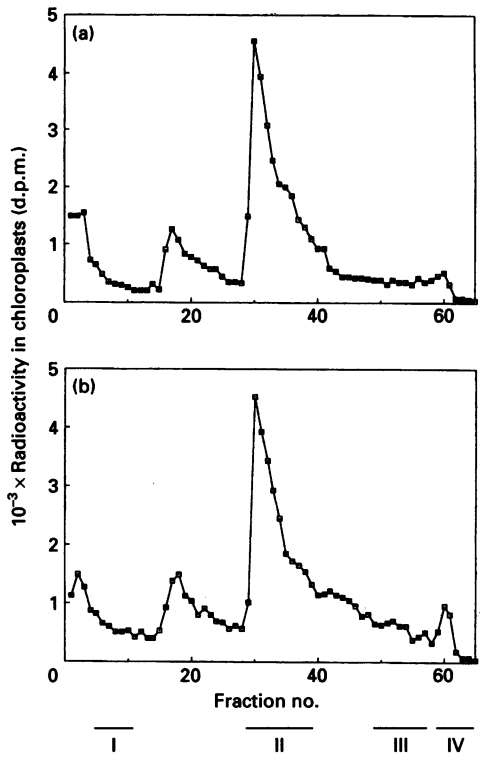

Figure 2 Distribution of radioactivity in chloroplasts incubated in the presence of tritiated polyamines (trichloroacetate-insoluble fraction)

Chloroplasts were incubated in the presence of 0.2 mM spermidine or 0.2 mM putrescine + 0.2 mM spermidine. Proteins were precipitated with trichloroacetic acid. Portions of the acid-insoluble fraction were proteolytically digested and analysed by automated ion-exchange chromatography. The retention time ranges indicated in the chromatograms are reported in the legend of Figure 1.

Table 1 Identification of *N*-acetyl[^{3}H]spermidine by acid hydrolysis

Fractions 52–56 eluted on ion-exchange chromatography (Figure 1b) and corresponding to N^1- and N^8-acetyl-SD were acid hydrolysed. Data are means ± S.D. of three determinations.

Compounds	Radioactivity (d.p.m./mg of protein)	Percentage of total radioactivity
Fraction 52–56	15000 ± 1200	100
Putrescine	800 ± 100	5
Spermidine	12500 ± 900	74
Spermine	–	–
Unknown	2800 ± 650	19

fractions 52–56 released spermidine (Table 1) and (ii) incubation of either of the two fractions with purified rat liver PAO led to the disappearance of the first peak, corresponding to N^1-acetyl-SD, leaving unchanged the second one corresponding to N^8-acetyl-SD (results not shown). These findings strongly suggest that the first chromatographic peak is really N^1-acetyl-SD and the second N^8-acetyl-SD (Figure 1a, elution region III). As a significant amount of free putrescine was found in the supernatant of chloroplasts incubated with spermidine only (Figure 1a, elution region IV), we concluded that catabolic conversion of polyamines with characteristics similar to that catalysed by rat liver PAO might take place in this organelle (such a PAO pathway has, as yet, never been detected in plants).

Table 2 Percentage distribution of γ-glutamylpolyamines in the proteolytic digest of acid-soluble and -insoluble proteins from chloroplasts incubated in the presence of tritiated polyamines

γ-Glutamylpolyamines were isolated from fractions eluted in region II of the ion-exchange chromatography as reported in Figures 1 and 2.

	Acid-soluble proteins		Acid-insoluble proteins	
	Mono-PU	Bis-SD	Mono-PU	Bis-SD
0.2 mM spermidine	84	16	80	20
0.2 mM putrescine + 0.2 mM spermidine	83	17	41	59

As shown in Figure 1(b), when chloroplasts were incubated with putrescine and spermidine together, the amount of presumptive N^1-acetyl-SD formed was higher than that obtained by incubation with spermidine only (Figure 1a). In contrast, the level of presumptive N^8-acetyl-SD was similar under both incubation conditions. As putrescine may inhibit a potential PAO activity by a feedback action, these data seem to provide further evidence for the presence of an enzymic pathway for the oxidation of polyamines in chloroplasts.

Even though most of the glutamylpolyamines were recovered, together with free polyamines, in the acid-soluble fractions (Figures 1a and 1b), mono-PU and bis-SD were also localized in the acid-insoluble fraction. The radioactivity recovered in the pellet obtained from chloroplasts incubated with either spermidine or spermidine plus putrescine is given in Figures 2(a) and 2(b) respectively. The profiles of the two chromatograms appear to be similar.

As the amount of these glutamyl derivatives in the pellet was enhanced by protease inhibitors, the presence of γ-glutamylpolyamines in the acid-soluble fraction may result from degradation by endogenous proteases of TGase-modified structural proteins.

The percentage distribution in the supernatant and pellet of mono-PU and bis-SD in chloroplasts incubated with spermidine in the presence or absence of putrescine is shown in Table 2. The distribution was similar in the two supernatants (approx. 80% mono-PU and 20% bis-SD) whereas in the pellets it appeared to be affected by the additional supply of putrescine. The relative percentages of mono-PU and bis-SD were similar to those found in the supernatant when only spermidine was supplied; in contrast, the relative percentages were 41 and 59 respectively when both putrescine and spermidine were supplied together. Derivatives formed after incubating chloroplasts with a higher polyamine concentration (4 mM) were very similar to those appearing in the chromatographic profiles shown in Figure 1, except that their levels were 20-fold higher (results not shown).

DISCUSSION

An extensive literature, mainly focused on animal cells, has emphasized the involvement of polyamines in post-translational modification of proteins in the reaction catalysed by TGases. Here we provide evidence for the formation also in plants of γ-glutamylpolyamine derivatives, which is a direct indication that polyamines are covalently bound to endoglutamyl residues of

proteins and thus that a true TGase is responsible for this modification.

Despite the common enzymic origin, the different γ-glutamylpolyamine derivatives found in chloroplasts differ significantly in several aspects. Mono-PU confers to the modified protein an additional positive charge located at the terminal primary amino group not involved in the linkage. This additional charge may cause a conformational rearrangement of the protein. A protein with this type of post-translational modification is highly susceptible to further modification by TGase leading to bis-PU. The final product of a TGase-catalysed reaction is a cross-link between two proteins. As more than one polyamine molecule can cross-link, a complex high-molecular-mass net may be formed.

The more extended chemical structure of the bis-SD bridge allows for a greater distance between chains and, because the specificity of TGase is limited to primary amines, the secondary amino groups of the spermidine moiety remain unconjugated and thus modify the overall charge on the proteins.

We have found most of the γ-glutamylpolyamines in the acid-soluble fraction from chloroplasts incubated with polyamines. This distribution may be the result of one of two conditions: (i) a strong protease activity, previously reported in chloroplasts (Matile, 1982; Lamppa and Abad, 1987), may give rise to the formation of small acid-soluble peptides. This hypothesis is supported by a significantly higher amount of γ-glutamylpolyamines recovered in the pellet on protease inhibition; (ii) several TGase substrates are acid-soluble peptides not derived from digestion.

The distribution of radioactive derivatives has previously been analysed by two-dimensional PAGE: in addition to label associated with some identified polypeptides of about 30 kDa, a considerable amount of radioactivity was also detected at the front of the SDS/polyacrylamide gel (Del Duca et al., 1994). The latter could be due to some of the derivatives detected in the present work.

Despite the possibility that the distribution of γ-glutamylpolyamines may be merely the result of experimental conditions, there is clear evidence for the formation of these derivatives in chloroplasts.

Analysis of the relative percentages of mono-PU and bis-SD, which are the most relevant derivatives found, shows that their distribution in the pellet, but not in the supernatant, appears to be regulated by the presence or absence of putrescine. This fact may be a consequence of the metabolic state of the biological system under investigation.

A comparison of the amounts of mono and bis derivatives of putrescine and spermidine indicates that, whereas spermidine tends to accumulate as the bis derivative, putrescine appears to remain as the mono derivative, at least in the fraction of TGase conjugates that, being solubilized by Pronase, can be analysed. Recovery of bis-SD in the pellet may result from the inability of endogenous proteases to digest the polymerized proteins.

The incubation of chloroplasts with putrescine or spermidine highlighted the presence of active polyamine metabolism in these organelles. We confirm earlier evidence [reported by Cohen et al. (1982)] for the conversion of putrescine into spermidine, but we also report the existence of a metabolic pathway that converts spermidine into putrescine, possibly via an acetylated intermediate, as previously described for yeasts and vertebrates (Gillyon et al., 1987; Bolkenius and Seiler, 1981). To our knowledge, this is the first time that acetylated polyamines have been detected in higher plants. Several lines of evidence appear to confirm our finding. The conversion of spermidine into putrescine in chloroplasts incubated in the absence of putrescine and the increased level of N^1-acetyl-SD, but not of N^8-acetyl-SD, obtained by adding putrescine to the incubation mixture are a clear indication of the presence of enzyme activity very similar to that of PAO, previously described in rat liver (Hölltä, 1977; Bolkenius and Seiler, 1981).

It has been reported that selective oxidation by FAD-dependent PAO may be one of the cellular mechanisms that regulates polyamine–protein cross-link levels (Beninati et al., 1992). Thus this finding seems to support the contention that the distribution of radioactivity in γ-glutamylpolyamines may depend on the catalytic activity of PAO, suggesting the existence of an oxidative mechanism that modulates formation of polyamine–protein linkages. This oxidative metabolism means that the post-translational modification of proteins catalysed by TGase may be subjected to further modification leading to modulation of bis-SD formation. It is reasonable to believe that the low recovery of this derivative in chloroplasts could be the result of the presence of this metabolic pathway. However, if this hypothesis is correct, in this case the modification by polyamines should be reversible. No information is available on the existence in plants of a γ-glutamylamino cyclotransferase, which is able to detach polyamines from the glutamyl residue. However, the polyamine-oxidizing activity reported above may release the positive charges of the polyamines.

Mono-PU formation has been reported in *Beta vulgaris* when extracts of leaves were incubated with *NN'*-dimethylcasein and radiolabelled putrescine (Signorini et al., 1991). A similar binding activity was observed in isolated chloroplasts of *H. tuberosus* and claimed to be responsible for the conjugation of polyamines to specific proteins, namely Rubisco and apoproteins of the chlorophyll *a*/*b* antenna complexes (Del Duca et al., 1994).

In the present report we have extended the investigation to provide unequivocal evidence for the formation of γ-glutamylpolyamine derivatives in isolated chloroplasts of *H. tuberosus*. The fact that the three different mono- and bis-(γ-glutamyl)-polyamine derivatives found were conjugated to endogenous chloroplast proteins suggests that this post-translational modification is physiologically significant.

Whereas polymerization of Rubisco subunits by polyamines, suggested by Klein et al. (1992), agrees with the proposed model of TGase stabilizing effects by cross-linkage formation, it is more difficult to interpret the effect of polyamine binding to some LHC proteins found by Del Duca et al. (1994). As it appears from the two-dimensional gels, the oligomeric form of the major LHC (II) is more heavily labelled by polyamines than the monomeric one. This suggests a role for polyamines in stabilizing oligomers by forming cross-links or by adding positive charges, which may have the opposite effect to that caused by their phosphorylation, which could favour the transfer of excitation energy between the photosystems (Mullet, 1983; Allen et al., 1981). Therefore one may speculate that, in chloroplasts, TGase may modify the apoproteins of the chlorophyll *a*/*b* antenna complex involved in the light-harvesting process of photosynthesis.

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