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PLANT TRANSGLUTAMINASES

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Abstract—The identification procedures, the characteristics and the potential function of the recently detected plant transglutaminases, are discussed in the light of the knowledge of animal transglutaminases. The enzyme has been studied occasionally in lower organisms (bacteria, fungi and green algae) and more extensively in Angiosperms.

INTRODUCTION

Cross-linking of proteins, an event which occurs post-translationally, is one of the vital physiological processes involved in the stabilization of tissue and cellular matrices. Among the various cross-links identified, ϵ -(γ -glutamyl)lysine and N,N -bis(γ -glutamyl)amine are the most abundant ones formed by enzymatic catalysis. The formation of these two types of cross-links is catalysed by Ca^{2+} -dependent acyl transferases (glutaminyl-peptide γ -glutamyltransferase, EC 2.3.2.13), known as transglutaminases (TGases). In the reaction catalysed by TGases the γ -carboxamide groups of peptide-bound glutamine residues are the acyl donors while primary amino groups in a variety of compounds may function as acyl acceptors with the subsequent formation of a mono-substituted γ -amide of peptide-bound glutamic acid. At less than saturating levels of a suitable primary amine or in its absence of an amine, water can act as the acyl acceptor with the formation of peptide-bound glutamic acid (Fig. 1).

Based on their distinct catalytic characteristics and distribution, several forms of transglutaminase have been identified to date and they have been found to exhibit differences in specificity. These differences are expressed in terms of variations in susceptibility of glutamine residues to catalytic modification and appear to be dependent, at least in part, upon amino acid residues surrounding a given glutamine. In contrast to their limited glutamine substrate specificity, TGases possess an exceptionally wide specificity for amine substrates. Among the latter, aliphatic polyamines (PA) are the more extensively studied. They are distributed in all living organisms, of which they regulate the growth [1, 2]. The most common ones are putrescine (1,4-diaminobutane) (PU), spermidine (N -[3-aminopropyl]-1,4-butanediamine) (SD) and spermine (N,N' -bis[3-aminopropyl]-1,4-butanedi-

amine) (SM) (Fig. 2). PU has an aliphatic tetramethylene backbone deriving directly from ornithine and indirectly from arginine depending on the organism. The biosynthesis of higher polyamines occurs by the addition of one or two aminopropyl groups to PU and thus SD and SM, respectively, are formed; these higher PA also bear protonated internal iminic groups. They are organic polycations at physiological pH, in fact the values of their pK are: PU $\text{pK}_2 = 9.04$, SD $\text{pK}_3 = 9.52$ and SM $\text{pK}_4 = 8.9$. [3]. PA are linear and flexible molecules of varying length and their backbone possesses low steric hindrance. Thus, they can interact precisely with several types of molecules and thereby affect their structure and function.

PA are covalently bound to proteins by TGase through a two-step reaction: attachment of the first primary amino group of the PA to the acyl donor substrate to form mono-(γ -glutamyl)-PA (mono-PA) and the binding of the second amine group to another glutamine residue to form bis-(γ -glutamyl)-PA (bis-PA) (Fig. 2) [4]. The first step is regulated by the PA concentration, since high levels of PA may saturate the acyl donor residues of the substrate proteins, thus preventing the second step.

TGases exhibit a different affinity for amine substrates: SD is preferred to SM and the latter is preferred to PU. This was observed in *Helianthus tuberosus* L. cv OB 1 etiolated sprout apices [5] and chloroplasts [6], in *Physarum polycephalum* M₃cV spherules [7] and in rat sperm [8].

The various γ -glutamyl-PA derivatives differ significantly under several aspects. Mono-(γ -glutamyl)-PU (mono-PU) confers to the modified protein an additional positive charge located at the terminal primary amino group not involved in the linkage. This further charge can cause a conformational re-arrangement of the protein. A protein having this type of post-translational modification is highly susceptible to a further modifica-

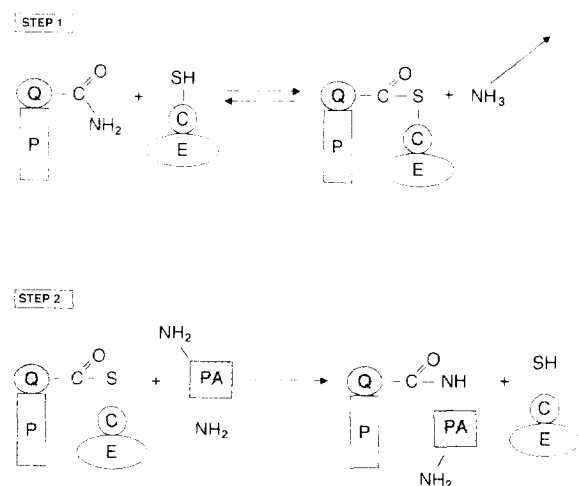


Fig. 1. Reaction scheme of the transglutaminase catalysis. It consists of the acyl-transfer reaction between γ -carboxamide groups of peptide-bound glutamine residues and polyamines (PA), resulting in the formation of new γ -amide-bonds. The reaction proceeds in two steps. Step 1: the intermediate acyl donor protein-enzyme is formed resulting in the release of ammonia. Step 2: nucleophilic attack by a primary amine group to the protein-enzyme complex, releasing the free enzyme. P = Protein, E = enzyme.

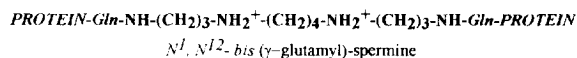
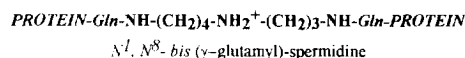
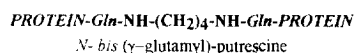
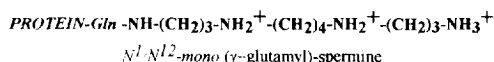
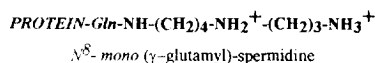
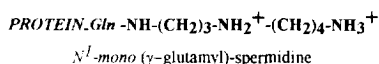
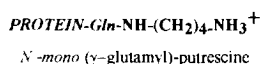


Fig. 2. PA derivatives resulting from the transglutaminase reaction. Different polyamines act as acyl acceptors substrates: putrescine, spermidine and spermine. The reaction of the first primary amine-group results in a mono-(γ -glutamyl) derivative formation, followed by the involvement on the second amine group with the production of a bis-(γ -glutamyl) derivative. Unlike putrescine and spermine, spermidine is an asymmetric molecule, therefore two sterically distinct mono-(γ -glutamyl)SD are produced. The total charge of the post-translationally modified protein is changed conforming to the PA bound to the protein.

tion by TGase leading to bis-(γ -glutamyl)-PU (bis-PU). The final products of a TGase-catalysed reaction is a cross-link between two proteins. Since more than a single polyamine molecule can cross-link, a complex high molecular mass network can be formed.

The more extended chemical structure of the bis-(γ -glutamyl)-SD (bis-SD) bridge and even more of the bis-(γ -glutamyl)-SM (bis-SM) bridge allow for a greater distance between chains. Because the specificity of TGase is limited to primary amines, the secondary amino groups of the SD and SM moiety remain unconjugated and thus modify the overall charge of the proteins.

The length of the PA molecule affects the structure and the properties of the network produced. This highly polymerized structure has been found in several mammalian tissues.

In conclusion, the results of TGase activity are: (i) a modification of the charge of the protein and (ii) a change in the conformation of the protein by formation of bridges within the protein itself or between different proteins, thus forming conjugates of higher mass up to supramolecular nets.

CHARACTERISTICS OF MAMMALIAN TGASES

Although the catalytic action of TGases and their limited specificity are known, much remains to be learned concerning tissue specificity regulation and structural relationships. TGases are widely distributed in various organs, tissues and body fluids. They are distinguishable from each other to a large extent by their physical properties and distribution.

Factor XIII is one of the best characterized TGases and its physiological role is well established. It is a plasma protein that circulates in blood as a tetramer of "a₂" "b₂" (*M_r* 320 000) and consists of two catalytic "a" subunits (*M_r* 75 000 each) and two non-catalytic "b" subunits (*M_r* 80 000 each). The "b" subunit is thought to stabilize the "a" subunit. Studies performed by X-ray crystallography revealed that the "a" chain is folded into four sequential domains and that the active site which is located in the central core domain, contains a triad of catalytic residues. A striking structural similarity in the active site region between factor XIII and the cysteine proteases has been noted, thus providing evidence for a TGase mechanism that is similar to the reverse of the hydrolysis mechanisms of proteases. Factor XIII also exists as a dimer of only "a" subunits in platelets, placenta, uterus, prostate, macrophages and other tissues and cells. Intracellular TGases differ in their sequence from the plasma enzyme and the so-called tissue type II enzyme (type C) is a ubiquitous monomer. A keratinocyte type I activity (TGase K) identified in cultured epidermal keratinocytes, in rat chondrosarcoma and in epithelial tissues, is known as TGase type B. Epidermal TGase (TGase E) occurs as a monomer (*M_r* 50 000–55 000), while hair follicle TGase exists as a dimer composed of two identical subunits (*M_r* 27 000). TGase E is neutral in humans and basic in rodents and is enriched in glycine, but other TGases are acidic and are

not glycine-rich. TGase K (M_r 90 000–92 000) is membrane-associated, while TGase C (M_r 75 000–80 000) and TGase E are soluble. TGase E is a zymogen like the plasma enzyme factor XIIIa. The properties and complete amino acid sequence of TGase C from guinea-pig liver, human factor XIIIa and TGase K have been determined. The amino acid sequences of the active sites of these three TGases are highly conserved (Fig. 3). Recently, it has been proposed that erythrocyte TGase is composed of two dense globular structures connected by a hinge region; Ca^{2+} binding affects the relative position of these globular structures favouring the exposition of the active site [9].

Three physiological roles have been unequivocally established for mammalian TGases: blood coagulation, construction of a cornified envelope in epidermal keratinocytes and apoptotic bodies and formation of a postejaculatory vaginal plug by prostate TGase in rodents. Some evidences has been found for additional functions that TGases may have. These include irreversible membrane stiffening of erythrocytes, opacification of eye lens, receptor mediated endocytosis, masking of the immunogenic power of spermatozoa surface, thus favouring fertilization, regulation of cell growth and differentiation, tumor metastasis and programmed cell death.

The TGase reaction catalysed by factor XIIIa leads to the cross-linking of a number of proteins in plasma. These include the dimerization of the γ -chains of two different fibrin molecules followed by the polymerization of the α -chains of fibrin. These reactions are critical to the blood coagulation cascade and result in the formation of a tough insoluble fibrin clot. A second important reaction catalysed by factor XIIIa is the cross-linking of an α_2 -plasmin inhibitor to the fibrin chains. This reaction plays a significant role in the regulation of fibrinolysis. A third reaction catalysed by factor XIIIa is the cross-linking of fibronectin to the α -chain of fibrin and to collagen, a reaction closely associated with wound healing. Accordingly, a deficiency of factor XIIIa can result in a lifelong bleeding tendency, defective wound healing and habitual abortion.

TGase is also present in nerve terminals and recognizes synapsin I, an abundant synaptic vesicle phosphoprotein involved in neurotransmission, as an excellent substrate. Activation of TGase stabilizes the vesicle-synapsin I-actin complex by covalent linkage and should

thereby block the phosphorylation-dependent release of synaptic vesicles from the cytoskeleton [10].

During terminal differentiation, mammalian epidermal cells acquire a deposit of protein on the intracellular surface of the plasma membrane which is termed 'cornified envelope'. This cross-linked structure is the most insoluble component of the epidermis owing to disulfide as well as ϵ -(γ -glutamyl)lysine isodipeptide bonds. Several proteins including involucrin, keratolinin and loricrin, are thought to be components of the epidermal envelope, but so far only loricrin has been shown to be cross-linked to these structures by ϵ -(γ -glutamyl)lysine isodipeptide bonds. Envelopes are highly resistant to chemical treatment and contribute to the protective function of the integuments. It has been reported that epidermal cell envelopes contain also cross-links of N^1, N^8 -bis(γ -glutamyl) SD. This additional PA linkage occurs at about one half the level of lysine cross-links in envelopes of normal individuals. In the skin of psoriatic patients, the levels of SD cross-links are much higher than those of normal individuals, resulting in imperfect cell envelopes, that do not achieve the biochemical prerequisite for the production of a normal stratum corneum. Protein cross-links by ϵ -(γ -glutamyl)lysine are irreversible and thus incompatible with most cell functions (proliferation, adhesiveness, movement, invasion etc.).

Cross-links have also been found in epidermal appendages such as wool, guinea-pig hairs and human nails.

TRANSGLUTAMINASES FROM OTHER SOURCES

In addition to the TGases extensively studied in mammals, other TGases have been studied in other organisms and in plants. TGase has been studied in *Streptovorticillum* sp. [11]; the enzyme has a M_r of 40 000 with an isoelectric point (IP) of 8.9. This enzyme is peculiar not only for the molecular mass, but also for its Ca^{2+} -independence, optimum pH range (6–7) and temperature (50°C). In filarial nematode, *Brugia malayi*, TGase has a M_r of 56 000 and an IP of 7.2; an optimum of pH at 8.5 and of temperature at 55°C [12]. Annulin, a homolog of mammalian TGase, is a protein of M_r 97 000 which is expressed in grasshopper embryo and seems to be related to cell stabilization under mechanical stress or to morphogenetic activities [13]. In *Limulus* hemocytes a TGase of M_r 86 000 been purified, having mammalian type II-TGase-like enzymatic properties. Two major substrates have been identified: one a proline-rich protein of M_r 80 000 and the other, a cysteine-rich 8600 protein able to form multimers. *Limulus* TGase shows significant sequence similarities with the mammalian TGase family; a phylogenetic tree representing an evolutionary relationship among the family members was inferred [14]. It has also been proposed that this TGase and its protein substrates may play an important role in the defence of this animal against invading microorganisms.

Other tissue-type TGases were studied in fish and that of *Pagrus major* was found to cross-link myosin heavy chains, thus forming a gel plug [15].

Human XIIIa	<u>Y G Q C W</u> V F A G V N T F L R C L G I P A R I V T N Y F S
Guinea pig TGase C	<u>Y G Q C W</u> V F A A V A C T V L R C L G I P T R V V T N F S
Rabbit TGase K	<u>Y G Q C W</u> V F A G V T T T V L R C L G L A T R I V T N F S

Fig. 3. Active site region of transglutaminases. The data are from factor XIIIa, guinea-pig liver TGase C and TGase K from rabbit tracheobronchial epithelial cells (from Kim *et al.* [72])

The active site is underlined.

PLANT TRANSGLUTAMINASES

Some TGases have been recently found in lower and higher plants, where they are present in different organs. Their presence was suggested by the recovery of TCA-pelletable polyamines whose linkage to proteins was not broken by treatment with surfactants. These conjugates have been frequently separated on SDS-PAGE and the molecular mass of the modified protein determined. The proof that they are formed by a TGase activity is supported by the recovery of labelled γ -glutamyl-PA after their proteolytic digestion. These conjugates have been obtained after an *in vitro* assay performed by incubating labelled PA with cell-free extracts of *Beta vulgaris* L. and *H. tuberosus* [16, 17]. The capacity of *H. tuberosus* TGase from sprout apices to recognize a synthetic dipeptide (Z-L-glutamyl-L-leucine), a specific substrate for TGase of animal origin, confirms that this PA-conjugat-

ing enzyme is a TGase [18]. Moreover, some recent reports indicated that higher and lower plant TGases cross-react with antibodies raised against TGases of animal origin [6, 19, 20].

Other characteristics, such as Ca^{2+} -dependence and dimethylated casein recognition as an exogenous substrate, are requirements not always or clearly met by plant TGases, as well as by some animal TGases.

Comparison of the characteristics of plant TGases

Several characteristics of plant TGases discussed below are comparatively presented in Tables 1 and 2 (references therein). Tables report the data obtained in non-photosynthetically competent (Table 1) and in photosynthetically competent cells (Table 2).

The cross-reactivity between polyclonal and monoclonal antibodies against TGases of animal origin was

Table 1. Transglutaminases in non-photosynthetically competent cells

	<i>Helianthus tuberosus</i> sprout apices [5, 18]	<i>Helianthus tuberosus</i> cycling cells [21]	<i>Pisum sativum</i> apices [22]	<i>Malus domestica</i> pollen [19]	<i>Physarum polycephalum</i> spherules [7]
Enzymes					
Immunorecognition	yes*†	n.d.	n.d.	yes‡	n.d.
M_r	75 000, 58 000			80 000	77 000
Localization		crude extract			crude extract
SN 22 000/27 000 g	65%		90%	100%§	
Pellet "	35%		3%		
Assay pH	7.8/8.4	7.6/8.4	8.5	7.8	7.5
Time dependence	hyperbolic	hyperbolic	linear	n.d.	n.d.
Ca^{2+}	0 or inhib	0 or inhib	0¶/stim**	stage dependent	stim.
Chelating agents	inhib††	inhib‡‡	inhib††‡‡	stage dependent	inhib††
Light dependence	n.d.	n.d.	n.d.	n.d.	n.d.
In function of [PU]:					
SN 22 000/27 000 g	sigmoidal/	linear	hyperbolic		hyperbolic¶
Pellet "	hyperbolic				
Pellet "	n.d.	n.d.	n.d.		
Protein substrates					
Recognition of substrates:					
N,N'-Dimethylcasein	no	no	yes	n.d.	yes
endog. proteins	yes	yes	yes	yes	yes
Endogenous substrates:					
M_r	high§§/high and low	high	n.d.	high and low	43 000
Identification	n.d.	n.d.	n.d.	actin + tubulin	actin

Activity in *in vitro* assay

* Polyclonal ab against rat prostatic gland TGase.

† Monoclonal ab against TGase K.

‡ Polyclonal ab against rat liver TGase.

§ SN obtained after the extraction with a Triton-containing buffer.

|| $\text{Ca}^{2+} > 5$ mM.

‡‡ EGTA.

†† EDTA.

** N,N'-Dimethylcasein added in the assay.

¶ N,N'-Dimethylcasein not added in the assay.

§§ Supernatant.

| | Crude extract.

Table 2. Transglutaminases in photosynthetically competent cells

	<i>Medicago sativa</i> floral buds [23, 24]	<i>Brassica pekinensis</i> leaves [25]	<i>Beta vulgaris</i> leaves [16]	<i>H. tuberosus</i> leaves [26]	<i>H. tuberosus</i> greening explants [27]	<i>H. tuberosus</i> chloroplasts [6]	<i>Dundaliella salina</i> [28]
Enzymes							
Immunorecognition	n.d.	n.d.	n.d.	n.d.	n.d.	yes**	yes*
M_r	39 000					58 000	70 000
Localization		chloroplasts			crude extract	thylakoids stroma?	chloroplasts
SN 22 000/27 000 g	100%†			20%			
Pellet "	n.d.		100%	78%			
Assay pH	7.9	8.5	7.8	8.2	7.8	8.3	8.5
Time dependence	n.d.	linear	linear	linear	n.d.	linear	n.d.
Ca^{2+}	n.d.	inhib	stim	inhib	n.d.	stim	n.d.
Chelating agents	inhib§	stim	inhib§	inhib	n.d.	n.d.	n.d.
Light dependence	yes	yes	n.d.	stim	yes	stim	n.d.
In function of [PU]:		linear			n.d.	hyperbolic	n.d.
SN 22 000/27 000 g	n.d.		n.d.	sigmoidal			
Pellet "	n.d.		linear*	hyperbolic			
Protein substrates							
Recognition of substrates:							
N,N' -Dimethylcasein	yes	n.d.	yes	no	n.d.	n.d.	n.d.
endog. proteins	yes	n.d.	yes	yes	yes	yes	yes
Endogenous substrates:							
M_r	high and low + 52 000–57 000	n.d.	about 66 000	high and low	high and low + < 36 000 + 50 000–60 000	high and low + 20 000–30 000 + 55 000	high and low + 55 000
Identification	Rubisco	n.d.	n.d.	n.d.	n.d.	LHC + CP + Rubisco	n.d.
Determination of γ - glutamyl-derivatives	n.d.	n.d.	yes	n.d.	n.d.	yes	n.d.

Activity in *in vitro* assay.

* Polyclonal ab against rat prostatic gland TGase.

† Monoclonal ab against TGase K.

‡ 100 000 g SN obtained after the extraction with a Triton-containing buffer.

§ EDTA.

|| EGTA.

¶ N,N' -Dimethylcasein added in the assay.

assayed only in some plants and was positive with, as for example, the enzyme of leaf chloroplasts, of sprout apices of *H. tuberosus* and of the unicellular alga *Dunaliella salina* Teodor. In germinating and ungerminated pollen of *Malus domestica* Borkh. cv Red Chief, polyclonal antibody recognized a single protein.

The molecular mass of the enzymes of other plants was determined by the presence of activity in chromatographic fractions. In unopened flower buds of *Medicago sativa* L., the catalytically active subunit has a M_r of 39 000. In the slime mould *P. polycephalum*, the value of the dimeric native enzyme is 77 000 and that of the monomer 39 600. In green leaves, either mature or developing, of *Brassica pekinensis* Skeels, *Beta vulgaris* L. and *H. tuberosus*, in *Dunaliella* cells as well as in pollen of *M. domestica*, most of the activity seems to be localized in the membranes, from which it can be released by surfactants. In etiolated apices of *H. tuberosus* and of *Pisum sativum* L., on the contrary, most of the activity is found in the 22 000 or 27 000 *g* supernatant. The differences observed in the subcellular distribution of this activity seems to be organ- and not species-specific.

The pH optima ranged between 7.6 and 8.5; these values are very similar to those of mammal TGases. Some features, such as the recognition of proteins of different M_r by antibodies, multiple pH optima and the different curves of activity as a function of PU concentration, suggest that multiple forms of the enzyme may exist in sprout apices and leaves of *H. tuberosus* and that some of them may be organ-specific. Different forms of TGases located in the same organ have also been reported to occur in animals [29, 30].

Ca^{2+} is a relevant parameter and, especially in the past, it was considered an essential factor for TGase. Generally, it must be supplied in the enzymatic assay even though, in some cases [8], it was found bound to the enzyme. In plants, perhaps owing to the higher availability of this cation in cell-free extracts, Ca^{2+} addition seems to be unnecessary; at times it can favour the activity, except at relatively high concentrations when it becomes inhibitory. Chelating agents generally lower the activity. However, the organ-related occurrence in *H. tuberosus* cycling cells of an aspecific or non-enzymatic binding, which takes place also after boiling, makes it difficult to completely clarify some of the properties of this activity, such as its pH- or Ca^{2+} -dependence.

Contrary to what happens in other plants, *N,N'*-dimethylcasein is not recognized as exogenous substrate by the TGase of *H. tuberosus*; this is also true for the enzyme of filarial parasites [31].

Light dependence has been studied only in green tissues and has been found to enhance the recovery of TCA-precipitable PA. The effect of light on thylakoid substrates is at present under investigation in our laboratory.

The conjugates formed with endogenous proteins and separated on SDS-PAGE appear to be more than one. In several organs very high molecular mass, or water-insoluble, conjugates are formed as products of TGase activity; these are also found in animal systems and, owing to

their mass, are difficult to analyse [32]. In green tissues or in etiolated apices of *H. tuberosus*, where leaf primordia and immature leaves are also present, substrates having a M_r lower than 30 000, were found in addition to the high M_r ones.

The exclusive property of TGase to form mono- and bis-glutamyl PA has been only rarely checked. Mono-glutamyl-PU was found in *Beta vulgaris* leaves after incubation of *N,N'*-dimethylcasein in the assay mixture [16]. More recently, in addition to mono-glutamyl-PU, bis-glutamyl-PU and -SD were also detected in *H. tuberosus* chloroplasts [17].

Polyamine conjugates formed in vivo

In addition to those reported in Tables 1 and 2 and obtained by cell free incubation, conjugates have been found also *in vivo*. When [^{14}C]PU was added to the incubation medium, it was metabolized by *H. tuberosus* cycling cells not only to SD and SM but also to other unknown derivatives [33]. The SDS-PAGE of the labelled proteins revealed that during G1 phase, radioactivity was present in two bands having an apparent M_r of 17 500 and 19 000, subsequently (from S phase onwards) the label appeared in bands having a M_r higher than 66 000. However, a large amount of radioactivity always remained in the wells, suggesting that mainly very high M_r or insoluble proteins had incorporated the label. Treatment of the TCA pellet with SDS ruled out the possibility that non-covalently linked PA were present therein [21, 33, 34].

Other authors also reported *in vivo* labelling of a M_r , 18 000 protein in several plant systems by supplying SD in the growth medium [35, 36]. In the early phases of cycling mammalian cells, only a single protein of 18 000, identified as the initiation factor for protein synthesis eIF-5A [37, 38], is modified by hypusine, an unusual amino acid derived from SD. The formation of this amino acid is not TGase-catalysed.

In protoplasts of *Petunia hybrida* Hort. ex Vilm. and *Avena sativa* L., after feeding [^3H] SD, label was incorporated into proteins having a M_r exceeding 45 000 [39]. This could be the product of an *in vivo* catalysis of TGase. Several plant proteins, as for example pea legumin are substrates of guinea-pig liver TGase [40].

Biological and functional aspects of plant transglutaminases

TGase in non-photosynthetically competent cells. Data from the literature about TGase activity in these plant cells is scanty [5, 22, 21, 41–43]. Two different mechanisms which bind PA to proteins occur in *H. tuberosus* cycling cells: one that is destroyed by boiling (Ts TGase), the other that is temperature-insensitive (Ti TGase) [21]. The latter binding reaction might be of a nonenzymatic nature and the complexes formed show a very faint radioactivity on SDS-PAGE autoradiography, suggesting that these PA are not covalently bound. On the contrary, the Ts TGase, whose activity is low during

dormancy, possesses enzymatic characteristics similar to those observed with animal TGase. After mid-G1, the T_s TGase activity increases considerably due to the synthesis of both the enzyme and/or its substrates, as demonstrated by supplying cycloheximide for different time periods and by immunoblot analysis of the enzyme [20, 43]. In S phase and in division phase, TGase activity reached the highest levels [21, 41]. In C6 glioma cells a similar system was studied: the enzyme appears to be constitutive, but inactive, in non-stimulated cells and synthesized *de novo* during the progression of the cell cycle. It was suggested that the enzyme was previously bound to cell membranes and then released, after growth stimulation, into the cytoplasm where it was subjected to proteolytic digestion. Thereafter a *de novo* synthesis compensated for this deficiency and a second peak in activity resulted [44]. Of course, the metabolic state of dormant tuber cells (out of the cycle) and of non-stimulated glioma cells (arrested during the cycle) are different whereas, after induction of cycling, their metabolisms are probably more comparable. In both *H. tuberosus* and in C6 glioma cells, a new (different ?) enzyme appears to be synthesised and thus the synthesis of these enzymes may be related to particular functions of cycling cells.

The amine partner of the products of TGase activity in *H. tuberosus* cycling cells were, apart from PU itself supplied to the medium, also PU derivatives, especially unknown compounds having a migration close to zero on TLC plates. Labelled bound SD, SM or γ -aminobutyric acid albeit in very low amounts were detected, indicating that PA metabolism occurred during the TGase assay [42].

At the end of the cell cycle of *H. tuberosus*, the electrophoretic pattern of proteins was quite different with respect to that observed in the G0 phase; in particular the percentage of high M_r protein bands increased considerably, whereas other protein bands completely disappeared. All these modifications were completely blocked by cycloheximide [43, 45]. The formation of these large complexes is accompanied by an heavier PU incorporation therein both *in vivo* and in cell-free extracts, suggesting that TGase activity might play a role in the formation of these large complexes. Some of them can be separated on 10% SDS-PAGE or by digesting them with cellulase and pectinase, in addition to protease [42]. In this phase it is known that fibrillar material from the cytoplasm migrates into the walls of *H. tuberosus* cells [46] and in *Catharanthus roseus* L. G. Don., wall protein synthesis has been reported [47]. Thus, it can be inferred that TGase is involved in cell wall organization.

Recently, TGases activity has also been studied in pollen of *Malus domestica* which represent a system of very rapid growth involving both cytoskeleton rearrangement and building of a new cell wall [19]. Actin and tubulin are known to play an important role in the cytoskeletal organization being closely involved in the pollen tube elongation [48]. During germination TGase activity is high when actin elements are organized [49] and markedly decreases at the end of germination. Labelled PA are bound to high and low M_r proteins but

especially to proteins of M_r 43 000 and 50 000–55 000. Incubation with purified actin and tubulin revealed that both are substrates for pollen TGase. In addition, in germinated pollen the supplied actin was modified giving rise to heavier labelled aggregates with respect to the unsupplemented samples. The conjugates formed in germinated and ungerminated pollen after actin supply have a different degree of polymerization.

Thus it can be inferred that in higher plants PA may be involved in the formation of cytoskeletal and of cell wall structures, whose formation is one of the main events of cytokinesis and cell enlargement.

Direct evidence that actin is a substrate for TGase also in fungi has been reported in *P. polycephalum* [7]. It was proposed that the role of TGase may be important for protein cross-linking during the conversion of microplasmodia to spherules, which can survive starvation and dessication. Actin modified *in vitro* by monodansyl-cadaverine via TGase of guinea-pig liver exhibits a lower critical concentration to polymerize and a faster rate of polymerization compared to those of the unmodified actin without affecting the binding to myosin heads [50]. In animals, a functional dependence on PA for the formation of cytoskeletal structures, like actin filaments and microtubules, has been shown in PA-deficient CHO cells even though the M_r was not clarified [51]; in addition, PA depletion caused a high incidence of binucleate fibroblasts [52]. Tubulin was reported to be a substrate of TGase in neuronal cytoskeleton of mammals [53].

The confirmation of the identity of the plant conjugating enzyme as being a TGase is provided by the following data [18]. The substrate for mammalian TGases, Z-L-glut-L-leu, is also a good substrate for TGase from etiolated apices of the sprouts growing from tubers of *H. tuberosus*. The activity is proportional to the concentration of PA supplied. The enzyme was partially purified by ion exchange chromatography and two forms were present, eluted at 0.1 and 0.3 M NaCl. Both fractions recognized the dipeptide forming γ -glutamyl-PA. A main band of M_r 75 000 in the 0.1 fraction and one of 58 000 in the 0.3 fraction cross-reacted both with a polyspecific antibody to prostate TGase and with a monospecific antibody to epidermal particulate TGase K.

These chromatographic characteristics are also shared by TGases from animal sources, as for example in rat chondrosarcoma [54]. The immunological cross-reactivity showed that common sequences are present in animal and plants. The recovery of two enzyme forms in the apices could suggest that they exert different functions. An enzyme form of M_r 58 000 was found also in thylakoids of chloroplasts of green leaves. In etiolated sprout apices, leaflets are present and, potentially, they may have the same form of enzyme as active chloroplasts (see below).

TGase in photosynthetically competent cells. When non-photosynthetic parenchyma of *H. tuberosus* tubers is stimulated *in vitro* to differentiate chloroplasts, TGase can be induced to a greater extent than in a hormone-stimulated control which grows actively but does not turn green [27]. The high M_r substrates observed in

non-photosynthetic cells are present in both explants, but in the green ones low M_r proteins were also modified by PA. The morphological characteristics of the two tissues are quite different: the non-green growing control consists of friable tissue whose cells have thin walls and empty intercellular spaces and resembles a tumour tissue (habituated callus); the green callus has well-developed chloroplasts, a compact consistency and dense material in the intercellular spaces. As a working hypothesis, it was inferred that a relationship exists between these morphological features and TGase activity. In animal systems (data in plants are too scanty), TGase activity is generally high in differentiated tissues, where it is involved in the organisation of protein structures responsible for the cohesion between cells and low in rapidly growing tumours in which the organisation of intra- and extra-cellular structural proteins is rather poor [29, 30, 55].

On the other hand, the presence of TGase in chloroplasts has been shown. Cohen *et al.* [25] observed that, in isolated chloroplasts of *B. pekinensis*, SD was 'fixed' with a higher efficiency in the light than in the dark. However, they concluded that the stimulation by EDTA and the inhibition by Ca^{2+} did not support the hypothesis of a typical TGase-catalysed fixation. This stimulation by light was also reported by other authors (see Table 1). On the contrary, in another system, namely *A. sativa* protoplasts, light markedly inhibited the uptake of label and consequently PA binding to TCA-precipitable material [39].

In isolated thylakoids and in entire chloroplasts from *H. tuberosus* leaves, label in TCA-precipitable material after the TGase assay was higher when they were incubated in the light rather than in the dark [6]. Autoradiography of a 2D gel of [^{14}C]SD- and [^{14}C]PU-labelled chloroplasts and thylakoids which separates, in the first dimension, chlorophyll-protein complexes and, in the second dimension, the relative apoproteins (first dimension, Deriphat-PAGE; second dimension, SDS-urea-PAGE) showed that a few and the same proteins (M_r 20 000–30 000) were labelled. The recognition of these proteins as the chlorophyll-*a/b* antenna complexes LHCII, CP24, CP26 and CP29 was ascertained by cross-reactivity with antibodies.

The fact that the oligomeric form of the major light-harvesting complex (LHCII) is a better substrate since it is more heavily labelled by PA than the monomeric form, suggests a role for PA in stabilizing oligomers. A detectable enhancement of the M_r or these proteins was not observed after a 30 min assay; thus, either the aggregation of these molecules does not take place or, possibly, polyamines are linked by only one terminal amino group or, finally, only intramolecular cross-bridges are formed. Concerning the role of PA in the post-translational modification of LHCII, it has been proposed that PA not only form cross-links but also add positive charges which may have the opposite effect to that of phosphorylation and thus could favour the transfer of excitation energy between the photosystems [56, 57]. Recently it was shown that another phosphorylation mechanism is re-

sponsible for the protection of the PSII reaction centre from photoinhibition by inducing a conformational change in an inner antenna complex called CP29 [58]. On the other hand, it has been observed that D_1 , D_2 , cytochrome *f* and the large Rubisco subunit are all stabilized by PA, by a not yet clarified mechanism [59].

In entire chloroplasts of *H. tuberosus*, an additional faint radioactive spot having a M_r of 55 000 was recognised by antibodies as the large subunit of Rubisco. The large Rubisco subunit was indicated as a substrate also in floral buds [23].

It has been proposed that the polymerization of Rubisco to a dimer is the initial step in the assembly of the catalytically active L8S8 Rubisco structure [7, 60].

TGase activity was detected in the green alga *D. salina* both in salt-stressed cells and in the controls. At 24 h after inoculation TGase activity was four-fold higher in the former and most of the radioactivity was localized in a high M_r region, in a protein of 55 000 and in the chlorophyll migrating zone [28]. The activity, also detected by immunocytochemical analysis, was mainly localized in the chloroplast, whereas the M_r of the enzyme evaluated by western blot analysis was *ca* 70 000. Proteolytic digestion of the products of TGase obtained by incubating chloroplasts of *H. tuberosus* with both SD and PU, leads to the recovery in both cases of *N*-(γ -glutamyl) PU, *N*¹,*N*⁴-bis-(γ -glutamyl) PU, *N*¹,*N*⁸-bis-(γ -glutamyl) SD. The presence of bis-derivatives represent a direct indication of the formation of cross-links via TGase. The most abundant derivatives are mono-PU and bis-SD in a ratio generally around 4:1, suggesting that the two PA may play a different role in the post-translational modification of proteins [17].

In addition to glutamyl-PA, free PA and their derivatives, *N*¹-(γ -acetyl) SD and *N*⁸-(γ -acetyl) SD, were detected in chloroplasts, incubated either with PU or with SD [17]. Whereas the existence of a metabolic pathway that converts SD into PU and SM into SD via an acetylated intermediate was previously described for invertebrates and yeasts [61, 62], the existence of this pathway in *H. tuberosus* indicates that a polyamine oxidase (PAO) similar to that of rat liver described by Hölltä [63] and by Bolkenius and Seiler [62] could also exist in plants. It has been reported that selective oxidation by FAD-dependent PAO may be one of the cellular mechanisms that regulates PA-protein cross-link levels [64]. This oxidative metabolism has as substrate *N*¹-(γ -glutamyl) SD and not *N*⁸-(γ -glutamyl) SD, leading to the formation of *N*-(γ -glutamyl)-3-amino-propionaldehyde and free PU (Fig. 4). The formation of bis-SD can thus be modulated. This oxidation, by releasing part of the PA molecule, determines the release of positive groups and thus a change in the total charge of the protein molecule. This is not a complete reversion of the protein to the initial state, which is achieved only by the activity of γ -glutamylamino cyclotransferase, which is able to detach the entire PA from the glutamyl residue. No information is available on the existence in plants of this enzyme, which is also little studied in mammals.

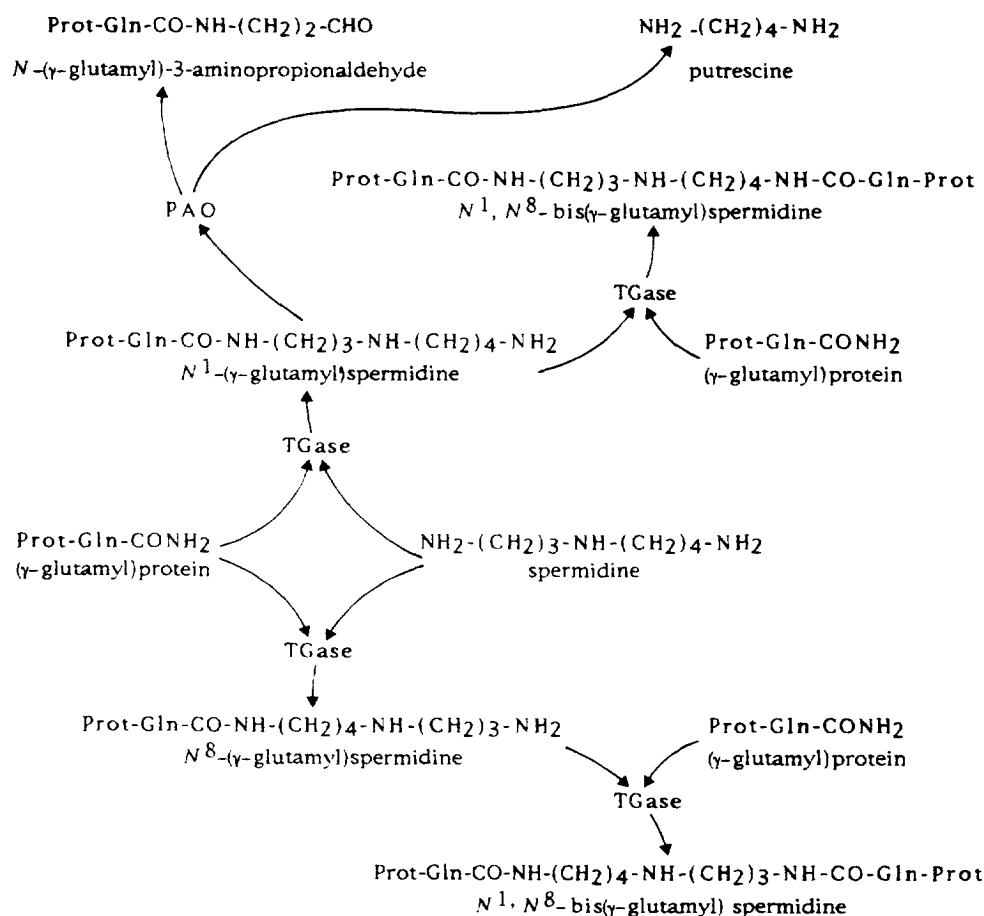


Fig. 4. Metabolic scheme of the potential modulation of transglutaminase action by FAD-dependent polyamine oxidase (PAO). The selective oxidation of N^1 -(γ -glutamyl) SD takes this substrate from the pool of mono derivatives. Therefore, N^8 -(γ -glutamyl) SD can preferentially form the sterically defined bis-derivative N^1, N^8 -(γ -glutamyl)SD.

CONCLUDING REMARKS

Information on TGase is relatively incomplete, but we can try to highlight its main aspects. The products of this enzymatic catalysis are different when PA or lysine residues are the amine donors. Thus two possible properties of the final product of the reaction should be considered; the first one is directly a structural consequence of the length of the molecule acting as amine-donor. In the light of this quality, the network produced by the extensive protein cross-linking would acquire various features of reactivity and plasticity depending on the substrate incorporated.

Highly polymerized structures are known to occur in collagen, elastin, fibrin and in the layers of epidermins. Thus the cellular or extracellular structural organization can be affected. The second property conferred by the modification by PA is the potential modulation of the cross-linked protein structure. In fact, the formation of mono-derivatives is regulated by the PA concentration since high levels of PA may saturate the acyl donor residues of substrate proteins, preventing the formation

of bis-derivatives and of glutamyl-lysine linkages. In this sense, the levels of PA have a critical role in the modulation of protein cross-links. The significance of this mechanism may be explained in the light of the suitable physical state requested for cell function. Moreover, the addition of positive charges due to the linkage with these polycations, could further affect the conformation and function of protein substrates.

Nevertheless, TGases seem to be a widespread class of enzymes with many characteristics in common among plants, animals and also bacteria [65], supporting the hypothesis of its involvement in important biological functions. A role of TGase shared by animals and plants seems to be related to the organization of structural proteins: for example in the differentiation of *P. polycephalum* microspherules, in pollen tube elongation and perhaps during cell division of *H. tuberosus* as well as differentiation of keratinocytes, modulation of metastatic potential of cancer cells, cataract formation, formation of apoptotic body and extracellular matrix [66–70]. A similar behaviour to TGase activity has also been reported in rapidly growing cells of plants and animals, like hor-

mone-stimulated callus or tumours. Besides these roles, others seem to be related to more specific functions, like photosynthesis in plants and receptor-mediated endocytosis in animals [71].

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