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Identification of the Large Subunit of Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase as a Substrate for Transglutaminase in *Medicago sativa* L. (Alfalfa)[1]

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ABSTRACT

Extracts prepared from floral meristematic tissue of alfalfa (*Medicago sativa* L.) were investigated for expression of the enzyme transglutaminase in order to identify the major protein substrate for transglutaminase-directed modifications among plant proteins. The large polymorphic subunits of ribulose 1,5-bisphosphate carboxylase/oxygenase in alfalfa, with molecular weights of 52,700 and 57,600, are major substrates for transglutaminase in these extracts. This was established by: (a) covalent conjugation of monodansylcadaverine to the large subunit followed by fluorescent detection in SDS-polyacrylamide gels; (b) covalent conjugation of [^{14}C]putrescine to the large subunit with detection by autoradiography; (c) covalent conjugation of monodansylcadaverine to the large subunit and demonstration of immunocross-reactivity on nitrocellulose transblot of the modified large subunit with antibody prepared in rabbits against dansylated-ovalbumin; (d) demonstration of a direct dependence of the rate of transglutaminase-mediated, [^{14}C]putrescine incorporation upon the concentration of ribulose, 1,5-bisphosphate carboxylase/oxygenase from alfalfa or spinach; and (e) presumptive evidence from size exclusion chromatography that transglutaminase may cofractionate with native molecules of ribulose 1,5-bisphosphate carboxylase/oxygenase in crude extracts. Analysis of the primary structure of plant large subunit has revealed numerous potential glutaminyl and lysyl sites for transglutaminase-directed modifications of ribulose 1,5-bisphosphate carboxylase/oxygenase.

Transglutaminases (TGase[2]; R-glutaminyl-peptide: amine γ-glutamyltransferase, EC 2.3.2.13) catalyze a number of conjugation and cross-linking reactions at glutaminyl amino acid side chains in select proteins (5). This element of specificity provides these enzymes the potential of mediating post-translational modifications in specific protein molecules. TGases are capable of catalyzing several different types of reactions. First, TGases catalyze cross-linking of select cytosolic and plasma membrane-associated proteins through ε-(γ-glutamyl-lysine) isopeptide bonds. The cross-link is formed through transamidation between a specific peptide-bound *endo* glutaminyl carboxamide carbonyl and the primary ε-lysyl amine group of a proximate amino acid side chain. Second, TGases catalyze an amine-exchange reaction between a variety of low mol wt, primary amine donor molecules and peptide-bound *endo* glutaminyl residues in selected proteins or peptides. Finally, enzymes of this group also catalyze hydrolysis and possibly transesterification reactions at specific *endo*-γ-glutaminyl sites on specific proteins. TGases have been investigated extensively in animals where they are widely distributed in various tissues, organs, and extracellular fluids (5). A number of biological functions involving protein cross-linking have been proposed for extracellular transglutaminases in animal systems including formation of the fibrin clot as a terminal step in the bloodclotting cascade (16), coagulation of seminal plasma in rodents (29), ionophore-induced hardening of the erythrocyte membrane (14), keratinization of the epidermis and hair structures (24), and participation in receptor-mediated endocytosis (4). In only a few cases have specific physiological protein substrates for TGases been identified and the function of the intracellular form of TGase is unknown.

Recently, presumptive evidence was reported for the occurrence of intracellular TGase in photosynthetic (19, 20), emergent sprout (25), and etiolated plant tissues (7). Earlier reports of the apparent covalent incorporation of polyamines, such as putrescine (26) and spermidine, into plant protein structures suggested the possible existence of this catalyst in plants (26). Polyamines can serve as primary amine donors in cross-linking and conjugating reactions catalyzed by TGase (5). Although the function of this enzyme in plants has not been established, the apparent induction of its enzymatic activity at wounded sites of excised or bruised leaves suggests a potential role in wound repair (19). Alternatively, the discovery of polyamines linked to cell wall polysaccharides or membranous fractions indicate prospects for a function in formation and anchoring of the plant cell wall polysaccharide net

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[2] Abbreviations: TGase, transglutaminase; Rubisco, ribulose, 1,5-bisphosphate carboxylase/oxygenase; L, large subunit of Rubisco; S, small subunit of Rubisco; TCEMTr, buffer containing 50 mM Tris-Cl (pH 8.5), 5 mM $CaCl_2$, 2 mM disodium EDTA, 4.5 mM 2-mercaptoethanol, 0.1% (v/v) Triton X-100; monodansylcadaverine, *N*-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide; TEMTr, buffer containing same components as TCEMTr except 5 mM $CaCl_2$ was omitted; TBMMTr, buffer containing 20 mM Tris-SO_4 (pH 8.1), 100 mM sodium bicarbonate, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 0.1% (v/v) Triton X-100; PBST, buffer containing 10 mM sodium phosphate (pH 7.5), 0.15 M NaCl, 0.05% (v/v) Tween 20.

to the plasma membrane, or in coupling membrane proteins to cytoplasmic structural proteins (26).

As part of an investigation to establish functional roles for TGase in plants, the present paper reports results on identification of a major *in vivo* substrate protein for TGase in the soluble fraction of alfalfa (*Medicago sativa* L.) meristematic floral tissue. The substrate protein which has been identified is Rubisco, the catalyst for primary carbon dioxide fixation via the reductive pentose phosphate cycle. Moreover, evidence is presented which shows that only the L, and not the S, serves as a substrate for TGase.

A preliminary report of this work has been presented (19).

MATERIALS AND METHODS

Plant Material

Field-grown alfalfa (*Medicago sativa* L.) plants, 30 to 90 cm in height, were grown in irrigated plots near New Mexico State University. Plants grown in greenhouses were approximately 30 cm in height. Several hundred grams of green, unopened flower buds were harvested and stored frozen at −80°C. These stored meristematic tissues retained full TGase enzymatic activity for at least 3 years.

Preparation of Alfalfa Extracts and Isolated Chloroplasts

Meristem floral tissue was pulverized to a fine powder with a Sorvall Omni-Mixer at dry-ice temperature. The powdered tissue was suspended in 2.5 volumes of ice-cold TCEMTr buffer. For some experiments, other buffers were used as indicated. The suspension was homogenized at full power in a Sorvall Omni-Mixer for 1 min at ice temperature. The homogenate was filtered through cheesecloth and the filtrate was centrifuged at 100,000*g* for 1 h at 3°C. The resulting supernatant solution served as the crude extract.

Intact chloroplasts were isolated from meristem tissue by the method of Saltz and Beckman (27).

Transglutaminase Assay

TGase catalysis was assayed by determination of the incorporation of [^{14}C]putrescine into TCA-insoluble protein adapted to the filter-paper assay method of Lorand *et al.* (15). The final reaction mixture in 105 μL final volume contained: 30 μL of Rubisco purified from spinach by the method of McCurry *et al.* (21), at 36 mg/mL in 25 mM Bicine (pH 7.9) with NaOH, 10 mM 2-mercaptoethanol, 1 mM disodium EDTA; 10 μL of 10 mM dithiothreitol; 10 μL of 0.12 mM putrescine, free base; and, 5 μL of [^{14}C]putrescine (about 90 mCi/mmol, 1.1×10^{-3} mmol/mL). The reaction was initiated by the addition of 50 μL of enzyme preparation. The reaction was conducted at 30°C for 30 min and quenched by pipeting a 75-μL portion onto a Whatman 3 MM paper disk of 23 mm diameter. The disk was immediately washed in 10% TCA for 15 min, 5% TCA for 15 min, and acetone for 1 min (15), dried, and counted in 3.5 mL of scintillation cocktail. Enzyme activities were usually determined in triplicate, normalized to protein content (1), and presented as the average of these determinations. Assays were always checked for linearity with time and the amount of added protein preparation.

Where indicated, *N,N'*-dimethylated casein (Calbiochem) stock solution at 11 mg/mL, or purified Rubisco from alfalfa at 14 mg/mL, was utilized as the protein substrate in place of spinach Rubisco.

Labeling and Detection of TGase Substrate Protein with [^{14}C]Putrescine

A TGase enzyme assay mixture was prepared containing 56 μM of [^{14}C] putrescine (0.5 μCi, 2.51×10^8 dpm/μmol), other components as described in the previous section, and 250 μg of crude extract protein. After reaction for 35 min at 30°C, the reaction was stopped by chilling on ice, dialyzed against 1000 volumes of TCEMTr buffer, then diluted with 210 μL of a quench buffer containing 62.5 mM Tris-Cl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 0.58 M 2-mercaptoethanol, 0.0013% (w/v) bromophenol blue dye, followed by heating at boiling water temperature for 3 to 4 min. A 50-μL sample (40 μg of protein) of this denatured protein solution was applied to a 16 × 18 cm, 3 mm thick, 15% (w/v) SDS-polyacrylamide slab gel, and was subjected to electrophoresis according to Laemmli (10). Potential labeling of the L and S subunits of Rubisco was analyzed by two methods. First, regions corresponding to the L and S subunits of Rubisco were cut from the slab gel and digested in 30% H_2O_2. The solubilized gel slices were mixed with 20 mL of aqueous scintillation cocktail and counted for [^{14}C]putrescine content. Second, the reaction mixture was dialyzed as indicated in the text, then was subjected to electrophoresis in an SDS-gel as described above. The separated protein bands were fixed by silver staining (22), treated with enhancer comprised of 0.4% (v/v) of 2,5-diphenyloxazole (Aldrich Chemical Co., Milwaukee, WI), 30% (v/v) xylene, 15% (v/v) absolute ethanol, 55% (v/v) glacial acetic acid, dried, then autoradiographed on X-Omat/AR diagnostic film (Kodak).

Dansylation and Immunodetection of TGase Substrate Protein

A sample of crude extract proteins from alfalfa meristem floral tissue was prepared in TEMTr buffer and transferred to 0.21-mL reaction mixture containing 18 mM Tris-Cl (pH 8.5), 1.2 mM dithiothreitol, 5 mM monodanyslcadaverine, 250 μg of protein. After incubation for 90 min at 30°C, the reaction was stopped by addition of two volumes of quench buffer described in the previous section. A 50-μL sample of this reaction mixture was applied and subjected to electrophoresis on a vertical 15% SDS-polyacrylamide slab gel, 3 mm thick, at 20 V for 15 h. The gel was transblotted to nitrocellulose (0.2 μm pore size; Bio-Rad Laboratories, Richmond, CA) essentially by the method of Towbin *et al.* (28). Transfers were conducted using 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, and 0.1% (w/v) SDS, and were subjected to electrophoresis for 20 h at about 20 V and 20°C. Dansylated proteins transferred to the nitrocellulose sheet were detected immunologically using a polyclonal antibody prepared in rabbits against dansylated chicken ovalbumin. Dansylated ovalbumin and antidansyl rabbit antibodies against dansylated chick ovalbumin were prepared by the method of Lorand *et al.* (17). ELISA were conducted on the transblotted

nitrocellulose sheet essentially as described by Lorand *et al.* (17) with two exceptions. First, the primary antibody, antidansyl rabbit antibody, was diluted 1:5000 with 10 mM sodium phosphate (pH 7.5), 0.15 M sodium chloride, 0.05% (w/v) Tween 20 (Sigma Chemical Co., St. Louis, MO) (PBST). Second, the secondary antibody, peroxidase-linked goat anti-rabbit antibody (Tago Inc., Burlingame, CA), was diluted 1:2000 with PBST. The peroxidase substrate solution was prepared by mixing 10 μL of 30% H_2O_2 and 12.5 mg of 3,3'-diaminobenzidine (Sigma) in 100 mL of 44 mM Tris-Cl (pH 7.5).

Coelution of TGase and Rubisco during Size Exclusion Chromatography

Alfalfa extracts were prepared as described in an earlier section in TEMTr buffer. To this crude extract was added dropwise with stirring to 10% (w/v) final concentration, PEG, mol wt 8000 (Sigma), from a stock solution at 50% (w/v) concentration. The protein mixture was stirred at room temperature for 30 min, then centrifuged at 27,000*g* for 10 min and 20°C. The supernatant fraction was discarded. The protein pellet, henceforth called the PEG precipitate, contained TGase and Rubisco enzymatic activities. PEG precipitates could be stored frozen indefinitely at −80°C with retention of both catalytic activities.

The frozen PEG precipitate was thawed and resolubilized at ice temperature with a minimum volume of the buffer indicated. A small amount of insoluble material was removed by centrifugation at 100,000*g* for 45 min. A 4-mL sample of the supernatant solution containing about 100 mg of protein was applied to a 2.5 × 98 cm column of Sepharose CL-6B (Pharmacia, Piscataway, NJ). The column was equilibrated in the same buffer as was used to resolubilize the respective PEG pellet. The column effluent was collected in a graduated cylinder until the void volume of the column, 169 mL, had eluted. Then, fractions of designated volume were collected. The absorbancies of fractions were measured at 290 rather than 280 nm in order to minimize interference by Triton X-100 absorption. Enzyme assays for TGase and Rubisco were conducted on eluted fractions.

Rubisco Assays

The procedure of Kuehn and McFadden (9) was followed for estimation of Rubisco enzymatic activity at 30°C and pH 8.0.

RESULTS

Fluorescence Detection of Dansylated Endogenous Protein Substrates for TGase

Crude extract proteins from alfalfa meristematic tissue were tested for conjugation to monodansylcadaverine in a TGase-mediated reaction. The reaction mixture was fractionated in an SDS-polyacrylamide gel and immediately viewed by irradiation with UV light. The fluroescent banding pattern in a gel is shown in Figure 1A. Numerous fluroescent bands were visible in the gel, but the dominant protein conjugated to

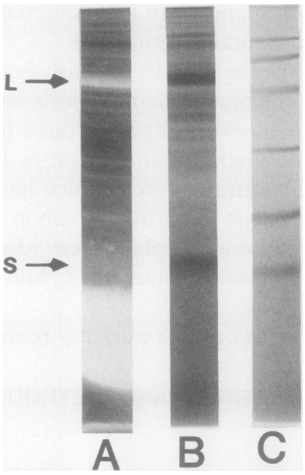

Figure 1. Fluorescent detection of TGase-directed, dansylation of endogenous proteins in a crude extract from alfalfa meristematic floral tissue. Lane A, Fluorescent protein bands viewed by illumination of the same gel shown in lane B, before staining, from the backside with a model TM-36 UV-transilluminator lamp (Chromato-Vue, San Gabriel, CA). Fluorescence was recorded by photography with a technical pan film 2415 (Kodak) through a No. 504 red filter lens. Whitened zones indicate fluorescence by the dansyl moiety. Left margin arrows locate the positions of the L and S subunits. Lane B, Crude extract proteins prepared in TEMTr buffer were incubated for 90 min at 30°C with monodansylcadaverine as described in the text. Samples of the reaction mixture (50 μg) were subjected to electrophoresis in a vertical 12.5% SDS-polyacrylamide slab gel, 3 mm thick, for 15 h at 20 V. Proteins were stained with Coomassie blue dye. Densely stained L and S subunits of Rubisco are dominant bands. Lane C, Standard mol wt marker protein samples (2 μg each) stained with Coomassie blue dye: phosphorylase B (top band), 92,500 M_r; bovine serum albumin, 66,200, M_r; ovalbumin, 45,000 M_r; carbonic anhydrase, 31,000 M_r; soybean trypsin inhibitor, 21,500 M_r; lysozyme (bottom band), 14,400 M_r.

monodansylcadaverine demonstrated a M_r of 57,600. This dominant band stained intensely with Coomassie blue dye (Fig. 1B), indicative of the L subunit of Rubisco. No fluorescent band was evident in the M_r region for the S subunit (Fig. 1A, bottom arrow). The large subunit of alfalfa Rubisco separated as a single band on 12.5% SDS-polyacrylamide gels. The intense fluorescence at the bottom of the electrophoretic gel (Fig. 1A), which apparently represented material conjugated to monodansylcadaverine, could not be stained with Coomassie blue or with silver staining. Thus, this material was not of proteinaceous composition.

Immunodetection of Dansylated Endogenous Protein Substrates

In an experiment similar to that described in the previous section, crude extract proteins were incubated in a TGase reaction mixture containing 5 mM monodansylcadaverine, and the reaction mixture was fractionated by electrophoresis in a 15% SDS-polyacrylamide vertical slab gel. The gel was then transblotted onto a nitrocellulose sheet. The nitrocellulose transblot was developed by a peroxidase-linked immunological procedure using rabbit antidansyl IgG as the pri-

mary antibody (Fig. 2A). Two proteins with M_r values of 52,700 and 57,600 cross-reacted with the antidansyl antibody probe. These M_r values coincided with the M_rs of two polymorphic L subunits for alfalfa Rubisco that were resolved into two polypeptides in 15% SDS-polyacrylamide gels. These proteins from alfalfa meristematic tissues were dominant bands in 15% polyacrylamide gels stained for protein with Commassie blue. No dansylated protein zone was observed in the predicted M_r region for the S subunit of Rubisco. Control transblots in which no dansylcadaverine had been provided in a reaction mixture, showed no staining upon immunoblotting (not shown). Moreover, the protein band patterns in gels containing dansylated samples, observed after staining with Commassie blue, were indistinguishable from the band patterns of protein samples that were not reacted with monodansylcadaverine (not shown).

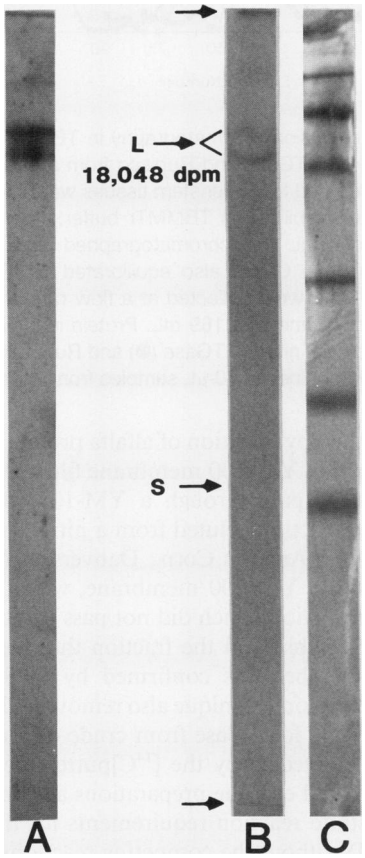

Figure 2. Lane A, Nitrocellulose transblot of crude extract proteins from meristem alfalfa tissue, which were conjugated to monodansylcadaverine by a TGase-directed reaction, followed by SDS-PAGE. Protein staining was achieved after successive treatment of the transblotted, dansylated extract proteins with rabbit antidansyloval-bumin antibodies, goat anti-rabbit IgG linked to peroxidase and 3,3′-diamino-benzidine tetrahydrochloride plus hydrogen peroxide as described in the text. Lane B, Profile of [^{14}C]putrescine-labeled proteins in a crude extract from meristematic alfalfa tissue. A 105-μL mixture containing crude extract proteins (about 250 μg), [^{14}C]putrescine, and other components was incubated as described in the text for the TGase assay. After reaction, the mixture was dialyzed, diluted with quench buffer as detailed in the text, and heated at boiling water temperature for 3 to 4 min. A 40-μg sample was subjected to SDS-PAGE on a 15% gel. After electrophoresis, autoradiography was performed on the gel and the autoradiogram is shown. On the left are displayed the counts of [^{14}C]putrescine incorporated into 3 mm regions of a second gel corresponding to M_r 52,700 to 57,600 and 14,600, respectively. Gel slices were hydrolyzed with 30% hydrogen peroxide prior to counting. Left margin arrows locate positions of L and S subunits as well as regions of radioactivity on the autoradiogram at the top origin and the bottom ion front. Lane C, Standard mol wt marker proteins as described in Figure 1.

Conjugation of [^{14}C]Putrescine to L Subunit of Rubisco

Crude extract proteinase were incubated in a TGase enzyme assay mixture containing [^{14}C]putrescine as detailed in an earlier section. After reaction, the mixture was dialyzed, subjected to electrophoresis in an SDS-polyacrylamide gel, the protein bands were fixed by silver staining, then the gel was dried and autoradiographed on film. The resulting fluorogram pattern exhibited four radiolabeled bands (Fig. 2B). The uppermost band was located at the origin of the polyacrylamide gel. This fraction was apparently of such high M_r that it failed to enter the electrophoretic gel. This is a common observation in TGase reaction mixtures. The high M_r material probably represents a protein(s) that was cross-linked intermolecularly, thus forming a large covalently linked macromolecular complex. The two lower bands on the fluorogram, in the region of 52,700-M_r and 57,600-M_r, were indicative of the polymorphic doublet characteristic of the L subunit of Rubisco in alfalfa. Alternatively, partial proteolysis of the larger 57,600-M_r subunit might also explain the occurrence of the 52,700-M_r peptide. A small amount of radioactivity was also evident at the ionic front near the bottom of the gel. From a second parallel gel which was not fixed or dried, the two regions of the electrophoretic gel corresponding to the 52,700-M_r and 57,600-M_r doublet and 14,600-M_r band, representing the L and S subunits of alfalfa Rubisco (Fig. 2B), respectively, were excised, digested individually in 30% H_2O_2, and counted for ^{14}C content. Figure 2B shows that the gel fraction corresponding to the L subunits of alfalfa Rubisco contained 120 pmol (18,048 dpm) of [^{14}C]putrescine. No detectable radiolabel was found in the gel fraction corresponding to the M_r of the S subunit of Rubisco (Fig. 2B, S arrow).

Cofractionation of TGase and Rubisco

An evaluation of potential methods for purification of TGase from alfalfa meristem tissues indicated that the plant TGase cofractionated with a spectrum of other cellular macromolecular structures. When the proteins that had been precipitated from crude extracts with 10% PEG were resolubilized with TEMTr buffer and fractionated by size-exclusion chromatography in the same buffer, TGase enzymatic activity was distributed over a broad mol wt range (Fig. 3, closed circles). TGase activity was detected in all fractions ranging from the first 290 nm-absorbing material to elute from the column, down to a size of about 60,000 M_r as indicated by a marker protein, bovine serum albumin. The major portion of the enzymatic activity was distributed in the M_r-range of about 700,000 to 60,000 (Fig. 3, fractions 42–70, closed circles). Subfractions indicated by shoulder inflections at frac-

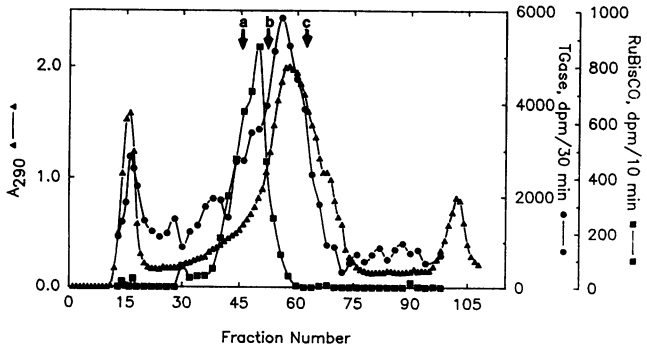

Figure 3. Size exclusion chromatography in TEMTr buffer of enzymatic activities for TGase and Rubisco from alfalfa. Proteins in a crude extract prepared from meristematic tissues were precipitated with 10% PEG and resolubilized in TEMTr buffer. Approximately 100 mg of protein in 4 mL was chromatographed on a 2.5 × 90 cm column of Sepharose CL-6B also equilibrated in TEMTr buffer. Fractions of 3.20-mL were collected at a flow rate of 0.80 mL/min. The column void volume was 169 mL. Protein in alternate fractions was measured at 290 nm (▲) instead of 280 nm in order to minimize interfering absorbance by Triton X-100 in the buffer. TGase (●) and Rubisco (■) enzymatic activities were determined in 50-μL samples from alternate fractions. Marker proteins indicated by the arrows are: a, thyroglobulin, M_r 669,000; b, apoferritin, M_r 443,000; c, bovine serum albumin, M_r 66,000.

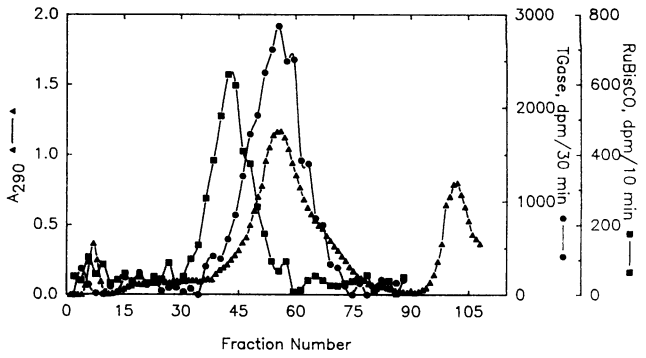

Figure 4. Size exclusion chromatography in TBMMTr buffer of enzymatic activities for TGase and Rubisco from alfalfa. Proteins in a crude extract prepared from meristem tissues were precipitated with 10% PEG and resolubilized in TBMMTr buffer. Approximately 100 mg of protein in 4 mL was chromatographed on a 2.5 × 90 cm column of Sepharose CL-6B also equilibrated in TBMMTr buffer. Fractions of 3.12-mL were collected at a flow rate of 0.78 mL/min. The column void volume was 169 mL. Protein in alternate fractions was measured at 290 nm (▲). TGase (●) and Rubisco (■) enzymatic activities were determined in 50-μL samples from alternate fractions.

tions 44 to 46 (closed circles) and 48 to 50 (closed circles) indicated cofractionation of TGase with a component of approximately 500,000 to 600,000 M_r.

Determination of Rubisco enzymatic activity in the same column fractions revealed two components with Rubisco enzymatic activity. This was indicated by the inflection at fractions 46 to 48 (Fig. 3, squares) and the peak fraction 56 (squares). This profile was observed in low ionic strength TEMTr buffer. Similar overlapping enzymatic activities for TGase and Rubisco were observed when PEG precipitates were resolubilized and fractionated on the same Sepharose CL-6B column in higher ionic strength TEMTr buffer containing 0.2 M NaCl (data not shown).

In contrast, chromatographic separations conducted in a buffer containing magnesium ion and bicarbonate, which optimally stabilized Rubisco enzymatic activity during chromatography, fractionated the TGase activity from the high M_r components (Fig. 4). Resolubilization of 10% PEG precipitates and chromatographic separation on Sepharose CL-6B with TBMMTr (Fig. 4) buffer abolished the high M_r, overlapping fractions of TGase and Rubisco observed under the conditions of Figure 3. Since 0.2 M sodium chloride in TEMTr buffer did not separate the overlapping TGase and Rubisco activities observed in Figure 3, we speculate that the bicarbonate-magnesium ion components in TBMMTr buffer may exert an important role in this separation which is unrelated to ionic strength.

Dependency of TGase Activity on Rubisco

Rubisco preparations, purified from both alfalfa and spinach, were found to serve as substrates for partially purified TGase from alfalfa. Rubisco and TGase enzymatic activities could be separated by filtration of alfalfa protein preparations through an Amicon YM-100 membrane filter. Proteins of M_r less than 100,000 pass through a YM-100 filter. Thus, a portion of TGase activity eluted from a nitrogen gas-pressurized filtration cell (Amicon Corp., Danvers, MA; cell model 3) equipped with a YM-100 membrane, while Rubisco was retained in the fraction which did not pass through the filter. The absence of Rubisco in the fraction that passed through the YM-100 member was confirmed by SDS-PAGE (not shown). This filtration technique also removed all endogenous protein substrate(s) for TGase from crude extracts of alfalfa, which could be detected by the [^{14}C]putrescine-conjugation assay. Thus, filtered enzyme preparations allowed a determination of substrate reaction requirements for the TGase reaction (Table I) without the competing reactions contributed by endogenous protein substrates. An absolute dependence of alfalfa TGase on Rubisco could be demonstrated using partially purified Rubisco from alfalfa or spinach. Calcium ion at 2.4 mM was inhibitory under the conditions of this study. EDTA at 1 mM was slightly stimulatory. 2-Mercaptoethanol at 2 mM was not essential for enzymatic activity. The protein *N,N'*-dimethylcasein served as a substrate for alfalfa TGase, although less efficiently than Rubisco.

Using optimal components for the TGase reaction as determined from Table I, the dependency of TGase on partially purified alfalfa and spinach Rubisco was examined (Fig. 5). Neither the alfalfa nor spinach Rubisco preparations that were used as substrates contained TGase activity. Interestingly, the TGase activity from alfalfa recognized and utilized a spinach Rubisco preparation as a substrate. Clearly, TGase enzymatic activity was dependent on the concentration of the Rubisco preparation used. Using a conservative estimate of 85% purity of the Rubisco preparations used as substrates, as judged from staining intensities of polyacrylamide gels containing samples of these preparations, maximum estimates of K_m values for alfalfa and spinach Rubisco were about 1.13

Table I. *Reaction Requirements for Transglutaminase from Alfalfa*

The complete assay in 105 μL contained: 59 μM [^{14}C]putrescine (1.79 × 10^8 dpm/μmol), 2.4 mM $CaCl_2$, 1.0 mM EDTA, 2.0 mM 2-mercaptoethanol, 0.05% (v/v) Triton X-100, 24 mM Tris-Cl (pH 8.5), 25 μg of TGase preparation from alfalfa, and 10.3 mg/mL purified RuBisCO from spinach or alfalfa. The reaction time was 30 min.

Components	Specific Activity	% of Complete
	pmol[^{14}C]putrescine/hr·mg protein	
Complete (+ alfalfa RuBisCO)	380	100
Complete (+ spinach RuBisCO)	272	72
− RuBisCO	0	0
− Ca^{2+}	534	140
− EDTA	286	75
− 2-Mercaptoethanol	394	104
− RuBisCO, + N,N′-dimethylcasein	167	44

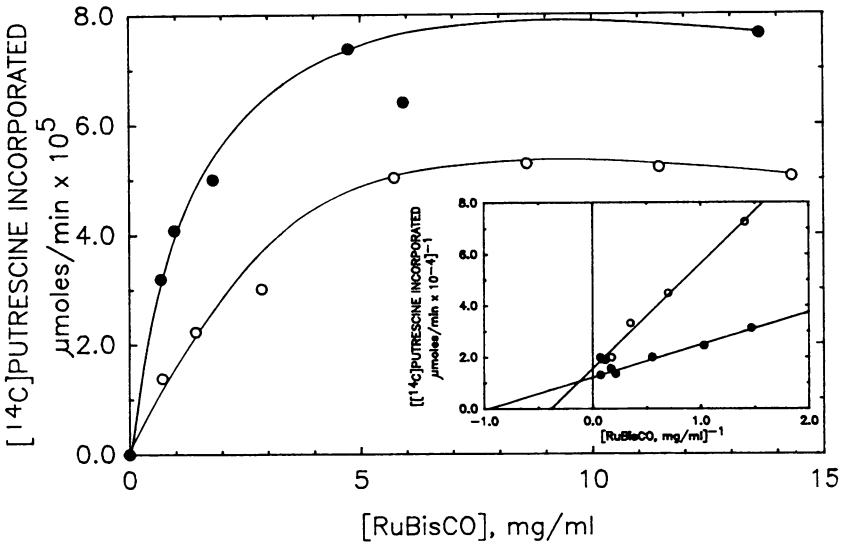

Figure 5. Michaelis-Menten plots for the rate of [^{14}C]putrescine incorporation by TGase as a function of Rubisco concentration. Rubisco substrate was partially purified from alfalfa (●) and from spinach (○). Partially purified, Rubisco-free, TGase was prepared by filtration of alfalfa extracts through a YM-100 membrane filter (Amicon Corp.). Reaction mixtures in 125 μL contained: 49 μM [^{14}C]putrescine (1.81 × 10^7 dpm/μmol), 0.8 mM EDTA, 1.8 mM 2-mercaptoethanol, 0.8 mM dithiothreitol, 0.04% (v/v) Triton X-100, 20 mM Tris-Cl (pH 8.5), 25 μg of TGase preparation from alfalfa and purified spinach Rubisco as indicated. Each data point is plotted as the mean of duplicate assays. Inset, a double-reciprocal plot for the relationship between the rate of [^{14}C]putrescine incorporation and Rubisco concentration.

mg/mL and about 2.68 mg/mL, respectively (Fig. 5, inset graph). Expression of K_m values in concentration terms of molarity was precluded by the heterogeneity of the L subunit M_r.

TGase Activity in Isolated Chloroplast Preparations

Intact, isolated chloroplasts from meristem tissue exhibited TGase enzymatic activity. As much as 30% of the total TGase activity in meristem tissue was associated with the chloroplasts. Data in Table II show that [^{14}C]putrescine was conjugated by chloroplasts into acid-insoluble components in a reaction that was stimulated by light. Analysis of the reaction product(s) by SDS-PAGE, followed by autoradiography, showed that Rubisco L subunit was the only detectable substrate in chloroplasts that conjugated to [^{14}C]putrescine (not shown).

Table II. *Light-Stimulated Incorporation of [^{14}C]Putrescine into Isolated Chloroplasts from Alfalfa*

Intact, isolated chloroplasts from alfalfa, containing 0.57 mg of total soluble protein, were incubated at 30°C for 30 min in reaction mixtures containing the following: 24 mM Tris-Cl (pH 8.5), 0.95 mM dithiothreitol, and 56 μM [^{14}C]putrescine (2.4 × 10^8 dpm/μmol). The volume was 105 μL. After the reaction time was completed, the chloroplast proteins were precipitated with trichloroacetic acid on paper discs and acid-washed as described in "Materials and Methods." The dried discs were counted. Illuminated reactions were conducted under 10,000 lux of white light. Nonilluminated reactions were conducted in tubes wrapped with tin foil.

Treatment	[^{14}C]Putrescine Incorporated
	pmol[^{14}C]putrescine/hr·mg protein
Zero reaction time	78
Illuminated chloroplasts	795
Nonilluminated chloroplasts	411

DISCUSSION

The existence of a TGase catalyst in plants, which may be similar to TGases found in animals, has only recently received scrutiny (7, 19, 20, 25). Definitive confirmation of the identity of the plant catalytic activity requires purification of the enzyme accompanied by structural determination of the ε-(γ-glutamyl)-lysine isopeptide cross-link and various γ-glutamylamine products which are formed through catalysis by the enzyme. Preliminary to these tasks, the reaction requirements for the TGase-like activity in plants must be identified. Here-

tofore, a homologous protein substrate had not been identified in plants for this enzymatic activity (19). Earlier communications defined the reaction requirements for this plant activity using a heterologous protein substrate, *N,N'*-dimethyl casein (7) or unfractionated and undefined, endogenous protein substrates (25). The present communication has identified a natural protein, the L subunit of Rubisco, as a major substrate protein for the enzymatic activity in alfalfa buds which conjugates primary amines to L in a TGase-like reaction. The identification of a natural plant protein substrate of such large abundance as Rubisco should now facilitate characterization of the chemical linkage which accounts for amine conjugation. It will also encourage possible identification of a putative ϵ-(γ-glutamyl)-lysine cross-link, incorporated into Rubisco, which would be indicative of true TGase catalysis.

The reaction requirements observed in this report for the putrescine-conjugating activity in alfalfa, using Rubisco as substrate, are in agreement with those reported previously in other plants (7, 25). Notably, exogenously supplemented calcium ion was seemingly not essential for catalytic activity and was actually inhibitory at millimolar levels. TGases from animal sources have been variously reported to be calcium ion-dependent (5), calcium ion-independent (23), or inhibited by high calcium ion concentrations (8). *N,N'*-Dimethylcasein is a model protein substrate used widely to assay animal TGases where it serves as an exogenous acyl donor. In this study, [^{14}C]putrescine incorporation was *N,N'*-dimethylcasein-dependent in plant protein preparation from which Rubisco had been removed by membrane filtration. No dependence of amine conjugation on *N,N'*-dimethylcasein was observed in plant extracts which simultaneously contained Rubisco and TGase activity. This observation probably explains the failure of Serafini-Fracassini *et al.* (25) to observe conjugation of [^{14}C]putrescine to *N,N'*-dimethylcasein in unfractionated extract preparations of shoot apices of *Helianthus* sprouts. When present, Rubisco appears to be the preferential endogenous acyl donor protein for TGase in plants. Similarly, *N,N'*-dimethylcasein was an efficient substrate for conjugation of [^{14}C]putrescine in extracts prepared from the apical meristematic tissue of etiolated pea seedlings, which lack the high concentration of Rubisco present in green plant tissues (7).

Dansylcadaverine has been used successfully as a convenient fluorescent tracer of TGase-directed, amine conjugation to substrate proteins in many types of biological systems (13). The reagent has proved to be an efficient substrate for all known TGases by virtue of its capacity to function as a lysine-substrate analog. The universal efficacy of dansylcadaverine to serve as a substrate for virtually all TGases investigated to date has led to its acceptance as an identification probe for the occurrence of TGase activity. The fact that plant extracts efficiently incorporated dansylcadaverine into Rubisco L subunit is further indirect evidence that this catalytic activity is attributable to a plant TGase. Detection of protein-bound dansylcadaverine in SDS-polyacrylamide gels by fluorescence of the dansyl moiety suggested dominant conjugation to Rubisco L subunit and lesser incorporations into a spectrum of other proteins. However, immunoblot detection of dansylated proteins on nitrocellulose transfers from SDS gels using a highly sensitive and specific antibody for the dansyl moiety, indicated selective dansylation of Rubisco L subunit. Presumably, many of the putative fluorescent bands observed in the SDS gel of Figure 1A was due to intrinsic fluorescence of the aromatic amino acids in the proteins and did not reflect conjugation with dansylcadaverine. These conclusions were corroborated by the incorporation of [^{14}C]putrescine into protein in extracts from alfalfa buds and fractionation of the extract proteins on SDS-polyacrylamide gels, followed by autoradiography (Fig. 2B). These experiments similarly showed that Rubisco L subunit was the preferred protein conjugated to [^{14}C]putrescine.

Results reported in this communication support the notion that TGase and Rubisco cofractionate in crude extracts and can interact with one another in intact, isolated chloroplasts. The gel filtration experiments on Sepharose CL-6B revealed macromolecular complexes in the range of 700,000-M_r, which simultaneously contained TGase enzymatic activity capable of modifying Rubisco L subunit. The minimum M_r species derived from gel filtration containing TGase enzymatic activity fractionated with a M_r approximating 60,000. TGase and Rubisco enzymatic activity profiles overlapped in separation trials which lacked bicarbonate and magnesium ion in the elution buffers (Fig. 3). In contrast, column fractionations conducted under conditions known to convert Rubisco into an enzymatically active form by carbamylation of a reactive lysine residue at its catalytic center, abolished the higher M_r fractions, which contained both Rubisco and TGase (Fig. 4).

The demonstration of a light-stimulated TGase enzymatic activity in isolated intact chloroplasts that conjugated [^{14}C]putrescine to Rubisco indicated a capacity for these two proteins to interact *in situ* in the organelle where Rubisco is located. Light-stimulation of [^{14}C]putrescine fixation by isolated chloroplasts has previously been observed (3). This enhancement could be due to stimulation of putrescine transport into chloroplasts, enhanced accessibility of Rubisco L subunit to TGase catalytic action, or direct stimulation of the TGase-catalyzed reaction. The data in this report do not allow identification of a mechanism for this effect. Regardless of which mechanism may be operative, the end result is increased conjugation of [^{14}C]putrescine to Rubisco L subunits. Plant chloroplasts presumably contain putrescine since the organelle can synthesize spermidine with methionine as an aminopropyl group donor (3). Chloroplasts also contain spermidine synthase (3), which required putrescine as an aminopropyl group acceptor.

There are currently limited data available on the primary protein sequences surrounding lysine and glutamine residues which govern the specificity of TGases. However, from that information which is available, a hypothesis can be deduced which may explain why Rubisco L subunit serves as an efficient substrate for TGase.

Regarding potentially reactive glutamine sites, Yan and Wold (30) identified the amino acid sequences around seven glutamine acceptor sites in β-casein, which were conjugated to glyco-amine substrates by the action of TGase from guinea pig. An amino acid with an electron-rich side chain was found immediately adjacent to each of the acceptor glutamine residues at five of the seven conjugation sites on β-casein. The

adjacent amino acids were all hydroxy amino acids—namely, serine, threonine, or tyrosine. The remaining two glutamine acceptor sites were located immediately adjacent to proline residues at β-bends in the secondary structure of β-casein. Moreover, participation of the glutamine positions adjacent to hydroxy amino acids in α-helical or β-sheet structures were likely to preclude recognition by TGase (30). Using these criteria for identification of potential conjugation sites in the highly conserved primary structure of the L subunit of plant Rubisco, five glutamine positions in Rubisco from tobacco (2) can be identified which match the type of TGase-specific sites found in β-casein. These are glutamines at positions 4, 30, 45, 209, and 366 (positions predicted from L subunit gene sequence given in ref. 2), or alternatively, at positions 2, 28, 43, 207 and 364 (positions predicted from posttranslationally modified sequence of the L subunit given in ref. 6). Glutamine 209 is located in a highly conserved decapeptide including lysine 201. Lys 201 is carbamylated in the presence of Mg^{2+}/HCO_3^- to form catalytically 'activated' Rubisco (18). Of these five glutaminyl sites, only position 209 is highly conserved in Rubiscos from all higher plants, *Anabaena*, and *Anacytis*.

Regarding potentially reactive lysine sites, there are no direct primary structure studies available which identify amino acid sequences that govern the specificity of TGases. However, TGases exhibit definite characteristic structural specificities for low M_r amine-donor substrates. In general, primary amines which bear aromatic, apolar substituents on an alkyl side chain of a length comparable to that of the lysine residue in proteins are preferred substrates (11). This was first demonstrated by Lorand *et al.* (12) with monodansylcadaverine, which was designed for the specific purpose of the TGase-directed labeling of reactive γ-glutaminyl sites in proteins with a dansyl fluorophore. This substrate preference suggests that amino acid sequences in proteins which are likely to serve as ε-amine donors are those in which a lysine and aromatic amino acid exist near one another. Ten lysine sites at positions 14, 18, 21, 81, 128, 146, 183, 201, 450, and 463 (positions predicted from L subunit gene sequence given in ref. 2), or alternatively, at positions 12, 16, 19, 79, 126, 144, 181, 199, 448 and 461 (positions predicted from post-translationally modified sequence of L subunit given in ref. 6) can be identified in the primary structure of tobacco Rubisco, which are proximate to an aromatic residue in first or second distal positions. Interestingly, lysine 201 is the amino acid which bears the carbamate activator carbon dioxide moiety (18).

In summary, select glutamine and lysine sites exist within the primary structure of plant Rubisco that have excellent potential to serve as cross-linking and conjugating substrates for TGase. The elucidation of these putative sites in TGase-modified products of Rubisco may lead to understanding their potential functions.

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