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Potato Tuber UDP-Glucose:Protein Transglucosylase Catalyzes Its Own Glucosylation[1]

Fernando J. Ardila and Juana S. Tandecarz*

Instituto de Investigaciones Bioquimicas "Fundación Campomar," Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Instituto de Investigaciones Bioquímicas Buenos Aires-Consejo Nacional de Investigaciones Científicas y Técnicas, Patricias Argentinas 435, (1405) Buenos Aires, Argentina

ABSTRACT

Potato (*Solanum tuberosum* L.) tuber UDP-glucose:protein transglucosylase (UPTG) (EC 2.4.1.112) is involved in the first of a two-step mechanism proposed for protein-bound α-glucan synthesis by catalyzing the covalent attachment of a single glucose residue to an acceptor protein. The resulting glucosylated 38-kilodalton polypeptide would then serve as a primer for enzymic glucan chain elongation during the second step. In the present report, we describe the fast protein liquid chromatography purification of UPTG from a membrane pellet of potato tuber. An apparently close association of UPTG, phosphorylase, and starch synthase was observed under native conditions during different purification steps. Enrichment of a 38-kilodalton polypeptide was found throughout enzyme purification. It is now shown that the purified UPTG, with an apparent molecular mass of 38 kilodaltons, undergoes self-glucosylation in a UDP-glucose- and Mn^{2+}-dependent reaction. Therefore, it is concluded that UPTG is the enzyme and at the same time the priming protein required for the biogenesis of protein-bound α-glucan in potato tuber.

The occurrence of an enzymic system in a potato (*Solanum tuberosum* L.) tuber particulate fraction, mainly constituted by proplastids and capable of synthesizing α-1,4 glucosidic chains covalently linked to protein, was reported in 1974 (7). This and subsequent reports (11, 25, 26) suggested that protein-bound glucan synthesis in potato tuber was involved in a two-step mechanism that formed a Ser/Thr-Glc linkage, the first glucosylating event catalyzed by UPTG[2] (11, 12). The resulting glucosylated 38-kD polypeptide served as the primer for the enzymic glucan chain elongation during the second step (12). Solubilization of all the protein components of the potato system was obtained by increasing the ionic strength of the medium (13). Further studies performed on this solubilized system allowed us to suggest that the acceptor and the enzyme would be two different proteins that copurified after each of the purification steps tested or that a single protein with enzymic activity and acceptor capability undergoes self-glucosylation (11).

Although it was not directly implicated in starch biosynthesis by other authors, a carrot glucosyltransferase composed of 40-kD polypeptides was reported to be the enzyme and, at the same time, the glucosyl acceptor (18). On the other hand, glycogenin, a protein with a molecular mass of 37 kD, was found to be a covalent component of rabbit muscle glycogen (2). A glycogen biosynthesis-related protein of similar M_r to glycogenin, which undergoes protein glucosylation when incubated with UDP-Glc and Mn^{2+}, was also described in muscle and other tissues (19). When purified to homogeneity, a muscle protein named self-glucosylating protein proved to be autocatalytic (8). A similar protein that primes the activity of glycogen synthase was found to form a complex with the catalytic subunit of this enzyme (15, 16). A rabbit liver enzyme was partially purified by Smythe *et al.* (22), and like the muscle protein, also catalyzed a Mn^{2+}- and UDP-Glc-dependent self-glucosylation reaction.

In this report we demonstrate the autoglucosylation of UPTG by showing that the 38-kD component is the active enzyme and, at the same time, the glucosyl acceptor required in the first of the proposed two-step mechanism of α-glucan formation in potato tuber. We also show that under native conditions, this activity is strongly associated with starch synthase and phosphorylase.

MATERIALS AND METHODS

Materials

UDP-[^{14}C]Glc (268 Ci/mol) and ADP-[^{14}C]Glc (262 Ci/mol) were obtained using the method described by Thomas *et al.* (27). [^{14}C]Glc-1-P (156 Ci/mol) was from New England Nuclear. Con A-Sepharose 4B and α-D-glucose-1-P (dipotassium salt, grade I) were from Sigma, and acrylamide and bisacrylamide were from Bio-Rad. All other reagents were of the highest quality available.

Enzyme Preparation

Solubilized preparations were made from membranes of potato (*Solanum tuberosum* L.) tuber (about 1 mg protein/mL) obtained as in Lavintman and Cardini (6) by keeping them in 100 mM Tris-HCl buffer, pH 7.4, and 5 mM 2-mercaptoethanol at 4°C for 48 to 72 h, as previously described (11). The mixture was centrifuged for 2 h at 140,000*g* and the

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[2] Abbreviations: UPTG, UDP-Glc:protein transglucosylase; FPLC, fast protein liquid chromatography; CTAB, cetyltrimethylammonium bromide.

supernatant containing the solubilized proteins was used as the enzyme source.

Enzyme Assays

UPTG

Transfer of glucose from UDP-[^{14}C]Glc to endogenous acceptor protein was measured as incorporation of [^{14}C]Glc into 10% TCA (w/v) pellet (7). Unless otherwise specified, the standard incubation mixture contained 10 mM $MnCl_2$, UDP-[^{14}C]Glc (0.2 nmol, 100,000 cpm), 100 mM Tris-HCl buffer, pH 7.4, and enzyme in a final volume of 0.1 to 0.25 mL. The incubations were performed at 30°C for 30 min.

Starch Phosphorylase

This was estimated in the direction of glucan synthesis. The reaction mixtures contained 100 mM Mes-NaOH buffer, pH 5.6, 10 μM [^{14}C]Glc-1-P (55,000 cpm), 3 mg/mL glycogen, and enzyme in a volume of 0.05 to 0.1 mL. After incubation for 60 min at 30°C, 1 mL of 70% methanol-1% KCl was added in order to precipitate glycogen, and [^{14}C]Glc incorporation in the methanol precipitate was measured (21).

Starch Synthase

The reaction mixtures contained 100 mM Gly-NaOH buffer, pH 8, 25 μM ADP-[^{14}C]Glc (95,000 cpm), 3 mg/mL glycogen, and enzyme in a final volume of 0.1 mL. After incubation for 60 min at 30°C, 1 mL of 70% methanol-1% KCl was added and radioactivity in the methanol precipitate was measured (11).

Chromatographic Methods

Mono Q (FPLC)

The solubilized enzyme preparations were chromatographed at room temperature on a Mono Q HR 5/5 anion exchange column (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris-HCl, pH 7.4, 20 mM 2-mercaptoethanol, or 100 mM Tris-HCl, pH 7.4, and 20 mM 2-mercaptoethanol (buffer A). The column was eluted with 15 mL of KCl linear gradient (0 to 0.5 M) in the corresponding buffer at 0.8 mL/min. Fractions of 0.5 mL were collected.

Con A-Sepharose 4B

The enzyme preparation was chromatographed at 4°C on a Con A-Sepharose 4B column (4 mL) equilibrated with 0.1 M NaCl and 1 mM each of $MgCl_2$, $MnCl_2$, and $CaCl_2$ in buffer A (buffer B). Buffer B, containing 0.2 M α-methylmannoside, was used as elution buffer. Fractions of 1 mL were collected.

Superose 6 and 12 (FPLC)

UPTG active fractions were chromatographed at room temperature in Superose 6 HR 10/30 or Superose 12 HR 10/30 gel filtration columns (Pharmacia) using buffer A containing 100 mM KCl at 0.5 mL/min. Gel filtration chromatographies were also performed in these columns using 1% Lubrol PX (Superose 6) or 10 mM CTAB (Superose 12) in the same buffer. Fractions of 0.5 mL were collected.

SDS-PAGE and Fluorography

SDS-PAGE analysis was performed using a Mini-Protean II electrophoresis system (Bio-Rad) on 10% acrylamide gels according to Laemmli *et al.* (4). Standard protein molecules (MW-SDS-200 and MW-SDS-70 L, Sigma) were coelectrophoresed. Protein bands were visualized either by silver staining (14) or by Coomassie brilliant blue R-250. Dried gels containing radiolabeled materials were exposed to Kodak X-OMAT AR-5 films at −70°C after 15 min in 1 M sodium salicylate (1).

Protein Assay

Protein concentration was determined by the method of Lowry *et al.* (9). Absorbance at 280 nm was also used to estimate protein concentration eluted from the column during enzyme purification.

RESULTS

When potato tuber solubilized membranes were submitted to FPLC-Mono Q chromatography, UPTG activity eluted with a wide range of salt concentrations (Fig. 1A). A similar broad pattern of radioactivity was obtained when an UPTG active fraction was rechromatographed in the same system after incubation in the presence of UDP-[^{14}C]Glc and Mn^{2+} (Fig. 1A). A 38-kD [^{14}C]polypeptide was found after fluorography of the SDS-PAGE-analyzed Mono Q radioactive fractions (data not shown). The charge heterogeneity observed was overcome by increasing the ionic strength of the buffer during Mono Q chromatography, as judged by the sharp peak of UPTG activity shown in Figure 1B. A 38-kD radioactive band was also observed upon fluorography of the SDS-PAGE-analyzed Mono Q pooled fractions after incubation in the presence of UDP-[^{14}C]Glc and Mn^{2+} (Fig. 1B, inset). Therefore, if the enzyme and acceptor protein were different proteins they copurify during this purification step. It is worth pointing out that although starch synthase and phosphorylase activities eluted from the Mono Q column above 0.35 M KCl, a considerable amount of these enzyme activities also coeluted with the UPTG-active Mono Q fractions (Fig. 1C).

To get rid of high amounts of a 40-kD polypeptide, presumably patatin (3), which was present in the Mono Q pooled fractions (data not shown), an affinity chromatography step on Con A-Sepharose 4B was performed. SDS-PAGE analysis of unbound and bound column fractions showed the binding of the 40-kD component to the lectin and its elution with α-methylmannoside (Fig. 2). As previously reported (11), UPTG activity eluted with the run-through (Fig. 2). Therefore, in further purification experiments, this fractionation step was always included.

To separate UPTG from accompanying proteins, aliquots of the Mono Q pooled fractions were applied to a Superose 6 column. UPTG activity exhibited considerably large heterogeneity when eluting in the range of 300 to 700 kD. On the

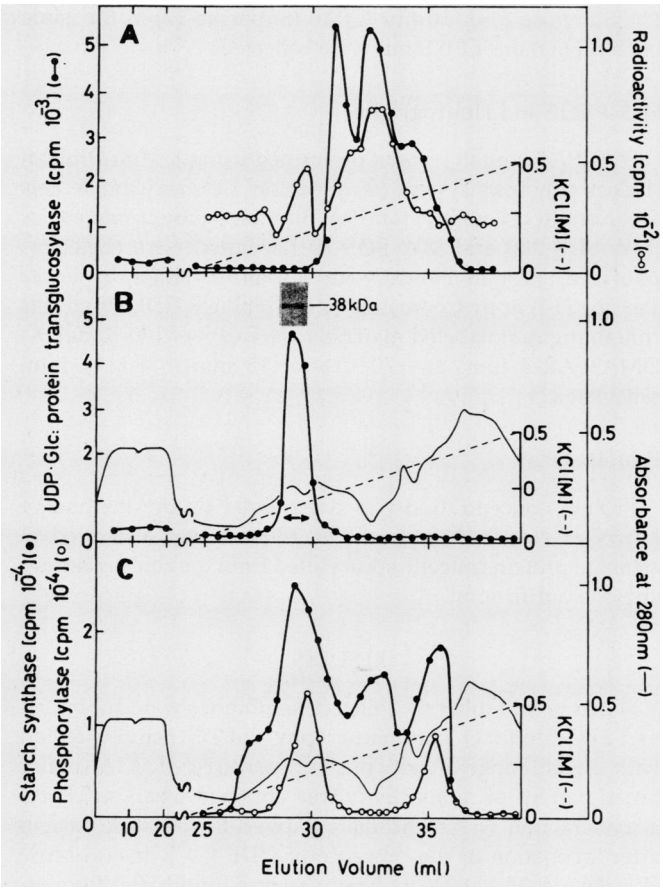

Figure 1. Mono Q chromatography. A, The solubilized preparation (5.5 mg of protein) was chromatographed on a Mono Q column equilibrated with 50 mM Tris-HCl buffer, pH 7.4, and 20 mM 2-mercaptoethanol. UPTG was assayed in 0.1-mL aliquots of each fraction as described under "Materials and Methods." TCA precipitable radioactivity was measured in each fraction after rechromatography in the same column of fraction 63 (0.4 mL) incubated in the presence of UDP-[^{14}C]Glc and Mn^{2+}. B, The solubilized preparation (10 mg of protein) was chromatographed on a Mono Q column equilibrated with buffer A, and UPTG activity was measured in 30-μL aliquots of each fraction. Pooled fractions are indicated by the double-headed arrow. The inset shows the 38-kD reaction product of UPTG on fluorography after SDS-PAGE of a 60-μL aliquot of the pooled fractions incubated in the presence of UDP-[^{14}C]Glc and Mn^{2+}. C, Starch synthase and phosphorylase elution profiles. Experimental conditions are as in B. Starch synthase and phosphorylase activities were measured in 50- and 25-μL aliquots, respectively, of each fraction.

other hand, phosphorylase activity was still present in the same fractions containing UPTG activity (Fig. 3A). From these results, we conclude that the hydrophobic nature of these proteins, which are normally found to be associated with proplastid membranes, could account for this behavior.

Affinity chromatography has been widely used for the purification of various mammalian glycosyltransferases (8, 10). We achieved the attachment of UPTG to reactive red agarose and UDP-GlcUA Sepharose columns, but these systems also failed to separate the enzyme from its acceptor as well as from phosphorylase (data not shown). Therefore, we tried to eliminate the putative protein interactions by using detergents. UPTG activity eluted as a sharp peak after gel filtration chromatography on Superose 6 in the presence of 1% Lubrol PX. However, in these conditions phosphorylase activity still coeluted with UPTG (Fig. 3B).

Next, we analyzed protein-to-protein interactions under stronger conditions. The effect of the cationic detergent CTAB on the enzymic activities was studied after a 1-h preincubation at 0°C with different concentrations of the ionic detergent. Complete inactivation of starch synthase (data not shown) and phosphorylase activities was observed even at the lowest detergent concentration tested, whereas a 70% increase in UPTG activity was reached at 6 mM CTAB (Fig. 4).

An aliquot of the Mono Q pooled fractions was then preincubated with 10 mM CTAB for 60 min and applied onto a Superose 12 column equilibrated with buffer B containing the same detergent concentration (Fig. 5A). UPTG activity eluted from this column at the same position as the 30-kD

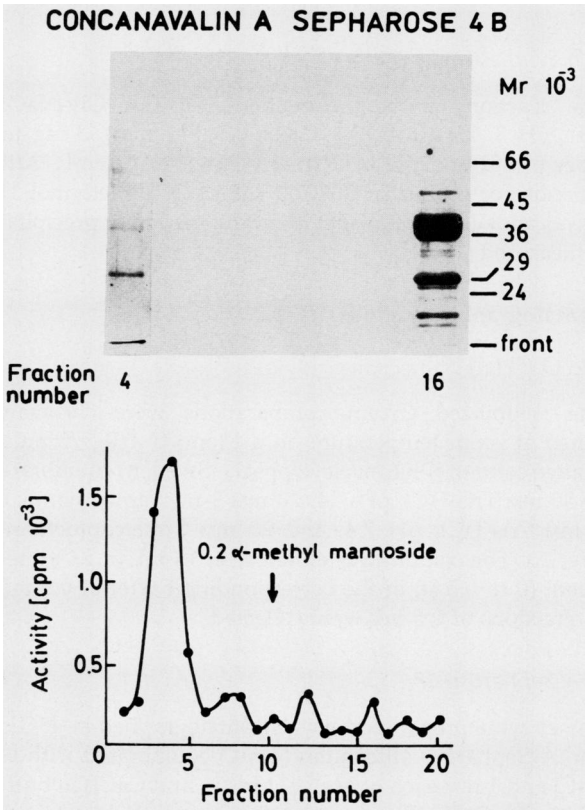

Figure 2. Affinity chromatography in Con A-Sepharose 4B. An aliquot (1 mg of protein) of Mono Q pooled fractions (Fig. 1B) in buffer B was applied to a Con A-Sepharose 4B column, which had been equilibrated with the same buffer. The column was first washed with buffer B and then eluted with 0.2 M α-methyl mannoside in the same buffer as indicated by the arrow. Fractions of 1 mL were collected, and UPTG activity was measured by using 0.1-mL aliquots of each fraction. The inset shows silver-stained SDS-PAGE gels of unbound fraction 4 (0.15 mL) and bound fraction 16 (0.9 mL). Note heavy bands of Con A at the bottom of gels.

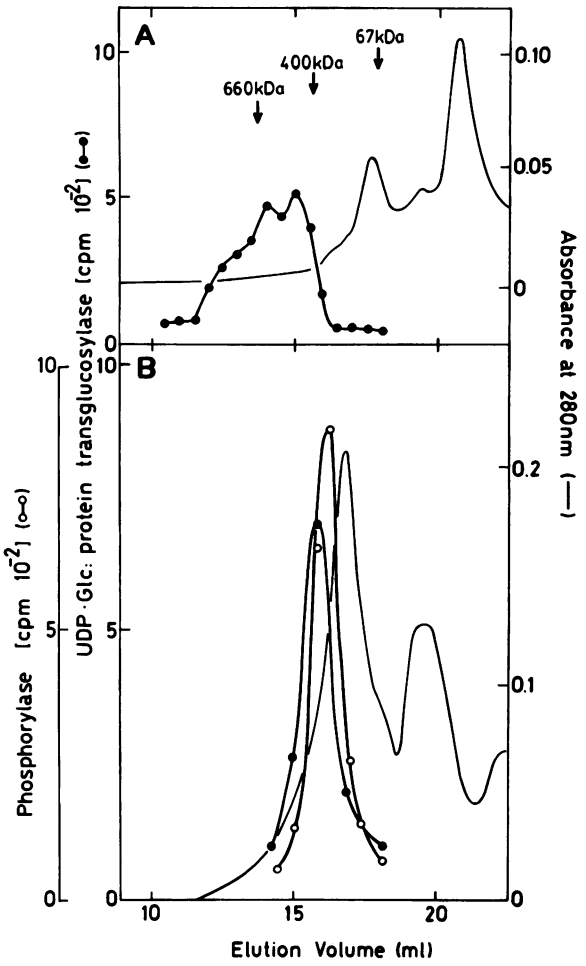

Figure 3. Superose 6 chromatography. Aliquots (0.3–0.5 mg of protein) of Mono Q pooled fractions (Fig. 1B) were submitted to Superose 6 chromatography in the absence (A) or in the presence (B) of 1% Lubrol PX. UPTG and phosphorylase activities were assayed in 50-μL aliquots of each fraction as described in "Materials and Methods." The elution volumes of marker proteins thyroglobulin (660 kD), ferritin (400 kD), and BSA (67 kD) are indicated by arrows in A.

mol wt marker carbonic anhydrase, indicating that a significant decrease in size took place under these conditions. SDS-PAGE analysis of the active fractions revealed the enrichment of a 38-kD band (Fig. 5B, lanes 14–16). This result suggested that the 38-kD polypeptide behaves as a monomer in the presence of CTAB. If CTAB succeeds in abolishing protein-to-protein interactions, then the inactivated phosphorylase will be expected to elute from the column as a 190-kD species (24). In fact, the presence of the 96-kD subunit of phosphorylase was verified on silver-stained SDS-PAGE gels of the corresponding column fractions (Fig. 5B, lanes 8 and 9). Moreover, antibody-to-potato tuber phosphorylase recognized a 96-kD polypeptide in a western blot analysis of the Mono Q pooled fractions (data not shown). It can be concluded that CTAB preincubation produced not only the inactivation of phosphorylase, but the physical separation of this enzyme protein from UPTG as well. However, a significant reduction in UPTG activity occurred during Superose 12 chromatography at room temperature in the presence of CTAB. The purified enzyme was extremely unstable and lost most of its activity within 24 h at 0°C.

Considering the results presented here, a sequential scheme of purification of UPTG was designed that started with the solubilized membrane preparation followed by chromatographies on Con A-Sepharose 4B, Mono Q, and Superose 12 columns, the last one in the presence of CTAB. SDS-PAGE of UPTG active fractions allowed us to establish the polypeptide composition of each purification step. As shown in Figure 6A, lane 5, Superose 12 was effective in removing accompanying bands still present after Mono Q chromatography, because only the 38-kD component can be observed. This result indicates this polypeptide as the only component of the UPTG enzyme. As previously reported for the potato particulate preparation (7), the purified enzyme was stimulated about threefold by 5 mM Mn^{2+}. Superose 12 active fraction was submitted to SDS-PAGE after incubation with UDP-[^{14}C]Glc and Mn^{2+}, and the fluorography demonstrated that the same 38-kD polypeptide became radioglucosylated (Fig. 6B).

DISCUSSION

The results presented here show that the most effective step in the separation and purification of UPTG from potato tuber was the use of FPLC-Superose 12 column chromatography in the presence of the cationic detergent CTAB. This

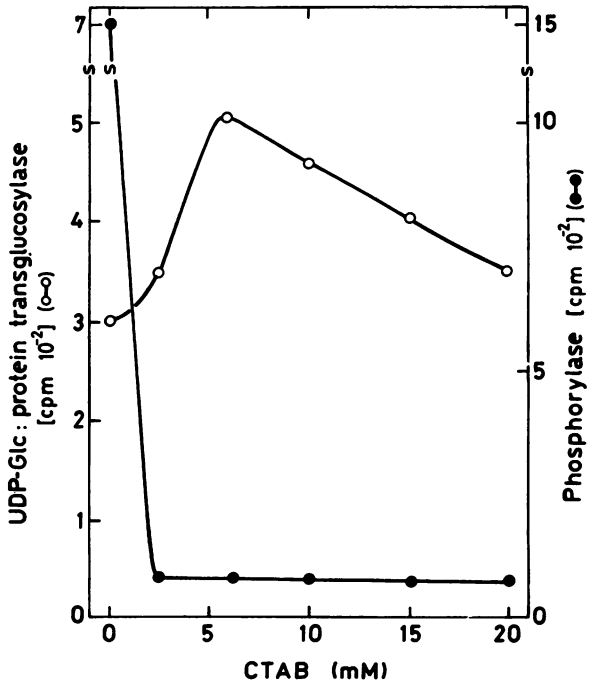

Figure 4. Effect of CTAB on UPTG and phosphorylase activities. Aliquots (6 μg of protein) of Mono Q pooled fractions (Fig. 1B) were incubated at 0°C in the presence of increasing amounts of CTAB. After a 60-min incubation, UPTG and phosphorylase activities were measured as described in "Materials and Methods."

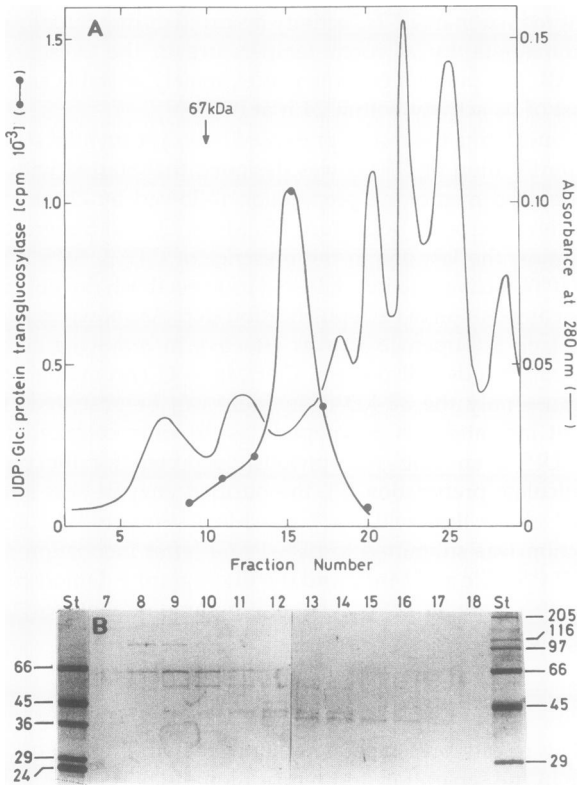

Figure 5. Superose 12 chromatography in the presence of CTAB. An aliquot (0.5 mg of protein) of Mono Q pooled fractions (Fig. 1B) was preincubated for 1 h at 0°C in the presence of 10 mM CTAB and applied to the column. The void volume (V_0) was at fraction 1 (6.75 mL) and the marker proteins BSA and carbonic anhydrase eluted at fractions 12 and 16, respectively. A, UPTG activity was assayed in 0.1-mL aliquots of each fraction as described in "Materials and Methods." B, Silver-stained SDS-gels of column fractions 7 to 18. Mol wt markers are shown at both sides of the gels.

procedure allowed the resolution of active UPTG and inactive phosphorylase (Fig. 5). Similarly, the separation of inactive starch synthase (85–90 kD) (17) from UPTG could be inferred by its elution ahead of the 67 kD marker, far enough from the elution of UPTG (Fig. 5).

The different purification steps used in this study to identify the UPTG protein structure indicate that the 38-kD polypeptide or its polymer undergoes self-glucosylation, thus showing that it is the glucosyl acceptor and the enzyme as well. If the acceptor protein and the enzyme were two different copurifying proteins, then protein separation during purification would lead to the abolition of enzyme activity. On the other hand, if a single protein contained both acceptor capability and enzymic activity, then UPTG activity would persist after each of the purification steps tested. Neither the solubilization of proplastid membranes nor the different chromatographic steps used here (Figs. 1–3), even in the presence of 1% Lubrol (Fig. 3B) or 10 mM CTAB (Fig. 5A), could lead to the abolition of UPTG activity. Moreover, a 38-kD polypeptide was the only polypeptide visible on a silver-stained SDS-PAGE gel of the Superose 12 active fractions (Fig. 6A, lane 5).

Charge and/or size heterogeneities observed during the present purification (Figs. 1A and 3A) may raise a question on the presence of larger forms of UPTG. At present we cannot exclude the possibility that *in vivo*, the purified protein may be component of a larger one. In fact, the formation of large-sized protein aggregates containing UPTG activity were observed not only in potato tuber during the present purification (Fig. 3A), but also in developing maize kernels (20) and in rabbit muscle (8, 15, 16) and liver (22) glycogenin preparations.

Interactions of potato tuber UPTG with other starch-related enzymes (starch synthase and phosphorylase) were observed under native conditions after anion exchange, affinity, and gel filtration chromatographies (Figs. 1–3). Even in the presence of 1% Lubrol PX, phosphorylase activity remained associated with UPTG (Fig. 3B). Superose 12 chromatography showed that preincubation of an active preparation in the presence of CTAB led to the separation of phosphorylase from UPTG (Fig. 5A). Presumably, the detergent weakened hydrophobic bonds involved in the interaction of UPTG with other proteins. Incubation with CTAB, which completely inactivated starch synthase (data not shown) and phosphorylase, stimulated UPTG activity (Fig. 4). To account for this observation, we proposed that the detergent would confer a positive charge on the latter enzyme. This would produce an increase in the local concentration of negatively charged sugar nucleotide and, hence, an increase in UPTG activity (5, 23). On the other hand, Pitcher *et al.* achieved in rabbit muscle preparations the deaggregation of the 86-kD catalytic subunit of glycogen synthase from the 38-kD component in the presence of 2M LiBr (15, 16).

Although CTAB succeeded in purifying UPTG, the study of some of the glucosylating properties of the 38-kD enzyme monomer was hampered by the presence of the cationic detergent. It is likely that the monomer may well be stabilized

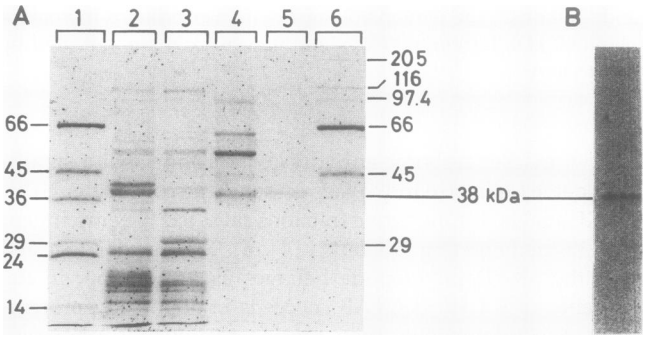

Figure 6. SDS-PAGE of the purification stages. A, Aliquots of UPTG active fractions from various stages of purification were electrophoresed on a 10% gel and stained with the silver reagent. Lane 1, mol wt standards; lane 2, solubilized preparation (8 μg); lane 3, Con A-Sepharose 4B pooled fractions (8 μg); lane 4, Mono Q pooled fractions (5 μg); lane 5, Superose 12 pooled fractions (1 μg); lane 6, mol wt standards. B, Fluorography after SDS-PAGE of the ^{14}C-labeled product obtained upon incubation of a Superose 12 UPTG active fraction (0.5 mL) in the presence of Mn^{2+} and UDP-[^{14}C]Glc. The 38-kD polypeptide (panel A, lane 5) and enzyme reaction product (B) are indicated.

by self-association or by its association with starch synthase and/or phosphorylase. It is also unknown whether the UPTG monomer constitutes the minimal unit required for all functional properties.

In summary, our results show that potato tuber UPTG catalyzes its own glucosylation, giving rise to the formation of the protein primer for α-1,4 glucan synthesis (11, 12). Although the environment of the *in vitro* experiments is quite different from the *in vivo* circumstance, the association of UPTG with starch synthase and phosphorylase would now become meaningful because they take part in a common metabolic pathway (12). Studies to assess the specificity of these enzyme interactions are currently underway.

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