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Evidence for an interaction between cytosolic aldolase and the ATP- and pyrophosphate-dependent phosphofructokinases in carrot storage roots

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Immunoaffinity chromatography was employed to identify potential plant cytosolic aldolase (ALD_c) binding proteins. A clarified homogenate of carrot storage root was chromatographed on a column of protein-A–Sepharose that had been covalently coupled to anti-(carrot root ALD_c) immunoglobulin G. The column was washed with phosphate-buffered saline (PBS), followed by step-wise elution with increasing concentrations of NaCl in PBS. Several proteins were eluted following application of the salt gradient. Western blotting identified the major eluting proteins to be the PP_i-dependent phosphofructokinase (PFP) and the cytosolic form of the ATP-dependent phosphofructokinase (PFK_c), enzymes that are metabolically sequential to ALD_c. The results suggest that ALD_c may specifically interact with PFP and PFK_c in carrots.

Enzyme–enzyme interaction; Glycolysis; Aldolase; PP_i:D-fructose-6-phosphate 1-phosphotransferase; ATP:D-fructose-6-phosphate 1-phosphotransferase

1. INTRODUCTION

An increasing body of evidence indicates that many so-called soluble enzymes which catalyze consecutive reactions of intermediary metabolism may be associated with each other, and/or with various structural elements in intact cells [1–9]. In particular, glycolytic enzymes in muscle cytoplasm are thought to form transient complexes wherein direct transfer (or channelling) of metabolites can occur [1–3,5–8]. In mammalian tissues, ALD has been proposed to function as a 'scaffold' upon which other sequential enzymes (i.e. PFK, FBPase, GAPDH, and GDH) bind in a metabolically dependent manner [5–8]. As well, evidence of a physical interaction between the chloroplastic forms of pea leaf ALD and triose-phosphate isomerase was recently reported [9]. This interaction was postulated to facilitate channelling of triose-phosphates between these two enzymes in the chloroplast [9]. This aspect of carbohydrate metabolism has not been examined with respect to plant cytosolic glycolysis or gluconeogenesis, nor have putative plant ALD_c 'binding proteins' been identified.

The findings of a preliminary study [10] led us to hypothesize that the stimulation of respiration which accompanies the slicing and subsequent aging of carrot storage roots may arise, in part, from an interaction between cytosolic glycolytic enzymes. The present investigation utilizes the techniques of immunoaffinity chromatography and immunoblotting to identify potential carrot ALD_c binding proteins. The results suggest that ALD_c may specifically interact with the PFP and PFK_c in carrots. Interestingly, PFP and PFK_c are both cytosolic enzymes that generate fructose-1,6-bisphosphate, the substrate for ALD_c.

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Abbreviations: ALD_c, cytosolic fructose-1,6-bisphosphate aldolase; PFP, PP_i-dependent phosphofructokinase; PFK_c, cytosolic ATP-dependent phosphofructokinase; PK_c, cytosolic pyruvate kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDH, glycerol-3-phosphate dehydrogenase; FBPase, fructose-1,6-bisphosphatase.

2. MATERIALS AND METHODS

Carrots (*Daucus carota* L.) were purchased at a local market and used the same day. Enzymes were assayed spectrophotometrically at 25°C as described in the following references, except for the variations given: ALD_c [11]; PFP and PFK_c [12]; GAPDH, 50 mM Bis-Tris-Propane-HCl (pH 8.5), 2 mM fructose-1,6-bisphosphate and 1 U rabbit muscle ALD [13]; GDH, 20 mM HEPES-NaOH (pH 7.0), 0.2 mM dihydroxyacetone phosphate, 0.1 mM NADH and 0.1% (v/v) Triton X-100 [14]; FBPase, 50 mM HEPES-NaOH (pH 7.5), 0.1 mM fructose-1,6-bisphosphate (acid treated according to [15]), 0.2 mM EGTA, 0.5 mM NAD^+; and 1 U *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase [16]; PK_c [17]. One unit of enzyme activity is defined as the amount of enzyme resulting in the production of 1 μmol product·min^{-1}. All assays were conducted in duplicate, and optimized with respect to pH and substrate concentration. Activity in all assays was proportional to the amount of extract added, and remained linear with respect to time. Protein concentrations were determined by the method of Bradford [18] using bovine γ-globulin as the protein standard. SDS-PAGE and Western blotting were performed as described previously [11]. Immunological specificities were confirmed by performing Western blots in which rabbit pre-immune serum was substituted for the various IgGs. An LKB Ultroscan XL Enhanced Laser Densitometer was used to scan Western blots. Densitometric data were analyzed and M_r estimates were made using the LKB Gelscan XL software (version 2.1). Immunoreactive polypeptides were quanti-

fied in terms of relative absorbance units at 633 nm. The immunoadsorbant was prepared by covalently coupling purified rabbit anti-(carrot ALD_c) IgG [11] (1 mg; prepared according to [19]) to protein-A–Sepharose (1 ml; Pharmacia) using dimethyl pimelimidate dihydrochloride as described by Harlow and Lane [20].

Carrots were homogenized (4:1 w/v) in 100 mM HEPES-NaOH (pH 7.5), containing 2 mM EDTA, 1 mM $MgCl_2$, 20 mM thiourea, 2 mM phenylmethylsulfonyl fluoride, 6 mM 1,10-phenanthroline, 1% (w/v) insoluble polyvinylpolypyrrolidone and 20% (v/v) glycerol at 4°C. The homogenate was squeezed through 6 layers of cheesecloth and 1 layer of Miracloth and centrifuged at 16,000 × *g* for 10 min. An aliquot (0.5 ml) of the clear supernatant was adjusted to 150 mM NaCl and 0.05% (v/v) Nonidet P-40, and incubated for 10 min at 4°C. The extract was diluted 2-fold with phosphate-buffered saline (PBS; 10 mM sodium phosphate, pH 7.4, 150 mM NaCl), and loaded at 0.1 ml·min^{-1} on to a column (0.7 × 2.5 cm) of underivatized protein-A–Sepharose that had been pre-equilibrated with PBS. The extract was cycled through the column for 30 min (to eliminate any proteins that might be non-specifically adsorbed on to protein-A–Sepharose). Non-bound proteins were eluted in 4 ml and applied at 0.1 ml·min^{-1} on to the anti-ALD_c IgG:protein-A–Sepharose column (0.7 × 1.0 cm) that had been pre-equilibrated with PBS and connected to an FPLC system. The extract was cycled through the immunoadsorbant column for 2 h. The column was washed with PBS until the A_{280} decreased to baseline, and then eluted in a step-wise fashion with increasing concentrations of NaCl (0.25, 0.5, and 1.0 M) in PBS. The column was subsequently eluted with 0.1 M glycine (pH 2.5), and the eluent immediately neutralized with 1 M Tris-HCl (pH 8.5). Fractions having an $A_{280} \geq 0.005$ were pooled, concentrated 4-fold using an Amicon YM-30 ultrafilter, and analyzed for their polypeptide composition by SDS-PAGE and Western blotting.

3. RESULTS AND DISCUSSION

The following maximal enzyme activities (expressed as U·g fresh wt.$^{-1}$) were assayed in the carrot extract: ALD, 0.14; PFP, 0.027; PFK, 0.12; GAPDH, 0.86; PK, 2.8. Activities of GDH and FBPase, however, were undetectable. The absence of GDH in some plant tissues has been noted previously [21].

This study was predicated on the assumption that polypeptides other than ALD_c that are retained by the anti-ALD_c IgG immunoaffinity column (following application of the carrot extract) could represent ALD_c binding proteins. Initial studies confirmed that the anti-ALD_c IgG column could bind purified carrot ALD_c [11], or the ALD_c present in the carrot crude extract. In both instances, the enzyme was not desorbed when the column was subsequently washed with PBS containing 1 M NaCl. However, ALD_c was eluted following application of 0.1 M glycine (pH 2.5) to the polyclonal antibody column (Figs. 1 and 2).

Fig. 1 presents an A_{280} elution profile that was obtained when the carrot extract was chromatographed on the anti-ALD_c IgG column. The vast majority of soluble proteins were not retained, and eluted in the flow-through fraction following the wash with PBS. When the column was subsequently eluted in a step-wise fashion with increasing concentrations of NaCl in PBS, several fractions were obtained that had an $A_{280} > 0.005$ (Fig. 1). SDS-PAGE revealed that each of the respective pooled fractions was enriched in a 65 kDa polypeptide(s) (Fig. 2A; lanes 1–3). Interestingly, immunoblotting of these fractions demonstrated that the 65 kDa silver-staining bands seen in Fig. 2A evidently represent carrot PFP and PFK_c (Fig. 3A and B). Wong and co-workers [12] have previously reported that the subunits corresponding to the carrot storage root PFP and PFK_c co-migrate following SDS-PAGE. The bulk of the PFK_c eluted at 0.25 and 0.5 M NaCl (Fig. 3B, lanes 2 and 3), whereas PFP was bound more tightly to the column, and was most abundant in the 0.5 and 1 M NaCl, and 0.1 M glycine (pH 2.5) pooled fractions (Fig. 3A, lanes 3–5). Despite similarities in reaction catalyzed and identical subunit sizes, carrot PFK_c and PFP are immunologically unrelated [12]. It is important to note that: (i) the anti-(carrot ALD_c) IgG showed absolutely no cross-reaction with denatured or native carrot PFK_c or PFP (Fig. 2B and data not shown), and (ii) the anti-(carrot PFK_c or potato PFP) IgG demonstrated no cross-reaction with denatured or native carrot ALD_c (Fig. 3A and B and data not shown). These controls ensured that neither PFP or PFK_c were bound to the anti-ALD_c IgG of the immunoadsorbant column, and that anti-(PFP or PFK_c) IgG was not present in the anti-ALD_c IgG preparation. Laser densitometric immunoquantification of the Western blots shown in Figs. 2 and 3 has revealed that approximately 24, 36 and 73% of the total ALD_c, PFK_c, and PFP, respectively, that were initially applied to the immunoaffinity column were retained and subsequently desorbed following step-wise elution as described in Fig. 1.

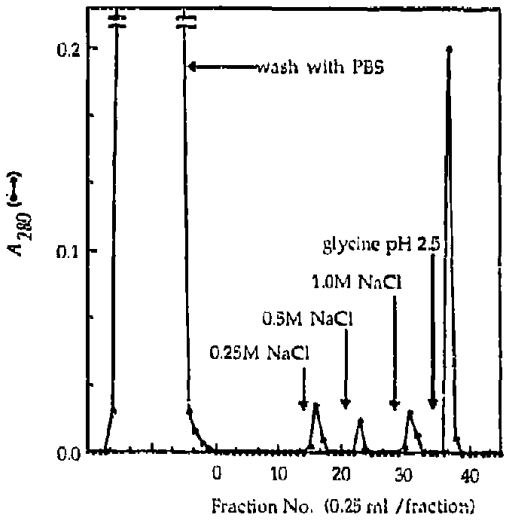

Fig. 1. A_{280} elution profile obtained following chromatography of a carrot storage root extract on the anti-(carrot ALD_c) IgG column. Details of the procedure appear in section 2.

Experiments utilizing rabbit anti-(castor oil seed GAPDH or PK_c) IgG [17] demonstrated that immunoreactive polypeptides corresponding to the subunits of GAPDH or PK_c could readily be detected on immunoblots of the carrot extract (data not shown).

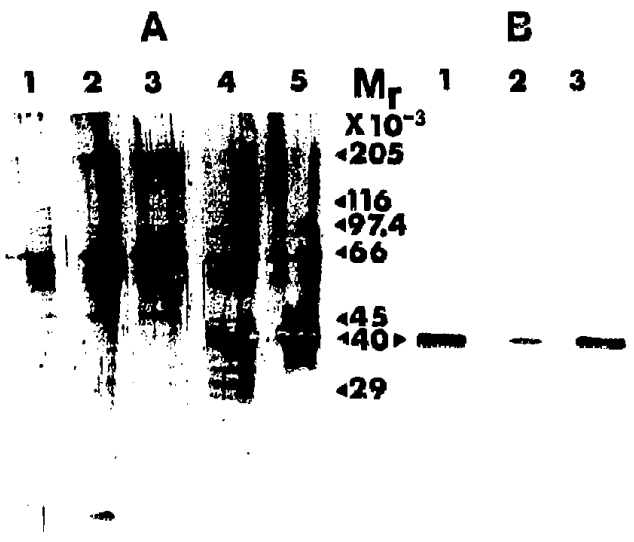

Fig. 2. SDS-polyacrylamide mini-gel electrophoresis (A) and immunoblot (B) analysis of pooled fractions obtained following chromatography of the carrot extract on the anti-(carrot ALD_c) IgG column. (A) The SDS-polyacrylamide gel (12% (w/v) separating gel) was stained with silver. Lanes 1, 2 and 3 contain the 0.25, 0.5, and 1.0 M NaCl concentrated fractions, respectively (20 μl each); lane 4 contains 20 μl of the 0.1 M glycine (pH 2.5) concentrated fraction; lane 5 contains 1.6 μg of purified carrot root ALD_c [11]. (B) Samples were subjected to SDS-PAGE and blot-transferred to a polyvinylidene difluoride membrane. Western analysis was performed using affinity-purified rabbit anti-(carrot ALD_c)IgG [11], and antigenic peptides were visualized using an alkaline phosphatase-tagged secondary antibody as in [11]; phosphatase staining was for 10 min at 30°C. Lane 1 contains 65 ng of purified carrot ALD_c [11]; lane 2 contains 3 μg of protein from the carrot clarified extract; lane 3 contains 5 μl of the 0.1 M glycine (pH 2.5) concentrated fractions.

However, these antigenic polypeptides were undetectable on the respective immunoblots of the various pooled fractions obtained following application of the stepwise NaCl gradient, or 0.1 M glycine (pH 2.5) to the anti-ALD_c IgG column (data not shown). Physicochemical and kinetic studies have shown GAPDH to interact with ALD in mammalian muscle tissue, thus allowing channelling of the shared intermediate, glyceraldehyde-3-phosphate [5–8]. This interaction is believed to prevent the unfavourable aldehyde–diol interconversion of glyceraldehyde-3-phosphate that occurs in aqueous medium [5,6,8]. Under the conditions employed in this study, GAPDH does not appear to associate with ALD_c, suggesting that in carrot roots these proteins may not interact.

The results outlined above provide the first indication that ALD_c may specifically interact with the PFK_c and PFP in a plant tissue. This interaction could have important implications with respect to the metabolism of fructose-1,6-bisphosphate within the plant cytosol. Mammalian muscle PFK is known to change from an active tetramer to a less active dimer upon incubation with calmodulin or various negative effectors [22]. Orosz and co-workers [22] have demonstrated that the calmodulin-induced inactivation of muscle PFK is arrested by addition of an equimolar amount of muscle ALD. The effect was attributed to an apparent competition between calmodulin and ALD for the dimeric form of PFK. Moreover, the direct binding of muscle ALD to calmodulin was demonstrated, which resulted in a significant decrease in the k_{cat} of ALD [22]. Carrot PFK_c is also known to exist in various oligomeric states [23], but whether these interconversions can be medi-

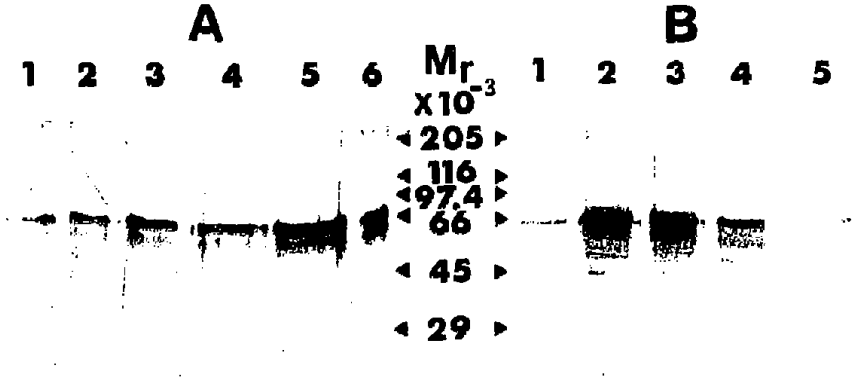

Fig. 3. Immunoblot analysis of pooled fractions obtained following chromatography of the carrot extract on the anti-(carrot ALD_c) IgG column. Immunoblotting and visualization of antigenic peptides was performed as described in Fig. 2. (A) The blot was probed with rabbit anti-(potato tuber PFP) immune serum (1:1,000 dilution) [26]. Lane 1 contains 30 μg of protein from the carrot extract; lanes 2, 3 and 4 contain the 0.25, 0.5 and 1.0 M NaCl concentrated fractions, respectively (20 μl each); lane 5 contains 20 μl of the 0.1 M glycine (pH 2.5) concentrated fraction; lane 6 contains 100 ng of purified potato tuber PFP [26]. (B) The blot was probed with rabbit anti-(carrot PFK_c) immune serum (1:500 dilution) [23]. Lane 1 contains 30 μg of protein from the carrot clarified homogenate; lanes 2, 3 and 4 contain the 0.25, 0.5 and 1.0 M NaCl concentrated fractions, respectively (20 μl each); lane 5 contains 20 μl of the 0.1 M glycine (pH 2.5) concentrated fraction.

ated by an interaction of the enzyme with ALD_e and/or calmodulin has yet to be determined.

PFP is unique to the plant cytosol and some microbes, and is believed to be important in plant metabolism owing to its widespread distribution, its potent activation by the regulatory metabolite, fructose-2,6-bisphosphate, and its high activity (which often exceeds that of PFK_c) [24]. The metabolic function of PFP is still controversial, partly because the reaction it catalyzes is close to equilibrium in vivo [24]. Recently, Hatzfeld and Stitt [25] demonstrated a high rate of cycling between triose-phosphates and hexose-phosphates in the cytosol of non-photosynthetic plant cells. The interaction of ALD_e with PFP (and PFK_c) could possibly facilitate the observed recycling of triose-phosphates through channeling of the shared intermediate, fructose-1,6-bisphosphate. Further investigation of the association of PFP with ALD_e may provide additional insights into the role of PFP in plant metabolism.

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