Communication

Binding of Glycolytic Enzymes to a Particulate Fraction in Carrot and Sugar Beet Storage Roots

DEPENDENCE ON METABOLIC STATE

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ABSTRACT

Numerous studies have demonstrated a rapid increase in the respiration rate during aging of slices of tuber and storage roots. To determine the molecular mechanisms of this phenomenon, the role of enzyme binding to the subcellular particulate fraction has been assessed in carrot (Daucus carota L.) and sugar beet (Beta vulgaris L.). Soluble versus particulate fractions were separated by centrifugation at 16,000g and both fractions assayed for the activities of six glycolytic enzymes. Preparations from sliced and aged tissues showed elevated percentages of five enzymes associated with the particulate fraction as compared with controls. The stimulation of respiration which occurs during aging of underground storage organ slices may result, in part, from an association of enzymes with the particulate fraction of the cell promoting an elevated glycolytic rate.

Recent studies have provided convincing evidence for subcellular structuring of metabolic pathways which were once thought of as being soluble. Various workers have reported associations between many of the so-called 'soluble' enzymes of glycolysis and membrane fractions or structural proteins in animal cells (4, 7, 9, 10, 12–14, 17, 18, 22). As yet such associations have not been documented for higher plant glycolytic enzymes. The microcompartmentation of enzymes and metabolic pathways which can result from these types of interactions could provide an effective means of metabolic control via (a) channeling of substrates between consecutive enzymes, and (b) altering enzyme kinetic properties due to conformational changes occurring during binding. It has been suggested, therefore, that metabolic activation of glycolysis can occur not only by allosteric regulation and covalent modification of key enzymes, but also by altering the partitioning of enzymes from the soluble to particulate phase (4, 7, 9, 10, 13, 14, 17, 22).

The dramatic stimulation of metabolism which accompanies the slicing of underground storage organs and subsequent aging has been studied for many years and includes a significant rise in the respiratory rate (termed 'induced respiration'). This is paralleled by an elevation in the concentration of Fru2,6P2 (11, 19, 20), and activation of both the oxidative pentose-P and glycolytic pathways (1–3, 5). It has been suggested that allosteric activation of PFK and PK may contribute to the stimulation of glycolysis in thin slices of aged carrot root tissue (1, 5). In the present study we have adopted the methodology of Clarke et al. (4) to measure glycolytic enzyme partitioning between soluble and particulate phases in dormant versus sliced and aged carrot and sugar beet storage roots. The results demonstrate that slicing and aging, with its concomitant reactivation of metabolism, leads to a significant elevation in binding of several glycolytic enzymes.

MATERIALS AND METHODS

Chemicals and Plants. All biochemicals and coupling enzymes were purchased from Sigma. All other chemicals were obtained from BDH Chemicals at the highest possible purity. Mature storage roots of carrot, Daucus carota L., and sugar beet, Beta vulgaris L., were purchased from a local market in June and July, stored at 4°C, and used within 1 week. Each individual experiment was performed using a separate carrot or sugar beet root.

Enzyme Assays. All enzyme assays had a final volume of 1.0 ml and were performed in duplicate at room temperature by monitoring NADH oxidation or NAD+ reduction at 340 nm using a Gilford recording spectrophotometer. Enzymes were assayed using the following conditions: HK: 50 mM Hepes-NaOH (pH 7.5), 7 mM D-glucose, 1 mM ATP, 0.2 mM NAD+, 5 mM MgCl2, and excess ALD, triose-P isomerase, and glyceral-3-P dehydrogenase; PPK: 50 mM Tris-HCl (pH 8.0), 1 mM fructose-6-P, 0.2 mM ATP, 0.1 mM NADH, 5 mM MgCl2, 1 mM EDTA, and excess ALD, triose-P isomerase, and glyceral-3-P dehydrogenase; PFP: 50 mM Tris-HCl (pH 8.0), 5 mM fructose-6-P, 2.5 mM Pi, 0.1 mM Fru2,6P2, 0.15 mM NADH, 5 mM MgCl2, and excess ALD, triose-P isomerase, and glyceral-3-P dehydrogenase; ALD: 50 mM Tris-HCl (pH 8.0), 1 mM fructose-1,6-P2, 0.1 mM NADH, 5 mM MgCl2, and excess triose-P isomerase and glyceral-3-P dehydrogenase; HK: 50 mM Hepes-NaOH (pH 7.0), 2 mM PEP, 2 mM ADP, 0.15 mM NADH, 50 mM KCl, 10 mM MgCl2, 2 mM DTE, 0.2 mg/ml BSA, and excess LDH; ADH: 50 mM Tris-HCl (pH 8.8), 100 mM ethanol, 1 mM NAD+, LDH: 50 mM Hepes-NaOH (pH 7.0), 1.5 mM pyruvate, 0.12 mM NADH. PK assays were corrected for contaminating PEP phosphatase activity by omitting ADP from the PK reaction mixture. All

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2 Abbreviations: Fru2,6P2, fructose-2,6-bisphosphate; PFK, ATP:fructose-6-phosphate 1-phosphotransferase; PK, pyruvate kinase; HK, hexokinase; ALD, aldolase; PFP, PPI:fructose-6-phosphate 1-phosphotransferase; PEP, phosphoenolpyruvate; LDH, lactate dehydrogenase; ADH, alcohol dehydrogenase; PMSF, phenylmethylsulfonyl fluoride.

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assays were corrected for blank NAD$^+$ reductase or NADH oxidase activity and were initiated by the addition of enzyme extract except the PFP assay which was initiated by the addition of PPI.

**Tissue Preparation and Measurement of Enzyme Binding.** Cylinders of phloem parenchyma tissue were removed from freshly topped carrot or sugar beet storage roots using a No. 4 cork borer. Control (metabolically dormant) tissues were sampled directly from these cylinders. Induced respiration was attained by slicing and incubation essentially as described by ap Rees and Beevers (2). Discs approximately 0.5 mm thick were cut into distilled water, rinsed twice, dried on paper towels, weighed, and placed in 20 mM K-phosphate (pH 5.0), 30 ml/g fresh weight, and maintained at 25°C. The incubation buffer was changed three times during the 17 h incubation period. The discs were continuously aerated and agitated by bubbling filtered air through the incubation medium.

Measurement of enzyme binding to the particulate fraction was based on the procedure as described (4). Both control and sliced/aged tissues were quickly washed in distilled water, blotted and homogenized (1:1 w/v) at 4°C in 300 mM sucrose containing 0.5 mM DTE using a chilled mortar and pestle with a small amount of sand. An aliquot (1.0 ml) of each homogenate was centrifuged for 5 min at 4°C in an Eppendorf microcentrifuge at 16,000g. The supernatant fraction was diluted with 1.5 volumes of a stabilization buffer which contained 150 mM K-phosphate (pH 7.0), 1 mM EDTA, 1 mM EGTA, 30 mM NaF, 0.2 mM ATP, 0.2 mM fructose-6-P, 2% (w/v) PVP, 1.5% (v/v) Triton X-100, 20% (v/v) glycerol, 2 mM DTE, 1 mM PMSF, and 10 μg/ml leupeptin. This diluted fraction was stored on ice for later measurement of soluble enzyme activity. The pellet was twice extracted with 0.5 ml stabilization buffer by resuspension, gentle vortexing, and centrifugation as above. The combined extracts were stored on ice and the enzyme activity in this fraction was taken to be a measure of the amount of bound enzyme. As a control, a further 0.5 ml of the homogenate was diluted with 2 volumes of stabilization buffer, gently vortexed, and centrifuged as above. The supernatant was kept as a measurement of total enzyme activity to check on recovery in soluble and bound fractions.

**RESULTS**

Initial tests were run to compare the distribution of three glycolytic enzymes (PFK, ALD, and PK) between soluble and particulate fractions after the tissue had been extracted in 0, 150, 300, and 500 mM (carrot) or 0, 300, 500, and 750 mM (sugar beet) sucrose containing 0.5 mM DTE. Control or sliced and aged carrot root discs both showed respective optimal binding to the particulate fraction in the 150 to 300 mM sucrose concentration range (data not shown). In carrot roots, binding of these enzymes to the particulate fraction appears to be sensitive to osmotic strength. Sucrose at 300 mM is isosmotic in many plant cells and is commonly used for preparation of intact mitochondria or plastids from plant tissues. Carrot tissues were therefore subsequently extracted with 300 mM sucrose. In contrast, varying the sucrose concentration in the extraction medium had no effect on the degree of binding of the three glycolytic enzymes to the particulate fraction in control or sliced and aged sugar beet roots. It is possible that the high intracellular sucrose concentration of sugar beets (500–600 mM) (15) is sufficient to maintain any weak interactions of bound enzymes during tissue extraction with no added sucrose. Sugar beets were also subsequently extracted with 300 mM sucrose.

The partitioning of enzyme activity between the soluble and particulate phases in carrots and sugar beets was examined for six glycolytic enzymes: HK, PFK, ALD, PK, ADH, and LDH. Maximal enzyme activities are reported in Table I. Recovery of enzyme activity was 90 to 110% when the sum of activity in the soluble and particulate fractions was compared with the total activity measured in the original homogenate. With the exception of carrot ADH, and sugar beet ADH and LDH, slicing and aging caused no significant change in the maximal enzyme activity obtained with respect to control tissues (Table I). Typical percentages of total activity in the particulate (bound) fraction are

**FIG. 1.** Percentage of recovered enzyme activity associated with the particulate fraction of the cell in carrot (A) and sugar beet (B) storage root tissue under two metabolic states: dormant (■) and sliced and aged (□). Separation of enzyme activities into soluble and bound fractions was based on a procedure (4) in which tissue extracted in a sucrose medium preserves enzyme associations with subcellular particles. All data are the means ± SE for n = 4 individual experiments. All values for the sliced and aged condition are significantly different from the corresponding dormant values, P < 0.05, using the Student's t-test, except those denoted ns (not significant).
shown in Figure 1A for carrot and Figure 1B for sugar beet. Two metabolic states are compared: dormant (i.e., controls taken directly from freshly excised tissue cylinders) and sliced and aged (i.e. metabolically reactivated). In carrot, PFK, ALD, PK, LDH, and ADH showed significant elevations in the percentage associated with the particulate fraction as a result of tissue slicing and aging (Fig. 1A). PFP activity was very low (Table I) and no activity was ever detected in the particulate fraction of extracts prepared from control or sliced and aged carrots. HK showed a uniformly high degree of association with the particulate fraction independent of the metabolic state of the tissue (Fig. 1A).

As with the carrot, aging sugar beet slices resulted in a significant increase in the percentage of total activity of PFK, ALD, PK, LDH, and ADH associated with the particulate fraction (Fig. 1B). HK behaved differently, however, showing a decrease in the percentage of enzyme bound in response to tissue slicing and aging (Fig. 1B). Sugar beet PFP activity was undetectable (Table I); therefore, no assessment of its distribution between soluble and bound phases was possible.

**DISCUSSION**

The purpose of the present study was to examine the potential binding of so-called 'soluble' enzymes to the particulate fraction of plant cells and to determine if glycolytic enzyme binding might play a role in the reactivation of metabolism which accompanies slicing and aging of plant underground storage organs. Clarke et al. (4) used homogenization in a sucrose medium to disrupt cells in an effort to leave intact enzyme associations with the particulate structure of cells which are normally disturbed by homogenization at high dilution in the low ionic strength buffers normally used to extract soluble enzymes. This technique was adopted not only for the rapid separation of soluble and particulate fractions, but also because of the high percentage of total enzyme activity recovered.

Results presented in Figure 1, A and B, demonstrate that slicing and aging of carrot and sugar beet storage root tissue promotes an increase in the percentage of five glycolytic enzymes associated with the particulate fraction. This is the first demonstration of metabolically dependent glycolytic enzyme binding to a particulate fraction in higher plants. Thus, one manner in which plants might regulate glycolytic flux is by altering the partitioning of enzymes between the soluble and bound states. The microcompartmentation of the glycolytic pathway which may result from such an association could act as an effective mechanism of metabolic control along with allosteric modulation and covalent modification of key regulatory enzymes.

The site of glycolytic enzyme binding in carrot and sugar beet storage roots has not yet been determined, but should be resolved through a combination of subcellular fractionation and immunocytological studies. Glycolytic enzyme binding sites in animal systems can include membranes and actin containing filaments (4, 7, 9, 10, 12, 13, 17, 22). In addition to the close proximity of consecutive enzymes which could be brought about by such a mechanism, the binding of glycolytic enzymes is known to alter kinetic properties via effects on enzyme conformation (4, 7, 9, 10, 12, 13, 22).

The molecular mechanisms which control the extent of glycolytic enzyme binding in vivo are not yet fully understood. It is clear that changes in ionic and/or osmotic strength can influence binding. Binding of animal glycolytic enzymes in vitro can also be influenced by concentrations of H+ ions, substrates, products, and metabolite effectors (4, 9, 10, 12, 13, 17, 22), as well as by enzyme phosphorylation (7).

The existence of known stable multienzyme complexes provide favorable evidence for the interaction of apparently soluble metabolically sequential enzymes. Due to high intracellular protein concentrations, such interactions are far more likely in vivo than in dilute in vitro enzymological studies (16–18). The limited solvent capacity of the cell also supports interactions between sequential enzymes of a metabolic pathway as the need for a large pool of free intermediates would be eliminated (16–18).

Although there is now compelling evidence for complexing and/or binding of so-called soluble enzymes and the direct transfer of substrates between sequential enzymes of various pathways in animal tissues these possibilities have only recently been addressed in plant systems. For example, Marques et al. (8) have provided evidence which points to direct channeling of substrates between Calvin cycle enzymes in illuminated pea leaf chloroplasts. This corroborates an earlier study by Wah Kow and Gibbs (21) which demonstrated that a spinach leaf chloroplast particulate fraction could assimilate CO2 in the absence of added stromal protein, yielding the intermediates of the Calvin cycle. Similarly, using an immunocytological approach, Hrazdina et al. (6) have
reported that the biosynthesis of flavanoids may take place in a multienzyme complex wholly or partially associated with the cyttoplasmic face of the endoplasmic reticulum membrane. The above studies, along with those of the present communication, indicate that before an overall understanding of metabolic control in plants can be achieved we must determine not only how, where, and when soluble plant enzymes of a given pathway might be microcompartmented into an organized multienzyme system, but also how such associations may alter individual enzyme kinetic/regulatory properties.

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LITERATURE CITED

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