Subcellular Localization of Hexokinase in Pea Leaves

EVIDENCE FOR THE PREDOMINANCE OF A MITOCHONDRIALLY BOUND FORM

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(Received for publication, February 22, 1984)

Hexokinase (ATP : d-hexose-6-phosphotransferase, EC 2.7.1.1) activity was determined in subcellular fractions prepared from pea (Pisum sativum) leaf homogenates. About 60% of the total detectable activity of hexokinase was found associated with a particulate fraction consisting essentially of mitochondria and chloroplasts and free of cytosol contamination. The hexokinase specific activity of the particulate fraction was 2-fold higher than that of the homogenate and about 4-fold higher than that of the cytosol.

Using a specially designed isokinetic-isopycnic sucrose density gradient centrifugation method, the distribution of hexokinase activity correlated with that of the mitochondrial marker (cytochrome oxidase) and not with that of the chloroplast membrane marker (chlorophyll) or that of the cytosol marker (phosphoenolpyruvate carboxylase). Thus, the hexokinase/mitochondria ratio was close to 1.0 along the entire gradient, while the hexokinase/chloroplast ratio varied over a 10-fold range.

The results strongly suggest that hexokinase is predominantly bound to mitochondria of pea leaves, and that pea leaf chloroplasts are essentially devoid of any specifically associated hexokinase activity. This work provides the first demonstration of a specific association of hexokinase with mitochondria from photosynthetic tissues of higher plants.

The subcellular localization of hexokinase in plant cells has been a matter of neglect or of controversy (1–7). Particulate hexokinase activity in plants was originally reported almost 3 decades ago (8, 9) but, to date, little is known about its precise subcellular localization in nonphotosynthetic and photosynthetic plant tissues, or of the physiological role of particulate hexokinase in the plant cell. The subcellular localization of hexokinase in animal cells has also been a matter of controversy until recently. Despite the fact that in 1945 Utter et al. (10) observed that hexokinase activity found in homogenates of rat brain is markedly reduced upon centrifugation, and that in 1953 Crane and Sols (11) partially characterized particulate hexokinase, it was not until 1967 that Rose and Warns (12) firmly established that the particulate behavior of hexokinase was due to an association of this enzyme with the outer surface of the external mitochondrial membrane.

Mitochondrial hexokinase in animal cells is now known to fall within the category of "ambiguous" enzymes as proposed by Wilson (13, 14). Thus, mitochondrial hexokinase can remain bound to mitochondria or become detached according to the relative concentrations of specific metabolites such as ATP, Mg\(^2+\), or Glc-6-P\(^3\) (12, 14–16). Significantly, mitochondrial hexokinase has been shown to be primarily responsible for the high aerobic glycolytic rate characteristic of many animal tumor cells (15, 17–20). The regulation of this key enzyme appears to be critical in shifting the cell from a low to a high glycolytic state or vice versa (15, 17–20).

The role of hexokinase in the compartmentalization of function in the photosynthetic plant cell has been obscured by the lack of experimental information on its precise subcellular localization. There seems to be a possible association of hexokinase with mitochondria in nonphotosynthetic cells according to recent reports which describe hexokinase activity in particulate fractions prepared from homogenates of Allium sativum bulbs (21) and lentil roots (22). With respect to hexokinase activity associated with particulate fractions of photosynthetic tissues such as leaves, a recent report by Stitt et al. (4) showed very conclusively that hexokinase was absent from the stroma of pea chloroplasts, and suggested that it might be associated with the external surface of the chloroplast envelope.

The main objective of the work presented here was to ascertain the precise subcellular localization of hexokinase in pea leaf homogenates. This objective has been accomplished via the use of an isokinetic-isopycnic sucrose density gradient centrifugation technique to partition the homogenate into regions where the mitochondria/chloroplasts ratios vary markedly. Through the use of appropriate marker enzymes, the results presented in this paper provide strong evidence for a predominant association of hexokinase with the mitochondria of pea leaves.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**DISCUSSION**

Results presented in this paper provide the first demonstration of an association of hexokinase with the mitochondria.

1 The abbreviations used are: Glc-6-P, glucose 6-phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

2 Portions of this paper including "Experimental Procedures," "Results," and Figs. 1–5 are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-0559, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
from photosynthetic organs of plants. Several lines of experimental evidence point toward the predominance of a mitochondrial bound form of hexokinase in the pea leaf. (i) About 60% of the hexokinase activity detectable in pea leaf homogenates was found associated with a particulate fraction. (ii) The hexokinase specific activity of the particulate fraction is 2-fold higher than that of the homogenate and about 4-fold higher than that of the cytosol. (iii) The particulate fraction was found to be enriched in both mitochondria and chloroplasts and devoid of cytosol markers. (iv) The mitochondria/chloroplast ratio was found to vary markedly when the pea leaf homogenate was centrifuged in isokinetic-isopycnic sucrose density gradients. (v) The hexokinase/chloroplast ratio was found to vary dramatically over a 10-fold range. (vi) The hexokinase/mitochondria ratio was found to be constant along the entire gradient.

The specific association of hexokinase with the outer surface of the external mitochondrial membrane is a firmly established phenomenon in many animal cells (10–17). Mitochondrial hexokinase can exist in the animal cell either bound to the mitochondrion or released from them (12, 13, 16) (the so-called ambiguous behavior proposed by Wilson (14)). The bound hexokinase form and the soluble form differ in their apparent affinities for their substrate MgATP (16).

Importantly, such a bound to soluble shift can be effected by specific metabolites such as ATP, Glc-6-P, or Mg²⁺ (12, 13, 16). It is interesting to note that hexokinase, which is the first enzyme in the glycolytic pathway, is actually capable of phosphorylating glucose present in the cytosol at the expense of mitochondrally synthesized ATP (15, 32). Thus, mitochondrially bound hexokinase seems to be more efficient in phosphorylation of glucose than the soluble form since (i) it has a higher apparent affinity for MgATP, and (ii) it is located adjacent to the major ATP source of the cell, thus gaining increased accessibility to this substrate.

However, extrapolation to plant cells of the important regulatory role exerted by hexokinase on energy metabolism in animal cells is not automatic. Photosynthetic cells contain chloroplasts in addition to mitochondria. Moreover, chloroplasts are organelles that are known to catalyze ATP synthesis driven by dissipation of the electrochemical potential generated mainly by the light reactions of photosynthesis (33). Therefore, if hexokinase were associated with the chloroplast membrane, it would also gain preferential access to chloroplast-synthesized ATP; conceivably, such a membrane-bound hexokinase form could have a kinetic advantage as well.

Consequently, a primary step in the understanding of the regulatory aspects of the interaction between the glycolytic and oxidative pathways of plant energy metabolism seemed to ascertain the precise subcellular location of hexokinase in photosynthetic tissues. Results presented in this paper provide strong experimental support for the predominance of a mitochondrially bound form of hexokinase in the pea leaf. In addition, our results do not indicate a specific association of hexokinase with chloroplasts of pea leaves. Thus, it could be conceived that mitochondrial hexokinase might play a role in the regulation of energy metabolism in the pea leaf perhaps similar to that described for animal tissues.

Acknowledgments—We wish to thank Dr. M. Stitt from the University of Göttingen (Germany), Dr. D. A. Walker from the University of Sheffield (U. K.), and Dr. J. McClure from Miami University (Oxford, OH) for valuable and helpful discussions during the course of this work.

REFERENCES

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**Mitochondrial Localization in Pea Leaves**

**Evidence for the Presence of a Mitochondrially-Bound Form**

**ERIC GOOL and ERNESTO BASTAMENTE**

**EXPERIMENTAL PROCEDURES**

**Materials**

The following reagents were purchased from Sigma Chemical Co.: cytochrome c type VII, DPsntiment, Negel, reduced nicotinamide adenine dinucleotide (NAD), NADP, ATP, Commerce brilliant red (C, B, R), phospho-6-phosphate (from *Leuconostoc mesenteroides*), which can also inhibit NADPH oxidase, and malate dehydrogenase. The TCA was obtained from the Bio-Rad Laboratories, Inc. Tropica, Ltd., 2,4,5-tribromophenol, and 2,6-di-tert-butyl-4-methylphenol (BHT) grade. [3H]-Nicotinate was purchased from American Radiolabeled Centre. Other reagents were from the ordinary commercial sources. The [3H]-nicotinate and all other labeled seeds were provided by the Department of Horticulture of the University of Illinois at Urbana-Champaign.

**Methods**

**Cloning of Plants and Homogenization of Leaves.** Growth was initiated by soaking one week after germination water for 24 hours, then placed in water at 22°C and illuminated for 16 hours. Plants were watered daily. The leaves of fully-expanded leaves were removed 30 days after planting. Approximately 4 g of fully-expanded leaves were homogenized at 4°C using a polytron for 15 seconds in 10 ml of buffer (0.1 M sucrose, 5 mM MgCl₂, 0.1 mM EDTA, pH 7.5). The resulting suspension was filtered through two layers of cheesecloth to separate the plant material from the homogenate. The supernatant was referred to as the mitochondrial fraction.

**Nucleic Acid Preparations.** Gel filtrafon centrifugation. Gel filtration was used to separate macromolecules. Macromolecules were subjected to Sephadex G-200 columns (Bio-rad Laboratories). The column was eluted with 100 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) at 4°C. The column was equilibrated with the same buffer and eluted at a flow rate of 3 ml/hr. The fractions were collected into chilled tubes and aliquots of 15 ml were collected. Each tube was subjected to electrophoresis on a 0.5% agarose gel. The gels were stained with ethidium bromide and photographed.

**Biochemical Procedures.** Assay of chlorophyll was carried out according to the method of Arnon (1949). Assay of phosphoenolpyruvate carboxylase (PEP) was determined according to the method of Stoner and Whitney (1970). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goot and Kalmus (1953). Assay of malate dehydrogenase was carried out according to the method of Carr (1965). Assay of NADH oxidase was carried out according to the method of van der Goo...
Mitochondrial Hexokinase in Pea Leaves

Fig. 1 Subcellular distribution of hexokinase activity in pea leaves. Pea leaves were homogenized and fractionated by the differential centrifugation procedure described under Experimental Procedures. The subcellular fractions were assayed for hexokinase activity by the spectrophotometric procedure described under Experimental Procedures. (A) Distribution of total hexokinase activity. (B) Distribution of relative specific activity. Results from homogenate (1) were used for calculations. Results from homogenate (2) were used for the particulate fraction (P) and the soluble fraction (S). In terms of chlorophyll content, the hexokinase activity of the homogenate was 100% using chlorophyll. Results reported in this figure represent the average of 5 independent experiments and the bars indicate SD.

Fig. 3 Distribution of proteinaceous material in sucrose-isopycnic sucrose density gradients. Panel (A): Distribution of cytochrome oxidase activity (○) and chlorophyll concentration (●). Panel (B): Distribution of PEP carboxylase activity (○) and protein concentration (●). Results from homogenate (1) were used for calculations. Results from homogenate (2) were used for the particulate fraction (P) and the soluble fraction (S). In terms of protein content, the PEP carboxylase activity of the homogenate was 100% using protein concentration. Results reported in this figure represent the average of 5 independent experiments and the bars indicate SD.

Fig. 5 Correlation of distribution of hexokinase with that of organic markers in sucrose-isopycnic sucrose density gradients. Results shown in Figs. 3 and 4 were used for this correlation. The xylose was concentrated at 0.5 in the homogenate (1) and at 0.25 in the homogenate (2). Results from homogenate (1) were used for calculations. Results from homogenate (2) were used for the particulate fraction (P) and the soluble fraction (S). In terms of concentration, the hexokinase activity of the homogenate was 100% using xylose concentration. Results reported in this figure represent the average of 5 independent experiments and the bars indicate SD.

Fig. 6 Distribution of hexokinase activity in isopycnic sucrose density gradients. Panel (A): Distribution of hexokinase total activity (○). Panel (B): Distribution of hexokinase specific activity (●). Results from homogenate (1) were used for calculations. Results from homogenate (2) were used for the particulate fraction (P) and the soluble fraction (S). In terms of concentration, the hexokinase activity of the homogenate was 100% using hexokinase activity. Results reported in this figure represent the average of 5 independent experiments and the bars indicate SD.