

Spinach hexokinase I is located in the outer envelope membrane of plastids

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Received 25 August 1999; received in revised form 12 October 1999

Abstract The subcellular localization of hexokinase activities in plant cells has been a matter of debate for a long time. We have isolated a hexokinase cDNA fragment from glucose-fed spinach leaves using a differential display reverse transcription-PCR approach. The corresponding cDNA was expressed in *Escherichia coli* and an antiserum, raised against the recombinant protein, was used in subcellular localization studies. The spinach hexokinase could be localized primarily to the outer envelope membrane of chloroplasts where it is inserted via its N-terminal membrane anchor. We suggest that the chloroplast envelope hexokinase is involved in the energization of glucose export from plastids rather than in the sugar-sensing pathway of the plant cell.

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Key words: Hexokinase; Plant; Glucose transport; Plastid envelope membrane; Sugar signalling; Subcellular localization

1. Introduction

Hexokinases play a central role in cellular sugar metabolism by converting glucose and, with lower efficiency, fructose into energy-rich phosphorylated hexoses. In mammalian tissues, four different hexokinases have been identified, two of which are apparently associated with the porin of the outer mitochondrial membrane and the other two are found in the cytosol. In yeast, two hexokinases and one glucokinase exist that are all cytosolic [1].

Several groups have studied the presence and localization of hexokinases in plant tissues. Using cell fractionation procedures and activity measurements, hexokinase and/or fructokinase activities have been found both in soluble fractions and/or associated with particulate fractions, probably either mitochondria or plastids or both types of organelles [2,3]. It has been proposed recently that a hexokinase from *Arabidopsis* might be involved in the sugar-sensing pathway [4–6].

In the present study, we describe the isolation of a cDNA encoding a hexokinase from spinach leaves (HxK1) that obviously possesses a N-terminal membrane anchor. HxK1 was heterologously produced in *Escherichia coli* and a specific antiserum was prepared directed against the recombinant protein. The corresponding IgGs were subsequently used in sub-

cellular localization studies. HxK1 was found associated with the outer envelope membrane of chloroplasts. The characterization of this envelope membrane-bound hexokinase is described.

2. Materials and methods

2.1. Glucose feeding of spinach leaves, RNA isolation and differential display reverse transcription-polymerase chain reaction (DDRT-PCR)

Feeding of detached spinach leaves was performed as described by Quick et al. [7]. RNA was isolated from spinach leaves as described by Logemann et al. [8]. DDRT-PCR of total RNAs from control and glucose-fed spinach leaves was performed as described by Liang et al. [9] and as modified by Bauer et al. [10].

2.2. Isolation and sequencing of the hexokinase I cDNAs

The HxK-DDRT-PCR fragment was labelled by the random oligonucleotide priming method [11] and used for a plaque hybridization screening of a spinach leaf cDNA library in λ ZAPII (Stratagene, La Jolla, CA, USA). Positive clones were isolated and the cDNA clone containing the longest insert was sequenced on both strands. The SoHxK1 cDNA was used to screen a cDNA library from tobacco under low stringency conditions ($6\times$ SSC, 54°C). Positive clones were purified and sequenced on both strands.

2.3. Heterologous expression of the spinach HxK1 in *E. coli* cells, affinity purification of the recombinant protein and preparation of an anti-HxK1 antiserum

A 1288 bp *SacI* fragment of spinach HxK1 cDNA encoding amino acid residues 32–460 was excised from the phagemid and inserted into the *SacI*-cleaved pQE31 plasmid (Qiagen, Hilden, Germany), yielding the plasmid pQE31-HxK1. This construct was transformed into *E. coli* TG1 cells. Expression of the recombinant protein was done as described before [12,13]. The protein was found in the insoluble fraction of *E. coli* and purified under denaturing conditions by Ni^{2+} -NTA-agarose chromatography (Qiagen, Hilden, Germany). The purified recombinant protein was subjected to preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein band containing the recombinant HxK1 protein was cut from the gel, homogenized to fine powder, resuspended in $1\times$ PBS and used for immunization of rabbits. For further experiments, the anti-HxK1 antiserum was affinity-purified using recombinant HxK1 protein immobilized on a PVDF membrane. The antiserum was incubated with the immobilized protein, the membranes were washed and the bound IgGs were eluted by a pH step.

2.4. Deletion of the spinach HxK1 membrane anchor

For the deletion of the first 30 amino acids including the membrane anchor, a *NotI*-*MunI* fragment of pBluescript-SoHxK1 was deleted by restriction and replaced by a *NotI*-*NcoI* PCR fragment starting at nucleotide 368 and ending with the *MunI* restriction site at nucleotide 417. The following primers were used:

SoHxK1-forw: GCGCGCGGCCCATGGGCTCAAGCAAG-TGGGGTCGTGTAATGGCA

SoHxK1-rev: AGGGTCCCACAATTGTCATCTAA

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2.5. Enzyme activity measurements

One g of plant material was ground to a fine powder in liquid nitrogen and further homogenized in 5 ml buffer AT (50 mM Tris-Cl, pH 8.0, 5 mM MgCl₂, 1 mM EDTA, 1% Triton X-100). Insoluble debris was removed by centrifugation (20 000 × g, 10 min, 4°C). For the isolation of total membranes, the plant material was homogenized in buffer A (buffer AT without Triton X-100). After centrifugation at 1000 × g (10 min, 4°C), the resulting supernatant was centrifuged at 100 000 × g (20 min, 4°C). For activity measurements, the membrane fractions were solubilized in buffer AT.

Hexokinase activity (EC 2.7.1.1) was measured at 30°C in a 96-well plate reader (Spectrafluor Plus, Tecan) with a 340 nm absorption filter in a total volume of 200 µl. Either glucose, fructose or mannose were used as substrates. The reaction mixture contained 100 mM HEPES-KOH pH 7.8, 10 mM MgCl₂, 2 mM ATP, 0.8 mM NADP, 0.2 U glucose-6-phosphate-dehydrogenase (EC 1.1.1.49) and either 5 mM glucose (for the measurement of glucokinase activity, EC 2.7.1.2) or 5 mM fructose and 0.2 U glucose phosphate isomerase (EC 5.3.1.9) (for the measurement of fructokinase activity, EC 2.7.1.4) or 10–200 µM mannose, 0.2 U phosphoglucomutase and 0.2 U phosphomannose isomerase (EC 5.3.1.8) (for the measurement of mannokinase activity, EC 2.7.1.7).

Cytochrome *c* oxidase activity was measured at room temperature in a spectrofluorometer (Spectronic 1201, Milton Roy, Rochester, USA) [14].

Antimycin A-insensitive NADH-cytochrome *c* reductase activity (EC 1.6.99.3) was measured as described [15].

Inosine 5'-diphosphatase activity (EC 3.6.1.6.) was measured according to [16,17].

2.6. Isopycnic sucrose density gradient centrifugation of cellular membranes

Isopycnic sucrose density gradient centrifugation of cellular membranes was performed as described by Schnarrenberger and Burkhard [18] using glycylglycine extraction buffer (20 mM glycylglycine, pH 7.5, 10 mM DTT, 0.4 M sucrose). The extracts were depleted from intact chloroplasts by centrifugation (1000 × g, 10 min, 4°C) and then layered on top of a continuous sucrose gradient (20–60%, w/w, in 20 mM glycylglycine, pH 7.5) for centrifugation at 70 000 × g for 3 h. The gradient was fractionated into 1 ml aliquots and the membranes were recovered from these fractions after dilution (40-fold, 20 mM glycylglycine, pH 7.5) by centrifugation (100 000 × g, 45 min, 4°C).

2.7. Isolation of chloroplasts and chloroplast envelope membranes

Intact spinach chloroplasts were isolated from spinach leaves and purified by Percoll gradients as described by Douce et al. [19]. Chloroplast envelope membranes were isolated from intact, Percoll-purified spinach leaf chloroplasts by a discontinuous sucrose gradient as described previously [19,20].

2.8. Protein import assay

Intact spinach leaf chloroplasts were isolated as described previously [19]. The cloned cDNA of the spinach HxK1 protein from spinach leaves was transcribed and translated using the TNT reticulocyte lysate system (Promega, Madison, WI, USA) with [³⁵S]methionine as labelled amino acid. Protein import assays, subfractionation of plastids and analysis of the protein import reactions was done as described by Flüggé et al. [21].

2.9. Alkaline extraction of membranes

Alkaline extraction of membrane fractions was done as described by Fujiki et al. [22].

Both, membrane and supernatant fractions, were analyzed by SDS-PAGE [23] and Western blot.

3. Results

3.1. Isolation of hexokinase 1 cDNAs and analysis of the sequences

It has been shown that glucose feeding of spinach leaves induced a switch in the function of the chloroplasts from carbon-exporting source-organelles to carbon-importing sink-organelles with a concomitant increase in plastidic starch biosynthesis [7,24,25]. A DDRT-PCR approach with RNAs isolated from spinach leaves that have been fed with either glucose or water was used to identify putative genes that are involved in this source-sink transition. A cDNA fragment encoding a spinach leaf hexokinase was obtained, which was then used to isolate the corresponding full-length cDNA clone (HxK1, accession no. AF118132). This clone was 1971 bp in length, encoding a protein of 498 amino acids with a calculated molecular mass of 54.1 kDa. The spinach HxK1 amino acid sequence shows about 73% identity to the already known HxK1 and HxK2 from *Arabidopsis* [5] and about 30% identity to hexokinases from *Saccharomyces cerevisiae* and *Homo sapiens* (Table 1). Identities up to 68% were found in regions that are presumably involved in sugar and ATP-binding [26]. The spinach HxK1 cDNA was further used to isolate the corresponding cDNA from tobacco (accession no. AF118133). The cDNA clone from tobacco (1843 bp) encodes a protein of 497 amino acids residues with 72% identity to spinach HxK1 and 65% identity to *Arabidopsis* HxK1 (Table 1). Analysis of the hydropathy plot of hexokinase protein sequences from spinach and tobacco [27] revealed the presence of a hydrophobic stretch of 20 amino acids at the very N-terminus of the protein (residues 4–24), which is long enough to span a membrane in an α -helical conformation. These analyses also revealed the presence of such membrane stretches at the N-termini of *Arabidopsis* HxK1 and HxK2, which, however, were not realized [5,28]. In the following, we address the question as to whether the hydrophobic region within the spinach HxK1 serves to anchor the protein in a particular cellular membrane. For this purpose, IgGs directed against spinach HxK1 were used.

3.2. Subcellular localization of spinach HxK1

In a first attempt, spinach leaf extracts were subjected to differential centrifugation and both, the resulting pellets and

Table 1

Percentage of amino acid identities of hexokinase proteins from various sources (spinach, (SoHxK1, this paper), tobacco (NtHxK1, this paper), potato (StHxK1, accession no. X94302), *Arabidopsis thaliana* 1 and 2 (AtHxK1, AtHxK2, accession no. U28214 and U28215), *S. cerevisiae* (ScHxK1, accession no. M14410) and human (HsHxK1, accession no. X66957))

	SoHxK1	NtHxK1	StHxK1	AtHxK1	AtHxK2	ScHxK1	HsHxK1
SoHxK1	–	72	65	74	72	31	34
NtHxK1		–	82	69	69	31	31
StHxK1			–	65	64	28	25
AtHxK1				–	84	31	29
AtHxK2					–	30	31
ScHxK1						–	31

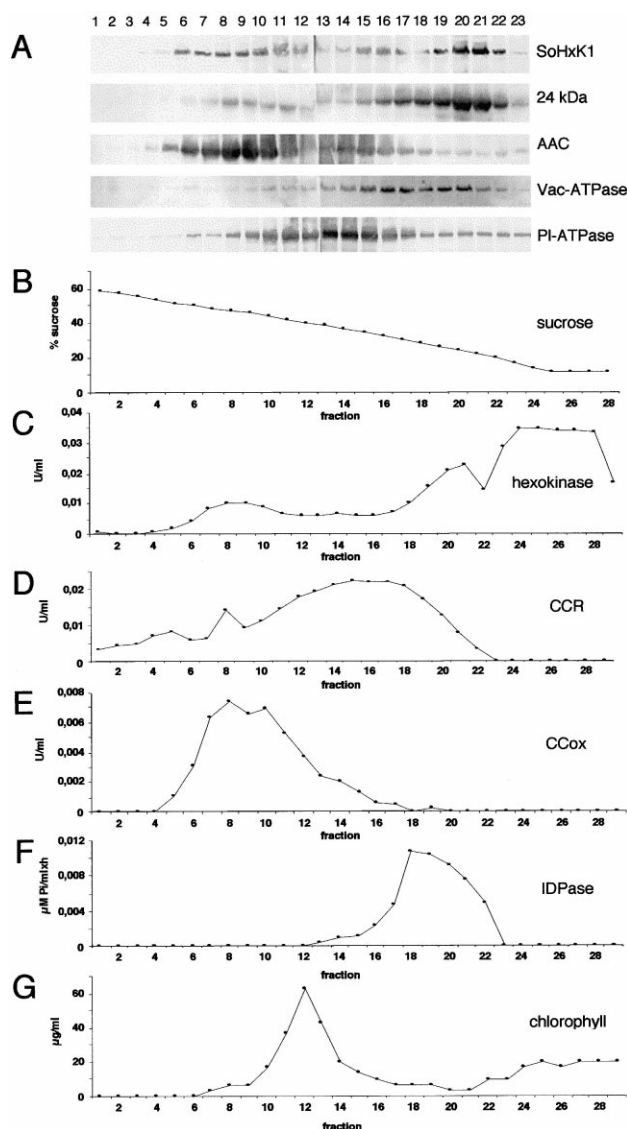


Fig. 1. Intracellular localization of HxK1 from spinach. A spinach leaf extract was subjected to isopycnic sucrose density centrifugation as described in Section 2 and the resulting fractions were analyzed (A) by Western blots using antibodies directed against the spinach HxK1 (SoHxK1), the 24 kDa protein of the outer envelope membrane (24 kDa), the mitochondrial adenylate translocator (AAC), the vacuolar ATPase (Vac-ATPase) and the plasma membrane ATPase (PI-ATPase), (B) for sucrose concentrations given in percentages (w/v), (C–F) by activity measurements: (C) glucose phosphorylating activity, (D) antimycin A-insensitive NADH-cytochrome *c* reductase, CCR, (E) cytochrome *c* oxidase, CCoX, (F) inosine 5'-di-phosphatase, IDPase, and (G) by chlorophyll concentrations.

the supernatants, were analyzed by SDS-PAGE and Western blots. Increasing relative centrifugation forces result in a shift of the HxK1 signal from the supernatant to the membrane fraction, indicating that spinach HxK1 is membrane-associated. This membrane association was not affected by treatment of the membranes with carbonate and could only be (partially) disrupted by treatment with NaOH, showing that the HxK1 protein is not only attached but inserted into the membrane bilayer. Signals for HxK1 were detected in membrane fractions of source leaves, sink leaves, petioles and roots, but not in the corresponding soluble fractions of these tissues (not shown).

To analyze the intracellular localization of HxK1 in more detail, chloroplast-depleted extracts from spinach leaves were subjected to isopycnic sucrose gradient centrifugation. The resulting gradient fractions were subsequently assayed for both the distribution of subcellular compartments and for the presence of HxK by Western blot analysis and activity measurements (Fig. 1). Mitochondrial fractions were identified by cytochrome *c* oxidase activity (Fig. 1E) and an antiserum directed against the mitochondrial adenylate transporter (AAC), plasma membranes by an antiserum directed against the plasma membrane ATPase, endoplasmic reticulum by cytochrome *c* reductase activity (Fig. 1D), Golgi fractions by IDPase activity (Fig. 1F), vacuolar fractions by an antiserum directed against the vacuolar ATPase [29], thylakoids by chlorophyll measurements (Fig. 1G, [30]) and chloroplast envelope membranes by an antiserum directed against the chloroplast outer envelope 24 kDa protein [12]. Hexokinase activity was present in two peaks (Fig. 1C): the stronger one in the low density fractions at the top of the gradient co-fractionated with chloroplast envelope membranes. The weaker peak at the higher density near the bottom of the gradient presumably represents mitochondrial fractions. The soluble fraction on top of the gradient also showed a high hexokinase activity. However, no signals for HxK1 were detected in these fractions using HxK1-specific IgGs. Together, these results reveal the presence of three different glucose phosphorylating activities in spinach leaves: a soluble hexokinase activity that is different from HxK1 and which is not recognized by the SoHxK1 antiserum and hexokinase activities (HxK1) associated with mitochondria and chloroplast envelope membranes.

To further characterize the association of HxK1 with chloroplasts, intact chloroplasts were subfractionated into thylakoids, the stroma fraction, and envelope membranes. These chloroplast subcompartments were analyzed for the presence of HxK1 by HxK1 IgGs and activity measurements. As shown in Fig. 2, a HxK1-specific signal and HxK activity (Fig. 3) were both absent from the stroma and the thylakoids and could only be detected in envelope membranes. Alkaline extraction demonstrates that HxK1 is inserted into the envelope membrane (Fig. 2B). Fig. 3C shows that the presence of HxK1 IgGs in the HxK activity assays is able to effectively inhibit HxK activity in a dose-dependent manner. The inhibition is specific, as the activity is not inhibited at the highest concentration of pre-immune IgGs included in the assay. It may be noted that the stroma fraction contains, instead of a HxK activity, a fructose phosphorylating activity (Fig. 3A).

To analyze to whether the HxK1 protein can be directed to

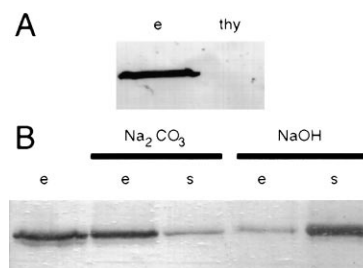


Fig. 2. (A) Immunoblots of chloroplast envelope and thylakoid membrane proteins separated by SDS-PAGE and using the SoHxK1 IgGs. (B) Alkaline extraction of spinach chloroplast envelope membranes. e, envelope membrane; thy, thylakoid membrane; s, supernatant of alkaline extractions.

chloroplasts, we studied the *in vitro* import of the HxK1 protein into chloroplasts. HxK1 protein was *in vitro* synthesized in the presence of [35 S]methionine and added to isolated spinach chloroplasts. Subsequently, the chloroplasts were subfractionated into the different chloroplast subcompartments and the envelope membranes were subjected to SDS-PAGE and fluorography. Fig. 4 shows that the insertion of HxK1 is independent of ATP, either added externally in the dark or produced via photophosphorylation in the light (lanes 2–4). Pre-treatment of the chloroplasts with the protease thermolysin, which is not able to penetrate the chloroplast outer envelope membrane, did not decrease the import efficiency. This indicates that proteinaceous receptors are not required for the binding/insertion process, as is the case for other proteins of the outer envelope membrane [12,31]. The inserted HxK1 protein was not affected by carbonate treatment of envelope membranes (Fig. 4C), which procedure is known to extract only soluble and peripheral membrane proteins. We therefore suggest that HxK1 is inserted into the outer envelope membrane, independent of the presence of receptor proteins and energy. Insertion into the membrane obviously proceeds via the N-terminal membrane anchor since a truncated HxK1 without the membrane anchor does not show any association with chloroplast envelope membranes (Fig. 4B).

As shown in Fig. 4A (lane 5), post-treatment of the chloroplasts with thermolysin led to an almost quantitative digestion of the imported HxK1, suggesting that the large C-terminal part of the protein is located at the cytoplasmic site of the outer envelope membrane. In addition, rupturing of intact chloroplasts by an osmotic shock did not cause an increase of the glucose phosphorylating activity, whilst the fructose phosphorylating activity increased by a factor of three due to the above described stroma-localized fructokinase (Fig. 3B).

3.3. Kinetic properties of the chloroplast envelope hexokinase

The chloroplast HxK1 was further characterized by the determination of its kinetic properties and its susceptibility to known inhibitors of hexokinases. Using purified envelope membranes, K_M values for glucose, mannose and fructose

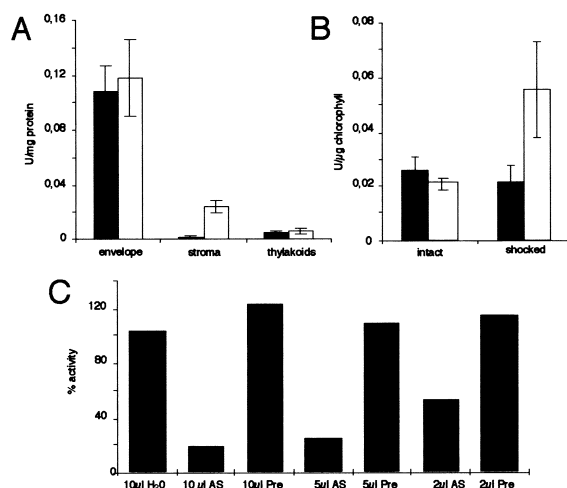


Fig. 3. (A) Hexokinase activities of isolated chloroplast compartments. (B) Hexose phosphorylating activities of intact and osmotically ruptured spinach chloroplasts. (C) Inhibition of hexokinase activity in the chloroplast envelope by IgGs directed against spinach HxK1 (AS) and the corresponding pre-immune serum (Pre). ■, glucose phosphorylating activity; □, fructose phosphorylating activity.

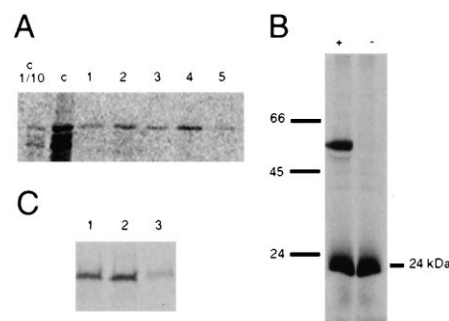


Fig. 4. Import of *in vitro*-translated SoHxK1 into isolated intact spinach chloroplasts. (A) Binding of SoHxK1 to envelope membranes under different conditions. c, translation control; 1, thermolysin pre-treatment; 2, binding in the dark, without ATP; 3, as in 2, with addition of 10 µM CCCP; 4, binding in the light, with additional ATP; 5, thermolysin post-treatment. (B) Binding of *in vitro*-translated, authentic (+) and truncated (without the N-terminal membrane anchor) (–) SoHxK1 to isolated, intact spinach chloroplasts. The 24 kDa outer envelope membrane protein was used as a control. (C) Alkaline extraction of chloroplast envelope membranes after binding of *in vitro*-translated SoHxK1. 1, envelope membranes; 2, envelope membranes after carbonate extraction; 3, supernatant of carbonate extraction.

were determined to be 35 µM, 21 µM and 3.8 mM, respectively. These values are comparable to those from other sources.

It has been shown that glucosamine and trehalose 6-phosphate serve as inhibitors of various hexokinases [32,33]. We therefore tested the glucose and fructose phosphorylating activities of spinach chloroplast envelopes, of crude spinach leaf extracts and of a commercially available soluble hexokinase from yeast (Roche Molecular Biochemicals) for the inhibition by glucosamine and trehalose 6-phosphate. As shown in Fig. 5, the glucose phosphorylating activities of the hexokinase activities from all three sources were inhibited by glucosamine to about the same extent. In addition, the fructose phosphorylating activity of chloroplast envelope membranes was strongly affected by glucosamine with an almost complete inhibition at glucosamine concentrations of 5 mM (Fig. 5A–C). However, even at glucosamine concentrations up to 100 mM, the fructose phosphorylating activity present in the leaf extract was only reduced to about 40%, indicating the presence of a glucosamine-insensitive fructokinase activity in the leaf extract.

Trehalose 6-phosphate, an inhibitor of yeast hexokinases [33], effectively inhibits, as expected, both the fructose or glucose phosphorylating activities of the yeast hexokinase but does not show a marked effect on the hexose phosphorylating activities present in leaf extracts or chloroplast envelopes (Fig. 5D–F). Thus, plant hexokinases, including HxK1, differ from the yeast hexokinase with respect to their susceptibility towards trehalose 6-phosphate.

4. Discussion

In this report, we describe the isolation and characterization of a hexokinase from spinach leaves (HxK1). The corresponding cDNA was obtained using a DDRT-PCR approach to identify differentially expressed genes during a source-sink transition induced by glucose feeding of detached leaves. Since plant hexokinases have been supposed to be involved in the

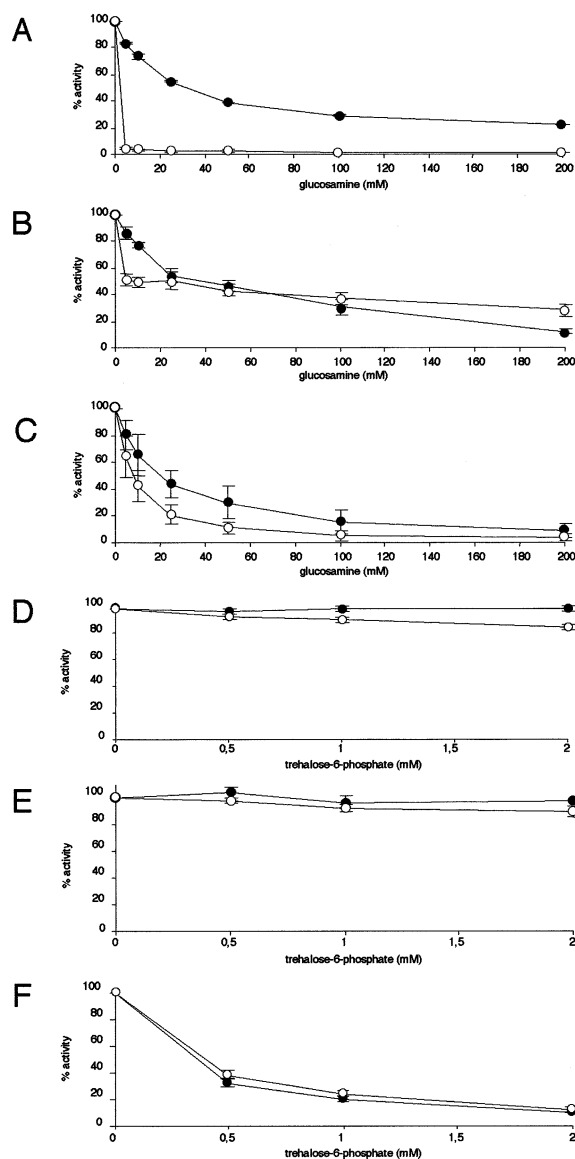


Fig. 5. Inhibition of hexokinase activities in chloroplast envelope membranes (A,D), crude leaf extracts (B,E) and of yeast hexokinase (C,F) by glucosamine (A–C) or trehalose 6-phosphate (D–F). Trehalose 6-phosphate inhibition was determined according to [34]. ●, glucose phosphorylating activity; ○, fructose phosphorylating activity.

glucose-sensing, the isolation of a hexokinase cDNA fragment in the DDRT-PCR was not unexpected. The corresponding full-length cDNA clone encodes a hexokinase containing a N-terminal hydrophobic stretch that possibly anchors HxK1 into a cellular membrane. We then addressed the question of the subcellular localization of HxK1. Hexokinase activities have been found associated with various cellular compartments in the past. All these experiments were based on activity measurements. For the first time, we could localize a hexokinase protein by using an antiserum raised against recombinant spinach HxK1.

Several lines of evidence indicate that HxK1 is a component of the outer envelope membrane: (i) in subcellular fractionation studies, HxK1 co-migrated with the 24 kDa protein of the outer envelope membrane (Fig. 1A), (ii) in vitro protein import studies revealed typical characteristics of an outer en-

velope membrane protein. HxK1 is carbonate-insensitively inserted into the outer envelope membrane without the need of import receptors and energy. (iii) HxK1 can be immunologically detected in isolated envelope membranes (Fig. 2) and (iv) treatment of intact chloroplasts with the protease thermolysin, which cannot penetrate the outer envelope membrane, led to a digestion of the radiolabelled, in vitro-translated HxK1.

Spinach HxK1 and corresponding cDNAs from tobacco and potato are highly homologous to *Arabidopsis* HxK1 and HxK2, which also possess a N-terminal membrane anchor and which have been supposed to be involved in the perception of external sugar signals [5,6]. In analogy to the yeast system, such sugar sensors are expected to be soluble factors of the cytosol, from where they can be transported into the nucleus [34,35]. However, spinach HxK1 could be localized to internal membranes, the chloroplast outer envelope membrane and, partially, also to mitochondrial membranes. A function of the membrane-bound spinach HxK1 in the sugar signalling pathway is therefore unlikely. Instead, we propose a role for HxK1 in the energization of glucose export from plastids.

During photosynthesis, part of the fixed carbon is shuttled into the biosynthesis of starch serving as a temporary carbohydrate deposit. The mobilization of this transitory starch during the following night period results in the production of either triose phosphates or glucose by phosphorylases or amylolytic starch breakdown, respectively. There is increasing evidence that the main route of transitory starch breakdown proceeds via the hydrolytic pathway, yielding glucose [36] that is subsequently exported from the chloroplasts via a glucose translocator [37]. This view is also supported by the observation that transgenic tobacco plants with reduced activities of the chloroplast triose phosphate translocator (TPT, exporting triose phosphates from phosphorytic starch breakdown) possess enhanced activities of α -amylase, hexokinase and a glucose transporter, thus bypassing the reduced activity of the TPT [38]. Non-aqueous fractionation of spinach leaves demonstrated that the cytosolic glucose concentration is kept constant at about 0.4 mM, whereas the cytosolic concentration of glucose 6-phosphate is about 10 times higher [39]. One putative function of the plastid envelope hexokinase could be the maintenance of a steep concentration gradient for glucose across the chloroplast envelope membrane. This requires that glucose is directly converted to glucose 6-phosphate by the outer envelope membrane-bound HxK1 during export from the chloroplasts. Due to the lack of a glucose 6-phosphate transport activity in photosynthetically active tissues [40], glucose 6-phosphate cannot be re-imported into the plastid and is fed either into glycolysis or sucrose biosynthesis. It is thus assumed that the main role of HxK1 in both chloroplasts and plastids from non-green tissues is to directly phosphorylate glucose when leaving the chloroplasts as a product of hydrolytic starch breakdown. The function of HxK1 might be comparable to that of the mammalian mitochondria-associated hexokinase, which couples its ATP consumption to the ATP production from mitochondria.

The identity of the mitochondria-associated hexokinase from spinach will be further investigated.

Acknowledgements: This work was funded by the Deutsche Forschungsgemeinschaft (GRK 306/1, Leibniz-Programm) and the Fonds der Chemischen Industrie.

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