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Substrate specificity and product inhibition of different forms of fructokinases and hexokinases in developing potato tubers

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Abstract. The substrate dependence and product inhibition of three different fructokinases and three different hexokinases from growing potato (*Solanum tuberosum* L.) tubers was investigated. The tubers contained three specific fructokinases (FK1, FK2, FK3) which had a high affinity for fructose (K_m = 64, 90 and 100 μM) and effectively no activity with glucose or other hexose sugars. The affinity for ATP (K_m = 26, 25 and 240 μM) was at least tenfold higher than for other nucleoside triphosphates. All three fructokinases showed product inhibition by high fructose (K_i = 5.7, 6.0 and 21 mM) and were also inhibited by ADP competitively to ATP. Sensitivity to ADP was increased in the presence of high fructose, or fructose-6-phosphate. In certain conditions, the K_i (ADP) was about threefold below the K_m (ATP). All three fructokinase were also inhibited by fructose-6-phosphate acting non-competitively to fructose (K_i = 1.3 mM for FK2). FK1 and FK2 showed very similar kinetic properties whereas FK3, which is only present at low activities in the tuber but high activities in the leaf, had a generally lower affinity for ATP, and lower sensitivity to inhibition by ADP and fructose. The tuber also contained three hexokinases (HK1, HK2, HK3) which had a high affinity for glucose (K_m = 41, 130 and 35 μM) and mannose but a poor affinity for fructose (K_m = 11, 22 and 9 mM). All three hexokinases had a tenfold higher affinity for ATP (K_m = 90, 280 and 560 μM) than for other nucleoside triphosphates. HK1 and HK2 were both inhibited by ADP (K_i = 40 and 108 μM) acting competitively to ATP. HK1, but not HK2, was inhibited by glucose-6-phosphate, which acted non-competitively to glucose (K_i = 4.1 mM). HK1 and HK2 differed, in that HK1 had a narrower pH optimum, a higher affinity for its substrate, and showed inhibition by glucose-6-phosphate. The relevance of these properties for the regulation of hexose metabolism in vivo is discussed.

Key words: Fructokinase – Hexokinase – *Solanum* – Sucrose metabolism

Introduction

Since hexokinase and fructokinase catalyse irreversible reactions in vivo (Kruger 1990), excess activity will presumably lead to an accumulation of phosphorylated intermediates and a depletion of inorganic phosphate (P_i) in the metabolic compartment in which they are located. Regulation will be needed, otherwise P_i will become limiting, for example, for ATP synthesis. In addition, the relative activity of the hexokinases and the transport carriers on the vacuole tonoplast will determine whether hexoses are respired, or are accumulated for storage or osmotic purposes. It is at present not known how plants regulate the flux at this important branch point.

Previous studies of hexose-phosphorylating enzymes have shown that they are usually relatively specific for fructose (and related ketoses) or for glucose (and related aldoses like mannose). The K_m for their preferred substrate usually lies in the submillimolar range (Turner and Turner 1980; Baldus et al. 1981; Turner and Copeland 1981; Miernyk and Dennis 1983; Copeland and Turner 1987; Doehlert 1989; Schnarrenberger 1990). Since the overall tissue concentration of hexoses in plant tissues is usually much higher (see e.g. values for potato tuber in Renz et al. 1993), it is unlikely that these enzymes will be limited by the concentration of their sugar substrate, unless the local concentration is lower as a result of metabolic compartmentation.

Plant hexose-phosphorylating enzymes often have a wide specificity for nucleotides (Doehlert 1989 and 1990; Schnarrenberger 1990). However, their K_m for ATP usually lies in the range of 50–100 μM (see above references, also Turner and Turner 1980; Miernyk and Dennis 1983;

Abbreviations: FK = fructokinase; Fru6P = fructose-6-phosphate; Glc6P = glucose-6-phosphate; HK = hexokinase; NTP = nucleoside triphosphate; P_i = inorganic phosphate; UDPGlc = uridine-5′-diphosphoglucose

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Copeland et al. 1984), making it unlikely that they will be ATP-limited under normal conditions. The estimated ATP concentrations in the cytosol, plastid and mitochondria in leaves are approx. 3, 1, and 0.5 mM respectively (Stitt et al. 1982), and comparable overall amounts of ATP are found in a wide range of other tissues (Wagner and Backer 1992). The discovery of UTP-utilizing fructokinases (Huber and Akazawa 1986; Doehlert 1990) led to the suggestion that an UTP-utilizing fructokinase operates in a cycle with sucrose synthase and uridine-5′-diphosphoglucose pyrophosphorylase (UDPGlc pyrophosphorylase) to utilize the UTP which is generated during sucrose mobilization by the latter two enzymes (Huber and Akazawa 1986). It is controversial whether the in vivo UTP concentrations are high enough to allow rapid operation of this pathway (see Baysdorfer 1989; Doehlert 1989 and 1990; Dancer et al. 1990a; Schnarrenberger 1990).

Relatively little is known about the susceptibility of plant hexose-phosphorylating enzymes to product or feedback inhibition. ADP inhibits fructokinases and glucokinases in pea seeds (Turner et al. 1977a; Copeland et al. 1978; Turner and Copeland 1981), maize (Doehlert 1989) and wheat seedlings (Higgins and Easterby 1974) but the concentrations needed were often unphysiologically high. There have been relatively few investigations of the response to their sugar-phosphate products. Fructokinase from pea seeds (Copeland et al. 1984) and spinach leaves (Schnarrenberger 1990) is inhibited by fructose-6-phosphate (Fru6P), but only at relatively high concentrations. Hexokinase is apparently unaffected by glucose-6-phosphate (Glc6P; Higgins and Easterby 1974; Turner et al. 1977a; Turner and Copeland 1981; Doehlert 1989). No evidence exists for significant inhibition of hexokinase or fructokinase by other metabolic intermediates. There is, therefore, little evidence to indicate that these enzymes are subject to feedback regulation in vivo. Several studies have reported that fructokinase is inhibited by high concentrations of its substrate, fructose (Higgins and Easterby 1974; Turner et al. 1977b; Copeland et al. 1978; Baysdorfer et al. 1989; Doehlert 1989), but the physiological significance of this effect is not clear.

The following paper investigates the substrate specificity and product inhibition of the six forms of hexokinase and fructokinase which we have separated from potato tubers. Our main aims were (i) to establish whether physiological concentrations of metabolites are likely to exert significant feedback regulation over their activity in vivo and (ii) to ask whether there are differences in their kinetic properties which could provide insights into their respective roles in vivo. The reasons for our choice, of potato tubers as an experimental system are given in the previous article (Renz et al. 1993).

Material and methods

Hexokinases and fructokinases were partially purified from large growing tubers of *Solanum tuberosum* L. cv. Desirée, as in Renz et al. (1993). Hexokinase and fructokinase were assayed by three different methods. (i) Glc6P or Fru6P production was coupled to $NADP^+$ as in Renz et al. (1993), modifying the concentrations of substrates as in the table and figure legends. (ii) The phosphorylation of other sugar substrates, and product inhibition by Glc6P or Fru6P was investigated using an assay modified from Turner et al. (1977a), which contained (in 1 ml) 50 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate, 17 nkat pyruvate kinase, 17 nkat lactate dehydrogenase, and ATP and hexose sugar as in the table and figure legends. (iii) To allow study of the simultaneous presence of ADP and hexose-6-phosphate, a radioactive assay was modified from Newsholme et al. (1967). The assay contained (in 100 µl), 20 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, D-[U-^{14}C] glucose or -fructose (105 Bq · µmol), ADP and Glc6P or Fru6P as stipulated in the figure legend. The assay was started by adding 10 µl hexokinase (HK) or fructokinase (FK) preparation, and terminated after 4 min by adding 50 µl 0.6 N HCl. In control incubations, ATP was not added until after the acid-stop. An aliquot (100 µl) was then applied to a micro anion-exchange column (BioRad AG 1–X2 200–400 mesh; Richmond, Va., USA; Quick et al. 1989), washed with 2×1000 µl H_2O, and then the ^{14}C-hexose-phosphate eluted with 1×100 and 2×1000 µl 2 N HCl, and an aliquot counted. The commercial preparations of ^{14}C-hexose sugar contained traces of acid contaminants and were passed over anion-exchange columns before use to decrease the background. The incorporation of radioactivity was linear for 5–6 min with this assay when crude extracts were used, and for at least 15 min for all partially purified preparations. In all incubations, the amount of enzyme added was adjusted so that less than 30% of the ^{14}C-hexose substrate was utilized.

To illustrate the relative specificity of an enzyme form for different hexose sugars compared with glucose, the phosphorylation coefficient (Walker 1966; Turner and Copeland 1981) was calculated as

$$\text{Phosphorylation coefficient} = \frac{V_{max}\,(\text{hexose})}{V_{max}\,(\text{glucose})} \times \frac{K_m\,(\text{glucose})}{K_m\,(\text{hexose}).}$$

To illustrate the relative specificity of an enzyme form for different nucleoside triphosphates (NTPs) compared with ATP, the dephosphorylation coefficient was calculated,

$$\text{Dephosphorylation coefficient} = \frac{V_{max}(\text{NTP})}{V_{max}(\text{ATP})} \times \frac{K_m(\text{ATP})}{K_m(\text{NTP}).}$$

Results

Hexose-dependence. FK1 and FK2 are the two major enzymes in the growing tuber, whereas FK3 is only

Table 1. Kinetic constants of the potato-tuber hexokinases for glucose, mannose and fructose. All assays contained 1 mM ATP. The K_m and V_{max} values were calculated from Eadie-Hofstee plots. Each value is the mean of three separate determinations (SE was 4–12% of the mean)

Parameter	Sugar	Hexokinase		
		HK1	HK2	HK3
$K_{m\,app}$ (mM)	Glucose	0.041	0.13	0.035
	Fructose	11	22	8.7
	Mannose	0.052	0.29	0.038
V_{max} [nkat · (mg protein)$^{-1}$]	Glucose	15	15	0.22
	Fructose	18	8.8	0.28
	Mannose	10	7.2	0.11
Phosphorylation coefficient	Glucose	1.0	1.0	1.0
	Fructose	0.0046	0.0036	0.0053
	Mannose	0.53	0.22	0.45

Table 2. Kinetic constants of the potato-tuber fructokinases for fructose. All assays included 1 mM ATP. K_m, V_{max}, and K_i values were estimated from Eadie-Hofstee plots. Each value is given as mean ± SE ($n = 3$)

Parameter	Fructokinase		
	FK1	FK2	FK3
$K_{m\,app}$ (mM)	0.064 ± 0.010	0.090 ± 0.014	0.10 ± 0.01
V_{max} [nkat · (mg protein)$^{-1}$]	183 ± 33	333 ± 33	13 ± 2
K_i (fructose) (mM)	5.7 ± 0.5	6.0 ± 0.5	21 ± 2

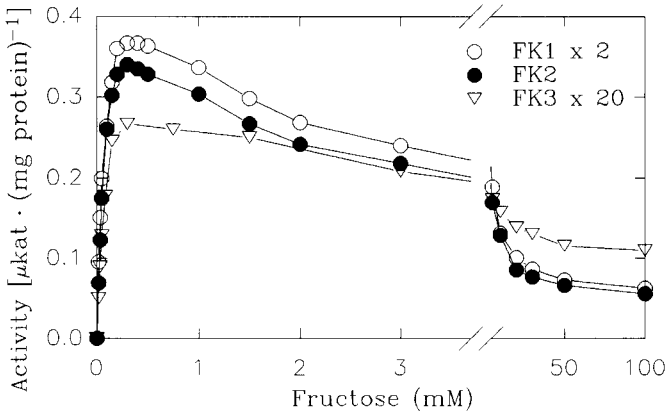

Fig. 1. Fructose dependence of FK1 (○, all values multiplied by 2), FK2 (●) and FK3 (▽, all values multiplied by 20), assayed at 1 mM ATP

present in minor activities in potato tubers, but represents a major activity in leaves. The glucose-specific forms HK1 and HK2 are present at low activities in growing tubers, and increase during storage and sprouting. HK3 was only present at minor activities at all stages of tuber development (Renz et al. 1993).

HK1 and HK3 showed a high affinity for glucose (K_m values are 41 and 35 μM) whereas the affinity of HK2 was threefold lower (Table 1). All three forms showed a relatively high affinity for mannose. In contrast, K_m (fructose) was 200–300 times higher. The V_{max} activities of HK1, HK2 and HK3 were in a similar range for all three sugars. The estimated values for the phosphorylation coefficient (Walker 1966; Turner and Copeland 1981) emphasize the high selectivity of all three enzyme forms for glucose, compared with fructose.

The fructokinases showed no detectable activity with glucose or mannose up to concentrations of 50 mM (data not shown). Instead, they had a high affinity for fructose [apparent K_m in the range of 0.06–0.1 mM (Table 2)]. All three forms were inhibited by high fructose (Fig. 1), with a K_i of about 6 mM for FK1 and FK2, and 21 mM for FK3. The inhibition by fructose was partial.

Nucleotide dependence. It is known that hexokinases are Mg^{2+} dependent, but are also inhibited by excess free Mg^{2+} (Turner and Turner 1980). In preliminary experiments in which we varied the ATP concentration between 0.1 and 1 mM we found that all hexokinase and fructokinase forms showed their maximal activity when the total Mg^{2+} concentration in the assay was about 1.5 mM higher than the concentration of ATP (data not shown). The following analysis of nucleotide specificity was carried out with a 1.5 mM excess of Mg^{2+} above ATP in all assays, plus 1 mM glucose or 0.4 mM fructose.

All three hexokinase forms had a distinctly higher affinity for ATP than for other NTPs, and also showed a higher V_{max} with ATP (Table 3). The dephosphorylation coefficient (see *Methods*) was 13- to 50-fold higher with ATP, emphasizing that ATP is the clearly preferred substrate. Interestingly, the ATP affinity of HK1 was higher than that of HK2.

All three fructokinases also had a higher affinity for ATP than for other NTPs. (Table 3). The V_{max} (NTP) was slightly higher for UTP in the case of FK1 and FK2, but calculation of the dephosphorylation coefficients again emphasized that ATP is the preferred substrate. It might be noted that the tuber-specific forms, FK1 and FK2,

Table 3. Kinetic constants of potato-tuber hexokinases and fructokinases for nucleoside triphosphates. All assays were carried out with 1 mM glucose or 0.4 mM fructose, and with a total Mg^{2+} concentration 1.5 mM above the added NTP concentration. The values of K_m and V_{max} were estimated from Eadie-Hofstee plots, and are the mean of three (for ATP) or two (for UTP, GTP and CTP) determinations

Parameter	NTP	Hexose kinases					
		HK1	HK2	HK3	FK1	FK2	FK3
$K_{m\,app}$ (mM)	ATP	0.090	0.28	0.56	0.026	0.025	0.24
	UTP	3.5	3.0	4.5	0.65	0.64	1.6
	GTP	2.2	0.75	1.3	0.31	0.20	0.64
	CTP	3.1	1.0	3.2	0.52	0.29	2.3
V_{max} [nkat · (mg protein)$^{-1}$]	ATP	13	15	0.27	167	317	13
	UTP	5.5	6.2	0.22	250	450	8.3
	GTP	5.5	2.8	0.040	300	517	11
	CTP	9.2	3.7	0.082	145	300	9.2
Dephosphorylation coefficient	ATP	1.0	1.0	1.0	1.0	1.0	1.0
	UTP	0.026	0.040	0.10	0.060	0.056	0.098
	GTP	0.017	0.073	0.065	0.15	0.20	0.30
	CTP	0.020	0.071	0.054	0.044	0.082	0.072

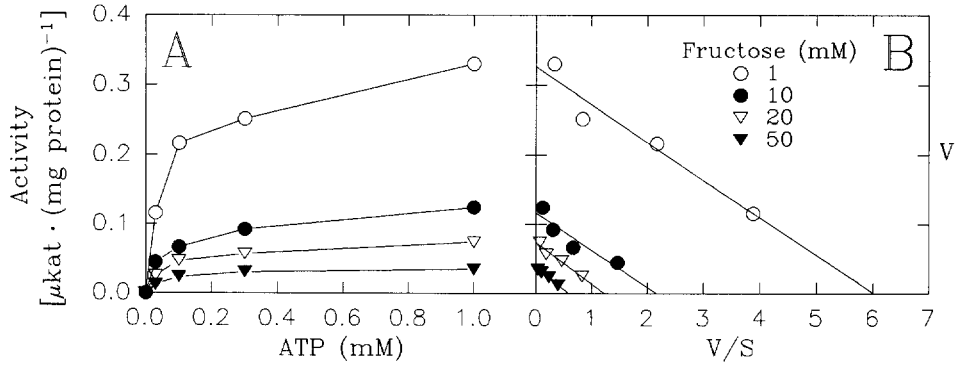

Fig. 2A, B. Influence of fructose concentration on the affinity of potato-tuber FK2 for ATP. **A** Saturation curves for ATP at 1 (○), 10 (●), 20 (▽) or 50 (▼) mM fructose, including Mg^{2+} at 1.5 mM in excess of ATP in all assays. **B** Eadie-Hofstee plot

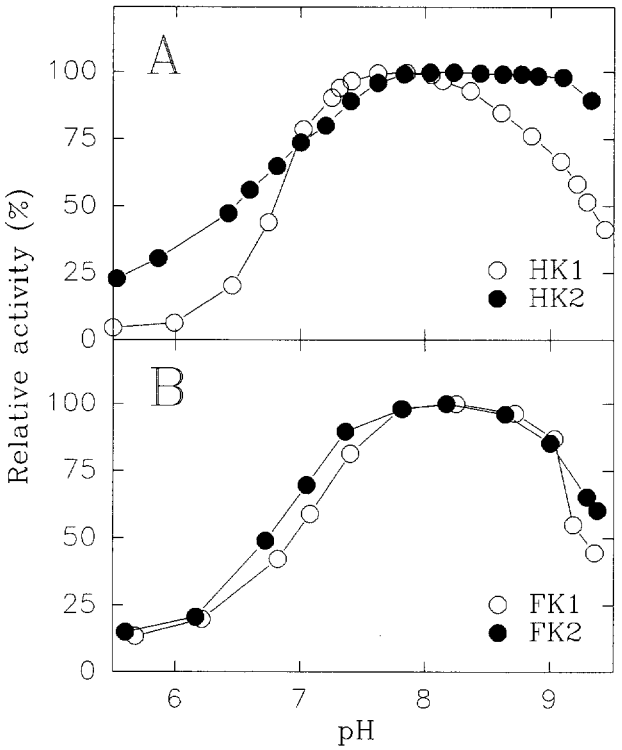

Fig. 3A, B. Dependence on pH of hexokinases and fructokinases from potato tubers. **A** HK1 (○) and HK2 (●). **B** FK1 (○) and FK2 (●). The assays were carried out with 50 mM Mes-KOH (pH 5.0–6.5), 50 mM Imidazole-HCl (pH 6.0–7.4) or 50 mM Tris-HCl (pH 7.0–9.5). All activities are normalized as a percentage of the activity shown by that particular enzyme form at its pH optimum

show very similar kinetic properties whereas the leaf-specific FK3 differs, having a tenfold lower affinity for ATP, and a two- to three-fold lower affinity for UTP.

Since fructose is a strong substrate inhibitor, we investigated the ATP saturation at four different fructose concentrations for FK2 (Fig. 2) and FK1 (data not shown, similar results were obtained as for FK2). A strong fructose inhibition was found at all ATP concentrations. The apparent K_m (ATP) was largely independent of the fructose concentration. Similar results have been obtained for pea seed fructokinase (Turner et al. 1977b).

pH-dependence. Whereas HK2 had a broad pH response, the activity of HK1 decreased quite strongly between pH 7.5 and pH 6.5 (Fig. 3A). Both FK1 and FK2 also showed a marked decline in activity over this range (Fig. 3B).

Effect of nucleotides and hexosephosphates on hexokinases. In an initial screening experiment, a range of metabolites and nucleotides were added to a standard assay containing 1 mM ATP and 1 mM glucose at pH 8 (Table 4). HK1, HK2 and HK3 were all inhibited four- to fivefold by 0.5 mM ADP. They were inhibited more weakly by UDP, AMP and UMP, 10 mM UDPGlc inhibited by 10–20% (data not shown), but 10 mM Glc6P or Fru6P had no effect. The assays were repeated at pH 7. Simular results were obtained at this pH, except that HK1 (but not HK2) was inhibited by $69 \pm 5\%$, ($n = 3$) by 10 mM Glc6P at pH 7.

Table 4. Influence of metabolites and nucleotides on activity of hexokinases and fructokinases from potato tubers. All measurements were carried out at pH 8 and 2.5 mM total $MgCl_2$, 1 mM ATP and 1 mM glucose or either 50 μM or 5 mM fructose. All values are the mean of two determinations. All nucleoside diphosphates or monophosphates were added with equimolar $MgCl_2$

Metabolite	Relative activity (%)								
	HK1	HK2	HK3	FK1		FK2		FK3	
				50 μM Fru	5 mM Fru	50 μM Fru	5 mM Fru	50 μM Fru	5 mM Fru
10 mM Glc6P	100	100	100	100	100	100	100	100	100
10 mM Fru6P	100	100	100	36	56	37	34	39	38
1 mM MgADP	21	25	29	87	31	79	43	90	81
1 mM MgAMP	71	77	80	90	64	85	74	96	84
1 mM MgUDP	51	49	62	88	70	95	81	95	84
1 mM MgUMP	89	97	92	93	81	97	91	97	90
10 mM UDPGlc	88	79	91	100	100	102	102	99	100

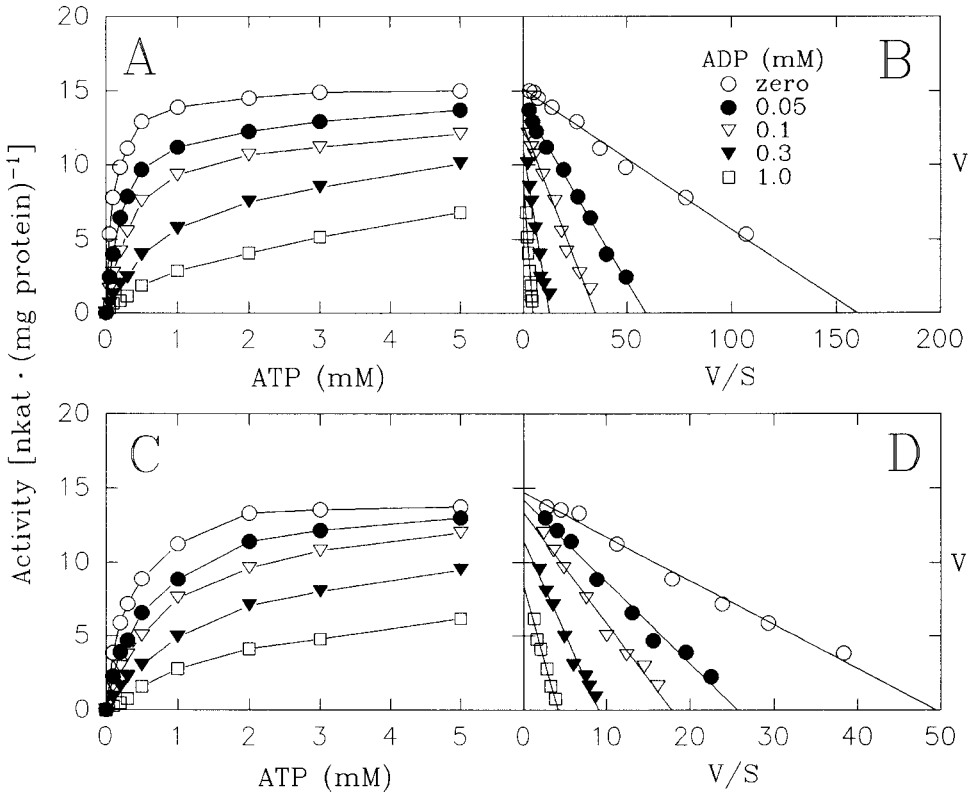

Fig. 4A–D. Inhibition by ADP of HK1 and HK2 from potato tubers. **A** ATP saturation curves for HK1 at zero (○), 0.05 (●), 0.1 (▽), 0.3 (▼) or 1 (□) mM ADP and **B** Eadie-Hofstee plot of the data. **C** ATP saturation curves for HK2 at different ADP concentrations (symbols as in **A**) and **D** Eadie-Hofstee plot of the data. All assays included 1 mM glucose and 1.5 mM more Mg^{2+} than the sum of ATP plus ADP

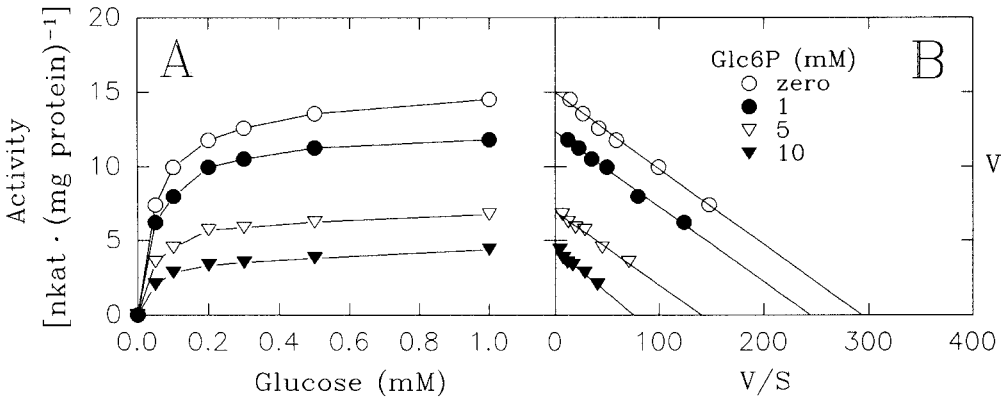

Fig. 5A, B. Inhibition of HK1 by Glc6P. **A** Glucose saturation curves at zero (○), 1 (●), 5 (▽) or 10 (▼) mM Glc6P and **B** Eadie-Hofstee plot of the data. All assays included 1 mM ATP at pH 7.0

Figure 4 shows the product inhibition of HK1 and HK2 by ADP in more detail. As already stated, the affinity for ATP is two- to threefold higher in HK1 than HK2. For both forms, ADP inhibited competively to ATP, with a small non-competitive component (Fig. 4) The K_i (ADP) values of 40 μM and 108 μM for HK1 and HK2, respectively, are two- to threefold below the K_m (ATP) for each enzyme, emphasizing that both enzymes will already be about 50% inhibited at an ATP/ADP ratio of 3, and will be increasingly strongly inhibited at lower ratios.

Glucose-6-phosphate inhibits HK1 non-competitively to glucose (Fig. 5) with a K_i value of 4.1 mM. The Glc6P content in the cytosol of spinach leaves has been estimated to be about 6–12 mM (Gerhardt et al. 1987). The overall content in potato tubers [100–200 nmol · (g FW)$^{-1}$, see Hajirezaei and Stitt 1991; Jelitto et al. 1992] would be equivalent to a concentration of 3–7 mM if we assume it to be equally distributed in the cytosol and the plastid occupying a volume similar to that estimated for leaves. To our knowledge, this is the first report that a plant hexokinase is inhibited by near-physiological concentrations of Glc6P.

Effects of nucleotides and hexose phosphates on fructokinases. The three fructokinase forms were screened in a standard assay at pH 8 with 1 mM ATP and either 50 μM or 5 mM fructose (Table 4). Similar results were also obtained for FK1 and FK2 at pH 7 (data not shown). FK3 was weakly inhibited by 1 mM ADP and other nucleotides, whereas FK1 and FK2 were more strongly inhibited. In all cases, ADP was the most effective inhibitor, UDP, AMP and UMP were less effective, and UDPGlc was without effect. When 1 mM ATP was

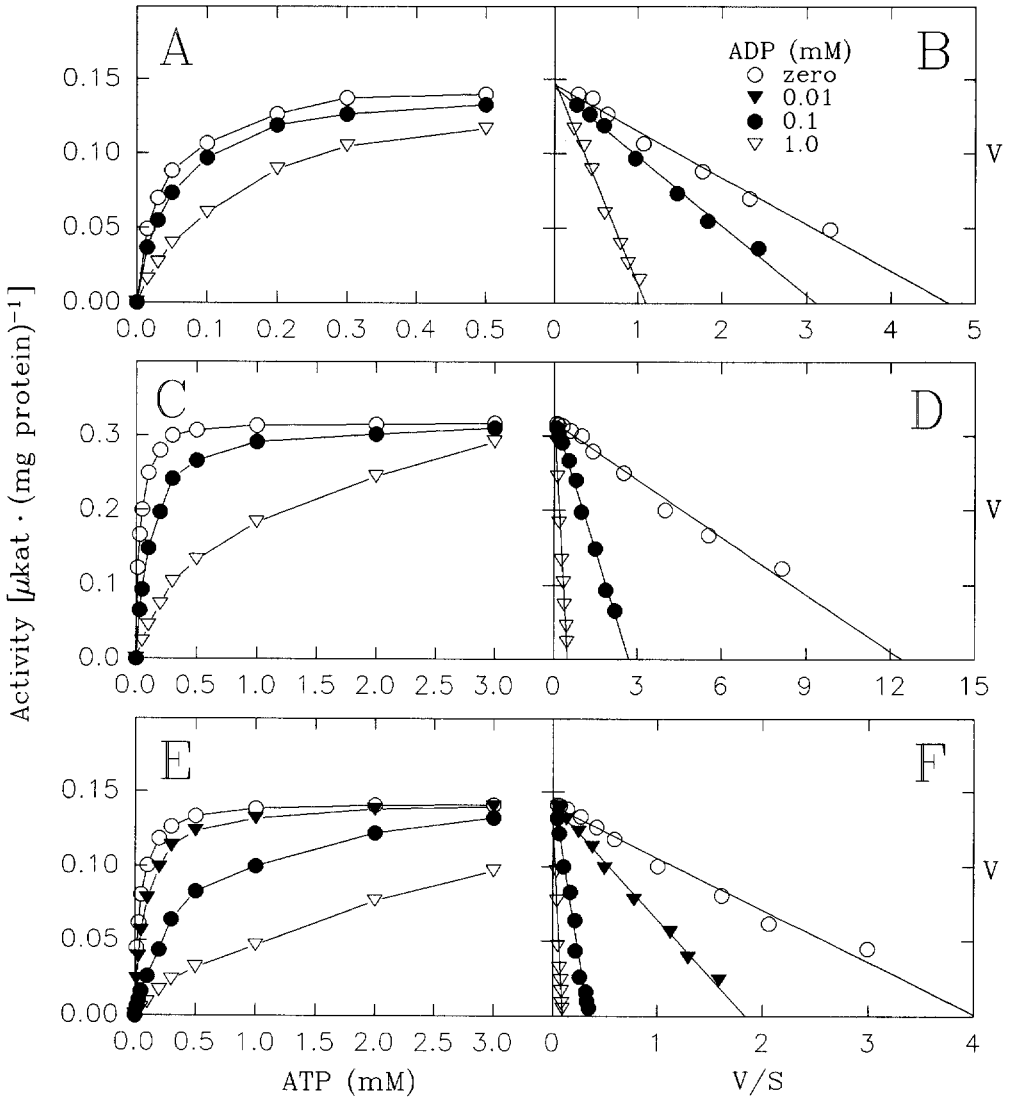

Fig. 6A–F. Inhibition of potato-tuber FK2 by ADP at different fructose concentrations. **A** ATP saturation curves for FK2 at 50 μM fructose with zero (○), 0.1 (●) or 1 (▽) mM ADP and **B** Eadie-Hofstee plot of the data. **C** ATP saturation curves for FK2 at 0.4 mM fructose with different ATP concentrations (symbols as in **A**) and **D** Eadie-Hofstee plot of the data. **E** ATP saturation curves for FK2 at 5 mM fructose with different ATP concentrations [symbols as in **A** plus (▼) for 0.01 mM ADP] and **F** Eadie-Hofstee plot of the data. The Mg^{2+} concentration was always 1.5 mM higher than the sum of ATP and ADP

replaced by 0.2 mM UTP, we found a 40% inhibition with 1 mM UDPGlc (data not shown). Surprisingly, the inhibition by ADP was much stronger when the assay included high fructose (see below for more data). A similar trend was also observed for the other nucleotides (see Table 4). The fructokinases were unaffected by 10 mM Glc6P, but 10 mM Fru6P led in all cases to a 50–70% inhibition of activity. The inhibition by Fru6P was largely independent of the fructose concentration (see below for more data). A 30–45% inhibition was already observed at 1 mM Fru6P (data not shown).

Figure 6 investigates the product inhibition by ADP in more detail for FK2, carrying out the measurements at limiting (50 μM), optimal (0.4 mM) and inhibitory (5 mM) fructose concentrations. As already noted, K_m (ATP) was only slightly affected by the fructose concentration. The inhibition by ADP was competitive to ATP, and became stronger as the fructose concentration increased. A K_i (ADP) of 260 ± 80, 29 ± 1 and 13 ± 5 μM ($n = 3$) was calculated in the presence of 0.05, 0.4 and 5 mM fructose. This can be compared with an apparent K_m (ATP) of 34, 28 and 35 μM in these conditions. It is apparent that ADP will be a poor inhibitor of FK2 at low fructose concentrations, but will become increasing effective at concentrations of 0.4 mM fructose or above. Copeland et al. (1984) reported a mainly non-competitive and weak ($K_i = 0.4$ mM) inhibition of pea seed fructokinase I by ADP with respect to ATP in the presence of low (0.12 mM) fructose. Turner et al. (1977b) observed that ADP inhibits the pea seed enzyme more strongly in the presence of high fructose.

The product inhibition of FK2 by Fru6P was investigated at 0.1 (data not shown) and 1 mM ATP (Fig. 7). Similar results were obtained at both concentrations of ATP (data not shown), indicating that the inhibition is non-competitive to ATP. The interaction of Fru6P was investigated over the range from limiting to super-optimal (inhibitory) fructose. Fructose-6-phosphate led to a partial inhibition of FK1 and FK2 (see Fig. 7). The Eadie-Hofstee plot in the region corresponding to limiting fructose concentrations reveals a largely uncompetitive inhibition with a small competitive component and a K_i (Fru6P) of 1.2 ± 0.3 mM can be estimated. Interestingly, this is about threefold lower than the K_i

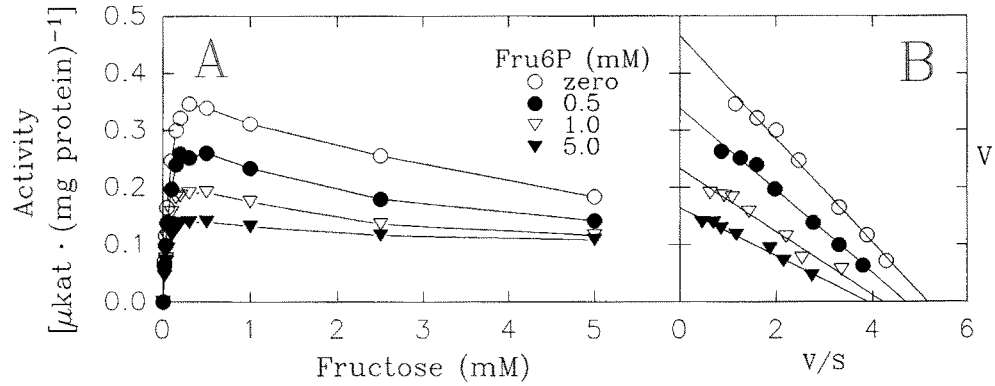

Fig. 7A, B. Inhibition of FK2 by Fru6P: dependence on the fructose concentration. **A** Fructose dependence at zero (○), 0.5 (●), 1 (▽) and 5 (▼) mM Fru6P and **B** Eadie-Hofstee plots of the non-inhibitory (0–0.25 mM fructose) part of the saturation curve. All assays included 1 mM ATP

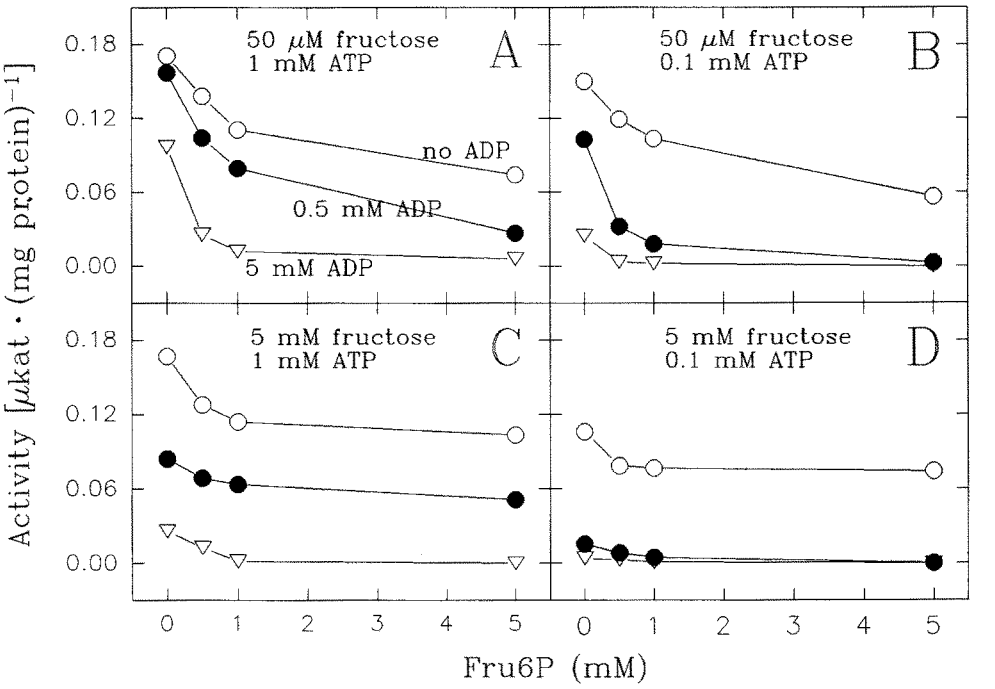

Fig. 8A–D. Inhibition of FK2 by an interaction between fructose, Fru6P and ADP. Fru6P inhibition curves were carried out in the presence of zero (○), 0.5 (●) and 5 (▽) mM ADP, at **A** 50 μM fructose and 1 mM ATP, **B** 50 μM fructose and 0.1 mM ATP, **C** 5 mM fructose and 1 mM ATP, and **D** 5 mM fructose and 0.1 mM ATP. The Mg^{2+} concentration was always 1.5 mM higher than the sum of ATP and ADP

(Glc6P) for HK1. In vivo, Fru6P and Glc6P are present in a ratio of about 1:3, due to the equillibrium constant of the reaction catalyzed by phosphoglucose isomerase. At high (inhibitory) fructose concentrations the Fru6P inhibition is lessened; correspondingly, at high Fru6P concentrations the substrate inhibition by fructose weakened. This might indicate that Fru6P and fructose (as inhibitor) bind at the same site. A similar type of inhibition has been reported for pea seed fructokinase II (Copeland et al. 1984).

The above assays on FK2 were carried out using an assay in which ADP formation was measured via a coupled assay with pyruvate kinase and lactate dehydrogenase. To confirm the inhibition by Fru6P, the assays were repeated on FK1 using a radioactive assay, in which conversion of [^{14}C]-fructose to [^{14}C]-Fru6P was assayed, using anion-exchange columns to separate the [^{14}C]- Fru6P. An identical inhibition was obtained with a partial, non-competitive inhibition and a K_i (Fru6P) of 1.3 ± 0.3 mM (data not shown). The inhibition of FK3 by Fru6P showed a similar pattern, but with a higher K_i (4.0 ± 0.3 mM; data not shown).

In a final set of measurements we investigated the interaction between fructose, ATP, ADP and Fru6P for FK2, using the radioactive assay (Fig. 8). Activity was assayed with limiting (50 μM) and inhibitory (5 mM) fructose, and with 0.1 mM (just saturating) and 1.0 mM (super-saturating) ATP. In each condition we added 0, 0.5 or 5 mM ADP, and tested the effect of increasing Fru6P between 0 and 5 mM. The results confirm that Fru6P inhibits mainly independently of the ATP concentration, that high fructose increases sensitivity to ADP, and that high fructose and Fru6P do not inhibit in an additive manner. It also shows that Fru6P and ADP inhibit in an additive, or in some conditions, synergistic fashion.

Effects of other metabolites. The following metabolites were tested with all three hexokinases and all three fructokinases, and were without effect: 3-phosphoglycerate

(10 mM), phosphoenolpyruvate (5 mM), fructose-1,6-bisphosphate (5 mM), P_i (10 mM), inorganic pyrophosphate (1 mM), fructose-2,6-bisphosphate (Fru2, 6bisP) (10 μM). We also added 10 μM Fru2, 6bisP to a desalted crude extract and found no inhibition of activity, indicating that these enzymes are not regulated via a Fru2, 6bisP-binding protein of the type found in liver (Niemeyer et al. 1987).

Discussion

Implications of the specificity for hexose. The enzymes in potato tuber possess a very high selectivity between glucose and fructose. We were not able to detect any activity of the fructokinases with glucose, even at high concentrations. The hexokinases reacted with mannose at a slightly lower affinity than glucose, but had an extremely low affinity for fructose. For this reason we refer to these enzymes as hexokinases, rather than glucokinases (Doehlert 1989). A similar high selectivity between glucose and fructose has been seen in all previous studies which have achieved an exhaustive separation of enzymes (Higgins and Easterby 1974; Turner and Copeland 1981; Doehlert 1989) and is functionally important because it allows the use of the two major hexose sugars to be regulated independently.

All of the enzymes had an extremely high affinity for their preferred hexose substrate with a K_m near or below 100 μM (Tables 1, 2). This is much lower than the overall concentration of glucose and fructose in potato tubers (see Table 5 in Renz et al. 1993). Taken in conjunction with the high activities of hexokinases and fructokinases, relative to metabolic fluxes and pools in the tuber (Renz et al. 1993), it is evident that the hexokinases and fructokinases must either (i) be very effectively down-regulated by metabolic control, or (ii) the concentrations of glucose and fructose must be maintained at a very low concentration in the metabolic compartments, due to active compartmentation into the vacuole.

Implications of the nucleotide specificity. All six enzyme forms used ATP in preference to the other NTPs. For the four major tuber enzyme forms (HK1, HK2, FK1, FK2), the K_m (ATP) was at least tenfold lower than the K_m for any other adenine nucleotide. The fructokinases from spinach leaves (Schnarrenberger 1990), barley leaves (Baysdorfer et al. 1989) and avocado fruit (Copeland and Tanner 1988) also show a much higher affinity for ATP than UTP.

A growing potato tuber is an ideal example of a tissue which needs to recycle UTP to UDP at high rates, because it degrades sucrose via sucrose synthase and UDPGlc pyrophosphorylase (Morrell and ap Rees 1986). The relative levels of ATP and UTP are not known in potato tubers, but comparison with other tissues (Dancer et al. 1990a; Wagner and Backer 1992) indicates that ATP is likely to be severalfold higher than UTP, and that activity of FK1 or FK2 with UTP will therefore be insignificant in vivo. Our results do not support for the scheme which was proposed for cycling of UTP to UDP by Huber and Akazawa (1986) and Xu et al. (1989). Spinach leaves contain enough nucleoside-5′-diphosphate kinase in their cytosol to allow direct equilibration of the adenine- and uridine-nucleotide pools (Dancer et al. 1990a). More studies are needed to establish whether growing potato tubers also contain nucleoside-5′-diphosphate kinase.

Implications of differences between enzyme forms. There were some clear differences between the properties of the various hexokinases and fructokinases. HK1 had a threefold higher substrate affinity and sharper pH optimum than HK2, and was inhibited by Glc6P, whereas HK2 was not. Pairs of hexokinases with high and low affinity for both substrates have also been reported in maize endosperm (Doehlert 1989) and spinach leaves (Schnarrenberger 1990). Hexokinase I and II of pea seed differed in their K_m (ATP) (Turner et al. 1977a; Turner and Copeland 1981). The presence of high-affinity and low-affinity hexokinase forms therefore appears to be a widespread phenomenon in plants. HK3 which was a very minor form, differed from HK1 and HK2 in having a sugar affinity similar to HK1 and a low affinity to ATP like HK2.

The two major tuber fructokinases, FK1 and FK2, had remarkably similar properties with respect to substrate affinity, specificity, fructose inhibition, and product inhibition. Doehlert (1989) also found two very similar fructokinases in maize seedlings. The leaf isoenzyme, FK3 differed in having a relatively higher affinity for UTP, and higher inhibition constants for fructose and Fru6P.

Possible significances of the regulatory properties of the hexokinases. For both HK1 and HK2, K_i (ADP) is only threefold lower than K_m (ATP). It is therefore unlikely that ADP will be an effective inhibitor in aerobic conditions, when high ATP/ADP ratios are expected in the cytosol (Stitt et al. 1982; Wagner and Backer 1992). The inhibition by ADP would, however, provide an effective mechanism to prevent overconsumption of ATP under anaerobic conditions, when the ATP/ADP ratio falls below unity in the cytosol (see above references).

HK1 is non-competitively inhibited by Glc6P, with a K_i (Glc6P) of about 4 mM at pH 7. Since the in-vivo concentration of Glc6P may be in or above this range, (see *Results*), it is possible that HK1 is subject to feedback regulation in vivo. Intriguingly, HK1 increased selectively in cold-stored and especially, in sprouting tubers. The physiological significance is difficult to assess at present, because the Glc6P inhibition depends on the pH, and because the tubers always contained some HK2 (which is apparently non-regulated). More information is needed about the molecular basis of the difference between HK1 and HK2, and their subcellular location.

Possible significance of the regulatory properties of the fructokinases. Provided that fructose was above 0.4 mM the K_i (ADP) of FK1 and FK2 was about twofold lower than the K_m (ATP). This resembles HK1 and indicates (see discussion above) that product inhibition by ADP is

unlikely to regulate fructokinase activity under aerobic conditions, but could regulate ATP consumption in anaerobic conditions. We will return to the case of low fructose later.

The K_i (Fru6P) of about 1 mM is low enough to allow feedback inhibition of the fructokinases in vivo. The inhibition is independent of pH and affects both major fructokinases in potato tubers. However, since Fru6P acts as a partial non-competitive inhibitor with respect to fructose, it seems unlikely that rising Fru6P, on its own, would ever inhibit the enzyme totally. A complete inhibition is possible (see Fig. 8) in combination with a low ATP/ADP ratio. This indicates that the feedback regulation of fructokinase would become very effective in anaerobic conditions if hexose-phosphates were being rapidly generated from another source, and were not being removed by phosphofructokinase. Usually, however, the Fru6P level falls during anaerobiosis (Turner and Turner 1980).

Interpretation of the product-regulation of the fructokinases is complicated by the substrate inhibition, which develops at high fructose. This is a very general property of plant fructokinases (see also Copeland et al. 1984; Baysdorfer et al. 1989; Doehlert 1989; Schnarrenberger 1990). The complication arises because high fructose concentrations modify the susceptibility of FK1 and FK2 to inhibition by their products. For Fru6P, the inhibition becomes somewhat weaker at high fructose; for ADP, the reverse occurs: ADP is an effective inhibitor (K_i = 13–25 μM) at high (0.4–5 mM) fructose, but is a rather ineffective inhibitor at low (50 μM) fructose [K_i = 260 μM, which is tenfold higher than the K_m (ATP)]. It should, however, be noted that FK2 will still be sensitive to inhibition by ADP at low fructose concentrations, provided 1–5 mM Fru6P is also present (Fig 8A, 8B). This indicates that a low ATP/ADP ratio will be able to modulate fructokinase activity in vivo at low, as well as high, fructose concentrations.

Does the fructose inhibition itself play a role in vivo? Evidence indicating that excessive release of fructose in the cytosol can inhibit fructokinase activity and lead to accumulation of fructose has been provided by studies of transgenic plants which express invertase from yeast in their cytosol; in spinach leaves (Sonnewald et al. 1991), and potato leaves and tubers (Heineke et al. 1992) fructose accumulated to concentrations of 20–50 mM, whereas glucose did not accumulate. A selective accumulation of fructose was not observed when the sucrose was hydrolysed by invertase expressed in the cell wall or vacuole, possibly because the rate of entry of hexoses into the cytosol was limiting and a high fructose concentration did not develop.

Accumulation of fructose will not be a very effective way of decreasing sucrose breakdown, when sucrose is being degraded via an irreversible reaction catalyzed by an alien invertase. However, in wildtype tubers, where sucrose is degraded via a reversible reaction catalyzed by sucrose synthase, an increase of the fructose concentration would provide a very effective mechanism to slow down or even reverse sucrose breakdown (Geigenberger and Stitt 1993). It can be envisaged that substrate inhibition of fructokinase by fructose could provide a mechanism to coordinate the use of fructose with the rate of sucrose breakdown via sucrose synthase. However, studies of compartmentation are still be needed to confirm this idea; the overall fructose content (0.25–1 mM) in growing tubers would not be high enough to strongly inhibit FK1 or FK2 unless the fructose is excluded from the vacuole. On the other hand, in cold-stored and sprouting tubers considerably higher concentrations of fructose are found and would have a considerable inhibitory effect on FK1 and FK2 unless they were actively accumulated in the vacuole.

Concluding remarks. Potato tubers contain a spectrum of six hexokinases and fructokinases which have a very high affinity and selectivity between glucose and fructose. These enzymes are probably regulated in a very effective manner by changes of the ATP/ADP ratio under anaerobic conditions, but not during normal aerobic metabolism. Some potential for inhibition by accumulating hexose-phosphate exists, but it appears unlikely that these properties alone could reduce their activity by 10- to 20-fold, the amount which seems necessary to decrease their activity in line with the fluxes occuring in growing tubers, or to allow the large accumulation of free hexoses which is observed in stored tubers. It is possible that metabolic cycles (Dancer et al. 1990b; Geigenberger and Stitt 1991) play a role in adjusting the net flux into the pool of phosphorylated metabolites. However, it will also be important in the future to learn more about the compartmentation of hexoses, and about how the uptake and release of hexose from the vacuole is regulated.

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