The mechanism of guanosine triphosphate depletion in the liver after a fructose load

The role of fructokinase

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A Sephadex G-25 filtrate of a 100000g supernatant of rat liver homogenate was shown to be able to phosphorylate fructose, with GTP as the phosphate donor. Attempts to separate ATP- and GTP-dependent fructokinase activities failed, indicating that there is a single enzyme able to use both nucleotides. With a partially purified enzyme, K_m values for fructose of 0.83 and 0.56 mM were found with ATP and GTP as substrates respectively. K_m values of 1.53 and 1.43 mM were found for GTP and ATP respectively. Both ADP and GDP inhibited the GTP- and ATP-dependent fructokinase activity. We conclude that the depletion of hepatic GTP caused by intravenous administration of fructose to mice and rats can be explained simply by the utilization of the nucleotide by fructokinase.

The depletion of hepatic ATP as the result of the parenteral administration of D-fructose is a well-known phenomenon (for review see Van den Berghe, 1978). The explanation for this observation is that the high rate of utilization of the sugar by fructokinase results in an intracellular accumulation of fructose 1-phosphate and a consequent depletion of P_i . The latter effect is thought to limit the rephosphorylation of ADP by the mitochondria.

The hepatic content of GTP is also reported to be lowered by the intravenous administration of D-fructose to mice or rats (Van den Berghe *et al.*, 1977, 1980). The depletion of GTP is assumed to be the result of the utilization of the nucleotide by triokinase for the phosphorylation of D-glyceraldehyde formed by the aldolytic cleavage of fructose 1-phosphate. The involvement of fructokinase in the depletion of GTP has been ruled out (Van den Berghe *et al.*, 1977; Van den Berghe, 1978), because Adelman *et al.* (1967) have reported that the enzyme has an absolute specificity for adenine nucleotides.

We have re-investigated the latter claim and have found that GTP is indeed a substrate for fructokinase. On the basis of the kinetic properties

of the enzyme with GTP as a substrate, we believe that the use of hepatic GTP by fructokinase *in vivo* is much more likely to result in the depletion of the nucleotide than its utilization by triokinase.

Materials and methods

Animals and diets

Mature male Wistar rats (250–350 g) were fed *ad libitum* on a normal laboratory diet.

Reagents

All enzymes and substrates were obtained from Sigma Chemical Co., Poole, Dorset, U.K., or Boehringer Corp., Lewes, Sussex, U.K. Substrates were in the form of their sodium salts. Inorganic reagents and solvents were of AnalaR grade from BDH Chemicals, Poole, Dorset, U.K. [U - ^{14}C]-Fructose (sp. radioactivity 330 Ci/mol) was from Amersham International, Amersham, Bucks., U.K.

Isolation and incubation of hepatocytes

Hepatocytes were prepared, incubated in the presence and absence of fructose, and the incubations terminated with $HClO_4$ as described previously (Mapungwana & Davies, 1982).

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Partial purification of fructokinase

Fructokinase was purified essentially by the method described by Sánchez *et al.* (1971a), except that a DEAE-cellulose-chromatography step (Adelman *et al.*, 1967) was added after the $(\text{NH}_4)_2\text{SO}_4$ -precipitation step and before the Sephadex G-100 chromatography. The 30%-satd. $(\text{NH}_4)_2\text{SO}_4$ -extraction step described by Sánchez *et al.* (1971a) was omitted. The purified enzyme preparation was stable for several months at -20°C . The preparation was free of adenylate kinase and nucleoside diphosphate kinase activities, measured by the methods of Bergmeyer *et al.* (1974) and Mourad & Parks (1966) respectively. The acid and heat treatment used in the purification procedure destroys ATPase and sorbitol dehydrogenase activities (Adelman *et al.*, 1967). There was no evidence for any breakdown of [^{14}C]fructose 1-phosphate by phosphatase in the purified enzyme preparation.

Analytical methods

(a) *Assays for fructokinase.* In the enzyme purification, the assay procedure used was that described by Sánchez *et al.* (1971a) involving the coupling of the fructose-dependent generation of ADP with phosphoenolpyruvate and pyruvate kinase. The pyruvate formed was assayed colorimetrically after treatment of the mixture with dinitrophenylhydrazine.

For all other studies, a radiochemical assay method was used based on that described by Sánchez *et al.* (1971a). Unless otherwise stated, the incubation mixture (total vol. 0.05 ml) contained 10 mM-Tris/HCl buffer, pH 7.4, 100 mM-KCl, 2 mM-[U- ^{14}C]fructose (2.2×10^5 d.p.m.), ATP or GTP and an equimolar amount of MgCl_2 adjusted to pH 7.4, and enzyme was added to start the reaction. The reaction was stopped by the application of samples to Whatman DE81 filter discs and drying them rapidly. After drying, each disc was washed with 50 ml of distilled water in a Buchner funnel. The discs were then allowed to dry and the radioactive material bound to each disc was assayed by liquid-scintillation counting in a medium (10 ml) containing 5 g of 2,5-diphenyl-oxazole/litre of toluene.

In some cases, the reaction was stopped by the addition of 0.5 vol. of 4.2 M- HClO_4 . In these cases the protein precipitates were removed by centrifugation at 3000 g, for 10 min, the supernatant was neutralized with 4.2 M- K_2CO_3 and re-centrifuged. Portions of the resulting supernatant were applied to Whatman no. 3 chromatography paper, and developed by descending chromatography for 16 h in methoxyethanol/methyl ethyl ketone/3 M- NH_3 (7:2:3, by vol.; Mortimer, 1952). The region

corresponding to fructose 1-phosphate was cut out and the radioactivity associated with the area was determined by liquid-scintillation counting as described above.

Both methods of separating the radioactive product from the substrate gave identical results, except when 0.5 M-KCl was present in the incubation medium, when the high salt content prevented the quantitative adsorption of fructose 1-phosphate to the DE81 discs. In such cases the chromatographic separation method was used routinely.

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

(b) *Determination of metabolites.* Fructose 1-phosphate and ATP were determined by the methods of Eggleston (1974) and Jaworek *et al.* (1974) respectively.

Statistical methods

Where appropriate, the results are expressed as means $\pm$ S.E.M. for three separate liver preparations.

Results

We have previously shown that fructose 1-phosphate accumulates and ATP is depleted very rapidly in isolated hepatocytes incubated with 10 mM-fructose. An initial rate of fructose 1-phosphate accumulation in isolated hepatocytes over a 30 s period was found to be $8.62 \pm 0.90 \mu\text{mol/min per g}$ of cells ($n=3$), whereas the ATP concentration declined from 2.42 ± 0.05 to $1.31 \pm 0.13 \mu\text{mol/g}$ of cells ($n=3$) during the same time period (S. M. Maswoswe & D. R. Davies, unpublished work). The maximum rate of ATP-dependent phosphorylation of 2 mM-[^{14}C]fructose catalysed by a Sephadex G-25 filtrate of a 100000 g supernatant of a rat liver homogenate (Table 1) is somewhat lower than the rate of accumulation of fructose 1-phosphate in isolated hepatocytes ($3.2 \mu\text{mol/min per g}$ of cells after a similar fructose load), but in agreement with estimates of hepatic fructokinase activity in animals fed on a normal laboratory diet previously observed in this laboratory (Pridham & Davies, 1978) and by other workers (Adelman *et al.*, 1967; Sánchez *et al.*, 1971a).

In the course of this study it was found that GTP was an effective substrate for fructokinase (Table 1). Both ATP- and GTP-dependent activities were increased by KCl, but the latter activity was stimulated to a greater extent by the salt. Under the conditions used for the assay, the rate of accumulation of fructose 1-phosphate was linear with time and with protein concentration. The identity of the

Table 1. Phosphorylation of 2mM-[^{14}C]fructose catalysed by a Sephadex G-25 filtrate of a 100000g supernatant of a rat liver homogenate

Substrate	Fructose 1-phosphate produced ($\mu\text{mol}/\text{min}$ per g of liver)		
	No KCl	0.1M-KCl	0.5M-KCl
ATP (6mM)	0.71 ± 0.03	1.46 ± 0.06	1.87 ± 0.08
GTP (6mM)	0.14 ± 0.02	0.47 ± 0.02	0.84 ± 0.10

Table 2. Co-purification of ATP- and GTP-dependent fructokinase

The activity and specific activity of fructokinase were determined by the colorimetric assay described in the Materials and methods section. The activity with GTP was assayed by the radiochemical method and is expressed as a percentage of that with ATP as a substrate, also determined by the radiochemical method. The results of the radiochemical and colorimetric assays were in close agreement. Abbreviation: N.D., not determined.

Purification stage	Volume (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity (units/ mg of protein)	Purification (fold)	Yield (%)	Activity with GTP (% of that with ATP)
Liver homogenate	144	0.434	26	0.017	1	100	N.D.
20000g supernatant	110	0.544	14	0.039	2.3	96	32
pH 5 supernatant	98	0.581	12	0.048	2.9	91	N.D.
Supernatant after heat treatment	85	0.572	6	0.095	5.7	78	N.D.
0-45% satd.-(NH_4) $_2\text{SO}_4$	4.4	6.742	29	0.233	13.9	47	34
DEAE-cellulose	20	1.086	2.8	0.388	22.8	35	34
Sephadex G-100	8	0.824	0.4	2.060	121.2	10	36

[^{14}C]fructose 1-phosphate generated by the GTP-dependent phosphorylation of [^{14}C]fructose was confirmed by column chromatography of the reaction products on a Dowex 1 (X4; 200-400 mesh; borate form) ion-exchange column (Lefewinae *et al.*, 1964). The radioactive product co-chromatographed with an unlabelled fructose 1-phosphate standard and could not be resolved from the product of the ATP-dependent fructokinase reaction (results not shown). No labelled fructose 6-phosphate was detected. Fructose 1-phosphate was also the only labelled product detected when the partially purified enzyme was incubated with [^{14}C]fructose and GTP.

Fructokinase was partially purified by a combination of established procedures (Adelman *et al.*, 1967; Sánchez *et al.*, 1971a). The enzyme was purified 121-fold compared with the crude homogenate, and throughout the purification procedure the ratio of GTP- to ATP-dependent fructokinase activity remained constant at approx. 1:3, when the enzyme was assayed in the presence of 0.1M-KCl (Table 2). Furthermore the ratio of the two activities was constant in all fractions containing fructokinase activity eluted from the DEAE cellulose and the Sephadex G-100 columns (results not shown). Thus there is good evidence to suggest that the two activities are functions of a single protein.

The properties of the partially purified fructokinase were further investigated, and K_m values of 0.83mM for fructose (Fig. 1) and 1.43mM for ATP (results not shown) were found. These values are in good agreement with those obtained by other workers. For example, Sánchez *et al.* (1971a) reported K_m values of 0.80 and 1.33mM for fructose and ATP respectively, determined at KCl concentrations similar to those used in the present study. When GTP was used as the phosphate donor, the K_m values were found to be 0.56mM for fructose (Fig. 1) and $1.53 \pm 0.10\text{mM}$ ($n=3$) for GTP. A typical kinetic analysis with GTP as a substrate is shown in Fig. 2. Neither CTP nor UTP was an effective substrate for the purified fructokinase assayed under similar conditions.

ADP is reported to be a strong competitive inhibitor of ATP-dependent fructokinase activity, with K_i values reported to be between 1.3 and 1.7mM (Parks *et al.*, 1957; Sánchez *et al.*, 1971b). This inhibition was confirmed in the present study (Fig. 2) and a K_i value of 1.1mM was found (Fig. 3). ADP was also found to inhibit the GTP-dependent reaction ($K_i = 0.78\text{mM}$). Adenylate kinase was shown to be absent from the enzyme preparation, eliminating the possibility of the generation of ATP from GTP and ADP during the course of the reaction. We noted that, with less-purified enzyme preparations containing adenylate kinase activity,

Fig. 1. Effect of fructose concentration on (●) ATP- and (○) GTP-dependent fructokinase activity.

The enzyme was purified to the DEAE-cellulose stage and assayed by the radiochemical technique described in the Materials and methods section. The nucleotide triphosphate concentration was 6 mM in each case. (a) Rate plot; (b) Lineweaver-Burk plot.

Fig. 2. Effect of GTP concentration on fructokinase activity assayed in the absence (○) or in the presence of 2 mM-ADP (●).

See Fig. 1 for other details. (a) Rate plot; (b) Lineweaver-Burk plot.

ADP apparently stimulated GTP-dependent fructose phosphorylation, presumably as the result of formation of ATP.

GDP was also found to be an inhibitor of both ATP- and GTP-dependent activities, with inhibition constants of 1.2 mM in each case. Under similar conditions CDP and UDP were without effect on enzyme activity with either phosphate donor (results not shown). Thus there is no evidence from the inhibition studies that there are two different fructokinases. Indeed, since ADP inhibits the GTP-dependent activity and GDP inhibits the ATP-dependent enzyme, there is further evidence for the existence of a single fructokinase with a specificity for both nucleotides.

Discussion

In contrast with the work of Adelman *et al.* (1967), we have obtained good evidence to suggest

that GTP can be utilized at a substantial rate by fructokinase. The failure of previous workers to demonstrate any fructokinase activity in the presence of GTP led Van den Berghe *et al.* (1977) to propose that the fructose-induced fall in GTP concentration is the consequence of the utilization of the guanine nucleotide in the triokinase reaction. In the latter reaction, GTP is used at only 10% of the rate of ATP utilization (Frandsen & Grunnet, 1971). The evidence presented here suggests that the utilization of GTP by fructokinase is much more likely to be the major cause of the depletion of the nucleotide.

The K_m value of the enzyme for GTP is similar to that for ATP, and the tissue concentration of GTP is approx. 1 mM (Van den Berghe *et al.*, 1980). We have shown that in isolated hepatocytes ATP concentrations fall to approx. 1 mM after a very short time interval, and thus, under such conditions, GTP may become an important substrate.

Fig. 3. Effect of ADP concentration on (●) ATP- and (○) GTP-dependent fructokinase activity

The enzyme was purified to the Sephadex G-100 stage. See Fig. 1 for other details. (a) Rate plot; (b) Dixon plot.

The observation that the K_m of the enzyme for fructose is lower with GTP than ATP as substrate also strengthens this view. However, the greater susceptibility of the GTP-dependent phosphorylation reaction to inhibition by ADP would result in a greater diminution of the GTP-dependent activity compared with the ATP-dependent activity *in vivo*. The inhibition of the enzyme by GDP is unlikely to be an important physiological phenomenon, since the tissue concentration of GDP is reported to be $0.04 \mu\text{mol/g}$ of liver (Söling, 1982), a value well below the K_i observed in the present study.

Under physiological conditions of substrates and effectors, 3mM-ATP, 2mM-fructose, 1mM-ADP, 1mM-GTP and 100mM-KCl, it is obvious that ATP would be the preferred substrate for the enzyme, but one would expect a significant

reaction with GTP, which would increase relative to the ATP-dependent reaction as the adenine nucleotide was depleted. Under these conditions we would expect the GTP-dependent activity of fructokinase to be sufficient to explain the depletion of hepatic GTP content observed by Van den Berghe *et al.* (1977, 1980), which was about 13% of the rate of ATP depletion.

Conclusion

It is clear from the evidence presented that GTP is an effective substrate for rat liver fructokinase. Furthermore, under physiological conditions there is sufficient enzyme activity, even in the presence of ADP, to account for the depletion of GTP as a result of a fructose load.

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