Fructose 2,6-bisphosphate and its phosphorothioate analogue

Comparison of their hydrolysis and action on glycolytic and gluconeogenic enzymes

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Purified chicken liver 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase was phosphorylated either from fructose 2,6-bis[2-³²P]phosphate or fructose 2-phosphorothio[³⁵S]thioate 6-phosphate. The turnover of the thiophosphorylated enzyme intermediate as well as the overall phosphatase reaction was four times faster than with authentic fructose 2,6-bisphosphate. Fructose 2-phosphorothioate 6-phosphate was 10–100-fold less potent than authentic fructose 2,6-bisphosphate in stimulating 6-phosphofructo-1-kinase and pyrophosphate:fructose 6-phosphate phosphotransferase, but about 10 times more potent in inhibiting fructose 1,6-bisphosphatase. The analogue was twice as effective as authentic fructose 2,6-bisphosphate in stimulating pyruvate kinase from trypanosomes.

INTRODUCTION

Fructose 2,6-bisphosphate (Fru-2,6- P_2) is a potent stimulator of mammalian and yeast 6-phosphofructo-1-kinase (PFK-1), of plant pyrophosphate:fructose 6-phosphate phosphotransferase (PP_i:PFK) and of trypanosome and leishmania pyruvate kinase; it is also an inhibitor of fructose 1,6-bisphosphatase (FBPase-1). Fru-2,6- P_2 has been detected in all mammalian tissues studied so far, as well as in yeast, fungi and higher plants, but not in prokaryotes. Because of its stimulatory effect on PFK-1, it has been attributed a role in the control of glycolysis, at least under certain conditions. In the liver, the decrease in Fru-2,6- P_2 concentration by glucagon participates in the control of gluconeogenesis by this hormone (for reviews, see [1,2]).

Fru-2,6- P_2 is synthesized by 6-phosphofructo-2-kinase (PFK-2) and hydrolysed by fructose 2,6-bisphosphatase (FBPase-2), which represent different functions of a single protein. The bifunctional enzyme has an M_r of 110 000 and is composed of identical M_r 55 000 subunits. The enzyme can be phosphorylated from [2-³²P]Fru-2,6- P_2 on His-258, suggesting that the bisphosphatase reaction involves a phosphoryl-enzyme intermediate (E-P) [3]. Moreover, the rate of enzyme phosphorylation is more than 100 times the overall rate of the bisphosphatase reaction, consistent with its formation in the reaction mechanism and with the fact that E-P hydrolysis is rate limiting [4].

Phosphorothioate analogues of second messengers and metabolites have proved to be useful tools in the study of metabolism and enzyme function [5]. These include the phosphorothioate analogues of cyclic AMP [5] and inositol 1,4,5-trisphosphate [6] which are resistant to hydrolysis, and guanosine 5'-[γ-thio]triphosphate, which is not hydrolysed by the GTPase activity of G-

proteins [7]. These also include adenosine 5'-[γ-thio]triphosphate (ATPγS), which is hydrolysed 650 times slower than ATP by myosin ATPase [8] and phenylazophenylphosphorothioate, whose hydrolysis by alkaline phosphatase is 1/100 of that of phenylazophenylphosphate [9]. The S_{α} isomer of phosphorothioate cyclic AMP is not hydrolysed by beef heart cyclic AMP phosphodiesterase, and yet it binds to, and activates, cyclic AMP-dependent protein kinase [5]. Several protein kinases catalyse phosphorylation reactions with ATPγS as substrate, but the phosphorylated proteins are often resistant to phosphoprotein phosphatase activity [10]. The sarcoplasmic reticulum ATPase, however, in which an aspartyl phosphate is formed [11], has been reported to hydrolyse ATPγS 5–10 times faster than ATP ([10]; see however [12]).

The phosphorothioate analogue of Fru-2,6- P_2 , fructose 2-phosphorothioate 6-phosphate (thio-Fru-2,6- P_2), was synthesized enzymically. Its hydrolysis by FBPase-2 was compared with that of authentic Fru-2,6- P_2 , as were its effects on the activities of PFK-1, PP_i:PFK-1, FBPase-1 and pyruvate kinase from trypanosomes. The results of this study are presented.

MATERIALS AND METHODS

Materials

[γ-³²P]ATP and [³⁵S]ATPγS were from Amersham International. Rabbit muscle PFK-1, FBPase-1, ATPγS and all other biochemicals were from Boehringer.

Purification of enzymes

PFK-2/FBPase-2 was purified from chicken livers essentially as described [13,14]. The preparation was homogeneous and the specific activity of PFK-2 was 160 munit/mg of protein.

Abbreviations used: Fru-2,6- P_2 , fructose 2,6-bisphosphate; thio-Fru-2,6- P_2 , fructose 2-phosphorothioate 6-phosphate; Fru-6-P, fructose 6-phosphate; Fru-1,6- P_2 , fructose 1,6-bisphosphate; PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PP_i:PFK, pyrophosphate:fructose 6-phosphate phosphotransferase (EC 2.7.1.90); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); FBPase-2, fructose 2,6-bisphosphatase (EC 3.1.3.46); FBPase-1, fructose 1,6-bisphosphatase (EC 3.1.3.11); ATPγS, adenosine 5'-[γ-thio]triphosphate.

Potato PP₁:PFK was purified up to the poly(ethylene glycol) precipitation step as in [15] and rat epididymal adipose tissue PFK-1 was purified as described [16].

Synthesis of [2-³²P]Fru-2,6-*P*₂ and [2-³⁵S]thio-Fru-2,6-*P*₂

To synthesize [2-³²P]Fru-2,6-*P*₂ and [2-³⁵S]thio-Fru-2,6-*P*₂, 2 mM-Fru-6-*P* and 0.2 mM of either [γ -³²P]ATP or [³⁵S]ATP γ S (each at a specific radioactivity of 500 μ Ci/ μ mol) were incubated for 2 h at 25 °C in a final volume of 1 ml containing 50 mM-Hepes, 100 mM-KCl, 20 mM-KF, 5 mM-potassium phosphate, 1 mM-dithioerythritol, 5 mM-MgCl₂, pH 7.1, 1 mg of bovine serum albumin/ml and 5 munit of purified chicken liver PFK-2. For the synthesis of nonradioactive thio-Fru-2,6-*P*₂ the same conditions were used, except that the incubation volume was 2 ml, 10 munit of PFK-2 was included with 5 mM-ATP γ S and the reaction mixture was left overnight at 25 °C. The reaction was stopped by the addition of 1 M-NaOH (final concentration 0.2 M) and the mixture was applied to a column (0.5 cm $\times$ 4 cm) of Dowex AG-1 X8 (Cl⁻ form). The column was eluted with 40 ml of a linear gradient of NaCl (0–0.5 M in 20 mM-NH₄OH). [2-³²P]-Fru-2,6-*P*₂ was eluted at 0.35 M-NaCl, whereas [2-³⁵S]-thio-Fru-2,6-*P*₂ was eluted at 0.45 M-NaCl. The concentration of [2-³²P]Fru-2,6-*P*₂ was estimated by the stimulation of potato PP₁:PFK [15], whereas the [2-³⁵S]-thio-Fru-2,6-*P*₂ concentration was calculated from the specific activity of [³⁵S]ATP γ S. Alternatively, the preparation was hydrolysed [30 min in 7.5 % (v/v) HClO₄ at 25 °C] and, after neutralization, Fru-6-*P* was measured in the supernatant [17].

Labelling of PFK-2/FBPase-2 from [2-³²P]Fru-2,6-*P*₂ and [2-³⁵S]thio-Fru-2,6-*P*₂

Purified chicken liver PFK-2/FBPase-2 (20 μ g) was incubated at 25 °C in a final volume of 0.2 ml containing 20 mM-Tes, 100 mM-KCl, 5 mM-MgCl₂, 1 mM-dithioerythritol, pH 7.5. The reaction was started by the addition of 4 μ M-[2-³²P]Fru-2,6-*P*₂ or [2-³⁵S]thio-Fru-2,6-*P*₂ and aliquots (20 μ l) were removed at the indicated times for the determination of radioactivity covalently bound to the enzyme [18].

The acid hydrolysis of the phosphoryl intermediate of PFK-2/FBPase-2 was measured on enzyme preparations which were labelled as described above, but at 0 °C. Immediately after the addition of radioactive Fru-2,6-*P*₂, the reaction was stopped with 0.1 ml of ice-cold 0.4 M-HClO₄/4 M-urea, to give a pH of 4. The mixture was then transferred to a water bath at 25 °C and aliquots (20 μ l) were removed at the indicated times for the determination of phosphoryl enzyme [18].

Enzyme assays

PFK-1 [19], potato PP₁:PFK [15] and PFK-2 [20] were assayed as described. Crude cytosolic extracts of *Trypanosoma brucei* were prepared as described [21] except that a 40 000 g supernatant was used. Pyruvate kinase was assayed spectrophotometrically at 25 °C [50 mM-triethanolamine/HCl (pH 7.2), 30 mM-KCl, 22.5 mM-MgSO₄, 18 mM-KHCO₃, 2.25 mM-ADP, 2.2 mM-phosphoenolpyruvate, 5 mM-potassium phosphate, 0.42 mM-NADH and 4 units of lactate dehydrogenase/ml]. FBPase-1 was assayed fluorometrically at 20 °C [50 mM-Hepes (pH 7.2), 100 mM-KCl, 2 mM-MgCl₂, 0.5 mM-EDTA, 0.25 mM-NADP⁺, 1.75 units of glucose-6-phos-

phate dehydrogenase/ml, 3.5 units of phosphoglucose isomerase/ml and 15 μ M-Fru-1,6-*P*₂].

For the measurement of FBPase-2 activity, purified chicken liver PFK-2/FBPase-2 (20 μ g) was depleted of bound Fru-6-*P* [4] by incubation (10 min at 30 °C) in 0.2 ml with 2.5 mM-NADP⁺, 17.5 units of glucose-6-phosphate dehydrogenase and 35 units of phosphoglucose isomerase. PFK-2/FBPase-2 was further diluted and 0.2 μ g of enzyme was assayed for FBPase-2 at 30 °C in a final volume of 0.5 ml of 20 mM-Tes (pH 7.5)/100 mM-KCl, 5 mM-MgCl₂, 1 mM-dithioerythritol containing [2-³²P]Fru-2,6-*P*₂ or [2-³⁵S]thio-Fru-2,6-*P*₂ (20 000 c.p.m.) at concentrations indicated in the Figure legends. The reactions were stopped after 15 min with 0.1 ml of 1 M-NaOH. The reaction mixture was heated at 80 °C for 5 min, cooled and applied to a column (1.8 ml) of Dowex AG-1 X8 (Cl⁻ form). [³²P]P_i was eluted from the column with 7 $\times$ 1 ml of 0.15 M-NaCl/20 mM-NH₄OH, whereas [³⁵S]P_i was eluted with 7 $\times$ 1 ml of 0.25 M-NaCl/20 mM-NH₄OH. The first 2 ml of eluate, which did not contain radioactivity, were discarded and radioactivity of the following 5 ml was measured. K_m^{app} and V_{max} were estimated by computer fitting [22]. One unit of activity represents the amount of enzyme which catalyses the formation of 1 μ mol of product per min under the stated conditions. The results are typical of two or three independent experiments.

Protein determination

Protein was estimated by the method of Bradford [23] using bovine serum albumin as standard. To determine the concentration of purified PFK-2/FBPase-2, protein was precipitated with 10 % (w/v) trichloroacetic acid, collected by centrifugation (5000 g $\times$ 5 min) and measured [24] with bovine serum albumin as standard.

RESULTS AND DISCUSSION

FBPase-2 activity towards authentic and thio-Fru-2,6-*P*₂

Quite unexpectedly, thio-Fru-2,6-*P*₂ was found to be a good substrate of chicken liver FBPase-2, but the kinetics of its hydrolysis were different from those of authentic Fru-2,6-*P*₂. Compared with the results obtained with authentic Fru-2,6-*P*₂, the V_{max} and K_m^{app} of FBPase-2 for thio-Fru-2,6-*P*₂ were about 4 and 6–7 fold greater respectively (Fig. 1a).

The addition of the ligands GTP, *sn*-glycerol 3-phosphate and P_i modified FBPase-2 activity on both authentic and thio-Fru-2,6-*P*₂ (compare Figs. 1a and 1b). With authentic Fru-2,6-*P*₂, the K_m^{app} increased from 0.15 to 0.23 μ M and the V_{max} increased by about 50 %. With thio-Fru-2,6-*P*₂, the saturation curve was shifted towards higher concentrations of substrate and the activity measured at 2 μ M was not maximal although it was still about 40 % greater than with authentic Fru-2,6-*P*₂.

The calculated k_{cat} values of chicken liver FBPase-2 for authentic and thio-Fru-2,6-*P*₂ (2.6 min⁻¹ and 10.2 min⁻¹ respectively; no ligands present) were similar to the value (6 min⁻¹) reported by Pilkis' group [4]. The k_{cat}/K_m values calculated with the K_m^{app} obtained from Fig. 1(a) are 1.7×10^7 M⁻¹·min⁻¹ for authentic and 1.1×10^7 M⁻¹·min⁻¹ for thio-Fru-2,6-*P*₂, indicating that the enzyme efficiency is the same with the two substrates. The 100-fold difference between these values and that reported by Pilkis [4] is due to the differences in K_m^{app} . It is interesting to point out that the k_{cat}/K_m for phosphoglycerate

Fig. 1. Saturation curves of liver FBPase-2 for authentic and thio-Fru-2,6- P_2

Purified chicken liver FBPase-2 was assayed in the absence of ligands (a), and in the presence of 1 mM-GTP, 1 mM-*sn*-glycerol 3-phosphate and 1 mM-potassium phosphate (b), with either authentic (●) or thio-Fru-2,6- P_2 (■) as substrate.

mutase acting as a phosphatase on 2,3-bisphosphoglycerate is $5.9 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ [25]. The mechanisms of the phosphoglycerate mutase and FBPase-2 reactions are probably identical [26].

Labelling of purified chicken liver PFK-2/FBPase-2 from [2-³²P]Fru-2,6- P_2 and [2-³⁵S]thio-Fru-2,6- P_2

Fig. 2(a) shows that incubation of purified chicken liver FBPase-2 with [2-³²P]Fru-2,6- P_2 or [2-³⁵S]thio-Fru-2,6- P_2 resulted in the formation of labelled E-P, which persisted longer with authentic than with thio-Fru-2,6- P_2 . This cannot be explained by a difference in lability of the two E-P species. Indeed, no difference in non-enzymic acid hydrolysis of E-P at 25 °C and pH 4 could

be detected (results not shown). Fig. 2 also shows that the steady state concentration of E-P was smaller with thio-Fru-2,6- P_2 than with authentic Fru-2,6- P_2 . The difference is probably due to a faster turnover of E-P in the enzyme that was thiophosphorylated and it may reflect an increased V_{max} , since E-P turnover is directly related to the reaction rate. Consistent with this interpretation and with the data in the literature [4], comparison of Figs. 2(a) and 2(b) shows that ligands such as GTP, glycerol 2-phosphate and P_i stimulate E-P breakdown and decrease the steady state concentration of E-P.

Pilkis and co-workers have shown that Fru-6- P -depleted rat liver PFK-2/FBPase-2 can be phosphoryl-

Fig. 2. Labelling of liver FBPase-2 from [2-³²P]Fru-2,6- P_2 and [2-³⁵S]thio-Fru-2,6- P_2

Purified chicken liver FBPase-2 (20 μg) was incubated with 4 μM-[2-³²P]Fru-2,6- P_2 (●) or 4 μM-[2-³⁵S]thio-Fru-2,6- P_2 (■) either in the absence of ligands (a), or in the presence of 1 mM-GTP, 1 mM-*sn*-glycerol 2-phosphate and 1 mM-potassium phosphate (b).

Fig. 3. Sensitivity of potato PP_i:PFK and adipose tissue PFK-1 to stimulation by authentic and thio-Fru-2,6-P₂.

(a) The stimulation of PP_i:PFK by authentic (●) and thio-Fru-2,6-P₂ (■) was measured in the presence of 1 mM-Fru-6-P and 1 mM-sodium pyrophosphate. (b) Adipose tissue PFK-1 was assayed with 0.1 mM-Fru-6-P, 1.5 mM-MgATP, 0.1 mM-AMP, 5 mM-potassium phosphate and either authentic (●) or thio-Fru-2,6-P₂ (■).

ated to the maximum degree of 1.8 mol/mol of holoenzyme [4], suggesting that Fru-2,6-P₂ phosphorylates each subunit to the extent of 1 mol/mol. However with Fru-6-P-depleted chicken liver PFK-2/FBPase-2, the maximum phosphorylation was 1 mol/mol of holoenzyme (Fig. 2) suggesting that phosphorylation of one subunit inhibits that of the other. Interestingly, treatment of dephosphorylated chicken liver enzyme with cyclic AMP-dependent protein kinase resulted in 0.8 mol of phosphate incorporated per mol of holoenzyme (results not shown and [14]), suggesting that the enzyme is phosphorylated on only one subunit.

The results described represent the first observation of an increased rate of breakdown of a thiophosphorylated enzyme. Phosphoglycerate mutase and bisphosphoglycerate mutase have primary sequence and functional homologies with the FBPase-2 domain of rat liver PFK-

2/FBPase-2 [3], and 3-phosphohistidine can be formed from 1,3-bisphosphoglycerate [26]. Interestingly, however, the mutase does not catalyse phospho transfer between the phosphorothioate analogues of phosphoglycerate [27], suggesting that the enzyme will not tolerate thiophosphorylation of histidine. If, as the acid lability studies suggest, phosphorylation of PFK-2/FBPase-2 involves 3-thiophosphohistidine formation, then this may represent the first example of thiophosphorylation of a histidine residue in a protein.

Comparison of the effects of authentic and thio-Fru-2,6-P₂ on target enzymes

Thio-Fru-2,6-P₂ was almost 100 times less potent than authentic Fru-2,6-P₂ in stimulating PP_i:PFK (Fig. 3a) and, therefore, the usual method of detection of Fru-2,6-P₂ by PP_i:PFK stimulation [15] cannot be applied to this

Fig. 4. Effect of authentic and thio-Fru-2,6-P₂ on the activities of rabbit muscle FBPase-1 and trypanosome pyruvate kinase

(a) The inhibition of rabbit muscle FBPase-1 by authentic (●) and thio-Fru-2,6-P₂ (■) was measured with 15 μM-Fru-1,6-P₂. (b) Trypanosome pyruvate kinase activity was measured in the presence of either authentic (●) or thio-Fru-2,6-P₂ (■).

analogue. The sensitivity of PFK-1 (purified from adipose tissue) to authentic and thio-Fru-2,6- P_2 stimulation was also measured. As was the case for PP_i :PFK, thio-Fru-2,6- P_2 was nearly two orders of magnitude less potent than authentic Fru-2,6- P_2 (Fig. 3*b*). However, thio-Fru-2,6- P_2 was only ten times less potent than authentic Fru-2,6- P_2 when assayed on rabbit muscle PFK-1 (results not shown).

Fig. 4(*a*) shows that, in contrast to its effect on PP_i :PFK and PFK-1, thio-Fru-2,6- P_2 was about 10 times more potent than authentic Fru-2,6- P_2 in inhibiting rabbit muscle FBPase-1. Comparison of Figs. 3(*b*) and 4(*a*) shows that, under the assay conditions used, 0.5 μ M-thio-Fru-2,6- P_2 did not stimulate PFK-1 but caused 50% inhibition of FBPase-1. Studies of the Fru-6- P /Fru-1,6- P_2 futile cycle existing in the liver have shown that such a cycle occurs in the fed state [28]. Therefore, the Fru-2,6- P_2 concentration (10–20 μ M) in the livers of fed rats is not sufficient to inhibit FBPase-1 completely. Fig. 4(*a*) suggests that concentrations of thio-Fru-2,6- P_2 larger than 1 μ M would almost completely block FBPase-1. If substitution of oxygen for sulphur renders thio-Fru-2,6- P_2 permeable to the cell, as is the case for phosphorothioate cyclic 3',5'-AMP [5], then the analogue would be a unique tool to inhibit glucose production by isolated hepatocytes. In such a system, the intracellular hydrolysis of thio-Fru-2,6- P_2 , which occurs faster than that of authentic Fru-2,6- P_2 (see above), would be compensated by maintaining a large extracellular concentration.

Fig. 4(*b*) shows that thio-Fru-2,6- P_2 was almost twice as effective in stimulating trypanosome pyruvate kinase as authentic Fru-2,6- P_2 . This effect was consistently observed.

It is surprising that replacement of an oxygen atom for sulphur on the C-2 phosphate group of Fru-2,6- P_2 causes such profound changes in its biochemical properties. Since sulphur is twice as big as oxygen, steric hindrance at the Fru-2,6- P_2 binding site of PFK-1 may explain the decreased potency of thio-Fru-2,6- P_2 . Other analogues of Fru-2,6- P_2 , such as α - and β -arabinose 1,5-bisphosphate [29] and Fru-2,6- P_2 in which the phosphoryl group on the second carbon is replaced by a methylphosphonoyl or phosphonoyl group, are less effective than authentic Fru-2,6- P_2 [30]. Taken with the results presented here, these findings suggest that the Fru-2,6- P_2 binding domains of PFK-1, FBPase-1 and trypanosome pyruvate kinase are different.

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