Fructose 2,6-bisphosphate in rat erythrocytes

Inhibition of fructose 2,6-bisphosphate synthesis and measurement by glycerate 2,3-bisphosphate

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The concentration of fructose 2,6-bisphosphate found in freshly isolated erythrocytes was below the limit of detection (20 pmol/ml of packed cells). However, it increased to about 250 pmol/ml of cells when erythrocytes were incubated with glucose at pH 6.9, but not at pH 7.4 or 8.2. This could be explained by variations in the content of glycerate 2,3-bisphosphate, which was found to inhibit 6-phosphofructo-2-kinase, the enzyme responsible for fructose 2,6-bisphosphate synthesis. Glycerate 2,3-bisphosphate was also found to inhibit the potato enzyme (pyrophosphate:fructose-6-phosphate 1-phosphotransferase) used for the measurement of fructose 2,6-bisphosphate.

INTRODUCTION

Fructose 2,6-bisphosphate (Fru-2,6- P_2) is a potent stimulator of 6-phosphofructo-1-kinase (PFK-1), which has been detected in all mammalian tissues studied so far [1], with the only exception of erythrocytes [2]. In the present work we studied whether the absence of Fru-2,6- P_2 could be explained either by a lack or by an inhibition of 6-phosphofructo-2-kinase (PFK-2), the enzyme responsible for the synthesis of Fru-2,6- P_2 . Our results support the latter possibility and show that glycerate 2,3-bisphosphate (Gri-2,3- P_2) inhibits not only PFK-2 but also pyrophosphate:fructose-6-phosphate 1-phosphotransferase (PP_i-PFK), the enzyme purified from potato tubers and used to measure Fru-2,6- P_2 in extracts [3].

MATERIALS AND METHODS

Incubation of rat erythrocytes

Blood was collected in heparinized tubes from anaesthetized (Nembutal; 60 mg/kg body wt., intraperitoneally) male Wistar rats. The cells were centrifuged (5 min, table centrifuge) and the buffy coat was removed. The erythrocytes were washed three times in a solution containing 50 mM-glycylglycine/30 mM-NaCl/5 mM-KCl/10 mM-sodium phosphate/1 mM-CaCl₂/1 mM-MgCl₂, at pH 6.9, 7.4 or 8.2 as indicated. After suspension in 4 vol. of the same medium, the cells were incubated at 40 °C in the presence of various concentrations of glucose at the indicated pH. At the end of the incubation, the cells were collected by centrifugation and lysed by the addition of 1 vol. of 15 mM-Tris/HCl (pH 9.1)/10 mM-NaOH/20 mM-KF/0.3% (w/v) sodium deoxycholate at 0 °C. The lysate was frozen at –20 °C, thawed and centrifuged (10000 g, 15 min). A sample (1 ml) of the supernatant was filtered through a column (1.5 cm × 17 cm) of

Sephadex G-25 (fine grade) equilibrated in 20 mM-Tris/HCl, pH 9.1. The fractions (1 ml) that were eluted after the protein peak were collected for Fru-2,6- P_2 determination [3]. For measurement of metabolites other than Fru-2,6- P_2 , the cells were deproteinized with 1 M-HClO₄.

Purification of erythrocyte PFK-2

About 30 ml of packed erythrocytes were washed in 0.15 M-NaCl and lysed in 1 vol. of water, followed by the addition of 4 vol. of a buffer containing 20 mM-Hepes, 50 mM-KCl, 2 mM-EDTA, 5 mM-MgCl₂, 5 mM-potassium phosphate, 15 mM-2-mercaptoethanol and 0.1 mM-phenylmethanesulphonyl fluoride, pH 7.5. After centrifugation (10000 g, 15 min), the supernatant was stirred for 2 h at 4 °C with 30 ml of Affi-Gel Blue (equilibrated in the same buffer). The gel was extensively washed on a Büchner filter until the A_{280} of the filtrate decreased to zero. The gel was then packed in a column (2.5 cm × 6 cm), and PFK-2 activity was eluted as described previously [4].

Analytical methods

Fru-2,6- P_2 was measured as described in [3]. Under these assay conditions, the amount of Fru-2,6- P_2 that gives a doubling of the blank value is 0.5 pmol. We considered this value as the limit of detection, which corresponds to 20 pmol/ml of packed erythrocytes when corrected for dilution of the cell extracts. Gri-2,3- P_2 [5], Glc-6- P [6], ATP [7] and protein [8] were measured as indicated in the references. For all measurements of Fru-2,6- P_2 , internal standards were included to check for the possible presence of inhibitors in the assay. PP_i-PFK was purified and assayed as described in ref. [3].

Separation of Fru-2,6- P_2 and Gri-2,3- P_2

Fru-2,6- P_2 was separated from Gri-2,3- P_2 on columns (0.5 cm × 4 cm) of Dowex AG1 X8 (Cl[–] form; 200–400

Abbreviations used: Fru-2,6- P_2 , fructose 2,6-bisphosphate; Fru-6- P , fructose 6-phosphate; Glc-6- P , glucose 6-phosphate; Gri-2,3- P_2 , glycerate 2,3-bisphosphate; PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); PP_i-PFK, pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90).

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mesh). A 40 ml gradient (0–0.5 M-NaCl in 10 mM-Mes, pH 6.9) was applied, which eluted Fru-2,6- P_2 at 0.23 M-NaCl and Gri-2,3- P_2 at 0.27 M-NaCl.

RESULTS AND DISCUSSION

Inhibition of PP_i-PFK and PFK-2 by Gri-2,3- P_2

The measurement of the recovery of Fru-2,6- P_2 standards added to extracts of erythrocytes revealed that the extracts contained a potent inhibitor of the assay of Fru-2,6- P_2 by PP_i-PFK. Since Gri-2,3- P_2 is known to inhibit several enzymes [9] and to be present in erythrocytes in millimolar concentrations, the possible inhibition of PP_i-PFK by this intermediate was tested. Fig. 1 shows that, under the usual assay conditions [3], Gri-2,3- P_2 inhibited PP_i-PFK and that the measurement of Fru-2,6- P_2 in the presence of Gri-2,3- P_2 was not reliable. The inhibition was apparently non-competitive ($K_i = 0.13$ mM; Dixon plot).

Moreover, Gri-2,3- P_2 inhibited purified erythrocyte PFK-2 (apparent $K_i = 0.5$ mM), assayed at sub-saturating concentrations of substrates (Fig. 2). Similar results were obtained with PFK-2 purified from liver and muscle. Since in erythrocytes the concentration of Gri-2,3- P_2 is about 10-fold greater than the K_i for inhibition of PFK-2, one would expect almost complete inhibition of PFK-2 in intact erythrocytes.

The two inhibitory effects of Gri-2,3- P_2 explain why Fru-2,6- P_2 was not detected in erythrocytes. Conversely, Fru-2,6- P_2 should be present in erythrocytes that do not contain Gri-2,3- P_2 , as for example in avian erythrocytes, and recent studies indeed showed that this was the case

Fig. 1. Inhibition PP_i-PFK by Gri-2,3- P_2

Enzyme activity was measured at pH 8 in the presence of 1 mM-PP_i, 1 mM-Fru-6- P and the indicated concentrations of Fru-2,6- P_2 .

Fig. 2. Inhibition of purified erythrocyte PFK-2 by Gri-2,3- P_2

Enzyme activity was measured at pH 7.1 in the presence of 0.1 mM-Fru-6- P , 5 mM-MgATP and 5 mM-P_i. The means $\pm$ S.E.M. for three different experiments are shown.

[10]. Fru-2,6- P_2 should also be present in mammalian erythrocytes treated to deplete their Gri-2,3- P_2 concentration.

Fru-2,6- P_2 in intact erythrocytes

In the first series of experiments, efforts were devoted to the detection of Fru-2,6- P_2 in deproteinized extracts of erythrocytes. Since Gri-2,3- P_2 was found to inhibit the assay of Fru-2,6- P_2 , these two phosphoesters were separated by ion-exchange chromatography as described in the Materials and methods section. Under these conditions, the concentration of Fru-2,6- P_2 found in extracts of freshly isolated erythrocytes was consistently lower than 20 nmol/litre of packed cells. For comparison, the concentration of Fru-2,6- P_2 in skeletal muscle and liver of well-fed rats is 20 and 1000 times larger respectively [11,12].

If the inhibition of PFK-2 by Gri-2,3- P_2 also applies to intact cells, one would expect that conditions that decrease Gri-2,3- P_2 *in vivo* would result in a de-inhibition of PFK-2. Fru-2,6- P_2 should then accumulate and become detectable. Previous work by Rapoport *et al.* [13] demonstrated that there is a time-dependent fall in the concentration of Gri-2,3- P_2 in erythrocytes incubated in the presence of glucose at pH 6.9, but not at pH 7.4 or 8.2. We have confirmed these findings, and Table 1 shows that at pH 6.9 the concentration of Gri-2,3- P_2 fell dramatically, whereas at pH 7.4 or 8.2 it remained relatively stable. In agreement with previous data [14,15], the concentrations of Fru-6- P and ATP, the two substrates of PFK-2, remained relatively stable during a 4 h incubation at pH 6.9, but not at pH 7.4 or 8.2. As expected, Fru-2,6- P_2 was synthesized when the concentration of Gri-2,3- P_2 was decreased, i.e. at pH 6.9. It is noteworthy that, by contrast with Fru-2,6- P_2 synthesis, glycolysis is known to be stimulated when pH shifts from

Table 1. Concentrations of several metabolites in rat erythrocytes incubated at different pH values in the presence of 20 mM-glucose

The values shown for pH 6.9 are means $\pm$ S.E.M. for at least three different experiments. The other values are means for two different experiments. The results are expressed per ml of erythrocytes.

Metabolite concn.	Time of incubation (h)	pH 6.9	pH 7.4	pH 8.2
Glc-6-P (nmol/ml)	0	48 $\pm$ 6	43	41
	4	39 $\pm$ 8	13	4
Fru-6-P (nmol/ml)	0	20 $\pm$ 5	13	11
	4	25 $\pm$ 2	3	< 1
ATP (μ mol/ml)	0	1.11 $\pm$ 0.06	1.06	1.07
	4	0.89 $\pm$ 0.04	0.98	0.72
Gri-2,3- P_2 (μ mol/ml)	0	6.2 $\pm$ 0.3	5.8	6.6
	4	0.6 $\pm$ 0.1	5.1	5.5
Fru-2,6- P_2 (pmol/ml)	0	22 $\pm$ 2	—	—
	2	157 $\pm$ 10	—	—
	4	260 $\pm$ 20	< 20*	< 20*

* The concentration of Fru-2,6- P_2 was below 20 pmol/ml in two other experiments, and 28 (pH 7.4) and 25 (pH 8.2) in a third similar experiment.

Table 2. Influence of glucose concentration on Fru-2,6- P_2 content of rat erythrocytes incubated at different pH values

Erythrocytes were incubated for 4 h at pH 6.9 in the absence of glucose, and then washed and incubated at the indicated pH for 1 h in the presence of various concentrations of glucose. The values are means $\pm$ S.E.M. for three different observations.

Glucose concn. (mM)	Fru-2,6- <i>P</i> ₂ concn. (pmol/ml of cells)			
	pH . . .	6.9	7.4	8.2
0		< 20	< 20	< 20
5		86 ± 15	25 ± 3	23 ± 2
10		107 ± 9	46 ± 1	22 ± 4
20		116 ± 19	33 ± 1	22 ± 2
40		144 ± 21	—	23 ± 2

7 to 8. This might be related to the well-known pH profile of PFK-1 [2].

On the other hand, Fru-2,6- P_2 , which was synthesized in the presence of 10 mM-glucose during a 4 h preincubation at pH 6.9, persisted for at least 2 h when the pH of incubation was shifted to 7.4 or 8.2. Under the latter conditions, the concentration of Gri-2,3- P_2 , which was below 0.5 μ mol/ml of cells after 4 h at pH 6.9, rose to 2.5 μ mol/ml of packed cells after 2 h at pH 8.2 (results not shown). This indicates that Gri-2,3- P_2 does not favour the disappearance of Fru-2,6- P_2 , but rather prevents its synthesis.

When erythrocytes were first preincubated without glucose at pH 6.9 to allow for the disappearance of Gri-2,3- P_2 and then incubated at the same pH in the

presence of various concentrations of glucose, there was a glucose-dependent rise in the intracellular concentration of Fru-2,6- P_2 (Table 2). However, this rise was much smaller or absent when the cells were incubated at pH 7.4 or 8.2.

Since pH is important in regulating the Fru-2,6- P_2 content of intact cells, its direct effect on the activity of erythrocyte PFK-2 was tested. At pH 7.5 and 8.5, the activity was 93 and 66% respectively of that measured at pH 7.0 (results not shown).

General conclusion

The concentration of Fru-2,6- P_2 in erythrocytes depends on the pH of incubation. At physiological pH, the concentration is negligible, as it is in freshly isolated cells. However, at pH 6.9, a time- and glucose-dependent increase in Fru-2,6- P_2 is obtained. Whether this results from a direct pH effect on PFK-2 activity is unlikely, since the activity of PFK-2 assayed *in vitro* at pH 7 is about the same as that measured at pH 7.5. Thus the effect of pH is probably indirect, and results from the disappearance of Gri-2,3- P_2 . This compound is an inhibitor of PFK-2, and its concentration in freshly isolated cells is about 10 times the K_i for PFK-2. We suggest that the high physiological concentrations of Gri-2,3- P_2 found in intact erythrocytes offer an explanation for the very low concentration of Fru-2,6- P_2 in erythrocytes. But other factors cannot be ruled out.

Therefore, erythrocytes, which depend exclusively on glycolysis for their energy supply and which contain PFK-1 sensitive towards Fru-2,6- P_2 , do not appear to depend on Fru-2,6- P_2 for maintaining glycolysis.

In addition, the inhibition of PP_i-PFK by Gri-2,3- P_2 underlines the shortcomings and pitfalls of the assay for Fru-2,6- P_2 . This assay, which has the advantage of being very sensitive and allows a fraction of 1 pmol to be readily detected [16], is, however, influenced by the concentration of ions [16] and, as it now appears, by Gri-2,3- P_2 . This stresses the importance of including Fru-2,6- P_2 standards in the assay to monitor the presence of putative inhibitors.

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