IN VIVO AND IN VITRO INTERCONVERSIONS OF ACTIVE AND INACTIVE FORMS OF PHOSPHOFRUCTOKINASE IN RAT LIVER

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1. Introduction

Phosphofructokinase (PFK) from rat liver, the key enzyme in the regulation of glycolysis, is an interconvertible enzyme. It has been shown that the active form of PFK is inactivated by a phosphatase-dependent dephosphorylation and the inactive form is reactivated by a kinase-catalysed phosphorylation [1]. Although the reactivation velocity was extremely reduced in livers from starved rats in comparison to fed rats, until now the physiological efficiency of this system was unknown.

In the present paper it is shown, that also in vivo, there exists an active and an inactive form of PFK and that the proportion of both forms differs with the nutritional state of the animal, whereas the sum of both forms of PFK remains constant. Similar results were found also in vitro using the isolated liver perfusion technique.

2. Materials and methods

2.1. *Materials*

Sephadex G-25 and Sepharose 6-B were obtained from Pharmacia, Frankfurt; nucleotides, sugar phosphates, and auxiliary enzymes from Boehringer-Mannheim G.m.b.H.; bovine serum albumin from Miles, Kankahee/Ill.; all other chemicals came from E. Merck A. G., Darmstadt.

Male Wistar rats (Zentralinstitut für Versuchstierzucht, Hannover-Linden) were used for experiments with a weight of 190–200 g. Rats were allowed free access to water and food (Altromin 1324, Altroge/Lage, Germany). For starvation rats were deprived of food at 9 a.m. for indicated hours.

2.2. *Preparation of the liver extract*

The rat was sacrified by decapitation, the liver removed and homogenized with a teflon homogenizer (1/3, w/v) in 20 mM potassium-phosphate, 100 mM $(NH_4)_2SO_4$, 0.5 mM $MgCl_2$, 5 mM mercaptoethanol, pH 7.4. The homogenate was centrifuged at 42 500 *g* for 45 min at 4°C. The supernatant was used for the determination of PFK activity and for gel filtration.

2.3. *Sephadex G-25 filtration*

The supernatant (0.5 ml) was applied to a Sephadex G-25 column (1 × 25 cm) equilibrated with 20 mM potassium phosphate, 0.5 mM $MgCl_2$, 5 mM mercaptoethanol, pH 7.6, and eluted with the same buffer. The main protein fraction (1.5 ml) was collected and assayed for active and inactive PFK.

2.4. *Determination of the active and the inactive form of PFK*

The active PFK was tested under optimized conditions as described earlier [2]. To determine the inactive form of PFK, the gel filtrate was preincubated with 6 mM ATP for 15 min at 25°C. The total activity of PFK was tested as above. The difference of the PFK activity before and after activation represents the inactive form of PFK.

One unit of enzyme was defined as the amount of enzyme that catalysed the formation of 1 μmol of fructose-1,6-P_2 per min at 30°C. Protein was determined by the Lowry [3] method.

2.5. *Separation of the active and the inactive form of PFK*

The preparation of the liver extract was done as described in 2.2 with the exception that the livers were homogenized 1/2 (w/v) instead of 1/3. 4 ml of the centrifuged supernatant was applied to a Sepharose 6-B column (2.5 × 90 cm) equilibrated with the same buffer as was used for the Sephadex G-25 filtration (2.3). Successive 5 ml fractions were collected and determined for active and inactive PFK.

2.6. *Isolated rat liver perfusion*

Rats starved for 48 h were used for the isolated liver perfusion. The perfusion technique was carried out as described by Söling et al. [4]. The liver was perfused first with a hemoglobin free Krebs-Ringer bicarbonate buffer containing 2% dialyzed bovine serum albumin without further additions for indicated periods. Liver tissue (about 500 mg) was removed prior to the addition of glucose (30 mM final concentration) to the medium and used for the determination of active and inactive PFK as described in 2.2–2.4. After perfusion with glucose for the indicated time, another tissue sample was assayed for both forms of PFK.

3. Results and discussion

3.1. *In vivo interconversion of active and inactive forms of PFK*

Kinetic experiments with purified PFK-phosphatase and inactive PFK associated with PFK-kinase showed that phosphate was a strong inhibitor of the phosphatase, whereas the activation of inactive PFK could be suppressed completely by ammonium sulfate. These findings made it possible to get a homogenate in which the proportion of active and inactive PFK was fixed in the in vivo state, because as well the kinase as the phosphatase could not affect their substrates. When livers from fed, starved, and refed rats were homogenized in the phosphate–ammonium sulfate medium (see 2.2), the specific activity of PFK showed remarkable differences (table 1): after starvation for 24, 30, and 48 h the specific activity of PFK was only one third of that in livers from fed rats. Refeeding for only 6 h was sufficient to recover the activity found in the fed state.

As the inactivated PFK could not be reactivated in the presence of ammonium sulfate, a part of the supernatant was filtrated on a Sephadex G-25 column to remove ammonium sulfate and endogenous low

Table 1

Activity of PFK and the proportion of its active and inactive form in high-speed supernatants (S_3) of liver homogenates from fed, starved, and refed rats. The inactive form of PFK has been activated in the Sephadex G-25 filtrate by preincubation with 6 mM ATP. For details see Material and methods.

Nutritional state	Liver wet weight	Spec. activity of PFK in S_3	Spec. activity of PFK in S_3 after Sephadex G-25 filtration		Factor of activation
			before activation	after activation	
	(g)	(mU/mg)	(mU/mg)	(mU/mg)	
fed	7.66	12.2	15.9	21.4	1.35
fed	10.7	13.0	13.7	19.6	1.43
starved, 6 h	9.72	15.9	15.9	19.5	1.23
starved, 24 h	4.61	4.57	4.88	19.1	3.91
starved, 24 h	6.24	5.49	5.27	17.2	3.27
starved, 24 h	6.37	5.73	5.68	18.2	3.20
starved, 30 h	5.47	4.27	6.51	21.9	3.36
starved, 48 h	4.75	7.43	6.99	17.3	2.47
starved, 48 h	5.53	4.30	5.84	18.7	3.21
starved, 48 h and refed					
for 6 h	6.80	15.5	18.3	18.8	1.03
for 20 h	9.80	10.1	17.3	19.9	1.15

molecular weight substances. During this procedure the phosphatase remained inhibited by phosphate and the kinase could not affect the inactive PFK because of the absence of nucleotide triphosphates. The differences in the PFK activity between liver extracts from fed and starved rats were the same as before gel filtration. After activation of the PFK with ATP the specific activity of PFK was found to be equal and independent from the nutritional state of the animals. This means that in livers from fed rats almost all PFK is present in its active form, whereas during starvation about two-thirds of the active form are converted to the inactive form, but without loss of PFK protein. Six hours of refeeding with standard food were sufficient to convert the inactive into the active PFK (table 1). These results fit very well with our previous findings, that the velocity of PFK reactivation is extremely reduced during starvation [1]. As we could not find any change in the catalytic activity of the phosphatase, it might be possible that the equilibrium between the active and the inactive form of PFK is determined mainly by changes in the kinase activity.

3.2. *Separation of active and inactive PFK present in vivo*

Both forms of PFK could be separated by Sepharose 6-B filtration because of their different molecular weights. Active PFK from rat liver has a molecular weight of 320 000 and consists of four subunits with a molecular weight of 82 000 each [2]. The inactive PFK exists as a dephosphorylated single subunit associated with the kinase, the complex having a molecular weight of 120–130 000 [1]. The proportion of active and inactive PFK was clearly dependent on the nutritional state as could be demonstrated by the fractionation of Sepharose (fig.1).

3.3. *Determination of interconversion of PFK in vitro*

When 48 h starved rats were used for isolated liver perfusion, again only one third of the PFK was present on the active form as long as no glucose was added to the perfusion medium. After 30 min perfusion with 30 mM glucose, almost all PFK had been converted into the active form (table 2).

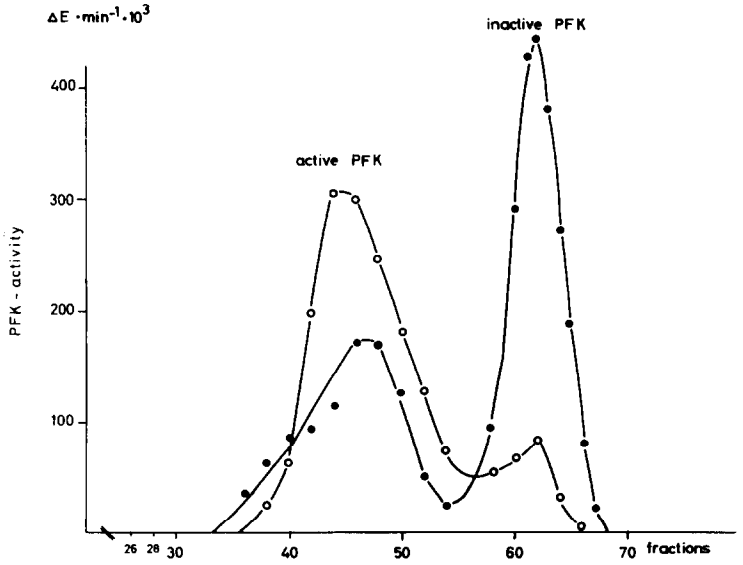

Fig.1. Separation of active and inactive PFK on Sepharose 6-B. High-speed supernatants of liver homogenates from fed (-o-o-) and 64 h starved (-●-●-) rats were used for gel filtration. For details see Materials and methods.

Table 2

Activity of PFK in the isolated perfused liver from 48 h starved rats and the proportion of active and inactive forms. First the livers were perfused without glucose. About 500 mg of liver tissue were removed and analysed for both forms of PFK. Then, the perfusion was continued in the presence of 30 mM glucose (final concentration). After the indicated times PFK activities were again determined. For details see Material and methods. (The roman numbers in the upper and the lower part of the table correspond to the same experiment.)

Experimental condition	Rat liver no.	Perfusion time	in S_3	PFK activity in Sephadex G-25 filtrate		Factor of activation
				− activation	+ activation	
		(min)	(mU/mg)	(mU/mg)	(mU/mg)	
Perfusion without glucose	I	30	4.5	4.6	14.3	3.10
	II	45	4.7	6.0	14.3	2.37
	III	60	4.8	5.4	17.1	3.14
30 mM glucose	I	30	10.8	12.8	13.6	1.06
	II	30	11.6	13.6	16.8	1.23
	III	60	8.1	6.8	8.6	1.24

3.4. *Conclusion*

For short time regulation of PFK activity up to now only allosteric effectors have been discussed. Especially for the liver enzyme the relative importance of allosteric regulation under physiological conditions is not yet clear. A regulation mechanism by changing the amount of active PFK was unknown, so far. Different activities of PFK were found in livers from fed, starved, and diabetic rat, but were refered to changes in protein turnover. For quite a while we could not detect any differences between the activity of livers from fed and starved rats, since we had worked with a medium which stabilized active PFK because only the phosphatase was inhibited. The results presented here, show clearly that the total amount of PFK protein stays rather constant under different metabolic conditions, whereas the actual enzyme activity is regulated by changes in the proportion of the active and the inactive form of PFK.

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