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ACTIVATION AND INACTIVATION OF RAT LIVER PHOSPHOFRUCTOKINASE BY PHOSPHORYLATION–DEPHOSPHORYLATION

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1. Introduction

Vinuela et al. described in 1964 [1] that yeast extract phosphofructokinase (PFK) could be desensitized against ATP inhibition by incubation with $MgATP^-$, NaF and cyclic 3′,5′-AMP. Afting et al. [2] demonstrated that protein is unnecessary for this interconversion, and eventually fluoride was identified as a factor stabilizing the insensitive form of PFK [3].

In the present paper it is shown, that crude rat liver PFK can be inactivated in the presence of high Mg^{++} concentrations and reactivated in the presence of $MgATP^{2-}$. Enzymes catalyzing the inactivation and reactivation reactions as well as active and inactive forms of rat liver PFK could be separated. Incorporation studies show, that rat liver PFK is activated by phosphorylation by a cyclic 3′,5′-AMP-independent kinase and inactivated by a phosphatase catalysed dephosphorylation.

2. Materials and methods

O-Phospho-L-serine was obtained from Sigma, St. Louis, Mo; [γ-^{32}P] ATP from Amersham Buchler, Braunschweig; anti rabbit γ-globulin serum (goat) from Behringwerke, Marburg/Lahn; Whatman DE-52 cellulose from Hormuth-Vetter, Heidelberg and Sephadex gels from Pharmacia, Frankfurt. Nucleotides, sugar phosphates, and auxiliary enzymes were purchased from Boehringer-Mannheim G.m.b.H., Mannheim; all other chemicals were from E. Merck, A. G., Darmstadt.

Liver PFK from male Wistar rats was purified and tested under optimized conditions as described earlier [4]. Rat liver PFK antiserum was prepared by injecting rabbits with pure enzyme (72 U/mg). The immuno-γ-globulin fraction was partially purified from this antiserum according to the method of Steinbuch and Audran [5].

2.1. *Preparation of the inactive form of PFK, and the activating and inactivating proteins*

Livers (200 g) from male Wistar rats were homogenized (1/4, w/v) in 0.2 M sucrose, 20 mM Triethanolamine-Cl, pH 7.6, 0.5 mM $MgCl_2$ and 5 mM mercaptoethanol. The homogenate was centrifuged at 3000 *g* for 10 min at 4°C, and then at 40 000 *g* for 30 min. The supernatant was poured through a funnel with glass wool to remove floating fat. The filtrate was adjusted to pH 5.5 with 0.5 N acetic acid and centrifuged at 20 000 *g* for 10 min. The supernatant was discarded, the pellet resuspended in 20 mM triethanolamine-Cl, pH 7.2, 0.5 mM $MgCl_2$, 5 mM mercaptoethanol (Medium A). The pH was adjusted to pH 7.2 and the suspension centrifuged at 40 000 *g* for 30 min. The supernatant was incubated in the presence of 20 mM $MgCl_2$ at room temperature until all PFK activity had disappeared from the solution. The pH was again adusted to 5.5. After centrifugation at 20 000 *g* for 10 min, the pellet was resuspended in 20 mM potassium phosphate, pH 7.6, 0.5 mM $MgCl_2$, 5 mM mercaptoethanol (Medium B). The resuspended acid precipitate was applied to a DEAE-cellulose column, equilibrated with medium B. After washing with the same buffer, a linear gradient (20 to 200 mM of medium B) was started. The eluate was collected in 4.7 ml fractions. When this gradient was finished, the column was washed with medium B and a second gradient of medium B,

contining 0–1 M NaCl was applied. The protein fractions containing inactive PFK and activating protein and the fractions containing the inactivating protein were concentrated by pressure filtration (Amicon Diaflo membrane PM 30 or PM 10). The inactivating protein was dialysed against medium A. A Sephadex G 100 column (0.9 × 180 cm) was equilibrated with medium B and charged with the protein solution (1.0–1.5 ml) containing inactive PFK and activating protein. Fractions (4 ml) containing inactive PFK plus activating protein were combined and concentrated by pressure filtration.

The fraction containing the inactivating protein was applied to a Sephadex G 200 column (1.9 × 90 cm), equilibrated with medium A. Fractions (4 ml) containing the inactivating protein were combined and concentrated by pressure filtration.

The inactivating protein was tested in a reaction mixture containing 0.2 U PFK (25 U/mg), 1% bovine serum albumin, 20 mM triethanolamine-Cl, pH 7.2, 5 mM mercaptoethanol, 15 mM $MgCl_2$. Reaction mixtures containing the inactivating protein but only 0.5 mM $MgCl_2$, or containing 15 mM $MgCl_2$, but no inactivating protein served as controls.

The activation of PFK was followed in a reaction mixture containing 20 mM potassium phosphate, pH 7.5, 0.5 mM $MgCl_2$, 2 mM ATP, 5 mM mercaptoethanol and inactivated PFK plus activating protein. The incubation temperature for inactivation and activation was 25°C.

2.2. *Phosphorylation of PFK by ATP*

Inactive PFK (5 U, when totally activated, plus activating protein, purified as described above, were incubated with 0.77 mM [γ-^{32}P] ATP (spec. act. 0.082 mCi/μmol) in the presence of 20 mM potassium phosphate, pH 7.6, 0.5 mM $MgCl_2$ and 5 mM mercaptoethanol at 25°C. After 1,2,5,10, and 20 min aliquots were diluted with cold ATP (20 mM), and then filtrated on Sephadex G-15 columns to remove free ATP. The filtrates were incubated for 1 hr at 37°C, then for 4 hr at 7°C with sufficient anti-PFK-γ-globulin to precipitate all PFK or with the same amount of control-γ-globulin.

Thereafter a ten-fold amount of anti-rabbit immunoglobulin serum was added. After an incuabtion for 5 hr as above the precipitates were sedimented by centrifugation and washed three times with 0.15 M NaCl. The pellet was solubilized with 0.1 N NaOH for liquid scintillation counting or suspended in H_2O and hydrolyzed under nitrogen in 6 N HCl at 110°C for 4.5 hr.

After hydrolysis HCl was removed by evaporation, and the residue dissolved in H_2O. Aliquots were applied to Whatman 3 MM paper together with cold *O*-phospho-L-serine and P_i. High voltage electrophoresis was carried out in 2 N acetic acid, pH 2.4 at 30 mA, 2900 V for 110 min.

2.3. *Dephosphorylation of PFK*

Labelled PFK (1.33 U; 0.068 μCi/U) was incubated with PFK inactivating protein purified as described above. The incubation mixture contained 20 mM triethanolamine-Cl, pH 7.2, 14.5 mM MgCl, 0.3% bovine serum albumin, and 0.9 mM F-6-P. Two control incubations were performed; one containing buffer instead of the inactivating protein, the other 0.5 mM instead of 14.5 mM $MgCl_2$. After 0,10.20, and 30 min min aliquots were transfered into 1/3 vol of 1 N $HClO_4$. After 20 min the precipitates were centrifuged. The supernatants were neutralized with KOH, and after treatment with charcoal (DARCO) counted for radioactivity. The pellets were washed with 0.25 N $HClO_4$, solubilized in 0.1 N NaOH and counted for radioacitivty.

3. Results and discussion

3.1. *Inactivation–activation*

Previous studies showed that PFK in the 100 000 *g* supernatant from rat liver homogenate could be inactivated in the presence of 20 mM $MgCl_2$ but could not be reactivated under our conditions by the addition of 20 mM ATP. Using the pH 5.5 precipitate a reactivation of inactivated PFK was possible when a concentration of ATP equimolar to the $MgCl_2$ concentration was added. The final PFK activity was 60% higher than the starting activity. An incubation with 20 mM $MgATP^{2-}$ without prior inactivation led to the same activation within 30 min min (fig.1).

The capacity for reactivation is dependent on the previous treatment of the rats. During starvation the velocity of reactivation was decreased to 20 percent of that in fed animals, but increased within 6 hr of refeeding to initial values (fig.2). Until now we have

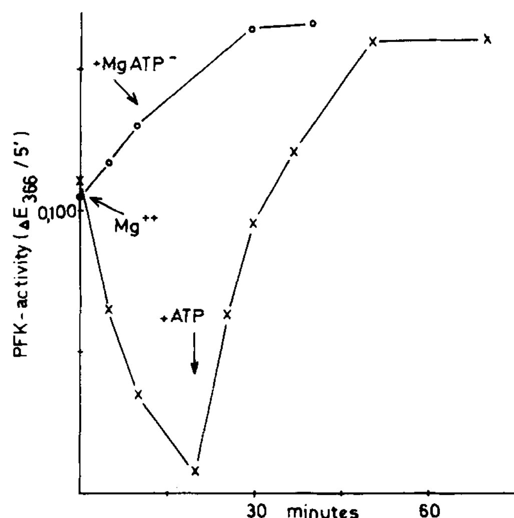

Fig.1. Time course of inactivation and reactivation of rat liver phosphofructokinase. The pH 5.5 pellet prepared as described in Materials and methods was used for incubation. The reaction mixture contained 20 mM triethanolamine-Cl, pH 7.2, 0.5 mM $MgCl_2$, 5 mM mercaptoethanol, and either 20 mM $MgCl_2$ (–x–x) or 20 mM $MgATP^-$(–o–o–). The incubation temperature was 25°C. Aliquots were removed at the times indicated and assayed for phosphofructokinase activity. At 20 min ATP (20 mM) was added to the incubation containing solely 20 mM $MgCl_2$.

not been able to determine whether there is a change in the amount of activating protein or if there exists an inhibitory factor which changes its capacity during feeding and starvation.

3.2. *Separation and partial purification of active PFK and inactivating protein from the activating protein–inactive PFK complex*

When a pH 5.5 precipitate containing active and inactivated PFK was fractioned on DEAE cellulose by a phosphate gradient the inactive PFK was eluted from the column by less than 100 mM phosphate, while the active form of PFK appeared at concentrations of 120 mM phosphate and higher. The inactivating protein was eluted with 0.5 M NaCl by a second gradient (fig.3).

The inactive PFK became activated after addition of 1.5 mM $MgATP^-$. The inactive PFK could not be separated from the activating protein by gel filtration on Sephadex G-100. The mol. wt of this fraction was estimated on Sephadex G-100 to be about 120 000, whereas the mol. wt of the PFK protomers is 82 000 [4]. We, therefore, assume that the fraction consists of a complex of a PFK protomer and the inactivating protein. This suggests an apparent mol.wt of about 40–50 000 for the activating protein.

Active PFK formed during the incubation of the inactivating protein–inactive PFK complex in the presence of $MgATP^-$emerged on Sephadex G-100 in the void volume indicating that the activation had resulted in the formation of the active monomer (mol. wt = 320 000 [4]) or polymers. We could demonstrate that a protein factor is necessary for activation.
Pure PFK, inactivated by the inactivating protein in the presence of 15 mM $MgCl_2$ could not be reactivated solely by the addition of $MgATP^-$.

3.3. *Phosphorylation and dephosphorylation of rat liver PFK*

To prove our assumption that activation and inactivation of PFK result from phosphorylation and dephosphorylation reactions, inactive PFK plus activating protein were incubated in the presence of [γ-^{32}P] ATP and $MgCl_2$. We found significant incorporation of ^{32}P

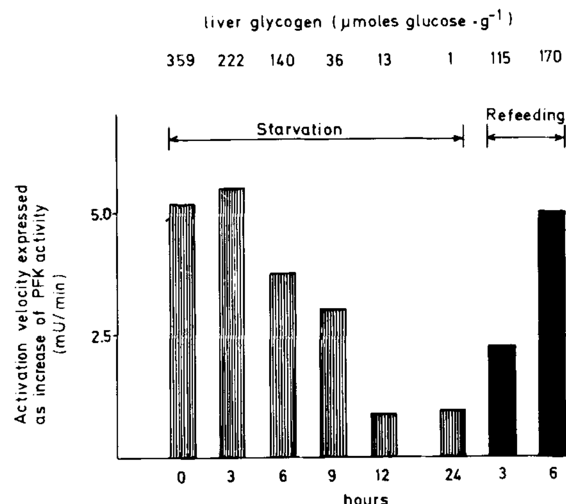

Fig.2. Initial volocity of reactivation of inactive rat liver phosphofructokinase in pH 5.5 sediments prepared from fed, starved, and refed rats. Phosphofructokinase was first inactivated in the presence of 20 mM $MgCl_2$. Reactivation was initiated by addition of ATP (20 mM). Incubation conditions were as described in the legend to fig.1.

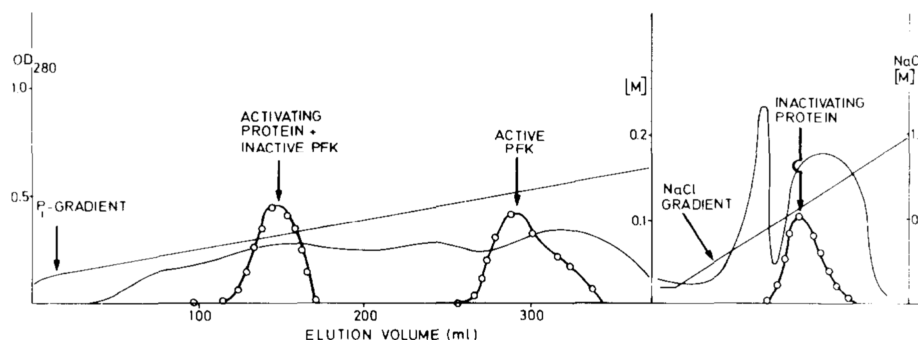

Fig.3. Fractionation of inactive phosphofructokinase (PFK) plus activating protein, active PFK, and inactivating protein on DEAE-cellulose. For details see Materials and methods.

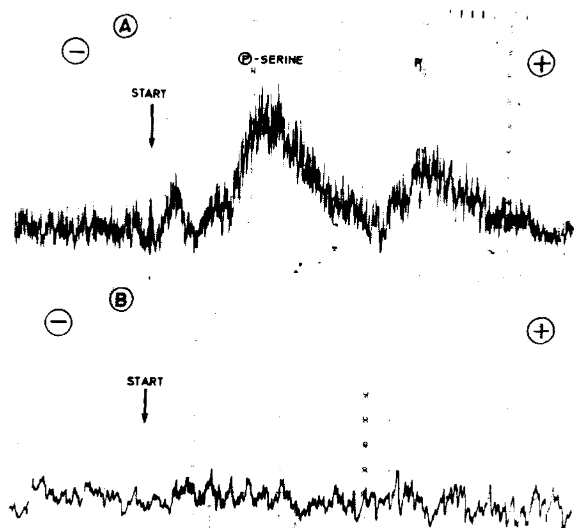

Fig.4. Original scan of a high voltage electrophoretogram of ^{32}P-labelled compounds. Inactivated purified rat liver phosphofructokinase (PFK) was reactivated in the presence of the activating protein and [γ-^{32}P] ATP. The ^{32}P-labelled PFK was reacted with a specific rabbit anti-rat liver PFK immunoglobulin and completely precipitated with anti-rabbit γ-globulin antiserum (Curve A of the figure). To exclude unspecific coprecipitation of radioactivity, the same ^{32}P-labelled PFK was incubated with a rabbit control immunoglobulin fraction and again a precipitation was initiated by the addition of anti-rabbit γ-globulin antiserum (curve B of the figure). The precipitates were hydrolyzed and the products of hydrolysis used for high voltage electrophoresis. For details see Materials and methods.

into the PFK protein precipitated by the specific antibody, whereas in the control immune precipitate no radioactivity was found. The high voltage electrophoretic (fig.4) analysis of hydrolyzed protein showed that the main radioactivity migrated with authentic *O*-phospho-L-serine. The free $^{32}P_i$ peak resulted from partial hydrolysis of phosphoserine during protein hydrolysis.

In time course experiments it was found that enzyme activation proceeded in parallel to the amount of radioactivity incorporated (fig.5). The relative increase of enzyme activity and of total ^{32}P incorporation were not identical. The amount of radioactivity incorporated into the protein was still increasing, when the enzyme was already fully activated (fig.5). A calculation of the time-dependent incorporation of ^{32}P per enzyme molecule helps to explain this finding. The incorporation (expressed as moles ^{32}P incorporated per mole protomer) varied from 0.22 in the first five min to 0.5 after 20 min. A double reciprocal plot of these values (fig.6) intersects the y-axis at a value of 1, which means that one mole of ^{32}P will be incorporated per protomer (or 4 P/monomer) when the incubation time is long enough to ensure complete phosphorylation. The smallest active form of liver PFK is a monomer consisting of 4 protomers [4]. Since incorporation of 1 molecule phosphate per active monomer leads already to full activation of the enzyme it can be deduced that one phosphorylated protomer can associate with 3 unphosphorylated protomers to form an enzymatically active monomer.

When ^{32}P-labelled PFK was incubated together with the inactivating protein in the presence of 11

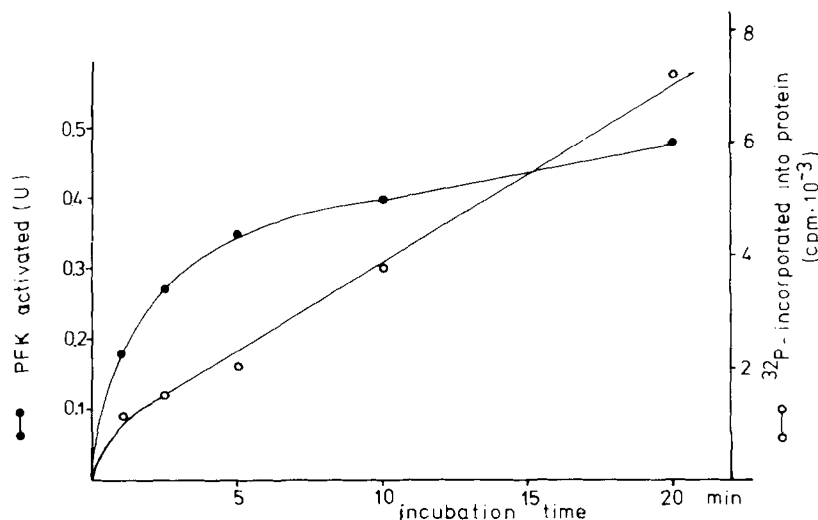

Fig.5. Time dependence of incorporation of $^{32}P_i$ into rat liver phosphofructokinase (PFK) (–o–o–) and of activation of PFK (–•–•–). Both parameters were measured during an identical incubation. For technical details see Materials and methods.

mM $MgCl_2$ the release of $^{32}P_i$ occurred in parallel with a decrease in PFK activity. No effect was seen in the presence of the inactivating protein with only 0.5 mM $MgCl_2$ or in the presence of 11 mM $MgCl_2$ without the inactivating protein (fig.7).

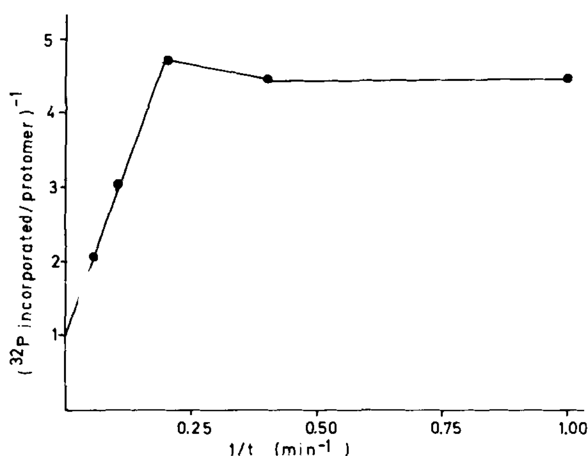

Fig.6. Double reciprocal plot of the number of ^{32}P-phosphate molecules incorporated per phosphofructokinase protomer against time. Data were taken from the experiment shown in fig.5. Calculations are based on a specific enzymatic activity of pure rat liver phosphofructokinase of 86 U/mg and a mol. wt of the protomer of 82 000 [4].

Upon incubation of inactivated PFK with high concentrations of fructose 1,6-diphosphate (FDP) (1 mM) the formation of active PFK occurred without phosphorylation. High concentrations of Mg^{++} (15 mM) inhibited this process. The FDP-activated PFK immediately inactivated upon removal of FDP (experiments not shown here). FDP also inhibited inactivation of [^{32}P] phosphorylated PFK by the inactivating protein but without inhibiting the release of ^{32}P.

According to our experiments there exist a kinase and a phosphatase in rat liver which are able to activate and inactivate liver PFK. A tentative model of the activation–inactivation reactions which best fits our data is shown in fig.8. We postulate that all aggregated forms of PFK are enzymatically active. A stable S-form exists when the protomers are phosphorylated, a labile L-form when they are dephosphorylated. The phosphatase hydrolyses a serine phosphoester in the presence of high Mg^{++} concentrations yielding the L-form which easily dissociates into the 4 inactive protomers. The kinase catalyzes phosphorylation of protomers thus enabling aggregation with unphosphorylated protomers to form active PFK. Preliminary data indicate that tetrameric forms of unphosphorylated PFK (e.g. in the presence of FDP) are more rapidly phosphorylated than the unphosphorylated protomers.

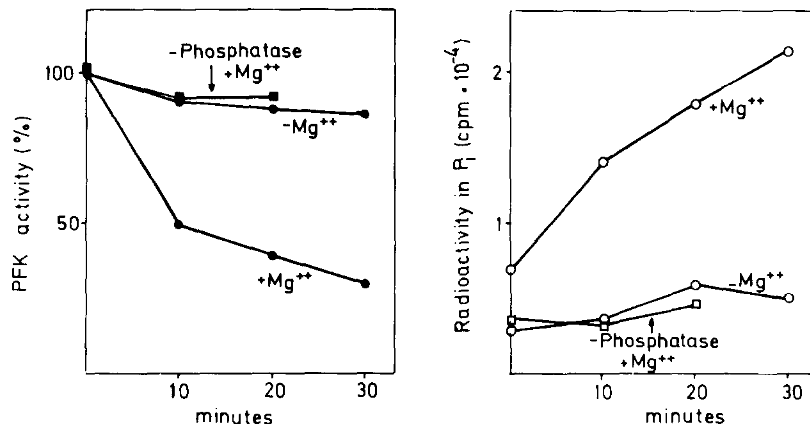

Fig.7. Inactivation of ^{32}P-labelled active rat liver phosphofructokinase by the inactivating protein (left) and release of free $^{32}P_i$ (right). The concentration of $MgCl_2$ was 20 mM. Controls contained either 20 mM $MgCl_2$ without inactivating protein or inactivating protein but only 0.5 mM $MgCl_2$.

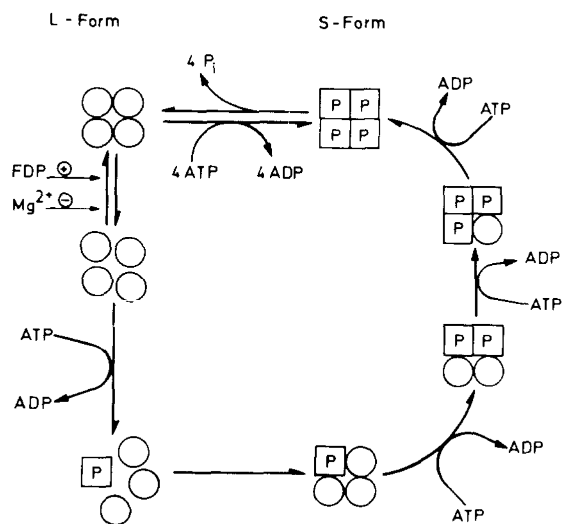

Fig.8. Tentative model of interconversion of rat liver phosphofructokinase between various active and inactive forms. For details see Results and discussion.

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