Interactions of antibodies against soluble phosphofructokinase with the soluble and particulate enzymes from yeast

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Antibodies obtained from rabbits against soluble yeast phosphofructokinase also react with the particulate yeast phosphofructokinase. Their effects on the activity of the soluble enzyme recognized as inactivation or slight activation depend on the specific immune response of an individual animal yielding antisera with different proportions of inactivating and activating antibodies. The availability of particulate phosphofructokinase to complex inactivating antibodies specifically allows a separation of activating and inactivating antibodies from each other by a simple extraction procedure.

Phosphofructokinase *Yeast, particulate enzyme* *Interaction, with antibodies*

1. INTRODUCTION

Phosphofructokinase (EC 2.7.1.11) from yeast, a key enzyme of glucose catabolism [1,2], is composed of two kinds of subunits, α and β, being present in a 1:1 ratio and forming an octameric assembly [3,4]. It was deduced that the larger subunit α does not participate in the catalytic process and the substrate binding sites being localized exclusively on the β-subunits [5].

In addition to this well-known cytosolic enzyme the presence of a membrane-bound phosphofructokinase was demonstrated in yeast [6]. This particulate enzyme is not inhibited by ATP but strongly inhibited by fructose 1,6-bisphosphate. With fructose 6-phosphate as well as with ATP the membrane-bound phosphofructokinase displays hyperbolic saturation curves; fructose 2,6-bisphosphate does not activate the enzyme. Furthermore, the gene responsible for the determination of the regulatory subunit of the soluble allosteric enzyme may be an essential determinant for the synthesis of the particulate enzyme [7,8].

In [9], immunization of rabbits with the soluble phosphofructokinase gave rise to distinct antibodies actually directed against the two subunits α and β. Here, we deal with the interaction of antibodies produced by rabbits after immunization with soluble phosphofructokinase with the cytosolic and the membrane-bound enzyme.

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid, disodium salt

2. EXPERIMENTAL

2.1. *Enzymes and chemicals*

Baker's yeast was purchased as Stellhefe I (exponentially growing yeast on sugar substrates) from VEB Backhefe (Leipzig). ATP, AMP, NADH, fructose 6-phosphate and auxiliary enzymes were products from Boehringer (Mannheim). Polyethylene glycol 4000 and agarose were obtained from Serva (Heidelberg). Buffer substances were of analytical grade. Soluble phosphofructokinase was prepared from baker's yeast as in [3].

2.2. *Enzyme assays*

Phosphofructokinase activity was measured as in [1]. Particulate phosphofructokinase was assayed by coupling the formation of fructose 1,6-bisphosphate from fructose 6-phosphate and ATP in a membrane suspension with the aldolase

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reaction and subsequent determination of triose phosphates: 0.5 ml membrane suspension (see below) were mixed with 1 ml assay buffer containing 3 mM fructose 6-phosphate, 0.6 mM ATP, 5 mM $MgSO_4$, 5 mM $(NH_4)_2SO_4$, 0.2 mM NADH, 0.05 mM PMSF and 0.2 units of aldolase in 0.1 M imidazole–HCl (pH 7.15). After 10 min the samples were centrifuged at 10000 × *g* and 1 ml supernatant was analyzed by adding 0.25 units of glycerol 1-phosphate dehydrogenase and 2.7 units of triose phosphate isomerase.

2.3. *Immunological methods*

For determination of antibody titers immunodiffusion was done as in [10] in 0.1 M sodium phosphate buffer (pH 7.5) and 1.5% agarose in the presence of 2.5% polyethylene glycol 4000. Purified phosphofructokinase was used as antigen at 1 mg/ml.

2.4. *Antibody preparation*

Phosphofructokinase (3 mg) was dialyzed against 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM PMSF, 1 mM fructose 6-phosphate, 5 mM 2-mercaptoethanol and 0.5 mM EDTA. A final volume of 0.5 ml was emulsified with an equal volume of complete Freund's adjuvant and injected i.m. at 2 sites in male rabbits of ~2 kg. After 10 weeks a second and after 15 weeks a third s.c. injection of 0.5 mg enzyme in complete Freund's adjuvant followed. One week later the antibody titer was ~1:64. The serum was fractionated with ammonium sulphate at 45% saturation and the precipitate was submitted to DEAE-cellulose chromatography at pH 8.2 in 50 mM sodium phosphate buffer. Protein which was not bound to DEAE-cellulose was concentrated by ammonium sulphate precipitation and stored at 4°C in 0.1 M sodium phosphate buffer (pH 7.2) containing 2.5 M ammonium sulphate. Before use, the immunoglobulin solution was dialyzed and diluted to equal titers.

2.5. *Preparation of yeast cell membrane suspension*

Yeast cells were washed and disrupted by ultrasonication as in [11]. Soluble phosphofructokinase was removed from the 10000 × *g* pellet of the cell homogenate by repeated washing with 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM PMSF and 0.5 mM EDTA. Each wash was checked by measuring phosphofructokinase activity in the 10000 × *g* supernatant. If they were free of soluble enzyme the pellet was suspended in an equal volume of washing buffer and designated as 'membrane suspension'. As a control, whole yeast cells were washed and suspended in the same way.

2.6. *Treatment of yeast membranes with antibodies*

Membrane suspension (0.5 ml) was mixed with 1 ml of antibodies of two rabbits A and B, diluted to a titer of 1:128. After incubation for 1 h and centrifugation at 10000 × *g* 1 ml of the supernatant was taken off and mixed again with 0.5 ml of a fresh membrane suspension. Incubation and centrifugation were done as before; the same process was repeated twice with 0.5 ml fresh membrane suspension for each cycle. After the fourth incubation the supernatant was used as 'membrane-treated anti-PFK I'. To discriminate unspecific antibody adsorption a second membrane treatment of antibodies was done: The 10000 × *g* pellet of the first incubation was washed and treated again with 1 ml antibody solution for 1 h. This supernatant was designated as yeast 'membrane anti-PFK II'. The precipitation titers of both the 'membrane-treated anti-PFK I and II' were brought to equal values by considering the dilution in the course of membrane treatment.

In a control experiment whole yeast cell suspension instead of disrupted cells was incubated with antibodies under the same conditions. Another control was done by treating the membrane suspension with purified immunoglobulins of a rabbit non-immunized with phosphofructokinase.

3. RESULTS

3.1. *Interaction of antibodies against soluble phosphofructokinase with particulate phosphofructokinase*

Table 1 shows the activity of particulate phosphofructokinase in yeast membranes. In whole cells, practically no phosphofructokinase activity could be detected in the suspension buffer in the absence or presence of added immunoglobulins. However, washed membrane pellets of ultrasonic homogenates, free of cytosolic

Table 1

Activities of particulate phosphofructokinase in yeast membranes untreated and treated with antibodies against soluble phosphofructokinase

Suspension of washed 10000 × g pellet of	Fructose 1,6-bisphosphate formed (μmol) (.min^{-1}. 1.5 ml suspension^{-1})	Activity (%)
Disrupted yeast cells	0.015	100
– treated with antibodies from rabbit A	0.009	60
– treated twice with antibodies from rabbit A	0.006	40
– treated with antibodies from rabbit A pre-treated 4 times with disrupted yeast cells (anti-PFK I)	0.012	80
– treated with antibodies from rabbit B	0.011	73
– treated twice with antibodies from rabbit B	0.009	60
– treated with antibodies from rabbit B pre-treated 4 times with disrupted yeast cells (anti-PFK I)	0.015	100
– treated with immunoglobulins from a non-immunized rabbit	0.014	93
Whole yeast cells	0.001	–
– treated with antibodies from rabbit A	0.002	–

phosphofructokinase, show the formation of fructose 1,6-bisphosphate, obviously due to the presence of the particulate phosphofructokinase [6]. This activity decreased partially if the membranes are incubated with antibodies against the soluble phosphofructokinase. The extent of inhibition depends on the source and the pretreatment of immunoglobulins with disrupted yeast cells. Immunoglobulins from non-immunized rabbits do not display any effect on the activity of particulate phosphofructokinase.

3.2. *Interaction of antibodies with soluble phosphofructokinase*

Purified soluble phosphofructokinase was incubated with anti-phosphofructokinase immunoglobulins, diluted to titers of 1:128. Fig.1 shows the changing in activity obtained with antibodies from rabbits A and B and with immunoglobulins of a non-immunized rabbit. Obviously, immunoglobulins of the latter do not influence phosphofructokinase activity. In the case of antibodies from rabbit A an inactivation of the enzyme was observed. If antibodies were diluted inactivation is delayed in a proportional manner (not shown). There is also no difference in the activities whether the samples are subjected to centrifugation or not.

In contrast, antibodies of rabbit B exhibiting the same titer as antibodies from rabbit A do not effect phosphofructokinase activity.

To elucidate the action of membrane-treated anti-PFK with soluble phosphofructokinase the enzyme solution was incubated with membrane-

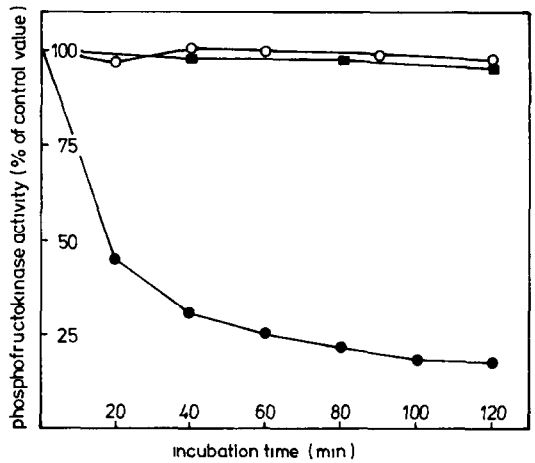

Fig.1. Time dependence of phosphofructokinase activity during incubation with antibodies from different animals. Phosphofructokinase solution (0.1 ml, 1 mg/ml) was incubated with 0.2 ml immunoglobulin solution from 2 immunized rabbits in 1.2 ml total vol. of 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM PMSF and 0.5 mM EDTA. Aliquots of 0.1 ml were taken at appropriate times and phosphofructokinase activity was estimated. Activities are compared with a control in which immunoglobulin solution is replaced by 0.2 ml buffer: (■—■) immunoglobulin from a non-immunized rabbit; (●—●) immunoglobulin from immunized rabbit A; (○—○) immunoglobulin from immunized rabbit B.

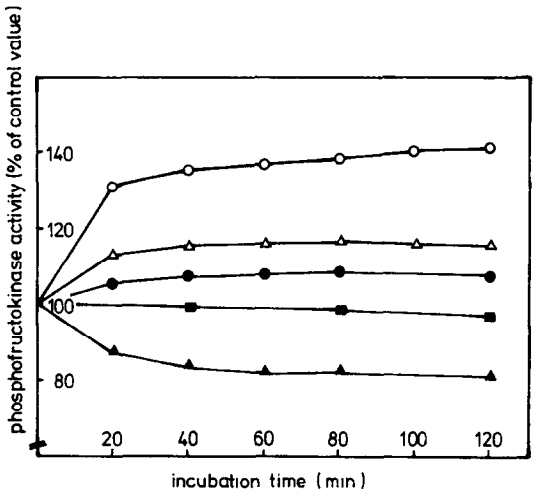

Fig.2. Time dependence of phosphofructokinase activity during incubation with yeast membrane-treated antibodies. Phosphofructokinase solution (0.1 ml, 1 mg/ml) was incubated with 1 ml 'membrane-treated anti-PFK I or II' or with 1 ml immunoglobulin solution from a non-immunized rabbit, treated 4 times with yeast membranes. Final volume was 1.2 ml. Aliquots (0.1 ml) were tested for phosphofructokinase activity. Activities are compared with a control in which immunoglobulin solution is replaced by buffer: (■—■) membrane-treated immunoglobulins from a non-immunized rabbit; (●—●) membrane-treated anti-PFK I from rabbit A; (▲—▲) membrane-treated anti-PFK II from rabbit A; (○—○) membrane-treated anti-PFK I from rabbit B; (△—△) membrane-treated anti-PFK II from rabbit B.

treated anti-PFK I or II, respectively, from the rabbits A and B or with membrane-treated immunoglobulins of a non-immunized rabbit (fig.2). The membrane-treated immunoglobulins of the control animal do not show any effect on phosphofructokinase activity. Antibodies of rabbit A, causing an inactivation if they are not pretreated with membranes, give rise to a slight increase of enzyme activity after treatment with yeast membranes. Under these conditions antibodies of rabbit B even cause a remarkable activation up to 140% comparing with the control activity. The membrane-treated anti-PFK II of rabbit A and B exhibit an intermediate action being between the original and the 4-times membrane-treated antibodies. If the antibodies are treated with whole yeast cell suspensions instead of disrupted yeast cells their effects on phosphofructokinase activity are identical with those obtained with the original immunoglobulins.

4. DISCUSSION

Immunization of rabbits with purified cytosolic yeast phosphofructokinase yields antisera which differ in their effects on phosphofructokinase activity. The diverse action of antibody populations from rabbits A and B on soluble phosphofructokinase can be explained by the presence of varying ratios and different affinities of inhibiting, non-inhibiting and activating antibodies in these antisera [12]. If antibodies are directed against the 2 subunits α and β of the soluble enzyme, one could expect that the particulate phosphofructokinase described in [6] should cross-react with these antibodies because one gene is assumed to be involved in the specification of both the particulate enzyme and the regulatory subunit of the soluble enzyme [7,8]. In fact, after treating disrupted yeast cells containing the particulate enzyme with immunoglobulins from rabbits immunized with cytosolic phosphofructokinase the activity of the membrane-bound enzyme decreases, indicating the expected cross-reactivity (table 1). Moreover, by treatment of the immunoglobulins with yeast membranes inactivating antibodies are removed from the antibody mixture and the remaining fractions are able to activate the soluble phosphofructokinase (rabbit B) or their inhibitory effect ceases (rabbit A). By considering also the control with non-disrupted cells and with membranes twice treated with antibodies the interaction of antibodies with the particulate phosphofructokinase seems to be specific. But if the common component of the soluble and the membrane-bound enzyme is the regulatory subunit of the cytosolic enzyme, it is surprising that anti-soluble phosphofructokinase causes an inactivation of both types of enzymes. An inactivation may be expected if the antibodies bind preferentially to the active center of the enzyme. Because the particulate enzyme and the β-subunit of the soluble enzyme (the nomenclature of cytosolic phosphofructokinase subunits is in accordance with [3,4,13] but in contrast to [8]), on which the catalytic centers are assumed to be localized, are products of distinct genes the cross-reactivity of inactivating antibodies may be due to their interactions with conforma-

tional determinants located closely to the active center both in the membrane-bound and in the soluble enzyme.

The activation of phosphofructokinase caused by membrane-treated antibodies has to be attributed to activating antibodies. A similar effect has been described for phosphofructokinase from rabbit skeletal muscle [14,15]. Due to the specific stability of the oligomeric structure of the yeast phosphofructokinase [3] the nature of its activation remains to be elucidated.

The possibility to remove inactivating antibodies from antisera containing both activating and inhibiting antibodies may be a useful tool for further experiments.

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