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INTERACTION OF PHOSPHOFRUCTOKINASE WITH ANTIBODIES

KINETIC PROPERTIES OF PHOSPHOFRUCTOKINASE IN COMPLEXES WITH ANTIBODIES

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Summary

The allosteric properties of phosphofructokinase (EC 2.7.1.11) from rabbit muscle are influenced by enzyme concentration, most probably due to changes in the association state of the enzyme. In this study, the behaviour of dispersed precipitates of phosphofructokinase as produced by treatment with antibodies has been investigated. The enzyme is not capable of rapid dissociation in the precipitated state as is confirmed by the lack of inactivation upon dilution and by the absence of shifts in substrate saturation curves as measured in the presence of different concentrations of the enzyme.

The Hill coefficient of phosphofructokinase is decreased from 1.96 to 1.04 by antibody treatment. The V at neutral pH is increased 3-fold while the $K_{0.5}$ for fructose 6-phosphate is reduced significantly. On the other hand, antibody-treated phosphofructokinase retains its sensitivity to allosteric activation by glucose 1,6-bisphosphate in the presence of high ATP concentrations.

Introduction

Phosphofructokinase (EC 2.7.1.11) from rabbit skeletal muscle exhibits cooperative saturation behaviour with its substrate fructose 6-phosphate at neutral pH. The apparent affinity of the enzyme toward its substrates is not only influenced by positive and negative effectors but also by the enzyme concentration [1,2] suggesting that the aggregation state of the enzyme modifies the kinetic properties. A model of association compatible with kinetic [1,3] and fluorescence [4] data was presented earlier. Kinetic conclusions [3] drawn from that model were recently supported by the results of Lad and Hammes [5] who used for their studies aggregates of phosphofructokinase which were cross-linked by superimidate. A disadvantage of this method for

preparing stable aggregates of phosphofructokinase, however, was a 60% loss of enzymic activity upon reaction of the probe with amino groups of the enzyme.

It has been shown previously [6] that the specific activity of phosphofructokinase is not changed by precipitation with specific antibodies from sheep serum. Antibodies, therefore, offered the possibility of studying the properties of phosphofructokinase in the rigid network of precipitated enzyme-antibody complexes. This state promised to be similar to that of the enzyme cross-linked by low-molecular bifunctional reagents.

Materials and Methods

Materials

Buffer substances were supplied in analytical reagent grade by Merck (Darmstadt, Germany). Enzyme substrates, and the enzymes aldolase, triosephosphate isomerase, and glycerol 3-phosphate dehydrogenase were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Phosphofructokinase from rabbit skeletal muscle was purified and crystallized as described previously [7]. Complete Freund's adjuvant was from Difco Laboratories (Detroit, U.S.A.). Immuno plates were products of Schwarz Bio Research (Orangeburg, N.Y., U.S.A.).

Immunization

Antibodies against crystalline phosphofructokinase from rabbit skeletal muscle were obtained by immunizing mature sheep (Württembergisches Landschaf) with a total of 25 mg enzyme. For each application, 5 mg phosphofructokinase were dissolved in 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.6) containing 0.2 M KCl and 0.5 mM fructose 1,6-bisphosphate and emulsified with 0.5 ml complete Freund's adjuvant.

Three doses were injected intramuscularly on successive days. After 1 week interval, the immunization was completed by two further injections. 250 ml of blood were obtained by puncture of the vena jugularis 10 days later. About 110 ml of serum were won in the usual way, frozen in liquid nitrogen, and stored at $-75°$ C.

In order to exclude interactions between phosphofructokinase and components of the antisera other than globulins, the γ-globulin fractions were isolated by repeated precipitation with Na_2SO_4. The antibody preparations obtained were free from albumin and showed a single precipitation band with phosphofructokinase in the double diffusion test.

As a control, the animals were bled before the beginning of the immunization procedure and the γ-globulin fraction was isolated as described above.

Assays

Phosphofructokinase activity was determined as described previously [7]. The protein concentrations were determined spectrophotometrically in 2 M NaOH by the absorbance at 290 nm, using an extinction coefficient of 1.09 for 1 mg phosphofructokinase/ml [8] and 1.3 for 1 mg γ-globulin/ml [9].

Results

Precipitation of phosphofructokinase by antibodies

The specific activity of phosphofructokinase was not altered when the enzyme was incubated with various amounts of γ-globulins from an antiserum for 15 min at 22°C in phosphate buffer containing 0.2 M KCl and 0.5 mM fructose 1,6-bisphosphate (pH 7.6) provided that the finely dispersed insoluble material was not removed from the samples by centrifugation. In this incubation medium, the enzyme obviously was not inhibited by the antibodies though it was completely precipitable in the presence of high antibody concentrations.

Dilution-dependent inactivation of phosphofructokinase

The specific activity of soluble phosphofructokinase decreases when the enzyme is diluted in the absence of either substrate or positive allosteric effector [10]. This decrease of the specific activity in the presence of low enzyme concentrations is illustrated by Curve A of Fig. 1. In this experiment, phosphofructokinase was diluted in the presence of purified γ-globulins obtained from the control serum.

In the series of experiments leading to Curve B of Fig. 1, the non-specific γ-globulins were replaced by the same concentration of globulins from the antiserum which was capable of converting all the enzyme into precipitable complexes. Under these conditions, the specific activity of the enzyme remained almost constant after dilution, showing that the dilution-dependent inactivation of phosphofructokinase was prevented by the antibodies.

The absence of inactivation in the case of the enzyme present in an insoluble state demonstrates that the loss of specific activity is the result of changes in the enzyme structure or conformation which is either suppressed by the antibodies or does only occur in solution. The specific activity of antibody-bound phosphofructokinase is exceeded by that of the free enzyme in the

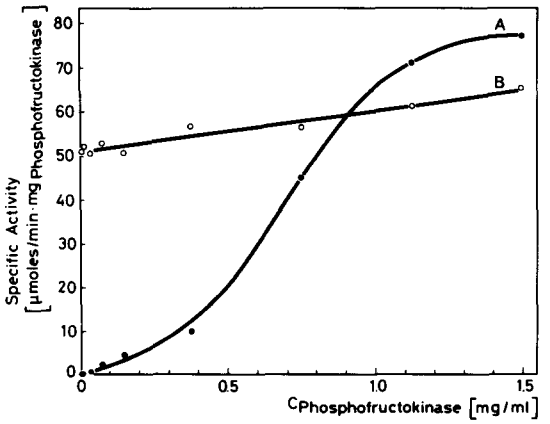

Fig. 1. Specific activities of phosphofructokinase after dilution in the presence of a constant concentration of antibodies (Curve B, o———o) or control globulins (Curve A, •———•). Different concentrations of phosphofructokinase as indicated by the abscissa were incubated in 0.1 M imidazole buffer (pH 7.5) containing 4.3 mg antibody-containing γ-globulins per ml or an equal amount of control globulins. The enzyme was diluted 25-fold upon addition to the assay system.

presence of high enzyme concentrations. This phenomenon may be due to the retardation of the substrate binding in the heterogeneous phase system.

Influence of antibodies on the behaviour of phosphofructokinase at suboptimal substrate concentrations

Phosphofructokinase shows sigmoidal substrate saturation curves for fructose 6-phosphate as demonstrated by Curve A of Fig. 2a. In the presence of 10 μg enzyme/ml and of 3.0 mM ATP, the Hill coefficient was 1.96 (cf. Fig. 2b). Curve B illustrates analogous experiments with the only difference being that the enzyme was incubated with antibodies prior to addition to the test cuvette. The optimum activity increased three times and the substrate saturation curve was shifted toward smaller concentrations of fructose 6-phosphate. The half-saturating concentration of the substrate decreased from 0.95 mM, as measured in the absence of antibodies, to 0.6 mM in the case of antibody-bound insoluble enzyme. Moreover, the sigmoid saturation curve for fructose 6-phosphate was converted to a hyperbolic function as indicated by the Hill coefficient of 1.06. Thus, antibodies not only led to an increase in the specific activity but also to a loss of cooperativity of substrate binding.

As will be shown below, cooperative fructose 6-phosphate saturation could be observed at pH 6.6 and 10 mM ATP also for insoluble phosphofructokinase antibody complexes. Thus, the allosteric properties of phosphofructokinase are weakened but not completely abolished by antibodies.

Action of allosteric effectors on phosphofructokinase in the presence of antibodies

The change in substrate saturation curves which occurred on preincuba-

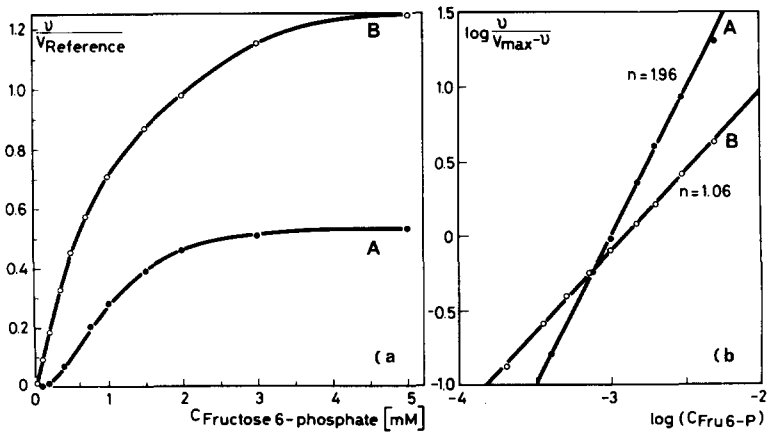

Fig. 2. (a) Saturation of phosphofructokinase with fructose 6-phosphate in the presence and absence of antibodies. 0.5 mg phosphofructokinase were incubated for 15 min at 22°C in 0.1 M imidazole · HCl buffer + 0.2 M KCl (pH 7.5) in the presence of 1.72 mg antibody-containing (Curve B, o——o) or control (Curve A, •——•) γ-globulins in a volume of 1 ml. The samples were diluted 100-fold upon addition to the assay. The enzyme activities V, as measured in the presence of different fructose 6-phosphate concentrations, 3.0 mM ATP and 4.0 mM Mg^{2+} (pH 6.9), were referred to the activities $V_{ref.}$ obtained at pH 7.6 under saturating substrate concentrations using an enzyme sample which was diluted to 0.5 mg/ml in phosphate buffer ($V_{ref.}$ = 140 units/mg phosphofructokinase). (b) Hill plots of the data presented in a.

tion of phosphofructokinase with antibodies closely resembled the action of positive effectors on the enzyme. This opened the question of whether the antibodies bind to the same site as the allosteric effectors, exerting their influence by the induction of conformational changes of the enzyme molecule. In this case, additive activation of phosphofructokinase by antibodies and allosteric effectors should not occur provided that the enzyme was saturated with the allosteric activator. The investigation of the influence of allosteric activators in the presence of antibodies is complicated by the fact that phosphofructokinase from rabbit skeletal muscle is an allosteric enzyme of the K-type [11] being sensitive to allosteric activation only under conditions which lead to cooperative fructose 6-phosphate saturation. As mentioned above, sigmoid substrate saturation curves were obtained at pH 6.6 and very high ATP concentrations (about 10 mM) also in the presence of antibodies (cf. Curve A, Fig. 3).

The influence of a saturating concentration (0.2 mM) of the allosteric activator glucose 1,6-bisphosphate on phosphofructokinase which was precipitated by antibodies is illustrated by Curve B of Fig. 3. Under the conditions of the experiment, glucose 1,6-bisphosphate acted as a strong allosteric activator shifting the saturation curve towards lower concentrations of the substrate. The effect of the allosteric activator was obviously not abolished by the antibodies. The activation of phosphofructokinase by the combined action of antibodies and glucose 1,6-bisphosphate was much stronger than the optimum activation by the allosteric effector alone; thus suggesting different mechanisms of activation of phosphofructokinase by antibodies and allosteric effectors in the presence of suboptimal substrate concentrations.

Effect of different enzyme concentrations on the substrate saturation of insoluble enzyme antibody complexes

The kinetic behaviour of phosphofructokinase precipitated by antibodies was similar to that observed in the presence of high concentrations of the enzyme. Highly concentrated phosphofructokinase showed substrate saturation

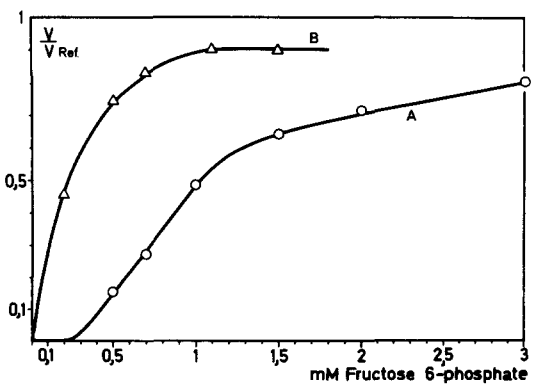

Fig. 3. Dependence of the enzymic activity of insoluble phosphofructokinase antibody complexes on fructose 6-phosphate concentration in the absence (Curve A, ○———○) and presence (Curve B, △———△) of 0.2 mM glucose 1,6-bisphosphate. The activities V were determined at pH 6.6 in the presence of 9.6 mM ATP (Mg^{2+}, 4.0 mM) and referred to the activity $V_{ref.}$ as described in the legend of Fig. 2.

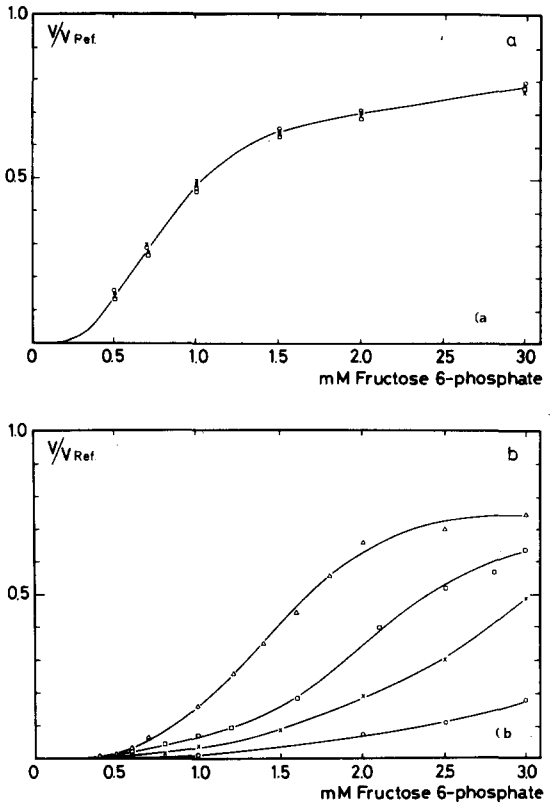

Fig. 4. (a) Saturation curves of insoluble phosphofructokinase antibody complexes for fructose 6-phosphate in the presence of various concentrations of the enzyme-antibody complex. Phosphofructokinase was completely precipitated with antibodies in the presence of 0.1 M imidazole · HCl buffer containing 0.2 M KCl (pH 7.5) and resuspended to different concentrations in the same medium. The concentrations of the phosphofructokinase moiety in the complexes were 0.05 mg/ml (△———△), 0.02 mg/ml (□———□), 0.01 mg/ml (X———X), and 0.005 mg/ml (○———○). The activities V were measured at pH 6.6 in the presence of 9.6 mM ATP (Mg^{2+}, 4.0 mM) and referred to the activity $V_{ref.}$ as determined at pH 7.6 in the presence of 2 mM fructose 6-phosphate and 1.5 mM ATP (Mg^{2+}, 4.0 mM). (b) Saturation curves of soluble phosphofructokinase for fructose 6-phosphate determined in the presence of the same enzyme concentrations as used in the experiments of a. The measurements were performed at pH 6.9 in the presence of 3.0 mM ATP and 4.0 mM Mg^{2+}.

curves which were weakly sigmoid and was only slightly sensitive to ATP inhibition [1—3]. Sigmoidicity of fructose 6-phosphate saturation curves became obvious on decreasing enzyme concentrations while the maximum specific activity of the enzyme also decreased. Curves of this type, as measured in the absence of antibodies are shown in Fig. 4b. Changes in the shape of substrate saturation curves in the presence of varying enzyme concentrations were not detected with phosphofructokinase which was insolubilized by antibodies as may be seen from Fig. 4a. The dependence of the specific activity of phosphofructokinase on the concentration of fructose 6-phosphate was identical for all enzyme concentrations.

Discussion

In preceding reports [1,3,4], a functional model of rabbit muscle phosphofructokinase has been suggested which referred some of the regulatory properties of the enzyme to transitions between phosphofructokinase species of different aggregation states in addition to the well-known allosteric effects of activators and inhibitors [12]. The model is based on the assumption that active phosphofructokinase molecules are able to dissociate reversibly into inactive subunits; in addition to this step which affects the optimum specific activity (V), the cooperativity of the saturation of the enzyme with fructose 6-phosphate is reduced by the association of the enzyme to high aggregates.

As changes of the molecular weight of phosphofructokinase during kinetic measurements could be hardly ruled out, it was desirable to fix a given association state by cross-linking reagents. Experiments with bifunctional probes reacting with amino acid residues like superimidate or glutaraldehyde suffer from the loss of phosphofructokinase activity upon modification of amino groups [5]. The use of antibodies seemed to be more promising for several reasons: (1) The enzyme is not modified by covalent linkages and retains its full activity. (2) The reaction of the enzyme with a given lot of antibodies is highly reproducible and the enzyme in finely dispersed precipitates behaves quite similar as in a solution when assayed under optimum conditions. (3) Phosphofructokinase fixed in the rigid network of insoluble enzyme antibody complexes is not likely to undergo rapid molecular weight transitions.

The primary disadvantage of the antibody cross-linking method lies in the impossibility of identifying defined structures in the precipitates. Yet, as the precipitation of phosphofructokinase was performed under conditions which favour association, e.g. in the presence of high enzyme concentration, we assume that the enzyme is present in the precipitates in a high molecular weight form.

In fact, the properties of phosphofructokinase in insoluble enzyme antibody complexes differ from the properties of dissolved enzyme of the same concentration in several respects: in contrast to the soluble enzyme, the insoluble phosphofructokinase in the complex is not inactivated by dilution. Moreover, it is less inhibited by ATP and the cooperativity of substrate saturation curves is greatly reduced and does no longer depend on phosphofructokinase concentration.

Acknowledgements

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