COLD LABILE PHOSPHOFRUCTOKINASE

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Summary

Phosphofructokinase from chicken liver undergoes reversible inactivation at low temperature. The rate of the inactivation increases with decrease in temperature. The cold inactivation is stimulated by monovalent anions or ATP, but prevented by polyvalent anions or fructose 1,6-diP. Results of sucrose density gradient centrifugation indicate that the cold inactivation is associated with dissociation of the phosphofructokinase (13.9S) to a protein with a S value of 5.4.

Phosphofructokinase, a key regulatory enzyme in glycolysis, has been investigated from a variety of sources (see reviews 1 and 2). The enzyme has been obtained in pure form from rabbit (3,4) and rat muscle (5), sheep heart (6), yeast (7), E. coli (8) and Clostridium pasteurianum (9). Pure liver phosphofructokinase was not available and as part of our studies on this enzyme we began purification of the enzyme from chicken liver. During the purification we found that the enzyme appears to be unstable at low temperature. This property has not been reported in any of other phosphofructokinases previously studied. This paper describes kinetics and reversibility of cold inactivation, and structural change accompanying the inactivation.

Materials and Methods

All enzymes except for phosphofructokinase were purchased from C. H.

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Boehringer and Mannheim, New York. All the chemicals were obtained from commercial sources as described previously (10).

Phosphofructokinase from chicken liver was purified to yield a homogeneous protein as judged by disc electrophoretic and ultracentrifugal techniques. The purification procedure involves activation of the crude extract of chicken liver by heat treatment, ammonium sulfate fractionation, DEAE-cellulose chromatography and molecular sieve filtration on Biogel 1.5 M. The specific activity of the purified phosphofructokinase is 114 units per mg. A detailed description of this purification procedure will be published elsewhere. The enzyme was usually stored at a protein concentration of 20 mg/ml in 0.2 M tris-P buffer (pH 8) containing 1 mM fructose 1,6-diP, 25 mM dithiothreitol, and 1 mM EDTA at 4°. Under these conditions the enzyme is stable for at least two weeks. To prepare fully active enzyme the enzyme was dissolved in 0.05 M glycylglycine buffer (pH 8.0) containing 25 mM dithiothreitol and 1 mM EDTA, and incubated at 25°. After a few hours the enzyme solution (0.2 ml, 1 mg/ml) was passed through a Sephadex G-25 (0.5 x 18 cm) at 25° which had been equilibrated with a solution containing 0.05 M glycylglycine buffer (pH 8.0), 20 mM potassium phosphate, 25 mM dithiothreitol and 1 mM EDTA.

Activity of phosphofructokinase was determined at 25° by measuring the rate of formation of fructose 1,6-diP as previously described (11), except glycylglycine at pH 8.2 instead of tris-HCl, and in addition 5 mM $MgCl_2$ and 1 mM each of fructose-6-P and ATP were used.

Sucrose density gradient centrifugation was performed according to the procedure of Martin and Ames (12).

Results and Discussion

Phosphofructokinase from chicken liver is rapidly inactivated when incubated at low temperatures as shown in Fig. 1. After 2 hours at 0° and 8° the enzyme loses 80% and 65% of its original activity respectively, whereas at 25° the enzyme is completely stable. A plot of these data as log. of

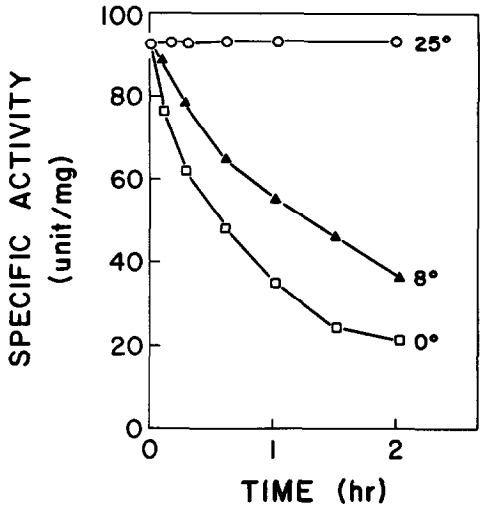

Fig. 1: Effect of temperature on the stability of phosphofructokinase. The enzyme at 0.01 mg/ml was incubated in 0.05 M glycylglycine at pH 8, 8 mM potassium phosphate, 2 mM ammonium sulfate, 25 mM dithiothreitol and 1 mM EDTA. The enzyme solution was incubated at 25° (o), 8° (▲) and 0° (□) and at given time interval aliquots were removed and assayed for enzyme activity as described in "Methods".

activity vs. time yields a biphasic time course of inactivation.

The cold inactivation depends on a variety of conditions such as protein concentration, pH of the medium, and the concentration and type of salt of the medium. For example, at 5 mg/ml or above the enzyme at 0° is completely stable for at least one day, but below 0.3 mg/ml the activity is lost very rapidly at this temperature. Thus, loss of the activity after 1 hour at 0° at protein concentrations of 30 μg/ml, 60 μg/ml, and 300 μg/ml is 70%, 55% and 35%, respectively.

The cold inactivation is enhanced by monovalent anions such as Cl^- or Br^-, but polyvalent anions such as phosphate or sulfate tend to protect against the inactivation. Fructose 1, 6-di-P also offers protection, but ATP enhances the rate of cold inactivation. The pH of the medium has a significant effect on the inactivation process. At 0° the enzyme is most stable at pH 8.3, but the stability decreased considerably above or below this pH.

As shown in Fig. 2 the cold inactivated phosphofructokinase can be reactivated by warming to 25°. In this experiment the enzyme which had been

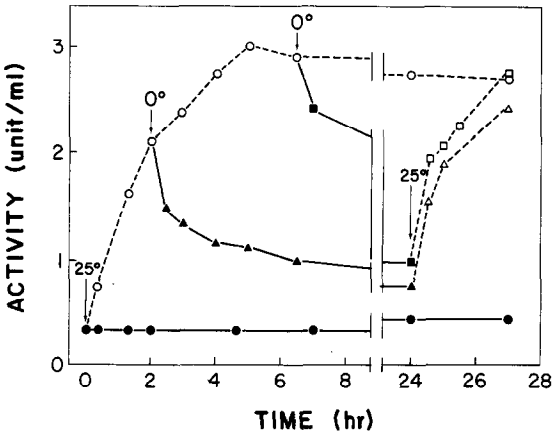

Fig. 2: Reversibility of cold inactivation. Cold inactivated enzyme prepared by storage at 4° C for 2 weeks at 5 mg/ml was diluted to 0.05 mg/ml in 0.2 M potassium phosphate pH 8.0 containing 0.8 M KCl, 5 mM dithiothreitol and 1 mM EDTA, and incubated at 0° (●—●) and 25° (o --- o). After 2 and 6.5 hours, aliquots were cooled to 0°C (▲-▲, ■-■) and heated again at 25°C (Δ---Δ, □---□).

inactivated at 0° was incubated at 25° to yield active enzyme. After 2 and 6.5 hours aliquots were removed and cooled to 0°, which resulted in loss of the activity. These results show that the cold inactivation is reversible and that the enzyme can be recycled between active and inactive forms by changes in temperature.

In order to determine if any structural changes occurred during the cold inactivation, samples of phosphofructokinase which had been incubated at 0° or 25° were subjected to sucrose density gradient centrifugation at 4° or 25°. The results are shown in Fig. 3. Aldolase (Ald.) and pyruvate kinase (PK) were used as molecular weight markers in these experiments. At 25° the enzyme sediments faster than pyruvate kinase and its S value is estimated as 13.9. On the other hand, the enzyme incubated at 0° and centrifuged at 4° the enzymic activity was not detectable in any of the fractions. Upon heating those fractions at 25° for 1 hour the enzymic activity appears as two peaks between fractions 18 and 30, and the corresponding S values are 7.5 and 5.4. The cold inactivated enzyme in the presence of 0.5 mM ATP (which enhances the rate of cold inactivation) shows only one peak corres-

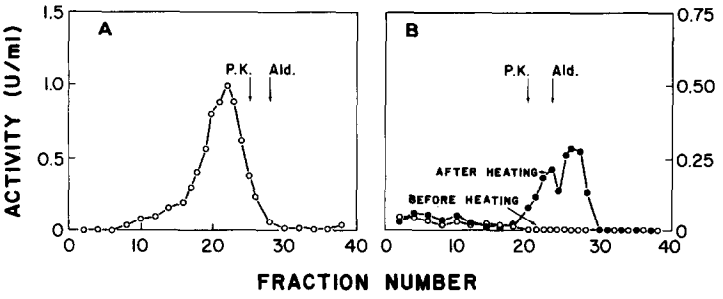

Fig. 3: Sucrose density gradient centrifugation of phosphofructokinase at 4° and 25°. The enzyme was incubated in 0.05 M glycylglycine, 20 mM potassium phosphate, 25 mM dithiothreitol and 1 mM EDTA pH 8.0 at the protein concentration of 0.2 mg/ml for 3 hours at 25° and 0°, respectively. At the end of the incubation an aliquot (0,1 ml) of the solution was placed on a sucrose gradient containing the above buffer mixture and subjected to centrifugation. (A) The enzyme incubated and centrifuged at 25° at 20,000 RPM for 15 hours. (B) The enzyme incubated at 0° and centrifuged at 4° at 31,000 RPM for 15 hrs. Pyruvate kinase (0.1 μg), "PK", and aldolase (10 μg), "Ald", were added in the gradient as internal marker proteins. The activity of the phosphofructokinase was determined as described in "Methods" before (o - o) and after heating (● - ●). The activities of pyruvate kinase and aldolase were determined according to the procedure of Wu and Racker (13).

ponding to 5.4S proteins. It appears, therefore, the 7.5S protein observed in the absence of ATP is probably due to the incomplete inactivation (or dissociation) of the enzyme at the beginning of centrifugation and it is formed during the centrifugation at 4°. In the presence of fructose 1,6-diP at 4° which protects against the cold inactivation, only one peak corresponding to 13.9S protein was observed and the dissociated enzyme was not detectable. It is clear from these results that the cold inactivation results in the dissociation of the native enzyme, which is probably a tetramer, to four protomers with a S value of 5.4. It is possible, however, that the dissociation of the enzyme is not a direct effect of low temperature, but rather temperature-induced conformational change which precedes the dissociation reaction.

The nature of the temperature sensitive bonds broken during dissociation of phosphofructokinase is not known. The observation that type and concentration of salt, pH and allosteric effectors all have a pronounced effect on the cold inactivation, and that the rate of inactivation is biphasic suggests that this is a complex phenomenon, and perhaps involves the

disruption of a variety of bonds which hold the polymeric form of the enzyme together.

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