ANALYSIS OF THE COLD LABILITY BEHAVIOR OF RABBIT MUSCLE PHOSPHOFRUCTOKINASE

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SUMMARY: Rabbit skeletal muscle phosphofructokinase has been previously shown to exhibit the characteristic of cold lability in phosphate buffers at pH values below pH 7 [Bock, P.E. and Frieden, C. (1974) Biochemistry 13, 4191-4196]. Studies of the residual activity as a function of pH, reflecting the equilibrium between active and inactive forms of the enzyme, have been performed. These experiments show that the cold lability can be ascribed to a shift of the apparent pK describing the pH-dependent inactivation to lower pH values at higher temperatures. The apparent pK, Hill interaction coefficient and heat of ionization for this process indicate that the equilibrium between the inactive and active forms of the enzyme may be controlled by the ionization of one or more histidine residues per enzyme subunit. In addition the apparent pK for the pH dependence of the residual activity at constant temperature is influenced by the presence of ligands which are substrates or effectors of the phosphofructokinase reaction.

It is well known that rabbit muscle phosphofructokinase undergoes a pH-dependent inactivation at pH values below about pH 7.2 (1,2). Aaronson and Frieden (3) and Pavelich and Hammes (4) showed quantitatively that the inactivation was a consequence of the active enzyme of four subunits dissociating to an inactive form containing two subunits. Bock and Frieden, in describing a mechanism for this process (5) observed that the enzyme showed the characteristics of cold lability at pH values low enough to involve the dissociation of the active enzyme to the inactive form.

In this paper, we will describe results which explain the cold lability characteristics of the enzyme.

MATERIALS AND METHODS

Phosphofructokinase: Crystalline rabbit muscle phosphofructokinase was obtained as an ammonium sulfate suspension in 1 mM ATP from the Sigma Chemical Co. (lot 102C-8720). The enzyme was charcoal treated to remove bound ATP as described previously (5). Most preparations of the enzyme used in these experiments had a specific activity of 160 units/mg measured by the rate of NADH oxidation coupled to fructose 1,6-bisphosphate formation by aldolase, triosephosphate isomerase and α-glycerophosphate dehydrogenase, where one enzyme unit is defined as the formation of 1 μmol of fructose 1,6-bisphosphate/min at 24° and pH 8 in the assay described below.

Assays were performed at 24° and pH 8 under the following conditions: 33 mM Tris-acetate (pH 8); 0.01 mM EDTA; 2 mM magnesium acetate; 2 mM ATP; 2 mM fructose 6-phosphate; 40 mM KCl; 4 mM NH_4Cl; 0.16 mM NADH; 2 units of aldolase; 25 units of triosphosphate isomerase and 2.5 units of α-glycerophosphate dehydrogenase in a final volume of 1 ml.

The reaction was started by the addition of the enzyme to the above assay mixture. The auxilliary enzymes were centrifuged and dissolved in 0.1 M Tris-acetate buffer (pH 8), 0.1 mM EDTA. They were dialyzed overnight against two changes of 100 volumes of this buffer to remove ammonium sulfate.

Enzymes, coenzymes and substrates were obtained from Sigma.

Residual Activity Experiments: Charcoal treated phosphofructokinase in 0.1 M sodium phosphate buffer (pH 8) containing 1 mM EDTA and 1 mM dithioerythritol was diluted from a concentrated stock solution (8-10 mg/ml) to 0.1 mg/ml in 0.13 M sodium phosphate buffers of known pH containing 1 mM EDTA and 0.2 mM dithioerythritol. Incubation mixtures of different pH values were held at the temperature of the experiment in a thermostatted water bath for three hours, sufficient time to obtain maximal inactivation under the particular conditions. Aliquots of the

incubation mixtures were diluted to 5 μg/ml at 0°C in 0.1 M sodium phosphate buffer (pH 8), 1 mM EDTA, 0.2 mM dithioerythritol, and assayed as described above. The activity measured in this way reflects the amount of active enzyme remaining at equilibrium in the incubation mixture at the specified pH, temperature and enzyme concentration (5). Since the enzyme is stable at pH 8 over the temperature range used in these experiments, the data is presented as the residual specific activity at pH values below 8 relative to the activity of the enzyme incubated at pH 8. The pH values reported in these experiments have been corrected to the temperature of the incubation.

RESULTS AND DISCUSSION

The final residual activity of enzyme allowed to undergo inactivation under given conditions reflects the equilibrium amount of enzyme in the active and inactive forms. Figure 1 shows the residual activity as a function of pH (measured as indicated in Methods) at three different

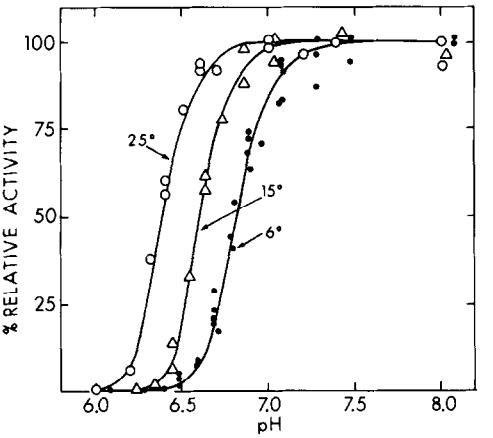

FIGURE 1. pH dependence of the residual activity of phosphofructokinase at 6°, 15° and 25°.

Rabbit muscle phosphofructokinase was incubated for three hours in 0.13 M sodium phosphate buffers containing 1 mM EDTA and 0.2 mM dithioerythritol at the pH and temperature shown and an enzyme concentration of 0.1 mg/ml. Enzymatic activity was measured as indicated in Methods.

temperatures. As has been previously shown (1,2) the residual activity is pH dependent. However, it is now observed that as the temperature is raised the residual activity curve shifts towards lower pH values. This observation explains the characteristics of cold lability described previously (5). Thus, for example, enzyme equilibrated at 6° and pH 6.6 will show considerably less activity compared to enzyme equilibrated at 25° and the same pH. Increasing the temperature beyond 30° yields plots similar to those shown in Figure 1. However, in these cases some irreversible inactivation appears to occur over the three hour period and the data are therefore not comparable to those shown. It would be expected, however, that this same process would occur even at physiological temperatures.

Analysis of any of the curves in Figure 1 shows that the residual activity is a highly cooperative function of pH yielding linear Hill plots with an apparent Hill interaction coefficient of between 4 and 5. The direction of the shift of the pH dependent residual activity curve with temperature shows that the apparent heat of ionization is relatively large and positive (8-10 kcal/mole). Thus the apparent pK value decreases as the temperature is increased. The values for the heats of ionization of carboxyl groups are not consistent with these results since they are either negative or quite small. Ionizable amino acids with large positive heats of ionization include histidine, lysine and N-terminal amino groups. The value of the apparent heat of ionization obtained at a given pH and the value of the apparent pK strongly imply that the ionization of histidine residues is responsible for the pH dependent behavior of the enzyme.

Studies of the time course of the inactivation of phosphofructokinase as a function of pH at 6° (5) have shown the kinetics of inactivation to be biphasic at certain pH values. This result implies that the equilibrium between active and inactive enzyme may not be strictly interpretable as a two state process. The equations for the apparent Hill interaction

coefficient and heat of ionization in these situations can be complex, and in some cases give rise to non-linear Hill plots (6). However, the data of Figure 1 suggest that under these conditions the temperature dependence of the apparent pK value for the dissociation to inactive enzyme may be dominated by the effect on the ionization of one or more histidine residues per enzyme subunit. While chemical modification of particular histidine residues of sheep heart phosphofructokinase does influence allosteric behavior, it is not clear what effect such modification has on the pH dependent molecular weight changes (7).

At a given temperature, ligands which are substrates for the reaction or effectors of the reaction also effect the pH dependence of the residual activity, that is influence the apparent pK value for the association-dissociation process. Thus, as shown in Figure 2, the substrate fructose-6-phosphate shifts the residual activity curve towards the left (i.e.,

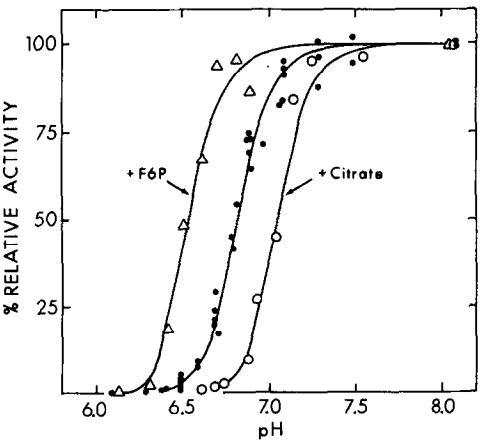

FIGURE 2. The effect of fructose 6-phosphate and citrate on the pH dependence of the residual activity of phosphofructokinase at 6°.

Rabbit muscle phosphofructokinase was incubated at 6° for three hours in 0.13 M sodium phosphate buffers containing 1 mM EDTA and 0.2 mM dithioerythritol at the pH values shown and an enzyme concentration of 0.1 mg/ml. Residual activity in the presence of 100 μM F6P (-Δ-), no additions (-●-), and 5 mM citrate (-O-).

lowers the apparent pK value) while citrate, an inhibitor, shifts the curve towards the right. The activator fructose 1,6-bisphosphate, like fructose 6-phosphate, also lowers the apparent pK value. The effect of ATP (not shown) is somewhat more complex and appears to involve more than one mode of action. This might be expected in light of the role of ATP as both a substrate and inhibitor of the reaction at pH values below 7.2. Residual activity curves in the presence of mixtures of ATP and fructose 1,6-bisphosphate or fructose-6-phosphate are very complex and indicate that the residual activity is not a simple function of the concentration of ligands.

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