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pH-Induced Cold Lability of Rabbit Skeletal Muscle Phosphofructokinase†

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ABSTRACT: In phosphate buffers below pH 7, rabbit skeletal muscle phosphofructokinase loses activity as a function of time. The inactivation is reversible and becomes more extensive at lower temperature, lower pH values, and lower enzyme concentrations. It is correlated with changes in the molecular weight of the enzyme as measured by light scattering. The effect of temperature between pH 6 and 7 shows the characteristics of enzyme systems described as cold labile. In general the loss of activity is biphasic but the phases do not appear to be directly correlated with individual molecular events. A mechanism consistent with the inactivation data as a function of enzyme concentration involves three reversible steps: dissociation of the active enzyme to an inactive form which can either repolymerize to an inactive form or isomerize to a different inactive form. ATP addition early in the inactivation process results in rapid partial inactivation (and depolymerization). ATP addition late in the inactivation process results in partial reactivation (and repolymerization). The results may be explained in terms of ATP binding with different affinities to the different forms of the enzyme arising in the inactivation process.

Phosphofructokinase is a complex regulatory enzyme of primary importance in the control of glycolysis in many tissues. The enzyme from rabbit skeletal muscle has been well studied and shown to exhibit a number of properties which are pH dependent. For example, at pH values below about 7–7.5, ATP is not only a substrate, but also an inhibitor of the reaction with the degree of inhibition increasing at lower fructose 6-phosphate levels. At inhibitory levels of ATP, sigmoidal plots of initial velocity *vs.* fructose 6-phosphate concentration are observed with the degree of sigmoidicity a function of the ATP level. Similarly, the effect of allosteric effectors like cAMP, AMP, and ADP is greater at the lower pH values. It has been shown that the overall activity of the enzyme is influenced by a pH-dependent conversion of active to inactive enzyme and it would be of interest to know if this property of the enzyme is related to its kinetic and regulatory behavior in terms of the ability of different ligands to affect this interconversion.

At low pH values, inactivation has been correlated with the conversion of the active enzyme to an inactive form of lower molecular weight for either the rabbit muscle (Paetkau and Lardy, 1967; Hofer and Pette, 1968) or sheep heart enzyme (Mansour, 1965). For the muscle enzyme in phosphate buffer, the process appears to involve dissociation to an inactive form of the enzyme containing two subunits and is cooperative with respect to the hydrogen ion concentration (Hofer and Pette, 1968; Pavelich and Hammes, 1973; Aaronson and Frieden, 1972). It has also been shown that this process can be reversed by raising the pH (Frieden, 1968; Hofer and Pette, 1968) and by the addition of ligands (Aaronson, 1971; Alpers *et al.,* 1971; Lad *et al.,* 1973), again with correlation between the increase in activity and the formation of the active enzyme of higher molecular weight.

It appears possible that the association–dissociation reaction involving active and inactive enzyme may be related to the role of various allosteric effectors in controlling enzyme activity. For example, Lad *et al.* (1973) have shown that citrate inhibition at pH 7 appears to be correlated with the ability of this compound to dissociate the enzyme.

The importance of this association–dissociation process in the regulation of the enzyme activity has therefore led us to a more careful study of the effect of pH as well as temperature on the rate of this process and its possible mechanism.

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). Most preparations of the enzyme used in these experiments had a specific activity of 170 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.

The enzyme was routinely charcoal treated to remove bound ATP by the following procedure: crystalline enzyme was centrifuged and the crystals were dissolved in 0.1 M sodium phosphate buffer (pH 8) containing 1 mM EDTA and

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‡ Predoctoral trainee supported by Training Grant GM-01311.

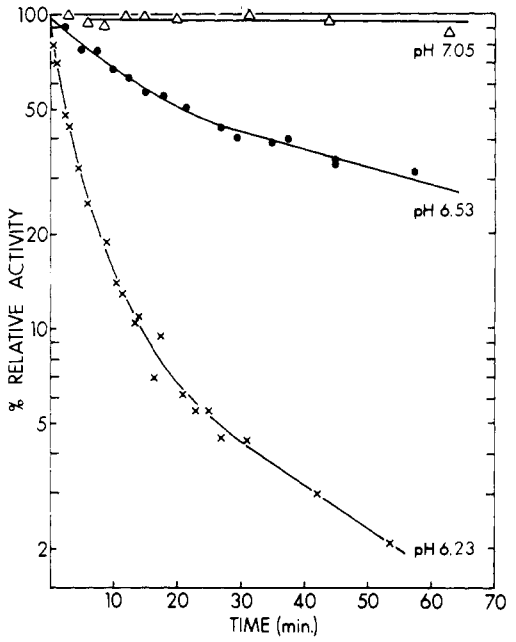

FIGURE 1: First-order plot of the inactivation of phosphofructokinase at different pH values. At zero time, the pH of a phosphofructokinase solution at pH 8 and 6° was lowered to the pH value indicated and a final enzyme concentration of 1 mg/ml in 0.1 M sodium phosphate buffer containing 1 mM EDTA and 2 mM dithiothreitol. The pH values 7.05 (△), 6.53 (●), and 6.23 (×) are those at 6°. Enzymatic activity as a function of time was measured as described under Materials and Methods.

5 mM dithiothreitol, to a final concentration of enzyme between 1 and 5 mg/ml. Norit A was added in a 1:1 (w/w) ratio and this suspension was kept on ice for 10 min before Millipore filtration (0.22 μ). The charcoal treatment was repeated twice. The enzyme was dialyzed overnight against two changes of 100 vol of 0.1 M sodium phosphate buffer (pH 8) containing 1 mM EDTA and 0.2 mM dithiothreitol. At this point the ratio of absorbance at 280 nm to that at 260 nm was not less than 1.65.

All assays were performed at pH 8 and 24° 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; 200 μg of aldolase; 10 μg of triosephosphate isomerase; and 25 μg of α-glycerophosphate dehydrogenase in a final volume of 1 ml. The reaction was started by addition of the enzyme to the above assay mixture. The auxiliary enzymes were centrifuged and dissolved in 0.1 M Tris-acetate buffer (pH 8), 10^{-4} M EDTA. They were dialyzed overnight against two changes of 100 vol of this buffer to remove ammonium sulfate. Absorbance changes observed in the assay were not constant as a function of time, but increased by about 10% within the first 2 min and more gradually thereafter. The velocity was measured between 60 and 90 sec after initiation of the reaction, where the activity is proportional to the concentration of added enzyme. Enzymes, coenzymes, and substrates were obtained from Sigma.

Inactivation Experiments. In order to perform the experiments described below, the pH of the enzyme solution was lowered by the following procedure: to a solution of known enzyme concentration at pH 8 in 0.1 M sodium phosphate buffer (containing 1 mM EDTA and 2 mM dithiothreitol) is added an equal volume of monobasic phosphate to give an incubation mixture of known final pH and enzyme concentration. All solutions were maintained at the given temperature of the experiment.

Assuming the time of mixing to be zero time, the kinetics of inactivation of the enzyme were followed by removing aliquots of the incubation mixture, diluting to 5 μg/ml in 0.1 M sodium phosphate at pH 8 and 0°, and finally diluting this enzyme 1:100 into the assay mixture. At the lowest enzyme concentration used (5 μg/ml) the enzyme was diluted directly into the assay mixture. Enzyme at pH 8 does not undergo dissociation into an inactive form even at high dilution (Pavelich and Hammes (1973) and higher molecular weight forms of the enzyme are all essentially equally active (Aaronson and Frieden, 1972)). Thus the method of activity measurement used reflects the concentration of active enzyme at the pH and temperature of the inactivation experiment. For the reference point to which activities as a function of time were compared, enzyme at pH 8 was diluted into pH 8 phosphate buffer at 0° to a final concentration of 5 μg/ml and then diluted 1:100 into the assay mixture. The per cent activity shown in the figures is the observed activity relative to the reference activity at pH 8. As will be shown, the loss of activity was not a simple first-order process. Assuming two phases, the data were initially analyzed by determining the half-time and extent of each phase with semilogarithmic plots. Later the data were analyzed using a computer program which simulated the time course of inactivation assuming the given mechanism (Bates and Frieden, 1973). Data describing the time course of inactivation were traced with a ρ-θ analog to digital device for storage on an IBM 360 and the digitized data transferred to LINC tape on a PDP-12 computer for visual comparison to the simulated curve on an oscilloscope screen.

Light Scattering. Light scattering measurements were performed in a thermostated Phoenix-Brice light scattering photometer equipped with digital readout. All solutions were filtered through 0.22-μ Millipore filters before mixing. Mixing was accomplished with a thermostated rapid mixing device capable of delivering 3 ml with a dead time of 200–300 msec. Equal volumes of enzyme at pH 8 and monobasic phosphate were mixed in the light scattering cell and, at known time intervals, aliquots were withdrawn and assayed as described above. Buffers mixed in the same way were used as blanks.

Results

Effect of pH on Inactivation at 6°. Figure 1 shows first-order plots of the loss of activity at varying pH values at 6° in 0.1 M sodium phosphate buffer. These data show that the rate of inactivation is highly sensitive to pH at this temperature. Thus, after 60 min at pH 6.23, the enzyme is almost completely inactive while less than 10% activity loss occurs at pH 7.05. The process is not a simple first-order one, but involves at least two phases. Examination of the semilogarithmic plots of Figure 1 in a qualitative way shows that the extent of the fast phase becomes a larger portion of the total inactivation as the pH is lowered. At sufficiently low pH values (about pH 6.0 at this temperature), the inactivation is essentially first order, represented by an apparent single fast phase. The half-time for this fast phase also becomes shorter as the pH is lowered. The second phase is more obvious at the higher pH values with the extent and half-time also pH dependent. As will be shown later, these apparent phases are difficult to correlate directly with specific molecular changes in the enzyme.

Inactivation as a Function of Temperature at pH 6.4.

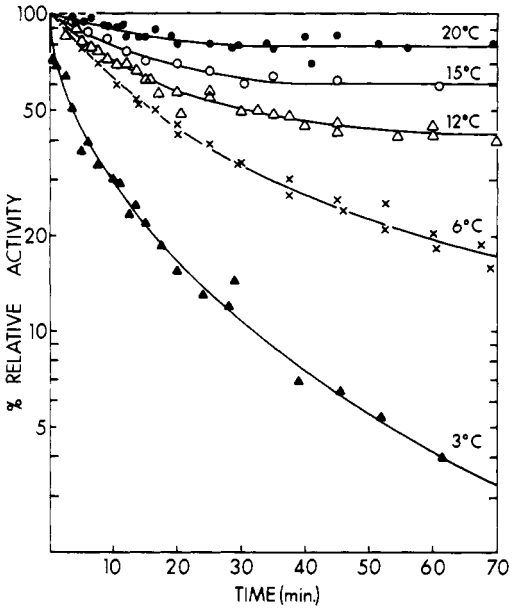

FIGURE 2: First-order plot of the inactivation of phosphofructokinase at different temperatures. At zero time, the pH of a phosphofructokinase solution at pH 8 and the proper temperature was lowered to pH 6.4 and a final enzyme concentration of 1 mg/ml. The final buffer concentration was 0.15 M sodium phosphate and contained 1 mM EDTA and 2 mM dithiothreitol. Enzymatic activity as a function of time was measured as described under Materials and Methods.

Figure 2 shows first-order plots of the time dependence of the activity loss at pH 6.4 as a function of temperature. Enzyme activity is lost faster and to a greater extent at lower temperatures, thus showing the characteristic of enzymes classified as cold labile. Again, these plots are not simple first order but can be analyzed qualitatively in terms of the extents and half-times of two phases which are dependent on temperature. It appears that not only the apparent half-times but also the extents of the fast and slow phases change as the temperature is lowered. At temperatures above 15° and at pH 6.4 the half-times and extents are such that there is only one phase which accounts for the partial loss of activity. It should be noted that the pH of phosphate buffers changes slightly with temperature, decreasing approximately 0.1 pH unit when the temperature is raised from 3 to 20°. This change has not been taken into account in this figure. If the pH had been maintained constant as a function of temperature, the data of Figure 1 indicate that the temperature dependence of the inactivation would be somewhat greater than shown by the data of Figure 2.

Correlation of Cold Lability at pH 6.4 with Changes in Molecular Weight Properties. The kinetics of inactivation of phosphofructokinase after lowering the pH have been directly correlated with depolymerization of the enzyme by simultaneous light scattering and activity measurements. The results of such an experiment at two temperatures are shown in Figure 3. The curve representing the loss of activity at 6° is biphasic (similar to that observed in Figure 1) and a decrease in scattering ratio is clearly associated with both phases of inactivation. The apparent half-time for each phase is nearly identical by light scattering and activity measurements. However, because the enzyme consists of a number of different molecular weight species (Aaronson and Frieden, 1972; Leonard and Walker, 1972; Ling *et al.*, 1965; Parmeggiani *et al.*, 1966), the extent of these two curves cannot be directly compared. At a higher temperature (20°) the final extent of inactivation is considerably smaller, as is the extent of depolymerization. Again, the half-time for this process is approximately the same when measured by either method.

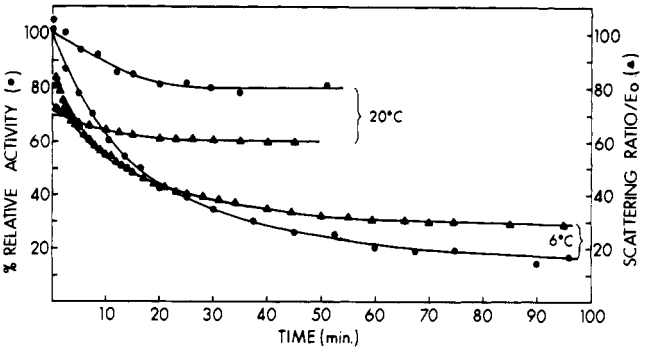

FIGURE 3: Comparison of activity loss and changes in light scattering at pH 6.4 and different temperatures. At zero time, the pH of a solution containing phosphofructokinase at pH 8 and the proper temperature was lowered to pH 6.4 as described under Materials and Methods. The final concentration of enzyme was 1 mg/ml in 0.15 M sodium phosphate buffer containing 1 mM EDTA and 2 mM dithiothreitol. Enzymatic activity as a function of time (●) was obtained by removing aliquots from the solution in the light scattering cell and measured as described under Materials and Methods. The values for the light scattering (▲) are given as the scattering ratio divided by the initial enzyme concentration. The upper curves are at 20° and the lower curves at 6°.

Effect of Enzyme Concentration on Inactivation at pH 6.5 and 6°. Figure 4 shows zero-order plots of the inactivation of phosphofructokinase at pH 6.5 and 6° at different

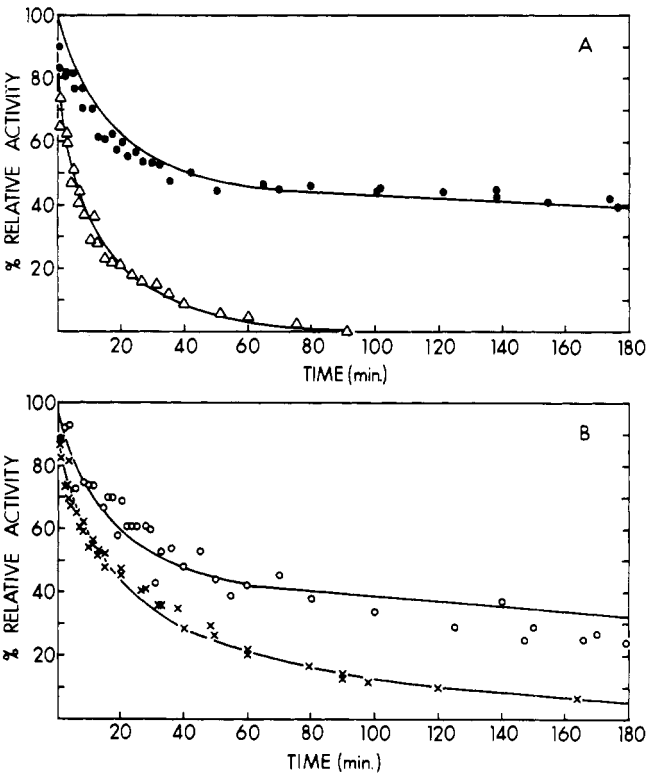

FIGURE 4: Plot of the inactivation of phosphofructokinase at different initial enzyme concentrations. At zero time the pH of a phosphofructokinase solution at pH 8 and 6° was lowered to pH 6.5 in a final buffer of 0.13 M phosphate containing 1 mM EDTA and 2 mM dithiothreitol. Enzymatic activity as a function of time was measured as described under Materials and Methods: (A) results obtained at final enzyme concentrations of 4 mg/ml (●) and 5 μg/ml (△); (B) results obtained at final enzyme concentrations of 1 mg/ml (○) and 50 μg/ml (×).

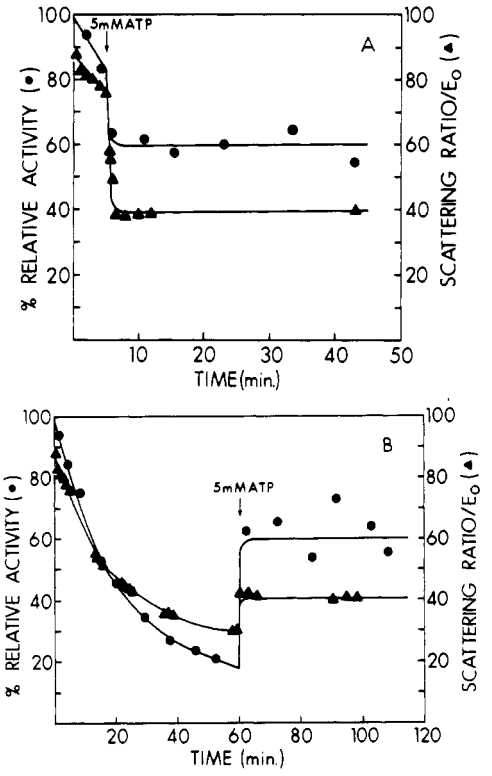

FIGURE 5: Plot of change of enzymatic activity and light scattering as a function of time before and after the addition of ATP. At zero time, the pH of a solution of phosphofructokinase at pH 8 and 6° was lowered to pH 6.4 as described under Materials and Methods. The final solution was 1 mg/ml of enzyme in 0.15 M sodium phosphate buffer containing 1 mM EDTA and 2 mM dithiothreitol. Activity (●) and scattering measurements (▲) were made as described in the legend to Figure 3: (A) the effect of 5 mM ATP added at 5 min; (B) the effect of 5 mM ATP added at 60 min after lowering the pH.

enzyme concentrations. It can be seen from these data that both the rate and extent of inactivation are protein concentration dependent with the loss of activity becoming faster and more extensive at lower enzyme concentrations. Examination of the first-order plots of these data with respect to the two phases of inactivation reveals that at lower enzyme concentrations the fast phase accounts for a greater portion of the activity loss. The apparent half-times of the two phases are also dependent on the phosphofructokinase concentration.

Effects of ATP on the Activity and Molecular Weight Properties of Phosphofructokinase. We have found that the pH-induced inactivation and concomitant depolymerization of phosphofructokinase are both modified by the presence of ATP. However, the observed effects are critically dependent on the pH, temperature, protein and ATP concentrations, and on the time of ATP addition.

The data in Figure 5 were obtained at pH 6.4, 6°, and at a protein concentration of 1 mg/ml. In this experiment, light scattering and activity measurements were obtained following ATP addition at different times during the inactivation.

Figure 5A shows that ATP added before completion of the fast phase of inactivation results in rapid depolymerization and activity loss. On the other hand, Figure 5B shows that ATP added at later times results in rapid reactivation and polymerization to the same final level as reached after the early addition. Hence ATP inactivates (depolymerizes) or reactivates (polymerizes) phosphofructokinase depending, under these conditions, on when it is added. Although not shown, it has been determined that the same result is observed at different pH values, temperatures, and enzyme concentrations.

Discussion

Cold lability of enzymes is not an uncommon phenomenon and appears in general to be related to dissociation of the enzyme into inactive subunits (Frieden, 1971). However, the effect of pH on this process has not been extensively investigated for any enzyme and frequently it is not reported whether the cold lability shows any pH dependence. While chicken liver phosphofructokinase has been observed to exhibit cold lability (Kono and Uyeda, 1973), this characteristic has not been reported for the rabbit muscle enzyme in spite of its extensive characterization. It is possible that previous investigators did not observe the cold lability because this property is extraordinarily pH sensitive as indicated in Figure 1 and shown by more extensive studies (unpublished results). Because cold lability of the rabbit muscle enzyme manifests itself under conditions which lead to dissociation of the enzyme into an inactive form, and it has been previously shown that the latter process is highly sensitive to pH either at high (Aaronson and Frieden, 1972) or low (Pavelich and Hammes, 1973) enzyme concentrations, changes in the rate and extent of inactivation are extremely sensitive to combined changes of pH and temperature.

Inactivation of the enzyme as a function of enzyme concentration was examined in detail because it was felt that it would reveal some of the characteristics of the mechanism of inactivation. Examination of Figure 4 shows that at pH 6.5 and 6°, inactivation was highly dependent on enzyme concentration. First-order plots of these data show that, at all but the lowest enzyme concentration, the inactivation is biphasic in nature indicating that at least two processes are involved. The simplest mechanisms which might give rise to such data involve at least two enzyme transformations. Computer simulation of the inactivation data, by methods described elsewhere (Bates and Frieden, 1973), using specific two-step mechanisms shows that although a given mechanism may be fit to an individual data curve, these mechanisms do not adequately describe the dependence of inactivation on enzyme concentration. Thus, any one of four mechanisms (listed in Scheme I) involving two reversible

SCHEME I: Two-Step Mechanisms for Describing the Inactivation of Phosphofructokinase.

$$E_4 \rightleftharpoons 2E_2 \qquad (I)$$
$$E_4' \rightleftharpoons 2E_2'$$

$$E_4 \rightleftharpoons 2E_2 \qquad (II)$$
$$E_4 \rightleftharpoons 2E_2'$$

$$E_4 \rightleftharpoons E_4' \rightleftharpoons 2E_2' \qquad (III)$$

$$E_4 \rightleftharpoons 2E_2 \rightleftharpoons 2E_2' \qquad (IV)$$

reactions could be fit to each data curve shown in Figure 4 with a given set of rate constants but when these rate constants are used at different initial protein concentrations in the simulation, one or more of the resulting simulated curves are clearly distinguishable from the experimental data. These mechanisms include (1) independent dissociation of two equally active noninterconvertable forms, (2)

dissociation of the active enzyme to two different inactive forms, (3) isomerization of active enzyme to an inactive form which then dissociates, and (4) dissociation of active enzyme to an inactive form which then isomerizes.

Mechanisms involving three enzyme transformations are therefore necessary. There are a number of possible mechanisms involving three steps which we have used to attempt to fit the data shown. Because of the number of parameters (six rate constants) and some ambiguity in the data itself, it appears that it may not be possible to uniquely define such a mechanism based on the inactivation results alone. However, these data in conjunction with light scattering data and the known characteristics of the protein under these conditions at both high and low concentrations (Aaronson and Frieden, 1972; Pavelich and Hammes, 1973) allow selection of one mechanism which appears to be consistent with all the known data.This is the mechanism shown (V).

$$\begin{array}{ccccc} & & E_4'' & & \\ & & k_{-3}\downarrow\uparrow k_3 & & \\ E_4 & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & 2E_2 & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & 2E_2' \end{array} \qquad \text{(V)}$$

This mechanism involves dissociation of the active monomer (consisting of four subunits) to an inactive species (consisting of two subunits), followed by isomerization of this form, assuming also that the nonisomerized enzyme can repolymerize to a form which is inactive. The curves drawn in Figure 4 are those generated by simulation with this mechanism using the rate constants given in Table I. One of the distinguishing features of this mechanism is that the inactive dissociated enzyme polymerizes to an inactive form, that is, a form different from the active form. This step is most important for fitting the data at higher protein concentrations. Sedimentation velocity and light scattering experiments at low pH as a function of protein concentration have demonstrated polymerization of the inactive dissociated enzyme to a species not present at pH 8 (Aaronson and Frieden, 1972). This observation supports the assumption that a polymer is formed at low pH values which is different from the active enzyme polymer and is therefore consistent with the proposed mechanism. Examination of the rate constants given in Table I reveals that the association constants for dimerization of E_2 to either E_4 or E_4'' are the same. While this may be coincidental, the forces contributing to the association of these forms appear to be of the same magnitude although only one product is fully active.

Since the mechanism is complex, the assignment of particular steps to phases of inactivation has not yet been achieved. The half-times of the two phases are probably functions of several of the six rate constants. Simulation of the loss of activity under other conditions of pH and temperature at several enzyme concentrations may allow the assignment of the effect of these variables to specific enzyme transformations.

In Figure 3 the rate of depolymerization as measured by light scattering is shown to be correlated with the loss of enzymatic activity. However, the extent of depolymerization under these conditions cannot be ascribed to dissociation of a particular polymeric species. At pH 8, phosphofructokinase is known to exist as a mixture of equally active polymers in equilibrium with the active monomer of molecular weight 320,000 (Ling *et al.*, 1965; Aaronson and Frieden, 1972). Dissociation of these higher molecular weight forms to the monomer by dilution at pH 8 or 7 has been observed to be fast relative to the dissociation of the monomer (Lad *et al.*, 1973). The dissociation of these higher molecular weight polymers, which are in rapid equilibrium with the monomer, followed by dissociation to the inactive form, may account for the large extent of depolymerization observed in our light scattering experiments and the agreement between the half-times for both phases of depolymerization and activity loss.

TABLE I: Rate Constants for Simulation of the Inactivation of Phosphofructokinase as a Function of Enzyme Concentration with Mechanism V.

Step	Rate Constant
$E_4 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2E_2$	$k_1 = 2.5 \times 10^{-3}$ sec^{-1} $k_{-1} = 5 \times 10^4$ M^{-1} sec^{-1}
$E_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2'$	$k_2 = 8 \times 10^{-4}$ sec^{-1} $k_{-2} = 3 \times 10^{-5}$ sec^{-1}
$E_4'' \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} 2E_2$	$k_3 = 6 \times 10^{-4}$ sec^{-1} $k_{-3} = 1.2 \times 10^4$ M^{-1} sec^{-1}

Of considerable importance in the study of this process are the observations related to the addition of ATP. Figure 5 shows that ATP may either inactivate or reactivate the enzyme depending on the time of addition of the ATP. This observation undoubtedly reflects the fact that ATP binds differently to the different forms which occur during the inactivation process. However, the final extent of activity is the same regardless of when the ATP is added, showing that the system always comes to the same equilibrium position. According to mechanism V, there are four different forms of the enzyme present during the inactivation process. Our data would indicate that ATP can bind to at least three of these forms.

The ATP effects, as well as the temperature dependence of the inactivation, should be considered when attempting to interpret some of the data in the literature dealing with phosphofructokinase. For example, Alpers *et al.* (1971) have, based on results obtained after changing the pH, ATP concentration, and buffer, proposed the concept of allosteric preconditioning in which a ligand stabilizes a metastable state in the maturation of a protein. Their data, with respect to phosphofructokinase and the idea that ATP might act catalytically in this process, may be explained on the basis that ATP binds with different affinities to different forms of the enzyme described in mechanism V and that these affinities are sensitive to pH, temperature, buffer, and so on.

In a similar way, the kinetic properties of phosphofructokinase may depend upon such preferential binding as well as the manner in which the reaction is run. It might be expected that the specific activity of the enzyme would be dependent on enzyme concentration at low pH and this has been observed (Hofer, 1971; Hulme and Tipton, 1971). In addition, we have recently obtained data on this point which show that the activity is markedly sensitive to temperature changes as might be expected from the data given in this paper.

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The Interaction of Cyclic 3′,5′-Adenosine Monophosphate with Yeast Glyceraldehyde-3-phosphate Dehydrogenase. I. Equilibrium Dialysis Studies†

Judi Milne and Robert A. Cook*

ABSTRACT: Binding studies with yeast glyceraldehyde-3-phosphate dehydrogenase indicate a maximum of four binding sites for the inhibitor cyclic 3′,5′-AMP and three binding sites for the substrate NAD^+. The binding data for cAMP indicate simple noncooperative binding with the binding affinity inversely related to temperature. The binding data for NAD^+, however, indicate positive and negative cooperative behavior, consistent with a ligand induced sequential model. Competition experiments indicate that NAD^+ inhibits the binding of cAMP in a noncompetitive manner. Similarly cAMP inhibits the binding of NAD^+ noncompetitively. The results indicate that cAMP and NAD^+ do not compete for the same binding site on the enzyme surface. The suggestion is made that cAMP inhibits enzyme activity by binding to a specific regulatory site rather than competing for the NAD^+ binding site.

Glyceraldehyde-3-phosphate dehydrogenase of yeast has been the subject of numerous physicochemical and kinetic studies due to the availability of large quantities of homogeneous enzyme and the central role this enzyme has played in the development of allosteric theory. The enzyme is generally accepted to be tetrameric in structure, composed of four apparently identical monomers (Harris and Perham, 1965). The binding of NAD^+ to the enzyme has been shown to be cooperative in nature by various techniques at several different temperatures (Cook and Koshland, 1970; Kirschner *et al.*, 1971; Ellenrieder *et al.*, 1972; Sloan and Velick, 1973). We have suggested, however, that the overall shape of the binding curves indicates greater complexity (Cook and Koshland, 1970). Specifically, distinct breaks occurred in the Hill plots at approximately 50% saturation and the Scatchard plots did not extrapolate to four which was expected on the basis of the number of subunits. The anomalies were explained by a ligand-induced sequential model (Koshland *et al.*, 1966; Kirtley and Koshland, 1967) with subunit interactions which led to a mixture of positive and negative cooperativity. Initial velocity kinetic studies with the yeast enzyme were consistent with this explanation (Koshland *et al.*, 1970). Qualitatively, similar subunit interactions resulting in mixed positive and negative cooperativity due to a single ligand binding have been observed with aspartate transcarbamylase (Cook, 1972).

The observation that cyclic 3′,5′-AMP was a potent inhibitor of the yeast enzyme (Yang and Deal, 1969) raised some interesting questions regarding the effect this inhibitor would have on the binding and kinetic behavior of the enzyme. This paper reports the stoichiometry of cAMP[1] binding to the yeast enzyme and some ligand binding interactions, as measured by equilibrium dialysis. The effect of cAMP on initial velocity kinetic studies of the enzyme are presented in the following paper (Rock and Cook, 1974).

Experimental Section

Materials. Glyceraldehyde-3-phosphate dehydrogenase (GPD) was purified from Red Star brand baker's yeast following the procedure of Krebs (1955). After four crystalliza-

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[1] Abbreviations used are: GPD, glyceraldehyde-3-phosphate dehydrogenase; cAMP, cyclic 3′,5′-adenosine monophosphate; n_H, Hill coefficient; n_m, maximum number of binding sites.