Temperature-Jump Study of the Interaction of Rabbit Muscle Phosphofructokinase with Adenylyl Imidodiphosphate and Adenosine 5′-Triphosphate†

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ABSTRACT: The interaction of ATP and adenylyl imidodiphosphate, an ATP analogue, with rabbit muscle phosphofructokinase was studied by using the temperature-jump method and monitoring fluorescence changes. In the absence of ligands, a relaxation process was observed at 12 °C in 20 mM imidazole, 100 mM KCl, 16 mM potassium phosphate, 1 mM dithiothreitol, 1 mM EDTA, and no $MgCl_2$ or 5 mM $MgCl_2$ and at pH 7.0 or 8.0. The relaxation time, about 20 ms, did not change with varying pH, with varying enzyme concentration (0.1–2.0 mg/mL), or upon addition of ATP, adenylyl imidodiphosphate, or cAMP to the reaction mixture. However, this relaxation process became undetectable at adenylyl imidodiphosphate concentrations greater than 10 μM. This relaxation process is attributed to a protein conformational change, but the significance of this change for the mechanism of action of the enzyme could not be assessed. In the presence of ATP and adenylyl imidodiphosphate at pH 7.0 and 6.5, but not at pH 8.0, a second relaxation process is observed with a larger relaxation amplitude and a relaxation time in the range of 0.6–5 ms. This relaxation time did not vary significantly with enzyme concentration over the range from 0.05 to 1.0 mg/mL. This relaxation process, under conditions where the enzyme is tetrameric (0.68 mg/mL), has been interpreted in terms of a Monod–Wyman–Changeux model with two types of ligand binding sites on the enzyme for ATP and adenylyl imidodiphosphate: a catalytic site and a regulatory site. The catalytic site is characterized by a dissociation constant of about 1 μM, with tighter binding to the active conformation than to the inactive conformation. The regulatory site is characterized by a dissociation constant of about 100 μM, with tighter ligand binding to the inactive conformation. The dissociation constants and the rate constants for the interconversion of the active and inactive conformations have been obtained under a variety of conditions. The relaxation amplitude is threefold greater for ATP than for adenylyl imidodiphosphate. The results obtained indicate that the presence of cAMP converts the enzyme to the active conformation. The presence of Mg^{2+}, lower pH, increased phosphate ion concentration, or increasing the temperature does not cause major changes in the mechanistic parameters although quantitative differences occur. The experimental evidence is consistent with this concerted conformational transition being important in the regulation of phosphofructokinase. The relaxation spectrum for a Monod–Wyman–Changeux model with multiple binding sites on the enzyme is derived in the Appendix.

The enzyme phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is important in the regulation of glycolysis. The activity of the enzyme from rabbit skeletal muscle is known to be influenced by various effectors (Passoneau & Lowry, 1962, 1963), as well as by the pH (Frieden et al., 1976) and the protein aggregation state (Aaronson & Frieden, 1972; Lad et al., 1973). A combination of the above factors is most probably responsible for regulating the activity of this multisubunit enzyme in vivo. The active tetramer has a molecular weight of about 320 000 (Coffee et al., 1973; Pavelich & Hammes, 1973), while both the monomer and dimer are essentially inactive (Lad et al., 1973).

Various models have been proposed for the regulation of phosphofructokinase (Goldhammer & Hammes, 1978; Pettigrew & Frieden, 1979). An important feature of these detailed models is a concerted conformational change between active and inactive states. In the work presented here, the interaction of ATP and adenylyl imidodiphosphate (AMP-PNP),[1] an ATP analogue, with rabbit muscle phosphofructokinase has been studied with the temperature-jump method. A relaxation process was observed in the presence of ATP and AMP-PNP by monitoring fluorescence changes which can be interpreted in terms of a concerted conformational change. Moreover, the dependence of the relaxation time on the concentrations of ATP, AMP-PNP, and cAMP and on pH is consistent with this conformational change being important in the regulation of phosphofructokinase.

Experimental Section

Materials. The AMP-PNP was obtained from ICN and purified according to Yount et al. (1971). The AMP-PNP solution was lyophilized and stored as a powder at −23 °C. The ATP, cAMP, fructose 6-phosphate, dithiothreitol, aldolase, α-glycerophosphate dehydrogenase, triose phosphate isomerase, and serum albumin (bovine) were purchased from Sigma Chemical Co. All other chemicals were the best available commercial grades, and all solutions were prepared with deionized, distilled water.

Phosphofructokinase. Rabbit skeletal muscle phosphofructokinase was purified by the method of Ling et al. (1966). The final ammonium sulfate precipitate was dissolved in 0.1 M potassium phosphate, 1.0 mM dithiothreitol, and 1.0 mM EDTA (pH 8.0) and dialyzed against the same buffer to give a stock solution of 8–18 mg/mL. The protein concentration was determined from the absorbance at 280 nm by using an extinction coefficient of 1.02 mL/(mg cm) (Parmeggiani et al., 1966).

Enzymatic activity was determined by using coupled enzyme reactions (Ling et al., 1966; Lad et al., 1973). Assays were carried out in the following mixture: 33 mM Tris-HCl (pH 8.0), 2 mM ATP, 5 mM $MgCl_2$, 2 mM fructose 6-phosphate, 0.1 mM NADH, 2 mM dithiothreitol, 0.20 units/mL aldolase, 35 units/mL triose phosphate isomerase, 3.2 units/mL α-

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[1] Abbreviations used: AMP-PNP, adenylyl imidodiphosphate; EDTA, ethylenediaminetetraacetic acid.

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glycerophosphate dehydrogenase, and 0.1–0.3 μg/mL phosphofructokinase in a total volume of 3.0 mL. Assays were initiated by the addition of phosphofructokinase, and the velocity of the enzymatic reaction was recorded spectrophotometrically at 340 nm on a Cary 118 spectrophotometer thermostated at 23 °C. The specific activity of the native enzyme in 0.1 M potassium phosphate, 1.0 mM dithiothreitol, and 1.0 mM EDTA (pH 8.0) was 90–120 units/mg where a unit of enzyme activity is defined as the production of 1 μmol of product per min.

Stopped-Flow Experiments. Stopped-flow measurements with fluorescence detection were performed on a Durrum D-10 stopped-flow apparatus. A 200-W Hanovia xenon–mercury arc lamp was used as the light source. An excitation wavelength of 280 nm was used, and the emitted light was filtered through a Corning 300-nm cutoff filter. The final protein concentration was 0.70 mg/mL in 20 mM imidazole, 100 mM KCl, 19 mM potassium phosphate, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol (pH 7.1), and the ATP concentration was varied from 0 to 200 μM. The sample syringes were thermostated at 12 °C.

Temperature-Jump Experiments. The temperature-jump apparatus with fluorescence detection is described elsewhere (del Rosario, 1970). The excitation wavelength of 280 nm was obtained from a 200-W Hanovia xenon–mercury arc lamp with a Bausch & Lomb monochromator. Changes in fluorescence were detected with an EMI 9635 QB photomultiplier tube equipped with a Corning 300-nm cutoff filter. Temperature jumps of 7.5–8.0 °C were applied to the solutions by discharge of a 0.2-μF capacitor which had been charged with 10 000 V. The thermostated temperature was generally 4 °C but was 17 or 23 °C in a few cases. Samples for temperature-jump experiments were prepared by combining aliquots of stock protein solution, stock ligand solution in buffer, and enough buffer for a 3.0-mL total volume. The sample was equilibrated in the thermostated apparatus for 5–10 min before the first temperature jump was applied and equilibrated for 1–2 min after mixing with fresh solution following each individual jump.

Similar experiments also were carried out with a temperature-jump apparatus detecting absorbance changes (Faeder, 1970). In this case, enzyme samples were dialyzed overnight against several changes of a solution containing 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.02 mM pH indicator dye at pH 7.0. Neutral red, phenol red, chlorophenol red, and *p*-nitrophenol were the indicators used to monitor pH changes. The temperature-jump cell was thermostated at 4 °C.

Data Analysis. The amplitudes and relaxation times were calculated by using a PDP-11 computer (Hilborn et al., 1973). Each data point reported represents the average obtained from a minimum of six oscilloscope traces. The concentration dependences of the relaxation times were fit to the specified equations on a PDP-11 computer or on an IBM 370/168 computer. The concentration of free ligand was calculated from the total concentration by assuming a dissociation constant of 1 μM and a stoichiometry of one binding site per polypeptide chain for the interaction of AMP-PNP or ATP with the enzyme.

Results

No change in protein fluorescence was detected when phosphofructokinase (final concentration 0.70 mg/mL) was mixed with ATP (up to a final concentration of 200 μM) in the stopped-flow apparatus. The buffer used was 20 mM imidazole, 100 mM KCl, 19 mM potassium phosphate, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.1 at 12 °C.

To successfully use the temperature-jump method for characterization of protein–ligand interactions, the stability of the enzyme to the electrical discharge and subsequent temperature jump must be established. Under conditions where the enzyme is predominantly tetrameric (0.68 mg/mL) in 20 mM imidazole, 100 mM KCl, 5 mM $MgCl_2$, 15 mM potassium phosphate, 1 mM EDTA, and 1 mM dithiothreitol (pH 7.0) at 4 °C, the specific activity of a 3-mL sample was reduced by 10–20% after about 30 temperature jumps over a period of 45 min. In the experiments reported here, a sample solution was jumped only 10–15 times over a 30-min period. Furthermore, the presence of ligands (cAMP, ATP, and AMP-PNP) increases the enzyme stability. Thus, enzyme inactivation during the course of the experiment can be safely neglected.

At 4 °C with 0.5 mg/mL phosphofructokinase in 100 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.0, no relaxation process was observed when pH indicators were used to monitor pH changes with the absorbance temperature jump in the presence or absence of ATP (0.5 mM) or fructose 6-phosphate (0.5 mM). Extensive experiments were not carried out because of the instability of the enzyme at low ligand concentrations in the absence of phosphate.

With the fluorescence temperature jump, a slow relaxation process with a small amplitude was observed with the enzyme at both pH 7.0 and 8.0, 4 °C, in 20 mM imidazole, 100 mM KCl, 5 mM $MgCl_2$, 16 mM potassium phosphate, 1 mM EDTA, and 1 mM dithiothreitol. A relaxation time of about 20 ms was associated with this process under all conditions, and the value of the relaxation time was independent of the enzyme concentration over a range from 0.1 to 2.0 mg/mL. When AMP-PNP was added at pH 7.0 or 8.0 to a solution containing 0.68 mg/mL enzyme, the relaxation process disappeared at nucleotide concentrations greater than about 10 μM; below this concentration the value of the relaxation time remained constant. The relaxation amplitude and time were unchanged by cAMP up to 100 μM. If both 100 μM AMP-PNP and 100 μM cAMP were present, no relaxation process was observed. In the presence of 50 μM cAMP, the relaxation amplitude became undetectable at AMP-PNP concentrations greater than 20 μM. This relaxation process appears to be associated with a slow ligand-dependent conformational change, but the data are insufficient to postulate a detailed mechanism.

No other relaxation processes were observed over the time range 5 μs–0.5 s for the enzyme in the absence of ATP and AMP-PNP. When AMP-PNP was present at a concentration of 5–200 μM at 4 °C, pH 7.0, in 20 mM imidazole, 100 mM KCl, 5 mM $MgCl_2$, 13–20 mM phosphate, 1 mM EDTA, and 1 mM dithiothreitol, a new relaxation process was observed. The dependence of the relaxation amplitude on the concentration of unbound AMP-PNP is presented in Figure 1. The dependence of the reciprocal relaxation time on the AMP-PNP concentration is presented in Figure 2. Data were obtained in the presence of 0, 10, or 50 μM cAMP, an allosteric activator of the enzyme. The relaxation amplitudes when 10 and 50 μM cAMP were present are qualitatively similar to those shown in Figure 1 except the rise in amplitude occurred at higher AMP-PNP concentrations. If $MgCl_2$ is omitted from the sample, the amplitudes and relaxation times are not significantly changed.

When ATP was substituted for AMP-PNP, a similar relaxation process again was observed over the accessible time range. The dependence of the relaxation amplitude on the ligand concentration was quite similar to that observed with

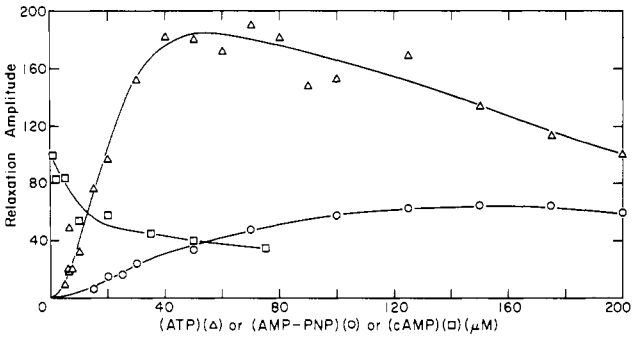

FIGURE 1: A plot of the relaxation amplitude (in arbitrary units) vs. the free concentration of ATP (△) or AMP-PNP (○) in 20 mM imidazole, 100 mM KCl, 15 or 16 mM potassium phosphate, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM EDTA, pH 7.0 at 12 °C, and vs. the total concentration of cAMP (□) in 20 mM imidazole, 100 mM KCl, 100 μM AMP-PNP, 13 mM potassium phosphate, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM EDTA, pH 7.0 at 12 °C. The enzyme concentration was 0.68 mg/mL. The curves have no theoretical significance.

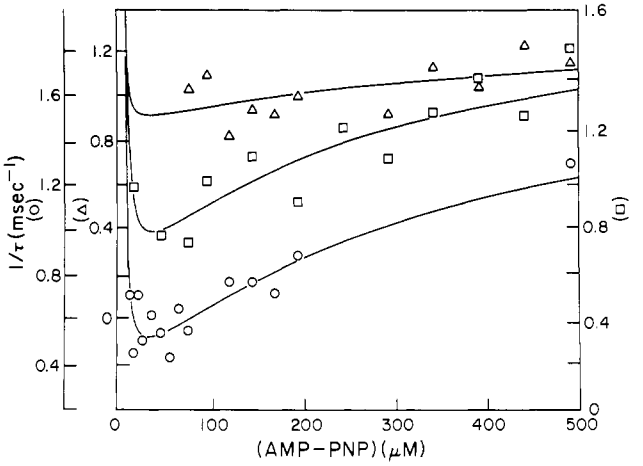

FIGURE 2: A plot of the reciprocal relaxation time, $1/\tau$ (ms^{-1}), vs. the free concentration of AMP-PNP in 20 mM imidazole, 100 mM KCl, 13–20 mM potassium phosphate, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, pH 7.0, 0.68 mg/mL phosphofructokinase, at 12 °C, and 0 μM cAMP (○), 10 μM cAMP (□), and 50 μM cAMP (△). The lines are calculated with eq 1 and the parameters in Table I.

AMP-PNP, except that the amplitude increased by about a factor of 3 (Figure 1). The variation of the reciprocal relaxation time with the free ATP concentration in the presence of 0, 50, or 100 μM cAMP is shown in Figure 3.

When the incubating temperature was raised from 4 to 17 °C, i.e., the final temperature was raised from 12 to 25 °C, the magnitude of $1/\tau$ increased, but the concentration dependence of the reciprocal relaxation time, which is shown in Figure 4, was not significantly altered. The results obtained when the enzyme was incubated at pH 6.5 and 23 °C (final temperature 31 °C) also are shown in Figure 4. Lower temperatures could not be used at this pH because of the cold lability of the enzyme (Bock et al., 1975). The effect of increased phosphate concentration was studied by using 0.1 M potassium phosphate, 100 mM KCl, 1 mM dithiothreitol, and 1 mM EDTA, pH 7.0, as the buffer medium at 4 °C. The reciprocal relaxation times at varying ATP concentrations are included in Figure 4.

The effect of cAMP on the relaxation process was studied further by incubating the enzyme with 100 μM AMP-PNP and varying the concentration of cAMP. Although the value of the reciprocal relaxation time remained constant up to 100 μM cAMP, the amplitude diminished substantially (Figure

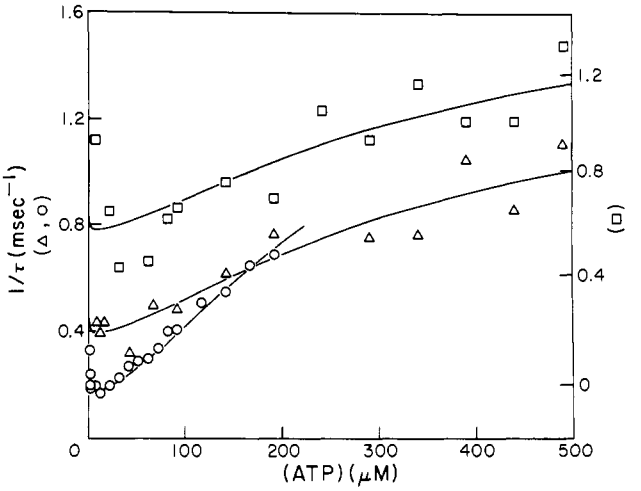

FIGURE 3: A plot of the reciprocal relaxation time, $1/\tau$ (ms^{-1}), vs. the free concentration of ATP in 20 mM imidazole, 100 mM KCl, 14–15 mM potassium phosphate, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, pH 7.0, 0.68 mg/mL phosphofructokinase, at 12 °C, and 0 μM cAMP (○), 50 μM cAMP (□), and 100 μM cAMP (△). The lines are calculated with eq 1 and the parameters in Table I.

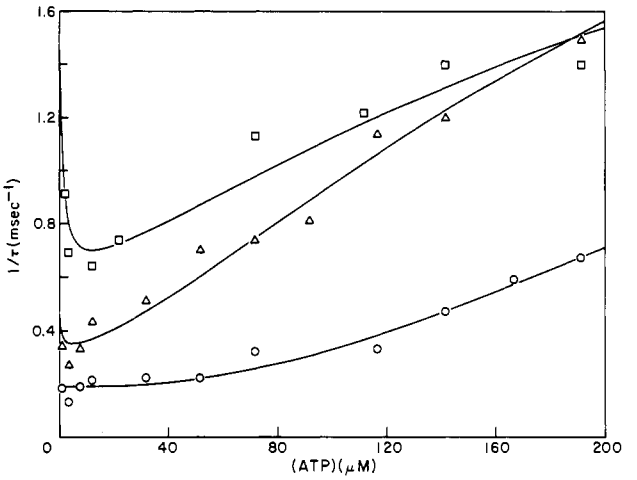

FIGURE 4: A plot of the reciprocal relaxation time, $1/\tau$ (ms^{-1}), vs. the free concentration of ATP in 20 mM imidazole, 100 mM KCl, 14–15 mM potassium phosphate, and 5 mM $MgCl_2$ at 25 °C (□), at pH 6.5, 30 °C (△) or in 0.1 M potassium phosphate, 100 mM KCl, 1 mM dithiothreitol, and 1 mM EDTA, pH 7.0 at 12 °C (○). The enzyme concentration was 0.68 mg/mL. The lines are calculated with eq 1 and the parameters in Table I.

1). In the presence of 10 μM AMP-PNP, the relaxation process could not be detected beyond a cAMP concentration of 2 μM. With cAMP alone as the ligand, the relaxation process was not observed over the concentration range 0–100 μM.

When the enzyme concentration was varied from 0.05 to 1.0 mg/mL with 50 μM AMP-PNP present, the value of the reciprocal relaxation time was not markedly changed (range 1.1–2.0 ms, average 1.6 ms). The relaxation process was not observed in the presence of AMP-PNP (up to 500 μM) at pH 8.0, in 20 mM imidazole, 100 mM KCl, 5 mM $MgCl_2$, 15 mM potassium phosphate, 1 mM EDTA, and 1 mM dithiothreitol. Furthermore, this relaxation process could not be detected at pH 7.0 in the presence of fructose 6-phosphate (up to 1.0 mM) or in the presence of 5 mM fructose 6-phosphate and AMP-PNP (up to 500 μM).

The interpretation of the data presented in mechanistic terms is difficult. However, some mechanisms can be excluded from consideration. The concentration dependences of both the amplitudes and the relaxation times indicate that a simple

Table I: Kinetic Parameters for the Monod-Wyman-Changeux Model

effector	pH[a]	final temp (°C)	k_{-00}/k_{00}	$10^{-3}\,k_{-00}$ (s^{-1})	$10^{-3}\,k_{00}$ (s^{-1})	K_T (μM)	K_T' (μM)	K_R (μM)	K_R' (μM)
MgAMP-PNP	7.0	12	0.0097	0.204	21.0	5.75	77.0	1.53	153
MgAMP-PNP + 10 μM cAMP	7.0	12	0.0247	0.498	20.2	5.75	77.0	1.53	153
MgAMP-PNP + 50 μM cAMP	7.0	12	0.133	0.834	6.25	5.75	77.0	1.53	153
MgATP	7.0	12	0.057	0.159	2.78	2.74	24.8	0.55	116
MgATP + 50 μM cAMP	7.0	12	0.42	0.569	1.35	2.74	24.8	0.55	116
MgATP + 100 μM cAMP	7.0	12	0.26	0.382	1.49	2.74	24.8	0.55	116
AMP-PNP	7.0[b]	12	0.030	0.453	15.3	4.74	37.1	0.61	180
MgATP	7.0	25	0.36	0.458	1.29	3.72	49.9	1.93	115
MgATP	6.5	31	1.14	0.277	0.244	1.77	30.1	1.05	99.8
ATP	7.0[c]	12	0.51	0.187	0.364	1.70	7.50	0.15	160

[a] In 20 mM imidazole, 100 mM KCl, 13–20 mM potassium phosphate, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM EDTA unless otherwise noted. [b] In 20 mM imidazole, 100 mM KCl, 16 mM potassium phosphate, 1 mM dithiothreitol, and 1 mM EDTA. [c] In 0.1 M potassium phosphate, 100 mM KCl, 1 mM dithiothreitol, and 1 mM EDTA.

bimolecular ligand–protein reaction is not being observed. The value of the relaxation time is not markedly dependent on the enzyme concentration so that protein aggregation also is unlikely to be a major factor in the mechanism: the enzyme is tetrameric in the experiments for which data are presented (Figures 1–4). The most reasonable hypothesis is that the relaxation process is associated with a conformational change, with the conformational equilibria being altered by a variety of ligands: AMP-PNP, ATP, cAMP, H^+, and P_i. A number of different mechanisms were tested; the simplest model consistent with the data is a modified version of the Monod–Wyman–Changeux model for allosterism (Monod et al., 1965; eq A1 and A2). In this model ATP and AMP-PNP are assumed to bind to two sites per polypeptide chain, a catalytic site and a regulatory site. The enzyme is assumed to exist in two conformations, designated R and T. Binding to the catalytic site by ATP and AMP-PNP shifts the enzyme toward the R state, while the binding of ATP and AMP-PNP at the regulatory site shifts the enzyme to the T state.

The relaxation time characterizing the interconversion of the R and T states becomes relatively simple if three conditions are satisfied: (1) the bimolecular ligand–protein reactions are rapid compared with the rate of equilibration of the R and T states; (2) the substrate concentration is much greater than the enzyme concentration; and (3) the rate constants characterizing the rate of equilibration of the R and T states are related as specified in the Appendix. The first two of these requirements are valid to a good approximation, while the validity of the third is unknown. If these conditions prevail, the relaxation time can be written as (see eq A9)

$$\frac{1}{\tau} = k_{-00} + \frac{k_{00}(1 + S/K_T)^4(1 + S/K_T')^4}{(1 + S/K_R)^4(1 + S/K_R')^4} \qquad (1)$$

where k_{00} and k_{-00} are the rate constants for the interconversion of the unliganded R and T states [$R \rightleftharpoons (k_{00}, k_{-00})\ T$], K_R and K_T are intrinsic dissociation constants for the binding of ATP or AMP-PNP at the catalytic site in the R and T states, respectively, and K_R' and K_T' are similar dissociation constants for nucleotide binding at the regulatory site. The data were fit to eq 1 by a nonlinear least-squares analysis, and the lines in Figures 2–4 represent the best fits of the data by using the parameters in Table I. In both Figures 2 and 3, all three curves were fit simultaneously by assuming that the dissociation constants for the binding of ATP and AMP-PNP to the enzyme were unaltered by the presence of cAMP. This was found to be approximately true in preliminary data analyses and considerably reduces the total number of parameters used in fitting the data. Also included in Table I are the parameters characterizing the dependence of the relaxation time on AMP-PNP concentration in the absence of $MgCl_2$ (data not presented).

Discussion

The two observed relaxation processes appear to be associated with conformational changes. The slower process has a relaxation time that is unchanged by substrates and effectors and by increasing the pH. The relaxation amplitude is altered by the addition of AMP-PNP. The specific nature of the conformational transition is impossible to assess, but it is unlikely to be of major importance either in catalysis, because it occurs too slowly, or in regulation, since the relaxation time and amplitude are similar at pH 7.0 and 8.0. The other relaxation process possesses the necessary characteristics for involvement in regulation: both the relaxation time and amplitude vary with changes in the concentrations of the substrates and/or allosteric ligands and with changes in pH. Since this relaxation process is not detected at pH 8.0, it is unlikely to be associated with the catalytic mechanism. Neither of the relaxation effects observed is related to the association–dissociation of the MgATP or MgAMP-PNP complex, as the relaxation time associated with this process would be very short (<50 μs; Diebler et al., 1960).

The basic mechanism proposed is a concerted conformational transition with preferential ligand binding to the two conformations that are in equilibrium. This type of mechanism and the existence of a catalytic binding site and regulatory binding site for AMP-PNP are consistent with binding measurements (Wolfman et al., 1978) and steady-state kinetics (Goldhammer & Hammes, 1978). The experimental conditions used in the temperature-jump experiments are similar to those used in the binding studies although the KCl and potassium phosphate concentrations and the temperature are somewhat higher in this work. The data are fit quite well by the proposed mechanism (Figures 2–4); however, six adjustable parameters are available, and the assumption of special relationships among the rate constants is certainly an oversimplification. Moreover, in such a complex equation the six parameters cannot be regarded as entirely independent. Thus, the constants in Table I should only be accorded semiquantitative significance. Obviously, other, more complex, mechanisms cannot be excluded.

The relaxation processes observed with AMP-PNP– and ATP–enzyme interactions are quite similar. Equilibrium binding measurements have shown that two binding sites per polypeptide chain exist for AMP-PNP which are characterized by dissociation constants of about 1 and 100 μM (Wolfman et al., 1978). The catalytic site is associated with the dis-

sociation constant of about 1 μM, and the regulatory site is associated with the dissociation constant of about 100 μM. Therefore, the parameters K_R and K_T in Table I can be identified with binding to the catalytic site, while K_R' and K_T' are characteristic of binding to the regulatory site. In all cases, AMP-PNP and ATP show a marked preference for binding to the catalytic site in the R state and to the regulatory site in the T state. The ratio k_{-00}/k_{00}, which is included in Table I, is equal to the ratio (R)/(T) for the unliganded enzyme. The primary effect of cAMP is to increase this ratio, which is in accord with its role as an activator. The binding of both nucleotides seems to be enhanced in the absence of Mg^{2+}, but this effect may be an artifact of the data fitting. The enzyme appears consistently to bind ATP more strongly than AMP-PNP. Raising the temperature increases the dissociation constants, while lowering the pH decreases them. Increased phosphate ion concentration causes quantitative changes in the parameters with no clear-cut mechanistic pattern.

A major inconsistency in terms of the proposed mechanism is that k_{00} and k_{-00} should be the same for AMP-PNP and ATP under a given set of conditions. This can be partially attributed to the uncertainty in obtaining a unique set of six parameters to fit the data but also probably is an indication that the proposed mechanism is oversimplified. However, introduction of a more complex mechanism (i.e., rate constants for the interconversion of R and T forms in various states of ligation) would not be meaningful.

The observed relaxation amplitudes are qualitatively consistent with the data. The relaxation amplitude for the proposed mechanism should reach a maximum when the population of R and T states is about equal and then should reach a constant value when the enzyme is fully saturated with AMP-PNP or ATP. (The observed decrease in amplitude at very high nucleotide concentrations is most likely due to an inner-filter effect.) Either cAMP and fructose 6-phosphate cannot shift the equilibrium between R and T states sufficiently to give a detectable relaxation amplitude or the observed fluorescence change only occurs when AMP-PNP or ATP is bound to the enzyme. If the enzyme is largely in the T state in the absence of ATP or AMP-PNP, as suggested by the ratio k_{-00}/k_{00}, the latter explanation is more likely. Previous work has shown that the fluorescence change associated with the interaction of enzyme with ATP or AMP-PNP is primarily due to binding at the regulatory site (Wolfman et al., 1978; Pettigrew & Frieden, 1978). The relaxation amplitude is much larger with ATP than with AMP-PNP; this can be attributed either to enhanced fluorescence changes with ATP or to larger enthalpy changes being associated with the ATP–enzyme interaction.

Although no apparent pH change was detected with the absorbance temperature jump, a detailed experimental investigation was not possible because of enzyme instability. Since no fluorescence change was detected when the enzyme was mixed with ATP in the stopped-flow apparatus, major fluorescence changes associated with ATP binding are complete within 5 ms. The relaxation process having a 20-ms relaxation time apparently is associated with too small a fluorescence change for detection with the stopped-flow apparatus.

Several models have been proposed to explain the regulatory behavior of rabbit muscle phosphofructokinase (Kemp et al., 1976; Bock & Frieden, 1976; Goldhammer & Hammes, 1978; Pettigrew & Frieden, 1979). The temperature-jump method has permitted for the first time direct study of a concerted conformational change, which is an essential part of previously proposed mechanisms. Although all of the studies, steady-state kinetics, equilibrium binding, and activity–pH measurements, suggest the same type of mechanism with similar equilibrium and kinetic parameters, a quantitative agreement between the results obtained with different methods still is lacking. For example, the cooperativity seen in binding measurements is not the same as that derived from steady-state kinetics and the temperature-jump experiments; also, the temperature-jump measurements suggest that ATP binds much more tightly to the catalytic site in the R state, whereas the steady-state kinetics suggests little preference of ATP for the R and T states. Thus, while a broad picture for the mechanism of regulation of phosphofructokinase has emerged, more work is needed to establish the quantitative aspects of the mechanism.

Acknowledgments

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Appendix

Relaxation Spectrum for Multisubstrate Monod–Wyman–Changeux Mechanism. The Monod–Wyman–Changeux mechanism for a single substrate S can be represented as

$$\begin{array}{ccc} S + R_0 & \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} & T_0 + S \\ \downarrow\uparrow & & \downarrow\uparrow \\ S + R_1 & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & T_1 + S \\ \vdots & & \vdots \\ \downarrow\uparrow & & \downarrow\uparrow \\ R_n & \underset{k_{-n}}{\overset{k_n}{\rightleftharpoons}} & T_n \end{array} \qquad \text{(A1)}$$

where R is an active conformation of the enzyme and T is an inactive conformation. If the rates of the binding processes are assumed to be rapid compared with the rate of the interconversion of R and T states, the three relaxation times characterizing this mechanism can be easily obtained (cf. Hammes & Wu, 1974).

For the case of two substrates, A and B, the Monod–Wyman–Changeux mechanism is more complex since the number of different liganded states is greatly increased. The interconversion of R and T states can be represented as

$$R A_i B_j \underset{k_{-ij}}{\overset{k_{ij}}{\rightleftharpoons}} T A_i B_j \qquad \text{(A2)}$$

There are $(n + 1)^2$ such equations coupled by the binding equilibria ($i = 0, 1,...n$; $j = 0, 1,...n$). From detailed balance

$$\frac{k_{ij}}{k_{-ij}} = \left(\frac{K_{RA}}{K_{TA}}\right)^i \left(\frac{K_{RB}}{K_{TB}}\right)^j \frac{k_{00}}{k_{-00}} = c^i d^j k_{00}/k_{-00} \qquad \text{(A3)}$$

where K_{RA} and K_{TA} are the intrinsic dissociation constants for the binding of A to the R and T states, K_{RB} and K_{TB} are the corresponding constants for the binding of B, and $K_{RA}/K_{TA} = c$ and $K_{RB}/K_{TB} = d$. The relaxation spectrum for this complex mechanism can be readily calculated if the assumptions are made that the two ligands A and B bind independently and that these binding processes occur more rapidly than the interconversion of R and T states. With two ligands, four fast bimolecular reactions occur: A reacting with all R states, A reacting with all T states, and two similar reactions of B with the R and T states. Therefore, four relaxation times are obtained: two for the binding of A and two

for the binding of B. The rate equation for the interconversion of R and T states is

$$\frac{-\mathrm{d}[\sum_{i,j}(\mathrm{RA}_i\mathrm{B}_j)]}{\mathrm{d}t} = \sum_{i,j}k_{ij}(\mathrm{RA}_i\mathrm{B}_j) - \sum_{i,j}k_{-ij}(\mathrm{TA}_i\mathrm{B}_j) \quad \text{(A4)}$$

In the neighborhood of equilibrium eq A4 can be rewritten as

$$\frac{-\mathrm{d}\{\delta[\sum_{i,j}(\mathrm{RA}_i\mathrm{B}_j)]\}}{\mathrm{d}t} = \frac{1}{\tau}\delta[\sum_{i,j}(\mathrm{RA}_i\mathrm{B}_j)]$$

where δ represents the deviation from equilibrium and τ, the longest relaxation time for the mechanism, is

$$\frac{1}{\tau} = \frac{\sum_{i,j}k_{ij}\dfrac{\delta(\mathrm{RA}_i\mathrm{B}_j)}{\delta(\mathrm{R}_0)}}{\sum_{i,j}\dfrac{\delta(\mathrm{RA}_i\mathrm{B}_j)}{\delta(\mathrm{R}_0)}} + \frac{\sum_{i,j}k_{-ij}\dfrac{\delta(\mathrm{TA}_i\mathrm{B}_j)}{\delta(\mathrm{T}_0)}}{\sum_{i,j}\dfrac{\delta(\mathrm{TA}_i\mathrm{B}_j)}{\delta(\mathrm{T}_0)}} \quad \text{(A5)}$$

By assumption that the substrate concentrations are greater than the enzyme concentration, $\delta(\mathrm{A}) = \delta(\mathrm{B}) = 0$ and the expressions for the equilibrium constants can be differentiated to give

$$\frac{\delta(\mathrm{RA}_i\mathrm{B}_j)}{\delta(\mathrm{R}_0)} = \left[\frac{(\bar{\mathrm{A}})}{K_{\mathrm{RA}}}\right]^i\left[\frac{(\bar{\mathrm{B}})}{K_{\mathrm{RB}}}\right]^j\frac{n!}{(n-i)!i!}\frac{n!}{(n-j)!j!}$$

$$\frac{\delta(\mathrm{TA}_i\mathrm{B}_j)}{\delta(\mathrm{T}_0)} = \left[\frac{(\bar{\mathrm{A}})}{K_{\mathrm{TA}}}\right]^i\left[\frac{(\bar{\mathrm{B}})}{K_{\mathrm{TB}}}\right]^j\frac{n!}{(n-i)!i!}\frac{n!}{(n-j)!j!} \quad \text{(A6)}$$

Substituting eq A6 into eq A5 gives

$$\frac{1}{\tau} = \frac{\sum_{i,j}k_{ij}\left[\dfrac{(\bar{\mathrm{A}})}{K_{\mathrm{RA}}}\right]^i\left[\dfrac{(\bar{\mathrm{B}})}{K_{\mathrm{RB}}}\right]^j\dfrac{n!}{(n-i)!i!}\dfrac{n!}{(n-j)!j!}}{[1+(\bar{\mathrm{A}})/K_{\mathrm{RA}}]^n[1+(\bar{\mathrm{B}})/K_{\mathrm{RB}}]^n} + \frac{\sum_{i,j}k_{-ij}\left[\dfrac{(\bar{\mathrm{A}})}{K_{\mathrm{TA}}}\right]^i\left[\dfrac{(\bar{\mathrm{B}})}{K_{\mathrm{TB}}}\right]^j\dfrac{n!}{(n-i)!i!}\dfrac{n!}{(n-j)!j!}}{[1+(\bar{\mathrm{A}})/K_{\mathrm{TA}}]^n[1+(\bar{\mathrm{B}})/K_{\mathrm{TB}}]^n} \quad \text{(A7)}$$

Two limiting cases result in relatively simple expressions for $1/\tau$. If $k_{00} = k_{ij}$ for all i and j, then $k_{-ij} = c^{-i}d^{-j}k_{-00}$ (eq A3) and

$$\frac{1}{\tau} = k_{00} + k_{-00}\frac{[1+(\bar{\mathrm{A}})/K_{\mathrm{RA}}]^n[1+(\bar{\mathrm{B}})/K_{\mathrm{RB}}]^n}{[1+(\bar{\mathrm{A}})/K_{\mathrm{TA}}]^n[1+(\bar{\mathrm{B}})/K_{\mathrm{TB}}]^n} \quad \text{(A8)}$$

Also, if $k_{-00} = k_{-ij}$ for all i and j, then $k_{ij} = c^id^jk_{00}$ and

$$\frac{1}{\tau} = k_{-00} + k_{00}\frac{[1+(\bar{\mathrm{A}})/K_{\mathrm{TA}}]^n[1+(\bar{\mathrm{B}})/K_{\mathrm{TB}}]^n}{[1+(\bar{\mathrm{A}})/K_{\mathrm{RA}}]^n[1+(\bar{\mathrm{B}})/K_{\mathrm{RB}}]^n} \quad \text{(A9)}$$

Equation 1 can be derived directly from eq A9 if n is set equal to 4 and ligands A and B represent ATP or AMP-PNP binding to the catalytic and regulatory sites, respectively.

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