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A Kinetic Study of the Subunit Dissociation and Reassembly of Rabbit Muscle Phosphofructokinase†

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ABSTRACT: The kinetics of dissociation and reassembly of rabbit skeletal muscle phosphofructokinase has been studied using fluorescence, stopped-flow fluorescence and enzyme activity measurements. The dissociation of the fully active tetramer in 0.8 M guanidine hydrochloride (0.1 M potassium phosphate, pH 8.0) occurs in three kinetic phases as measured by changes in the protein fluorescence emission intensity: dissociation of tetramer to dimer with a relaxation time of a few milliseconds; dissociation of dimer to monomer with a relaxation time of a few seconds; and a conformational change of the monomer with a relaxation time of a few minutes. All three phases exhibit first-order kinetics; ATP (0.05 mM) retards the second step but does not influence the rate of the other two processes. The rate of the second process increases with decreasing temperature; this may be due to the involvement of hydrophobic interactions in the stabilization of the dimeric enzyme. A further unfolding of the monomer polypeptide chain occurs at higher guanidine concentrations, and the relaxation time associated with this process was found to be 83 ms in 2.5 M guanidine, 0.1 M potassium phosphate (pH 8.0) at 23 °C. The phosphofructokinase monomers were reassembled from 0.8 M guanidine chloride by 1:10 dilution of the guanidine hydrochloride concentration and yielded a protein with 70–94% of the original activity, depending on the protein concentration. The reactivation process follows second-order kinetics; ATP (5 mM) increases the rate of reactivation without altering the reaction order, while fructose 6-phosphate does not influence the rate of reaction. The rate-determining step is probably the association of monomers to form the dimer.

The experimental and theoretical aspects of folding and unfolding processes of single chain globular proteins are currently under intensive investigation in a number of laboratories (cf. Anfinsen and Scheraga, 1975; Pace, 1975; Tsong, 1973; Ikai and Tanford, 1973; Wetlaufer and Ristow, 1973; Tsong et al., 1972; Tanford, 1968, 1970). Several recent studies have shown that, after extensive denaturation in high concentrations of urea or Gdn·HCl,[1] oligomeric proteins may also regain substantial enzymatic activity (Teipel and Koshland, 1971a,b; Bornmann et al., 1974; London et al., 1974). The reactivation of multisubunit proteins represents an extension of the basic chain folding process in that reassembly of fully folded or partially folded protomers must be considered. In the case of rabbit muscle phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11), the dissociation process can be isolated from the gross unfolding of the polypeptide chains (Parr and Hammes, 1975). Thus dissociation–reassociation phenomena under the influence of a denaturant such as Gdn·HCl may be studied without the added complication of extensive protomer unfolding and refolding.

Rabbit muscle phosphofructokinase is composed of identical subunits (Pavelich and Hammes, 1973; Coffee et al., 1973), and aggregates smaller than the tetramer possess little activity (Pavelich and Hammes, 1973; Lad et al., 1973). Furthermore, dissociation–reassociation equilibria appear to be relevant to the regulation of enzymatic activity (Hofer and Krystek, 1975; Lad et al., 1973; Hill and Hammes, 1975).

In this work some of the kinetic parameters for dissociation and reassembly of phosphofructokinase are reported. On addition of the phosphofructokinase tetramer to 0.8 M Gdn·HCl, three transitions well resolved in time are observed. The first transition has a relaxation time of a few milliseconds and is related to the dissociation of tetramer to dimer. The second and third transitions have relaxation times of a few seconds and a few minutes, respectively, and represent dissociation of dimer to monomer followed by a conformational alteration of the monomer. These results are in agreement with the mechanism proposed previously for the equilibrium denaturation of phosphofructokinase by Gdn·HCl (Parr and Hammes, 1975).

Under suitable conditions, the monomer units may be reassembled to yield almost fully active enzyme. This renaturation process is found to obey second-order kinetics. The presence of ATP accelerates the regain of activity, and the monomer-to-dimer association appears to be the rate-limiting step in reactivation.

Experimental Section

Reagents. The ATP, fructose 6-phosphate, aldolase, α-glycerophosphate dehydrogenase, triosephosphate isomer-

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[1] Abbreviations used are: Gdn·HCl, guanidine hydrochloride; EDTA, ethylenediaminetetraacetic acid.

ase, and albumin (bovine) were purchased from Sigma Chemicals. UltraPure Gdn·HCl (lots ZZ1127 and ZZ1833) was obtained from Schwarz/Mann and used without further purification. All other chemicals were the best available commercial grades. Distilled deionized water was used in all experiments. Solutions of Gdn·HCl in phosphate buffer were adjusted to pH 8.0 with KOH and used within 12 h of preparation.

Phosphofructokinase. Rabbit skeletal muscle phosphofructokinase was prepared by the method of Ling et al. (1966). The ammonium sulfate precipitate was dissolved in 0.1 M potassium phosphate, 1 mM EDTA, pH 8.0, and dialyzed against the same buffer to give a stock solution of 7–14 mg/ml. The enzyme concentration was determined from the absorbance at 280 nm using an absorptivity of 1.02 ml/(mg cm) (Parmeggiani et al., 1966). The specific activity of the enzyme at 23 °C, 0.1 M phosphate (pH 8.0), was 100–120 units/mg. Phosphofructokinase solutions were prepared by dilution of concentrated enzyme to the appropriate concentration in 0.1 M potassium phosphate, pH 8.0, 1 mM EDTA, 5 mM dithiothreitol, and allowed to equilibrate for 1–2 h to ensure that only the tetrameric species was present (Lad et al., 1973).

Assays. Enzymatic activity was determined by the coupled assay procedure of Ling et al. (1966). Standard assays were run under the following conditions: pH 8.0, 33 mM Tris-Cl, 2 mM ATP, 5 mM $MgCl_2$, 2 mM fructose 6-phosphate, 0.1 mM NADH, 2 mM dithiothreitol, 0.20 units/ml of aldolase, 35 units/ml of triosephosphate isomerase, 3.2 units/ml of α-glycerophosphate dehydrogenase, and ≤ 0.1 μg/ml of phosphofructokinase in a total volume of 3.0 ml. All assays were performed at 23 °C. Assays were initiated by the addition of phosphofructokinase to the assay mixture, and the change in absorption with time was recorded spectrophotometrically using a Cary 14 spectrophotometer. Assay velocities were unaltered by increasing the concentrations of the auxiliary enzymes, and the concentration of NADH was sufficiently low to avoid inhibition of the auxiliary enzymes (Newsholme et al., 1970).

Kinetic Measurements. Stopped-flow kinetic measurements were performed on a Durrum Model D-110 stopped-flow apparatus equipped with a fluorescence attachment. The instrument dead time is estimated to be 2–3 ms. The excitation wavelength was 280 nm, and a Dow-Corning type 0-52 filter was used to limit the detected fluorescence emission to wavelengths greater than about 340 nm. All solutions were filtered through a 0.22 μm millipore filter prior to use. In all cases equal volumes of protein and Gdn·HCl solutions were mixed and the instrument was thermostatted at the temperature indicated in the text. Blank experiments were conducted by mixing Gdn·HCl with free tryptophan at a concentration approximately equal to that in the protein solution. No change in fluorescence intensity was observed at times <2 s/div, while at longer time scales a small decrease in intensity was evident, presumably due to an instrumental artifact. This artifact had no influence on the kinetic measurements reported here. Blank reactions were also run in the absence of Gdn·HCl by mixing protein solution with phosphate buffer; no changes in fluorescence intensity were observed.

Data acquisition was through a Biomation Model 802 analogue to digital converter interfaced to a PDP-11 computer. The kinetic data were fit to a single exponential equation, and the amplitude and relaxation time, τ_i, of the relaxation processes were determined (cf. Hilborn, 1973; Hilborn et al., 1973).[2] The computer routines allow signal averaging of multiple kinetic traces and the parameters of the resulting signal averaged curve to be computed. The data reported here were derived from an average of at least ten curves in the case of τ_1 values and at least five curves in the case of τ_2 values. In addition, the parameters for an averaged curve were determined in at least two separate experiments with freshly prepared solutions.

Changes in fluorescence intensity with time for the slowest reaction were observed on a Perkin-Elmer MPF-3 fluorescence spectrophotometer. Measurements were carried out in a 1-cm path-length cuvette thermostated at the appropriate temperature. An excitation wavelength of 295 nm was used in conjunction with a 310-nm cutoff filter. The excitation and emission slit widths gave a band-pass of 6 and 20 nm, respectively, and a ×3 scale expansion was employed. Concentrated Gdn·HCl was added to protein solution in the cuvette to give a final Gdn·HCl concentration of 0.8 M. The dead time between mixing and recording of data was about 10 s.

Reactivation. Monomeric phosphofructokinase was prepared by addition of concentrated Gdn·HCl to a protein solution at 5 °C to obtain a final Gdn·HCl concentration of 0.8 M. Addition of the Gdn·HCl was performed slowly and with stirring to prevent concentration gradients. After incubation for about 1 min, an aliquot was withdrawn and diluted 1:10 into the reactivation solution at 23 °C. The reactivation solution was composed of 0.1 M potassium phosphate (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol, 0.016 mg/ml bovine serum albumin, and, in some cases, 5 mM ATP or 5 mM fructose 6-phosphate. The reactivation mixture was then placed in a temperature bath at 23 °C and assayed as described previously. The percent reactivation was determined with respect to a reference solution prepared by diluting an aliquot of undenatured phosphofructokinase solution into an appropriate amount of reactivation mixture such that the final concentration of phosphofructokinase in the reference solution was identical with that in the reactivated solution. Two such reference solutions were generally prepared, maintained at 23 °C, and assayed periodically throughout the course of the reactivation experiment. The specific activity of the reference solutions was independent of protein concentration in the range 0.004–0.15 mg/ml and was identical in 0 and 0.08 M Gdn·HCl.

Results

The dissociation and unfolding of tetrameric phosphofructokinase was investigated by mixing protein solution at an initial concentration of about 0.16 mg/ml in 0.1 M potassium phosphate (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol, with Gdn·HCl to yield a final Gdn·HCl concentration of 0.8 M. The Gdn·HCl concentration was chosen to correspond to the midpoint of the plateau region between the two major transitions observed previously under equilibrium conditions (Parr and Hammes, 1975). Three well-separated kinetic events can be observed. All three transitions result in an increase in fluorescence intensity.

The fastest observable reaction occurs with a relaxation time, τ_1, of a few milliseconds. A kinetic trace of the relaxa-

[2] The relaxation time normally is defined only close to chemical equilibrium where the rate equations can be linearized. In this work since only first-order rate processes are observed (except for reactivation which is treated separately), the relaxation time is taken to be the reciprocal first-order rate constant obtained from the kinetic plots, although not all of the data are obtained close to equilibrium.

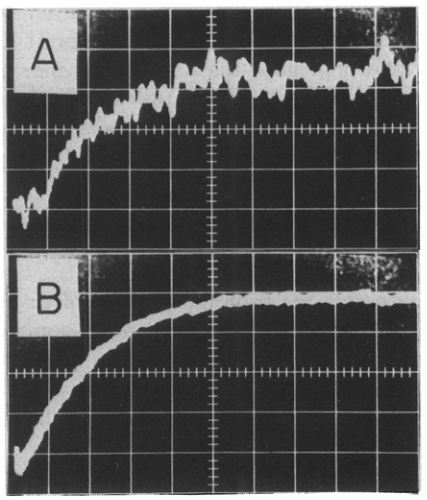

FIGURE 1: Oscilloscope traces of the change in fluorescence emission intensity associated with τ_1 (A) and τ_2 (B) obtained by mixing phosphofructokinase at a concentration of about 0.16 mg/ml in 0.1 M potassium phosphate (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol with Gdn·HCl in a stopped-flow apparatus to obtain a final protein concentration of about 0.08 mg/ml and a final Gdn·HCl concentration of 0.8 M. Results shown were obtained at a temperature of 14 °C. The vertical scale is 10 mV/div in both cases. Horizontal scales are 10 ms/div (A) and 1 s/div (B). Other details are as given in the Experimental Section.

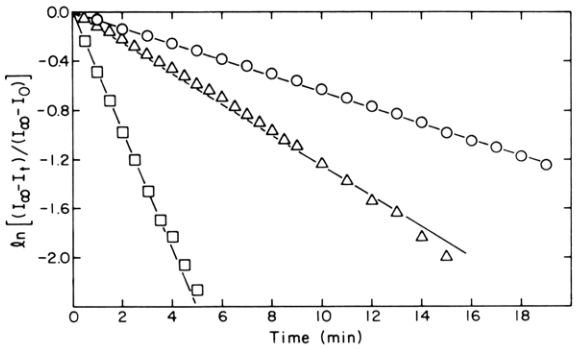

FIGURE 2: First-order kinetic plots of the kinetic process associated with τ_3 obtained on mixing a solution of phosphofructokinase (0.18 mg/ml, 0.1 M potassium phosphate (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol) with concentrated Gdn·HCl to yield a final Gdn·HCl concentration of 0.8 M. The final concentration of phosphofructokinase was 0.144 mg/ml. Each set of data represents the average of three separate runs at 31 (□), 23 (△), and 14 °C (○). I_0, I_t, and I_∞ represent the relative fluorescence emission intensity at $t = 0$, $t = t$, and $t = \infty$, respectively. Data were obtained by fluorescence measurements as described in the Experimental Section.

tion process associated with τ_1 is shown in Figure 1A. Values of τ_1 were determined at final phosphofructokinase concentrations of 0.08 and 0.04 mg/ml, and the results were identical within experimental error. The upper limit of the concentration range is determined by the fact that the starting material must consist entirely of the tetrameric species, while the lower limit is determined by the sensitivity of the detection system. The effect of ATP on τ_1 was investigated by adding 0.1 mM ATP to the protein solution, yielding a final ATP concentration of 0.05 mM after mixing. (Significantly higher concentrations of ATP resulted in a substantial decrease in fluorescence intensity, presumably due to absorption of the excitation beam by the ATP.) No effect due to the added ATP was observed. Data were obtained over a temperature range of 5–23 °C, and the relaxation times obtained are summarized in Table I. A plot of ln $(1/\tau)$ vs. the reciprocal absolute temperature was used to determine an Arrhenius activation energy of about 8 kcal/mol.

The second observable reaction has a relaxation time, τ_2, of a few seconds. An oscilloscope trace of the progress of this reaction is shown in Figure 1B. τ_2 also was found to be independent of phosphofructokinase concentration in the range 0.04–0.08 mg/ml. However, the presence of ATP has a significant effect on τ_2 (Table I). The addition of ATP reduces the reaction rate at all temperatures which were studied, although the magnitude of the effect increases at lower temperatures. No data are reported for τ_2 at higher temperatures because the processes associated with τ_2 and τ_3 are no longer well separated in time. The temperature dependence of τ_2 in the absence of ATP indicates an apparent activation energy of about −6 kcal/mol.

The third observable reaction has a relaxation time, τ_3, of a few minutes. First-order plots of the data taken at different temperatures are shown in Figure 2. The presence of up to 5 mM ATP in the reaction mixture was found to have no significant effect on the rate of reaction. τ_3 is difficult to observe quantitatively at temperatures lower than about 14 °C due to a reduction in the amplitude of the total change in fluorescence intensity and to instrumental instabilities over long periods of time. The Arrhenius activation energy for τ_3 was calculated from the data in Table I to be about 25 kcal/mol.

Table I: Relaxation Times for the Dissociation and Unfolding of Phosphofructokinase in 0.8 M Gdn·HCl.[a]

T (°C)	τ_1 (ms)	τ_2 (s)	$\tau_{2,\ ATP}$[b]	$10^{-2}\,\tau_3$ (s)
31				1.25 ± 0.15
23	8 ± 2			4.8 ± 0.5
14	16 ± 2	2.23 ± 0.1	2.47 ± 0.1	9.2 ± 1.5
10	28 ± 4	1.30 ± 0.05	1.69 ± 0.05	
5	43 ± 4	1.03 ± 0.05	1.54 ± 0.05	

[a] Experimental details are described in the text and in the legends for Figures 1 and 2. [b] The phosphofructokinase solution was 0.1 mM in ATP, yielding an ATP concentration of 0.05 mM after mixing.

Stopped-flow kinetic experiments were also performed at final Gdn·HCl concentrations less than 0.8 M. It was found that at Gdn·HCl concentrations less than about 0.6 M at either 14 or 5 °C only the most rapid transition (τ_1) could be observed. Both the amplitude and apparent rate constant $(1/\tau_1)$ increased with Gdn·HCl concentrations between 0.2 and 0.6 M and remained approximately constant at Gdn·HCl concentrations greater than 0.6 M. The weight average molecular weight ratio, M_i/M_o, where M_o represents the tetramer, under similar conditions varies from about 0.7 to 0.3 (Parr and Hammes, 1975). This suggests that the process observed at lower Gdn·HCl concentrations may be an equilibrium between different molecular weight species of phosphofructokinase, and thus the relaxation time should exhibit a dependence on protein concentration. Quantitative results were obtained at a final Gdn·HCl concentration of 0.3 M at 5 °C and at final protein concentrations of 0.087 and 0.04 mg/ml in 0.1 M potassium phosphate (pH 8.0). At the higher protein concentration the relaxation time was found to be 66 ± 4 ms, while at the lower concentration the relaxation time was 81 ± 4 ms. Thus the concentration dependence observed suggests a dissociation–reassociation equilibrium between different molecular weight species of the enzyme.

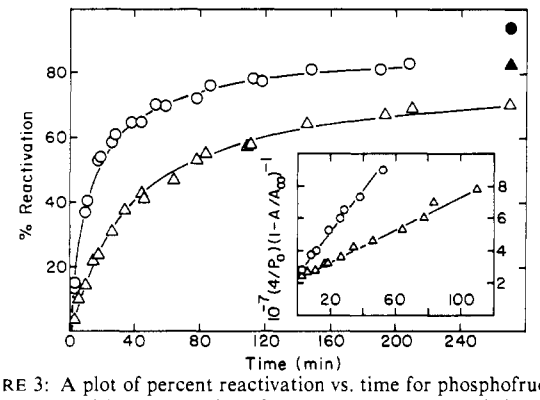

FIGURE 3: A plot of percent reactivation vs. time for phosphofructokinase dissociated in 0.8 M Gdn·HCl. Dissociation was carried out at 5 °C, while reactivation was at 23 °C following a tenfold dilution of the denatured protein into 0.1 M potassium phosphate (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol. The data shown are for a final phosphofructokinase concentration of 0.014 mg/ml in the absence of ATP (△) and in 5 mM ATP (□). Each plot includes data from two separate experiments. The filled symbols represent the percent reactivation at 24 h. Additional details are given in the Experimental Section. Second-order kinetic plots for the same data according to eq 6, 8, and 9 are shown in the inset.

The major unfolding transition of the phosphofructokinase monomer, which occurs between Gdn·HCl concentrations of about 1.0 and 3.5 M (Parr and Hammes, 1975), has also been studied with the stopped-flow fluorescence technique. A solution of 3.0 M Gdn·HCl, 0.1 M potassium phosphate (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol, and 0.18 mg/ml phosphofructokinase was mixed with an equal volume of 2.0 M Gdn·HCl, 0.1 M potassium phosphate (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol at 23 °C giving final concentrations of 0.09 mg/ml protein and 2.5 M Gdn·HCl. A single kinetic process was observed with a relaxation time of 83 ± 4 ms. (The relaxation time was computed from a signal averaged curve consisting of ten individual kinetic traces.) The final Gdn·HCl concentration was chosen to correspond to the midpoint of the unfolding transition as determined by the wavelength of maximum fluorescence emission. If the unfolding–refolding reaction can be described as a two-state transition, the kinetic parameters at 2.5 M Gdn·HCl should be identical whether the protein is initially present in the 3.0 M Gdn·HCl solution or the 2.0 M Gdn·HCl solution. However, when the protein is initially in the 2.0 M Gdn·HCl solution, no reaction is observed. This is probably due to the fact that in this range of Gdn·HCl concentrations, the largest changes in fluorescence emission intensity occur at wavelengths less than 340 nm which are unobserved due to the cutoff filter. Use of a filter with a lower wavelength cutoff value results in instrumental difficulties due to stray light and light scattering. Nonetheless, the unsymmetrical behavior of the intensity changes with respect to the apparent midpoint of the transition suggests that the reaction may be more complex than can be accounted for by a two-state mechanism.

Reactivation experiments were carried out as described in the Experimental Section. The rationale for this procedure is that dissociation to monomer is related to the kinetic phase associated with τ_2, while the kinetic process associated with τ_3 is irreversible. Thus reactivation must be carried out under conditions where the process associated with τ_2 is complete while that associated with τ_3 has not proceeded to any significant extent. The experimental procedure satisfies these criteria. The concentration of phosphofructokinase in reactivation experiments varied from about 0.004 to 0.02 mg/ml. Bovine serum albumin was used to prevent surface denaturation which occurs at low concentrations of phosphofructokinase in the absence of albumin. Typical plots of percent reactivation vs. time are shown in Figure 3. These data are inconsistent with first-order kinetics but can be fit to second-order kinetics. If the reassembly is assumed to obey the mechanism given by eq 1

$$4P \xrightarrow{k_1} 2P_2 \xrightarrow{k_2} P_4 \tag{1}$$

where P represents the protomer unit (mol wt 80 000) and the enzymic activity is proportional to the amount of tetramer present, then two limiting cases can give rise to simple second-order kinetics: $k_2 \gg k_1$ and $k_1 \gg k_2$. These situations may be analyzed mathematically as follows. The production of tetramer is given by eq 2.

$$d(P_4)/dt = k_2(P_2)^2 \tag{2}$$

If $k_2 \gg k_1$, P_2 is present in a steady state so that

$$(P_2)^2 = (k_1/k_2)(P)^2 \tag{3}$$

and substituting eq 3 into eq 2 gives eq 4.

$$d(P_4)/dt = k_1(P)^2 \tag{4}$$

If the mass balance is written as in eq 5

$$(P_0) = (P) + 2(P_2) + 4(P_4) \tag{5}$$

and (P_2) is approximately equal to zero, then eq 4 can be integrated to give eq 6.

$$-\frac{4}{(P_0)} + \frac{4}{(P_0) - 4(P_4)} = k_1 t \tag{6}$$

In the second limiting case $k_1 \gg k_2$; (P) then can be approximated as equal to zero in the mass balance equation so that eq 2 can be written as eq 7 which can be integrated to give eq 8.

$$\frac{d(P_4)}{[(P_0) - 4(P_4)]^2} = (k_2/4)dt \tag{7}$$

$$-\frac{4}{(P_0)} + \frac{4}{(P_0) - 4(P_4)} = (k_2/4)t \tag{8}$$

Thus in either case a plot of $4/[(P_0) - 4(P_4)]$ vs. t should yield a straight line whose slope is directly related to the rate constant. Furthermore, if the activity is assumed to be entirely due to the tetramer, it is apparent that

$$\left(\frac{4}{(P_0) - 4(P_4)}\right) \equiv \frac{4}{(P_0)}\left[1 - \frac{A_t}{A_\infty}\right]^{-1} \tag{9}$$

where A_t and A_∞ represent the percent reactivation at time t and at infinite time, respectively. Typical second-order plots are shown in the inset of Figure 3. The results obtained at various protein concentrations both in the presence and absence of ATP are listed in Table II. The percent of activation achieved relative to the original activity also is given in this table. The presence of ATP accelerates the reaction without changing the reaction order. At 23 °C, ATP appears to have a small effect on the final yield of active enzyme. However, the amount of reactivation drops sharply at lower temperatures and displays a definite dependence on ATP (e.g., ~65% in the presence of ATP and ~25% in the absence of ATP at 5 °C). The presence of fructose 6-phosphate at a concentration of 5 mM has no significant effect on reactivation at either 23 or 5 °C (cf. Table II).

Discussion

The results of kinetic studies following addition of the native phosphofructokinase tetramer to 0.8 M Gdn·HCl show the existence of three consecutive first-order processes. Additional information provided by the previously reported equilibrium study of the interaction of Gdn·HCl with phosphofructokinase (Parr and Hammes, 1975) allows the following interpretation of the observed kinetic processes to be made. The shortest relaxation time, τ_1, is associated with the conversion of tetramer (mol wt 320 000) to dimer (mol wt 160 000); τ_2 is associated with the subsequent dissociation of dimer to monomer (mol wt 80 000); and the longest relaxation time, τ_3, is associated with a conformational change of the monomer. This mechanism may be represented by eq 10.

$$P_4 \xrightarrow{\tau_1} 2P_2 \xrightarrow{\tau_2} 4P \xrightarrow{\tau_3} 4P' \qquad (10)$$

Light scattering studies under conditions similar to those employed here showed that dissociation to the monomer was complete within 1 min (Parr and Hammes, 1975). Since τ_3 is much longer (Table I), it must be associated with a change in the structure of the monomer. Such a conformational change had been predicted from the results of the equilibrium study. Correlation of the molecular weight ratio at low Gdn·HCl concentrations (Parr and Hammes, 1975) with kinetic observations at a final Gdn·HCl concentration of 0.3 M is consistent with an interpretation of this process as being due to a tetramer–dimer equilibrium. The similarity of the relaxation times in 0.3 M Gdn·HCl to τ_1 (0.8 M Gdn·HCl) also is consistent with τ_1 being related to the dissociation of tetramer to dimer. Since it is known that the monomer is produced in 0.8 M Gdn·HCl (Parr and Hammes, 1975), τ_2 can be reasonably associated with the dissociation of dimer to monomer.

The observed changes in the fluorescence emission intensity associated with τ_1 and τ_2 may be directly related to the respective dissociations, or may be due to conformational changes either preceding or following the dissociation. Attempts to gain further insight into this problem by using the stopped-flow apparatus in a light scattering mode proved unsuccessful due to the low concentrations of protein which must be employed.

The apparent negative activation energy for the dissociation of dimer to monomer is unusual. However, a similar phenomenon has been observed previously for the denaturation of proteins in urea or Gdn·HCl (Simpson and Kauzmann, 1953a,b; Pace and Tanford, 1968; Pohl, 1968; Tanford and Aune, 1970). Tanford (1968, 1970) has suggested that such behavior is a result of an unusually large change in heat capacity associated with the transfer of hydrophobic residues to a more aqueous environment. Thus the temperature dependence of τ_2 may indicate that the monomer–dimer interface of phosphofructokinase contains a large number of hydrophobic contacts. The Arrhenius activation energy for $1/\tau_3$ suggests that the native structure of the phosphofructokinase monomer is changed substantially in the reaction characterized by τ_3.

Only τ_2 is altered significantly in the presence of ATP; this suggests that, under the experimental conditions utilized, ATP has a stabilizing effect on the dimer species relative to the monomer.

From the values of τ_2 and τ_3 listed in Table I it is clear that, in 0.8 M Gdn·HCl at 5 °C, dissociation to monomer is complete within a few seconds, while the amount of monomer undergoing irreversible conformational change is negligible up to at least 1 min. Thus the procedure used in the reactivation experiments is quantitatively justified by the kinetic measurements. The mechanism in eq 1 can be justified on the grounds that the trimer is unstable relative to dimeric and tetrameric species (Lad and Hammes, 1974) and that no evidence exists for additional processes. Since only a single second-order reaction is observed on reactivation (reassembly) of the monomer, either k_1 or k_2 (eq 1) may be rate limiting. However since ATP accelerates reactivation and retards the dimer to monomer dissociation, it would appear that k_1, dimerization of monomers, probably is the limiting step in the reassembly resulting in active enzyme. This provides additional support for the assumption that only the tetrameric species contributes to the observed activity during reactivation even though the dimer is thought to have some activity (Pavelich and Hammes, 1973; Lad et al., 1973). If k_1 is the rate-limiting step, significant amounts of P_2 would not accumulate during the course of the reaction and the contribution of dimeric species to the observed activity would be negligible. The average value of the rate constant, k_1, is 7.9 (± 1.5) × 10^3 M^{-1} s^{-1} in the absence of ATP and 18.4 (± 3.0) × 10^3 M^{-1} s^{-1} in 5 mM ATP. The scatter in the values of the rate constants is a reflection of the difficulty in obtaining reproducible results in the reactivation experiments, although why this is the case is unclear.

The mechanism proposed here for the denaturation and reactivation of phosphofructokinase at low concentrations of Gdn·HCl is similar to a mechanism recently proposed for the denaturation of lactate dehydrogenase at low pH (Vallee and Williams, 1975). For lactate dehydrogenase, however, the conformational change following dissociation is at least partially reversible.

Table II: Data Summary for the Reactivation of Phosphofructokinase.[a]

Protein (mg/ml)	ATP (mM)	Slope 10^{-3} (M^{-1} s^{-1})	% Reactivation
0.0141	5.0	19.3	94
0.0126	5.0	22.3	83
0.0076	5.0	17.0	85
0.0040	5.0	15.0	81
0.0210	0.0	7.20	86
0.0141	0.0	9.08	82
0.0084	0.0	6.91	71
0.0084[b]	0.0	8.61	73

[a] Reactivation was carried out in 0.1 M potassium phosphate, pH 8.0, 23 °C, 1 mM EDTA, 5 mM dithiothreitol, 0.016 mg/ml bovine serum albumin. Experimental procedure for dissociation and subsequent reactivation is described in the text. [b] 5 mM fructose 6-phosphate added to reactivation mixture.

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Rat Liver Cytoplasmic Glucose-6-phosphate Dehydrogenase. Steady-State Kinetic Properties and Circular Dichroism†

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ABSTRACT: Steady-state kinetic studies including initial velocity, NADPH product inhibition, dead-end inhibition, and combined dead-end and product inhibition measurements with purified rat liver glucose-6-phosphate dehydrogenase indicate a sequential and obligatory addition of substrates in the order of $NADP^+$, glucose-6-P for the catalytic pathway at pH 8.0. Although instability of 6-phosphoglucono-δ-lactone precluded product inhibition experiments which might directly exclude an enzyme-6-phosphoglucono-δ-lactone complex, the absence of an enzyme-glucose-6-P complex suggests that the enzyme-lactone product is unlikely and the release of products is also ordered, with NADPH released last. Consideration of the kinetic constants (K_a = 2.0 μM, K_{iq} = 13 μM) and cellular concentration of the substrates and products suggests extensive inhibition of the enzyme in vivo and control by the NADPH/$NADP^+$ ratios. Circular dichroism spectra of the enzyme in 20 mM phosphate buffer at pH 7.0 and 25 °C indicate 51% helix and 33% pleated sheet structures which is considerably different from results (14% helix) with yeast enzymes.

This paper reports steady-state kinetic and secondary structure analyses of rat liver glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) to further characterize the liver enzyme's physical properties and to provide estimates of catalytic activities with cellular substrate and product concentrations. Steady-state kinetic mechanisms have been reported for the enzymes from *Candida utilis* (Afolayan, 1972), human erythrocyte (Soldin and Balinsky, 1968), *Leuconostoc mesenteroides* (Olive et al., 1971), and human blood platelets (Kosow, 1974). Enzymes from these sources represent each of the three classes of nucleotide specificity (Olive et al., 1971). An ordered sequential mechanism with addition of coenzymes to the free enzyme forms is indicated in each case. In addition, the NAD^+-linked reaction with *L. mesenteroides* (Olive et al., 1971) appears to require an isomerization of free enzyme, and the $NADP^+$-linked reactions of *C. utilis* and erythrocyte enzymes (Afolayan, 1972; Afolayan and Luzzatto, 1971; Luzzatto and Afolayan, 1971) exhibit sigmoidal kinetics.

Although subunits of the enzyme are thought to be identical in all tissues of a given animal (Yoshida, 1966), considerable tissue differences are observed in both the polymerization (Schmukler, 1970) and the microheterogeneity of enzyme within a given molecular weight form.[1] Previous

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