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Subunit Dissociation and Unfolding of Rabbit Muscle Phosphofructokinase by Guanidine Hydrochloride†

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ABSTRACT: The denaturation of rabbit skeletal muscle phosphofructokinase by guanidine hydrochloride has been studied using fluorescence, light scattering, and enzyme activity measurements. The transition from fully active tetramer (0.1 *M* potassium phosphate (pH 8.0) at 10 and 23°) to unfolded polypeptide chains occurs in two phases as measured by changes in the fluorescence spectrum and light scattering of the protein: dissociation to monomers at low guanidine hydrochloride concentrations (~0.8 *M*) followed by an unfolding of the polypeptide chains, which presumably results in a random coil state, at high concentrations of denaturant (>3.5 *M*). The initial transition can be further divided into two distinct stages. The native enzyme is rapidly dissociated to inactive monomers which then undergo a much slower conformational change that alters the fluorescence spectrum of the protein. The dissociation is complete within 2 min and is reversible, but the conformational change requires about 2 hr for completion and is not reversible under a variety of conditions, including the presence of substrates and allosteric effectors. The conformationally altered protomer reaggregates to form a precipitate at 23°, but is stable below 10°. The second major phase of the denaturation is fully reversible. A simple mechanism is proposed to account for the results, and its implications for the corresponding renaturation process are discussed.

Rabbit skeletal muscle phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is composed of identical subunits (Pavelich and Hammes, 1973; Coffee et al., 1973). No disulfide bonds exist (Younathan et al., 1968; Coffee et al., 1973), and aggregates smaller than the tetramer appear to possess little activity (Pavelich and Hammes, 1973; Lad et al., 1973). Under defined conditions the enzyme exists essentially entirely as the tetramer (Pavelich and Hammes, 1973). Both association-dis-

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sociation equilibria and allosteric interactions appear to be important in the regulation of enzymatic activity (Lad et al., 1973; Hill and Hammes, 1975). The folding and unfolding of simple globular proteins have been extensively studied (cf. Wetlaufer and Ristow, 1973). Rabbit muscle phosphofructokinase provides a good prototype for studying the processes of both polypeptide chain folding and unfolding and subunit assembly and disassembly.

In this work, the influence of guanidine hydrochloride (Gdn · HCl)[1] on the state of rabbit skeletal muscle phosphofructokinase is reported. The equilibrium denaturation by Gdn · HCl is clearly divided into two stages: dissociation to inactive monomers at Gdn · HCl concentrations below 1.0 *M* followed by an apparently complete unfolding of the polypeptide chains at higher Gdn · HCl concentrations. The dissociation process is further separable into a kinetically rapid phase which is reversible and a much slower, apparently irreversible, conformational change. The conformationally altered monomer aggregates at 23° but is stable at 10°. A simple mechanism for the denaturation of phosphofructokinase by Gdn · HCl is presented.

Experimental Section

Reagents. The ATP, fructose 6-phosphate, fructose 1,6-biphosphate, aldolase, α-glycerophosphate dehydrogenase, triose phosphate isomerase, and albumin (bovine) were purchased from Sigma Chemicals. Ultra-pure Gdn · HCl (lots Y3825 and ZZ1127) 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 48 hr of preparation.

Phosphofructokinase. Rabbit skeletal muscle phosphofructokinase was purified by the method of Ling et al. (1966). The ammonium sulfate precipitate was dissolved in pH 8.0, 0.1 *M* potassium phosphate–1.0 m*M* EDTA, and dialyzed against the same buffer to give a stock solution of 9–14 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°, 0.1 *M* phosphate (pH 8.0) was 110–125 units/mg. (A unit of enzyme activity is defined as the production of 1 μmol of product/min.)

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 m*M* Tris-Cl, 2 m*M* ATP, 5 m*M* $MgCl_2$, 2 m*M* fructose 6-phosphate, 0.1 m*M* NADH, 2 m*M* dithiothreitol, 0.20 unit/ml of aldolase, 35 units/ml of triose phosphate isomerase, 3.2 units/ml of α-glycerophosphate dehydrogenase, and 0.1 μg/ml of phosphofructokinase in a total volume of 3 ml. Measurements were performed at either 5 or 23°. Assays were initiated by the addition of phosphofructokinase to the assay mixtures, 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).

Fluorescence Measurements. Fluorescence emission spectra were determined with a Perkin-Elmer MPF-3 fluorescence spectrophotometer. Measurements were performed in a 1-cm path-length cuvet thermostated at 23 or 5°. In all cases, an excitation wavelength of 295 nm was used in conjunction with a 310-nm cut off filter. Excitation and emission slit widths varied from about 1.3 to 2.0 mm. No photodecomposition of the protein was detected under these conditions. All fluorescence spectra were uncorrected.

Light Scattering Measurements. Light scattering was measured with a Bausch and Lomb photogonio diffusometer thermostated at 10 or 23°. The incident wavelength was 546 nm. Scattering cells and glassware were cleaned with chromic acid and extensively rinsed with dust-free water. Solutions were additionally filtered with a 0.22-μ Millipore filter before use.

Molecular weight ratios were determined by use of eq 1.

$$\frac{M_i}{M_0} = \frac{(n_0)^2(dn/dc_0)^2(c_0)(I_{i,90})}{(n_i)^2(dn/dc_i)^2(c_i)(I_{0,90})} \quad (1)$$

In this equation, M is the molecular weight, n is the refractive index, dn/dc is the refractive index increment, c is the protein concentration, and I_{90} is the light scattering increment at 90° (cf. Timasheff and Townend, 1970). The subscripts 0 and i refer to some specified reference state and test state, respectively. In the light scattering experiments the reference state was a 0.15 mg/ml solution of phosphofructokinase in 0.1 *M* potassium phosphate (pH 8.0), 1 m*M* EDTA, and 10 m*M* dithiothreitol. Under these conditions the enzyme is known to be tetrameric with a molecular weight of 320,000 (Pavelich and Hammes, 1973). The test state is identical with the above except for the addition of varying concentrations of Gdn · HCl. The use of molecular weight ratios eliminates the need for calibration of the instrument, and the ratios can be determined with reasonable accuracy even at low protein concentrations. Equation 1 is derived assuming that the second and higher virial coefficient terms are small. At the low concentrations employed ($\leq$0.15 mg/ml), this approximation is quite reasonable. Refractive index increment values were taken from the data of Noelken and Timasheff (1967), assuming that the refractive index increments of all globular proteins exhibit similar values under similar conditions (cf. Huglin, 1972). While the data used were obtained at an incident wavelength of 436 nm and a temperature of 25°, reasonable estimates of the change in these parameters at 546 nm and lower temperatures result in virtually no change in the ratio $(dn/dc_0)^2/(dn/dc_i)^2$ (cf. Huglin, 1972). Refractive index values were measured with an Abbé type refractometer. A calibration curve for the scattering of buffer and buffer plus Gdn · HCl solutions was constructed and subsequently used to determine the scattering increment of phosphofructokinase.

Results

Fluorescence. The fractional change in wavelength at which the fluorescence emission spectrum of phosphofructokinase is maximal at 23 and 5° is plotted against the concentration of Gdn · HCl in Figure 1. The values of the wavelength maximum in the absence of Gdn · HCl, F_0, and at Gdn · HCl concentrations greater than 4 *M*, F_∞, are 331.8–332.0 nm and 348.7–348.9 nm, respectively. Two well-separated transitions are observed. The first transition is quite sharp and occurs between about 0.25 and 0.50 *M* Gdn · HCl. A plateau region exists from about 0.5 to 1.0 *M* Gdn · HCl, and is followed by a second transition which is essentially complete at 3.5 *M* Gdn · HCl. The protein con-

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

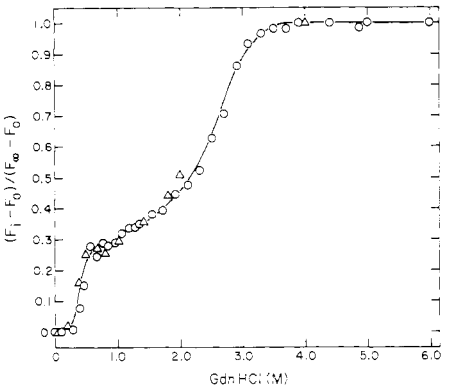

FIGURE 1: A plot of the fractional change in the wavelength of maximum fluorescence emission of phosphofructokinase, $(F_i - F_0)/(F_\infty - F_0)$, vs. the molarity of Gdn · HCl. F_i is the wavelength for a particular sample, F_0 is the wavelength in the absence of Gdn · HCl, and F_∞ is the wavelength at Gdn · HCl concentrations greater than 4 *M*. The protein concentration was 0.15 mg/ml in 0.1 *M* potassium phosphate (pH 8.0), 1 m*M* EDTA, and 10 m*M* dithiothreitol. The excitation wavelength was 295 nm and the emission and excitation slits were about 1.5 mm. Data were obtained at 23° (○) and 5° (△).

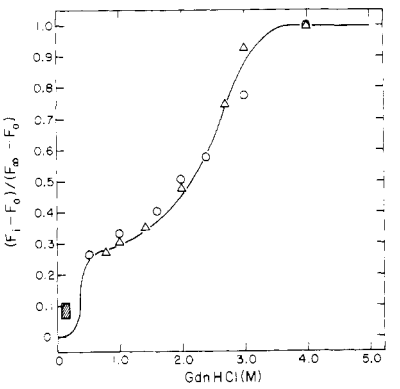

FIGURE 2: The reversibility of the Gdn · HCl induced fluorescence transitions of phosphofructokinase. The ordinate and abscissa are the same as in Figure 1. The solid line is a smooth curve drawn through the data points of Figure 1. The enzyme was incubated in 4.0 *M* Gdn · HCl, 0.1 *M* potassium phosphate (pH 8.0), 1 m*M* EDTA, and 10 m*M* dithiothreitol for 1–3 hr followed by rapid dilution into 0.1 *M* potassium phosphate (pH 8.0), 1 m*M* EDTA, and 10 m*M* dithiothreitol to obtain the final concentration of Gdn · HCl given on the abscissa. Fluorescence spectra were recorded within 10 min of dilution at 23° (○) or 5° (△). The initial enzyme concentration was either 0.39 mg/ml of 0.5 mg/ml. The rectangular shaded area represents the results of numerous measurements of solutions diluted to very low Gdn · HCl concentrations (see text for details).

centration, 0.15 mg/ml, was chosen to ensure that the starting material was the tetrameric enzyme (Pavelich and Hammes, 1973).

In the plateau region, aggregation is observed to occur when the experiment is carried out at 23° either by the presence of visual turbidity or by the presence of a large light scattering peak centered at about 295 nm in the fluorescence spectrum. This precipitate could be dissolved in higher concentrations of Gdn · HCl, suggesting that the formation of covalent bonds is not involved in the aggregation process. When the experiment is carried out at 5°, no evidence of aggregation is found. The changes in the wavelength of maximum fluorescence emission with Gdn · HCl are essentially identical at both 23 and 5° (Figure 1).

The reversibility of the fluorescence transitions is shown in Figure 2. In these experiments, the enzyme was incubated in 4.0 *M* Gdn · HCl, 0.1 m*M* potassium phosphate (pH 8.0), 1 m*M* EDTA, and 10 m*M* dithiothreitol for 1–3 hr followed by rapid dilution with the same buffer (not containing Gdn · HCl) to the final concentration of Gdn · HCl indicated on the abscissa. The fluorescence spectrum of the protein was then measured at 23 or 5° within 10 min of dilution. The protein concentration in the incubation mixtures was either 0.39 or 0.50 mg/ml. The second transition appears to be fully reversible at both 23 and 5°. As with the denaturation, a final concentration of 0.5–1.0 *M* Gdn · HCl resulted in turbidity at 23° but no turbidity or light scattering peak was observed when the dilution was carried out at 5°. The initial transition is only partially reversible following incubation in 0.8, 1.0, 2.0, or 4.0 *M* Gdn · HCl. The rectangular shaded area in Figure 2 represents the results of numerous attempts to regain the starting material by dilution from the various Gdn · HCl concentrations at 5°. Dilution to low Gdn · HCl concentrations invariably results in the appearance of a light scattering peak at 295 nm in the fluorescence emission spectrum, suggesting the presence of highly aggregated species.

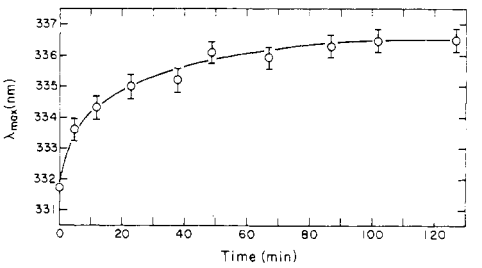

FIGURE 3: A plot of the change in the wavelength of the fluorescence emission maximum of phosphofructokinase in 0.8 *M* Gdn · HCl at 5° vs. time. Concentrated Gdn · HCl was added to phosphofructokinase at a concentration of 0.17 mg/ml in 0.1 *M* potassium phosphate (pH 8.0), 1 m*M* EDTA, and 10 m*M* dithiothreitol to give final concentrations of 0.8 *M* Gdn · HCl and 0.14 mg/ml of enzyme. Each data point represents the average of two spectra. Approximately 90 sec was required for recording a single spectrum.

The time course of the initial fluorescence transition following addition of Gdn · HCl, to a final concentration of 0.8 *M*, to 0.15 mg/ml of phosphofructokinase in 0.1 *M* potassium phosphate (pH 8.0), 1 m*M* EDTA, and 10 m*M* dithiothreitol at 5° is shown in Figure 3 as a plot of the wavelength of maximum fluorescence emission vs. time. The reaction is relatively slow, requiring nearly 2 hr to reach completion. A very similar result is obtained when the final concentration of Gdn · HCl is 0.6 *M*. In 2.0, 3.0, or 4.0 *M* Gdn · HCl, equilibrium as judged by fluorescence is complete within a few minutes after addition of denaturant.

Activity. The percent activity of phosphofructokinase (0.15 mg/ml, 0.1 *M* potassium phosphate, pH 8.0, 1 m*M* EDTA, and 10 m*M* dithiothreitol) vs. Gdn · HCl concentration is shown in Figure 4. Solutions were incubated for 2–3 hr at 5° before being assayed. The resulting curve corresponds closely to the first fluorescence transition (Figure 1). However, if concentrated Gdn · HCl is added to the enzyme at 5° to give a final concentration of 0.8 *M* Gdn · HCl, complete loss of activity occurs within 2.5 min. This is in marked contrast to the fluorescence transition which, under identical conditions, requires nearly 2 hr to reach completion (Figure 3), and suggests the occurrence of at least two distinct processes.

Light Scattering. The ratio of the molecular weight (M_i) of phosphofructokinase in Gdn · HCl (0.1 *M* potassium phosphate, pH 8.0, 1 m*M* EDTA, 10 m*M* dithiothreitol) to the molecular weight (M_0) of the enzyme at 0.15 mg/ml

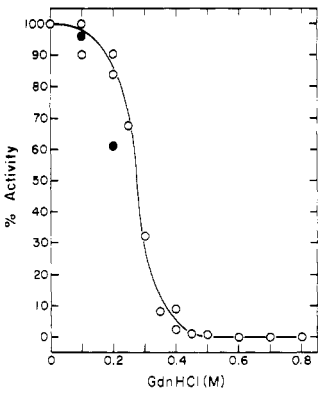

FIGURE 4: A plot of the percent activity of phosphofructokinase vs. the concentration of Gdn · HCl. Phosphofructokinase was added from a stock solution (9–14 mg/ml) to Gdn · HCl in 0.1 *M* potassium phosphate (pH 8.0), 1 m*M* EDTA, and 2–10 m*M* dithiothreitol and incubated for 2–3 hr at 5°. The final concentration of enzyme was 0.15 mg/ml. Assays were performed at both 5 and 23° with no appreciable difference in the results. The filled circles represent the activity after 24-hr incubation at 5°.

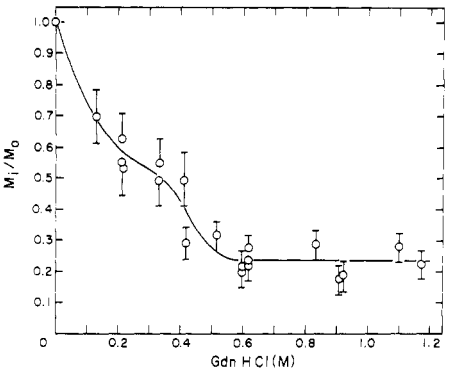

FIGURE 5: A plot of the ratio of the molecular weight of phosphofructokinase in Gdn · HCl (M_i) to the molecular weight of the phosphofructokinase tetramer (M_0) vs. the molarity of Gdn · HCl. The light scattering intensity at 90° of a solution of phosphofructokinase (0.14–0.16 mg/ml) in 0.1 *M* potassium phosphate (pH 8.0), 1 m*M* EDTA, and 10 m*M* dithiothreitol was measured at a temperature of 10°. Concentrated Gdn · HCl was then added to yield the molarity indicated on the abscissa and the resulting scattering intensity was determined. The scattering due to the solution in the absence of enzyme was determined from a calibration curve and subtracted from the meter reading to give the scattering increment due to protein. The molecular weight ratio was calculated according to eq 1.

(0.1 *M* potassium phosphate, pH 8.0, 1 m*M* EDTA, and 10 m*M* dithiothreitol) is shown in Figure 5. Under the reference state conditions (M_0) the enzyme is known to exist as a tetrameric species with a molecular weight of approximately 320,000 (Pavelich and Hammes, 1973). Since the subunits of phosphofructokinase are identical (Pavelich and Hammes, 1973; Coffee *et al.*, 1973), the molecular weight ratio is a direct measure of the extent of dissociation of the tetramer. At concentrations of Gdn · HCl greater than about 0.5 *M*, the enzyme is fully dissociated to monomeric subunits. (The average molecular weight ratio between 0.6 and 1.2 *M* Gdn · HCl is 0.24.) At concentrations of Gdn · HCl less than 0.5 *M*, a complex equilibrium among the tetramer, dimer, and monomer probably exists. (The trimer is unstable relative to these three species (Lad and Hammes, 1974).) Comparison of the data in Figures 4 and 5 indicates the existence of a species which is smaller than the tetramer yet has some residual enzymatic activity. Since the results of this and previous work indicate that the monomer has no activity while earlier results (Pavelich and Hammes, 1973; Lad et al., 1973) demonstrate that the dimer may have 10–20% activity at pH 8.0, appreciable concentrations of dimeric intermediates must be present during the dissociation process.

The molecular weight ratio at several Gdn · HCl concentrations was also determined under conditions of constant chemical potential (Timasheff and Townend, 1970). That is, the protein was dialyzed against the Gdn · HCl solution and the light scattering intensity of the resulting protein solution was compared with that of the dialysate. The results were identical with those shown in Figure 5.

The change in the aggregation state of phosphofructokinase on addition of Gdn · HCl to the protein (0.15 mg/ml) to yield a final Gdn · HCl concentration of 0.8 *M* is shown in Figure 6, in which the molecular weight ratio (M_i/M_0) is plotted vs. time. The experiment was carried out at 23 and 10°. In both cases, dissociation to monomer is complete within the time required to obtain the first measurement (about 1 min). This is in good agreement with the observation that a complete loss of activity under the same conditions occurs within 2.5 min. Thus the active tetramer undergoes at least two consecutive processes on addition of Gdn · HCl to a final concentration of 0.8 *M:* rapid dissociation to inactive, though presumably highly folded, monomers followed by a much slower conformational change which alters the fluorescence emission spectrum of the enzyme.

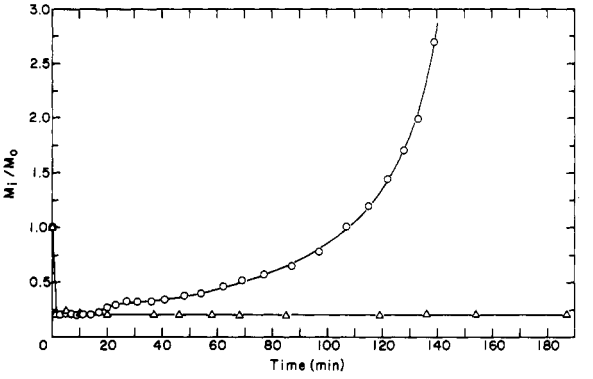

FIGURE 6: A plot of the molecular weight ratio of phosphofructokinase in 0.8 *M* Gdn · HCl, 0.1 *M* potassium phosphate (pH 8.0), 1 m*M* EDTA, and 10 m*M* dithiothreitol vs. time. Molecular weight ratios were determined as described in the Experimental Section and in the legend for Figure 5. Data were obtained at 23° (○) and 10° (△).

When the experiment is carried out at 23°, reaggregation begins to occur after a lag period, causing an apparent increase in the ratio M_i/M_0 with time (Figure 6). Ultimately a precipitate is produced. The lag period suggests that the conformationally altered monomer is the aggregating species. On the other hand, when the experiment is carried out at 10° the molecular weight ratio remains constant at a value consistent with dissociation to the monomer, and thus no reaggregation occurs at low temperatures.

Reactivation. The reactivation of phosphofructokinase denatured in 0.8 *M* Gdn · HCl also was studied. Aliquots of the denaturation mixture were withdrawn after a specified incubation time (T_D) and diluted 20:1 in a renaturation mixture consisting of 0.1 *M* potassium phosphate (pH 8.0), 1 m*M* EDTA, 10 m*M* dithiothreitol, and 5 m*M* ATP. The resulting activity was determined at specified time intervals (T_R) after dilution, and the results are presented in Figure 7 as plots of percent activity vs. T_R for a series of values of

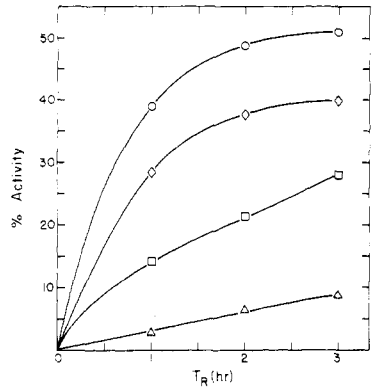

FIGURE 7: A plot of the percent reactivation of phosphofructokinase after incubation in 0.8 *M* Gdn · HCl vs. time. Concentrated Gdn · HCl was added to a solution of phosphofructokinase (0.17 mg/ml) at 5° in 0.1 *M* potassium phosphate (pH 8.0), 1 m*M* EDTA, and 10 m*M* dithiothreitol to give a final concentration of 0.8 *M* Gdn · HCl and 0.135 mg/ml of enzyme. After a specified time in the denaturant (T_D), an aliquot was withdrawn and diluted 20:1 in a reactivation solution consisting of 0.1 *M* potassium phosphate (pH 8.0), 1 m*M* EDTA, 10 m*M* dithiothreitol, and 5 m*M* ATP, resulting in final concentrations of 0.04 *M* Gdn · HCl and approximately 0.007 mg/ml of enzyme. T_D was 10 min (○), 20 min (◇), 40 min (□), and 80 min (△). Immediately after dilution at 5°, the reactivation mixtures were placed in a water bath thermostated at 23°. Assays were performed at 23° at specified intervals after dilution (T_R). The percent activity was determined with respect to a solution of phosphofructokinase (0.007 mg/ml) in 0.1 *M* potassium phosphate (pH 8.0), 1 m*M* EDTA, 10 m*M* dithiothreitol, and 5 m*M* ATP.

T_D ranging from 10 to 80 min. A clear correlation is observed between the extent of reactivation and the time of withdrawal suggesting that the monomer initially formed will readily undergo reassembly on removal of the denaturant, while the conformationally altered monomer cannot be converted to active enzyme.

If the enzyme is allowed to remain in 0.8 *M* Gdn · HCl for 2 hr (the time required for completion of the fluorescence transition) or longer, no reactivation is obtained. Numerous attempts to reactivate phosphofructokinase under varying conditions after prolonged incubation (≥2 hr) in 0.8 *M* Gdn · HCl, or after short exposure to 2.0 or 4.0 *M* Gdn · HCl, have proved unsuccessful. The reactivation conditions employed included removal of denaturant by both dilution and dialysis; use of a wide range of protein concentration (0.06–0.003 mg/ml); 0.1 *M* and 0.01 *M* potassium phosphate and 0.1 *M* Tris-Cl at both pH 8.0 and pH 7.0; the presence of a variety of known effectors, such as ATP, MgATP, fructose 6-phosphate, and citrate; the addition of various amino acids and KF (which is used to stabilize the enzyme during its preparation); and incubating the reactivation mixture for varying periods of time at 5°.

Discussion

The ability of single chain globular proteins to spontaneously assume their native structure after extensive denaturation is well established. The renaturation of multi-subunit proteins represents an extension of this process in that reassembly of fully folded or partially folded protomers must be considered. Several studies have shown that after extensive denaturation in high concentrations of Gdn · HCl or urea, oligomeric proteins may regain substantial enzymatic activity (Epstein et al., 1964; Deal, 1969; Teipel and Hill, 1971; Teipel and Koshland, 1971a,b; Teipel, 1972; Shifrin and Parrot, 1974; Bornmann et al., 1974; London et al., 1974). The results reported thus far suggest that the renaturation of oligomeric proteins may exhibit interesting features not normally associated with the renaturation of single chain globular proteins.

The renaturation of oligomeric proteins must involve several stages including refolding, reassembly, and possibly reactivation. Thus the renaturation process can be best studied if the renaturation can be experimentally divided into its component phases. The detailed study of the corresponding denaturation process can provide some insight into the conditions under which such a separation might be achieved. The fluorescence data show that the denaturation of phosphofructokinase can clearly be separated into two phases: an initial transition at low Gdn · HCl concentrations in which at least some tryptophan residues acquire a less hydrophobic environment, followed by a more general unfolding in which the tryptophan residues exhibit the fluorescence emission properties of free tryptophan (cf. Chen et al., 1969). The second transition can be interpreted as the unfolding of some reasonably compact globular species to a random coil. While the existence of a true random coil has not been proven here, the assumption of a random coil state is reasonable in light of previous results utilizing Gdn · HCl as the denaturing agent (Tanford, 1968).

The results of the light scattering experiments demonstrate that the intermediate species produced at Gdn · HCl concentrations between 0.5 and 1.0 *M* is the phosphofructokinase monomer (molecular weight 80,000). Furthermore, comparison of the time required for dissociation (Figure 6) with the time required for completion of the fluorescence transition (Figure 3), both in 0.8 *M* Gdn · HCl, indicates that formation of the intermediate monomer species occurs in at least two consecutive steps: rapid dissociation of the tetramer followed by a slow conformational change. The regain of activity following denaturation in 0.8 *M* Gdn · HCl is strongly dependent on incubation time (Figure 7), suggesting that the dissociation itself is readily reversible but that the slow conformational change is not reversible, at least with respect to simple dilution of the denaturant. The lack of complete reversibility of the initial fluorescence transition is also in accord with this point of view.

The simplest mechanism consistent with the experimental observations for the denaturation of phosphofructokinase by Gdn · HCl is summarized in eq 2. In this equation P

$$P_4 \rightleftharpoons 2P_2 \rightleftharpoons 4P \longrightarrow 4P' \rightleftharpoons \text{random coil} \quad (2)$$

represents the protomer species (molecular weight 80,000). The above mechanism assumes that the transition from the conformationally altered protomer, P′, to the random coil is reversible, and that P′ represents a true, identifiable intermediate rather than a collection of many structurally different, partially folded species. This is very likely an oversimplification of the actual mechanism. The fact that the fluorescence emission spectrum of the species designated P′ is independent of how this state is achieved (i.e., starting from native tetramer, or from random coils in 4.0 *M* Gdn · HCl) provides support for the assumption of reversibility. While it would be desirable to characterize the secondary structure of P′, attempts to use circular dichroism for this purpose have proved unreliable due to the low concentrations of enzyme which must be employed.

The lack of reactivation of fully unfolded phosphofructokinase can be attributed to a failure to reverse the process P → P′. Therefore, in light of previous results for multi-subunit proteins (Bornmann et al., 1974; Deal, 1969), it seems quite possible that some small molecule, as yet unidentified,

exists which can induce the transition P′ → P and thus prevent nonspecific aggregation of P′ on removal of the denaturant. In any case, it appears that the amino acid sequence alone of the enzyme as isolated does not contain sufficient information to produce fully active phosphofructokinase from random coil polypeptide chains in Gdn · HCl.

Although ATP was used in the reassembly experiments (Figure 7), it does not appear to have the critical effect on reactivation reported for the pH induced denaturation–renaturation process (Alpers et al., 1971). Preliminary results indicate that ATP enhances the rate of reassembly of P protomers after dissociation in 0.8 *M* Gdn · HCl, but it is not clear that it has a significant effect on the ultimate extent of reactivation. The denaturation mechanism presented here bears some similarity to the mechanism proposed to account for the pH induced cold lability of rabbit muscle phosphofructokinase (Bock and Frieden, 1974).

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