Subunit Dissociation in the Allosteric Regulation of Glycerol Kinase from *Escherichia coli*. 2. Physical Evidence†

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ABSTRACT: The dependence of the molecular weight of glycerol kinase on enzyme concentration and on binding of fructose 1,6-bisphosphate has been examined by velocity sedimentation, gel filtration, and polyacrylamide gel electrophoresis. The sedimentation coefficient and Stokes radius decrease as a consequence of dilution in a manner consistent with dissociation into half-molecules, with limiting values suggesting molecular weights of about 218 000 and 136 000 for the associated and dissociated species, respectively. Fructose 1,6-bisphosphate (5 mM) prevents the decrease in sedimentation coefficient brought about by dilution, suggesting a decrease in the apparent subunit dissociation constant of at least four orders of magnitude. Electrophoretic mobility in polyacrylamide gels increases as a consequence of dilution in the absence, but not in the presence, of fructose 1,6-bisphosphate. Ferguson plots indicate that glycerol kinase has the same molecular weight in the presence of fructose 1,6-bisphosphate as the covalently cross-linked tetramer and is substantially smaller in the absence of fructose 1,6-bisphosphate. These results are consistent with the model of glycerol kinase proposed in the preceding paper of this issue [de Riel, J. K., and Paulus, H. (1978), *Biochemistry 17*] relating subunit dissociation and ligand binding.

In the preceding paper of this issue (de Riel and Paulus, 1978a), we presented kinetic evidence that glycerol kinase from *E. coli* can undergo reversible subunit dissociation accompanied by release from allosteric inhibition by fructose 1,6-bisphosphate (FBP).[1] We proposed a model to account for the observed kinetic properties of the enzyme, incorporating the assumption of tetrameric and dimeric structures for the associated and dissociated species, respectively, based on the earlier determination of a tetrameric structure for glycerol kinase at high enzyme concentrations (Thorner and Paulus, 1971). The model ascribes the allosteric desensitization at low enzyme concentrations to the inability of the dimer to bind FBP.

This model leads to a number of predictions concerning the effects of enzyme concentration and of FBP on the molecular weight of glycerol kinase. We expect the enzyme to be a tetramer (M_T = 220 000) at high enzyme concentrations and a dimer (M_T = 110 000) at low enzyme concentrations. At intermediate enzyme concentrations, the molecular weight is expected to vary between these two limits in a manner laid down by the equilibrium expression for an $A_2 \rightleftharpoons 2A$ dissociation (de Riel and Paulus, 1978a). If FBP binds only to the tetramer, its presence should cause a decrease in the observed subunit dissociation constant, thereby increasing the molecular weight at intermediate and low enzyme concentrations toward a limiting value of 220 000.

We have tested these predictions using velocity sedimentation in sucrose gradients (Martin and Ames, 1961) and gel filtration (Andrews, 1965). Although these techniques are expected to perturb the subunit dissociation equilibrium, they have the advantage of sensitivity well below the μg/mL range, where dissociation was found to occur in the kinetics studies (de Riel and Paulus, 1978a). The parameters measured by these methods, viz., sedimentation coefficient and Stokes radius, bear no necessary relationship to molecular weight; however, by combining them, a reliable estimate of molecular weight can be made (Siegel and Monty, 1966). We have also examined the effect of FBP on the electrophoretic behavior of glycerol kinase, estimating molecular size by comparing mobility at various polyacrylamide concentrations (Ferguson, 1964; Hedrick and Smith, 1968). We have been able to confirm that a dissociation of glycerol kinase occurs, that it involves dimeric and tetrameric species, and that FBP promotes tetramer formation by binding at least ten times more strongly to the tetramer than to the dimer.

Materials and Methods

Glycerol kinase from strain 72 of *E. coli* K12 (obtained from Dr. E. C. C. Lin) was purified as described by Thorner and Paulus (1971). Lactate dehydrogenase (rabbit muscle), malate dehydrogenase (pig heart mitochondria), catalase (beef liver), NADH, and sodium pyruvate were purchased from Boehringer; β-galactosidase (*E. coli*) was from Worthington; FBP (tricyclohexylammonium salt), ATP, phosphoenolpyruvate, oxaloacetate, and *o*-nitrophenyl β-galactoside were from Calbiochem; and sucrose (ultraPure) was from Schwarz-Mann. Dimethyl suberimidate was prepared by the procedure of Davis and Stark (1971).

Enzyme Assays. Glycerol kinase was assayed by the radiometric procedure described in the preceding paper of this issue (de Riel and Paulus, 1978a).

All marker enzymes were assayed photometrically on a

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[1] Abbreviations used: FBP, fructose 1,6-bisphosphate; NADH, reduced nicotinamide adenine dinucleotide; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; TMG buffer, 50 mM triethanolamine hydrochloride, 5 mM $MgCl_2$, 2 mM glycerol (pH 7.0); p_D and p_T, parameters (e.g., sedimentation coefficient) applying to pure dimer and tetramer, respectively, of glycerol kinase.

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Gilford 222-A photometer equipped with Beckman DU optics and a Gilford 6040 recorder, and activities are expressed in international units (IU) unless otherwise stated. Lactate dehydrogenase activity was assayed by measuring the disappearance of NADH (0.3 mM) at 340 nm in the presence of pyruvate (0.75 mM) in 50 mM triethanolamine hydrochloride (pH 7.0). Activity was calculated using ϵ_{340} of 6.22×10^3 M^{-1} cm^{-1} for NADH (Kornberg, 1957). Malate dehydrogenase activity was assayed by measuring the disappearance of NADH (0.3 mM) in the presence of oxaloacetate (0.5 mM) in 0.1 M potassium phosphate (pH 7.5). Catalase activity was assayed by measuring the disappearance of hydrogen peroxide (0.07% solution in H_2O) at 240 nm. Activity was calculated using ϵ_{240} 43.6 M^{-1} cm^{-1} for H_2O_2 (Beers and Sizer, 1952). β-Galactosidase activity was assayed by measuring the appearance of *o*-nitrophenol at 405 nm in a solution containing 50 mM NaCl, 2 mM *o*-nitrophenyl galactoside, and 100 mM triethanolamine hydrochloride (pH 7.6). Activity was calculated using ϵ_{405} 3.1×10^3 M^{-1} cm^{-1} for *o*-nitrophenol (Wallenfels and Malhotra, 1960).

Zone Sedimentation. Sedimentation velocity was measured by centrifugation in sucrose gradients (Martin and Ames, 1961) in a Beckman L5-65 ultracentrifuge using an SW41 rotor. Linear 5–30% sucrose gradients (12 mL) were prepared in a solution composed of TMG buffer [50 mM triethanolamine hydrochloride, 5 mM $MgCl_2$, 2 mM glycerol, (pH 7.0)], 1 mM 2-mercaptoethanol, and FBP as indicated and were found to be approximately isokinetic in the SW 41 rotor (de Riel, 1976). Samples (0.2 mL) were loaded in the same buffer without sucrose and contained 0.01 μg to 10 mg of glycerol kinase, 5 μg of malate dehydrogenase, 10 μg of lactate dehydrogenase, and 12 μg of catalase. Following centrifugation at 39 000 rpm for 13 h at 25 °C, fractions (0.3 mL) were collected from the bottom of the tube by oil displacement. Samples of each fraction were removed for assay of lactate dehydrogenase (10 μL), malate dehydrogenase (10 μL), catalase (0.1 mL), and glycerol kinase (volume depending on enzyme concentration). In order to avoid interference by reducing sugars produced from sucrose during the radiometric assay procedure for glycerol kinase, 50 μL of 0.14% H_2O_2 was added just before the molybdate reagent. Sedimentation coefficients for glycerol kinase were calculated by comparing its sedimentation velocity with that of the reference proteins. The following sedimentation coefficients were used: catalase, $s_{20,w}$ = 11.3 S (Martin and Ames, 1961); lactate dehydrogenase, $s_{20,w}$ = 7.6 S (Jaenicke and Knopf, 1968); and malate dehydrogenase, $s_{20,w}$ = 4.3 S (Thorne, 1962).

Gel Filtration. Stokes radii were estimated by gel filtration through columns of Bio-Gel A-0.5m (Bio-Rad). After repeated decantation with TMG buffer to remove fine particles, the gel was packed into Plexiglas columns (0.9 × 60 cm) and washed with 2 column volumes of TMG buffer containing 0.1 mg/mL gelatin to prevent losses by adsorption of small amounts of glycerol kinase. Samples (0.5 mL) were layered onto the gel surface in TMG buffer supplemented with 5% sucrose; they contained 0.022 to 220 μg of glycerol kinase, 12 μg of β-galactosidase, 30 μg of catalase, and 25 μg of lactate dehydrogenase. The columns were developed at 25 °C and a flow rate of about 10 mL/h, and fractions (0.5 mL) were collected and assayed for glycerol kinase and the marker enzyme activities. Void volumes (v_0; about 19 mL) and included volumes (v_i; about 40 mL) were measured with Blue dextran (Pharmacia) and Bromphenol blue, respectively. The partition coefficient (K_d) was calculated from the elution volume (v_e) by the formula:

$$K_d = (v_e - v_0)/(v_i - v_0)$$

Stokes radii for glycerol kinase were estimated by comparing its partition coefficient with those of the reference proteins, using values of 69, 52, and 41 Å, respectively, for the Stokes radii of β-galactosidase, catalase, and lactate dehydrogenase (Andrews, 1965; Siegel and Monty, 1965).

Polyacrylamide Gel Electrophoresis. Electrophoresis in nondenaturing gels was performed essentially by the method of Clarke (1964). For electrophoresis at pH 7.6, gels and buffer contained 2 mM glycerol, 44 mM glycylglycine, and 5 mM Tris. For electrophoresis at pH 6.5, gels and buffer contained 2 mM glycerol, 44 mM 3-(*N*-morpholino)propanesulfonic acid, and 10 mM imidazole. For electrophoresis in FBP, 2 mM FBP was present in the gels and in the cathode buffer and was not significantly depleted during electrophoresis. Samples (25 μL) containing 1–30 μg of protein, 1 μg of Bromphenol blue, and 5% sucrose in electrophoresis buffer were layered onto the gels and subjected to electrophoresis at 4 °C. Electrophoresis was terminated by pipetting Freon 214 (tetrachlorotetrafluoropropane) onto the top of each gel when the dye marker was 0.5 cm from the bottom of the gel. The center of the dye band was marked with India ink before fixing and staining with Coomassie brilliant blue R-250 by the procedure of Dunker and Rueckert (1969). Electrophoresis in gels containing 0.1% sodium dodecyl sulfate was carried out essentially as described by Weber and Osborn (1969) in a buffer containing 0.2 M sodium phosphate and 0.2% sodium dodecyl sulfate at pH 7.2. Protein samples were diluted with 0.1 M sodium phosphate buffer (pH 7.2) containing 1% sodium dodecyl sulfate, 10% glycerol, and 10% 2-mercaptoethanol and heated at 65 °C for 15 min before layering onto the gels.

Cross-linking with Dimethyl Suberimidate. Glycerol kinase (1 mg/mL) in 0.2 M triethanolamine hydrochloride (pH 8.5) was treated with solid dimethyl suberimidate (0.4 or 10 mg/mL) at room temperature. The reagent was added slowly with stirring in several small portions; after 3 h the reaction products were dialyzed overnight against TMG buffer and centrifuged at 10 000*g* to remove any precipitate before being subjected to electrophoresis.

Protein Determination. Glycerol kinase concentration was measured photometrically using an $A_{280nm}^{0.1\%}$ of 1.4 (Thorner and Paulus, 1973).

Results

Effect of Enzyme Concentration on Sedimentation Velocity. Glycerol kinase was sedimented in sucrose gradients at six concentrations ranging over five orders of magnitude (0.5 μg/mL to 50 mg/mL) in the presence of constant amounts of catalase, lactate dehydrogenase, and malate dehydrogenase as reference proteins. The sedimentation velocity of glycerol kinase decreased as concentration decreased, whereas the sedimentation of the marker enzymes was unaffected by the amount of glycerol kinase in the gradient. The sedimentation coefficient of glycerol kinase was estimated from a standard curve based on the known $s_{20,w}$ of the reference proteins (not shown). The results for duplicate experiments are plotted as a function of glycerol kinase concentration in Figure 1. The curve drawn through the points (curve A) is a theoretical equilibrium curve for an $A_2 \rightleftharpoons 2A$ dissociation (de Riel and Paulus, 1978a), fitted to the data by least-squares analysis. The best fit was achieved with sedimentation coefficients for presumed dimer and tetramer of p_D = 6.6 S and p_T = 9.3 S, and with a dissociation constant K_D = 2000 μg/mL. The curve matches the data closely, indicating agreement of the data with the postulated stoichiometry of dissociation.

Effect of FBP on Sedimentation Velocity. To test the effect of FBP on the putative dissociation reaction, an analogous set

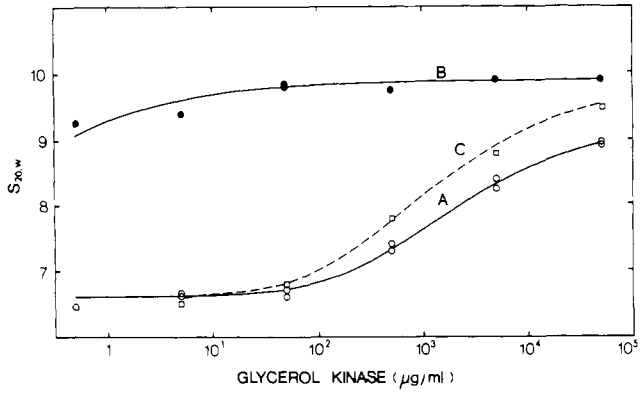

FIGURE 1: Effect of enzyme concentration on the sedimentation coefficient of glycerol kinase in the absence and presence of FBP. Sedimentation was done in 5-30% sucrose gradients as described under Materials and Methods: (○) centrifugation at 39 000 rpm for 13 h in the absence of FBP; (●) centrifugation at 39 000 rpm for 13 h in the presence of 5 mM FBP; (□) centrifugation at 20 000 rpm for 48 h in the absence of FBP. The curves were generated and plotted by computer from the equilibrium expression for an $A_2 \rightleftharpoons 2A$ dissociation (de Riel and Paulus, 1978a), using the following parameters: (A) p_D = 6.6 S, p_T = 9.3 S, and K_D = 2000 μg/mL; (B), p_D = 6.6 S, p_T = 9.9 S, and K_D = 0.086 μg/mL; (C), p_D = 6.6 S, p_T = 9.9 S, and K_D = 1160 μg/mL.

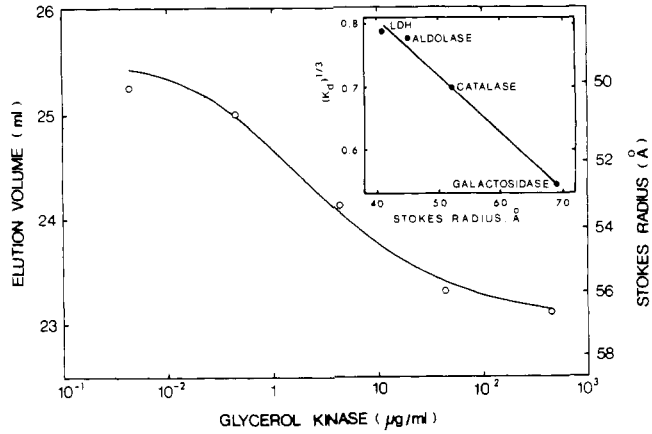

FIGURE 2: Effect of enzyme concentration on the Stokes radius of glycerol kinase. The partition coefficient (K_d) at different glycerol kinase concentrations was calculated as described under Materials and Methods, and the Stokes radii were estimated by comparison with the values of the reference proteins as shown in the insert. The curve was generated and plotted by computer from the equilibrium expression for an $A_2 \rightleftharpoons 2A$ dissociation (de Riel and Paulus, 1978a), using p_D = 49.4 Å, p_T = 57.0 Å, and K_D = 2.7 μg/mL.

of experiments was done in sucrose gradients containing 5 mM FBP. The same reference proteins were included, and FBP had no effect on their sedimentation. Values of $s_{20,w}$ for glycerol kinase determined from these experiments are plotted in Figure 1 (curve B). The $s_{20,w}$ of glycerol kinase at high concentrations in the presence of 5 mM FBP was 9.9 S. This rapidly sedimenting form persisted as enzyme concentration decreased until the two lowest concentrations, where a slight decrease in the sedimentation coefficient was seen and the sedimenting zones of glycerol kinase exhibited a broad trailing edge (not shown), consistent with a slowly reversible dissociation. For purposes of comparison, a best-fit dissociation constant in the presence of 5 mM FBP was calculated, based on a p_D and p_T of 6.6 and 9.9 S, respectively. This value of K_D' (0.086 μg/mL) suggests that 5 mM FBP promoted a shift of over four orders of magnitude in the dissociation equilibrium under the conditions of these experiments.

Effect of Rotor Speed on the Sedimentation Coefficient. In order to examine the effect of pressure on the dissociation equilibrium of glycerol kinase, we also examined the concentration dependence of the sedimentation coefficient of glycerol kinase in the absence of FBP upon centrifugation at 20 000 rpm for 48 h instead of at 39 000 rpm for 13 h. The dissociation curve fitted to these data (Figure 1, curve C) showed that the lower rotor speed caused a twofold decrease in the observed dissociation constant (K_D = 1160 μg/mL), indicating that the dissociation reaction involves an increase in molar volume of about 0.1 L or 0.1% (Kegeles et al., 1967). It is of interest that at the higher rotor speed p_T was 9.3 S in the absence of FBP, compared to 9.9 S at the lower speed or at high speed in the presence of 5 mM FBP.

Effect of Enzyme Concentration on the Stokes Radius. Glycerol kinase was subjected to gel filtration on agarose beads at five enzyme concentrations ranging over four orders of magnitude (0.044-440 μg/mL), together with β-galactosidase, catalase, and lactate dehydrogenase as reference proteins. Glycerol kinase eluted ahead of catalase at high enzyme concentrations and slightly after catalase at very low enzyme concentrations with a net change in elution volume of 2.0 mL (0.05 column volume). By comparing the partition coefficients of glycerol kinase and the reference proteins with known Stokes radii (Figure 2, insert), the Stokes radius of glycerol kinase could be calculated as a function of concentration and is shown in Figure 2. The data points could be fitted to a theoretical equilibrium curve for an $A_2 \rightleftharpoons 2A$ dissociation by least-squares analysis. The best fit employed the parameters p_D = 49.4 Å, p_T = 57.0 Å, and K_D = 2.7 μg/mL and matches the data fairly well (Figure 2).

Calculation of Molecular Weights. Assuming that the limiting parameters, p_T and p_D, measured at high and low enzyme concentration in velocity sedimentation and in gel filtration experiments refer to the same molecular species, the molecular weights of these species can be calculated as proposed by Siegel and Monty (1965) using the relationship

$$M_r = \frac{6\pi\eta N a s_{20,w}}{1 - \bar{v}\rho}$$

where η = viscosity of water at 20 °C, N = Avogadro's number, $\bar{v}$ = partial specific volume of the protein, and ρ = density of water at 20 °C. Using the values for the partial specific volume of glycerol kinase obtained experimentally (0.724 mL/g) or calculated from its amino acid composition (0.732 mL/g) (Thorner and Paulus, 1971), the limiting parameters at high enzyme concentration ($s_{20,w}$ = 9.3 S and a = 57 Å) yield a molecular weight of 215 000-220 000, whereas the limiting parameters at low enzyme concentration ($s_{20,w}$ = 6.6 S and a = 49.4 Å) give a molecular weight of 134 000-138 000. The higher molecular weight agrees well with the molecular weight of the tetramer, as determined by Thorner and Paulus (1971) from sodium dodecyl sulfate electrophoresis (M_r = 4 × 57 000) or equilibrium sedimentation in the native (M_r = 210 000-217 000) or denatured state (M_r = 4 × 55 000). The lower molecular weight is 20-25% higher than the value expected for the glycerol kinase dimer (M_r = 110 000).

The frictional ratios (f/f_0) of the high- and low-molecular-weight forms of glycerol kinase were calculated from the relationship $f/f_0 = a(3\bar{v}M_r/4\pi N)^{-1/3}$ (Siegel and Monty, 1965) and were found to be 1.42 and 1.45, respectively, suggesting that both molecules are ellipsoidal.

Effect of FBP on Electrophoretic Behavior. Glycerol kinase (0.5 mg/mL) was subjected to electrophoresis at pH 7.6 under nondenaturing conditions in the absence and presence of 2 mM

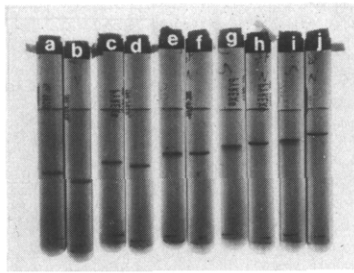

FIGURE 3: Electrophoresis of glycerol kinase at different polyacrylamide concentrations in the absence and presence of FBP. Glycerol kinase (12.5 μg in 25 μL) was subjected to electrophoresis in nondenaturing gels at pH 7.6 without (a, c, e, g, i) or with (b, d, f, h, j) 2 mM FBP, as described under Materials and Methods: (a, b) 4% polyacrylamide; (c, d) 5% polyacrylamide; (e, f) 6% polyacrylamide; (g, h) 7% polyacrylamide; (i, j) 8% polyacrylamide. The position of the Bromophenol blue marker dye has been indicated with India ink.

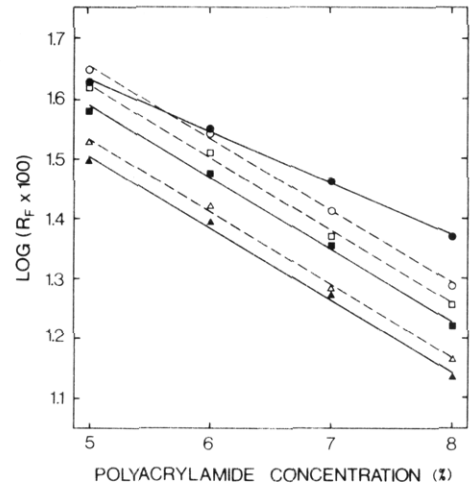

FIGURE 4: Ferguson plot of unmodified glycerol kinase and covalently cross-linked tetramer in the absence and presence of FBP. Unmodified glycerol kinase (circles) and glycerol kinase cross-linked with 0.4 (squares) or 10 mg/mL dimethyl suberimidate (triangles) as described under Materials and Methods were subjected to electrophoresis in nondenaturing polyacrylamide gels at pH 7.6 in the absence (closed symbols) or the presence (open symbols) of 2 mM FBP. The lines have been drawn by the method of least squares.

FBP in gels with an acrylamide content ranging from 4 to 8% (Figure 3). In the absence of FBP a band with a diffuse leading edge was observed, particularly at the higher gel concentrations, whereas in the presence of FBP the band appeared sharp and symmetrical. The effect of FBP on the electrophoretic mobility of the enzyme depended strikingly on acrylamide concentration: mobility was decreased in gels with more than 6% acrylamide and increased in gels of lower concentration. Thus, electrophoretic mobility of glycerol kinase was more sensitive to gel concentration in the presence of FBP than in its absence. The molecular weights of the predominant forms of glycerol kinase in the absence and presence of FBP were estimated by the use of Ferguson plots (Ferguson, 1964) with ovalbumin and bovine serum albumin monomer, dimer, and trimer as standards (not shown) and were found to be 124 000 and 172 000, respectively. Similar experiments carried out at pH 6.5 revealed an analogous behavior and yielded molecular weights in the absence and presence of FBP of 138 000 and 173 000, respectively.

The molecular weight thus determined for glycerol kinase in the presence of FBP is not in accord with the results from velocity sedimentation and gel filtration, which indicated a tetrameric species of molecular weight of about 220 000. This could mean either that the glycerol kinase tetramer was not stable under the conditions of electrophoresis even in the presence of 2 mM FBP or that the calibration of the Ferguson plots with proteins of known molecular weight was invalid on account of large differences in molecular shape between the reference proteins and glycerol kinase. In order to examine the second possibility, we compared the electrophoretic behavior of glycerol kinase in the presence of FBP with covalently stabilized glycerol kinase tetramer, prepared by cross-linking with dimethyl suberimidate as described by Thorner and Paulus (1971). Glycerol kinase was cross-linked at a relatively high concentration (1 mg/mL) with the bifunctional imidoester, and electrophoresis of the cross-linked enzyme in the presence of sodium dodecyl sulfate revealed that at least 90% of the enzyme was present as a covalently cross-linked tetrameric species. The slopes of the Ferguson plots for the cross-linked glycerol kinase tetramer were unaffected by the presence of FBP and were identical to the slope obtained with unmodified glycerol kinase (0.25 mg/mL) in the presence of FBP (Figure 4).

Discussion

The results of the physical studies on glycerol kinase reported here are generally consistent with the model proposed in the preceding paper of this issue (de Riel and Paulus, 1978a) and bear out its predictions concerning the existence of a dissociation, the molecular weights of the enzyme species involved, and the effect of FBP on the dissociation. The molecular weights obtained from an analysis of the velocity sedimentation and gel-filtration experiments validate the assumption that the associated and dissociated species are tetramer and dimer, respectively. The molecular weight of the associated species (M_r = 218 000) is in good accord with that expected for the glycerol kinase tetramer. The dissociated species had a molecular weight (136 000) which is about 25% too high for the dimer. The reason for this discrepancy is not clear; it may in part reflect the uncertainty in the determination of the elution volume from agarose gels of glycerol kinase at the lowest concentration used.

Theoretical dissociation curves appropriate for a tetramer $\rightleftharpoons$ dimer equilibrium fit the data quite well. This is particularly noteworthy in the velocity sedimentation experiments, where at least one asymptote was firmly defined by the data and did not need to be obtained by curve fitting. It is of general interest that dissociation curves that correspond well to the postulated stoichiometry could be obtained by zone fractionation techniques which are known to perturb the dissociation equilibrium.

Because zone fractionation procedures are expected to shift the equilibrium in the direction of dissociation, it is not surprising that considerable differences were observed in the dissociation constant (K_D), as measured by kinetic analysis (de Riel and Paulus, 1978a) and by the physical techniques reported here. The theory of gel filtration for dissociating systems developed by Ackers (1970) indicates that narrow-zone gel chromatography of the sort used in our experiments may yield an apparent dissociation constant which is orders of magnitude too large. A computer-simulated gel-filtration experiment for a hypothetical $A_4 \rightleftharpoons 4A$ system yielded a dissociation curve of the appropriate shape but with an "observed" K_D that was five orders of magnitude larger than the "real" value (Zimmerman and Ackers, 1971). The corresponding error for an $A_2 \rightleftharpoons 2A$ system would be about 50-fold, because in this case the calculation of K_D is much less sensitive to shifts in the enzyme concentration at which 50% dissociation is observed. The

precise shift expected in a given case is rather sensitive to such details as the flow rate of the column and is very difficult to predict (Ackers, 1970). No comparable analysis of the effects of zone sedimentation or electrophoresis on the apparent dissociation constant has been published; however, one would predict qualitatively that these zone fractionation methods should also cause an increase in the apparent dissociation constant. If we assume that the subunit dissociation constant obtained from the enzyme kinetic studies described in the preceding paper of this issue (de Riel and Paulus, 1978a) represents the true, unperturbed equilibrium constant (K_D = 3.15 μg/mL), then K_D derived from the gel-filtration experiments (2.7 μg/mL) is essentially unperturbed, whereas K_D deduced from velocity sedimentation (2000 μg/mL) is shifted upwards by nearly three orders of magnitude. This large discrepancy between the apparent dissociation constants observed in gel filtration and velocity sedimentation raises the possibility that different dissociation reactions occurred in the two types of experiment. Since both dissociation processes accorded with an $A_2 \rightleftharpoons 2A$ equilibrium expression, this would require the occurrence of a monomer $\rightleftharpoons$ dimer transition in one case and a dimer $\rightleftharpoons$ tetramer transition in the other. However, this is unlikely, since the larger species observed by both techniques appears to be the tetramer. In the case of velocity sedimentation, the sedimentation coefficient of the limiting species at high enzyme concentration was very close to that observed in the presence of FBP (9.3 and 9.9 S, respectively). Since the gel-electrophoresis experiments clearly demonstrate that FBP stabilizes the tetrameric form of glycerol kinase, the species with $s_{20,w}$ = 9.9 S must be a tetramer. In the case of gel filtration, the limiting species at high enzyme concentration had a Stokes radius of 57 Å, compared to a value of 52 Å for catalase. Since catalase has a molecular weight of 235 000, it is rather unlikely that the glycerol kinase species with a larger Stokes radius than catalase could be a dimer with less than half the molecular weight. It seems, therefore, that the same dissociation process is seen in velocity sedimentation and gel filtration, but that the two methods perturb the equilibrium to different extents. In fact, two aspects unique to the sedimentation experiments are known to have a perturbing influence on K_D, pressure and the presence of sucrose. A twofold reduction in rotor speed was found to decrease the apparent K_D by a factor of 2, showing that pressure causes a slight increase in the dissociation constant. Studies of the effect of 5% sucrose on inhibition of glycerol kinase by FBP (not shown) showed a decreased inhibition, which could be accounted for by a twofold increase in K_D. Since glycerol kinase migrated through sucrose concentrations ranging from 5 to 20% in the course of a sedimentation experiment, a significant increase in the observed K_D might be expected. Even though these factors can account for only a small part of the observed discrepancy, in combination with the equilibrium perturbations characteristic of transport processes discussed earlier, they might well account for the abnormally high values of K_D seen in the sedimentation experiments.

The sedimentation behavior of glycerol kinase was strikingly affected by FBP. In the presence of the inhibitor, the enzyme sedimented as a tetramer even at concentrations where dissociation would ordinarily have been complete. Curve fitting showed that 5 mM FBP reduced the apparent dissociation constant by more than a factor of 10^4. This provides strong support for our model and implies that the affinity of FBP for the dimer is at most one-tenth of that for tetramer, extending the limit of one-third estimated from kinetic data (de Riel and Paulus, 1978a). The electrophoretic results also indicate that FBP promotes the formation of tetramer, since glycerol kinase in the presence of FBP produced Ferguson plots with the same slope as the covalently cross-linked tetramer. However, the molecular weight calculated for this authentic tetramer by the use of reference proteins of known molecular weight was 20% lower than expected. The reason for the anomalously shallow Ferguson plot for glycerol kinase is not clear, but it may perhaps be related to the large frictional ratio of the enzyme (f/f_0 = 1.42), which implies an ellipsoidal structure with an axial ratio of 8–10. In any case, glycerol kinase represents an apparent exception to the generalization that slopes of Ferguson plots are a reliable measure of molecular weight, independent of molecular shape (Ferguson, 1964; Hedrick and Smith, 1968; Rodbard and Chrambach, 1971). In view of this anomaly, it may not be justified to assume that the form of glycerol kinase which predominates in the presence of FBP is of lower molecular weight because its Ferguson plot slope is less. On the other hand, the alternative is very unlikely. If we were to postulate that a conformational change rather than a dissociation is responsible for the more shallow Ferguson plot slope in the absence of FBP, then we would have to assume that glycerol kinase migrated as a tetramer both in the presence and absence of FBP. This assumption would intensify rather than resolve the anomaly in the Ferguson plot. Moreover, cross-linking of the enzyme, in the absence of FBP, converted it into a form that closely resembled the native enzyme in the presence of FBP, despite the lack of a structural resemblance between FBP and the cross-linker. The hypothesis of a dissociation provides a simple explanation for this, whereas very special assumptions would be required to explain it on the basis of a conformational change.

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Subunit Dissociation in the Allosteric Regulation of Glycerol Kinase from *Escherichia coli*. 3. Role in Desensitization†

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ABSTRACT: The mechanism of desensitization of glycerol kinase to allosteric inhibition by fructose 1,6-bisphosphate caused by salt, urea, and high pH has been examined in the light of the model proposed in an earlier paper [de Riel, J. K., and Paulus H. (1978), *Biochemistry 17*] relating subunit dissociation and ligand binding. KCl (0.4 M) causes a tenfold decrease in the affinity of tetrameric glycerol kinase for fructose 1,6-bisphosphate but has no significant effect on the dissociation process itself. Urea (2 M) causes a large increase in the equilibrium constant for the dissociation of the glycerol kinase tetramer to dimer but has no effect on the affinity of the tetramer for the allosteric inhibitor. High pH (9-10) has only a small effect on the subunit dissociation constant but greatly reduces the rates of subunit association and dissociation. Desensitization of glycerol kinase to allosteric inhibition can thus occur by three different mechanisms, two of which are directly related to the polysteric nature of the enzyme.

On the basis of their studies on mutants of *Escherichia coli* with lesions in fructose 1,6-biphosphate aldolase, Bock and Neidhardt (1966a,b) predicted that glycerol kinase should be inhibited by fructose 1,6-bisphosphate (FBP).[1] However, they were unable to demonstrate such inhibition in vitro using the conventional photometric assay for glycerol kinase which had to be conducted at pH 9.5. Subsequently, Zwaig and Lin (1966), by using an assay procedure that could be carried out at pH 7.5, showed that glycerol kinase was indeed inhibited by FBP at this physiological pH but that the enzyme was densitized to allosteric inhibition at pH 9.5. This result was confirmed by Thorner and Paulus (1973) who used a new radiometric assay for glycerol kinase in which the pH could be adjusted over a wide range, and they found that other agents such as salt and guanidium chloride also brought about a loss of sensitivity to inhibition by FBP.

In the first paper of this series (de Riel and Paulus, 1978a), we described another mode of desensitization of glycerol kinase to inhibition by FBP which was brought about by dilution of the enzyme. Kinetic studies suggested that desensitization was due to the dissociation of glycerol kinase tetramer to an enzymatically active dimeric form which is unable to bind FBP, and such a model was supported by direct physical evidence (de Riel and Paulus, 1978b). Since, under certain conditions, dilution of glycerol kinase led to a small increase in specific activity (de Riel and Paulus, 1978a) and treatment by high pH, guanidium chloride, and salt had a similar activating effect (Thorner and Paulus, 1973), it was tempting to speculate that all desensitizing treatments involved the same mechanism, viz., dissociation into glycerol kinase dimers.

In this paper, we examine the effects of salt, urea, and high pH on the subunit dissociation of glycerol kinase and on the inhibition by FBP. The results of these studies revealed not one but three distinct mechanisms of desensitization to allosteric inhibition. One of these was the "classical" mode of desensitization in which the affinity of the glycerol kinase tetramer for the inhibitor FBP was reduced. The other mechanisms involved two different kinds of perturbation of the tetramer-dimer dissociation process: an increase in the equilibrium constant for dissociation and a decrease in the rates of the association and dissociation reactions.

Materials and Methods

Urea (ultraPure) was obtained from Schwarz-Mann, and solutions were freshly prepared before use. All other reagents and experimental procedures have been described in an earlier paper (de Riel and Paulus, 1978a).

Glycerol kinase was assayed either by a radiometric procedure or spectrophotometrically (de Riel and Paulus, 1978a). Assays at neutral pH were carried out in TMG buffer [50 mM triethanolamine hydrochloride, 5 mM $MgCl_2$, 2 mM glycerol (pH 7.0)]; assays at alkaline pH were buffered with 50 mM ethanolamine hydrochloride.

The extent of subunit dissociation in the absence of FBP was measured by first equilibrating tne enzyme under desensitizing conditions in the absence of FBP and then diluting it into complete assay solution (in the absence of densitizing agent) with and without 2 mM FBP for immediate determination of

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[1] Abbreviations used: FBP, fructose 1,6-bisphosphate; TMG buffer, 50 mM triethanolamine hydrochloride, 5 mM $MgCl_2$, 2 mM glycerol (pH 7.0); p_D and p_T, parameters (e.g., percent inhibition) applying to pure dimer and tetramer, respectively, of glycerol kinase.

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