Subunit Dissociation in the Allosteric Regulation of Glycerol Kinase from *Escherichia coli*. 1. Kinetic Evidence†

Jon K. de Riel*,‡ and Henry Paulus§

ABSTRACT: Glycerol kinase from *Escherichia coli* is a tetrameric protein composed of identical subunits, which is subject to allosteric inhibition by fructose 1,6-bisphosphate. In order to explain the dependence of enzyme inhibition on glycerol kinase concentration, a model is proposed in which the tetrameric form of the enzyme is presumed to be able to dissociate into half molecules which are unable to bind fructose 1,6-bisphosphate. The dimeric and tetrameric forms of the enzyme are assumed to have approximately equal catalytic activities, but the tetramer can rapidly bind fructose 1,6-bisphosphate and undergo a transition to a totally inactive conformation. Kinetic evidence is presented in support of this model: (1) The inhibition of the enzyme following equilibration with 1 mM fructose 1,6-bisphosphate was found to depend on enzyme concentration, approaching zero at very low concentrations. The nature of the dependence is approximately that expected for a reaction of the type $2A \rightleftharpoons A_2$, with an apparent dissociation constant of 3.5×10^{-10} M. (2) The inhibition of enzyme equilibrated in the absence of fructose 1,6-bisphosphate was also found to be dependent on enzyme concentration with a dissociation constant of 2.8×10^{-8} M. Thus, 1 mM fructose 1,6-bisphosphate decreased the equilibrium constant nearly two orders of magnitude, indicative of selective binding to the more highly associated state of the enzyme. (3) Plots of inhibition as a function of fructose 1,6-bisphosphate concentration were nearly hyperbolic at high enzyme concentrations and became increasingly sigmoid at lower enzyme concentrations, with a concomitant increase in K_i and in the Hill coefficient for inhibition. (4) Upon addition of fructose 1,6-bisphosphate to enzyme not previously exposed to it, the acquisition of sensitivity to inhibition was sufficiently slow to be measured by conventional techniques. The rate of sensitization to inhibition depended on enzyme concentration, but not fructose 1,6-bisphosphate concentration, and followed a second-order time course, identifying as the rate-limiting step an association of enzyme species with no direct involvement of fructose 1,6-bisphosphate. The second-order rate constant was 5×10^7 M^{-1} min^{-1}; the independently measured first-order rate constant for dissociation to dimers was 1.2 min^{-1}. The possible physiological significance of the modulation of feedback inhibition of glycerol kinase by subunit dissociation is discussed.

Glycerol kinase (EC 2.7.1.30) catalyzes the first reaction in the bacterial catabolism of glycerol. Its regulation in bacteria has been extensively investigated (Lin, 1976). In *Escherichia coli*, glycerol kinase is subject to three independent modes of regulation: (a) induction in the presence of exogenous glycerol or glycerol phosphate, the actual inducer being glycerol phosphate, the product of the reaction catalyzed by the enzyme; (b) catabolite repression; and (c) feedback inhibition by the glycolytic intermediate, fructose 1,6-bisphosphate (FBP).[1] This stringent regulation is thought to be related to the exceptional cytotoxicity of metabolites which accumulate during overproduction of glycerol phosphate (Freedberg et al., 1971).

The enzyme from *E. coli* has been purified to homogeneity and shown to be a tetramer composed of identical 55 000-dalton subunits, each containing a single catalytic site and a single allosteric binding site (Thorner and Paulus, 1971). Thorner and Paulus (1973) also investigated the allosteric properties of the enzyme and reported a number of discrepancies between the inhibitory effects of FBP, measured in kinetic studies, and its binding to the enzyme, measured in physical studies. The K_i for FBP was substantially larger than its dissociation constant, and treatment at pH 10 desensitized the enzyme to allosteric inhibition but did not prevent binding of FBP. Moreover, the maximum inhibition obtainable with high FBP concentrations was 80%. These findings were rationalized (Thorner and Paulus, 1973) by an allosteric model incorporating the assumption of nonexclusive binding (Rubin and Changeux, 1966).

We now demonstrate that at low enzyme concentrations the tetramer of glycerol kinase from *E. coli* undergoes a reversible dissociation to a dimeric species possessing full catalytic activity but unable to bind the allosteric inhibitor FBP. This paper describes the evidence from kinetic studies. The two following papers in this issue present physical evidence for the dissociation (de Riel and Paulus, 1978a) and investigate the role of subunit dissociation in the desensitization of glycerol kinase to allosteric inhibition by a number of other treatments (de Riel and Paulus, 1978b).

Materials and Methods

Glycerol kinase from strain 72 of *E. coli* K-12 (obtained from Dr. E. C. C. Lin) was purified as described by Thorner

† From the Department of Metabolic Regulation, Boston Biomedical Research Institute, Boston, Massachusetts 02114, and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115. *Received March 31, 1978; revised manuscript received August 11, 1978.* This investigation was supported by Grants BMS 72-02323 and PCM 76-03910 from the National Science Foundation. This work is part of a dissertation presented by J. K. de Riel to the Graduate School of Arts and Sciences of Harvard University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

‡ Present address: Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn. 06510. Supported by a Predoctorial Traineeship GM-00451 from the United States Public Health Service.

§ Recipient of Career Development Award 1-K3-GM-9848 from the National Institute of General Medicinal Sciences.

[1] Abbreviations used: FBP, fructose 1,6-bisphosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; 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.

0006-2960/78/0417-5134$01.00/0

and Paulus (1971). Rabbit muscle lactate dehydrogenase and pyruvate kinase were obtained from Boehringer, as was NADH. FBP (tricyclohexylammonium salt), ATP (sodium salt), and phosphoenolpyruvate (sodium salt) were obtained from Calbiochem. [γ-^{32}P]ATP was from New England Nuclear.

Radiometric Assay. Glycerol kinase activity was measured as conversion of [γ-^{32}P]ATP to an acid-stable phosphate ester in the presence of glycerol by the procedure of Thorner and Paulus (1971). All radiometric assays were at 25 °C in a volume of 1 mL of TMG buffer [50 mM triethanolamine hydrochloride, 5 mM magnesium chloride, 2 mM glycerol (pH 7.0)] containing 1 mM [γ-^{32}P]ATP. In experiments in which very low concentrations of enzyme were to be assayed, 0.1 mg/mL gelatin was also present; at this concentration, gelatin had no effect on the activity or sensitivity of glycerol kinase. Under these conditions, the enzyme was stable for hours, both in the absence and presence of FBP. The length of the assay varied from 1.5 min to 4 h in the conventional radiometric assay. One unit of glycerol kinase activity is defined as the amount of enzyme catalyzing the formation of 1 μ-mol of glycerol phosphate in 15 min under these conditions of assay.

Rapid Radiometric Assay. In order to assay enzyme concentrations higher than 10 μg/mL an apparatus was designed to give reliably timed assays as short as 1 s in duration. It consists of two disposable 10-mL syringes mounted in tandem on a Lucite frame. The two syringes are connected with a 14-gauge needle which is inserted into the lower syringe through an airtight stopper. Enzyme is inserted into the lower syringe and substrate into the upper syringe (or vice versa). Rapid depression of the plunger in the upper syringe mixes the two solutions, places them under slight pressure, and simultaneously activates a timer. After a preset time of 1 s or more, a solenoid-driven stopcock is opened and the reaction mixture is expelled into a tube containing 1 N $HClO_4$. The assay is linear with respect to both time and enzyme concentration and shows a 0.3-s lag (de Riel, 1976).

Photometric Assay. Glycerol kinase activity was measured at 25 °C as conversion of ATP to ADP in the presence of glycerol by enzymatically coupling ADP production to the disappearance of NADH, using pyruvate kinase and lactate dehydrogenase. The reaction was monitored photometrically at 340 nm on a Gilford 222-A photometer equipped with Beckman DU optics and a Gilford 6040 recorder. The assay was carried out in TMG buffer containing 1 mM ATP, 5 mM phosphoenolpyruvate, 0.3 mM NADH, 20 mM KCl, 0.067 mg (10 IU) of rabbit muscle pyruvate kinase, and 0.071 mg (25 IU) of rabbit muscle lactate dehydrogenase. The measured relaxation time (McClure, 1969) for the coupling reactions under these conditions was about 9 s (de Riel, 1976).

Measurement of Rate of Sensitization to FBP. Glycerol kinase was first equilibrated in TMG buffer for 30 min in the absence of FBP at the enzyme concentration at which sensitization was to be carried out. Control activity was measured by diluting a sample from this solution to an enzyme concentration of 0.4 μg/mL in a mixture containing all components of the radiometric assay but lacking FBP. Initial sensitivity to FBP (at zero time on the sensitization curve) was measured in the same way, except that FBP was present at the appropriate final concentration in the assay mixture. To the remaining portion of the enzyme solution, 0.11 volume of a tenfold concentrated solution of FBP in TMG buffer was added to initiate sensitization, and at timed intervals samples were removed and diluted to 0.4 μg/mL glycerol kinase in assay solution containing the same final concentration of FBP.

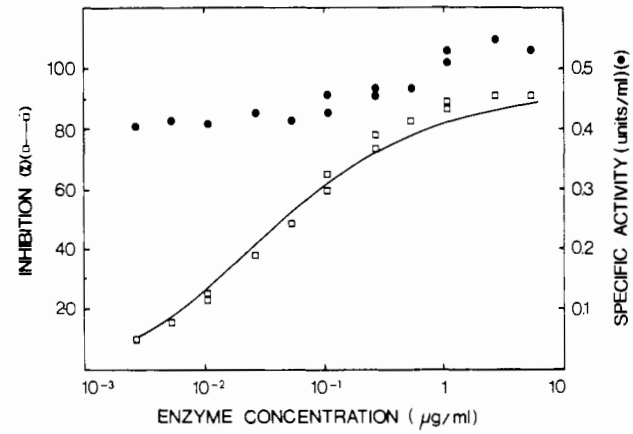

FIGURE 1: Dependence on glycerol kinase concentration of inhibition by FBP and specific activity, measured by the radiometric assay. Glycerol kinase was diluted to the indicated concentrations and split into two portions which were incubated for 120 min at 25 °C with or without 1 mM FBP and then assayed for periods ranging from 1.5 to 240 min after the addition of 0.04 volume of 25 mM [γ-^{32}P]ATP: (●) specific activity; (□) percent inhibition by 1 mM FBP. The curve was generated and plotted by computer from the equilibrium expression for an $A_2 \rightleftharpoons 2A$ dissociation with p_D = 0% inhibition, p_T = 94% inhibition, and K_D = 0.038 μg/mL (see Appendix).

Each assay was quenched with acid after 5 min. All procedures were carried out at 25 °C. The data were analyzed by plotting inhibition by FBP against time of exposure to the inhibitor. In order to fit the resulting points to theoretical curves for first- and second-order reactions, inhibition was normalized by expressing it as "percent completion" of the reaction. For example, in an experiment where inhibition was 66% at the moment of FBP addition and reached a limiting value of 97% after exposure to FBP, 66% inhibition was defined as "0% completion" and 97% inhibition was defined as "100% completion". The "percent completion" values so obtained were fitted to the theoretical time courses for first- and second-order reactions derived in the Appendix. The fitted parameters were $k_2' = c_0 k_2$ for the second-order curve and k_1 for the first-order curve, where k_1 and k_2 are the first- and second-order rate constants, respectively, and c_0 is the initial concentration of reactant. To obtain the true k_2 in each case, c_0 was calculated by assuming that percent inhibition is a measure of the proportion of product species present. Thus, in the example given above, where the total enzyme concentration was 10 μg/mL, c_0 was calculated as $10(1 - 66/97) = 3.19$ μg/mL.

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

Results

Dependence of Inhibition on Enzyme Concentration. The inhibition of glycerol kinase by FBP was found to depend on enzyme concentration. Figure 1 shows inhibition by 1 mM FBP over a 2000-fold range of enzyme concentrations, in the standard radiometric assay. As will be described later, the response of the enzyme to FBP is relatively slow or hysteretic (Frieden, 1970) under some conditions; accordingly, in order for the enzyme to achieve a steady-state sensitivity, each tube was incubated for 120 min under the conditions of assay prior to the addition of ATP to start the reaction. Inhibition was 93% at 5.4 μg/mL of enzyme and fell to 10% at 0.003 μg/mL. The curve drawn through the points is a theoretical equilibrium curve for a dissociation with the stoichiometry $A_2 \rightleftharpoons 2A$ (see Appendix), fitted to the data by least-squares analysis. In this instance, the lower limit of inhibition (p_D, the inhibition of pure

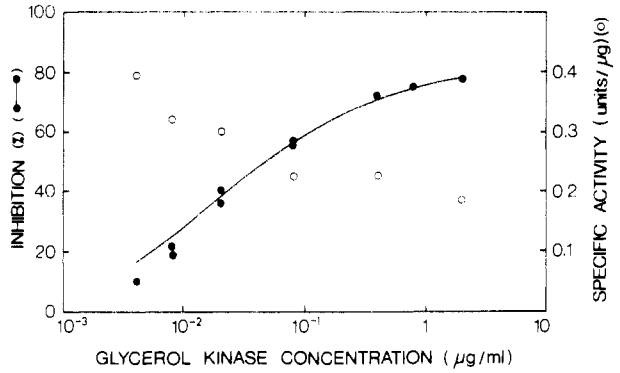

FIGURE 2: Dependence on glycerol kinase concentration of inhibition by FBP and specific activity, measured by the photometric assay. Glycerol kinase was diluted to the indicated concentrations in photometric assay solution without ATP and split into two portions which were incubated for 45 min at 25 °C with or without 10 mM FBP and then assayed photometrically after the addition of 0.01 volume of 100 mM ATP: (○) specific activity; (●) percent inhibition by 10 mM FBP. The curve was generated and plotted by computer from the equilibrium expression for an $A_2 \rightleftharpoons 2A$ dissociation with p_D = 0% inhibition, p_T = 85% inhibition, and K_D = 0.027 μg/mL.

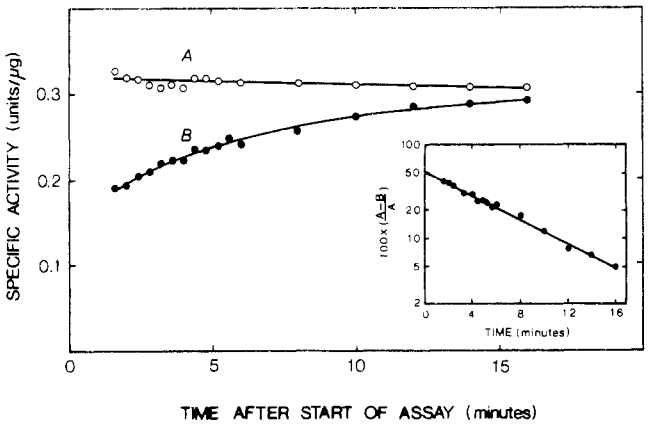

FIGURE 3: Specific activity of glycerol kinase in the photometric assay after dilution from different buffers. Glycerol kinase (8 μg/mL) was incubated for 30 min at 25 °C in (A) storage buffer [20 mM triethanolamine hydrochloride, 10 mM glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol (pH 7.0)] or (B) photometric assay solution without ATP. The enzyme was then diluted 100-fold into photometric assay solution containing 1 mM ATP to start the assay. Specific activities at different times were calculated from the slopes of the tangents to the recorder tracing. Insert: Replot of the same data as log $[(A - B)/A]$ against time.

dimer) was assumed to be 0%; the best fit was achieved with an upper limit of inhibition (p_T, the inhibition of pure tetramer) of 94% and an apparent dissociation constant in the presence of 1 mM FBP (K_D') of 0.038 μg/mL. Comparison of the best-fit theoretical curve with the data indicates that the measured inhibition fell off slightly more steeply with dilution than expected. Figure 1 also shows the specific activity in the absence of FBP over this range of enzyme concentration, determined from the same experiment. Specific activity decreased only slightly as enzyme concentration was reduced.

This experiment was repeated using the photometric assay procedure, in which ADP production was enzymatically coupled to DPNH oxidation (Figure 2). Higher concentrations of FBP were necessary to produce comparable levels of inhibition because the photometric assay solution partially desensitized the enzyme. Nevertheless, dependence of inhibition on enzyme concentration was qualitatively similar, with a fitted dissociation constant of comparable magnitude (K_D' = 0.027 μg/mL). Under the conditions of the photometric assay, specific activity in the absence of FBP increased twofold as the enzyme was diluted from 2.7 to 0.005 μg/mL. This increase appeared to be real because dilution simultaneous with the start of the assay was followed by a slow activation if the enzyme had previously been incubated in photometric assay solution (but not if it had been incubated in a simpler buffer) (Figure 3).

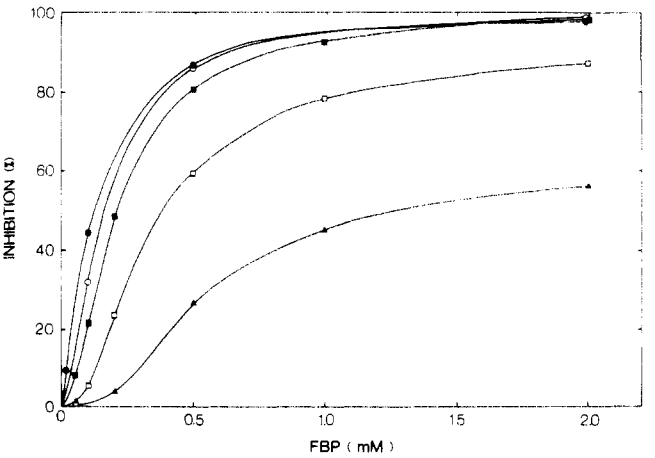

FIGURE 4: Inhibition of glycerol kinase at several different concentrations by FBP. Glycerol kinase was incubated at 25 °C in TMG buffer at the indicated concentrations of FBP before assay by the radiometric method: (▲) glycerol kinase was incubated for 120 min at 0.011 μg/mL and assayed for 60 min at the same concentration; (□) glycerol kinase was incubated for 120 min at 0.27 μg/mL and assayed for 15 min; (■) glycerol kinase was incubated for 120 min at 5.4 μg/mL and assayed for 1.5 min; (○) glycerol kinase was incubated for 30 min at 50 μg/mL and assayed for 5 s; (●) glycerol kinase was incubated for 30 min at 500 μg/mL and assayed for 1.5 s.

Dependence of Inhibition on FBP Concentration. The inhibition of sensitized glycerol kinase by different concentrations of FBP was measured at five enzyme concentrations (Figure 4). The K_i for FBP was 0.12 mM at the highest enzyme concentration (500 μg/mL) and increased progressively with dilution of the enzyme to a value greater than 0.5 mM at 0.011 μg/mL. Maximal inhibition approached 100% at the three highest enzyme concentrations (at 500 μg/mL, glycerol kinase was 99.8% inhibited by 10 mM FBP) but not at the lower two enzyme concentrations (0.27 and 0.011 μg/mL). Plots of inhibition against FBP were nonhyperbolic at all five enzyme concentrations, becoming progressively more sigmoid at the lower concentrations. The degree of sigmoidicity was estimated by the use of Hill plots of −log [1 − 100/% inhibition] against log [FBP] (not shown). The Hill coefficient n_H was found to increase from 1.3 at 500 μg/mL to about 3–4 at the lower enzyme concentrations (0.27 and 0.011 μg/mL).

Kinetics of Sensitization. Preliminary experiments had shown that, at low enzyme concentrations, sensitivity to FBP was acquired only gradually following exposure of glycerol kinase to FBP. To determine the mechanism of this hysteretic response, the kinetics of sensitization by FBP were measured at several different enzyme concentrations from 0.5 to 40 μg/mL (Figure 5). In this concentration range, the enzyme is essentially fully sensitized in the steady state (see Figure 1), making it possible to treat the sensitization as an irreversible reaction. In each case, the experimental data were fitted to theoretical curves for first- and second-order reactions (see Appendix and Materials and Methods). The results are summarized in Table I. In every case, the best second-order curve fit the data closely, whereas the best first-order curve did not (de Riel, 1976). Moreover, the best first-order rate constant (k_1) varied with enzyme concentration, whereas the second-

TABLE I: Summary of Analysis of Sensitization Kinetics.

enz concn (μg/mL)	% inhibit at 0 time	init dimer concn[b] (c_0, μg/mL)	parameter for best 1st-order fit (k_1, min^{-1})	parameter for best 2nd-order fit (c_0k_2, min^{-1})	rate const for best 2nd-order fit (k_2, mL μg^{-1} min^{-1})	Recalcd c_0k_2 for overall best fit[e] (min^{-1})
0.5	40 (23.2)[a]	0.294 (0.380)[a]	0.075 (4.30)[c]	0.128 (2.35)[c] (0.162)[a]	0.436 (0.426)[a]	0.128
2.0	51.9	0.93	0.23 (6.20)[c]	0.373 (2.66)[c]	0.401	0.406
4.0	59.8	1.53	0.43 (8.35)[c]	0.745 (2.87)[c]	0.486	0.668
10.0	66.1	3.19	0.74 (5.75)[c]	1.32 (2.05)[c]	0.414	1.39
20.0	73.8	4.78	1.09 (7.42)[c]	2.13 (1.50)[c]	0.445	2.09
40.0	81.8	6.31	1.61 (7.17)[c]	3.42 (2.41)[c]	0.542	2.75
					Av: 0.436[d]	

[a] Corrected for sensitization during assay. [b] Based on assumption of 97% inhibition of tetramer, 0% inhibition of dimer, and equal specific activities, but uncorrected for sensitization during assay. [c] Root mean square deviation. [d] Excluding the value obtained for 40 μg/mL enzyme. [e] Based on average k_2 of 0.44 mL μg^{-1} min^{-1}, and used to generate the curves in Figure 5.

order rate constant remained approximately the same over the concentration range studied (Table I). Theoretical curves generated with a single average second-order rate constant (k_2 = 0.44 mL μg^{-1} min^{-1}) fit the data well at all enzyme concentrations (Figure 5).

In spite of this excellent agreement, the preceding analysis must be considered approximate, since further sensitization of the enzyme undoubtedly occurred during the 5-min period of enzyme assay at 0.4 μg/mL in the presence of 2 mM FBP. The observed levels of inhibition, therefore, represent averages over the course of the assay and must be corrected accordingly. Fortunately, the calculated rate constant (k_2 = 0.44 mL μg^{-1} min^{-1}) can be used as a first approximation to calculate the actual extent of inhibition at the start of each assay, based on the observed average inhibition (see Appendix). The results of such a correction for the lowest enzyme concentration (0.5 μg/mL) are included in Table I. Both the initial concentration of reactant (c_0) and the fitted parameter (c_0k_2) were increased proportionately as a result of the correction, so that the corrected k_2 derived from this procedure (0.426 mL μg^{-1} min^{-1}) was not significantly different from the k_2 originally obtained for this reaction (0.436 mL μg^{-1} min^{-1}). The error for the reactions at higher enzyme concentrations was even less, since the concentration of reactant was lower during the assay.

Dependence of Sensitivity on Enzyme Concentration in the Absence of FBP. The data in Figure 5 and Table I can also be used to determine the sensitivity to FBP inhibition of glycerol kinase in the absence of FBP, that is, the inhibition at the moment of FBP addition before any FBP-induced sensitization has taken place. The zero-time sensitivities (i.e., the intercepts along the vertical axis in Figure 5) at various enzyme concentrations were corrected for sensitization occurring during the assay and plotted as a function of enzyme concentration (insert in Figure 5), together with a few additional points obtained at high enzyme concentrations in similar experiments. The line drawn through the points is a theoretical equilibrium curve for an $A_2 \rightleftharpoons 2A$ dissociation, fitted to the data by least-squares analysis. In this case, all three parameters were varied simultaneously to obtain the best fit, which was achieved with p_D = 0% inhibition, p_T = 97% inhibition, and K_D = 3.15 μg/mL. The experimental points show no systematic deviation from the theoretical curve.

Effect of FBP on the Rate of Sensitization. In a separate experiment, sensitization of glycerol kinase, at 10 μg/mL, was carried out in the presence of three different concentrations of FBP (1, 2, and 5 mM). In each case, assays were carried out

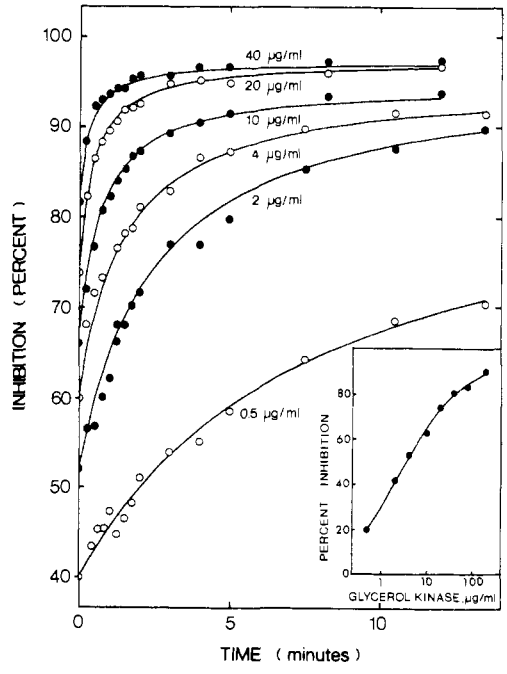

FIGURE 5: Time course of sensitization of glycerol kinase at various concentrations to inhibition by 2 mM FBP. Sensitization and assay were carried out as described under Materials and Methods at the enzyme concentrations indicated. The curves are second-order kinetics generated and plotted by computer as described in the Appendix, using the best average second-order rate constant (k_2 = 0.44 mL μg^{-1} min^{-1}) from Table I. Insert: Effect of glycerol kinase concentration on the sensitivity to inhibition by 2 mM FBP after equilibration in the absence of FBP. The zero-time inhibitions from the sensitization reactions have been corrected for sensitization that occurred during the assay as described in the Appendix. The curve was generated and plotted by computer from the equilibrium expression for an $A_2 \rightleftharpoons 2A$ dissociation with p_D = 0% inhibition, p_T = 97% inhibition, and K_D = 3.15 μg/mL.

at the same concentration of FBP as the sensitization reaction. The rates of sensitization were virtually identical over this range of FBP concentrations, and the curves (not shown) fitted to values of c_0k_2 of 1.42 (1 mM FBP), 1.56 (2 mM FBP), and 1.42 mL μg^{-1} min^{-1} (5 mM FBP).

Rate of Desensitization. Desensitization to inhibition by FBP was measured after dilution from 200 to 0.5 μg/mL in the absence of FBP. As with the sensitization experiment, sensitivity was monitored by assay at 0.4 μg/mL in the presence of 2 mM FBP and was corrected for resensitization occurring

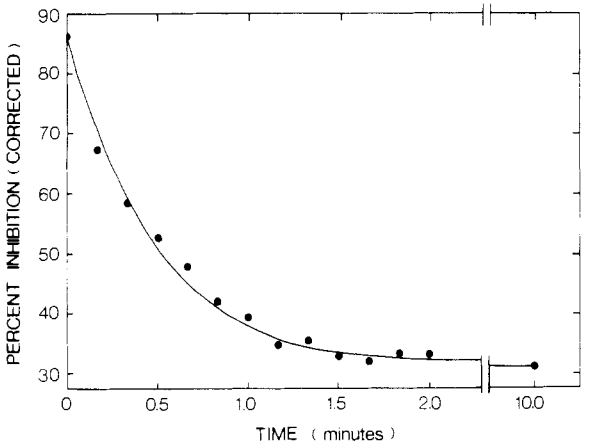

FIGURE 6: Time course of desensitization of glycerol kinase to inhibition by FBP following dilution. Glycerol kinase (200 μg/mL) was equilibrated in TMG buffer for 30 min at 25 °C and then diluted to 0.5 μg/mL. After the indicated time intervals, samples were diluted to 0.4 μg/mL and assayed immediately by the radiometric assay with and without 2 mM FBP. The data have been corrected for sensitization occurring during the 5-min assay period. The curve was generated by computer as described in the text, with $k_1 = 1.2$ min^{-1} and $k_2 = 0.86$ mL μg^{-1} min^{-1}.

during the assay. The time course of the reaction is shown in Figure 6. Sensitivity to inhibition by 2 mM FBP decreased from 87 to 31% as a result of the dilution. Because the reaction approached an equilibrium position without reaching completion, it was analyzed as a first-order reaction accompanied by a second-order reverse reaction. To avoid dealing with the cumbersome rate equation for such a situation, the reaction was simulated by iteration on the computer, and the forward (first order) and reverse (second order) rate constants were varied to achieve the best fit to the data. The best-fit curve, defined by $k_1 = 1.2$ min^{-1} and $k_2 = 0.86$ mL μg^{-1} min^{-1}, is shown in Figure 6.

Discussion

We propose the model shown in Figure 7 for the interaction of FBP with glycerol kinase. The tetrameric form of the enzyme is presumed to be able to dissociate reversibly into half molecules which are unable to bind FBP. The dimeric and tetrameric species are assumed to have approximately equal catalytic activities. Binding of FBP to the tetramer is assumed to be rapid and to be accompanied by a transition to a totally inactive conformation, although the details of this transition are left unspecified. The essential features of this model—the existence of a dissociation and its stoichiometry, and the behavior of the two different enzyme species with respect to FBP—can be deduced solely from the kinetic evidence presented here. All analyses of these results have been carried out without any assumptions about molecular weights. The assignment of tetramer and dimer for the associated and dissociated species is based on earlier work (Thorner and Paulus, 1971) and is confirmed in the following paper in this issue (de Riel and Paulus, 1978a).

The existence of a dissociation is indicated by two general observations. First, the enzyme is desensitized to FBP by dilution of the enzyme without concomitant loss of activity in the absence of inhibitor. This situation is diagnostic for change in the multiplicity of subunit association (Dunne and Wood, 1975). Second, the rate of sensitization after addition of FBP depends on the enzyme concentration. Moreover, the fact that the rate-limiting step in the sensitization reaction was clearly second order with respect to enzyme permits the assignment of a $2A \rightleftharpoons A_2$ stoichiometry for the reassociation of enzyme

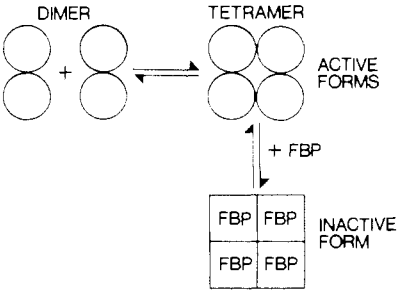

FIGURE 7: Proposed model for glycerol kinase.

subunits. Introducing the molecular weight of the dimer (110 000) for the dissociated species A, we can convert the rate constant for reassociation into more conventional units: $k_2 = 0.44$ mL μg^{-1} min^{-1} $= 0.8 \times 10^6$ M^{-1} s^{-1}. This is the same order of magnitude as association rate constants in a number of other interacting protein systems (Koren and Hammes, 1976).

The steady-state results also corroborate the assignment of an $A_2 \rightleftharpoons 2A$ stoichiometry for the dissociation. For such a stoichiometry, in the steady-state situation, one can predict the dependence on enzyme concentration of the fraction of enzyme in the tetrameric form (see Appendix). Assuming equal specific activities for dimer and tetramer, inhibition by FBP is expected to be proportional to the fraction of enzyme in the tetrameric form and thus should satisfy eq 5 (Appendix). (Even should the specific activities of dimer and tetramer differ by a factor of two, computer simulation indicates that the *shape* of the curve of inhibition against enzyme concentration would be relatively little affected.) Inhibition as a function of enzyme concentration was determined under two sets of conditions: for enzyme equilibrated in the absence of FBP and for enzyme equilibrated in the presence of 1 mM FBP. The data for enzyme equilibrated in the absence of FBP fit the hypothesis of an $A_2 \rightleftharpoons 2A$ dissociation exactly. The best-fit theoretical curve has plausible asymptotal values (p_D and p_T) and is of the same steepness as the data. The close correspondence of the experimental points to the theoretical curve also suggests that no other dissociation or aggregation reactions occur with dissociation constants in the same range, since the formation of octamer or monomer species, for example, would be expected to perturb the tetramer–dimer equilibrium, even if there were no associated changes in the catalytic properties of the enzyme. On the other hand, the possibility of multiple conformational states of either dimer or tetramer cannot be excluded by this or any other evidence presented here. The dissociation constant obtained from these data ($K_D = 3.15$ μg/mL $= 3.8 \times 10^{-8}$ M) is the true dissociation constant for the dimer–tetramer equilibrium, since it refers to enzyme equilibrated in the absence of ligand perturbation.

The data shown in Figures 1 and 2, for enzyme incubated with 1 mM FBP, are in less exact agreement with theory. The deviation can be explained on the basis of hysteresis. Equilibrium in this case was approached from the direction of the dimer, so that failure to attain equilibrium would result in observed inhibitions less than the true steady-state levels, and the error would be greater at lower enzyme concentrations, giving rise to a curve that falls off too steeply. Thus, the experiments shown in Figures 1 and 2 do not satisfy the conditions required for a decisive quantitative test of the prediction embodied in eq 5 of the Appendix because steady-state has not been attained. Had complete equilibration occurred in the experiment described in Figure 2, the curve would be shifted toward lower enzyme concentrations, and the apparent dis-

sociation constant for enzyme equilibrated with 1 mM FBP would be lower. Accordingly, the K_D' fitted to the data in Figure 1 (0.038 μg/mL = 3.5×10^{-10} M) represents an upper limit. It might seem preferable to attempt to approach this equilibrium from the direction of tetramer, i.e., by dissociation of fully sensitized enzyme following dilution in 1 mM FBP. However, attempts to measure the rate of dissociation in 1 mM FBP were unsuccessful, presumably because this rate is very small as suggested by our model, which predicts that binding of FBP to the tetramer will block dissociation.

Several lines of evidence suggest that FBP does not bind to the dimer. First, the dimer appeared not to be significantly inhibited by 2 mM FBP. In the insert in Figure 5, the best fit was obtained with a value for p_D, the inhibition of pure dimer by 2 mM FBP, of 0%. This implies that the dimer is inhibited at most a few percent by 2 mM FBP, a concentration more than tenfold above the K_i for the tetramer. Second, the rate of sensitization was not affected by FBP concentration in the range of 1 to 5 mM, indicating that the rate-limiting step for sensitization is independent of FBP concentration. If the dimer were able to bind FBP, two or more species of dimer (with and without bound FBP) would be present in varying proportions, depending on FBP concentration, and might be expected to associate at different rates (Dunne and Wood, 1975). Finally, comparison of the apparent dissociation constants for enzyme equilibrated in the absence and presence of FBP (K_D and K_D', respectively) shows clearly that FBP shifts the equilibrium in the direction of tetramer and must, therefore, bind less well to the dimer. Assuming equivalent binding constants for FBP to each subunit of an oligomeric species, it can be shown that the ratio of affinities for dissociated and associated species, c, is related to the apparent dissociation constants K_D and K_D' by the expression $c \leq (K_D'/K_D)^{1/n}$, where n is the number of subunits in the associated species (Colosimo et al., 1976; de Riel, 1976). From the present results, $K_D'/K_D \leq 1/80$ and $c \leq 1/3$ for the dimer and tetramer of glycerol kinase. The results described in the following paper in this issue (de Riel and Paulus, 1978a) show that the actual value of c must be much lower. The data in Figure 4 support the conclusion that dissociation and FBP binding are at least partially competitive. The observed increase in K_i and in the Hill coefficient n_H as a consequence of dilution confirms the conclusion that FBP binds preferentially to the tetrameric form of glycerol kinase. However, a test of the prediction of our model that, under steady-state conditions, high concentrations of FBP should be able to effect complete inhibition even at very low glycerol kinase concentrations has not been possible because of the experimental difficulty of achieving steady state under these conditions. The failure to approach 100% inhibition at low enzyme concentration is an artifact due to incomplete equilibration, as discussed above, and does not necessarily imply binding of ligand to dimer.

On the other hand, the tetramer can be completely inhibited by FBP. The maximum inhibition of 80%, previously observed by Thorner and Paulus (1973), probably resulted from incomplete sensitization of the enzyme due to hysteresis. Moreover, the K_i for FBP determined by Thorner and Paulus (1973) under their assay conditions is several-fold higher than the value of 0.12 mM observed at very high enzyme concentrations in the present work. The earlier reported discrepancy (Thorner and Paulus, 1973) between the kinetically determined K_i for FBP and the dissociation constant obtained from binding studies was most likely due to the fact that the two kinds of measurements were made at different enzyme concentrations. It appears, therefore, that the results obtained by Thorner and Paulus (1973) can be explained in terms of a slow dimer-tetramer equilibrium of glycerol kinase without the necessity of invoking nonexclusive binding (Rubin and Changeux, 1966) of FBP to the active and inactive states of the enzyme.

We have assumed in the foregoing analyses that specific activities of dimer and tetramer are approximately equal, at least in the radiometric assay. A 20% difference in specific activity was observed in the radiometric assay across the range of enzyme concentrations studied, but this may reflect artifacts of dilution. On the other hand, the twofold increase seen with dilution in the photometric assay appears to be significant, since it can be observed as a time-dependent process. At the lowest enzyme concentration studied, the specific activities (0.4 unit/μg of enzyme) were the same in the radiometric and the photometric assays but then diverged as enzyme concentration increased. This suggests that the specific activity of the dimer, but not that of the tetramer, may be relatively independent of assay conditions. The fact that a time-dependent increase in specific activity was observed in the photometric assay after dilution from the photometric assay solution but not after dilution from a simpler buffer (Figure 3) supports such a possibility.

Changes in the multiplicity of enzyme association in connection with ligand binding are quite common (Dunne and Wood, 1975) and a number of theoretical discussions have appeared (see, for example, Frieden, 1967; Nichol et al., 1967; Colosimo et al., 1976). The physiological significance of such polysteric phenomena remains, however, unclear. That they have a function is suggested by their preservation across wide evolutionary gaps; for example, dCMP deaminase is activated by a dCTP-induced oligomerization in chick embryo (Maley and Maley, 1968), in *Staphylococcus aureus* (Duncan et al., 1972), and in bacteriophage T2 infected cells of *E. coli* (Maley et al., 1972), despite large differences in other properties of the enzymes from these sources. A major consequence of ligand-induced oligomerization is a dependence of the regulatory properties of an enzyme on its concentration. Such a characteristic might be of physiological significance for enzymes whose intracellular concentration varies either with time or on account of differential distribution in various tissues in higher organisms, provided there is some need for different modes of regulation at different enzyme levels. Glycerol kinase provides a good example of an enzyme for which this might be advantageous. When the enzyme is induced to high levels by glycerol, feedback inhibition is essential to prevent overproduction of glycerol 3-phosphate (Zwaig et al., 1970). On the other hand, when the enzyme is present at low levels in the uninduced state, some glycerol kinase activity is necessary for the induction of the glycerol regulon in response to exogenous glycerol, because the actual inducer is the product of the glycerol kinase reaction (Hayashi and Lin, 1965). In this situation, feedback inhibition serves no purpose and may well be counterproductive by interfering with the acquisition of the capacity to utilize glycerol. The polysteric behavior of glycerol kinase may thus have evolved specifically in order to cope with the problem of induction of a pathway by an inducer which is one of its products.

Appendix

Tetramer-Dimer Equilibrium. For a tetramer-dimer equilibrium of the type proposed in this paper, the mass-action expression is

$$K_D = D^2/T \quad (1)$$

where D is the molar concentration of dimer, T the molar concentration of tetramer, and K_D the dissociation constant.

If E, the total enzyme concentration, is defined as the total concentration of *dimer equivalents*, then the mass conservation expression is

$$E = D + 2T \tag{2}$$

Combining eq 1 and 2 and solving the resulting quadratic yields

$$T = [4E + K_D - (8EK_D + K_D^2)^{1/2}]/8 \tag{3}$$

The weight fraction of enzyme in the form of tetramer, X_T, is then

$$X_T = 2T/E = [4E + K_D - (8EK_D + K_D^2)^{1/2}]/4E \tag{4}$$

In this equation and the following one, E and K_D may be expressed in any units.

Any weight-average parameter, p, should vary with enzyme concentration according to the equation

$$p = p_D + (p_T - p_D)X_T$$

or

$$p = p_D + (p_T - p_D)[4E + K_D - (8EK_D + K_D^2)^{1/2}]/4E \tag{5}$$

Equation 5 describes a theoretical relationship containing three adjustable parameters (p_D, p_T, and K_D), none of which need be known, although in many cases values will be predicted or at least constrained by a particular model. In order to compare data with eq 5, it is first necessary to assign values to these parameters in order to arrive at a specific curve. In general, this is done by least-squares analysis in which all three parameters are iterated simultaneously to discover the values which give the best fit of eq 5 to the data. The fitted optimal values can then be compared to predicted values for these parameters and the resultant curve compared to the data for systematic deviations.

Second-Order Kinetics. For a reaction of the form $2C \rightarrow C_2$, the rate equation may be written as the rate of disappearance of the reactant:

$$-dc/dt = k_2c^2 \tag{6}$$

where c is the concentration of the reacting species C, t is time, and k_2 is the second-order rate constant. (Here, c and t may be expressed in any units as long as k_2 is expressed in the reciprocal units.) Integrating eq 6,

$$1/c - 1/c_0 = k_2t \tag{7}$$

where c_0 is the concentration of C at $t = 0$. The ratio c/c_0 is related to the fractional extent of completion of the reaction, x, *by*

$$x = 1 - c/c_0 \tag{8}$$

Combining eq 7 and 8 yields

$$x = c_0k_2t/(1 + c_0k_2t) = k_2't/(1 + k_2't) \tag{9}$$

where $k_2' = c_0k_2$.

Equation 9 was used for least-squares fitting of kinetic data to a second-order reaction. The fitted parameter is k_2' which has the dimensions of reciprocal time (e.g., min^{-1}). Thus, no knowledge or assumption about absolute concentrations is required to fit the data to a second-order curve, but c_0 must be known to calculate the actual rate constant.

If reactant concentration is measured in an assay of duration T, during which the reaction continues, the average concentration obtained from the assay, $\bar{c}$, can be expressed by

$$\bar{c} = (1/T)\int_0^T c\,dt \tag{10}$$

where c is the instantaneous concentration of reactant at time t. If c_0 is the concentration at the start of the assay, and if C continues to react in a second-order fashion as described by eq 7, the concentration of C at time t after the start of the assay is given by

$$c = c_0/(c_0k_2t + 1) \tag{11}$$

Combining eq 10 and 11,

$$\bar{c} = \frac{1}{k_2T}\ln(c_0k_2T + 1) \tag{12}$$

and

$$c_0 = [\exp(\bar{c}k_2T) - 1]/k_2T \tag{13}$$

Equation 13 allows the initial concentration of C (at the start of the assay) to be calculated from the observed average concentration, provided the value of k_2 is known. If necessary, eq 13 can be used in cycle with eq 9 to provide successive approximations of k_2.

First-Order Kinetics. The first-order analogue of eq 9, obtained by integrating the corresponding rate equation, is

$$x = 1 - \exp(-k_1t) \tag{14}$$

where x is the fractional extent of completion of the reaction and k_1 the first-order rate constant. In the case of a first-order fit, the rate constant is obtained directly as the fitted parameter.

References

Boyer, P. D. (1962), *Enzymes, 2nd Ed. 6,* 95–113.

Colosimo, A., Brunori, M., and Wyman, J. (1976), *J. Mol. Biol. 100,* 47–57.

de Riel, J. K. (1976), Ph.D. Thesis, Harvard University.

de Riel, J. K., and Paulus, H. (1978a), *Biochemistry 17* (second paper in a series of three in this issue).

de Riel, J. K., and Paulus, H. (1978b), *Biochemistry 17* (third paper in a series of three in this issue).

Duncan, B. K., Diamond, G. R., and Bessman, M. J. (1972), *J. Biol. Chem. 247,* 8136–8138.

Dunne, C. P., and Wood, W. A. (1975), *Curr. Top. Cell. Regul. 9,* 65–101.

Freedberg, W. B., Kistler, W. S., and Lin, E. C. C. (1971), *J. Bacteriol. 108,* 137–144.

Frieden, C. (1967), *J. Biol. Chem. 242,* 4045–4052.

Frieden, C. (1970), *J. Biol. Chem. 245,* 5788–5799.

Hayashi, S.-I., and Lin, E. C. C. (1965), *J. Mol. Biol. 14,* 515–521.

Koren, R. and Hammes, G. G. (1976), *Biochemistry 15,* 1165–1171.

Lin, E. C. C. (1976), *Annu. Rev. Microbiol. 30,* 535–578.

Maley, G. F., MacColl, R., and Maley, F. (1972), *J. Biol. Chem. 247,* 940–945.

Maley, G. F., and Maley, F. (1968), *J. Biol. Chem. 243,* 4506–4512.

McClure, W. R. (1969), *Biochemistry 8,* 2782–2786.

Nichol, L. W., Jackson, W. J. H., and Winzor, D. J. (1967), *Biochemistry 6,* 2449–2456.

Rubin, M. M., and Changeux, J.-P. (1966), *J. Mol. Biol. 21,* 265–274.

Schwert, G. W., and Winer, A. D. (1963), *Enzymes, 2nd Ed.* 127–148.

Thorner, J. W., and Paulus, H. (1971), *J. Biol. Chem. 246,* 3885–3894.

Thorner, J. W., and Paulus, H. (1973), *J. Biol. Chem. 248,* 3922–3932.

Zwaig, N., Kistler, W. S., and Lin, E. C. C. (1970), *J. Bacteriol. 102,* 753–759.