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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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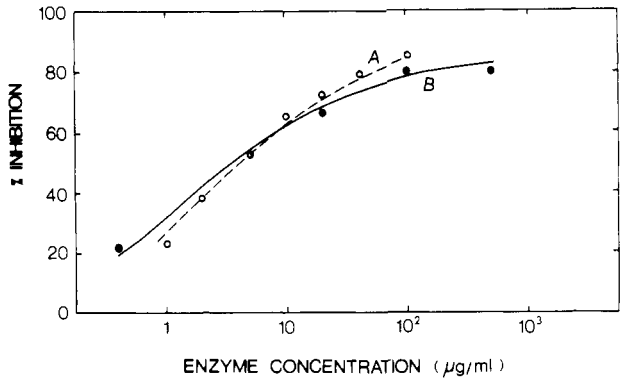

FIGURE 1: Dependence on glycerol kinase concentration of inhibition by FBP after equilibration at 0.4 M KCl in the absence or presence of FBP. Open circles: Glycerol kinase at the indicated concentrations was incubated for 30 min at 25 °C in TMG buffer containing 0.4 M KCl but no FBP and then diluted to an enzyme concentration of 0.4 μg/mL in TMG buffer without KCl containing 1 mM [γ-^{32}P]ATP for an immediate 5-min radiometric assay in the absence and presence of 2 mM FBP. Inhibition was corrected for changes occurring during the assay period (de Riel and Paulus, 1978a). Full circles: Glycerol kinase at the indicated concentrations was incubated for 60 min at 25 °C in TMG buffer containing 0.4 M KCl without or with 5 mM FBP and then assayed by the radiometric procedure after the addition of [γ-^{32}P]ATP to 1 mM. The curves were generated and plotted by computer from the equilibrium expression for an $A_2 \rightleftharpoons 2A$ dissociation, using the following parameters: curve A, p_D = 0% inhibition, p_T = 97% inhibition, K_D = 3.65 μg/mL; curve B, p_D = 0% inhibition, p_T = 87% inhibition, K_D' = 2.25 μg/mL.

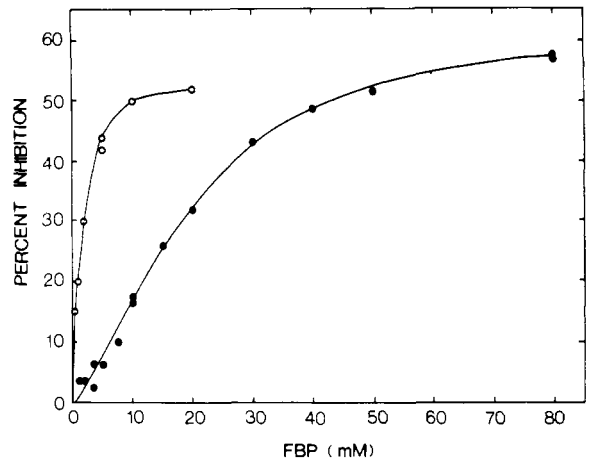

FIGURE 2: Inhibition of glycerol kinase by FBP at 20 mM KCl and at 0.4 M KCl. Glycerol kinase (0.08 μg/mL) was incubated for 45 min at 25 °C in photometric assay solution without ATP (de Riel and Paulus, 1978a) at the indicated concentrations of FBP and (○) 20 mM or (●) 0.4 M KCl and then assayed by the photometric procedure after the addition of 0.01 volume of 0.1 M ATP.

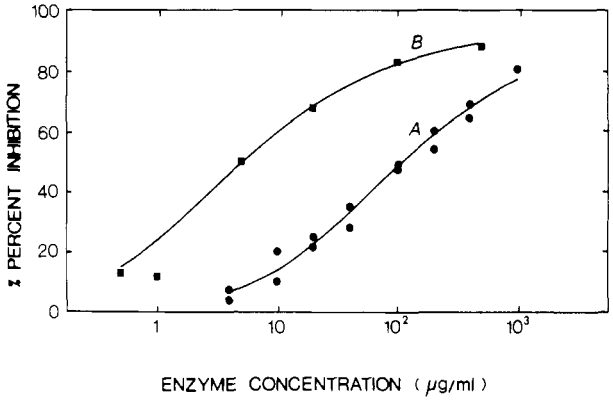

FIGURE 3: Dependence on glycerol kinase concentration of inhibition by FBP after equilibration in 2 M urea in the absence and presence of FBP. Circles: Glycerol kinase at the indicated concentrations was incubated for 15 min at 25 °C in TMG buffer containing 2 M urea and then diluted to an enzyme concentration of 0.4 μg/mL in TMG buffer without urea containing 1 mM [γ-^{32}P]ATP for an immediate 5-min radiometric assay in the absence and presence of 2 mM FBP. Inhibition was corrected for changes occurring during the assay period (de Riel and Paulus, 1978a). Squares: Glycerol kinase at the indicated concentrations was incubated for 18 min at 25 °C in TMG buffer containing 2 M urea without or with 2 mM FBP and then assayed by the radiometric assay after the addition of [γ-^{32}P]ATP to 1 mM. The curves were generated and plotted by computer from the equilibrium expression for an $A_2 \rightleftharpoons 2A$ dissociation, using the following parameters: curve A, p_D = 0% inhibition, p_T = 97% inhibition, K_D = 100 μg/mL; curve B, P_D = 0% inhibition, p_T = 96% inhibition, K_D' = 4.5 μg/mL.

sensitivity. Percent inhibition by FBP was used as a measure of percent tetramer (de Riel and Paulus, 1978a). Procedures for correcting for sensitization which occurred during the assay and for fitting the data to a dissociation curve to determine the subunit dissociation constant have been described (de Riel and Paulus, 1978a). The extent of subunit dissociation in the presence of FBP was measured by first incubating the enzyme for an appropriate period at the experimental concentration under desensitizing conditions in the presence and absence of FBP and then assaying by adding a small volume of [γ-^{32}P]-ATP. Rates of subunit association were measured by following the time course of acquisition of sensitivity to FBP after the addition of FBP to enzyme previously unexposed to it and deriving a second-order rate constant by curve fitting (de Riel and Paulus, 1978a).

Results

Desensitization by KCl. At a concentration of 0.4 M, KCl completely desensitized glycerol kinase to 1 mM FBP and activated the enzyme about 20% above low salt conditions (Thorner and Paulus, 1973). The effect of 0.4 M KCl on the dimer–tetramer equilibrium in the absence of FBP is shown in Figure 1 (curve A). The corrected data points have been fitted by a theoretical dissociation curve for an $A_2 \rightleftharpoons 2A$ reaction to yield a best-fit dissociation constant K_D = 3.6 μg/mL. This value is close to K_D = 3.2 μg/mL obtained previously in the absence of KCl (de Riel and Paulus, 1978a), suggesting that KCl had little effect on the dissociation equilibrium of glycerol kinase in the absence of ligand perturbation. A dissociation curve was also constructed for enzyme equilibrated at various concentrations in 0.4 M KCl in the presence of FBP (Figure 1, curve B). The best-fit apparent dissociation constant in the presence of 5 mM FBP was K_D' = 2.2 μg/mL, almost two orders of magnitude higher than the K_D' measured in the absence of salt at only 1 mM FBP (0.038 μg/mL; de Riel and Paulus, 1978a). The relatively low value of the upper asymptote of the fitted curve (p_T = 87% inhibition by 5 mM FBP) suggests that high salt affects the binding of FBP by the tetramer. Comparison of the effect of FBP concentration at low and high levels of KCl showed that high salt concentrations increased the FBP concentration required for half-maximal inhibition about tenfold but had no effect on the maximum level of inhibition or on the shape of the inhibition curve (Figure 2).

Desensitization by Urea. At a concentration of 2 M, urea desensitized low levels of glycerol kinase almost completely to inhibition by 2 mM FBP and activated the enzyme 50%. The effect of 2 M urea on the dimer–tetramer equilibrium in the absence of FBP is shown in Figure 3 (curve A). The corrected data points could be fitted by a theoretical curve for an $A_2 \rightleftharpoons 2A$ reaction with K_D = 100 μg/mL, a value about 30-fold higher than the K_D measured in the absence of urea. When glycerol kinase was equilibrated in 2 M urea in the presence

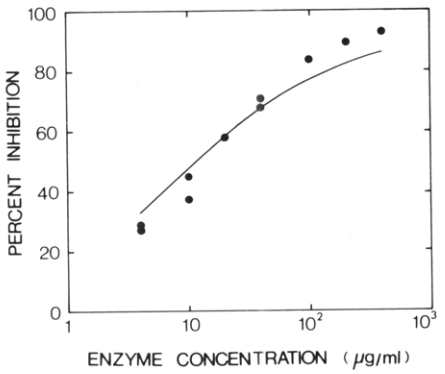

FIGURE 4: Dependence on glycerol kinase concentration of inhibition by FBP after equilibration at pH 10 in the absence of FBP. Glycerol kinase at the indicated concentrations was incubated for 15 min at 25 °C in buffer at pH 10.0 (50 mM ethanolamine hydrochloride, 5 mM $MgCl_2$, and 2 mM glycerol) and then diluted to an enzyme concentration of 0.4 μg/mL in TMG buffer at pH 7.0 containing 1 mM [γ-^{32}]ATP for an immediate 5-min radiometric assay in the absence and presence of 2 mM FBP. Inhibition was corrected for changes occurring during the assay period (de Riel and Paulus, 1978a). The curve was generated and plotted by computer from the equilibrium expression for an $A_2 \rightleftharpoons 2A$ dissociation, using p_D = 0% inhibition, p_T = 97% inhibition, and K_D = 11 μg/mL.

of 2 mM FBP, the inhibition data could be fitted by a dissociation curve with K_D' = 4.5 μg/mL (Figure 3, curve B). This value of K_D' was about 120-fold higher than the apparent dissociation constant in the presence of 1 mM FBP measured in the absence of urea. The upper asymptote of curve B in Figure 3 (p_T = 96% inhibition by 2 mM FBP), for enzyme equilibrated and assayed in 2 M urea, is close to the comparable value of 97% for enzyme assayed in the absence of urea (curve A in Figure 3; see also de Riel and Paulus, 1978a). The agreement indicates that the sensitivity of the tetramer to FBP is not significantly affected by FBP.

Urea also destabilized glycerol kinase. In the presence of 2 M urea, enzyme activity decayed irreversibly in a simple first-order fashion (not shown). The rate of inactivation depended on enzyme concentration, the first-order rate constant increasing from 1.5×10^{-3} min^{-1} at 4 mg/mL glycerol kinase to a maximum of about 9×10^{-3} min^{-1} at enzyme concentrations of 40 μg/mL and below. FBP protected glycerol kinase from inactivation, reducing the first-order rate constant for inactivation fourfold at 2 mM and an enzyme concentration of 20 μg/mL. In order to avoid artifacts caused by differential inactivation when the enzyme was incubated with and without FBP (Dunne and Wood, 1975), the time of incubation in the experiment shown in Figure 3 was kept short enough that inactivation never exceeded 15%.

Desensitization at High pH. When glycerol kinase (0.4 μg/mL) was assayed in the absence and presence of 2 mM FBP after a 15-min equilibration period in buffers of pH 7 to 10, enzyme activity was found to increase up to pH 9 and then decline, while inhibition decreased progressively to zero at pH 10 (not shown), confirming the original observation of Thorner and Paulus (1973). The effect of high pH on the dimer–tetramer equilibrium is shown in Figure 4. The corrected data points have been fitted by a theoretical dissociation curve for an $A_2 \rightleftharpoons 2A$ reaction with a dissociation constant K_D = 11 μg/mL. This dissociation constant is about three times larger than that observed at pH 7, but the difference is too small to account for the complete desensitization observed at high pH. To explore this discrepancy further, the rate of sensitization of the enzyme following the addition of FBP was measured at pH 7.0, 9.0, and 9.5 in parallel experiments. The time courses of these experiments revealed that sensitization at high pH was

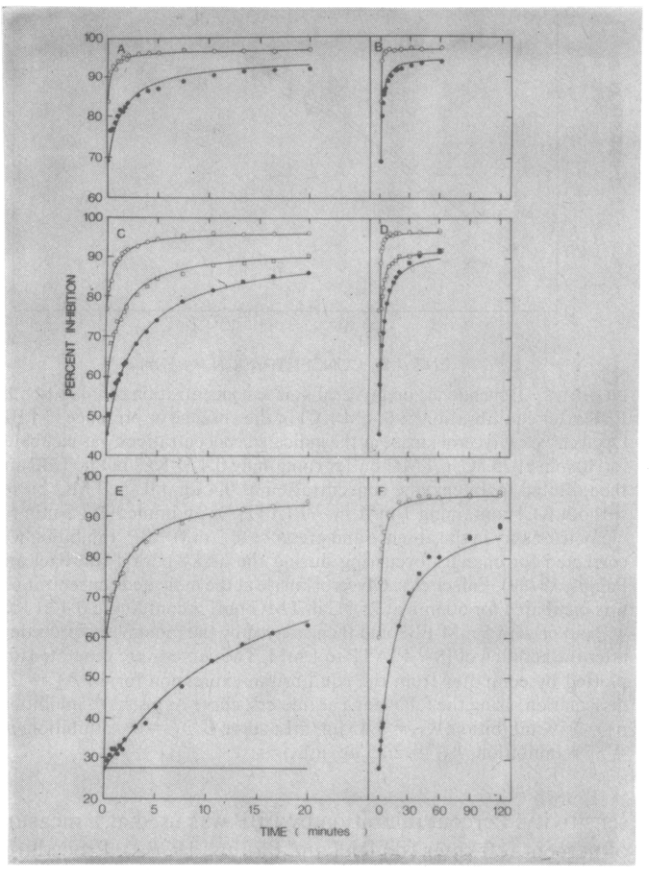

FIGURE 5: Time course of sensitization of glycerol kinase at various concentrations to inhibition by 2 mM FBP at pH 7.0, 9.0, and 9.5. Sensitization and assay were carried out as described previously (de Riel and Paulus, 1978a) in TMG buffer at pH 7.0 (○), or 50 mM ethanolamine hydrochloride, 5 mM $MgCl_2$, and 2 mM glycerol at pH 9.0 (□) or 9.5 (●). Glycerol kinase concentrations were 40 (A and B), 10 (C and D), or 2 μg/mL (E and F). Panels B, D, and F show the same data as panels A, C, and E, respectively, on a different time scale. The curves are best second-order fits to the data generated and plotted by computer as described earlier (Appendix in de Riel and Paulus, 1978a), using the parameters summarized in Table I.

considerably slower (Figure 5); the best-fit second-order rate constant k_2 was approximately tenfold less at pH 9.5 than at pH 7.0 (Table I). Since k_2 (the rate constant for subunit association) changed more rapidly than the equilibrium constant K_D, a decrease would also be expected in k_1 (the rate constant for subunit dissociation). Comparison of the rates of desensitization following dilution in the absence of FBP at pH 7.0 and 9.0 confirmed that k_1 was about threefold smaller at the higher pH (not shown). Even at pH 9.5, fully sensitized enzyme was inhibited at least 94.5% by 2 mM FBP, indicating that the tetramer is still highly sensitive at that pH, although somewhat less so than at neutral pH.

Like 2 M urea, pH 10 conditions destabilized the enzyme, particularly at low enzyme concentrations in the absence of FBP. Measured rate constants for inactivation at pH 10 were 8×10^{-3} and 1.3×10^{-3} min^{-1} at 1 μg/mL in the absence and presence of FBP, respectively, and 1.5×10^{-3} min^{-1} at 100 μg/mL enzyme in the absence of FBP (data not shown). These rates were sufficiently slow so as not to perturb significantly the measurement of the subunit association and dissociation rates under the conditions used.

Discussion

In the first paper of this series (de Riel and Paulus, 1978a),

TABLE I: Summary of Analysis of Sensitization Kinetics at pH 7.0, 9.0, and 9.5.

pH	enz concn (μg/mL)	% inhibit at 0 time	initial dimer concn (c_0, μg/mL)	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})
7.0	2	59.9	0.764[a]	0.56	0.733
7.0	10	76.7	2.09[a]	1.45	0.693
7.0	40	83.9	5.41[a]	3.42	0.632
9.0	10	54.0	4.16	0.85	0.204
9.5	2	27.5	1.42[b]	0.065	0.046
9.5	10	45.5	5.19[b]	0.325	0.063
9.5	40	69.3	10.67[b]	0.65	0.061

[a] Based on 97% inhibition of pure tetramer. [b] Based on 94.5% inhibition of pure tetramer.

we proposed a model for the interaction of FBP with glycerol kinase, which involved two types of reactions. One of these was an equilibrium between the tetrameric and dimeric forms of glycerol kinase, which were assumed to have approximately equal catalytic activities. The second was the binding of FBP to the glycerol kinase tetramer (but not to the dimer), leading to enzyme inhibition. This model predicts that the sensitivity of glycerol kinase to FBP inhibition can be modulated in at least two ways: by the perturbation of the tetramer–dimer equilibrium and by the modification of the affinity of the tetramer for the inhibitor. These possibilities can be distinguished by examining the effect of desensitizing conditions on the magnitudes of K_D and K_D', the observed subunit dissociation constants in the absence and the presence of FBP, respectively. Whereas K_D concerns solely the tetramer–dimer equilibrium of glycerol kinase unperturbed by ligand, K_D' is a composite dissociation constant for the linked equilibria dimer $\rightleftharpoons$ tetramer and tetramer + nFBP $\rightleftharpoons$ tetramer·FBP$_n$, and thus depends on K_D as well as on the association constant of FBP with the tetramer. Accordingly, a perturbation of K_D will lead to parallel changes in the magnitudes of both K_D and K_D', but an alteration in the affinity of the tetramer for FBP will affect K_D only and not K_D'. Our experimental approach for analyzing desensitization caused by salt, urea, and high pH was to measure K_D and K_D' under the assay conditions where desensitization itself was observed, using the kinetic approaches described earlier (de Riel and Paulus, 1978a). The behavior of glycerol kinase in these studies illustrates the diversity of mechanisms by which desensitization of a polysteric enzyme can occur.

KCl was found to affect K_D' but not K_D, indicative of direct interference with FBP binding. This is probably a general ionic-strength effect, since other salts (including triethanolammonium chloride) had comparable effects (de Riel, 1976). FBP bears a substantial negative charge, and its binding to the enzyme probably involves electrostatic interactions which would be diminished in a medium of high ionic strength. Desensitization by KCl is of physiological interest because the intracellular milieu of *E. coli* contains approximately 0.2 M KCl (Lubin and Ennis, 1964). Since KCl has a large effect on the position of the subunit dissociation equilibrium in the presence of ligand, its presence inside the cell will affect the range of enzyme concentrations over which the polysteric transition of glycerol kinase occurs at physiological levels of FBP.

In contrast, urea affected K_D and K_D' to similar extents, indicating a direct effect on subunit equilibrium. The observed promotion of subunit dissociation by urea is consistent with its well-known tendency to weaken protein–subunit interactions in general. A small effect of urea on the binding of FBP by the tetramer cannot be ruled out, but this cannot be a major factor in the desensitization because the level of inhibition by 2 mM FBP at high enzyme concentration was the same as that observed in the absence of urea.

At high pH, K_D was altered only slightly. The major consequence of increasing pH appeared to be a reduction in the rates of both subunit association and dissociation, i.e., a hysteretic effect (Frieden, 1970). The decreased rate of approach to equilibrium from both directions at high pH explains an earlier puzzling observation that previously sensitized enzyme remained sensitive and previously desensitized enzyme remained insensitive in the presence of FBP at pH 10 (de Riel, 1976). It also precluded direct measurement of K_D' because the required equilibration time in the presence of FBP would have been long compared to the rate of inactivation under these conditions; however, the fact that the level of inhibition by 2 mM FBP at high enzyme concentration was not altered at pH 10 suggests that the ability of the tetramer to bind FBP was not significantly affected. A plausible explanation for the effect of high pH on the rates of dimer–tetramer interconversion but not on the equilibrium position or on FBP binding is that formation of the transition state for the dimer–tetramer interconversion requires the protonation of a group on the enzyme, so that the transition state becomes less accessible under alkaline conditions.

The results described in this paper show that desensitization of glycerol kinase to inhibition by FBP under three different conditions manifested itself in three different ways. Our model for the allosteric regulation of glycerol kinase provided a powerful conceptual tool for exploring and classifying these diverse effects. Whereas salt acted by reducing the affinity of the tetrameric form of glycerol kinase for FBP in what might be considered the "classical" mechanism of allosteric desensitization, urea and high pH caused desensitization by a mechanism which involved perturbation of the subunit dissociation reaction with indirect effects on the allosteric transition. The desensitizing effects of urea and high pH illustrate the unique susceptibility of a polysteric enzyme, in which a dissociation equilibrium is linked to the allosteric transition, to perturbation of its regulatory properties. Under some circumstances, this increased sensitivity to perturbation may well serve a regulatory function. One such situation has already been discussed in the first paper of this series (de Riel and Paulus, 1978a) in relation to the variation of the sensitivity to allosteric inhibition with intracellular enzyme concentration. Any other factor which might perturb the dimer–tetramer equilibrium, such as hydrogen ion concentration, ionic strength, or more specific ligands, could similarly modulate the sensitivity of glycerol kinase to feedback inhibition by FBP. Moreover, the occurrence of hysteresis in the dimer–tetramer transition opens further possibilities for enzyme regulation (Frieden, 1970).

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Effects of Mg^{2+} and Substrates on the Conformation of Ribulose-1,5-bisphosphate Carboxylase†

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ABSTRACT: Evidence for conformational changes in pea and spinach ribulose-1,5-bisphosphate carboxylase, caused by interaction of the enzyme with ribulose 1,5-bisphophate (RuP_2) or with $MgCl_2$ and $NaHCO_3$, was obtained by measurements of chemical cross-linking and circular dichroism. Samples of the enzyme were incubated with RuP_2 or with $NaHCO_3$ and $MgCl_2$ and then cross-linked with tetranitromethane $C(NO_2)_4$ or dimethyl suberimidate. Cross-linked subunits were detected by dodecyl sulfate-polyacrylamide gel electrophoresis. The formation of most cross-links between subunits with either $C(NO_2)_4$ or dimethyl suberimidate was enhanced by pretreatment with $MgCl_2$ and $NaHCO_3$ and inhibited by pretreatment with RuP_2. A protein species with a slightly faster electrophoretic mobility than the large subunit was formed by treatment with $C(NO_2)_4$. This species is derived from the large subunit, possibly through an intrachain cross-link. The formation of this species was markedly stimulated by pretreatment with $MgCl_2$ and $NaHCO_3$ and almost completely inhibited by pretreatment with RuP_2. The carboxylating activity of the enzyme was inhibited substantially by pretreatment with $C(NO_2)_4$ and slightly by pretreatment with dimethyl suberimidate. Preincubation with $NaHCO_3$ and $MgCl_2$ increased the extent of inactivation by either cross-linking reagent. Preincubation with RuP_2 protected the enzyme activity. The circular dichroism spectrum of the enzyme was altered in magnitude by the addition of $MgCl_2$ and $NaHCO_3$. The greatest shift was at 275 nm. Treatment of RuP_2 carboxylase with $MgCl_2$ and $NaHCO_3$ activates its enzymatic activity. These measurements of chemical cross-linking and circular dichroism indicate that a conformation change occurs in the enzyme upon activation.

Ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39] catalyzes the carbon dioxide fixation reaction of the photosynthetic carbon reduction cycle and is also possibly involved in glycolate production. The purified enzyme can form two molecules of 3-phosphoglycerate from CO_2 and ribulose 1,5-bisphosphate (RuP_2)[1] (carboxylase activity) or one molecule of phosphoglycolate and one of 3-phosphoglycerate from O_2 and RuP_2 (oxygenase activity). Both reactions of the enzyme are activated by exposure of the protein to $NaHCO_3$ and $MgCl_2$ (Pon et al., 1963; Chu and Bassham, 1973; Lorimer et al., 1976; Badger and Lorimer, 1976; Laing and Christeller, 1976). RuP_2 can bind to the enzyme and inhibit the interconversion of inactive and active forms (Laing and Christeller, 1976).

Evidence that $MgCl_2$, $NaHCO_3$, and RuP_2 cause conformational changes in the enzyme has come from studies using difference spectrophotometry (Rabin and Trown, 1964; Kwok and Wildman, 1974), fluorometry of added probes (Wildner, 1976; Vater et al., 1977), and measurement of inactivation of the enzyme by chemical modifiers (Schloss and Hartmann, 1977).

In eukaryotic plants, the enzyme is comprised of two types of subunit, L (M_r 55 000) and S (M_r 12 000-14 000) (Kung, 1976). Earlier work in this laboratory has shown that it is possible to probe the association of these subunits through the use of chemical cross-linking reagents (Roy et al., 1978). The present study shows that this kind of structural analysis can be used to detect conformational changes in the enzyme caused by binding of substrate molecules, providing information of a different sort than the probes mentioned above yield. Additionally, conformational changes were detected by circular dichroism. The circular dichroism of RuP_2 carboxylase is altered by $MgCl_2$ and $NaHCO_3$.

Experimental Procedures

Enzyme Isolation. RuP_2 carboxylase was purified from homogenates of pea seedling shoots (*Pisum sativum*, variety Progress no. 9, Agway, Buffalo, N.Y.) as described previously (Roy et al., 1978), except for the final chromatographic step. The enzyme was loaded onto a 2.5 × 10 cm DEAE-Sephadex A-50 column which had been equilibrated with 0.05 M Tris-HCl (pH 7.5), 0.05 M NaCl. A 300-mL linear gradient of 0.05 to 0.30 M NaCl in the same buffer was used to elute the en-

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[1] Abbreviations used: RuP_2, ribulose 1,5-bisphosphate; L, large subunit of RuP_2 carboxylase; S, small subunit of RuP_2 carboxylase; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

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