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Inactivation of *Escherichia coli* Glycerol Kinase by 5,5′-Dithiobis(2-nitrobenzoic acid) and *N*-Ethylmaleimide: Evidence for Nucleotide Regulatory Binding Sites†

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ABSTRACT: Glycerol kinase (EC 2.7.1.30, ATP:glycerol 3-phosphotransferase) from *Escherichia coli* is inactivated by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and by *N*-ethylmaleimide (NEM) in 0.1 M triethanolamine at pH 7 and 25 °C. The inactivation by DTNB is reversed by dithiothreitol. In the cases of both reagents, the kinetics of activity loss are pseudo first order. The dependencies of the rate constants on reagent concentration show that while the inactivation by NEM obeys second-order kinetics (k_2^{app} = 0.3 M^{-1} s^{-1}), DTNB binds to the enzyme prior to the inactivation reaction; i.e., the pseudo-first-order rate constant shows a hyperbolic dependence on DTNB concentration. Complete inactivation by each reagent apparently involves the modification of two sulfhydryl groups per enzyme subunit. However, analysis of the kinetics of DTNB modification, as measured by the release of 2-nitro-5-thiobenzoate, shows that the inactivation is due to the modification of one sulfhydryl group per subunit, while two other groups are modified 6 and 15 times more slowly. The enzyme is protected from inactivation by the ligands glycerol, propane-1,2-diol, ATP, ADP, AMP, and cAMP but not by Mg^{2+}, fructose 1,6-bisphosphate, or propane-1,3-diol. The protection afforded by ATP or AMP is not dependent on Mg^{2+}. The kinetics of DTNB modification are different in the presence of glycerol or ATP, despite the observation that the degree of protection afforded by both of these ligands is the same. Initial velocity studies show that the kinetics with respect to ATP are complex, in agreement with previous observations [Thorner, J. W., & Paulus, H. (1973) *J. Biol. Chem. 248*, 3922–3932]. AMP is an inhibitor and displays complex inhibition behavior with respect to ATP. These results suggest the presence of at least one regulatory binding site for adenine nucleotides on the enzyme.

Glycerol kinase (EC 2.7.1.30, ATP:glycerol 3-phosphotransferase) from *Escherichia coli* catalyzes the MgATP-dependent phosphorylation of glycerol to yield *sn*-glycerol 3-phosphate (Lin, 1976). It is an inducible catabolic enzyme whose metabolic role is to mobilize glycerol as a carbon source. The activity of glycerol kinase is rate-limiting in the metabolism of glycerol by *E. coli* (Zwaig et al., 1970). The reaction proceeds with net inversion of the configuration at the phosphorus, and it is postulated that this may require a general base to deprotonate the attacking glycerol hydroxyl group and a general acid to assist in the departure of ADP (Blättler & Knowles, 1979). The enzyme is subject to feedback inhibition by Fru-1,6-P_2[1] (Zwaig & Lin, 1966). Little is known, however, about roles of specific amino acids in the catalytic and regulatory properties of this enzyme. It has been reported that treatment of the enzyme with different sulfhydryl reagents results in different extents of inactivation at the end of an incubation period (Thorner & Paulus, 1973a). In the absence of ligands, it was reported that DTNB modifies three sulfhydryl groups per subunit, while only 0.5 group is modified in the presence of glycerol (Thorner & Paulus, 1973b). It was postulated that this change in the number of DTNB–titrable sulfhydryl groups upon glycerol binding reflects a substantial

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; Fru-1,6-P_2, fructose 1,6-bisphosphate; Gdn-HCl, guanidine hydrochloride; MMTS, methylmethanethiosulfonate; NEM, *N*-ethylmaleimide; NTCB, 2-nitro-5-(thiocyano)benzoic acid; SDS, sodium dodecyl sulfate; TNB^-, 2-nitro-5-thiobenzoate.

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conformational change. This paper extends these studies of chemical modification of glycerol kinase sulfhydryl groups. Results presented here show that DTNB and NEM inactivate glycerol kinase by modification of a single sulfhydryl group per subunit. Ligand protection and steady-state kinetics studies indicate there are regulatory ligand binding sites for adenine nucleotides.

Materials and Methods

Materials. The following materials were purchased from Sigma Chemical Co. of St. Louis, MO: ATP, AMP, ADP, cAMP, Fru-1,6-P_2, NAD, NADH, DTT, DTNB, NTCB, phosphoenolpyruvate, ammonium sulfate suspensions of α-glycerophosphate dehydrogenase, pyruvate kinase, and lactate dehydrogenase, ampicillin, 2-mercaptoethanol, streptomycin sulfate, Gdn-HCl, triethanolamine hydrochloride, and Sephadex G-200. DEAE-cellulose was a product of Whatman. Electrophoresis reagents, including SDS, were purchased from Bio-Rad. Enzyme-grade ammonium sulfate was purchased from BRL. NEM was obtained from Pierce Chemical Co., and *N*-ethyl[2,3-^{14}C]maleimide was purchased from Amersham. 2-(Chloromercuri)-4-nitrophenol was a product of Eastman Kodak and was generously lent by Dr. C. N. Pace of the Department of Biochemistry and Biophysics. MMTS and 4-vinylpyridine were purchased from Aldrich. Prepacked PD-10 columns containing Sephadex G-25 were purchased from Pharmacia. Components for bacterial culture media were products of Difco.

Glycerol Kinase. Glycerol kinase was purified from a strain of *E. coli* carrying the gene on plasmid pCJ102. This strain was constructed and kindly supplied by Dr. James R. Johnson, Department of Microbiology, Miami University, Oxford, OH. The plasmid was constructed by cloning the *Hin*dIII fragment from the glycerol kinase transducing phage λglpK100 (Conrad et al., 1984) into pBR322 (J. R. Johnson, personal communication). This plasmid was used to transform JJ161, a strain of *E. coli* that is Hfr, RelA1, glpk1, and metB1. Stock cultures of these cells were maintained at −20 °C in Luria broth containing 40% glycerol and 75 μg/mL ampicillin. Overnight cultures were started with 0.05 mL of the stock cultures to inoculate 10 mL of Luria broth. This and all subsequent cultures contained 75 μg/mL ampicillin. After overnight incubation at 37 °C with vigorous shaking, the cultures were used to inoculate 500 mL of Luria broth in a 1-L Erlenmeyer flask. The 500-mL cultures were incubated at 37 °C with vigorous shaking for 8 h. These cultures were used to inoculate 5 L of Luria broth in a 12-L glass carboy. The 5-L cultures were incubated overnight at room temperature with vigorous stirring with a magnetic stir bar and vigorous aeration with filtered compressed air. Two of these 5-L cultures were used to inoculate 180 L of Luria broth in the New Brunswick Fermenter located in the Department of Biochemistry and Biophysics. The cultures were grown overnight, and the cells were chilled and harvested in a continuous-flow centrifuge. Typically, 300 g of cells (wet weight) is obtained. The cells were stored at −20 °C until used.

Glycerol kinase was purified from the cells according to the procedures outlined by Thorner and Paulus (1971) with the following changes: streptomycin sulfate was used rather than 6,9-diamino-2-ethoxyacridine lactate, and the enzyme was crystallized by proceeding directly to the second crystallization step. Crystallization was effected by seeding with glycerol kinase (Calbiochem) that was crystallized as described by Hayashi and Lin (1967). Typically, 200 mg of crystalline glycerol kinase was obtained from 110 g of cells (wet weight). Specific activity of the purified enzyme is 32–37 IU/mg. Electrophoresis in polyacrylamide gels containing SDS (not shown) shows the enzyme to be greater than 95% homogeneous; on overloaded gels, two faint bands of lower mobility than glycerol kinase are visible. The subunit molecular weight, steady-state kinetic properties, and inhibition by Fru-1,6-P_2 were found to be the same as previously described (Thorner & Paulus, 1973b). Thus, the cloning of the enzyme has produced no detectable alterations in its properties.

Crystalline glycerol kinase was stored at 4 °C in the second crystallization buffer of Thorner and Paulus (1971). For these studies, the glycerol and 2-mercaptoethanol were removed by dialysis or Sephadex G-25 gel-permeation chromatography at room temperature with 0.1 M triethanolamine, pH 7. It was found that the enzyme is quite stable under these conditions; the specific activity remains constant for at least 12 h after glycerol removal. The concentration of the enzyme was determined from the absorbance at 280 nm, with an extinction coefficient of 1.4 $(mg/mL)^{-1}$ cm^{-1} (Thorner & Paulus, 1973b). The molar concentration of the enzyme was calculated on the basis of a subunit molecular weight (M_r) of 55000 (Thorner & Paulus, 1971).

Enzyme Assays. The activity of glycerol kinase was routinely determined with an ADP-coupled assay containing 25 IU of pyruvate kinase, 10 IU of lactate dehydrogenase, 5 mM phosphoenolpyruvate, 0.3 mM NADH, 10 mM $MgCl_2$, 20 mM KCl, 2 mM glycerol, and 1 mM ATP in 0.05 M triethanolamine, pH 7. Reactions were initiated by the addition of glycerol kinase to a final concentration of 0.5 μg/mL to cuvettes that were thermostated at 25 °C. Reaction velocities were determined from the rate of decrease of absorbance at 340 nm in a Beckman DU-6 spectrophotometer equipped with the kinetics software package. One unit of glycerol kinase activity is defined as the amount of enzyme catalyzing formation of 1 μmol of product in 1 min under these conditions.

For ligand protection experiments involving ADP, glycerol kinase activity was determined with α-glycerophosphate dehydrogenase to couple the phosphorylation of glycerol to the reduction of NAD. The assay used was essentially that described by Hayashi and Lin (1967) and contained the following components in a final volume of 1.0 mL: 0.15 M glycine buffer, pH 9.5, 11 mM $MgCl_2$, 0.27 M hydrazine, 1.2 mM NAD, 5 mM ATP, 2 mM glycerol, 20 units of α-glycerophosphate dehydrogenase, and glycerol kinase.

Chemical Modification of Glycerol Kinase. In studies of the inactivation of glycerol kinase by sulfhydryl reagents, reactions were initiated by the addition of the reagent. Details of particular experiments are given in the legends to the tables and figures. Aliquots of the reactions were removed for determination of enzyme activity by the assay described above. In the case of DTNB, the kinetics of chemical modification were also followed by the rate of change in absorbance at 412 nm. The concentration of released TNB^- was calculated with the extinction coefficients given by Riddles et al. (1983).

The kinetics of DTNB modification, as determined by the release of TNB^-, were analyzed with the chemical kinetics program KINSIM (Barshop et al., 1983), which was kindly supplied by Dr. Carl Frieden of Washington University Medical School in St. Louis, MO. The following mechanism was used for the simulations:

$$E \xrightarrow{k_1} M$$

$$E' \xrightarrow{k_2} M'$$

$$E'' \xrightarrow{k_3} M''$$

where E, E′, and E″ represent different sulfhydryl groups on

the enzyme and M, M′, and M″ represent enzyme modified at each of those sulfhydryl groups. This mechanism represents the independent modification of the three different groups. The rate of modification is given by the rates of appearance of M, M′, M″, which are governed by the three pseudo-first-order rate constants k_1, k_2, and k_3. In the simulations, the values of these rate constants were adjusted to obtain the best fit by eye to the experimental data. Efforts to use nonlinear least-squares methods to estimate the rate constants (Turner et al., 1981) were unsuccessful due to the similarity of the values of the rate constants.

Incorporation of [^{14}C]NEM. Glycerol kinase at about 1 mg/mL was incubated at room temperature in 0.1 M triethanolamine, pH 7, with 5 mM NEM containing *N*-ethyl-[2,3-^{14}C]maleimide. The decrease in catalytic activity was monitored until about 95% inactivation, at which point 2-mercaptoethanol was added to a final concentration of 0.14 M. The sample was then dialyzed 4 times against 250 volumes of 0.1 M sodium borate buffer at pH 8.8, which contained 6 M Gdn-HCl and 10 mM EDTA, with 6–8 h between changes of the buffer. The protein concentration of the dialyzed sample was determined from the absorbance at 280 nm, and an aliquot was removed for scintillation counting. The specific radioactivity of the [^{14}C]NEM (1.5×10^6 cpm/μmol) was determined by scintillation counting of an aliquot. The concentration of the NEM was determined from the absorbance at 305 nm, with an extinction coefficient of 620 M^{-1} cm^{-1} (Riordan & Vallee, 1967). Liquiscint (National Diagnostics) scintillation cocktail was used in counting of these aqueous samples on a Beckman LS-6800 counter. Aliquots of stock solutions were added to the samples such that the concentrations of Gdn-HCl, triethanolamine, and protein were the same in all the samples. The radioactivity of the final change of dialysis buffer was the same as the background.

Results

Inactivation of Glycerol Kinase by Sulfhydryl Reagents. Incubation of glycerol kinase with DTNB results in a time-dependent loss of enzyme activity. Figure 1A shows a first-order plot of the kinetics of inactivation observed at different concentrations of DTNB. Over this range of DTNB concentrations and under these conditions, the inactivation obeys pseudo-first-order kinetics, and the rate of inactivation increases with increasing DTNB concentration. In the absence of DTNB, no activity is lost over the time course of this experiment. In fact, no activity is lost for up to 12 h, even in the absence of reducing agents, such as DTT.

Figure 1B shows the DTNB concentration dependence of the pseudo-first-order rate constant for the inactivation. That dependence is not linear but appears to be hyperbolic. The inset to the figure shows that a double-reciprocal plot of these data is linear. Thus, the inactivation by DTNB is saturable with respect to the reagent, indicating the equilibrium formation of an enzyme–DTNB complex prior to the reaction that results in loss of catalytic activity. An apparent dissociation constant of 0.4 mM for DTNB binding and an apparent maximum rate constant for inactivation of 0.11 min^{-1} are estimated from the double-reciprocal plot.

Incubation of glycerol kinase with NEM also results in a time-dependent loss of enzyme activity. Figure 1C shows a first-order plot of the kinetics of inactivation observed at different concentrations of NEM. As with DTNB, the inactivation obeys pseudo-first-order kinetics, and the rate of inactivation increases with increasing NEM concentration. There are, however, striking differences between the inactivations by these two reagents. First, as shown in Figure 1D,

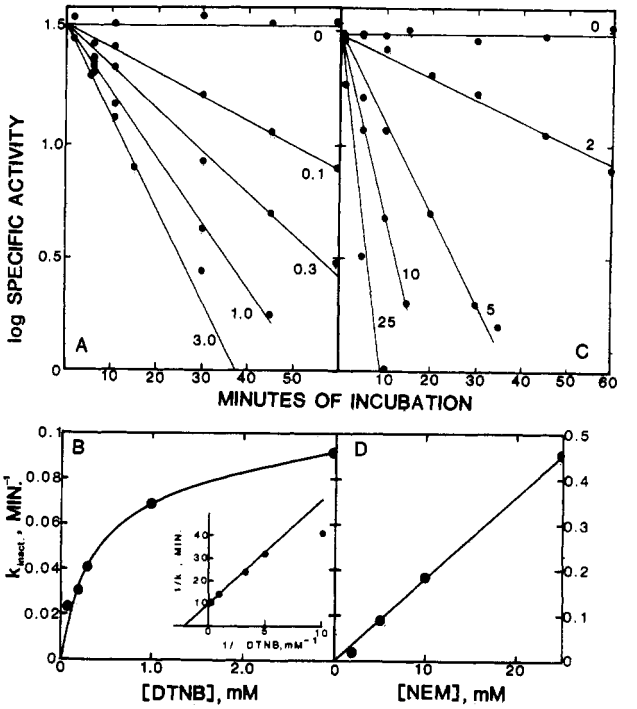

FIGURE 1: Inactivation of glycerol kinase by DTNB and NEM. Enzyme was incubated at 25 °C with DTNB or NEM at the concentrations (mM) indicated in the figure. At the indicated times after reagent addition, 0.01-mL aliquots were removed for determinations of glycerol kinase activity with the ADP-coupled assay described under Materials and Methods. (A) First-order plot of the inactivation by DTNB. (B) DTNB concentration dependence of the pseudo-first-order rate constants for inactivation. Pseudo-first-order rate constants were derived from the slopes of the lines shown in panel A. The inset shows a double-reciprocal plot of these data. (C) First-order plot of NEM inactivation. (D) NEM concentration dependence of the pseudo-first-order rate constants for inactivation. Pseudo-first-order rate constants were derived from the slopes of the lines shown in panel C.

the pseudo-first-order rate constant for inactivation by NEM is linearly dependent on reagent concentration. Thus, the inactivation by NEM appears to obey strictly second-order kinetics. The apparent second-order rate constant under these conditions is 0.30 M^{-1} s^{-1}. Second, enzyme that is inactivated by NEM slowly precipitates, while enzyme modified by DTNB remains in solution.

Inactivation of glycerol kinase by other sulfhydryl reagents was investigated under these same experimental conditions. No inactivation was observed after 60 min of incubation with 0.1 mM NTCB. On the other hand, the enzyme is completely inactivated in less than 1 min of incubation with 3 μM 2-(chloromercuri)-4-nitrophenol. However, the modified enzyme precipitates rapidly. The enzyme activity is not decreased by incubation for 30 min with 0.1 M iodoacetic acid or 0.3% 4-vinylpyridine under these conditions.

Reversal of DTNB Inactivation. The inactivation of glycerol kinase by DTNB is reversible. Incubation of inactivated enzyme at 25 °C with 10 mM DTT in 0.1 M triethanolamine, pH 7, results in the recovery of 86 ± 9% (n = 4) of the initial specific activity. Under these conditions, the recovery of activity is completed within 10 min. This result is consistent with inactivation due to DTNB modification of cysteine sulfhydryls in a disulfide interchange reaction.

Stoichiometries of Modifications by DTNB and NEM. The relationship between loss of enzyme activity and the extent of modification by DTNB, as measured by TNB^- release, is presented in Figure 2. The relationship is linear, and 1.7 cysteine sulfhydryls are modified per subunit upon complete

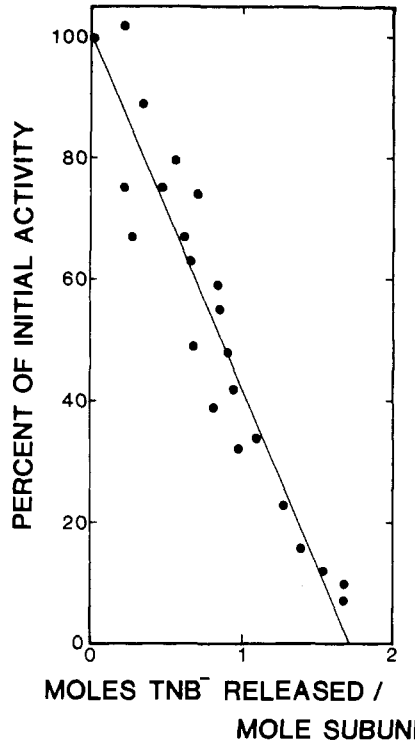

FIGURE 2: Relation between DTNB inactivation of glycerol kinase and release of TNB^-. Glycerol kinase was incubated at 0.2 mg/mL in 0.1 M triethanolamine, pH 7, at 25 °C with 1 mM DTNB. In separate experiments, the absorbance change at 412 nm was recorded or aliquots were removed for determination of glycerol kinase activity with the coupled assay. The number of moles of TNB^- released at the time at which the activity was assayed was calculated from the absorbance change at 412 nm (ϵ = 14.1 mM^{-1} cm^{-1}; Riddles et al., 1983).

Table I: Stoichiometry of NEM Modification

expt	sp act. (% unmodified control)	enzyme concn (mg/mL)	cpm per 0.1 mL	mol of NEM/mol of subunit
1	4	1.42	7685	1.86
2	5	0.91	4930	2.04

inactivation of the enzyme. In our hands, there are 4.5 ± 0.2 (n = 4) DTNB-titrable sulfhydryl groups per subunit, and the same number is obtained in both SDS and Gdn-HCl. This is in good agreement with the reported value of five sulfhydryl groups per subunit (Thorner & Paulus, 1971). Thus, complete inactivation by DTNB appears to involve the modification of 40% of the titrable sulfhydryl groups per subunit, i.e., two of the five groups.

The extent of modification by NEM was determined from the incorporation of [^{14}C]NEM as described under Materials and Methods. Results of these experiments are shown in Table I. In two separate experiments, enzyme that was 95% inactivated was found to contain an average of 1.95 mol of nondialyzable [^{14}C]NEM/mol of subunit. Thus, there is good agreement between the results obtained for the stoichiometries of the modifications by DTNB and NEM.

Kinetics of Modification by DTNB. The kinetics of modification of glycerol kinase by 1 mM DTNB, as measured by the rate of release of TNB^-, are presented in Figure 3. The upper curve shows the rate of modification in the absence of added ligands. The points are the experimentally observed absorbance at 412 nm, corrected for the autooxidation of DTNB. The solid line was calculated as described under Materials and Methods, with the rate constants given in the figure legend. The data are well described in terms of inde-

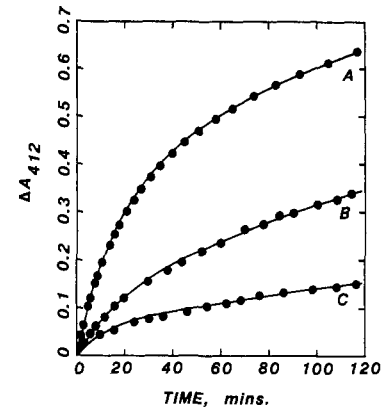

FIGURE 3: Kinetics of DTNB modification of glycerol kinase. Enzyme was incubated at 1.15 mg/mL (0.021 mM subunit) in 0.1 M triethanolamine, pH 7, at 25 °C, with other additions as indicated. The absorbance at 412 nm was recorded following the addition of DTNB to a final concentration of 1 mM at zero minutes. The absorbance shown was corrected for autooxidation of DTNB by subtraction of the absorbance of a blank that contained no enzyme. (A) No other additions. The points are the experimentally observed absorbances, and the solid line was calculated as described under Materials and Methods with the following rate constants (min^{-1}): k_1, 0.065; k_2, 0.01; k_3, 0.005. (B) Experimentally observed absorbance with 5 mM ATP added. (C) Experimentally observed absorbance with 2 mM glycerol added.

pendent modifications of three sulfhydryl groups per subunit, in agreement with an earlier report (Thorner & Paulus, 1973b). Thus, there are three phases of equal amplitude. The rate constant for the fastest of these phases, 0.065 min^{-1}, is the same as the rate constant for the inactivation by 1 mM DTNB (Figure 1B). The rate constants of the other two phases are about 6 and 15 times slower than the rate of inactivation. Attempts to fit the data on the basis of the modification of two sulfhydryl groups per subunit were unsuccessful. Furthermore, a change of 10% in the value of any of the rate constants results in a poorer fit to the data. These results strongly suggest that the inactivation by DTNB is due to the modification of a single sulfhydryl group per subunit. They also indicate that the apparent modification of a second sulfhydryl group upon complete inactivation actually reflects partial modifications of two other sulfhydryl groups.

Effects of Ligands on Inactivations by DTNB and NEM. The effects of various ligands on the inactivations of glycerol kinase by DTNB and NEM are presented in Table II. The same pattern is observed for both reagents. The enzyme is protected from inactivation by glycerol, propane-1,2-diol, ATP, ADP, AMP, and cAMP. The protection afforded by ATP or AMP is not affected by the addition of Mg^{2+}. The following ligands do not protect the enzyme from inactivation: propane-1,3-diol, Mg^{2+}, and Fru-1,6-P_2.

The results obtained for glycerol and Fru-1,6-P_2 are consistent with those reported previously for effects of these ligands on the modification of glycerol kinase by DTNB, as determined by the absorbance at 412 nm (Thorner & Paulus, 1973b). Thus, glycerol decreases the number of sulfhydryl groups that are modified, while Fru-1,6-P_2 has no effect on the DTNB titration.

The protection experiments using ADP provide indirect information about the effects of the DTNB modification on the pH dependence of glycerol kinase kinetic properties. For these experiments, the enzyme activities were determined with the α-glycerophosphate dehydrogenase assay at pH 9.5, rather than the ADP-coupled assay at pH 7. This was necessary because the significant carryover of ADP from the incubation to the assay gave artifactually high reaction velocities. In

Table II: Effects of Ligands on Inactivation of Glycerol Kinase by DTNB and NEM[a]

ligand (final concn)	remaining activity (% control) DTNB (1 mM)	NEM (5 mM)
none	11	13
glycerol (2 mM)	85	63
propane-1,2-diol (10 mM)	23	16
propane-1,3-diol (10 mM)	7	12
$MgCl_2$ (2 mM)	8	11
ATP (1 mM)	68	85
ATP (5 mM)	80	nd
ATP (1 mM) + $MgCl_2$ (2 mM)	72	88
ADP (5 mM)	83[b]	nd
AMP (5 mM)	50	73
AMP (5 mM) + $MgCl_2$ (2 mM)	47	76
cAMP (5 mM)	46	nd
Fru-1,6-P_2 (2 mM)	7	11

[a] Glycerol kinase was incubated at 0.05 mg/mL in 0.1 M triethanolamine, pH 7, at 25 °C with the indicated concentration of DTNB or NEM and other additions as shown. After 30 min, 0.01 mL was removed from the incubation for determination of glycerol kinase activity. The remaining enzyme activity is expressed as a percentage of that of a control incubation to which no additions were made. nd, not determined. [b] Determined with the α-glycerophosphate dehydrogenase assay at pH 9.5 as described under Materials and Methods.

addition to examination of the protection by ADP, the entire series of ligands was repeated. While the specific activity is higher at pH 9.5 than at pH 7, the remaining activities, expressed as a fraction of that of the unmodified control, are the same at both values of pH for all of the ligands. Thus, the inactivation observed at pH 7 does not appear to reflect a shift in the pH dependence of the catalytic properties toward more alkaline values.

AMP Inhibition of Glycerol Kinase. The ligand protection studies show that adenine nucleotides protect glycerol kinase from inactivation by sulfhydryl modification. Since it is a substrate, the protection afforded by ATP may be due to its binding at the active site. This is consistent with the protection observed for glycerol and propane-1,2-diol. However, the basis for the protection afforded by the other adenine nucleotides is less clear. AMP is an inhibitor of glycerol kinases from a variety of sources, and the type of inhibition observed with respect to ATP appears to be uncompetitive in the case of the enzyme from *Candida mycoderma* (Grunnet & Lundquist, 1967). At a concentration of 1 mM, ADP, AMP, and cAMP are inhibitors of the *E. coli* enzyme, and the degree of inhibition decreases in the order ADP > AMP > cAMP (Thorner, 1972). Thus, the relative degree of protection from inactivation that is afforded by these nucleotides appears to be correlated with their effectiveness as inhibitors. However, the type of inhibition by these adenine nucleotides has not been characterized for the *E. coli* enzyme. Consequently, studies were undertaken to define the type of inhibition by AMP.

The effects of AMP on the steady-state kinetic behavior with respect to ATP are presented in Figure 4. In the absence of AMP, the double-reciprocal plot of the ATP concentration dependence of the initial velocity is concave downward, as previously described (Thorner & Paulus, 1973b). This result is consistent with either negative cooperativity in the binding of ATP or the presence of more than one type of ATP binding site on the enzyme. In either case, the ATP dependence of the kinetic behavior is not simple. The data that are obtained in the presence of different concentrations of AMP also show complex kinetic behavior in that the curves are concave downward. AMP is an inhibitor, and the degree of inhibition increases with increasing AMP concentration. However, the

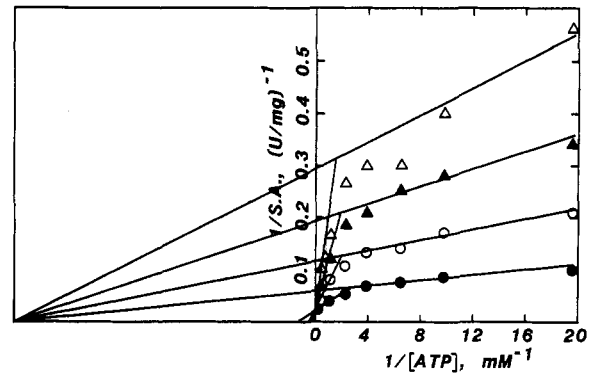

FIGURE 4: AMP inhibition of glycerol kinase. The ATP concentration dependence of the initial velocity was determined with the ADP-coupled assay. Conditions: 0.5 μg/mL glycerol kinase, 25 °C, 0.1 M triethanolamine, pH 7, 2 mM glycerol, and 6 mM $MgCl_2$. AMP concentrations: (●) 0, (○) 1, (▲) 2.5, and (△) 5 mM.

type of inhibition by AMP is complex. The nature of the complexity can be demonstrated by considering a limiting case. The lines shown in Figure 4 are for the limiting case of two classes of ATP binding sites. The data show that the apparent Michaelis constants of the sites are 0.043 mM and 0.83 mM. The data also show that the type of inhibition by AMP is different for each of the two classes of binding sites. The inhibition with respect to the higher affinity site appears to be noncompetitive, while the inhibition with respect to the lower affinity site appears to be competitive. In other studies, AMP was observed to be a noncompetitive inhibitor with respect to glycerol (not shown). In the context of this limiting case, the inhibitory action of AMP is due to binding at a regulatory site, since the apparent noncompetitive inhibition requires that the inhibitor and substrate are simultaneously bound to the enzyme. In the case where the curved double-reciprocal plot reflects negative homotropic interactions between ATP binding sites on different subunits, the complex inhibition by AMP is also consistent with its binding to a regulatory site. Thus, these results suggest the presence of at least one adenine nucleotide regulatory binding site on glycerol kinase.

The presence of a regulatory adenine nucleotide binding site is supported by results of sulfhydryl titrations in the presence of different ligands. The two lower curves in Figure 3 show the kinetics of modification by 1 mM DTNB with either 5 mM ATP or 2 mM glycerol added to the reaction. The points are the experimentally observed absorbances at 412 nm, corrected for DTNB autooxidation. The kinetics of modification by DTNB are significantly different in each case. On the other hand, results presented in Table II show that both ligands afford the same degree of protection from inactivation by DTNB. The different modification kinetics in the presence of these ligands indicates that the enzyme conformation with bound glycerol is different from that with bound ATP.

Modifications with Smaller Reagents. It has been shown (Degani & Patchornik, 1974) that effects of the introduction of the small and uncharged cyano group as an S substituent may be used as a measure of how essential a sulfhydryl group is for enzyme function. Thus, it was of interest to determine whether cyanylation of DTNB-modified glycerol kinase restored activity. Glycerol kinase was incubated with DTNB until more than 99% inactivated. The enzyme was chromatographed on Sephadex G-25 to remove unreacted DTNB and TNB^-. KCN was added to a final concentration of 0.1 M to cyanylate the modified sulfhydryls (Jacobson et al., 1973). Measurements of the absorbance at 412 nm show that the cyanylation, i.e., release of TNB^-, is completed within 5 min

and 1.6 mol of TNB^- is released per mole of subunit. The cyanylated enzyme has 10% of the specific activity of native enzyme, and this is unchanged by dialysis against 0.1 M triethanolamine, pH 7. However, incubation of the cyanylated enzyme with DTNB resulted in inactivation. Furthermore, initial velocity studies showed that the Michaelis constants for both substrates and the complex ATP kinetics were the same for the cyanylated enzyme as for native enzyme. These results suggest that the activity of the cyanylated enzyme may be due to residual unmodified enzyme. This suggests that the introduction of the small cyano group at the modified sulfhydryl group is sufficient to inactivate the enzyme.

Preliminary investigations were also conducted with MMTS. This reagent also inactivates glycerol kinase. However, the kinetics of inactivation are biphasic, and only about one-third of the activity is restored by DTT. Thus, inactivation by this reagent is more complex than that observed for DTNB or NEM. However, it appears that introduction of a mixed disulfide with a thiomethyl group at the modified sulfhydryl is also sufficient to inactivate glycerol kinase. These results on modifications with smaller reagents suggest that a sulfhydryl group is essential for activity and that any modification of this residue inactivates the enzyme.

Discussion

This study is the first using enzyme purified from the cloned copy of the gene that codes for *E. coli* glycerol kinase. As discussed under Materials and Methods, the properties of this enzyme are the same as those reported earlier for the chromosomal enzyme (Thorner & Paulus, 1973b). Incubation of this glycerol kinase with DTNB, NEM, or 2-(chloromercuri)-4-nitrophenol results in inactivation of the enzyme. Under the conditions used in these studies, these reagents are highly specific for the modification of sulfhydryl groups. Furthermore, the inactivation by DTNB is reversed by DTT. Thus, the loss of activity involves the modification of enzyme sulfhydryl groups. Determinations of the stoichiometries of the modifications by DTNB and NEM show that about two sulfhydryl groups per subunit are modified upon inactivation of the enzyme. However, other lines of evidence strongly suggest that the inactivation is due to the modification of a single sulfhydryl group per subunit. For both DTNB and NEM, the inactivation obeys pseudo-first-order kinetics. Second, the kinetics of modification by DTNB, as measured by TNB^- release, show that one group is modified at a rate equal to that of the loss of activity, while two additional groups are modified more slowly. The observation of the same pattern of ligand protection for both DTNB and NEM suggests that the same sulfhydryl group is modified by both reagents.

The reactions of both DTNB and NEM with glycerol kinase are significantly slower than their reactions with model compounds. For DTNB, a second-order rate constant of 367 M^{-1} s^{-1} was observed for reaction with a peptidyl sulfhydryl group at pH 7 and 23 °C (Snyder et al., 1981). An apparent second-order rate constant for the reaction of DTNB and glycerol kinase at pH 7 and 25 °C can be estimated by dividing the maximum pseudo-first-order rate constant for the inactivation by the apparent dissociation constant for DTNB binding in a manner analogous to the use of k_{cat}/K_m in enzyme kinetics (Fersht, 1977). This treatment gives an apparent rate constant of 4.6 M^{-1} s^{-1}, which is 80-fold less than that for reaction with the model compound. In the case of NEM, the second-order rate constant for reaction with cysteine at pH 7 is estimated to be 1500 M^{-1} s^{-1} (Gorin et al., 1966). The rate constant estimated for the reaction of NEM with glycerol kinase is 0.3 M^{-1} s^{-1}, which is 5000-fold less than that for the model compound. This slower rate of reaction with NEM has been observed for sulfhydryl groups in several enzymes, and it has been concluded that addition of sulfhydryl groups at the surface of an enzyme to the double bond in NEM is hindered for electronic and/or steric reasons (Brubacher & Glick, 1974). Decreased reactivity of enzyme sulfhydryl groups may reflect several factors (Torchinskii, 1974). These include steric screening by neighboring groups and effects of nearby charged groups on the approach of ionized reagents and on the ionization of the sulfhydryl group. In the case of disulfide interchange reactions in proteins, it has been shown that local electrostatic effects dominate the reactivities of the disulfides (Shaked et al., 1980; Snyder, 1984).

The different kinetic behavior of DTNB and NEM with respect to inactivation of glycerol kinase may reflect different affinities for a binding site near the modified group. Saturation kinetics have been observed in the modification of bovine serum albumin with fluorodinitrobenzene (Green, 1963) and in the modification of D-amino acid oxidase with *N*-alkylmaleimides (Fonda & Anderson, 1969) or 1,2-cyclohexanedione (Ferti et al., 1981). In each of these cases, the observed saturation kinetics has been interpreted in terms of a two-step mechanism for the modification, where the first step is binding of the reagent to a nonpolar region of the protein in the vicinity of the modified amino acid. Ornithine transcarbamoylases are inactivated by DTNB, and DTNB binds to the enzymes prior to the inactivation (Marshall & Cohen, 1980). In this case, the binding is thought to reflect specific interactions between the reagent and the sulfhydryl group, which result in its enhanced reactivity. Thus, the saturation kinetics observed in the inactivation of glycerol kinase by DTNB indicate that the reagent binds to a region near the modified sulfhydryl group. The apparent absence of binding of NEM may reflect differences in polarity and/or size. If binding of DTNB is due to the aromatic ring, then saturation kinetics may be observed with other N-substituted maleimides, as is the case with D-amino acid oxidase (Fonda & Anderson, 1969). On the other hand, the binding of DTNB may involve electrostatic interactions with the ionized carboxyl groups on the reagent.

The observation that modification of a cysteine side chain affects the kinetic properties of glycerol kinase is consistent with results obtained for other kinases. In the cases of pyruvate kinase (Flashner et al., 1972) and hexokinase (Otieno et al., 1977), the modified groups are at the active sites of these enzymes. This does not appear to be the case for creatine kinase (Degani & Degani, 1979) or adenylate kinase (Whitesides et al., 1979). For both of these enzymes, modification with DTNB causes inactivation, but modification with smaller groups (cyanylation or methylmethanethiosulfonate, respectively) only partially decreases the enzyme activity. In the case of rabbit skeletal muscle phosphofructokinase (Kemp, 1969), the modified cysteine is not located at the active site of the enzyme.

The protection from inactivation of glycerol kinase afforded by glycerol and its analogues suggests that the modified cysteine is located at or near the active site. Since glycerol is a substrate, it binds at the active site. Under the conditions used in these studies, propane-1,2-diol acts as a competitive inhibitor with an apparent K_i of 3.6 mM.[2] It should be noted, however, that the propane-1,2-diol used in these experiments is a racemic mixture. It has been shown for glycerol kinase from *C. mycoderma* that (−)-propane-1,2-diol (1-deoxy-*sn*-glycerol) is a substrate (K_m = 45 mM), while (+)-propane-

[2] Unpublished experiments.

1,2-diol (3-deoxy-*sn*-glycerol) is a competitive inhibitor (K_i = 4.6 mM) (Eisenthal et al., 1972). Thus, this ligand also binds at the active site. In the case of the *C. mycoderma* enzyme, propane-1,3-diol is a substrate with an apparent K_m of 88 mM (Eisenthal et al., 1972). At the concentration used in these studies (10 mM), it is neither a substrate nor an inhibitor of the *E. coli* enzyme.[2] This is consistent with its lack of protection against inactivation of the enzyme. The degree of protection afforded by these active site ligands is correlated with their apparent affinities for binding.

The protection from the inactivation that is afforded by the adenine nucleotides is somewhat more complex. The Mg^{2+} complex of ATP is the true substrate (Hayashi & Lin, 1967). Steady-state kinetic studies of the glycerol kinases from *C. mycoderma* (Janson & Cleland, 1974) and *E. coli* (Thorner & Paulus, 1973b) are consistent with an ordered kinetic mechanism with glycerol binding to the enzyme first. It is possible, however, that MgATP can bind to the active site in the absence of glycerol. In this case, the protection obtained with MgATP would be consistent with the protection obtained with glycerol, suggesting that the modified sulfhydryl group may be located at or near the active site of the enzyme. However, several considerations suggest that the modified group may not be at the active site. The ATP protection from inactivation is not dependent on Mg^{2+}. The results of the DTNB titrations described above show that the conformations of glycerol kinase are different with bound glycerol or bound ATP. Finally, the complex nature of the inhibition observed with AMP is consistent with binding at adenine nucleotide regulatory binding sites.

In the initial report of the effect of glycerol upon the titration of glycerol kinase by DTNB, it was postulated that the large decrease in the number of titrable sulfhydryl groups observed upon the binding of glycerol reflected an enzyme conformational change (Thorner & Paulus, 1973b). Results of the ligand protection experiments described here are consistent with the postulated conformational change. Such a conformational change could explain how binding of ligands at either the active site, e.g., glycerol, or the putative adenine nucleotide binding sites, e.g., ATP or AMP, affords protection from the inactivation. Binding of these ligands at separate sites is also consistent with the observation of different conformations with glycerol or with ATP bound (Figure 3). While results presented here are not sufficient to define the relation between location of the modified sulfhydryl and ligand binding sites on glycerol kinase, it is clear that the sulfhydryl is located in a region of functional importance. It appears that *E. coli* glycerol kinase may be grouped with creatine kinase, adenylate kinase, and phosphofructokinase in that the cysteine whose modification inactivates the enzyme may not be located at the active site. Furthermore, glycerol kinase appears to be similar to phosphofructokinase with respect to adenine nucleotide regulatory binding sites (Pettigrew & Frieden, 1978).

It has recently been shown that there is a regulatory site on *E. coli* glycerol kinase that mediates inhibition by enzyme III^{Glc} of the bacterial phosphotransferase system (Novotny et al., 1985). This site is distinct from the site for Fru-1,6-P_2. Results presented here indicate that the putative regulatory sites for adenine nucleotides are also distinct from the site for Fru-1,6-P_2, since this inhibitory ligand affords no protection from the inactivation. These results indicate that glycerol kinase is subject to several types of regulation involving both protein–ligand and protein–protein interactions. These findings are consistent with the role of glycerol kinase as the focal point of regulation of glycerol metabolism in *E. coli* (Zwaig et al., 1970). Since it catalyzes the first reaction in the pathway, albeit a catabolic pathway, it is not surprising that it appears to be subject to a variety of controls. Furthermore, since the pathway for glycerol metabolism enters glycolysis after the phosphofructokinase reaction, modulation of glycerol kinase could serve to control the flux through the second portion of the glycolytic pathway.

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Registry No. DTNB, 69-78-3; NEM, 128-53-0; ATP, 56-65-5; ADP, 58-64-0; AMP, 61-19-8; cAMP, 60-92-4; EC 2.7.1.30, 9030-66-4; $HOCH_2CH(OH)CH_2OH$, 56-81-5; $HOCH_2CH(OH)Me$, 57-55-6.

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Effects of Diethylstilbestrol, 2,2′-Dithiodipyridine, and Chloral Hydrate on the Esterase Activity of Sheep Liver Cytoplasmic Aldehyde Dehydrogenase

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ABSTRACT: The binding of diethylstilbestrol (DES) to aldehyde dehydrogenase (ALDH) has a very similar effect on the dehydrogenase activity of the enzyme as has modification of the enzyme by 2,2′-dithiodipyridine [Kitson, T. M. (1982) *Biochem. J. 207*, 81-89]. The latter modification may occur at the site of the esterase activity of the enzyme [Kitson, T. M. (1985) *Biochem. J. 228*, 765-767]. This suggests that DES might be a competitive inhibitor of the esterase reaction. However, in the absence of oxidized nicotinamide adenine dinucleotide (NAD^+) or reduced nicotinamide adenine dinucleotide (NADH), and at low concentrations of substrate (4-nitrophenyl acetate, PNPA), DES is a potent partial noncompetitive inhibitor. It is concluded therefore that DES binds at a site different from the esterase active site and that the enzyme–DES complex retains some ability to act as an esterase. High concentrations of PNPA appear to displace DES from its binding site. In the presence of NAD^+, DES is a weaker inhibitor, and in the presence of NADH, DES has very little effect. Esterase activity is enhanced by NADH when PNPA concentrations are high but is inhibited when they are low. The rate of reaction of ALDH with 2,2′-dithiodipyridine is only slightly reduced by DES, suggesting that the site at which thiol modifiers react and the DES binding site are different. When ALDH is modified by 2,2′-dithiodipyridine, it has reduced esterase activity, which declines further as the modified enzyme loses its 2-thiopyridyl label. In the presence of NAD^+, chloral hydrate is a simple competitive inhibitor of the esterase reaction. The results are consistent with a single site on ALDH being responsible for the dehydrogenase and esterase activities, the binding of aldehydes at high concentration, and the reaction of the enzyme with thiol modifiers. A second site, the physiological significance of which is unknown, has the ability to bind DES and steroids.

The cytoplasmic aldehyde dehydrogenase (EC 1.2.1.3, ALDH[1]) of sheep liver has received considerable attention from a number of aspects. These include its dehydrogenase activity [e.g., MacGibbon et al. (1977a)], its esterase activity (Blackwell et al., 1983b), the effect of thiol-modifying reagents such as disulfiram and 2,2′-dithiodipyridine (Kitson, 1982a, 1984), the effect of steroid hormones and diethylstilbestrol (DES) (Kitson, 1982b; Kitson & Crow, 1982), and the observation of acyl-enzyme intermediates during its catalytic cycle (Dunn & Buckley, 1985).

On the basis of previous work [see Kitson (1985)] it has been concluded that disulfiram and 2,2′-dithiodipyridine oxidize a particular pair of enzymic thiol groups to a disulfide and that the site where this happens has the ability to bind aldehydes at high concentrations and is also probably the active site for the enzyme's esterase action. That this should also be the enzyme's dehydrogenase active site is a natural assumption [see, for example, Eckfeldt and Yonetani (1976) and Takahashi and Weiner (1981)], and evidence in support of this has been presented (Duncan, 1979). However, other

[1] Abbreviations: ALDH, aldehyde dehydrogenase; PNPA, 4-nitrophenyl acetate; DES, diethylstilbestrol; E-S-S-(2-pyridyl), aldehyde dehydrogenase covalently modified by reaction with 2,2′-dithiodipyridine; NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide.

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