# Inactivation–Reactivation of Two-Electron Reduced *Escherichia coli* Glutathione Reductase Involving a Dimer–Monomer Equilibrium†

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ABSTRACT: Glutathione reductase from *Escherichia coli* is inactivated when incubated with either NADPH or NADH. The process is inversely dependent on the enzyme concentration. Inactivation is rapid and monophasic with 1 $\mu$M NADPH and 1 nM enzyme FAD giving a $t_{1/2}$ of 1 min. Complex formation between NADPH and the two-electron reduced enzyme ($EH_2$) at higher levels of NADPH protects against rapid inactivation. $NADP^+$, produced in a side reaction with oxygen, also protects by forming a complex with $EH_2$. These complexes make analysis of the concentration dependence of the inactivation process difficult. Inactivation with NADH, where complexes do not interfere, is slower but can be analyzed more readily. With 152 $\mu$M NADH and 5.4 nM enzyme FAD, the time required for 50% inactivation is 17 min. The process is markedly biphasic, reaching the final inactivation level after 5–7 h. Analysis of the relationship between the final level of inactivation with NADH and the enzyme concentration indicates that inactivation is due to dissociation of the normally dimeric enzyme. Thus, the position of the dimer–monomer equilibrium between an active dimeric two-electron reduced species and an inactive monomeric two-electron reduced form determines the enzyme activity. An apparent equilibrium constant ($K_d$) for dissociation of dimer obtained from the anaerobic concentration dependent inactivation curves is 220 nM. Enzyme inactivated with NADH can be reactivated with glutathione, and the reactivation kinetics are second order, monomer–monomer over 75% of the reaction with an average apparent association rate constant ($k_a$) of 13.1 ($\pm$5.5) $\times$ $10^6$ $M^{-1}$ $min^{-1}$.

The flavoenzyme glutathione reductase (EC 1.6.4.2) catalyzes the NADPH-dependent reduction of oxidized glutathione to two molecules of glutathione. The enzyme belongs to the pyridine nucleotide–disulfide oxidoreductase family which includes lipoamide dehydrogenase and thioredoxin reductase, each of which is dimeric (Williams, 1976; Schirmer & Schulz, 1987). Glutathione reductase from yeast and from human erythrocytes has been studied extensively and compared mechanistically with lipoamide dehydrogenase with which it is homologous in all three domains (Williams et al., 1982; Rice et al., 1984). The X-ray crystal structure of the human erythrocyte enzyme is known at high resolution and shows that the isoalloxazine ring is positioned between the two independent substrate binding sites. Furthermore, it reveals that elements from both polypeptides, including the redox-active disulfide and a histidine residue, contribute to each active site and to the oxidized glutathione binding site (Thieme et al., 1981). The structure suggests that the monomeric species should be inactive in the glutathione reductase reaction which involves elements from both subunits but should be active in reactions involving only the flavin such as diaphorase and oxidase reactions.

Veeger and his associates were the first to propose a dimer–monomer equilibrium affecting activity in the physiological and diaphorase reactions of pig heart lipoamide dehydrogenase (Kalse & Veeger, 1968; Visser & Veeger, 1968). They observed a decrease of the dihydrolipoate dehydrogenase activity and of the molecular weight (based on light-scattering measurements) upon enzyme dilution. The diaphorase activity, on the other hand, increased upon dilution.

Worthington and Rosemeyer (1976) observed the inactivation of human erythrocyte glutathione reductase in the presence of NADPH and associated the process with aggregation.

Lopez-Barea and his colleagues have studied the inactivation of mouse liver (Lopez-Barea & Lee, 1979), yeast (Pinto et al., 1984, 1985), and *Escherichia coli* (Mata et al., 1985) glutathione reductase by NADPH and NADH. Their data provide some basic properties of the inactivation process, especially for the yeast enzyme. They propose that the process involves intramolecular thiol oxidation. Since they have suggested that the inactivation is important for physiological control, we feel that the mechanism should be more completely understood.

We have studied the inactivation of *E. coli* glutathione reductase by NADPH, the physiological substrate, and by NADH. We find that the extent to which the inactivation proceeds is inversely related to the enzyme concentration, suggesting that dimer dissociation is involved.

## Materials and Methods

Glutathione reductase from *E. coli* was purified and characterized as described by Williams and Arscott (1970). Enzyme concentration is expressed as the concentration of enzyme-bound FAD using an extinction coefficient of 11 300 $M^{-1}$ $cm^{-1}$ at 462 nm (Massey & Williams, 1965). NADPH, NADH, $NADP^+$, $NAD^+$, oxidized glutathione (GSSG),[1] and GSH were of analytical grade and obtained from Sigma Chemical Co.

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[1] Abbreviations: $E_{ox}$, oxidized enzyme; $EH_2$, two-electron reduced enzyme; GSH, reduced glutathione, GSSG, oxidized glutathione.

*Enzyme Inactivation–Reactivation and Catalytic Assay.* For all inactivation reactions with either NADH or NADPH, a predetermined volume of concentrated (ca. 10–12 μM FAD) *E. coli* glutathione reductase was added to a large volume of appropriate buffer (see figure legends) to give the final preincubation concentration desired. At zero time, a concentrated solution of either NADH or NADPH was added to the diluted enzyme to start the inactivation. The total preincubation volume was sufficient for the number of aliquots to be withdrawn for subsequent activity measurements. For reactivation experiments (with $NAD^+$, $NADP^+$, or GSH), 8–10 mL of the preincubation mixture was brought to the desired concentration of reactivator by addition from a concentrated solution. Catalytic activity was measured, either in a Cary 118C spectrophotometer or in a Beckman DU output through a log convertor to a recorder, by following the decrease in absorbance at 340 nm at 25 °C. The assay buffer concentrations and pH values were identical with the preincubation conditions (see Figure legends); assays were initiated by adding an aliquot of the preincubation mixture to give a final volume of 1.6 mL and final concentrations of 100 μM NADPH and 3.5 mM GSSG. The standard deviation of these assays was ±3%. An optimum enzyme concentration in this assay was found to be 1–2 nM enzyme-bound FAD. Activity is defined as the moles of NADPH oxidized per minute per mole of enzyme-bound FAD. Anaerobic inactivations were performed after six cycles of evacuation and flushing with oxygen-free $N_2$ of the inactivation mixture. Anaerobic aliquots were withdrawn from the apparatus (Williams et al., 1979) and assayed aerobically as described above. The buffers used in both the preincubation and catalytic assay solutions for the effect of pH on activity and inactivation were as follows: pH 5.8–8.0, 94 mM mixtures of $NaH_2PO_4$ and $K_2HPO_4$; pH 8.3–9.3, 94 mM Tris (adjusted with concentrated HCl); all buffers contained 0.3 mM EDTA. No attempt was made to control ionic strength or specific ions in the pH study. The complexity of these effects in yeast glutathione reductase has been studied (Moroff & Brandt, 1973, 1975).

*NADPH–Oxidase Measurements.* The change in absorbance at 340 nm due to the oxidation of NADPH by oxygen catalyzed by *E. coli* glutathione reductase was recorded with time in a Cary 118C thermostated at 25 °C. To increase the signal of a 1.2 μM NADPH solution, 10-cm path-length cells holding ca. 30 mL were used. Fluorescence measurements were done on a G.K. Turner Model 111 fluorometer with a temperature-stabilized sample holder maintained at 25 °C. The decrease in NADPH fluorescence was recorded with excitation at 340 nm (Corning 7-60 narrow-pass filter) and emission at 456 nm (combination Kodak Wratten 2A sharp-cut and Corning 48 narrow-pass filters). Final buffer concentration in both methods was 0.1 M sodium/potassium phosphate, pH 7.6, and 0.3 mM EDTA.

## Results

*Inactivation of Glutathione Reductase with NADH: Effect of Enzyme Concentration.* When glutathione reductase is preincubated with 150 μM NADH and subsequently assayed by the addition of saturating substrates to aliquots of the preincubation mixture, activity decreases biphasically and reaches a semistable value after about 5–7 h of incubation. Figure 1 shows the percent remaining activity as a function of the preincubation time at various concentrations of enzyme. The $t_{1/2}$ is ca. 17 min for the lowest concentration of enzyme. The inverse influence of the initial enzyme concentration on the level of inactivation throughout the time course is notable. If the assumption is made that catalytic activity reflects a

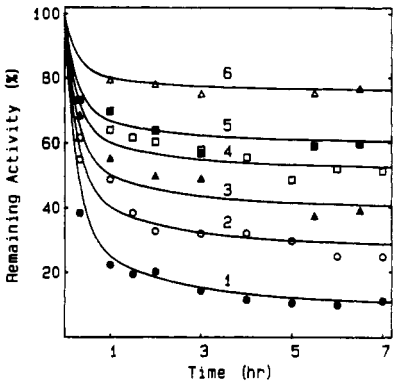



FIGURE 1: Time course of inactivation of *E. coli* glutathione reductase by incubation with NADH: dependence on enzyme concentration. Various concentrations of enzyme (curves 1–6: 5.4, 26.8, 53.4, 85.1, 127, and 372 nM, respectively) were preincubated at 25 °C in 150 μM NADH, 50 mM Tris buffer, and 0.3 mM EDTA, pH 7.5. Catalytic activity was determined by adding aliquots of the preincubation mixture to a standardized NADPH/GSSG assay solution (see Materials and Methods). The diluted enzyme concentration in the assay was 1.8 nM.

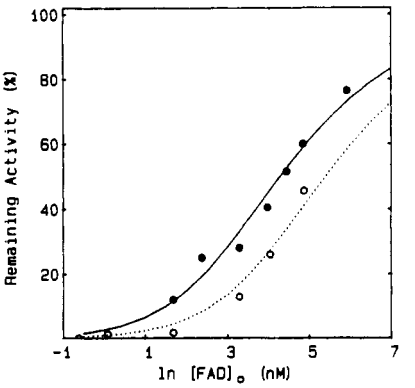



FIGURE 2: Dependence of the remaining activity level on the ln of the initial enzyme concentration. The level of remaining activity at 5–7 h for each enzyme concentration of Figure 1 (●). The solid line represents a theoretical dimer to monomer $K_d$ of 73 nM. Conditions as in Figure 1 except that the preincubation mixture was anaerobic (see Materials and Methods) (○). Dotted line assumes a $K_d$ of 220 nM.

dimer–monomer equilibrium in which dimer is active and monomer is not active catalytically, the expressions can be used to analyze the data of Figure 1:

$$\text{dimer} \rightleftharpoons 2 \text{ monomer} \quad (1)$$

$$K_d = [\text{monomer}]^2/[\text{dimer}] \quad (2)$$

where dimer is the native two-electron reduced dimeric enzyme and monomer is the putative two-electron reduced monomer. The total enzyme concentration, $E_t$, is equated with the $[FAD]_0$, synonymous with active-site concentration. The conservation equation applies:

$$E_t = 2[\text{dimer}] + [\text{monomer}] \quad (3)$$

Activity or remaining activity is expressed as turnover number, moles of pyridine nucleotide oxidized per minute per mole of FAD, and the fraction of remaining activity, $A_r$, is

$$A_r = 2[\text{dimer}]/E_t \quad (4)$$

Rearranging eq 3 and eq 4 to solve for [monomer] and [dimer], respectively, and substituting into eq 2 give

$$K_d = 2E_t(1 - A_r)^2/A_r \quad (5)$$

Figure 2 (closed circles) shows the remaining activity as a function of ln $E_t$ (to compress the FAD concentration range)

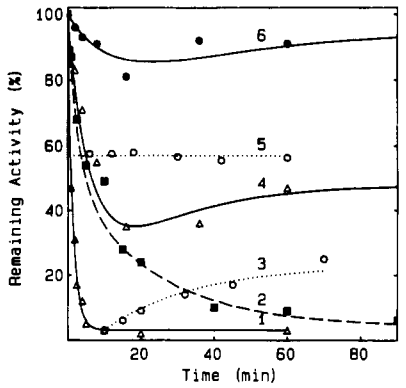
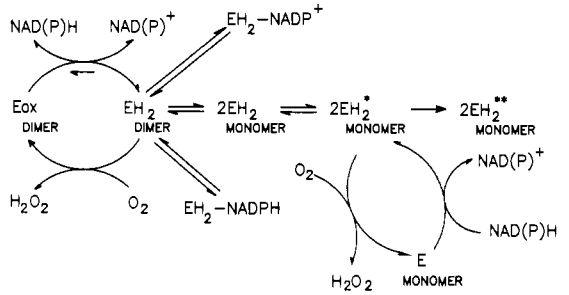



FIGURE 3: Inactivation of *E. coli* glutathione reductase by incubation with NADPH in the presence and absence of $NADP^+$. All preincubations at 25 °C in 67 mM phosphate buffer/0.3 mM EDTA, pH 7.6, with enzyme at a concentration of 1.2 nM. Preincubate with 1 μM NaDPH: curve 1, no $NADP^+$; curve 4, 0.5 μM $NADP^+$; curve 6, 5.0 μM $NADP^+$; curve 5, made 10 μM $NADP^+$ after 0.5-min preincubation; curve 3, made 20 μM $NADP^+$ after 10-min preincubation. Curve 2, preincubated enzyme with 100 μM NADPH, no $NADP^+$ added.

from the data of Figure 1. The solid line represents a theoretical $K_d$ of 73 nM. The aerobic inactivation profiles, using NADH as reductant, at enzyme concentrations less than 5.0 nM were very erratic, and it was suspected that at these enzyme concentrations the side reaction of $EH_2$ dimer with oxygen might be the source of the problem. When the preincubation solution was made anaerobic (see Materials and Methods), the final level of remaining activity was 10–20% lower (open circles, Figure 2). The kinetics were still biphasic. The apparent $K_d$ for dissociation of dimer to monomer in the anaerobic inactivation with NADH was 220 nM or more than 4-fold less than the concentration of glutathione reductase in the cell [as calculated from Williams and Arscott (1970)]. Thus, the side reaction of $EH_2$ dimer (and possibly E monomer) with oxygen leads to an underestimation of $K_d$ due to competition between $EH_2$ dimer to monomerize or to reoxidize with $O_2$. This becomes readily apparent only at the lowest enzyme concentrations used in these experiments (oxidase activity has been measured, see below).

*Inactivation of Glutathione Reductase with NADPH.* Glutathione reductase at a concentration of 1 nM is inactivated, in a monophasic process, by preincubation with 1 μM NADPH with an apparent $t_{1/2}$ of 1 min (Figure 3, curve 1). This is in contrast to the inactivation with NADH where 80–150 μM reductant is needed to effect similar final levels of activity and the process is much slower; the reason for this is not obvious. At higher concentrations of NADPH, the rate of inactivation is much slower. As shown in Figure 3, curve 2, after 90 min with 100 μM NADPH only 6% activity remains, associated with a $t_{1/2}$ of 7 min. This 7-fold decrease in the initial rate of inactivation is ascribed to the formation of the dimeric $EH_2$–NADPH complex at the higher NADPH concentration, thus removing free $EH_2$-dimer from equilibrium with $EH_2$-monomer (see Scheme I). If the $K_d$ of 12 μM for the dissociation of the $EH_2$–NADPH complex (Williams et al., 1976) is applied to the preincubation conditions of 100 μM NADPH and 1 nM glutathione reductase, 89% of the rapidly formed $EH_2$ is immediately complexed with NADPH. Thus, at cellular concentrations of NADPH of 150 μM (Bochner & Ames, 1982), more than 90% of the enzyme would be complexed. On the other hand, when 1 μM NADPH and 1 nM glutathione reductase are used, only 8% of $EH_2$ is complexed, leaving 92% free to dissociate to a putative monomeric $EH_2$ form.

Scheme I



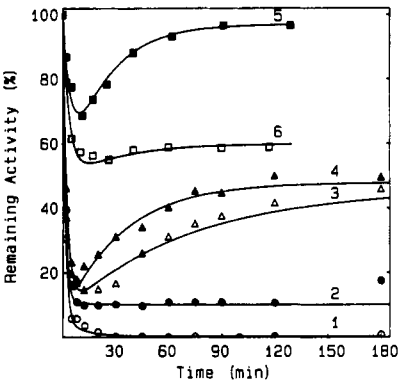




FIGURE 4: Inactivation of *E. coli* glutathione reductase by NADPH: dependence on enzyme concentration. All preincubations at 25 °C in 50 mM Tris-HCl buffer/0.3 mM EDTA, pH 8.0. The NADPH concentration in the preincubation mixture was 1.2 μM, and the enzyme concentration was 2.1, 5.3, 8.5, 11.6, and 42.7 nM for curves 1–5 respectively. The conditions for the experiment shown in curve 6 were identical with those of the inactivation given in curve 5 except that the preincubation was anaerobic.

Two-electron reduced glutathione reductase also forms a complex with $NADP^+$ (Williams et al., 1976). Not surprisingly then, the enzyme is protected from inactivation and reactivated by $NADP^+$, as is seen in Figure 3, curves 3 and 5, where the protector was added after 10 and 0.5 min of preincubation, respectively, and in curves 4 and 6, where $NADP^+$ was present at the beginning of the preincubation. The latter two traces also bring out the fact that as $NADP^+$ builds up as a product of the oxidase activity, protection–reactivation increases. It will be shown below that the NADPH oxidase activity of glutathione reductase is not inhibited in concert with the NADPH–GSSG activity. The reactivation is not evident in curve 5 since the level of $NADP^+$ added is high relative to that produced in the very slow reaction with oxygen. As expected, $NADP^+$ also protects against inactivation with NADH when added at the start of preincubation. It only minimally reactivates NADH-inactivated enzyme when added 2–6 h into preincubation (10% at 90 min; 5% at 4.5 h). Mata et al. (1985) have also observed $NADP^+$ protection in inactivations by various reductants. $NAD^+$, on the other hand, does not protect against inactivation with NADH even at concentrations as high as 1 mM. It does slow the process presumably by competition with NADH (time for 50% inactivation is 35 min with 100 μM $NAD^+$ and 120 min with 1.0 mM $NAD^+$ using 5.5 nM enzyme and 150 μM NADH).

The final level of inactivation with NADPH is strictly dependent on the enzyme concentration (Figure 4) just as is the case with NADH. The dependence only appears less marked due to protection by NADPH and $NADP^+$. The $NADP^+$ generation and protection phenomena are demonstrated in Figure 4, where the NADPH concentration was held constant and the enzyme concentration was increased to give

Table I: Oxidase Activity of *E. coli* Glutathione Reductase

| expt group | enzyme $[FAD]_0$ | [NADPH) ($\mu$M) | measurement[a] method | TN[b] |
|---|---|---|---|---|
| 1 | 11.9 nM | 1.24 | absorbance | 0.40 ± 0.06 |
| | 23.0 nM | 1.19 | absorbance | 0.41 ± 0.06 |
| 2 | 0.33 $\mu$M | 111.1 | absorbance | 0.45 ± 0.02 |
| | 0.66 $\mu$M | 111.1 | absorbance | 0.50 ± 0.02 |
| | 3.89 $\mu$M | 32.6 | absorbance | 0.82 ± 0.03 |
| 3 | 11.9 nM | 1.0 | fluorescence | 0.36 ± 0.05 |
| | 24.0 nM | 1.0 | fluorescence | 0.36 ± 0.04 |
| | 47.0 nM | 1.0 | fluorescence | 0.47 ± 0.03 |

[a] See Materials and Methods. [b] TN, turnover number, is the number of moles of NADPH oxidized per minute per mole of enzyme flavin, $[FAD]_0$.

NADPH:enzyme–FAD ratios ranging from 544:1 to 28:1 in curves 1–5. In an experiment using the same enzyme and NADPH concentrations as in the inactivation shown in curve 5 but preincubated under anaerobic conditions (curve 6), there is essentially no reactivation. Attempts to further demonstrate that aerobic $NADP^+$ production leads to reactivation using glucose 6-phosphate plus glucose-6-phosphate dehydrogenase or using NADase were not successful due to contamination of both enzymes with glutathione reductase. A concentration of 12 nM glutathione reductase (curve 4) leads to a remaining activity of 50%, whereas preincubation of 11 nM enzyme with NADH gives more extensive inactivation—25% remaining activity (Figure 1). However, if the NADPH to enzyme ratio is kept at 1000:1 (data not shown), the remaining activity with 11 nM glutathione reductase is close to that observed when the same concentration of enzyme is inactivated with 150 $\mu$M NADH. The complexity of the inactivation process when NADPH is used precludes the type of analysis carried out on the data obtained in preincubations with NADH, where complex formation is not a problem.

*Oxidase Activity of Glutathione Reductase.* Unlike yeast glutathione reductase where the two-electron reduced species $EH_2$ is very slow to react with oxygen (Bulger & Brandt, 1971; Williams, 1976), the *E. coli* enzyme has significant oxidase activity. If the NADPH oxidase activity of glutathione reductase is followed under conditions similar to those used in Figure 4, curve 4, and over a similar time course, the activity does not decrease as does the NADPH–GSSG activity. Therefore, over the time course of the inactivation experiments, $NADP^+$ builds up, and the protection–reactivation observed in the inactivation experiments (Figure 4) has been ascribed to it. Turnover numbers calculated from the initial rates are given in Table I, experiment group 1. Since these are quite low rates, the oxidation of NADPH was measured in two additional types of experiments covering ranges of enzyme concentration used in both catalytic and spectral experiments. The results shown in Table I define a narrow range of oxidase activity from 0.35 to 0.82 $min^{-1}$. These turnover numbers are a direct measure of the reoxidation of $EH_2$ by oxygen, remembering that the turnover with GSSG is 16 000 $min^{-1}$.

The amount of $NADP^+$ necessary to bring about substantial protection is impressively small—0.5–20 $\mu$M in the experiments of Figure 3. Moreover, it can be calculated (using the rates in Table I) that the $NADP^+$ concentration at 90 min in the experiment of Figure 4, curve 4, would be only 0.26 $\mu$M. It is suggested that this protection results from tight binding of $NADP^+$ to $EH_2$ (dimer or monomer). The amounts of $NADP^+$ produced during preincubation are too small to be inhibitory in the subsequent assay as shown in the next section.

*Inhibition of Glutathione Reductase in Steady-State Turnover by $NADP^+$.* It was suggested that the kinetic mechanism of glutathione reductase from yeast was ping-pong on the basis of observed parallel lines in inverse plots (Massey & Williams, 1965). A more extensive kinetic analysis of the enzyme from porcine erythrocytes (and yeast) indicates that the mechanism is branched; at high [GSSG], a sequential pathway is followed while at low [GSSG], a ping-pong pathway prevails (Boggaram et al., 1978). In steady-state kinetic experiments with the *E. coli* enzyme (data not shown), parallel Lineweaver–Burke kinetic behavior was observed. A $K_m$(NADPH) of 10–12 $\mu$M was determined, which is in close agreement with 16 $\mu$M found by Mata et al. (1984). An inhibition study using the product $NADP^+$ showed competitive inhibition toward NADPH with a $K_i$ of 88 $\mu$M for E–$NADP^+$. Both the parallel line behavior and the competitive product inhibition strongly indicate that this enzyme operates by a ping-pong mechanism (Northrop, 1969; Segel, 1975; Williams, 1976). The $K_i$ of 88 $\mu$M shows that the levels of $NADP^+$ generated in the preincubation are too low to inhibit glutathione reductase in the subsequent assay.

*Inactivation of Glutathione Reductase as a Function of pH.* The affect of pH on the extent of inactivation with NADPH has been studied; the pHs of the preincubation buffers and of the assay mixtures were varied from pH 5.8 to pH 9.3. In order to minimize $NADP^+$ production and maximize the inactivation rate, NADPH concentration was held at 1.35 $\mu$M and enzyme concentration at 1.45 nM. A plot of turnover number vs pH with no NADPH in the preincubation buffer (control, data not shown) was bell-shaped and showed turnover numbers of 16 200 $min^{-1}$ at pH 7.3 (maximum), 9550 $min^{-1}$ at pH 5.8, and 5660 $min^{-1}$ at pH 9.0. It should be noted that enzyme preincubated for up to 15 min in the absence of NADPH showed no loss of activity (±4%). However, when NADPH was included in the preincubation buffers over the same range of pHs, the enzyme lost activity. Plots similar to Figure 3, curve 1, were obtained with 98% of the activity lost within 10 min after the addition of enzyme to the preincubation buffers. Therefore, the extent of inactivation was not pH dependent under the conditions of this experiment which were designed to maximize inhibition and minimize exposure to extremes of pH. Mata et al. (1985) reported a pH effect on the extent of inactivation. Their conditions of NADPH and enzyme concentration and time of incubation would not lead to extensive inactivation. An alternative interpretation of their data suggests that their pH profile reflects the remaining activity after approach to dimer–monomer equilibrium observed at a single long time point.

*Reaction of NADH with Glutathione Reductase.* The interpretation of the data of Figure 1, inactivation with NADH, assumes that NADH and $NAD^+$ do not complex significantly with $EH_2$ as is the case with NADPH and $NADP^+$ (Figure 3). The lack of a significant complex between NADH and the oxidized enzyme is demonstrated in steady-state turnover experiments using a constant, saturating GSSG concentration (3.5 mM) and NADH concentrations of 25–200 $\mu$M. A plot of turnover number versus [NADH] (data not shown) is linear and shows no tendency to plateau, indicating a second-order reaction and an apparent Michaelis complex at least 2 times greater than the highest NADH concentration of 200 $\mu$M. These assays required an enzyme concentration of 600 nM, because the turnover number, for example, at 150 $\mu$M NADH, is only 3.3 mol of NADH oxidized $min^{-1}$ (mol of glutathione reductase–FAD)$^{-1}$. The rate constant derived from the slope is 20 800 $M^{-1}$ $min^{-1}$ which is 52 times slower than that obtained with the yeast glutathione reductase (Bulger & Brandt, 1971). Given the high turnover number of the *E. coli* glutathione

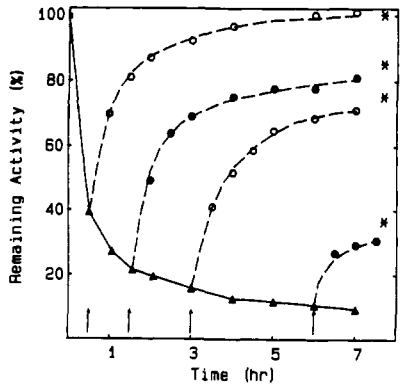



FIGURE 5: Time course of inactivation of *E. coli* glutathione reductase with NADH and subsequent reactivation with GSH. A 5.4 nM enzyme solution was preincubated with 152 μM NADH at 25 °C and in 50 mM Tris-HCl buffer/0.3 mM EDTA, pH 7.5. At the arrows (0.5, 1.5, 3.0, and 6.0 h), GSH was added to aliquots of inactivating enzyme to give a final concentration of 1 mM. The estimated final activity level, $A_\infty$, is indicated by an asterisk.

reductase in its physiological reaction, 16 000 $min^{-1}$, it seems safe to assume that the rate constant determined in the NADH–GSSG turnover represents the limiting rate of reduction of the enzyme by NADH. To confirm this, the rate constant of an anaerobic reduction of 15 μM glutathione reductase with 30 μM NADH was monitored at 530 nm using an extinction coefficient of 3200 $M^{-1}$ $cm^{-1}$ for $EH_2$ (determined in a separate titration experiment, see below); 50% reduction occurred in the first minute. The slope of a second-order plot, which is linear over 95% of its course, gives a rate of 26 850 $M^{-1}$ $min^{-1}$, comparing well with that of 20 800 $M^{-1}$ $min^{-1}$ found in steady-state turnover. The stoichiometry of the reaction of NADH with the enzyme was determined in an anaerobic titration and gave an end point of 1.2 mol of NADH/mol of enzyme and an extinction coefficient of 3200 $M^{-1}$ $cm^{-1}$. It is important to note that the final spectrum was that of uncomplexed $EH_2$; $EH_2$–NADPH and $EH_2$–$NADP^+$ complexes are readily distinguished from uncomplexed $EH_2$ (Williams et al., 1976). This observation further supports the assumption that neither NADH nor $NAD^+$ binds significantly to the pyridine nucleotide binding site of *E. coli* glutathione reductase at the concentrations used above. Similar data have been shown for yeast glutathione reductase (Bulger & Brandt, 1971).

*GSH Reactivation of Glutathione Reductase Inactivated with NADH.* When aliquots of glutathione reductase preincubating with NADH were made 1 mM in GSH (at various times in the inactivation), a reactivation of the enzyme was observed. As shown in Figure 5, 100% activity was regained when the GSH was added after 30 min of inactivation. However, as the inactivation proceeded, addition of GSH was less effective, and as seen at 6 h, only about 25% of the enzyme was reactivated over a 90-min period with a final level of 35%. This loss in reactivatable enzyme is thought to be the result of an irreversible step (see Scheme I and Discussion) following the dimer dissociation to monomer. It should be noted that the reactivation is specific for GSH; dithiothreitol at 100 μM did not effect any reactivation.

In a monomer–monomer reassociation, the integrated rate equation is unique in that the solution is not exponential as shown in eq 6, where $k_a$ is the monomer association rate

$$k_a t = 1/[\text{monomer}] - 1/[\text{monomer}]_i \qquad (6)$$

constant, $[\text{monomer}]_i$, the monomer concentration at the initiation of reactivation, and [monomer] the monomer concentration (as determined from eq 3 and 4) at some time $t$.

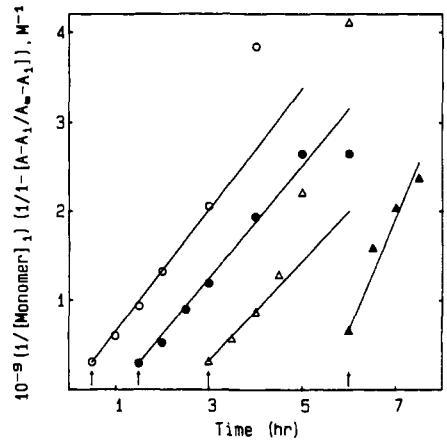



FIGURE 6: Second-order kinetic plot for reactivation of *E. coli* glutathione reductase from data of Figure 6. Arrows indicate the times in the preincubations at which GSH was added.

Table II: Reactivation of NADH-Inactivated *E. coli* Glutathione Reductase with 1 mM GSH

| start of reactivation (h) | enzyme[a] [monomer] (nM) | $k_a$[b] (×$10^{-6}$ $M^{-1}$ $min^{-1}$) | $t_{1/2}$[c] (min) | total % reactivated |
|---|---|---|---|---|
| 0.5 | 3.25 | 11.5 | 26.8 | 61 |
| 1.5 | 3.38 | 10.3 | 28.8 | 63 |
| 3.0 | 3.07 | 9.3 | 35.1 | 57 |
| 6.0 | 1.50 | 21.2 | 31.4 | 28 |

[a] Initial reactivatable monomer concentration is defined as the product of $[FAD]_0$ (5.4 nM glutathione reductase from Figure 5) and total percent reactivated glutathione reductase. [b] $k_a$ is the slope from the lines of Figure 6. [c] $t_{1/2}$ for monomer–monomer association is the reciprocal of the product of $k_a$ and the initial monomer concentration.

Again, it is assumed that catalytic activity is due to dimeric enzyme. The identity given below holds:

$$1/[\text{monomer}] - 1/[\text{monomer}]_i \equiv (1/[\text{monomer}]_i)\{1/[1 - (A - A_i/A_\infty - A_i)]\}$$

where $A_\infty$, $A_i$, and $A$ represent percent remaining activity at infinite time, at the initiation of reactivation, and at time $t$, respectively. Figure 6 shows plots of the disappearance of monomer reflected in the increase in enzymatic activity seen in Figure 5. All four reactivation curves are linear out to 75% of the reaction using monomer–monomer reaction kinetics (Figure 6) whereas pseudo-first-order plots (not shown), an alternative reactivation scheme, are nonlinear. The apparent monomer association rate constants in Table II were calculated from the slopes of the lines in Figure 6. The average of 13.1 (±5.5) × $10^6$ $M^{-1}$ $min^{-1}$ is similar to the second-order rate constants determined for other associating protein systems (Kanamura & Nakamura, 1983; Ip & Ackers, 1977; Parr & Hammes, 1976).

## Discussion

Glutathione reductase from *E. coli* is particularly sensitive to preincubation with its substrate, NADPH, under some conditions. Results with the human erythrocyte enzyme, on the other hand, are quite variable with the extent of inactivation ranging from 30% to 60% under conditions which, with the *E. coli* enzyme, give greater than 95% inactivation (data not shown). This is of interest since the erythrocyte enzyme appears to have an intersubunit disulfide bond (Thieme et al., 1981). It is possible that inactivation of the erythrocyte enzyme is a manifestation of a lessening of the interaction between subunits, seen in the X-ray crystal structure (Thieme

et al., 1981), without frank dissociation. We have therefore confined the data presented here to the *E. coli* enzyme.

The loss of activity with the *E. coli* enzyme is greatest when the enzyme concentration is low, and the process is fastest when the NADPH concentration is less than the $K_d$ of 12 $\mu$M for the $EH_2$–NADPH complex. A mechanism for inactivation which assumes that the native, dimeric enzyme is active while the monomer is not is given in Scheme I. Reduction to the $EH_2$ level is required for significant enzyme dissociation. It should be noted that native, diluted (1–5 nM), unreduced enzyme retains 94–98% actvity over 5–8-h periods of incubation and that both NADPH and NADH, at the concentrations used, reduce glutathione reductase within seconds to the $EH_2$ species. This reduction step is the first half-reaction of catalysis involving NADPH binding, electron transfer, and $NADP^+$ dissociation. $EH_2$ is known to form tight complexes with both NADPH and $NADP^+$ (Williams et al., 1976), and our results show that these complexes are protected from inactivation (Figures 3 and 4). $EH_2$ is a charge transfer complex in which the donor is one of the nascent thiolates and FAD is the acceptor (Massey & Ghisla, 1974; Wilkinson & Williams, 1979). For purposes of discussion, in Scheme I it is assumed that $EH_2$(monomer) is the FAD-$(SH)_2$ form while $EH_2$*(monomer) is the $FADH_2$-$S_2$ form. All of the species up to this point are in dynamic equilibrium. $EH_2$**(monomer) is irreversibly inactivated. The presence of such a species is indicated by the failure to reactivate completely after long periods of preincubation with GSH (Figure 6) and the slight continued inactivation at long times with NADH (Figure 1). Certain thiols in such a species might be oxidized beyond the disulfide level possibly by hydrogen peroxide produced in the oxidase reaction, or the conversion of $EH_2$* to $EH_2$** may simply be protein denaturation (see below).

Earlier work on yeast glutathione reductase and on the homologous, mechanistically related lipoamide dehydrogenase has shown that a group in the active site acts as an acid at the $EH_2$ level in the former enzyme and as a base at the E level in the latter; the two enzymes operate physiologically in opposite chemical directions (Matthews & Williams, 1976; Arscott et al., 1981). Furthermore, the nascent thiols have distinct functions—the thiol nearer the N-terminus interacting with the dithiol/disulfide substrate (interchange thiol) and the other thiol interacting with the FAD (electron-transfer thiol) (Thorpe & Williams, 1976; Arscott et al., 1981; Thieme et al., 1981). Sequence analysis revealed that the acid–base group was a histidine residue in human erythrocyte glutathione reductase, His-467′ (Untucht-Grau et al., 1979) and, by homology, His-452′ in lipoamide dehydrogenase (Williams et al., 1982; Adamson et al., 1984; Rice et al., 1984). The X-ray crystal structure of human erythrocyte glutathione reductase shows that this His is strongly hydrogen bonded to Glu-472′ via the N-1 while the N-3 faces the disulfide, somewhat closer to the interchange thiol. A fascinating feature of the structure is that the His-Glu pair is contributed to the active site by one polypeptide while the disulfide and the FAD binding site are formed from the adjacent chain (Thieme et al., 1981; Pai & Schulz, 1983). Thus, it is eminently reasonable to suggest that monomer would be inactive in the physiological reaction and active in the oxidase reaction which does not involve the thiol/disulfide or its base stabilizer.

The most notable characteristic of the inactivation process is that the final level of activity decreases as the enzyme concentration in the preincubation medium decreases (Figures 1 and 4). Efforts to model this process using data from inactivations with the physiological substrate, NADPH, were not successful. The presence of complexes of $EH_2$ with NADPH and $NADP^+$ made the system too complicated. The enzyme is also inactivated by preincubation with NADH, and since NADH does not form a significant complex with $EH_2$, data from these preincubations could be readily analyzed assuming an equilibrium between active dimer and a putative inactive monomer. As shown in Figure 2, an excellent correlation exists between the data points found from the NADH inactivation curves of Figure 1 and the theoretical curve which assumes an apparent $K_d$ of 73 nM for the dimer–monomer equilibrium. The inactivation is not dependent on the presence of oxygen (Figure 2), but oxygen does lead to an erroneously low dissociation constant for the monomerization. It is suggested that this is due to the fact that $EH_2$(dimer) can be reoxidized by oxygen and this reaction effectively lowers the $EH_2$(dimer) concentration (Scheme I).

Inactivation is more complete in the absence of oxygen. The apparent equilibrium constant for dimer dissociation ($K_d$) determined anaerobically is 220 nM. Monomer can also be reoxidized by oxygen as shown by the continuing oxidase activity of the enzyme after the glutathione reductase activity is largely gone. In inactivations with NADPH, this oxidase activity leads to $NADP^+$ production and to protection and reactivation, further demonstrating the dynamic nature of the equilibria involved in the inactivation (Scheme I). The amount of $NADP^+$ produced in the oxidase reaction and thus the amount needed to show reactivation is impressively small, and this is puzzling. It is possible that the binding constant for $NADP^+$ on one subunit is effected by the redox state of the other subunit.

Reactivation of NADH-inactivated enzyme is promoted by GSH as shown in Figure 5. If reactivation involves dimerization and the dimerization is rate limiting, then the kinetics will be second order in monomer, and this is indeed the case (Figure 6). The average apparent association rate constant of $13 \times 10^6$ $M^{-1}$ $min^{-1}$ is 3 orders of magnitude slower than a diffusion-controlled second-order reaction. This slow rate for monomer–monomer association of protein subunits is frequently observed (Kanamura & Nakamura, 1983; Ip & Ackers, 1977; Parr & Hammes, 1976). Creighton (1983) and Mrabet et al. (1986) discuss this kinetic aspect and ascribe the phenomenon to an "encounter complex". In the case of dimerization of monomers, it is proposed that these complexes are a summation of both productive and nonproductive monomer interactions. Thus, dimerization becomes a two-step process where the observed rate of formation of a productive dimer is slower than the rate of dissociation of the "encounter complex" to monomer.

The exact mechanism of reactivation of glutathione reductase by GSH cannot be ascertained from these studies since the initial kinetics of reactivation are second-order monomer–monomer and give no clue as to the function of GSH. Mata et al. (1985) have suggested that GSH could reduce a spurious disulfide formed between one of the nascent thiols and another thiol. In their proposal, the electron acceptor in the formation of the spurious disulfide is not obvious. It is difficult to see how disulfide formation would be dependent on the protein concentration. Two alternatives seem more logical, first, that GSH reduction of a reoxidized active-site disulfide monomer is followed by a slower rate-limiting monomer–monomer association to active dimer or, second, that the binding of GSH to monomer forms a species more geometrically favorable to interaction with a similar monomer–GSH complex. It should be pointed out in this connection that GSH binds across the monomer–monomer interface (Pai &

Schulz, 1983). Parr and Hammes (1976), in a similar study of the kinetics of reassembly of monomeric rabbit muscle phosphofructokinase to tetramer, observed a 2–3-fold increase in the measured association rate constant in the presence of the substrate ATP with no change in the reaction order. They suggested that this was due to a "...stabilizing effect on the dimer species relative to the monomer..." by the ATP. In this connection, we have attempted to separate dimer from monomer after reduction by NADPH using either Sepharcyl S-200 or a molecular sieve HPLC column (Waters, I-125) but have not been successful in spite of varying the time on the column extensively. This result may be ascribed to the inherent dynamics of gel chromatography when attempting to separate a kinetically controlled dimer–monomer system (Zimmerman, 1974). However, the emerging reductant-free enzyme fraction was observed to reactivate over the succeeding 60 min and at a faster rate when 0.9 mM GSH was added to this fraction prior to activity measurements. This shows that removal of the reduced pyridine nucleotide allows the equilibrium (Scheme I) to shift back to active dimeric $EH_2$ and $E_{ox}$.

Lopez-Barea and his colleagues have suggested that the inactivation of mouse liver, yeast, and *E. coli* glutathione reductase by NADPH in the cell provides a mechanism of physiological control (Lopez-Barea & Lee, 1979; Pinto et al., 1985; Mata et al., 1985). Supporting this suggestion is the fact that the glutathione reductase reaction is not at equilibrium [see pp 344–345 of Schirmer and Schulz (1987)]. However, we have shown that conditions promoting the inactivation of the *E. coli* enzyme (which are comparable to those in some of the studies of Lopez-Barea and his colleagues) are far removed from those present in the cell. At the concentration of glutathione reductase in *E. coli* (880 nM; Williams & Arscott, 1970) and using the $K_d$ of 220 nM determined anaerobically, the enzyme would be less than 30% dissociated. Moreover, inactivation is quite slow at the NADPH concentration reported in bacterial cells (150 $\mu$M, Bochner & Ames, 1982). This makes it unlikely that this mechanism is significant as a physiological control.

## Added in Proof

Scrutton et al. (1988) have engineered an *E. coli* glutathione reductase in which Thr-75 has been changed to Cys (T75C). (In the human erythrocyte enzyme, this residue is a Cys and is at the 2-fold axis.) They have shown that the T75C enzyme does not dissociate to monomer in SDS–PAGE. They have also shown that the T75C enzyme is heat activated at a lower temperature in the presence of 5 mM NADPH than in its absence. They have not, however, attempted the inactivation of the T75C enzyme under the conditions shown in the present paper to lead to dimer dissociation, that is, at low NADPH concentration where complex formation between NADPH and the enzyme is not a complication.

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**Registry No.** NADPH, 53-57-6; NADH, 58-68-4; GSH, 70-18-8; glutathione reductase, 9001-48-3.

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# Selective Modification of the Catalytic Subunit of cAMP-Dependent Protein Kinase with Sulfhydryl-Specific Fluorescent Probes†

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ABSTRACT: The catalytic subunit of cAMP-dependent protein kinase contains only two cysteine residues, and the side chains of both Cys 199 and Cys 343 are accessible. Modification of the catalytic subunit by a variety of sulfhydryl-specific reagents leads to the loss of enzymatic activity. The differential reactivity of the two sulfhydryl groups at pH 6.5 has been utilized to selectively modify each cysteine with the following fluorescent probes: 3,6,7-trimethyl-4-(bromomethyl)-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine, and 4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole. The most reactive cysteine is Cys 199, and exclusive modification of this residue was achieved with each reagent at pH 6.5. Modification of Cys 343 required reversible blocking of Cys 199 with 5,5'-dithiobis(2-nitrobenzoic acid) followed by reaction of Cys 343 with the fluorescent probe at pH 8.3. Treatment of this modified catalytic subunit with reducing reagent restored catalytic activity by unblocking Cys 199. In contrast, catalytic subunit that was selectively labeled at Cys 199 by the fluorescent probes was catalytically inactive. Even though Cys 199 is presumably close to the interaction site between the regulatory subunit and the catalytic subunit, all of the modified C-subunits retained the capacity to aggregate with the type II regulatory subunit in the absence of cAMP, and the resulting holoenzymes were dissociated in the presence of cAMP. The fluorescent properties of the modified catalytic subunits have been characterized and are consistent with both Cys 199 and Cys 343 being located in relatively hydrophobic environments. In addition, the Stokes radii of the modified catalytic subunits is 26 Å based on Perrin analysis of the polarized fluorescence emission.

During the past 20 years, fluorescence spectroscopy and the covalent attachment of fluorescent probes to macromolecules have been used extensively to investigate biological systems. Covalently bound fluorophores have been used to probe the local environment of the chromophore (McClure & Edelman, 1966; Brand et al., 1971; Lakowicz, 1980; MacGregor & Weber, 1981), to determine the rotational diffusion of the macromolecule to which the fluorophore is attached (Weber, 1952a,b, 1953), and to determine the distance between two fluorophores attached to different sites (Forster, 1948, 1966; Stryer, 1978). This latter application permits estimations of both intramolecular and intermolecular distances and provides a sensitive method for monitoring conformational changes that alter the distance between the two probes. A prerequisite for using fluorescence in structural studies is the ability to label specific sites on the macromolecule uniquely and quantitatively. We describe here a method for the selective modification of either Cys 199 or Cys 343 in the C-subunit of cAMP-dependent protein kinase by several different fluorescent probes.

The catalytic (C) subunit of cAMP-dependent protein kinase is a monomeric protein that contains only two cysteine residues, Cys 199 and Cys 343 (Shoji et al., 1983). These cysteines can be modified by a variety of reagents, including 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB),[1] *N*-ethylmaleimide (NEM), and iodoacetic acid (IAA) (Sugden et al., 1976; Bechtel et al., 1977; Armstrong & Kaiser, 1978; Nelson & Taylor, 1981). Modification of the C-subunit by sulfhydryl-reactive reagents inhibits catalytic activity but not the capacity to bind to the regulatory subunit (First & Taylor, 1988).

Jimenez et al. (1982) investigated the reaction of DTNB with the C-subunit and found that the kinetics of cysteine

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[1] Abbreviations: IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; IANBD, 4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole; monobromobimane, 3,6,7-trimethyl-4-(bromomethyl)-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione; DTNB, 5,5'-dithiobis(nitrobenzoic acid); IAA, iodoacetic acid; NEM, *N*-ethylmaleimide; MgATP, magnesium chelate of adenosine 5'-triphosphate.

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