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MODIFICATION OF AN ESSENTIAL AMINO GROUP OF GLUTATHIONE REDUCTASE FROM YEAST BY PYRIDOXAL 5′-PHOSPHATE

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Yeast glutathione reductase is inactivated by pyridoxal 5′-phosphate (PLP). The reactivation of the enzyme by dilution as well as a characteristic absorption peak at 325 nm exhibited by $NaBH_4$ – reduced – PLP modified enzyme show that the inactivation is due to the specific modification of the ε-amino group of lysine residue. The maximum of 70% inactivation was observed at 7 mM PLP and the equilibrium was reached within 3 min. Kinetic and equilibrium analysis of inactivation data derived at different PLP concentrations showed that a noncovalent intermediate is formed prior to inactivation. From the studies on the effect of pH on the inactivation rate, the pKa of ε-amino group of the reactive lysine residue was calculated to be 7.3. Among various protecting agents tried, only NADP was found to be effective. The apparent stoichiometry of the reaction was one to one as the incorporation of 0.65 mole PLP/mole of enzyme led to 70% inactivation at saturating PLP concentration.

Keywords: Pyridoxal 5′-phosphate; Glutathione reductase; Lysine

INTRODUCTION

Glutathione reductase is a FAD-containing homodimeric enzyme with a molecular weight of 108 kda and catalyses NADPH-dependent reduction of oxidized glutathione.[1] The mechanism of reduction has been shown to be a two-step process, with NADPH binding first to the enzyme forming reduced enzyme (EH_2). EH_2 then binds GSSG, which is reduced to GSH with the concommitant oxidation of the enzyme.[2] X-ray crystallographic

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studies have confirmed the presence of separate substrate binding sites for NADPH and GSSG. These sites are located on the opposite sides of the protein separated by the isoalloxazene ring of FAD.[3] The cellular function of glutathione reductase is to maintain high levels of reduced GSH, which plays an important role in DNA synthesis.[4,5] It may also have a role in the cell division cycle and stress adaptation at a cellular level.[6,7]

Chemical modification studies using group specific reagents have shown the presence of redox disulphide[8-10] arginine[11] and histidine[12,13] at or near the active site of glutathione reductase.

Although PLP is a coenzyme for many enzymes, particularly involving amino acid substrates, it has also been extensively used as a specific reagent for the modification of ε-amino groups in proteins.[14-18]

In this communication, we report the presence of an essential lysine residue in yeast glutathione reductase by chemical modification with PLP. Preliminary work is reported elsewhere.[19]

EXPERIMENTAL PROCEDURES

Materials

Purified yeast glutathione reductase, glutathione (oxidized), pyridoxal 5′-phosphate (PLP), NADPH, EDTA, sodium borohydride, Sephadex G-50 and buffer components were purchased from Sigma Chemical Company, USA. All other chemicals were of highest purity grade.

Method

The enzyme was dialysed for 16 h at 4°C against 0.05 M sodium phosphate buffer at pH 7.5, to remove ammonium sulphate. The protein content of dialysed enzyme was determined by the Lowry method using bovine serum albumin as the standard.[20]

Glutathione reductase activity was measured at 25°C by following the absorbance change in 60 s at 340 nm, which is due to NADPH oxidation. The assay mixture consisted of 50 mM phosphate buffer (pH 7.5), 1.5 mM GSSG, 1.0 mM EDTA, 0.25 mM NADPH and 2.35 μg enzyme in a total volume of 1 ml.

Chemical modification of glutathione reductase by PLP was carried out in an incubation medium containing 0.05 M phosphate buffer, the enzyme (0.11 μM) and varying concentrations of the modifying reagent. The incubation mixture was protected with aluminium foil to prevent

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photodestruction of PLP. Aliquots were withdrawn at different time intervals and the residual activity was determined immediately.

Reactivation of the enzyme was carried out by diluting the incubation mixture 100-fold with 0.05 M phosphate buffer. Kinetics of reactivation were followed by removing the aliquots at different time intervals and assaying for reductase activity.

Protection studies against PLP inactivation was carried out by preincubating the enzyme with the desired compound in the incubation mixture for 5 min prior to the addition of PLP.

For studying the effect of pH on the modification of the enzyme by PLP, the incubation mixture consisted of 0.05 M phosphate buffer of required pH, PLP (5 mM) and enzyme (0.11 μM). Aliquots were withdrawn at different intervals and assayed for residual glutathione reductase activity in the standard assay mixture described earlier. Appropriate controls without PLP were also run for each pH.

The stoichiometry of the enzyme modification reaction was determined by incubating the enzyme in 0.05 M phosphate buffer with 7 mM PLP for 15 min at 25°C. Modified enzyme was reduced by the addition of 20-fold excess $NaBH_4$ and kept for 15 min in an ice bath. Excess reagent was removed by filtering thrice through Sephadex G-50 columns according to the procedure described by Penefsky.[21] The moles of PLP incorporated per mole of enzyme was calculated by taking the ratio of concentrations of the N^6-pyridoxylphospholysine derivative and the enzyme. Concentration of lysyl-PLP derivative was determined by measuring the absorbance at 325 nm and using a molar extinction coefficient of 8500 $M^{-1}cm^{-1}$. Protein concentration was estimated by the method of Lowry *et al.*[20]

RESULTS AND DISCUSSION

Treatment of glutathione reductase with PLP resulted in the inactivation of the enzyme. The activity was reduced to a non-zero constant value within 3 min, which varied with the initial PLP concentration. A plot of residual activity versus different PLP concentrations is presented in Figure 1, which shows that the maximum inactivation of 70% was observed at 7 mM PLP which remained constant even when the concentration was increased to 20 mM. Similar incomplete inactivation with PLP has been observed with other enzymes.[15,16]

82% of initial glutathione reductase activity was regained on 100-fold dilution of the incubation mixture containing 5 mM PLP (Figure 2). This

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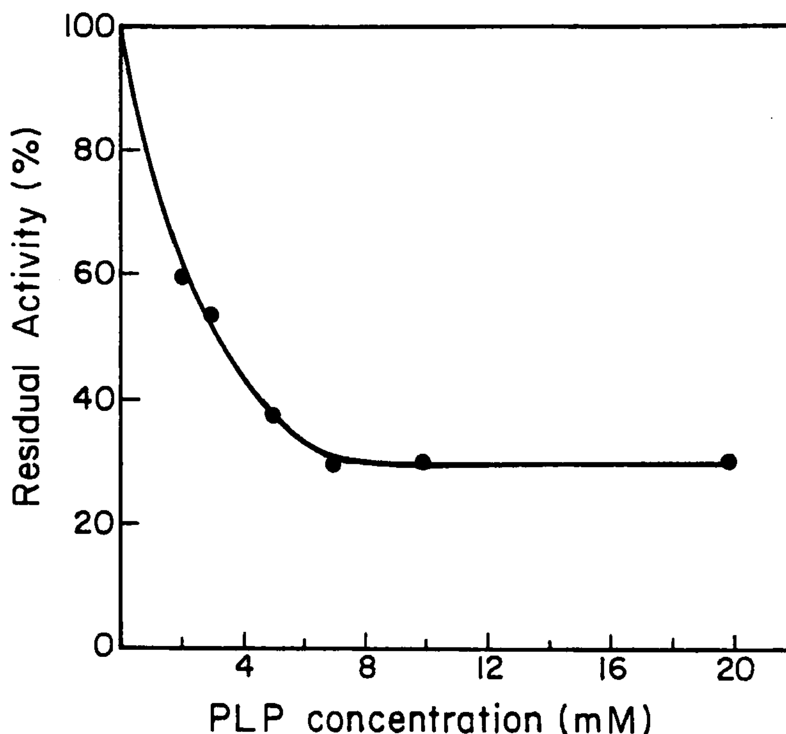
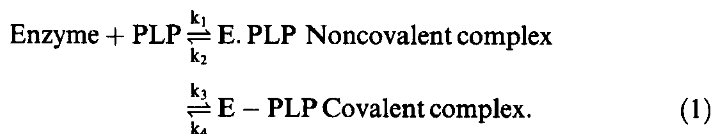


FIGURE 1 The concentration dependence of the PLP inactivation of glutathione reductase. The enzyme (0.11 μM) was incubated with different concentrations (2–20 mM) of PLP for 3 min. Aliquots (10 μl) were withdrawn for assay and residual activity was determined as described in "Experimental Procedure".

is in accordance with the reversal modification of the ε-amino group of lysine to form Schiff's base. Further, the reduction of modified enzyme with sodium borohydride led to irreversible modification. Reduced enzyme exhibited an absorption maximum at 325 nm which is characteristic of a N^6-pyridoxyllysyl derivative. These results showed that the inactivation of glutathione reductase by PLP is due to the specific modification of a lysine residue.

Chen and Engel[17] have proposed an equilibrium model to explain the residual activity remaining after modification with PLP.



$$\text{Enzyme} + \text{PLP} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{E.PLP Noncovalent complex} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{E} - \text{PLP Covalent complex.} \qquad (1)$$

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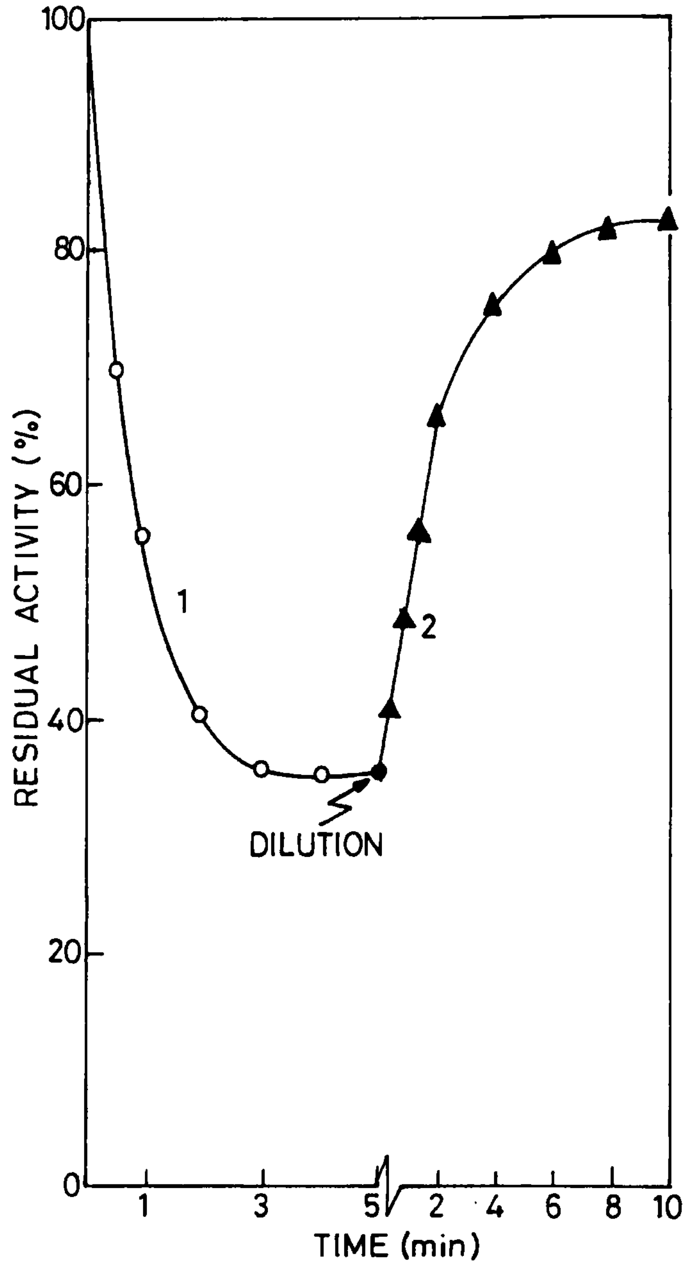

FIGURE 2 Reactivation of PLP modified enzyme on dilution. Enzyme (0.11 μM) was incubated with 5 mM PLP for 5 min and then diluted 100-fold with 0.05 M phosphate buffer. The activity was determined in an aliquot at the specified time intervals (1, 2) as described in Experimental Procedure.

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According to this model, residual activity is due to the equilibrium between inactive covalent Schiff's base derivative of the enzyme and the noncovalent E.PLP complex, which readily dissociates to give active enzyme. The inactivation data obtained at different PLP concentrations was subjected to equilibrium analysis as described in the above model to determine whether the residual activity is due to the unreacted active enzyme or partially active enzyme.

The pseudo first-order kinetic plot for the inactivation data obtained at different PLP concentrations is shown in Figure 3. A plot of the first-order rate constants against PLP concentrations yielded a hyperbola whereas a double reciprocal plot gave a straight line which did not pass through the origin (Figure 3). This indicated that a kinetically significant noncovalent

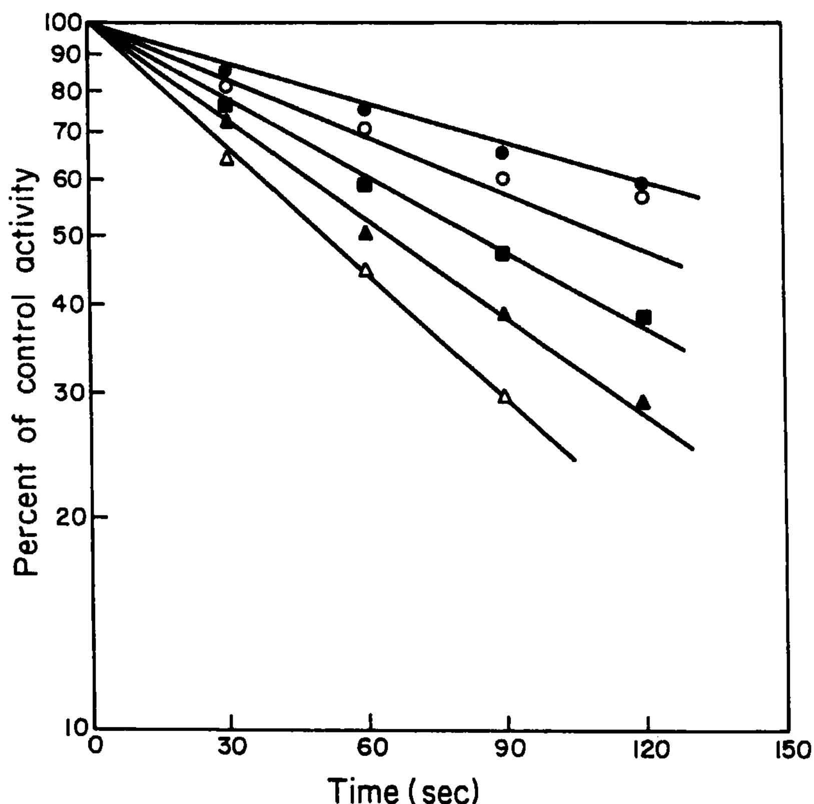

FIGURE 3 Pseudo First order kinetic plot for the inactivation of glutathione reductase. The enzyme (0.11 μM) was incubated with different concentrations of PLP (●) 2 mM; (○) 3 mM; (■) 5 mM; (▲) 7 mM; (△) 10 mM. Aliquots (10 μl) were withdrawn at different time intervals and the residual activity was determined as described in Experimental Procedure.

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enzyme. PLP complex is formed prior to inactivation. Since in the above model, a slow first-order interconversion is preceded by a rapid binding equilibrium with PLP, the observed forward rate constant for inactivation is given by

$$k_{app} = \frac{[PLP]k_3}{[PLP] + K_1}, \tag{2}$$

where $K_1 = k_2/k_1$ and k_4 is assumed to be negligibly slow as compared to k_1 and k_2.

$$\frac{1}{k_{app}} = \frac{1}{[PLP]}\frac{K_1}{k_3} + \frac{1}{k_3}. \tag{3}$$

A double reciprocal plot of first-order rate constants and PLP concentrations should yield a straight line with an intercept of $1/k_3$ and slope of k_1/k_3. Figure 4 shows that the derived data is in accordance with this and the values obtained of k_3 and k_1 are $0.83\,min^{-1}$ and 13.16 mM respectively.

The dissociation constant for the noncovalent and covalent enzyme-PLP complex formation can also be determined using the following relationship.[15]

$$R = \frac{1 + k_1/k_2[PLP]}{1 + (k_1/k_2)[PLP][1 + k_3/k_4]}, \tag{4}$$

where R is the fractional residual activity observed at a particular PLP concentration. Assuming that the covalent complex is completely inactive and the noncovalent complex rapidly dissociates into active enzyme on dilution of an assay mixture, the following relationship can be obtained for equation (4)

$$\frac{1}{[PLP]} = \frac{k_1 k_3}{k_2 k_4}\frac{1}{(1 - R)} - (1 + k_3/k_4)k_1/k_2. \tag{5}$$

Thus a plot of $1/[PLP]$ versus $1/(1 - R)$ should be linear. The abscissa intercept represents $1 + k_4/k_3$, which is the reciprocal of maximum inactivation obtained with an infinite concentration of PLP. Figure 5 shows that a straight line is obtained indicating that the equilibrium model is valid for glutathione reductase as observed with other enzymes modified with PLP.[15,16,18] The enzyme activity at saturating PLP concentrations was

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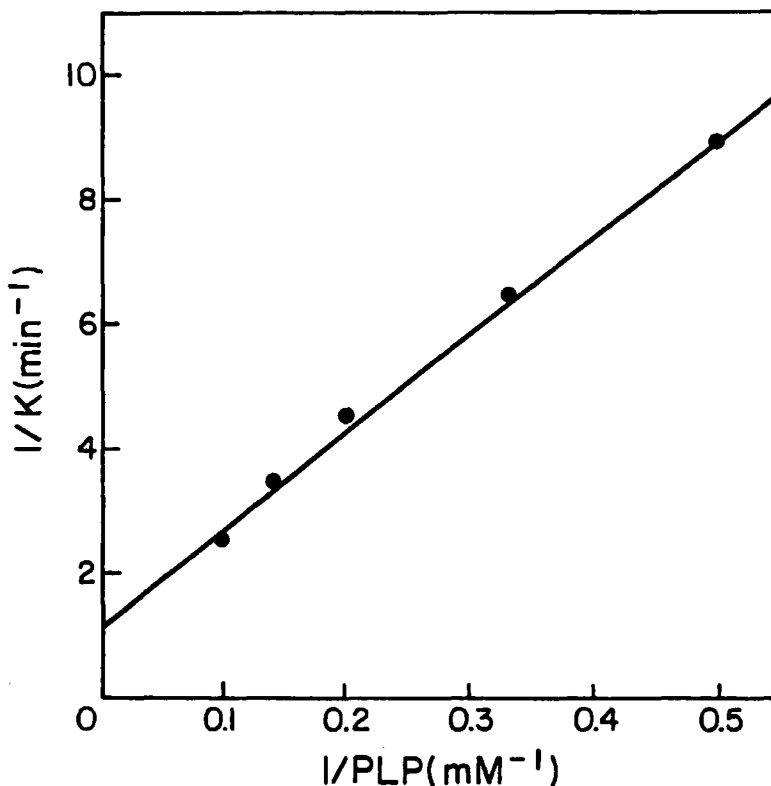

FIGURE 4 A double reciprocal plot of the concentration-dependence of the rate of PLP inactivation of yeast glutathione reductase.

calculated to be 26% which is in close agreement with the experimentally determined value. The values for k_4 and another estimation of k_1 (k_2/k_1) by non-linear regression analysis of equation (4) was determined to be 0.099 min^{-1} and 28.2 mM respectively. There is some discrepancy in K_1 values derived from the kinetic approach and the constant residual activity measurements. Results derived from equation (4) may be more reliable as compared to the one from the kinetic approach as residual activity can be more accurately determined.

In accordance with the equilibrium model, the reversal of activity by dilution should follow first-order kinetics at least initially, as the dissociation of noncovalent complex is very rapid when compared to reversal of Schiff's base. k_4 estimation from the data presented in Figure 2 yielded a value of 0.13 min^{-1} which is reasonably close to the one derived from non-linear regression analysis.

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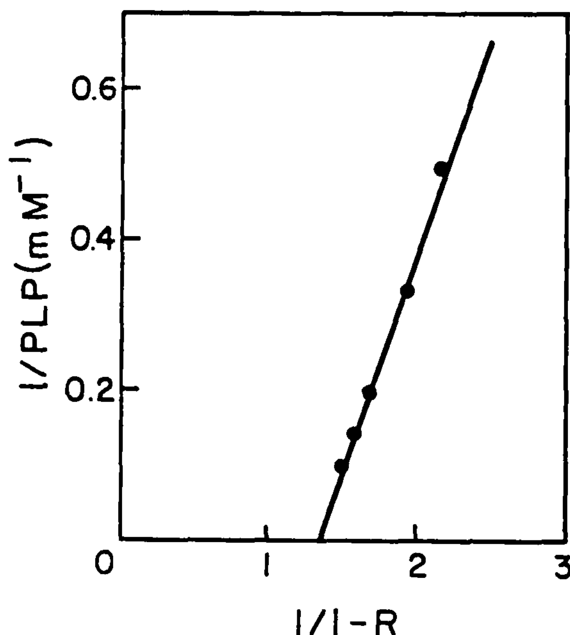

FIGURE 5 Double reciprocal plot of PLP concentration versus fractional residual activity of glutathione reductase.

pH Dependence of PLP Inactivation

The inactivation of glutathione reductase by PLP as a function of H^+ ion concentration was studied in the pH range 5.9–8.0. Pseudo first-order rate constants for inactivation were determined at each pH (Figure 6), which was then divided by PLP concentration to get the apparent second-order rate constants. Change in the second-order inactivation rate constant with pH is due to its dependence on the mole fraction of conjugate base form of the reactive functional group on the enzyme which reacts with the modifier. The pK_a of the functional group can be determined from the plot of 1/k versus $[H^+]$ using the following equation

$$\frac{1}{k_{inact}} - \frac{1}{k_{max}}\left(1 + \frac{H^+}{K}\right), \tag{6}$$

where k_{inact} is the second-order rate constant at a particular H^+ concentration, k_{max} is the maximal second-order rate constant and K is the proton dissociating constant of the ionising residue. The pK_a was calculated

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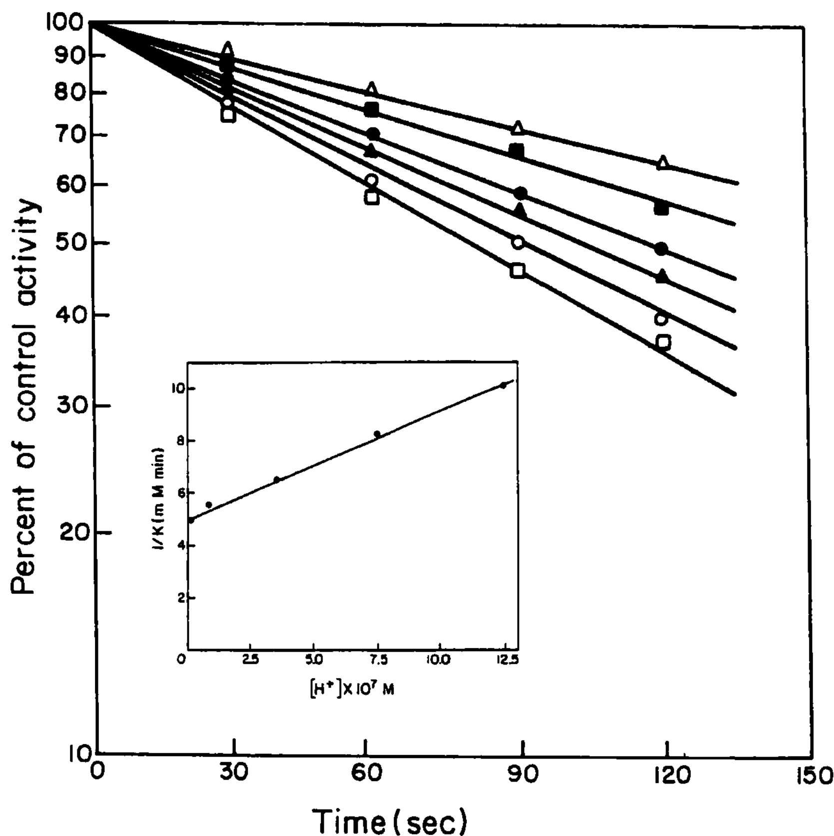

FIGURE 6 pH-dependence of inactivation of glutathione reductase. The enzyme (0.11 μM) was incubated with 5 mM PLP in 0.05 M phosphate buffer at the following pH values. (△) 5.9; (■) 6.12; (●) 6.5; (▲) 7.1; (○) 8.0; (□) 7.5. At the indicated time the aliquots were withdrawn and enzyme activity was determined. Inset shows 1/k mM min versus $[H^+]$.

to be 7.3 for ionisation of the ε-amino group of reactive lysine (Figure 6 inset). This is rather low as the reported value for the ε-amino group of lysine is around 10.4. This decrease may be due to the hydrophobic environment around the reactive lysine. Similar behaviour for active site lysines has been reported for other enzymes.[22–24]

GSSG, NADPH, NADP, NADH, AMP and NAD were used as protective agents against PLP inactivation. Although GSSG protected the enzyme, it is possible that this may be due to its direct reaction with PLP. Among the various nucleotides used, only NADP was effective. The extent of protection by NADP was dependent on the initial enzyme inactivation by PLP. When the inactivation of glutathione reductase was 40% almost

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TABLE I The inactivation of glutathione reductase by PLP in the presence and absence of NADP

Concentration of PLP (mM)	*% Residual Activity*	
	Absence of NADP	*Presence of NADP*
3	60	90
5	36	82
7	30	75

Glutathione reductase (0.11 μM) was preincubated with NADP (4 mM) for 5 min in 0.05 M sodium phosphate buffer (pH 7.5). The required concentration of PLP was added to this incubation mixture. After 5 min 10 μl was withdrawn from the mixture and the residual activity was determined as described in "Experimental Procdures".

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complete protection was observed in the presence of 4 mM NADP. On the other hand, when the residual activity was 30%, the same concentration of NADP afforded 75% protection. (Table I) The differential effect of NADPH and NADP as protecting agents has been observed earlier for glutathione reductase with N-ethylmaleimide inactivation by Dubler and Anderson.[8] The importance of the 2′-phosphate group in the binding of the nucleotide to the enzyme was shown by the ineffectiveness of NAD and AMP as protecting agents.

The stoichiometry of covalent incorporation of PLP into the enzyme was calculated by determining the concentration of N^6-pyrid oxyllysine formed after reducing the modified enzyme with $NaBH_4$. Results indicated that 0.65 mole PLP was incorporated per mole of the enzyme. Since 30% residual activity remained at the saturating PLP concentration, an apparent stoichiometry of 1:1 can be assumed.

Results presented in this communication conclusively prove the presence of a lysine residue at or near the active site of glutathione reductase. X-ray crystallographic studies have indicated the presence of lysine at the bottom of the nicotinamide binding pocket in glutathione reductase.[3] It may be possible that PLP reacts with this lysine thereby affecting the enzyme activity.

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