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INACTIVATION OF YEAST GLUTATHIONE REDUCTASE BY *O*-PHTHALALDEHYDE

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Yeast glutathione reductase was inactivated by the bifunctional reagent, *o*-phthalaldehyde. The initial rate of inactivation followed pseudo-first order kinetics. Fluorescence spectral properties of modified enzyme indicated the formation of an isoindole derivative from cysteine and lysine residues present in close proximity as shown by typical fluorescence emmision and excitation maximum at 410 nm and 337 nm, respectively. The fluorescence spectral studies with *o*-phthalaldehyde in the presence and absence of *N*-ethylmaleimide indicated that both the inhibitors react with the same cysteine residue, which is non-essential for enzyme activity. The coenzyme NADPH did not protect the enzyme against the *o*-phthalaldehyde reaction while oxidised glutathione prevented *o*-phthalaldehyde inactivation. This could be due to reaction of the amino group of glutathione with *o*-phthalaldehyde. Stoichiometry of the reaction showed that the formation of approximately 2 isoindole derivatives per subunit of glutathione reductase is accompanied by 75% loss of activity. The results suggest that *o*-phthalaldehyde binds to non-essential cysteine and lysine residues present in close proximity which results in conformational changes leading to enzyme inactivation.

Keywords: *O*-Phthalaldehyde; glutathione reductase; cysteine; lysine.

INTRODUCTION

Yeast glutathione reductase (GR) is a dimeric enzyme with a subunit molecular mass of 52.4 kDa.[1] Each subunit has one noncovalently bound FAD molecule.[2] Glutathione reductase catalyzes the NADPH dependent reduction of glutathione disulfide as follows,

$$H^+ + NADPH + E \text{ —— } NADP^+ + EH_2$$

$$EH_2 + GSSG \text{ —— } 2\ GSH$$

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where the stable EH_2, which is the 2 electron reduced form of the enzyme, is formed as an intermediate. The cellular function of glutathione reductase is to maintain the high levels of reduced cellular concentration of the reduced form of glutathione (GSH), which plays a critical role in the biosynthesis of DNA.[3,4]

X-ray diffraction studies have confirmed the presence of separate substrate binding sites for glutathione disulphide and NADPH. These sites are located on opposite sites of a subunit of the protein and separated by the isoalloxazime ring of FAD.[5] Recent studies on the enzyme-NADPH complex have indicated that the coenzyme binds to the enzyme in an extended conformation in a cleft that constitutes part of the active site.[6,7] A comparison of the primary structure of *E. coli* and human glutathione reductase has been shown to be homologous throughout.[8] Glycine[9] and tyrosine[10] at the NADPH binding site and histidine[10] in the glutathione binding pocket have been shown to be conserved in the *E.coli* and human enzymes. Various chemical modifiers have been used to identify the involvement of redox sulfide,[12–14] histidine[11,15] and arginine[2] residues at the catalytic centre of the enzyme. A histidine residue has been implicated as a base in the reduction of GSSG.[11] When the histidine was replaced by glutamine using site specific mutagenesis the activity of glutathione reductase was reduced to 1% of the control indicating its important role in catalytic activity of the enzyme.[16] However, in the case of yeast glutathione reductase the enzyme was inactivated only 10–15% by diethyl pyrocarbonate.[11]

We have studied the effect of the bifunctional reagent *o*-phthalaldehyde, which preferentially reacts with proximal cysteine and lysine residues on the glutathione reductase and the results are presented in this communication.

EXPERIMENTAL PROCEDURE

Materials

Yeast glutathione reductase, NADPH, *o*-phthalaldehyde, β-mercaptoethanol, glutathione oxidised (GSSG), *p*-chloromercuric-phenylsulphonic acid (PCMS), *N*-ethylmaleimide (NEM) and buffer components were obtained from Sigma Chemical Company, USA. All other chemicals were of highest purity grade.

Methods

Enzyme Preparation and Activity Measurements

The enzyme glutathione reductase was dialyzed at 4°C against 0.05 M sodium phosphate buffer at pH 7.5 for 16 h, with three changes of buffer. The protein content was measured by Lowry's method using bovine serum albumin as standard.[17]

Glutathione reductase activity was determined by measuring the decrease in the absorbance of NADPH at 340 nm on a spectrophotometer (Gilford Model 260) equipped with a recorder. All the components of the reaction mixture were prepared in 0.05 M sodium phosphate buffer (pH 7.5). The concentrations of the components in the assay mixture were as follows; 0.25 mM NADPH, 1.5 mM GSSG, 1 mM EDTA and 2.35 μg enzyme in a final volume of 1.0 ml.

Inactivation Studies of Glutathione Reductase

For inactivation studies the enzyme (0.11 μM) was incubated with different concentrations of *o*-phthalaldehyde at 25°C. The aliquots were withdrawn from the incubation mixture at different time intervals and added to the assay mixture to measure the remaining reductase activity. Controls without inhibitor were run concurrently.

Effect of Substrates on Glutathione Reductase Inhibition by o-Phthalaldehyde

For protection experiments, the enzyme was preincubated with varying concentrations of GSSG, NADPH, NADP and alanine for 5 min prior to the addition of *o*-phthalaldehyde (200 μM). After 15 min of addition of *o*-phthalaldehyde, aliquots were withdrawn and added to the assay mixture for determination of the residual activity.

Fluorescence Spectral Experiments

Fluorescence spectral measurements were made on a fluorometer (Perkin Elmer, model LS 50B). In this experiment the dialyzed enzyme (0.1 μM) was incubated with 200 μM *o*-phthlaldehyde for 30 min in 0.05 M sodium phosphate buffer (pH 7.5) at 25°C. The emission spectrum with excitation at 337 nm and at 280 nm and the excitation spectra at 410 nm with a peak at 337 nm were recorded.

Stoichiometry of the Reaction of o-Phthalaldehyde with Glutathione Reductase

For the stoichiometry of reaction of *o*-phthalaldehyde with glutathione reductase, the enzyme was incubated with 200 μM *o*-phthalaldehyde for different time intervals and the absorbance was recorded at 337 nm. The number of isoindole derivatives formed were estimated as described previously. [18] The concentration of isoindole derivative formed was calculated by using extinction coefficient of 7.66 $M^{-1}cm^{-1}$ at 337 nm and the moleculer weight of the dimeric enzyme was taken as 104,000.

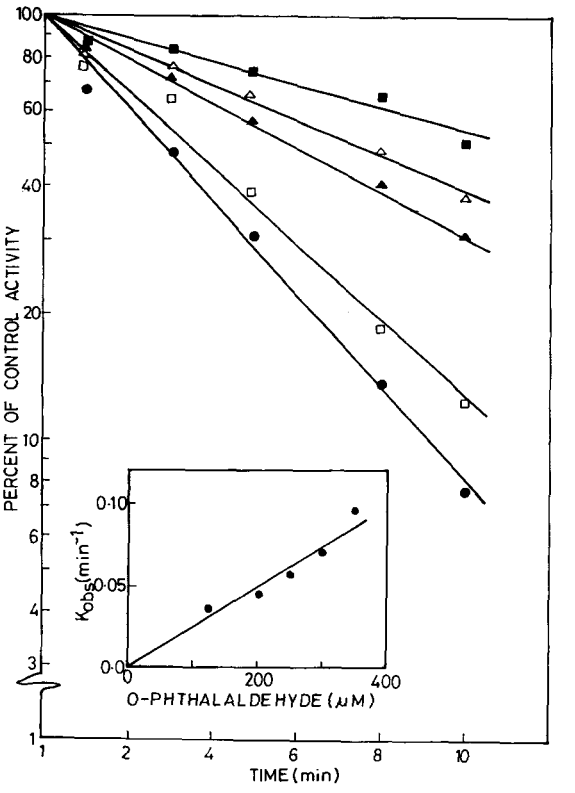

FIGURE 1 Time course of inactivation of glutathione reductase by *o*-phthalaldehyde. The enzyme (0.11 μM) was incubated with different concentrations of *o*-phthalaldehyde (■), 150 μM; (△), 200 μM; (▲), 250 μM; (□), 300 μM; (•), 350 μM. Aliquots (10 μl) were withdrawn at different time intervals and the residual activity was determined as described in the experimental procedure. (Inset). The linear relationship between the pseudo-first order rate constant (K_{obs}) for the inactivation and *o*-phthalaldehyde concentration.

RESULTS AND DISCUSSION

Inactivation of Glutathione Reductase by *o*-Phthalaldehyde

A progressive decrease in enzyme activity was observed when the enzyme was incubated with increasing concentrations of *o*-phthalaldehyde (Figure 1). The loss of enzyme activity followed pseudo-first order kinetics to 70% inactivation. Similar behaviour has been reported with other enzymes for *o*-phthalaldehyde inactivation.[19,20] K_{obs} was calculated for each concentration. A replot of K_{obs}

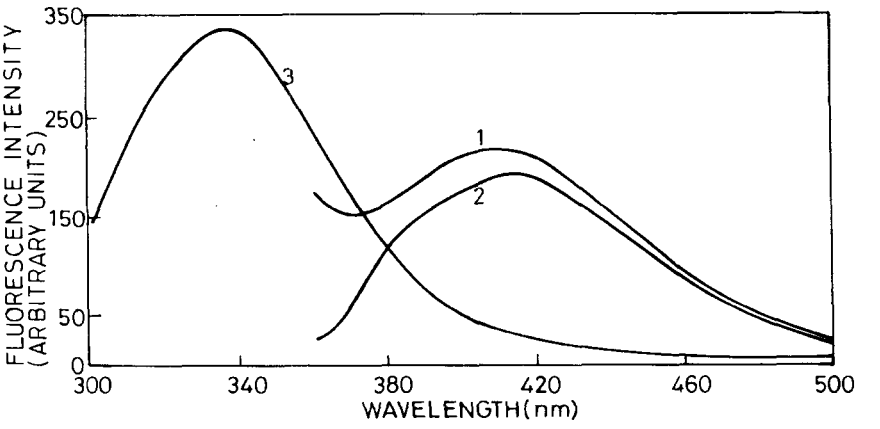

FIGURE 2 Fluorescence emission and excitation spectra of *o*-phthalaldehyde-modified glutathione reductase. The enzyme (0.1 μM) was incubated with 200 μM *o*-phthlaldehyde for 30 min in 0.05 M sodium phosphate buffer (pH 7.5) at 25°C. The emission spectrum with excitation at 337 nm (1) and at 280 nm, (2) and the excitation spectra at 410 nm with a peak at 337 nm (3) were recorded as described in the Experimental Procedure.

versus *o*-phthalaldehyde concentration was linear as shown in Figure 1 (inset) and the second order rate constant for the inactivation was calculated to be 15 $M^{-1}S^{-1}$. The overall activity of the enzyme decreased to 70% within a period of 10 min at 350 μM concentration of the reagent.

Characterization of the Modified Glutathione Reductase

o-Phthalaldehyde-modified glutathione reductase exhibited a typical fluorescence excitation and emission maxima. Excitation of glutathione reductase-*o*-phthalaldehyde adduct at either 280 or 337 nm led to the appearence of a fluorescence emission maximum at 410 nm (Figure 2). The excitation spectrum of the modified enzyme (emission wavelength at 410 nm) showed the presence of an excitation peak at 337 nm. These spectral characteristics of *o*-phthalaldehyde-modified glutathione reductase are consistent with the formation of an isoindole derivative by the reaction of *o*-phthalaldehyde with proximal-SH group of cysteine and ε-NH_2 of lysine of the enzyme. Formation of isoindole derivative showed that the distance between the cysteine and lysine residues is 3A°.[21,22] The molar transition energy (E_T) was calculated using the following equation.[23]

$$E_T = 2.985\lambda_{em} - 1087.28$$

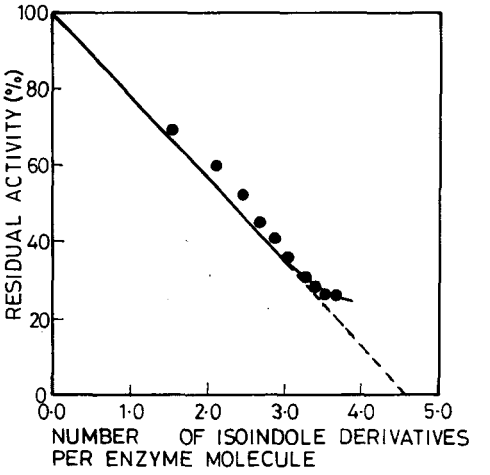

FIGURE 3 Correlation of the loss of glutathione reductase activity with the number of cysteine and lysine residues modified. Glutathione reductase (1 μM) in 0.05 M sodium phosphate buffer (pH 7.5) was treated with 200 μM *o*-phthalaldehyde as described in the Experimental Procedure. The enzyme activity was determined from 2–30 min and simultaneously the increase in the absorbance was monitored at 337 nm.

where λ_{em} is the fluorescence emission maxima exhibited by the modified enzyme. An E_T value of 145.55 kJ/mol was comparable to that reported for the synthetic isoindole derivative in diethylether (138 kJ/mol) indicating that the microenvironment of the site modified by *o*-phthalaldehyde is comparatively hydrophobic. A similar molar transition energy of 136.5 kJ/mol has been reported for aldolase.[23]

SDS-polyacrylamide gel electrophoretic studies showed no higher molecular weight species since both the native and modified enzyme showed identical mobilities. This indicated that there is no cross-linking between the monomers when the enzyme reacts with *o*-phthalaldehyde.

Number of Cysteine and Lysine Residues of Glutathione Reductase Modified by *o*-Phthalaldehyde

The stoichiometry of inactivation by *o*-phthalaldehyde was determined by absorbance measurement at 337 nm. The correlation between the loss of enzyme activity with increase in number of isoindole derivatives is shown in Figure 3. Extrapolation of the inactivation process to the complete inactivation of the enzyme

is accompanied by formation of 4 isoindole derivatives thereby showing that approximately 2 cysteine and 2 lysine residues per subunit of dimeric enzyme were essentially involved in the inactivation of glutathione reductase.

Effect of Substrates on Glutathione Reductase Inhibition by *o*-Phthalaldehyde

Effect of substrates viz. GSSG, NADPH and $NADP^+$ were carried out to ascertain the site of reaction of *o*-phthalaldehyde with the enzyme. It was observed that NADPH in the concentration range of 0.1–1 mM did not protect the enzyme against inactivation. However, GSSG afforded almost complete protection at a concentration of 12 mM. GSSG is a peptide having amino groups. It is reported that *o*-phthalaldehyde reacts with amino groups leading to the formation of nonfluorescent adducts.[24] In order to ascertain whether the protection afforded by GSSG was due to this reaction, the effect for alanine (12 mM), which is not a substrate for glutathione reductase, on the inactivation of enzyme was studied. Results indicated that alanine afforded complete protection to glutathione reductase against *o*-phthalaldehyde inactivation (Table I).

Effect of Pretreatment of Enzyme by PCMS and NEM on Inactivation by *o*-Phthalaldehyde

Two thiol specific reagents, viz. PCMS and NEM, were used to study the effect of blocking the -SH groups of enzyme on isoindole formation by *o*-phthalaldehyde. In the present study, it was observed that there is a significant decrease in fluorescence intensity of enzyme-*o*-phthalaldehyde adduct, if the enzyme is pretreated with NEM (Table II). This leads to the conclusion that both NEM and *o*-phthalaldehyde react with the same (-SH) group. However, NEM treatment does not lead to enzyme inactivation. Thus this cysteine is not essential for enzyme activity. Furthermore this non essential -SH group is proximal to lysine which on reaction with *o*-phthalaldehyde leads to isoindole derivative formation.

PCMS treatment on the other hand, leads to inactivation of the enzyme and its action is reversed by cysteine and β-mercaptoethanol. This shows the specific modification of an -SH group essential for enzyme activity. Further GSSG, the substrate, can protect the enzyme against this inactivation. There was no change in fluorescence intensity of enzyme-*o*-phthalaldehyde adduct even when the enzyme was treated with PCMS prior to the addition of *o*-phthalaldehyde. This clearly shows that NEM and PCMS react with different cysteines. The non-essential -SH group forms isoindole derivative with the ε-amino group of lysine in the *o*-phthalaldehyde reaction, resulting in inactivation due to conformational changes.

TABLE I Effect of substrates on the inhibition of glutathione reductase by *o*-phthalaldehyde. The enzyme (0.11 μM) was preincubated with GSSG, NADP and alanine for 5 min at indicated concentrations in 0.05 M sodium phosphate buffer (pH 7.5). *o*-Phthalaldehyde (200 μM) was then added and after 15 min an aliquot (10 μl) was withdrawn and the residual activity measured as described in the Experimental Procedure.

Protecting Substrate	*Residual Activity*
None	40
GSSG (12 mM)	94
NADP (2 mM)	48
NADP (4 mM)	50
Alanine (12 mM)	92
NADPH (0.1 mM)	30
NADPH (1.0 mM)	35

TABLE II Comparison of isoindole formation with native, PCMS-treated and NEM-treated glutathione reductase. Glutathione reductase (0.13 μM) was incubated with 200 μM *o*-phthalaldehyde in 0.05 M sodium phosphate buffer (pH 7.5) at 25°C for 15 min. The inactivated enzyme was dialyzed extensively in 0.05 M sodium phosphate buffer. In another experiment the enzyme (0.13 μM) was incubated with PCMS (200 μM) or NEM (1 mM) in 0.05 M buffer at 25°C and after extensive dialysis was treated with *o*-phthalaldehyde (200 μM) for 15 min. The fluorescence intensity of samples were determined as described in the Experimental Procedures.

Sample	*Fluorescence (arbitrary units) [Glutathione reductase] (μM)*
Native enzyme + *o*-phthalaldehyde	40
Native enzyme + PCMS	7
PCMS-treated enzyme + *o*-phthalaldehyde	40
Native enzyme + NEM	6
NEM-treated enzyme + *o*-phthalaldehyde	6

Our earlier studies which have shown that the inactivation of glutathione reductase by pyridoxal 5′-phosphate, a lysine specific reagent could be prevented by NADP, indicated the presence of an amino group at the coenzyme binding site.[25] However, the present investigations show that NADP did not offer any protection against *o*-phthalaldehyde inactivation. Thus it is possible that *o*-phthalaldehyde reacts with non-essential -SH and -NH_2 groups of glutathione reductase resulting in conformational changes leading to the enzyme inactivation. Similar observations have also been reported for octopine dehydrogenase inactivation by *o*-phthalaldehyde.[26]

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