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SIMULTANEOUS INACTIVATION OF THE CATALYTIC ACTIVITIES OF YEAST GLUTATHIONE REDUCTASE BY *N*-ALKYLMALEIMIDES

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Summary

A series of straight chain *N*-alkylmaleimides was shown to simultaneously inactivate the reductase, transhydrogenase and diaphorase activities of yeast glutathione reductase (NAD(P)H: oxidized-glutathione oxidoreductase, EC 1.6.4.2.) at pH 7.5 and 25°C. Apparent second-order rate constants for the inactivation of all enzyme activities exhibited parallel increases with increasing chainlength from C-2 through C-7 of the alkyl substituent of the maleimide. This positive chainlength effect is suggested to be due to an enhanced binding of maleimides through nonpolar interactions with the enzyme. Reduction of the active site disulfide with NADPH was required prior to addition of maleimide for inactivation to occur. NADP, AcPyADP, SNADP, AADP, and oxidized glutathione all protected the enzyme from inactivation. 2′AMP, 3′AMP, 2′-phospho-5′AMP, 2′-phospho-5′ADP and 2′-phospho-ADP-ribose although all coenzyme-competitive inhibitors failed to protect the enzyme from *N*-ethylmaleimide inactivation.

N-Phenyl and *N*-alkylmaleimides covalently modified two, of six available, sulfhydryl groups per subunit. No other amino acid residues were modified. The reactivity of these sulfhydryl groups was at least two orders of magnitude higher than any reported for the *N*-ethylmaleimide reaction with many other

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Abbreviations DTNB, 5,5′-dithiobis(2-nitrobenzoic acid), SNADP, thionicotinamide adenine dinucleotide phosphate, AAD, 3-aminopyridine adenine dinucleotide, AADP, 3-aminopyridine adenine dinucleotide phosphate, FMA, fluorescein mercuric acetate, AcPyADP, 3-acetylpyridine adenine dinucleotide phosphate, 2′,5′ADP, 2′-phospho-5′AMP

'essential sulfhydryl' enzymes. No change in the charge transfer band of the reduced enzyme was observed upon complete inactivation by *N*-ethyl, *N*-heptyl or *N*-phenylmaleimide. The retention of the charge transfer band after selective modification of two sulfhydryl groups suggests the involvement of a third reactive sulfhydryl group in the functioning of the yeast enzyme. No inactivation was observed when coenzymatically reduced enzyme was incubated with the site-specific sulfhydryl reagent, diazotized AADP.

Introduction

Yeast glutathione reductase catalyzes the NADPH-dependent reduction of oxidized glutathione. The enzyme has also been shown the possess a NADPH to SNADP transhydrogenase activity as well as a diaphorase activity [1,2]. The enzyme exists as a dimer of identical subunits containing 1 mol of tightly-bound FAD per subunit. Massey and Williams have demonstrated the presence of six cysteine residues per subunit [3]. Two of the cysteine residues in each subunit form an oxidation-reduction active disulfide which is essential for reductase activity.

The proposed mechanism of the reductase activity [4] involves the transfer of electrons from NADPH through bound FAD to the active site disulfide. The reduced disulfide has been implicated in the reduction of oxidized glutathione, through formation of an enzyme-glutathione mixed disulfide followed by ultimate reoxidation of the enzyme to the disulfide form. It is during the reduced disulfide step in the catalytic cycle that the enzyme was shown to be sensitive to sulfhydryl-modifying reagents [3,5]. The involvement of essential sulfhydryl groups in either the transhydrogenase or diaphorase activities has not been reported.

X-ray crystallographic studies of an analogous enzyme, erythrocyte glutathione reductase [6] indicated the presence of a small hydrophobic region near the active site thought to facilitate the binding of FAD If such a region is present in the yeast enzyme it could be expected to influence the reactivity of sulfhydryl groups nearby. In previous studies of enzyme inactivation by *N*-alkylmaleimides with various dehydrogenases [7—9], papain [10] and D-amino acid oxidase [11], facilitation of the rates of inactivation through nonpolar interactions of these reagents with the enzymes was demonstrated. Effective protection of these enzymes by either substrate, coenzyme or coenzyme analogs was observed in each case. It was of interest to study the effects of *N*-alkylmaleimides on yeast glutathione reductase in order to obtain further information concerning the reactivity of sulfhydryl groups of the enzyme towards alkylation, the involvement of nonpolar interactions in the functioning of these groups, the selectivity and stoichiometry of sulfhydryl modification and the possible involvement of sulfhydryl groups in the transhydrogenase and diaphorase activities.

Experimental procedures

Materials

N-Ethylmaleimide, fluorescein mercuric acetate, oxidized glutathione, 5,5'-dithiobis(2-nitrobenzoic acid), ammonium sulfamate, *N*-(1-naphthyl)-ethylene-

diamine dihydrochloride, sodium dodecyl sulfate (SDS), NADP, NADPH, NAD, NADH, FAD, SNADP, AcPyADP, 2′-monophosphoadenosine 5′-diphosphoribose, β-mercaptoethanol and Tris were purchased from Sigma 2′,5′ADP-Sepharose and Sephadex G-25 were purchased from Pharmacia. *N*-Phenylmaleimide and *N*-butylmaleimide were products of Nutritional Biochemicals, Inc. *N*-Pentyl, *N*-hexyl, *N*-heptyl, *N*-octyl, *N*-nonyl and *N*-decylmaleimides were synthesized according to Heitz et al. [9]. 3-Aminopyridine adenine dinucleotide (AAD) was prepared by the method of Fisher et al. [12] while the 2′-phosphate analog (AADP) was prepared by the method of Anderson et al. [13]

Yeast glutathione reductase was obtained either from Sigma or Calbiochem-Behring and further purified by a modification of the method of Carlberg and Mannervik [14]. All steps were performed at 4°C and all buffers contained 0.5 mM EDTA unless otherwise stated. Enzyme was dialyzed against two changes of 10 mM sodium phosphate buffer, pH 7.0, applied to a 2′,5′ADP-Sepharose column (1 × 5 cm) and washed with 50 ml of 50 mM sodium phosphate buffer, pH 7.5. A linear gradient of 0—500 mM NaCl in wash buffer was applied to the column. Eluted fractions were assayed for protein by absorbance at 280 nm. Enzymatic activity was assayed using the standard glutathione reductase assay outlined below. Fractions containing enzymatic activity were pooled, dialyzed against 10 mM sodium phosphate buffer, pH 7.0, and concentrated using a Diaflo PM-10 membrane. Protein concentrations were determined using $\epsilon^{1\%}_{280nm} = 18.6$ [3] and by the method of Lowry et al. [15] with bovine serum albumin as standard. Specific activity of the purified preparations ranged between 264 and 334 I.U./mg protein.

Methods

Glutathione reductase activity was assayed spectrophotometrically in 3 ml reaction mixtures containing 50 mM sodium phosphate buffer, pH 7.5/1.0 mM EDTA/0.67 mM oxidized glutathione/0.1 mM NADPH. After the addition of enzyme, the decrease in absorbance at 340 nm was monitored at 25°C using a Beckman Acta M-VI recording spectrophotometer

Transhydrogenase activity was assayed in 3 ml reaction mixtures containing 50 mM sodium phosphate buffer, pH 7.5/1.0 mM EDTA/0.2 mM SNADP/ 0.016 mM NADPH. The increase in absorbance at 399 nm at 25°C was recorded and the concentration of reduced SNADP was calculated using $\epsilon^{1\%}_{399nm} = 1.17 \cdot 10^4$ [16].

Diaphorase activity was assayed in 3 ml containing 50 mM sodium phosphate (pH 7.5)/1 mM EDTA/0.2 mM $K_3Fe(CN)_6$/0.07 mM NADPH. Prior to the addition of enzyme the non-enzymatic rate of ferricyanide reduction was recorded at 420 nm. The rate of non-enzymatic reduction was substracted from enzymatic rate of reduction to yield the net enzyme catalyzed rate.

Inactivation of glutathione reductase by *N*-alkylmaleimides was accomplished by first preincubating 4 μg (3 4 10^{-11}) mol enzyme at 25°C with a 500-fold excess NADPH in a volume of 0.1 ml. To the reduced enzyme was added 0.9 ml of 50 mM sodium phosphate buffer, pH 7.5/5% EtOH and the desired concentration of *N*-alkylmaleimide. After mixing, 50 μl aliquots were assayed for remaining reductase activity, whereas 150 μl aliquots were assayed

for transhydrogenase or diaphorase activities.

Studies of the protection of glutathione reductase from inactivation by *N*-alkylmaleimides were performed in the same manner as the inactivation experiments. The compound to be examined for protection was added simultaneously with the *N*-alkylmaleimide. Compounds were studied initially at 3-times their K_i or K_m values and if protection was observed, the concentration of protecting compound was decreased to either 100 or 50 μM to ascertain if protection was a concentration-dependent phenomenon.

DTNB titrations were performed by the method of Habeeb [17]. Reaction mixtures contained 2% SDS/5 mM EDTA/0.1 M Tris-HCl buffer, pH 8.0/0.67 mM DTNB in a total volume of 1.5 ml. Protein was denatured for 5 min at room temperature prior to addition of DTNB. After addition of DTNB, color was developed for 20 min at 25°C and absorbance at 412 nm recorded.

For aniline determinations, glutathione reductase (1.5 mg) was reduced with 500-fold excess NADPH. After approx. 97% inactivation by *N*-phenylmaleimide (usually 20 min), 2-fold molar excess β-mercaptoethanol was added to stop the reaction. The enzyme mixture was applied to a Sephadex G-25 column (1.7 × 45 cm) and eluted with 5 mM sodium phosphate buffer, pH 7.5, at 4°C. The fractions containing protein were pooled, concentrated using a Diaflo PM-10 membrane, and assayed by DTNB titration. The remaining enzyme was lyophilized to dryness, redissolved in 6 M HCl and hydrolyzed in vacuo for 72 h. The hydrolysate was air dried and the aniline released upon hydrolysis was determined according to Bratton and Marshall [18].

FMA titration of oxidized and reduced enzyme and maleimide-inactivated enzyme was performed as reported by Heitz and Anderson [19]. Stock solutions of FMA were prepared in 50 mM sodium phosphate buffer, pH 7.5. Exact concentrations of stock FMA were determined in 0.1 M NaOH at 499 nm using the molar extinction coefficient of $7.8 \cdot 10^4$ [19]. A standard curve was prepared in 2.9 ml of 50 mM sodium phosphate buffer, pH 7.5, by recording the relative fluorescence intensity with an Aminco-Bowman spectrophotofluorimeter. Solutions were excited at 495 nm and fluorescence recorded at 525 nm.

Spectral changes in FAD during the inactivation process were determined as follows. The spectrum of 4 μM glutathione reductase in 10 mM sodium phosphate (pH 7.0)/0.5 mM EDTA was recorded from 700—300 nm. The enzyme was reduced by addition of 0.1 ml of 6.7 mM NADPH and the resulting absorbance spectrum recorded. The reduced enzyme was then inactivated by 50 μM *N*-ethylmaleimide, 32 μM *N*-phenylmaleimide or 30 μM *N*-heptylmaleimide. Loss of enzymatic activity was monitored by assaying 2 μl aliquots directly from the cuvette. In all cases, the enzyme was 100% inactivated after 30 min at which time an absorbance spectrum of the completely inactivated enzyme was recorded.

Diazotized AADP was prepared according to the method of Anderson et al. [13]. To 0.065 ml of 60 mM AADP was added 0.25 ml of 1.0 M HCl, followed by the addition of 0.5 ml of 1.0 M $NaNO_2$. The resulting solution was incubated for 10 min prior to the addition of 0.5 ml of 2.0 M ammonium sulfamate and 10 min later the mixture was neutralized by addition of 0.25 ml of 1 M NaOH. The diazotized AADP was diluted with 10 mM sodium phosphate, pH 7.0 and the inactivation of glutathione reductase was performed in the same manner as for *N*-alkylmaleimides.

Results

Inactivation of reductase activity by N-alkylmaleimides

The oxidized form of the enzyme was reduced by incubation with 3 μM NADPH resulting in the appearance of two additional sulfhydryl groups as assayed by titration with DTNB. The reductase activity of the reduced enzyme was rapidly inactivated at pH 7.5 and 25°C by incubation with *N*-ethylmaleimide. Over the same time period, no inactivation of the oxidized form of the enzyme was observed. Inactivation of the reductase activity followed pseudo-first-order kinetics and the rates of inactivation for four concentrations of *N*-ethylmaleimide are shown in Fig. 1. In the absence of *N*-ethylmaleimide no loss in catalytic activity of the reduced enzyme was observed (Fig. 1). Pseudo-first-order rate constants for inactivation of the enzyme obtained for six concentrations of *N*-ethylmaleimide were shown to be linearly related to *N*-ethylmaleimide concentration. From these data, an apparent second-order rate constant of $2.3 \cdot 10^3\ M^{-1} \cdot min^{-1}$ was determined.

The reductase activity was shown to be effectively inactivated by a number of other *N*-substituted maleimides. Pseudo-first-order rates of inactivation of the reduced enzyme were obtained by incubating the enzyme with *N*-phenylmaleimide, *N*-benzylmaleimide and *N*-alkylmaleimides varying in chainlength from *N*-butyl to *N*-decyl, inclusive. The apparent second-order rate constants determined for these *N*-substituted maleimides are listed in Table I. The effectiveness of the straight chain *N*-alkylmaleimides in inactivating the enzyme increased to a degree with increasing chainlength of the alkyl substituent. A linear increase in these rate constants from the *N*-ethyl to *N*-heptyl derivative was observed. A further slight increase in rate constants was observed for the

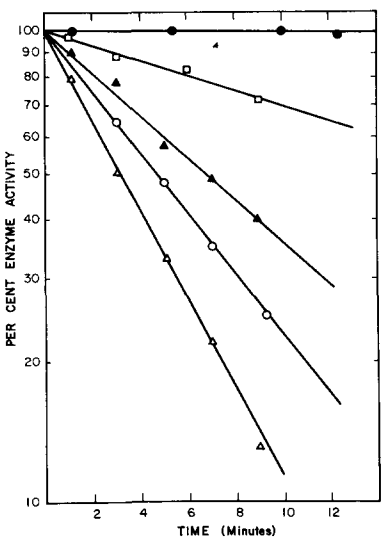

Fig 1 Time-dependent inactivation of yeast glutathione reductase (reduced form) by *N*-ethylmaleimide Reaction mixtures contained 4 μg reduced enzyme/50 mM sodium phosphate buffer, pH 7 5/5% ethanol/ *N*-ethylmaleimide in a total volume of 1 ml The concentrations of *N*-ethylmaleimide used were 0 (●———●), 40 μM (□———□), 60 μM (▲———▲), 80 μM (○———○) and 100 μM (△———△)

TABLE I

APPARENT SECOND-ORDER RATE CONSTANTS FOR INACTIVATION OF THE REDUCTASE AND TRANSHYDROGENASE ACTIVITIES OF YEAST GLUTATHIONE REDUCTASE

n d , not determined

Compound	Reductase k_2 (M^{-1} min^{-1})	Transhydrogenase k_2 (M^{-1} min^{-1})
N-Ethylmaleimide	2 3 10^3	2 9 10^3
N-Butylmaleimide	3 7 10^3	3 7 10^3
N-Pentylmaleimide	9 3 10^3	10 9 10^3
N-Hexylmaleimide	12 2 10^3	15 0 10^3
N-Heptylmaleimide	17 0 10^3	23 5 10^3
N-Octylmaleimide	17 4 10^3	20 9 10^3
N-Nonylmaleimide	17 8 10^3	23 2 10^3
N-Decylmaleimide	7 8 10^3	13 8 10^3
N-Benzylmaleimide	4 6 10^3	n d
N-Phenylmaleimide	30 2 10^3	36 6 10^3

N-octyl and *N*-nonyl derivatives followed by a pronounced decrease for the largest derivative, *N*-decylmaleimide.

Protection from inactivation by N-alkylmaleimides

The inactivation of reduced glutathione reductase by 50 μM *N*-ethylmaleimide and 7.5 μM *N*-heptylmaleimide was studied in the presence of oxidized glutathione, FAD, NADP and a number of monocleotides and dinucleotides having some structural analogy to the pyridine nucleotide coenzyme. The rates of maleimide inactivation in the presence of these compounds were compared to those observed with *N*-ethyl and *N*-heptylmaleimide in order to evaluate the effectiveness of these compounds in protecting the enzyme against maleimide inactivation. The results of the *N*-ethylmaleimide studies are shown in Table II. FAD and NAD were ineffective in protecting the enzyme against maleimide inactivation. The mononucleotides were likewise ineffective. NADP and the NADP analogs, AADP, AcPyADP and SNADP were very effective pro-

TABLE II

PROTECTION OF THE REDUCTASE ACTIVITY AND TRANSHYDROGENASE ACTIVITY OF GLUTATHIONE REDUCTASE FROM 50 μM *N*-ETHYLMALEIMIDE INACTIVATION

Protecting compound	Concentration (μM)	Reductase protection (%)	Transhydrogenase protection (%)
NADPH	12	0	0
NADP	81	33	67
AADP	84	76	81
SNADP	72	33	10
AcPyADP	90	75	67
GSSG	165	100	100
FAD	414	0	−3
NAD	1050	−3	−4
2′,5′ADP	3	−11	−13
2′-phospho-ADP-rib	6	−10	−2

tecting agents. The oxidized substrate was also observed to protect the enzyme against *N*-alkylmaleimide inactivation; however, in this specific case reoxidation of the enzyme must be considered.

Inactivation of transhydrogenase activity by N-alkylmaleimides

The kinetic properties of yeast glutathione reductase catalyzed NADPH to NAD transhydrogenase reaction have been reported [1], but involvement of sulfhydryl groups in this activity was not established. Such an involvement in the transhydrogenase activity was investigated by studying the effects of *N*-alkylmaleimides on this activity.

Incubation of oxidized enzyme with *N*-alkylmaleimides did not result in any inactivation of the transhydrogenase activity. However, incubation of reduced enzyme with *N*-ethylmaleimide resulted in a rapid, time-dependent pseudo-first-order inactivation process. Plots of pseudo-first-order rate constants vs. *N*-ethylmaleimide concentration were linear and an apparent second-order rate constant of inactivation of $2.9 \cdot 10^3\ M^{-1} \cdot min^{-1}$ was determined.

The transhydrogenase activity was also effectively inactivated by the series of *N*-substituted maleimides. The apparent second-order rate constants are listed in Table I. As was observed in the inactivation of the reductase activity the effectiveness of inactivation again increased as the number of carbons in the *N*-alkyl chain increased. A linear relationship was again observed in a plot of the logarithm of apparent second-order rate constant vs. carbon number as the *N*-alkyl chain increased from *N*-ethyl to *N*-heptylmaleimide.

Protection of transhydrogenase activity from inactivation by N-ethylmaleimide

Incubation of reduced enzyme with either AADP or NADP in the presence of *N*-ethylmaleimide effectively protected the enzyme from inactivation Therefore, a series of mono- and dinucleotides at concentrations 3-times their K_i or K_m values were evaluated for protection against 50 μM *N*-ethylmaleimide inactivation of yeast glutathione reductase transhydrogenase activity. These results are shown in Table II. The mononucleotides were again unable to protect the enzyme. Lack of protection was also observed with FAD and NAD. Only the complete dinucleotides were able to provide protection from *N*-ethylmaleimide inactivation of the transhydrogenase activity of the enzyme. The reduced coenzyme which is required for enzymatic activity was unable to protect the enzyme from inactivation. Oxidized glutathione which is not a substrate for the transhydrogenase reaction exhibited complete protection from inactivation.

N-Ethylmaleimide inactivation of the diaphorase activity

A third type of activity exhibited by yeast glutathione reductase is a reduced dinucleotide to ferricyanide diaphorase activity. The requirement for sulfhydryl groups in this activity was examined and only the reduced form was inactivated by *N*-ethylmaleimide and in a linear, time-dependent manner. A plot of the relationship of pseudo-first-order rates of inactivation as a function of *N*-ethylmaleimide concentration was linear and yielded an apparent second-order rate constant for inactivation of $2.7 \cdot 10^3\ M^{-1} \cdot min^{-1}$. Incubation of reduced enzyme with *N*-heptylmaleimide resulted in higher pseudo-first-order

rates of inactivation. These rates were linear with respect to concentration and gave an apparent second-order rate constant of $19.0 \cdot 10^3$ $M^{-1} \cdot min^{-1}$.

Inhibition studies

A number of the compounds used in the protection experiments had not been evaluated previously with respect to binding to the enzyme. Inhibition by these compounds was studied in two separate experiments. In the first case, inhibition was studied as a function of varying NADPH concentration. Initial velocity measurements made in the presence of NADP and plotted according to Lineweaver and Burk [20] are shown in Fig. 2. In a second set of experiments, inhibition of the enzyme by NADP was studied under conditions of constant NADPH and substrate, varying inhibitor concentration. Inhibition at six concentrations of NADP was plotted according to Dixon [21]. From these two types of inhibition studies, inhibitor dissociation constants were determined for a number of mono- and dinucleotides and are listed in Table III.

Sulfhydryl titration studies

The titration of SDS-denatured native oxidized enzyme with DTNB demonstrated the presence of four sulfhydryls per subunit of enzyme (59 000). After reduction of the enzyme with NADPH and subsequent SDS denaturation, DTNB titration revealed six sulfhydryls per subunit. Reduced enzyme more than 95% inactivated with *N*-phenylmaleimide was observed by DTNB titration to contain four sulfhydryls per subunit of enzyme. Two sulfhydryl groups were modified during the inactivation process. Oxidized enzyme was incubated with *N*-phenylmaleimide under conditions resulting in greater than 95% inactivation of the reduced enzyme and subsequent DTNB titration indicated the presence of four sulfhydryls per subunit. Therefore, *N*-phenylmaleimide at the low concentration used did not modify sulfhydryl groups in the oxidized form of the enzyme. These sulfhydryls were also observed to be unreactive toward *N*-heptylmaleimide

The sulfhydryl groups of yeast glutathione reductase were also titrated with fluorescein mercuric acetate (FMA) [19]. The fluorescence of 14 concentra-

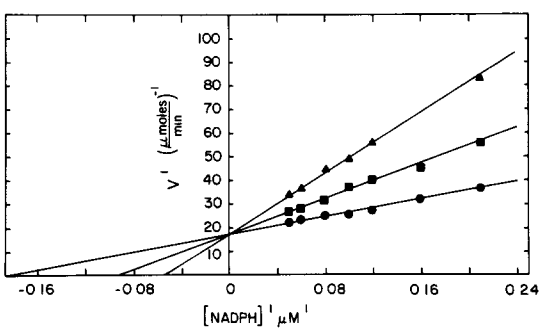

Fig 2 Competitive inhibition of yeast glutathione reductase by NADP NADPH concentrations varied from 3 6 to 19 μM Reaction mixtures contained 50 mM sodium phosphate buffer, pH 7 5/0 67 mM oxidized glutathione/0 8 mM EDTA/NADPH in a total volume of 3 ml 91 μM NADP (▲———▲), 27 μM NADP (■———■) and 0 NADP (●———●)

TABLE III

INHIBITION OF GLUTATHIONE REDUCTASE BY VARIOUS MONO- AND DINUCLEOTIDES

Compound	Type of inhibition *	K_I (μM)	
		Lineweaver-Burk	Dixon
NADP	competitive	27	26
SNADP	competitive	24	24
AADP	competitive	28	28
AcPyADP	competitive	30	27
Diazotized AADP	competitive	6 6	— **
2'-phospho-ADP-Rib	competitive	2 1	1 9
ADP-Rib	competitive	2300	2500
2'AMP	competitive	91	100
3'AMP	competitive	3500	3700
5'AMP	competitive	6800	9700
2'-phospho-5'-AMP	competitive	0 4	1
2'-mono-5'-diphosphoadenosine	competitive	1 9	0 5
5'ADP	competitive	438	375
3'-5'-cyclic AMP	competitive	8000	6000
NMN	competitive	— **	6600
FMN	non-competitive	— **	150
FAD	non-competitive	138	135

* Inhibition was measured relative to coenzyme

** Inhibition constant not determined by this method

tions of FMA was measured alone and in the presence of two concentrations of native oxidized enzyme. The fluorescence intensities obtained, plotted according to Hsu and Lardy [22], are shown in Fig. 3. From the inflection points of the lines seen in the presence of enzyme, the titration of one sulfhydryl group per subunit of oxidized enzyme was indicated. Titration of the reduced enzyme with FMA indicated the presence of three sulfhydryls per subunit. FMA titration of the *N*-phenylmaleimide-inactivated enzyme showed the

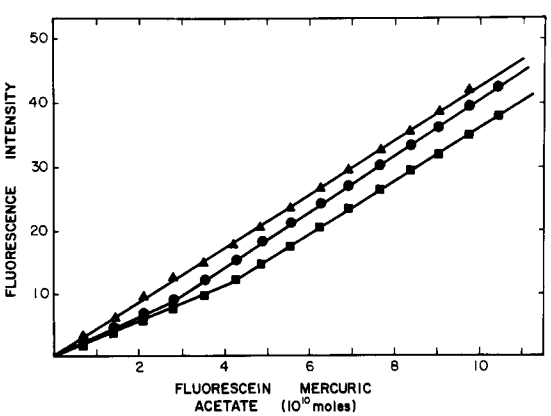

Fig 3 Fluorescence titration of yeast glutathione reductase with fluorescein mercuric acetate Reaction mixtures contained 50 mM sodium phosphate buffer, pH 7 5/fluorescein mercuric acetate as indicated and the following additions of glutathione reductase 0 (▲———▲), 1 1 10^{-10} mol (●———●), 1 6 10^{-10} mol (■———■)

TABLE IV

STOICHIOMETRY OF *N*-PHENYLMALEIMIDE INACTIVATION

Enzyme form	FMA titratable sulfhydryls/subunit	DTNB titratable sulfhydryls/subunit	Aniline released (mol/subunit)
Oxidized	1 1 ± 0 2	4 2 ± 0 1	0
Reduced	3 0 ± 0 4	5 9 ± 0 4	0
N-Phenylmaleimide	0 9 ± 0 1	3 9 ± 0 1	2 3 ± 0 3

presence of one available sulfhydryl group per subunit, again indicating that *N*-phenylmaleimide inactivation modified two sulfhydryls per subunit of enzyme. A summary of these results is listed in Table IV.

Concentrations of FMA sufficient to titrate three sulfhydryl groups per subunit of reduced glutathione reductase were observed to inactivate the reduced enzyme rapidly and irreversibly. Concentrations of FMA sufficient to titrate the one available sulfhydryl group per subunit in the oxidized enzyme did not cause inactivation when incubated with the oxidized enzyme.

DTNB and FMA titration studies demonstrated the modification of two sulfhydryl groups per subunit during the inactivation of the reduced enzyme by *N*-phenylmaleimide. The actual amount of *N*-phenylmaleimide covalently attached to the enzyme during inactivation required a separate analysis. Reduced glutathione reductase inactivated by *N*-phenylmaleimide was desalted and hydrolyzed in 6 M HCl to release aniline from amino acid residues modified by *N*-phenylmaleimide The aniline released was assayed as described by Bratton and Marshall [18]. 2 mol aniline were liberated per subunit of *N*-phenylmaleimide inactivated enzyme (Table IV) indicating the absence of modification of enzyme groups other than sulfhydryls.

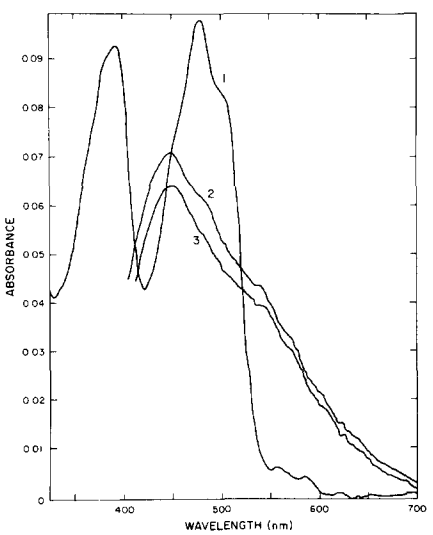

Fig 4 Absorbance spectrum of yeast glutathione reductase Line 1, oxidized enzyme, Line 2, coenzymatically reduced enzyme, Line 3, enzyme totally inactivated by *N*-phenylmaleimide Spectra have not been corrected for dilution

The loss of two sulfhydryls during inactivation by 20 μM *N*-ethylmaleimide was monitored by fluorescence titration with FMA. At 23, 57, 75 and 94% inactivation, 25.5, 43.4, 69.3 and 97.6%, respectively, of the two sulfhydryls were modified, indicating a correlation between inactivation and modification of the two reactive sulfhydryl groups.

Spectral changes of inactivated enzyme

Coenzymatic reduction of glutathione reductase yields a two-electron reduced form of the enzyme which exhibits an increased absorbance from 500 to 700 nm characteristic of a charge transfer complex [3]. The charge transfer band (line 2 of Fig. 4) was generated by reduction of $4 \cdot 10^{-9}$ mol enzyme with $4 \cdot 10^{-7}$ mol NADPH in 10 mM sodium phosphate, pH 7.9/0.5 mM EDTA. After recording the reduced spectrum the enzyme was inactivated by either *N*-ethyl, *N*-heptyl or *N*-phenylmaleimide. In each case, the spectrum of the completely inactivated enzyme (line 3, Fig. 4) did not change from that of the reduced enzyme indicating retention of the charge transfer complex.

Inactivation by diazotized AADP

The ability of diazotized AADP to bind to yeast glutathione reductase was examined at varying NADPH concentration and at fixed substrate concentration. Initial velocity measurements made in the presence of diazotized AADP were plotted according to Lineweaver and Burk. From the series of intersecting lines it was determined that diazotized AADP was a coenzyme competitive inhibitor of the enzyme with a K_i of 6.6 μM.

Diazotized AADP at concentrations 3-times the K_i value of 6.6 μM did not inactivate the reductase activity of either the oxidized or reduced yeast enzyme. Increasing the concentration of reagent to 100 μM resulted in slow inactivation of the reduced form of the enzyme. Inactivation occurred through a pseudo-first-order process with a pseudo-first-order rate constant of 0.038 min^{-1}. Concentrations of diazotized AADP greater than 100 μM no longer inactivated in a pseudo-first-order manner but instead biphasic inactivation was observed. When either 200 μM diazotized AAD or diazotized AADP were incubated with reduced enzyme equal rates of inactivation were observed.

Discussion

The reductase, dinucleotide transhydrogenase and ferricyanide diaphorase activities of yeast glutathione reductase have been shown in this study to be rapidly and irreversibly inactivated by a series of straight chain *N*-alkylmaleimides from *N*-ethyl to *N*-decylmaleimide, inclusive. Protection experiments and chainlength effects indicate that all three activities are altered to the same degree by the *N*-alkylmaleimide inactivation. Inactivation was only observed in the coenzymatically reduced form of the enzyme. Oxidized enzyme which contains four DTNB titratable sulfhydryl groups per subunit was neither inactivated nor modified by incubation with micromolar concentrations of *N*-heptyl and *N*-phenylmaleimide. One of the four sulfhydryl groups of oxidized yeast glutathione reductase was previously observed to be modified by *N*-ethylmaleimide [5], however, prolonged incubation with millimolar concentrations of

this reagent were required. Presumably, the remaining three sulfhydryl groups of the oxidized enzyme are buried in the enzyme molecule and inaccessible to these reagents in the absence of denaturing conditions. Incubation of the oxidized enzyme with nanomolar concentrations of FMA resulted in the modification of one sulfhydryl group per subunit of enzyme. The bulkiness of the FMA molecule and the inability of millimolar concentrations of *N*-ethylmaleimide to modify more than one sulfhydryl group per subunit argues for the location of one sulfhydryl group at or near the surface of the oxidized enzyme. Although this sulfhydryl group appears more accessible to the aqueous environment, it is not sufficiently reactive to be modified by micromolar concentrations of *N*-heptyl and *N*-phenylmaleimide.

Inactivation of the reductase, transhydrogenase and diaphorase activities of reduced glutathione reductase by *N*-alkylmaleimides was characterized by an increase in the rate of inactivation with an increase in the number of carbons of the *N*-alkyl substituent. A linear relationship between rate of inactivation and chainlength was observed between the *N*-ethyl and *N*-heptyl derivatives. The *N*-octyl and *N*-nonylmaleimides showed a less than projected effect on the rate of inactivation of these activities. Increasing the number of carbons as in *N*-decylmaleimide resulted in a pronounced decrease in effectiveness in inactivation. This decreased effectiveness could be due to either steric problems in the binding of longer chain derivatives or a limitation in the size of the nonpolar region of the enzyme. Limited solubility of the longer chain maleimides was ruled out as a factor in the decreased effectiveness since very low concentrations were used and longer chain derivatives were observed to be effective inactivators of other enzymes [10].

In previous studies [7—11] of the inactivation of enzymes by *N*-alkylmaleimides, positive chainlength effects have been attributed to an enhanced binding of these reagents to the enzyme. The ratio of second-order rate constants of inactivation by *N*-heptyl and *N*-ethylmaleimide, k_2 (heptyl)/k_2 (ethyl), has served as an index of the importance of nonpolar effects in the functioning of these compounds. A few representative values for this ratio are 8.6 for yeast alcohol dehydrogenase [9], 18.4 for papain [10], 11 for beef muscle lactate dehydrogenase [8] and 5.0 for ficin [23]. The values presented in Table V which were obtained for the reductase, transhydrogenase and diaphorase activities fall in the range obtained with the above enzymes. When nonpolar interactions are not important, a ratio close to unity has been observed, for example, 1.3 for yeast glucose-6-phosphate dehydrogenase [9], 0.6 for yeast 6-phosphogluconate dehydrogenase [24] and 1.0 for reactions with cysteine and glutathione [9]. From the ratios presented in Table V all three activities of

TABLE V

CHAINLENGTH EFFECTS FOR YEAST GLUTATHIONE REDUCTASE INACTIVATION

Activity	*N*-ethyl k_2 (M^{-1} min^{-1})	*N*-heptyl k_2 (M^{-1} min^{-1})	$\frac{k_2 \text{ (}N\text{-heptyl)}}{k_2 \text{ (}N\text{-ethyl)}}$
Reductase	2 3 10^3	17 0 10^3	7 4
Transhydrogenase	2 9 10^3	23 5 10^3	8.1
Diaphorase	2 7 10^3	19 0 10^3	7 0

yeast glutathione reductase have the same chainlength effect. This would suggest that a sulfhydryl group essential to all three activities is located in or very near a hydrophobic region on the enzyme. Schulz and coworkers [25] have proposed a hydrophobic region near the active site of human erythrocyte enzyme which is believed to facilitate binding of the benzene portion of the isoalloxazine ring of FAD. The possibility exists that a hydrophobic region may also occur in the yeast glutathione reductase and be responsible for the nonpolar effects in the functioning of the *N*-alkylmaleimides.

The inactivation of enzymes by *N*-alkylmaleimides cannot be attributed specifically to the modification of sulfhydryl groups without further experimentation Reactions of *N*-ethylmaleimide with histidine, lysine and N-terminal amino groups of proteins have been reported [26]. The inactivation of yeast glutathione reductase by *N*-phenylmaleimide was demonstrated to involve the modification of sulfhydryl groups only. This conclusion arises from the observation that the number of sulfhydryl groups disappearing during inactivation equalled the number of molecules of aniline released from the modified enzyme by acid hydrolysis. The rate of inactivation of the enzyme by *N*-ethylmaleimide equalled the rate of modification of the two reactive sulfhydryl groups of the enzyme.

Although the positive chainlength effects observed in the inactivation of the yeast enzyme by *N*-alkylmaleimides were consistent with those observed in the inactivation of several other enzymes, the reactivity of the glutathione reductase sulfhydryl groups was considerably higher. The apparent second-order rate constant for inactivation of yeast glutathione reductase by *N*-ethylmaleimide ($2.3 \cdot 10^3$ $M^{-1} \cdot min^{-1}$) was 173-times greater than that observed with yeast alcohol dehydrogenase and 14-times greater than that observed with papain. The latter comparison is of special interest since the sulfhydryl group of papain is a catalytically functional group. This higher reactivity in the *N*-alkylmaleimide inactivation of the reductase, transhydrogenase and diaphorase activities of glutathione reductase is consistent with the modification of an essential sulfhydryl group or groups involved in all three of these activities.

The two sulfhydryl groups of yeast glutathione reductase produced through reduction with NADPH have been localized with respect to N-terminal and C-terminal residues by Jones and Williams [27] indicating that the sulfhydryl closest to the N-terminus participates in disulfide interchange with the substrate, oxidized glutathione, and can be specifically alkylated by reaction with iodoacetamide [4]. The second sulfhydryl closer to the C-terminus was not modified by iodoacetamide. The iodoacetamide-inactivated enzyme was observed to retain diaphorase activity and exhibited a charge transfer band. It is therefore of special interest that the inactivation of reduced yeast glutathione reductase by *N*-alkylmaleimides resulted in the modification of two sulfhydryl groups per subunit of reduced enzyme and produced an inactivation of all three catalytic activities of the enzyme. An argument for the modification of both of the oxidation-reduction active sulfhydryl groups appears warranted, however, the retention of the charge transfer band during inactivation by maleimides would be inconsistent with this argument assuming the charge-transfer band is due solely to the sulfhydryl-FAD interaction. An alternate explanation would be the involvement of a third sulfhydryl group in the reduced enzyme, which

may or may not be necessary for transhydrogenase and diaphorase activity, but which is rapidly modified by reaction with maleimides.

Reversible inhibition of yeast glutathione reductase was observed with a number of mono- and dinucleotides (Table III). A comparison of the coenzyme-competitive inhibitors demonstrates the importance of a 2′-phosphate group in the binding of these compounds to the enzyme, consistent with the known coenzyme specificity of this enzyme. The same compounds were tested for protection of the enzyme against *N*-alkylmaleimide inactivation. Only the dinucleotides, SNADP, NADP, AcPyADP and AADP provided substantial protection against this inactivation (Table II). Thus, the positively charged pyridinium ring system appeared necessary for the protective effect. The requirement for a complete dinucleotide and not just the pyridinium ring system is evident by the inability of NMN to protect the enzyme. The simultaneous addition of NMN and 2′-phospho-5′AMP was unable to overcome this lack of NMN protection. If an active site geometry homologous to that observed in X-ray studies of erythrocyte glutathione reductase [6] is assumed, then a physical separation of the oxidation-reduction active disulfide and the pyridine nucleotide binding site, shielded by the isoalloxazine ring of FAD, would be expected. The requirement for an intact dinucleotide molecule for protection may indicate an induced conformational change on binding which results in the reactive sulfhydryl groups being less accessible to *N*-alkylmaleimides.

The suggestion that binding a dinucleotide elicits a conformational change is strengthened by reported dinucleotide titration of dithionite- or borohydride-reduced enzyme [28—30]. Spectral data from these studies indicated that chemically reduced enzyme exhibited a different charge-transfer band than that of coenzymatically reduced enzyme. Titration of chemically reduced two-electron reduced enzyme with NADPH caused a progressive increase in the 550—700 nm absorbance of the charge-transfer band which eventually became indistinguishable from a charge-transfer band produced by NADPH reduction. NADP titration of two-electron reduced enzyme on the other hand, resulted in a decrease of the 550—700 nm absorbance of the charge-transfer band resulting in an entirely different spectrum than the one obtained from NADPH titration. These data suggest that NADPH and NADP bind differently to the enzyme and result in different enzyme conformations.

Lack of protection by NADPH is difficult to explain in the light of the previous discussion. The data of Bulger and Brandt [28] suggest NADPH is capable of binding to the two-electron reduced form of the enzyme and therefore should have the ability to protect the enzyme. However, based on the X-ray data of Schultz et al. [25], the pyridine ring is not tightly bound to the enzyme and is therefore able to assume at least two orientations upon binding. The protection data suggest that in whichever orientation NADPH binds it does not protect the enzyme from *N*-ethylmaleimide inactivation. Protection of the enzyme by oxidized glutathione can be explained on the basis of reoxidation to the disulfide form of the enzyme. This was ruled out as a factor in the protection by dinucleotides since AADP has been demonstrated not to be chemically or enzymatically reduced [12,13], and spectral data do not indicate a reoxidation of the enzyme by AADP [31].

Diazotized AAD and AADP have been demonstrated to be site-labelling reagents for several pyridine nucleotide-requiring enzymes selectively modifying sulfhydryl groups located nearby the coenzyme binding sites of these enzymes [12,13,32—34].

If either of the two reactive sulfhydryl groups modified by maleimides was located near the pyridinium ring region of the NADP binding sites of yeast glutathione reductase, a site-directed inactivation by diazotized AADP would be expected. The inability of diazotized AADP to inactivate the enzyme suggests a different location for the two reactive sulfhydryl groups. This observation is consistent with the interpretation that the protection by dinucleotides against maleimide inactivation occurs through an induced conformational change of the enzyme rather than a direct blocking of the accessibility of these functional groups. Inactivation of the reductase by higher concentrations of diazotized AADP can be considered to be a nonspecific process as witnessed by the fact that diazotized AAD, which is not selectively bound to the enzyme, functions in an identical manner.

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