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Inactivation of Glutathione Reductase by 4-Hydroxynonenal and Other Endogenous Aldehydes

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ABSTRACT. 4-Hydroxynonenal, a product of oxidative degradation of unsaturated lipids, is an endogenous reactive α,β-unsaturated aldehyde with numerous biological activities. 4-Hydroxynonenal rapidly inactivated glutathione reductase in an NADPH-dependent reaction. Inactivation appears to involve the initial formation of an enzyme–inactivator complex, K_D = 0.5 μM, followed by the inactivation reaction, $k = 1.3 \times 10^{-2}$ min.$^{-1}$. α,β-Unsaturated aldehydes such as acrolein, crotonaldehyde, and cinnamaldehyde also inactivated glutathione reductase, although rates varied widely. Inactivation of glutathione reductase by α,β-unsaturated aldehydes was followed by slower NADPH-independent reactions that led to formation of nonfluorescent cross-linked products, accompanied by loss of lysine and histidine residues. Other reactive endogenous aldehydes such as methylglyoxal, 3-deoxyglucosone, and xylosone inactivated glutathione reductase by an NADPH-independent mechanism, with methylglyoxal being the most reactive. However, 2-oxoaldehydes were much less effective than 4-hydroxynonenal. Inactivation of glutathione reductase by these 2-oxoaldehydes was followed by slower reactions that led to the formation of fluorescent cross-linked products over a period of several weeks. These changes were accompanied by loss of arginine residues. Thus, the sequence of events is different for inactivation and modification of glutathione reductase by α,β-unsaturated aldehydes compared with 2-oxoaldehydes with respect to kinetics, NADPH requirements, fluorescence changes, and loss of amino acid residues. The ability of 4-hydroxynonenal at low concentrations to inactivate glutathione reductase, a central antioxidant enzyme, suggests that oxidative degradation of unsaturated lipids may initiate a positive feedback loop that enhances the potential for oxidative damage. BIOCHEM PHARMACOL 53;8:1133–1140, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. glutathione reductase; 4-hydroxynonenal; methylglyoxal; 3-deoxyglucosone; xylosone; acrolein

Glutathione reductase (EC 1.6.4.2), a flavoprotein that catalyzes the NADPH-dependent reduction of GSSG† to GSH, plays a key antioxidant role through its intracellular function of maintaining a high GSH/GSSG ratio [1, 2]. A high GSH/GSSG ratio is essential for protection against oxidative stress; GSH serves as a substrate for glutathione peroxidase and glutathione S-transferases in the decomposition of H_2O_2 and organoperoxides and the conjugation of reactive electrophiles, as a reductant to maintain ascorbic acid in the reduced state, as a substrate of thioltransferases, and as a radical scavenger [3, 4]. Decreases in the intracellular levels of glutathione reductase with aging, which may contribute to increased oxidative stress, have been reported [5–7]. Elevated levels of glutathione reductase are often found in drug resistant-tumors [8]. GSH levels are decreased in erythrocytes and lens in diabetes [9–11]. In addition, glutathione reductase levels are reduced in erythrocytes from diabetic adults and children [12, 13]. Glutathione reductase is inactivated by glycation [14] and by oxidative damage [15], which may contribute to the oxidative stress associated with diabetes.

A number of aldehydes are produced during oxidative stress or in response to various pathological conditions. 4-Hydroxynonenal and related alkenals are produced by oxidative degradation of lipids and are elevated in response to oxidative stress [16]. 2-Oxoaldehydes derived from glucose, including 3-deoxyglucosone [17], glucosone [18], and methylglyoxal [19], are produced in diabetes. The 2-oxoaldehydes xylosone and 3-deoxyxylosone are derived from oxidation of dehydroascorbic acid and ascorbic acid [20–22]. All of these endogenous aldehydes are especially reactive and may participate in cross-linking reactions due to their bifunctional reactive groups.

In the present study, we examined the abilities of these endogenous aldehydes to inactivate glutathione reductase. The question of interest is whether enhanced production of reactive aldehydes may contribute to the development of oxidative stress by inactivation of this central antioxidant enzyme. Glutathione reductase catalyzes a redox reaction in which the enzyme itself cycles between redox states involving reversible disulfide bond formation. Glutathione reduc-

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† *Abbreviations*: GSSG, oxidized glutathione; and GSH, reduced glutathione.

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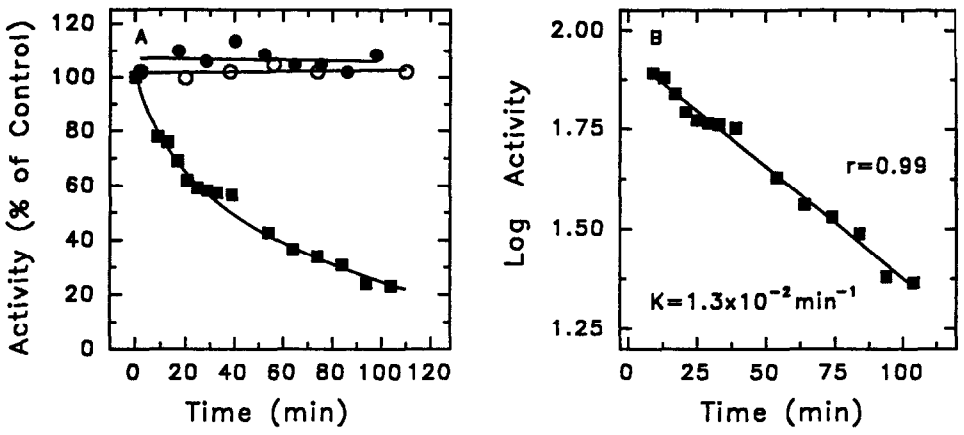

FIG. 1. Inactivation of glutathione reductase by 4-hydroxynonenal. (A) Experiments demonstrating the effect on 0.1 μM glutathione reductase of 10 μM 4-hydroxynonenal, pH 7, 37°. Key: (●) control, glutathione reductase without 4-hydroxynonenal or NADPH; (○) glutathione reductase incubated with 10 μM 4-hydroxynonenal; and (■) glutathione reductase incubated with 10 μM 4-hydroxynonenal and 0.1 mM NADPH. The K_m values for GSSG and NADPH of glutathione reductase after partial inactivation by 4-hydroxynonenal were unchanged. (B) Inactivation of glutathione reductase followed first order kinetics, k = 1.3 × 10^{-2} min^{-1}.

tase is known to be sensitive to chemical modification of the redox active thiol groups [23].

MATERIALS AND METHODS

Materials

Bovine glutathione reductase (Sigma, St. Louis, MO) was purified by HPLC on a Bio-Gel HPHT hydroxylapatite column (Bio-Rad, Richmond, CA). The diethylacetal of 4-hydroxynonenal was prepared according to Esterbauer and Weger [24]. 4-Hydroxynonenal was generated from the acetal with citric acid [25]. Acrolein, crotonaldehyde, and cinnamaldehyde (Aldrich, Milwaukee, WI) were purified by distillation. Methylglyoxal was generated from pyruvic aldehyde dimethylacetal (Aldrich) by treatment with sulfuric acid [26] followed by azeotropic distillation with water. Methylglyoxal was standardized using the glyoxalase system [27]. 3-Deoxy-D-glucosone, D-glucosone, 3-deoxy-L-xylosone, and L-xylosone were synthesized from D-glucose and L-xylose [28].

Enzyme Assays and Kinetics

The effects of treatment of glutathione reductase with various aldehydes were determined by monitoring changes in activity, kinetic properties, intrinsic fluorescence, cross-linking, and amino acid composition. Glutathione reductase was routinely assayed in 0.1 M potassium phosphate buffer, pH 7, containing 2 mM EDTA, 0.1 mM NADPH and 1 mM GSSG. Reactions were monitored at 340 nm, 25°, with a Perkin–Elmer Cetus Lambda 6 spectrophotometer. Kinetic analysis of glutathione reductase was carried out in the same buffer. K_m and k_{cat} values were determined by nonlinear regression analysis (ENZFITTER, Elsevier–Biosoft).

Fluorescence Studies

Glutathione reductase, 5 μg/mL, in the same buffer as above, was treated at 37° with 1 mM 4-hydroxynonenal, acrolein, methylglyoxal, 3-deoxyglucosone, or xylosone. Fluorescence emission and excitation spectra were taken at various times with a Perkin–Elmer LS 50 Luminescence Spectrometer: λ_{ex} 335 nm, λ_{em} 415 nm, slits 5 nm.

Cross-linking

Cross-linking of glutathione reductase by aldehydes was determined by SDS–PAGE under reducing conditions with 5% stacking gels and 20% running gels, followed by silver staining. Glutathione reductase, 100 μg/mL, was treated with 1 mM aldehydes for up to 6 weeks at 37°, under sterile conditions (filter sterilization). Cross-linking was also monitored by HPLC with a Bio-Sil TSK-250 gel filtration column.

Amino Acid Analysis

Changes in the amino acid composition of glutathione reductase upon reaction with 1 mM 4-hydroxynonenal or methylglyoxal were determined by taking samples of treated enzyme at various times and determining the amino acid composition after oxidation with performic acid, followed by acid hydrolysis. Untreated enzyme was used as the control. Amino acids were determined using a Waters Pico Tag system.

RESULTS

Inactivation of Glutathione Reductase by 4-Hydroxynonenal

The effects of 10 μM 4-hydroxynonenal, pH 7, 37°, on the activity of glutathione reductase, 0.1 μM, are shown in Fig. 1

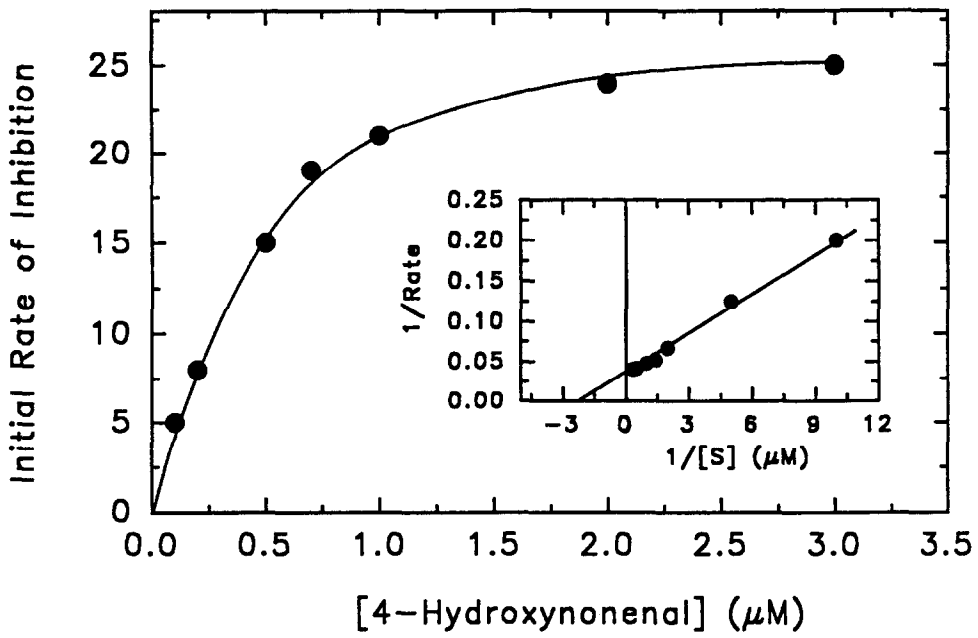

FIG. 2. Kinetic analysis of the initial rates of inactivation of glutathione reductase by 4-hydroxynonenal. Analysis of the initial rapid 20–25% inactivation of glutathione reductase (Fig. 1A) at lower concentrations of 4-hydroxynonenal.

There was a rapid inactivation of glutathione reductase only if NADPH was present. The inactivation reaction was first order, $k = 1.3 \times 10^{-2}$ min^{-1}, $T_{1/2} = 53$ min (Fig. 1B). However, there was a very rapid initial loss of 20–25% of the activity before the first order loss of the remaining activity. The kinetics of this initial loss of activity were evaluated at low concentrations of 4-hydroxynonenal, 0.1 to 3 μM, by measuring the initial rates of inactivation. Glutathione reductase with NADPH was incubated with 4-hydroxynonenal for 5 min, after which the residual activity was measured. The results (Fig. 2) suggest that 4-hydroxynonenal forms a complex with glutathione reductase, $K_D = 0.5$ μM; formation of this complex can be detected because it leads to rapid partial inhibition or inactivation of glutathione reductase. This is then followed by a slower first order inactivation reaction. To test whether partially inactivated forms of glutathione reductase could be detected, the enzyme was treated with 4-hydroxynonenal until 50% inactivation occurred, after which the sample was treated with dithiothreitol (DTT) to remove unreacted 4-hydroxynonenal by thiol addition to the α,β-unsaturated bond; the sample also was placed on ice. The treatment with DTT did not regenerate any of the lost enzyme activity. The kinetic properties of the remaining active enzyme were compared with untreated control. No differences in K_m values were observed, $K^{GSSG} = 42$ μM; $K^{NADPH} = 12$ μM.

Inactivation of Glutathione Reductase by Acrolein, Crotonaldehyde, and Cinnamaldehyde

The inactivation of glutathione reductase by the simple α,β-unsaturated aldehyde acrolein, 10 μM, is shown in Fig. 3. The results are similar to those observed with 4-hydroxynonenal (Fig. 1). Inactivation occurred rapidly only in the presence of NADPH. Similar studies were carried out with crotonaldehyde and cinnamaldehyde, as shown in Fig. 3. Rates of inactivation varied widely depending upon which α,β-unsaturated aldehyde was used. However, there is a consistent picture that supports the conclusion that the mechanism of inactivation of glutathione reductase by α,β-aldehydes is an NADPH-dependent process.

In the absence of NADPH, α,β-unsaturated aldehydes can produce a slow inactivation of glutathione reductase.

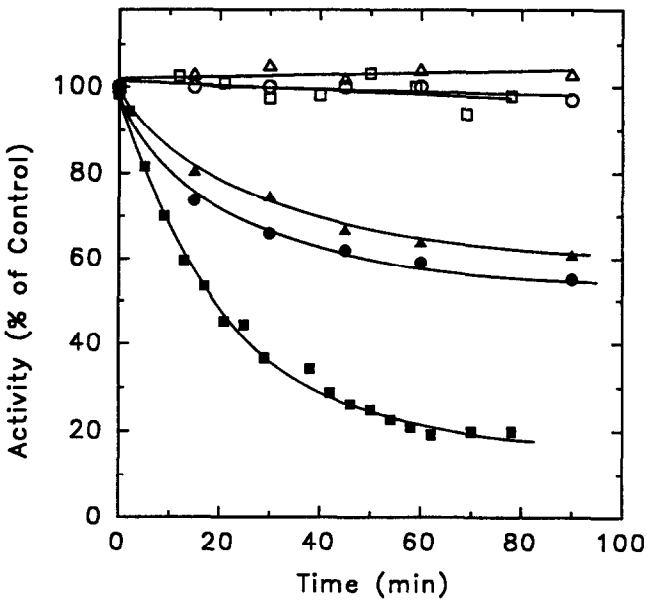

FIG. 3. Inactivation of glutathione reductase by acrolein, crotonaldehyde, and cinnamaldehyde. Shown are the results of experiments performed with 0.1 μM glutathione reductase in the presence of NADPH and (■) 10 μM acrolein, (▲) 100 μM crotonaldehyde, and (■) 500 μM cinnamaldehyde. Experiments performed in the absence of NADPH are shown by the open symbols.

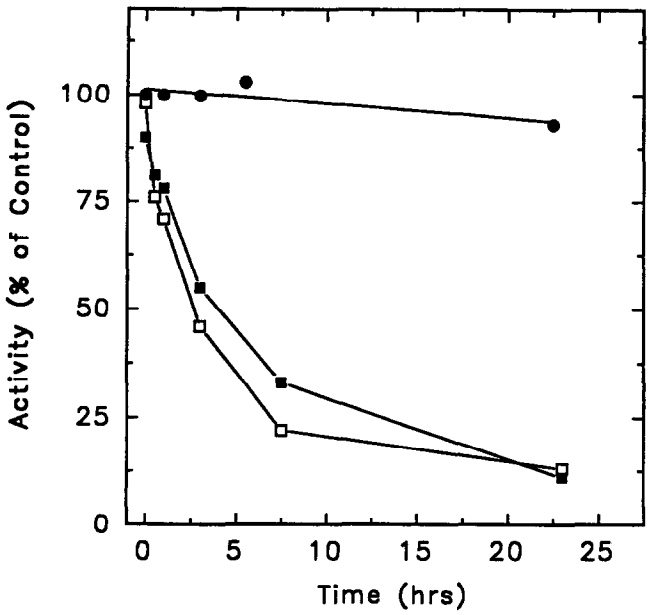

FIG. 4. Inactivation of glutathione reductase by methylglyoxal. Shown is the effect of 1 mM methylglyoxal on the rate of inactivation of 0.1 μM glutathione reductase, by methylglyoxal, 1 mM pH 7, 37°. Key: (●) control, glutathione reductase without methylglyoxal or NADPH; (■) glutathione reductase incubated with 1 mM methylglyoxal; and (□) glutathione reductase incubated with 1 mM methylglyoxal and 0.1 mM NADPH.

Treatment of glutathione reductase with 1 mM 4-hydroxynonenal or 1 mM acrolein, pH 7, 37°, resulted in 50% inactivation within 24 hr (data not shown). Thus, there are NADPH-dependent and -independent pathways of inactivation of glutathione reductase by α,β-unsaturated aldehydes.

Inactivation of Glutathione Reductase by Methylglyoxal

Glutathione reductase, 0.1 μM, was treated with 1 mM methylglyoxal, pH 7, 37°, with and without the addition of NADPH. Loss of activity was independent of the presence of NADPH. Glutathione reductase was 50% inactivated within 3 hr (Fig. 4). Glutathione reductase was treated with 1 mM methylglyoxal, as shown in Fig. 4, until 60% activity remained. The sample was then treated with GSH and glyoxalase-I to remove the residual methylglyoxal. The kinetic properties of the partially inactivated glutathione reductase were compared with control enzyme that was not exposed to methylglyoxal. Glutathione reductase that was partially inactivated by methylglyoxal exhibited hyperbolic kinetics, similar to the untreated enzyme, except that the Michaelis constant for GSSG for the partially inactivated enzyme increased 3-fold compared with the control (K_m 143 vs 42 μM). When the inactivation reaction was allowed to proceed to 80% inactivation, glutathione reductase still demonstrated hyperbolic kinetics, but K_m (GSSG) increased to 272 μM. Thus, the kinetic properties of the methylglyoxal-treated enzyme are altered even before there is complete loss of activity, unlike the situation with 4-hydroxynonenal. The increased K_m value for GSSG suggests that the modified glutathione reductase has lower affinity for GSSG. By comparison, there was no significant change in the K_m for NADPH upon treatment with methylglyoxal (K_m = 12 μM).

Inactivation of Glutathione Reductase by 3-Deoxyglucosone and Xylosone

Inactivation of glutathione reductase by 1 mM 3-deoxyglucosone or xylosone, under the same conditions used for inactivation by methylglyoxal, is shown in Fig. 5. The presence of NADPH did not affect the rates of inactivation. For 3-deoxyglucosone, 50% inactivation was achieved in approximately 150 hr, compared with 50 hr for xylosone. Thus, both 3-deoxyglucosone and xylosone are similar to methylglyoxal in effecting NADPH-independent inactivation of glutathione reductase but are 15- to 50-fold less reactive than methylglyoxal and several orders of magnitude less reactive than 4-hydroxynonenal.

Fluorescence Changes upon Modification of Glutathione Reductase with Endogenous Aldehydes

Incubation of glutathione reductase with 1 mM methylglyoxal resulted in the formation of a new absorption band at 330 nm that was fluorescent with an emission maximum at 400 nm. Changes in the emission spectrum that occurred over a 5-week period are shown in Fig. 6A (left) along with the excitation spectrum showing the new absorption band

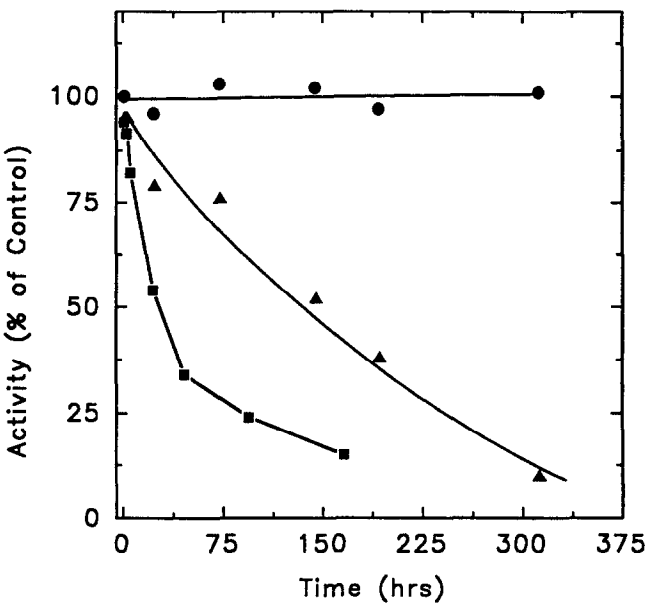

FIG. 5. Inactivation of glutathione reductase by 3-deoxyglucosone and xylosone. The inactivation of 0.1 μM glutathione reductase by 1 mM 3-deoxyglucosone (▲) and by 1 mM xylosone (■) compared with the untreated control (●) at pH 7, 37°.

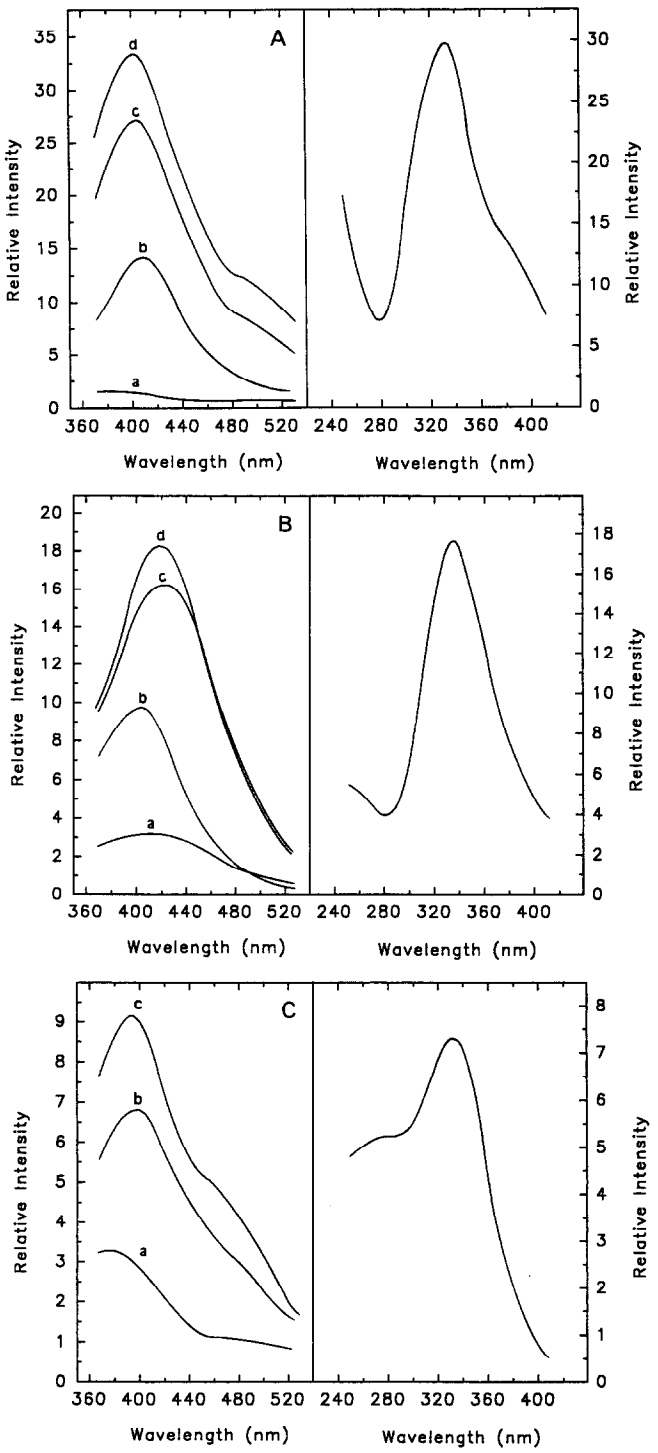

FIG. 6. Changes in the intrinsic protein fluorescence of glutathione reductase during modification by methylglyoxal, 3-deoxyglucosone, and xylosone. (A, left): Emission spectrum of 0.1 μM glutathione reductase during reaction with 1 mM methylglyoxal at 0, 1, 3, and 5 weeks (spectra a–d). λ_{max} = 400 nm; (A, right): excitation spectrum of glutathione reductase after reaction with methylglyoxal for 5 weeks. (B) Emission spectra during reaction with 1 mM 3-deoxyglucosone at 0, 1, 3, and 5 weeks (spectra a–d, left) and the excitation spectrum at 5 weeks (right). (C) Emission spectra during reaction with 1 mM xylosone at 0, 3, and 5 weeks (spectra a–c, left) and the excitation spectrum at 5 weeks (right).

at 330 nm (Fig. 6A, right). Incubation of glutathione reductase with 1 mM 3-deoxyglucosone or xylosone produced similar spectral changes. Upon treatment with 3-deoxyglucosone, the emission spectrum showed a maximum at 420 nm that resulted from a new absorption band at 340 nm (Fig. 6B). With xylosone treatment, the emission spectrum showed a maximum at 400-nm that resulted from a new absorption band at 335 nm (Fig. 6C). By comparison, there were no significant fluorescence changes upon modification of glutathione reductase with 1 mM 4-hydroxynonenal or acrolein.

Cross-linking of Glutathione Reductase by Endogenous Aldehydes

Treatment of glutathione reductase with 1 mM 4-hydroxynonenal or acrolein for up to 5 weeks produced extensive cross-linking, as shown in Fig. 7. For 4-hydroxynonenal, the cross-linking was so extensive that the cross-linked product did not enter the separating gel on SDS–PAGE. This was confirmed by analysis of the product by HPLC on a size exclusion column; all of the product appeared in the exclusion volume. Treatment of glutathione

FIG. 7. Cross-linking of glutathione reductase by treatment with 4-hydroxynonenal, acrolein, or methylglyoxal. Treatment of glutathione reductase, 100 μg/mL, with 1 mM 4-hydroxynonenal, acrolein, or methylglyoxal (lanes 3, 4, and 2, respectively) for 6 weeks at 37° resulted in extensive cross-linking. Purified glutathione reductase migrated as a doublet under the conditions used (lane 1). For 4-hydroxynonenal-treated glutathione reductase, the extensively cross-linked product did not enter the separating gel. Acrolein-treated glutathione reductase exhibited a wide spectrum of higher molecular weight products, reflected in the wide smear in lane 4. Samples were overstained to demonstrate this observation. For each lane, 0.6 μg protein was loaded onto the gel.

reductase with 1 mM methylglyoxal, 3-deoxyglucosone, or xylosone at 37° for up to 5 weeks resulted in extensive cross-linking, as shown in Fig. 7 for the reaction with methylglyoxal. The results with 3-deoxyglucosone and xylosone were similar to those with methylglyoxal (data not shown), indicating that the cross-linking reactions occurred at comparable rates although the initial rates of inactivation of glutathione reductase by these 2-oxoaldehydes varied by 50-fold.

Effects of Aldehyde Treatment on the Amino Acid Composition of Glutathione Reductase

Glutathione reductase was treated for up to 6 weeks at 37°, pH 7, with 1 mM methylglyoxal, as a representative 2-oxoaldehyde, and with 1 mM 4-hydroxynonenal, as a representative α,β-unsaturated aldehyde, to determine which amino acids appear to be lost during the reactions that lead to extensive cross-linking. Treatment of glutathione reductase with methylglyoxal for 6 weeks resulted in loss of approximately one-half of the arginine residues with little change in any other residues. By comparison, treatment of glutathione reductase with 4-hydroxynonenal for 6 weeks resulted in the loss of approximately one-third of the lysine residues and one quarter of the histidine residues. Clearly, the chemistry that results in the formation of cross-links by these aldehydes depends upon which aldehyde is used.

DISCUSSION

The present study demonstrated two different patterns of inactivation of glutathione reductase by endogenous aldehydes. Inactivation of glutathione reductase by the α,β-unsaturated aldehydes 4-hydroxynonenal and acrolein (Figs. 1 and 3) was a rapid, NADPH-dependent reaction. Subsequent slower reactions that produced cross-linking (Fig. 7) were accompanied by loss of lysine and histidine residues. By comparison, the 2-oxoaldehydes methylglyoxal, 3-deoxyglucosone, and xylosone inactivated glutathione reductase in an NADPH-independent reaction (Figs. 4 and 5). Subsequent reactions that produced fluorescent products (Fig. 6) and cross-linking (Fig. 7) were accompanied by loss of arginine residues. It is particularly noteworthy that the rapid inactivation of glutathione reductase by 4-hydroxynonenal occurred at low concentrations that are likely to be physiologically relevant. The mechanism of this inactivation by 4-hydroxynonenal likely involves modification of the redox-active cysteine residues that participate in the catalytic cycle of glutathione reductase and are sensitive to covalent modification [1]. The accepted mechanism of glutathione reductase can be considered to involve two half-reactions. In the first half-reaction, the binding of NADPH results in the reduction of enzyme-bound FAD. The binding of NADPH involves the sandwiching of the nicotinamide ring to the *re* face of the flavin ring and to a salt bridge comprised of Lys66 and Glu201 (numbered for the human enzyme) [1]. Transfer of reducing equivalents from NADPH to FAD occurs either as a hydride transfer or as electron flow through a charge transfer complex between the sandwiched rings. The reduced flavin, $FADH_2$, in turn, donates electrons to the active site internal disulfide (Cys58-Cys63) to form the stable reduced enzyme with two active site cysteine residues. Cys63, as the thiolate anion, forms a charge transfer complex with the oxidized flavin. Cys58 exists as a highly reactive nucleophile. In the second half-reaction, GSSG binds to reduced glutathione reductase. Cys58 attacks GSSG to form a mixed disulfide with liberation of GSH. Cys63 then attacks the mixed disulfide to liberate the second GSH and regenerate the initial oxidized glutathione reductase. Cys58 is in an environment that makes this a highly reactive thiol. Previous studies on the inactivation of glutathione reductase have shown that this is the residue that is often covalently modified [1]. The inactivation of glutathione reductase by 4-hydroxynonenal likely involves Michael addition of Cys58 to the α,β-unsaturated bond. NADPH is required for the inactivation (Fig. 1), consistent with the involvement of an active site cysteine. However, an explanation of the rapid initial loss of 20–25% activity upon the addition of 4-hydroxynonenal, which appears to involve addition of 4-hydroxynonenal to a saturable binding site (Figs. 1 and 2), is much more speculative. One possibility is that the initial binding of 4-hydroxynonenal produces a conformational change to a form with lower activity, and that this form of glutathione reductase is sufficiently stable that its activity can be determined in the general assay used in this study. A more likely possibility is that the binding of 4-hydroxynonenal at the active site may involve interaction of a nucleophile with the aldehyde functional group to hold 4-hydroxynonenal in place. This would still require that this complex have a reduced activity to explain the experimental observations of Fig. 2. In this scenario, formation of the partially inhibited complex would be followed by first order inactivation through addition of Cys58 to the α,β-unsaturated bond.

4-Hydroxynonenal and acrolein are two of many aldehydes formed by oxidative degradation of lipids [16]. 4-Hydroxynonenal has been studied extensively. This α,β-unsaturated aldehyde exhibits a range of biological activities at low micromolar to submicromolar concentrations. This includes inhibition of DNA synthesis [29], reduction of c-*myc* transcription [30], stimulation of adenylate cyclase, guanylate cyclase, and phospholipase C [31, 32], and stimulation of chemotaxis [33]. Basal levels in human serum are generally less than micromolar; however, serum levels can be much higher in experimental animal models of oxidative stress [34]. Recent studies by Stadtman and coworkers demonstrated that 4-hydroxynonenal can modify and inactivate a number of enzymes, leading to the formation of cross-linked products. In addition, 4-hydroxynonenal protein adducts can be detected immunochemically in hepatocytes subjected to oxidative stress [37]. This suggests that covalent modification of proteins by 4-hydroxynonenal may be physiologically important, especially if it occurs

in long-lived proteins where cross-links can accumulate over time. Thus, the slow cross-linking of enzymes such as glutathione reductase by 4-hydroxynonenal may be less important than the cross-linking of matrix proteins and lens proteins.

Glutathione reductase appears to be especially sensitive to oxidative stress. A recent study of the effects of depletion of GSH *in vivo* on the activities of GSH-requiring enzymes demonstrated that glutathione reductase is the most sensitive to loss of GSH [38]. The present study suggests that oxidative damage to lipids, resulting in the formation of 4-hydroxynonenal and related α,β-unsaturated aldehydes, may amplify the oxidative stress through a positive feedback mechanism in which the key antioxidant enzyme glutathione reductase is inactivated by these aldehydes at low, physiologically relevant concentrations.

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