Inactivation of Glucose-6-phosphate Dehydrogenase by 4-Hydroxy-2-nonenal

SELECTIVE MODIFICATION OF AN ACTIVE-SITE LYSINE*

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Inactivation of glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides with 4-hydroxy-2-nonenal (HNE) results in a pseudo first-order loss of enzyme activity. The pH dependence of the inactivation rate exhibits an inflection around pH 10, and the enzyme is protected from inactivation by glucose 6-phosphate. Loss of enzyme activity corresponds with the formation of one carbonyl function per enzyme subunit and the appearance of a lysine-HNE adduct. The data presented in this paper are consistent with the view that the ε-amino group of a lysine residue in the glucose 6-phosphate-binding site reacts with the double bond (C=) of HNE, resulting in the formation of a stable secondary amine derivative and loss of enzyme activity. We have described a mechanism by which HNE may, in part, mediate free radical damage. In addition, a method for the detection of the lysine-HNE adduct is introduced.

Reactive oxygen species readily interact with membrane lipids, often resulting in the formation of unsaturated aldehydes such as hydroxyalkenals (1). These aldehydes are more stable than free radical species and may more readily diffuse into cellular media, where they are available for facile reaction with various biomolecules. Modification of protein and other biomolecules by lipid peroxidation products is believed to play a role in various biological effects such as high toxicity to Ehrlich ascites tumor cells (6), effects such as the inhibition of enzyme-catalyzed reaction, performed at 25 °C, was initiated by the addition of a 50 μl aliquot of 10 mM NADP+ and 100 mM glucose-6-phosphate to 50 μl of enzyme solution. Glu-6-P dehydrogenase activity was then determined by following the appearance of NADPH absorbance at 340 nm with a molar absorptivity of 6.220 M⁻¹ cm⁻¹ for NADPH.

MATERIALS AND METHODS

Preparation of Glucose-6-phosphate Dehydrogenase—Glu-6-P dehydrogenase purified from L. mesenteroides was purchased from Sigma at a concentration of 8.8 mg/ml in 3.2 M (NH₄)₂SO₄. Prior to exposure of the enzyme to HNE, (NH₄)₂SO₄ was removed by passage through a Sephadex G-25M PD-10 column (Pharmacia LKB Biotechnology Inc.). The enzyme was eluted with 10 mM MOPS, 100 mM KCl at pH 7.4. This process was then repeated to ensure complete removal of (NH₄)₂SO₄. The enzyme, at a final concentration of ~2.0 mg/ml, was stable at room temperature for up to 12 h. 4-Hydroxy-2-nonenal Preparation—4-Hydroxy-2-nonenal dimethylacetal was synthesized as previously described (12). Prior to use, HNE was generated by acid treatment (1 mM HCI) of 4-hydroxy-2-nonenal dimethylacetal. The 1H NMR spectra of 4-hydroxy-2-nonenal dimethylacetal and 4-hydroxy-2-nonenal were in agreement with published results (12). The concentration of HNE was determined by measurement of UV absorbance at 224 nm with a molar absorptivity of 13,750 M⁻¹ cm⁻¹ for HNE.

Measurement of Glucose-6-phosphate Dehydrogenase Activity—Glu-6-P dehydrogenase was diluted in 10 mM MOPS, 100 mM KCl at pH 7.4 to give a final protein concentration of ~25 mg/ml. The enzyme-catalyzed reaction, performed at 25 °C, was initiated by the addition of a 50 μl aliquot of 10 mM NADP+ and 100 mM glucose-6-phosphate to 50 μl of enzyme solution. Glu-6-P dehydrogenase activity was then determined by following the appearance of NADPH absorbance at 340 nm with a molar absorptivity of 6.220 M⁻¹ cm⁻¹ for NADPH.

FIG. 1. Structure of 4-hydroxy-2-nonenal.
spectrophotometrically at 340 nm using a Hewlett-Packard Model 8452A diode array spectrophotometer. Enzyme activity was linear under all conditions tested over the time assayed.

Incubation of Glucose-6-phosphate Dehydrogenase with 4-Hydroxy-2-nonenal—Glu-6-P dehydrogenase (0.5 mg/ml) was incubated at 37 °C in 10 mM MOPS, 100 mM KCl at pH 7.4 (except where stated) with various concentrations of HNE for the indicated periods of time. Inactivation was arrested by diluting the inactivation mixture 20,000-fold in 10 mM MOPS, 100 mM KCl at pH 7.4. Enzyme activity was then determined as described above. Prior to amino acid analysis or treatment of the modified enzyme with 2,4-dinitrophenylhydrazine or NaBH₄, unreacted HNE was removed by passage over a Sephadex G-25M PD-10 column.

Treatment of Modified Glu-6-P Dehydrogenase with 2,4-Dinitrophenylhydrazine—The carbonyl content of the protein was determined using a variation of a previously described technique (13). Enzyme (0.25 mg), taken to dryness using a rotary evaporator, was resuspended in 500 μl of 10 mM 2,4-dinitrophenylhydrazine, 60 mM guanidine HCl, and 0.5 M K₂HPO₄, pH 2.5. The enzyme was incubated with 2,4-dinitrophenylhydrazine at 25 °C for 90 min. Derivatized protein was then separated from excess reagent by filtration of the reaction mixture through a Zorbax GF250 gel column (Du Pont-New England Nuclear) using a Hewlett-Packard Model 1090 high-performance liquid chromatograph equipped with a diode array UV detector. Derivatized enzyme was eluted with a solution containing 60 mM guanidine HCl and 0.5 M K₂HPO₄, pH 2.5. Elution profiles were detected at 276 and 370 nm. Molar ratios of carbonyl content to enzyme subunit were then calculated using molar absorptivities of 9500 and 22,000 M⁻¹cm⁻¹ for the hydrazone at 276 and 370 nm, respectively. The molar absorbivity for Glu-6-P dehydrogenase in 60 mM guanidine HCl and 0.5 M K₂HPO₄, pH 2.5, is 60,000 M⁻¹cm⁻¹ at 276 nm.

Treatment of Modified Glu-6-P Dehydrogenase with NaBH₄—A 400-μl aliquot of HNE-modified Glu-6-P dehydrogenase (0.25 mg/ml) was mixed with 0.1 M EDTA (40 μl) and 1 N NaOH (40 μl) in a 1.5-ml Sarstedt tube fitted with an O-ring and cap. A 40-μl aliquot of 0.1 M NaBH₄ in 0.1 N NaOH was then added, and the mixture was incubated at 37 °C for 1 h. The reaction was terminated by the addition of 100 μl of 1 N HCl. To separate "H-labeled protein from other radioactive contaminants, the reaction mixture was chromatographed over a Sephadex G-25M PD-10 column using 60 mM guanidine HCl as eluant. Recovery of protein was determined by the measurement of UV absorbance at 278 nm with a molar absorptivity of 60,000 M⁻¹cm⁻¹ for Glu-6-P dehydrogenase. The protein carbonyl content was calculated from radioactive measurement of the suitable aliquots. The specific radioactivity of NaB³H₄ was determined by reaction with 4.0 M acetone in 100 mM NaOH (14) and the subsequent measurement of incorporated ³H.

Amino Acid Analysis—A 100-μl aliquot of modified Glu-6-P dehydrogenase (0.25 mg/ml) was treated with 10 μl of 10 mM EDTA, 10 μl of 1 N NaOH, and 10 μl of 100 mM NaBH₄ in 0.1 N NaOH. After 1 h at 37 °C, the reaction was terminated by the addition of 40 μl of 1.0 N HCl. The solution was brought to dryness using a rotary evaporator, and the protein was subsequently hydrolyzed with 6 N HCl (200 μl) for 20 h at 110 °C under nitrogen atmosphere. The hydrolyzed sample was evaporated to dryness and resuspended in 2.0 ml of 50 mM sodium phosphate buffer, pH 8.0, containing 1 mM EDTA. A 10-μl aliquot of the hydrolyzed protein solution was labeled with o-phthalaldehyde (15). Derivatized amino acids were analyzed by reverse-phase HPLC using a C₁₈ column (Jones Chromatography) fitted to a Hewlett-Packard Model 1090 chromatograph equipped with a Hewlett-Packard Model 1046A programmable fluorescence detector.

Lysine-4-Hydroxy-2-nonenal Adduct—The standard sample of the HNE-lysine adduct was prepared by reaction of HNE with N-acetyllysine. N-Acetyllysine (50 μm) was treated with 5 μm HNE in 50 mM sodium phosphate buffer, pH 7.2, for 20 h at 37 °C. Formation of products was determined by HPLC. A linear gradient of 0.05% trifluoroacetic acid in water (solvent A)/acetonitrile (solvent B) (time 0, 100%; B) at a flow rate of 1 ml/min was used with a TSK-GEL ODS-80TM column (TOSOHASS). The major component was collected and further characterized by amino acid and mass spectral analyses. Amino acid analysis was performed as outlined above. A JEOL JMS-SX102 mass spectrometer was used for fast atom bombardment–mass spectrometry.

RESULTS AND DISCUSSION

Inactivation of Glu-6-P Dehydrogenase by 4-Hydroxy-2-nonenal—Incubation of Glu-6-P dehydrogenase with HNE resulted in a loss of enzyme activity. Approximately 70% of the initial activity was lost when the enzyme was exposed to 1.5 mM HNE at pH 7.4 and 37 °C for 90 min (Fig. 2). A semilogarithmic plot of remaining activity versus incubation time (Fig. 2, inset) is linear over the experimental interval examined, demonstrating that inactivation occurred by a pseudo first-order process. The fractional loss of Glu-6-P dehydrogenase activity was independent of enzyme concentration over
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Fig. 3. Observed rate constant \((k_{\text{obs}})\) for inactivation of glucose-6-phosphate dehydrogenase as function of 4-hydroxy-2-nonenal concentration. Enzyme at a concentration of 0.5 mg/ml was incubated at 37 °C for 0–30 min at the concentrations of 4-hydroxy-2-nonenal indicated on the abscissa. Inactivation was arrested by dilution, and remaining activity was determined as described under "Materials and Methods." The observed rate constant \((k_{\text{obs}})\) is equal to \(0.693/t_{1/2}/\text{min}\), where \(t_{1/2}\) is defined as the time at which half of the initial enzyme activity remains. The inset shows the initial rate of these data expressed as a double-reciprocal plot of the rate of inactivation versus 4-hydroxy-2-nonenal concentration.

Fig. 4. Double-reciprocal plot of difference between rate of glucose-6-phosphate dehydrogenase inactivation in absence and presence of glucose 6-phosphate as function of glucose 6-phosphate concentration. Enzyme at a concentration of 0.5 mg/ml was incubated with 1.0, 2.0, 4.0, and 8.0 mM 4-hydroxy-2-nonenal at 37 °C for 0–30 min with 0, 0.5, 1.0, 2.0, or 4.0 mM glucose 6-phosphate. Inactivation was arrested by dilution, and remaining activity was determined as described under "Materials and Methods." Fig. 3 (inset), the double-reciprocal plot of the initial rate versus HNE concentration is linear with an intercept near the origin. These data suggest that the \(K_d\) for the Glu-6-P dehydrogenase-HNE complex is much larger than 5.0 mM, if such a complex indeed forms for the inactivation reaction. It is likely that inactivation is the result of a bimolecular collision between an exposed nucleophilic residue on the protein and HNE, with a second-order rate constant of 11.0 min⁻¹ mM⁻¹.

Mechanism of Inactivation of Glu-6-P Dehydrogenase by 4-Hydroxy-2-nonenal—The effect of adding substrate to the
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Lysine Residue

\[
\begin{align*}
&\text{Lysine Residue} \\
&\text{4-Hydroxy-2-nonenal} \\
&\text{2,4-Dinitrophenylhydrazine}
\end{align*}
\]

**Fig. 6.** Proposed reaction of lysine residue with double bond of 4-hydroxy-2-nonenal and subsequent derivatization of lysine-4-hydroxy-2-nonenal adduct with 2,4-dinitrophenylhydrazine.

Lysine Residue

\[
\begin{align*}
&\text{Lysine Residue} \\
&\text{4-Hydroxy-2-nonenal} \\
&\text{2,4-Dinitrophenylhydrazine}
\end{align*}
\]

**Fig. 7.** Proposed reaction of lysine residue with carbonyl function of 4-hydroxy-2-nonenal and subsequent derivatization of 4-hydroxy-2-nonenal with 2,4-dinitrophenylhydrazine.

Inactivation mixture was tested to identify sites of modification. We found that glucose 6-phosphate protected Glu-6-P dehydrogenase from HNE inactivation. A double-reciprocal plot of the difference between the rate of inactivation in the absence and presence of glucose 6-phosphate versus glucose 6-phosphate concentration (Fig. 4) reveals that glucose 6-phosphate inhibits inactivation of the enzyme by HNE in a noncompetitive fashion with a \( K_i \) of \(-1.5 \text{ mM} \). Glucose 1-phosphate, glucose, phosphate, and NADP\(^+\) had no effect on inactivation of the enzyme by HNE, indicating that protection afforded by glucose 6-phosphate is highly specific. As previously noted, HNE does not appear to form a stable complex with Glu-6-P dehydrogenase. Therefore, the noncompetitive pattern obtained with glucose 6-phosphate does not exclude the possibility that HNE reacts with amino acid residue(s) in or near the glucose 6-phosphate-binding site. The observed \( K_i \) (1.5 mM) for glucose 6-phosphate inhibition of HNE inactivation is 10 times greater than the \( K_m \) (150 \text{ \mu M}) of Glu-6-P dehydrogenase for glucose 6-phosphate. This difference between \( K_i \) and \( K_m \) is not surprising since the \( K_m \) of Glu-6-P dehydrogenase for glucose 6-phosphate is not a measure of binding affinity (16).

In an attempt to provide information on the amino acid residue(s) involved in the inactivation of Glu-6-P dehydrogenase by HNE, we explored the effect of pH on this process. As shown in Fig. 5, the rate of inactivation increased with increasing pH. The inflection point was at a pH of 10, a value comparable to the \( pK_a \) values of lysine and arginine. Ionic strength had little or no effect on the rate of inactivation (data not shown). In the absence of HNE, no loss of enzyme activity was observed over the pH range used in this study throughout the 2-min incubation period. At pH values above 11.5, the enzyme was highly unstable. Furthermore, glucose 6-phosphate protected the enzyme from HNE inactivation at all pH values tested (data not shown). This fact and the demonstration that Glu-6-P dehydrogenase from \( L. \) mesenteroides contains a lysine residue required for the binding of the phosphate moiety of glucose 6-phosphate (17–19) are consistent with the view that loss of activity results from reaction of HNE with the lysine residue involved in the binding of glucose 6-phosphate.

Like other \( \alpha,\beta \)-unsaturated aldehydes, 4-hydroxy-2-nonenal should be susceptible to nucleophilic addition at both the double bond (C-3) and the carbonyl moiety (C\(_1\)). Accordingly, the \( \epsilon \)-amino group of lysine residues on a protein may react with HNE to form a secondary amine derivative with
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Fig. 8. Molar ratio of carbonyl to enzyme subunit as function of fractional loss of glucose-6-phosphate dehydrogenase activity. Following treatment of the enzyme as described for Fig. 2, 2,4-dinitrophenylhydrazine-detectable carbonyl content was determined as described under "Materials and Methods.

Fig. 9. Proposed structure of lysine-4-hydroxy-2-nonenal adduct.

Fig. 10. HPLC profile of amino acid analysis of native (---) and 4-hydroxy-2-nonenal-treated (----) glucose-6-phosphate dehydrogenase and lysine-4-hydroxy-2-nonenal adduct (.....). Samples were prepared as described under "Materials and Methods."

Retention of the aldehyde group (Fig. 6) or to form a Schiff base with retention of the double bond (Fig. 7). The relative contributions of these two kinds of enzyme modification may be deduced by studying the reactions of HNE-modified protein with 2,4-dinitrophenylhydrazine and with sodium borotritide. If the ε-amino group of lysine reacts with HNE at the double bond of HNE (Fig. 6), the carbonyl function of the protein adduct will react with 2,4-dinitrophenylhydrazine to form a nondissociable hydrazone derivative. However, the reaction of 2,4-dinitrophenylhydrazine with an enzyme-HNE Schiff base adduct will lead to cleavage of the protein-HNE adduct at the Schiff base bond and to the formation of the free HNE hydrazone derivative, which is readily separated from protein (Fig. 7). By contrast, sodium borotritide will react with both kinds of protein-HNE conjugates to yield stable 3H-labeled protein derivatives. It follows that the fraction of total HNE adduct formed by reaction of the ε-amino group of lysine with the double bond of HNE is given by the molar ratio of the protein-bound product formed in the reaction with 2,4-dinitrophenylhydrazine and with sodium borotritide, i.e. moles of protein-bound hydrazone/moles of protein-bound 3H. Reaction of 2,4-dinitrophenylhydrazine with Glu-6-P dehydrogenase that had been inactivated from 0 to 70% indicated a one-to-one relationship between the fraction of activity lost and the moles of hydrazone derivative incorporated per mole of enzyme subunit (Fig. 8). Treatment of the enzyme with HNE for extended periods of time resulted in a decrease in this ratio. Reaction of partially inactivated enzyme with sodium borotritide gave similar results: a one-to-one relationship between the fraction of activity lost and the moles of 3H incorporated per mole of enzyme subunit. Protection of Glu-6-P dehydrogenase from HNE inactivation by the addition of glucose 6-phosphate resulted in a level of
2,4-dinitrophenylhydrazine-detectable carbonyl which correlates with the degree of inactivation rather than the time of incubation. These data indicate that inactivation is the result of sealed, a modification of Glu-6-P dehydrogenase by HNE: 1 mol of HNE is bound per mol of enzyme subunit and appears to result in inactivation. The molar ratio of the product formed in the reaction of 2,4-dinitrophenylhydrazine and sodium borotritide is also 1, indicating that there is no detectable reaction between protein and the carbonyl group of HNE. Instead, a nucleophilic residue on the enzyme reacts with the double bond (C-3) of HNE, resulting in the incorporation of a stable carbonyl group on the protein and loss of enzyme activity.

It is difficult to determine which amino acid is lost upon HNE inactivation of Glu-6-P dehydrogenase by conventional amino acid analysis. This is particularly true for lysine, of which there are 36 residues/enzyme subunit (11, 19). We therefore performed a product analysis of the reaction of N-acetyllysine with HNE. Fast atom bombardment-mass spectrometry analysis of the major product of this reaction revealed a quasi molecular ion of 345, consistent with the formation of the lysine-HNE adduct shown in Fig. 9. Following NaBH₄ reduction, amino acid analysis of this adduct revealed a peak distinct from that of unreacted lysine (Fig. 19). In addition, incubation of this compound with NaB³H₄ resulted in the incorporation of 1 mol of d³H/mol of lysine-HNE adduct.

Amino acid analysis of Glu-6-P dehydrogenase inactivated to varying degrees by reaction with HNE followed by stabilization with NaBH₄ revealed a peak on the HPLC chromatogram with the same elution profile as that of the lysine-HNE adduct formed by reaction of N-acetyllysine with HNE (Fig. 10). The area of this peak increased with a corresponding increase in the level of inactivation. Protection from inactivation by the addition of glucose 6-phosphate to the inactivation mixture prevented the formation of this compound. Upon near complete inactivation, ~1 mol of lysine-HNE adduct was formed per mol of enzyme subunit. The levels of lysine-HNE adduct determined at various stages of inactivation were, however, difficult to quantify with much certainty due to the relatively low level of modification. As previously noted, HNE treatment of Glu-6-P dehydrogenase also resulted in a relatively small amount of histidyl modification. Since inactivation does not appear to involve strong binding of HNE to the enzyme (Fig. 3), it is likely that certain amino acid residues present in the glucose 6-phosphate-binding site are exposed to solvent, while other nucleophilic amino acid residues on the protein are not.

Structural and Functional Properties of 4-Hydroxy-2-nonenal-modified Glu-6-P Dehydrogenase—In an attempt to identify the properties of partially modified Glu-6-P dehydrogenase, we determined the molecular size, kinetic parameters, and thermal stability of enzyme inactivated to various degrees. As judged by gel filtration chromatography, modification did not result in cleavage of the peptide chain, dissociation of active dimer to inactive monomer, or any appreciable cross-linking under the conditions of our experiments. The Kₘ values of the remaining active catalytic sites for glucose 6-phosphate (~150 μM) and NADP⁺ (~15 μM) remained virtually unchanged at various stages of inactivation, indicating that loss of enzyme activity is likely caused by reduction of active enzyme population. In addition, enzyme inactivated to various degrees by HNE exhibited little change in the thermal stability of remaining activity, as determined by incubation at 47 °C for varying periods of time.

Modification of protein and other biomolecules by lipid peroxidation products is believed to play a central role in many pathophysiological conditions often associated with free radical damage. We have described, using a model system, a mechanism by which HNE may, in part, mediate free radical damage. In addition, a method for the detection of the lysine-HNE adduct was introduced. Our results will therefore be useful for future in vivo studies that attempt to define the mechanisms of free radical impairment of cellular function.

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