Inhibition of the multicatalytic proteinase (proteasome) by 4-hydroxy-2-nonenal cross-linked protein

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Abstract Oxidative modification of glucose-6-phosphate dehydrogenase (Glu-6-PDH), as observed for other proteins, increases the susceptibility of the protein to degradation by the multicatalytic proteinase/proteasome (MCP). Oxidized Glu-6-PDH is, however, more prone to cross-linking reactions by the lipid peroxidation product 4-hydroxy-2-nonenal (HNE), processes which render the protein resistant to proteolysis. In addition, HNE cross-linked protein inhibits the degradation of oxidatively modified glutamine synthetase by the MCP. In contrast to oxidized Glu-6-PDH, which inhibits the proteolysis of GS in a competitive manner, HNE cross-linked protein acts as a noncompetitive inhibitor. As judged by binding of the hydrophobic fluorescent probe 8-anilino-1-naphthalenesulfonic acid, a common structural feature of both macromolecular substrates and inhibitors of the MCP is an increased accessibility of hydrophobic regions on the protein.

Key words: 4-Hydroxy-2-nonenal; Lipid peroxidation; Free radicals; Glucose-6-phosphate dehydrogenase; Multicatalytic proteinase (20S proteasome); Hydrophobicity

1. Introduction

Protein can be oxidatively modified by a variety of cellular oxidation systems (for review see [1]). These forms of protein are generally degraded more rapidly than their native counterparts [2–4]. The steady-state level of oxidized protein therefore reflects the balance between the rates of protein oxidation and the rates of degradation of oxidized protein. In certain tissues, the level of oxidized protein increases with age and in response to oxidative stress, while age-related decreases in alkaline protease activity have been reported [5–7]. It has therefore been theorized that the age-related increase in the levels of oxidized protein is, at least in part, a result of a decline in its ability to be degraded [8].

A major cytosolic alkaline protease, the multicatalytic proteinase/proteasome (MCP), has been implicated in the degradation of various oxidatively modified forms of protein [9–11]. The activity of the MCP may decline due to a decrease in the amount or specific activity of the enzyme and/or to an increase in the level of endogenous inhibitors of the proteinase. We have recently shown that the model protein glucose-6-phosphate dehydrogenase (Glu-6-PDH) from the bacterium *Leuconostoc mesenteroides*, modified by the lipid peroxidation product 4-hydroxy-2-nonenal (HNE), was not degraded by the MCP and inhibited the ability of this proteinase to degrade oxidized forms of protein [12]. The inhibitory effect was associated with the formation of cross-linked Glu-6-PDH, which exhibited fluorescence reminiscent of that observed for lipofuscin (for review see [13]). Cross-linking occurs upon reaction of lysine residues with bifunctional HNE [14]. These observations therefore suggest that age- and disease-related increases in oxidized protein, lipofuscin, and/or ceroid pigments may be due, at least in part, to modification of protein by lipid peroxidation products [15–18]. Because of the potentially important implications of modification of protein by HNE, we have investigated the ability of oxidized forms of protein to be cross-linked by HNE and the mechanism(s) by which HNE-modified protein inhibits the MCP.

2. Materials and methods

2.1. 4-Hydroxy-2-nonenal preparation

HNE was prepared by acid treatment of 4-hydroxy-2-nonenal dimethylacetal which was synthesized as described by De Montarby et al. [19] and purified as previously reported [14]. The HNE concentration was determined by measurement of UV absorbance at 224 nm ($\varepsilon = 13\,750\ M^{-1}\ cm^{-1}$).

2.2. Glucose-6-phosphate dehydrogenase preparation and modification

Glu-6-PDH from *Leuconostoc mesenteroides* was purchased from Worthington Biochemical Corporation. The lyophilized enzyme powder was dissolved in 25 mM KH_2PO_4, 100 mM KCl, pH 7.4 and dialyzed overnight at 4°C against the same buffer using Spectra Por dialyzing membrane (MW cut-off 10 000). Oxidative modification of the enzyme was achieved by incubating Glu-6-PDH (20 μM) with $FeSO_4$ (1 mM) and citrate (1.2 mM) in 5 mM KH_2PO_4, pH 7.0, for 2 min at room temperature under aerobic conditions. The reaction was terminated by addition of 100 mM citrate, pH 7.4. Residual activity of the modified enzyme was determined using a spectrophotometric assay as previously described [20]. HNE treatment of native and oxidized Glu-6-PDH (2.5 mg/ml) was performed in 25 mM KH_2PO_4, 100 mM KCl, pH 7.4 with 0–4 mM HNE. At indicated times (0–4 h), the reaction was quenched with 10 mM $NaBH_4$ for 15 min at 25°C and the sample was passed over a PD-10 column (Pharmacia LKB Biotechnology Inc.) equilibrated with 50 mM HEPES, 100 mM KCl, pH 7.8. Intermolecular cross-linking of Glu-6-PDH by HNE was monitored by gel filtration chromatography on a Zorbax GF 250 column equilibrated with 1% SDS in 100 mM NaH_2PO_4, pH 7, at a flow rate of 2 ml/min using a Hewlett-Packard 1090 liquid chromatograph. The enzyme was diluted to a concentration of 4 μM in elution buffer prior to analysis of a 250 μl aliquot of the diluted solution.

2.3. 8-Anilino-1-naphthalene sulfonic acid binding

The various forms of Glu-6-PDH at a concentration of 0.4 μM were incubated with 100 μM 8-anilino-1-naphthalenesulfonic acid (ANSA) (Sigma Chem. Co.) at 37°C in 50 mM HEPES, 100 mM KCl, pH 7.8. The fluorescence emission spectra (excitation, 370 nm) were obtained using a Photon Technology International LS 100 spec-

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Abbreviations: MCP, multicatalytic proteinase (20S proteasome); Glu-6-PDH, glucose-6-phosphate dehydrogenase; GS, glutamine synthetase; HNE, 4-hydroxy-2-nonenal; ANSA, 8-anilino-1-naphthalenesulfonic acid; TCA, trichloroacetic acid

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trofluorimeter. Binding of ANSA to protein was observed after subtraction of the emission spectrum of ANSA from that of ANSA in the presence of enzyme.

2.4. Proteolytic assays

The MCP was purified from rat liver acetone powder (Sigma Chem. Co.) following the procedure of Skilton et al. [21] with minor modifications as previously described [22]. Proteolysis of various forms of Glu-6-PDH by the MCP was performed at 37°C in 50 mM HEPES, 100 mM KCl at pH 7.8 as previously described [22]. Following TCA precipitation of the protein, the rate of proteolysis was determined by monitoring increases in the concentration of small peptides in the supernatant. Upon reaction of peptide amino groups with fluorescamine (Sigma), the formation of fluorescent derivatives was monitored by measuring emission intensities at 475 nm (excitation, 375 nm). A calibration curve was obtained in parallel using glycine as the standard.

Proteolysis of GS was measured by incubating the MCP (0.3 μM) at 37°C with oxidized radio-labeled GS (8 μM) in 50 mM HEPES, 100 mM KCl, 0.4 mM $MgCl_2$, pH 7.8 in the absence or presence of native or HNE modified Glu-6-PDH. At appropriate times, 10 μl aliquots were removed and diluted with 12.5% TCA to a final volume of 50 μl. After centrifugation of the precipitated proteins for 10 min at 14000 rpm in an Eppendorf microfuge, the concentration of small peptides present in the supernatant was determined by measuring the radioactivity of each sample using a Tricarb 2200 CA liquid scintillation analyzer. ^{14}C-labeled GS was purified from the over-producing strain *E. coli* K12 pgln6 YMC10 grown in a synthetic medium supplemented with ^{14}C-amino acids (Dupont NEN and ICN) as previously described [23]. The specific radioactivity obtained was 4000 ± 300 cpm/μg of GS. Radio-labeled enzyme was oxidized by exposure to 0.1 mM $FeCl_3$ and 25 mM ascorbate for 8 h at 37°C [23].

3. Results

3.1. Cross-linking of native and oxidized glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal

The appearance of intermolecular cross-links during incubation of native and oxidized Glu-6-PDH with HNE was monitored by SDS gel filtration chromatography as previously described [12]. Prior to analysis, samples were reduced with sodium borohydride to quench the reaction and stabilize the cross-link. In the presence of 1.0% SDS, native dimeric enzyme dissociated and eluted in the monomeric form. As shown in Fig. 1A,B, treatment of both native and oxidized forms of Glu-6-PDH with HNE resulted in a time- and concentration-dependent increase in multimeric protein. Oxidized enzyme was more susceptible to cross-linking. Cross-linking resulted in the formation of primarily dimeric and tetrameric protein with no appearance of large aggregates or protein precipitates under the conditions used in these experiments.

3.2. Decrease in proteolytic susceptibility of oxidized Glu-6-PDH after treatment with HNE and inhibition of the multicatalytic proteinase

In the remainder of this report, the phrase 'HNE-treated Glu-6-PDH' refers to oxidatively modified Glu-6-PDH that was exposed to 4.0 mM HNE for 4 h. The rates of proteolysis for native, oxidized, and HNE-treated forms of the protein were determined by monitoring the increase in small peptide concentration after incubation with the MCP. As shown in Fig. 2, oxidation of native Glu-6-PDH by iron and citrate led to increased susceptibility of the protein to proteolysis by the MCP. It is interesting to note that enzyme modified in this manner lost 50% of its initial activity associated with oxidation of one active site lysine residue [20]. Treatment of oxidized Glu-6-PDH with HNE rendered the protein resistant to proteolysis (Fig. 2). In addition, this form of the enzyme prevented the degradation of oxidatively modified ^{14}C-labeled GS (Fig. 3), as has been reported for native Glu-6-PDH cross-linked by HNE [12]. Not surprisingly, oxidized Glu-6-PDH and to a lesser extent native enzyme also interfered with the degradation of oxidized GS likely due to substrate competition.

3.3. Noncompetitive inhibition of the MCP by HNE cross-linked Glu-6-PDH

To further investigate the mechanism of MCP inhibition, the rate of proteolysis of oxidized GS was determined at different substrate and inhibitor concentrations. As shown in Fig. 4A, oxidized Glu-6-PDH inhibited the proteolysis of oxi-

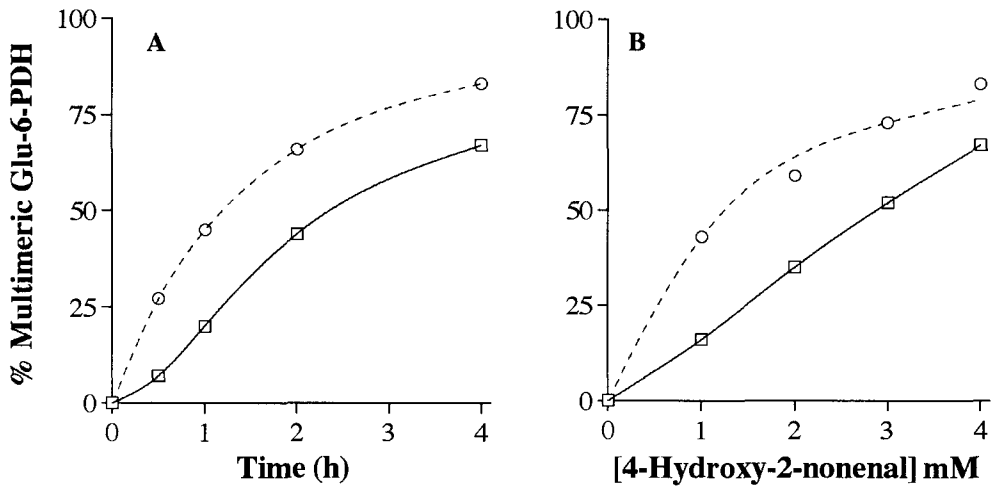

Fig. 1. Intermolecular cross-linking of native and oxidized Glu-6-PDH by HNE. Native and oxidized Glu-6-PDH (2.5 mg/ml) were incubated at 37°C in 25 mM KH_2PO_4, 100 mM KCl at pH 7.4 with 4 mM HNE for 0, 0.5, 1.0, 2.0, and 4.0 h or with 0, 1, 2, 3, and 4 mM HNE for 4 h. At indicated times, the sample was treated with 10 mM $NaBH_4$ for 15 min at 25°C. Protein was then diluted to 0.2 mg/ml in elution buffer followed by gel filtration chromatography of a 250 μl aliquot. Protein elution was monitored at 280 nm with 1.0% SDS in 100 mM NaH_2PO_4, at pH 7.0, as eluant at a flow rate of 2 ml/min. The peak area of the multimeric cross-linked protein (expressed as % of total, i.e. monomeric+multimeric peak areas) is plotted as a function of time (A) or HNE concentration (B) for native (□) and oxidized (○) Glu-6-PDH.

dized GS by the MCP in a competitive manner (Fig. 4A) as expected for substrate competition. In contrast, proteolysis of oxidized GS was inhibited by HNE cross-linked Glu-6-PDH in a noncompetitive manner (Fig. 4B). When native Glu-6-PDH is cross-linked by HNE it also acts as a noncompetitive inhibitor (data not shown). The observation that inhibition of the proteinase is noncompetitive invites speculation that the MCP reacts with HNE released from Glu-6-PDH and/or with free functional groups of the protein-HNE derivative. This does not appear to be the case since treatment of HNE-modified Glu-6-PDH with $NaBH_4$, which reduces remaining functional groups and stabilizes protein adducts to dissociation, does not alter the ability of HNE cross-linked protein to inhibit the MCP.

3.4. ANSA binding to HNE-treated forms of Glu-6-PDH

Binding of the hydrophobic probe ANSA to protein is a useful tool for assessing the accessibility of hydrophobic regions of protein to solvent [24]. When bound to protein, ANSA exhibits an increase in fluorescence. As previously reported [22], in contrast to native enzyme, oxidized Glu-6-PDH binds ANSA (Fig. 5). When native or oxidized Glu-6-PDH is exposed to HNE for extended periods of time, the protein becomes cross-linked and binds ANSA (Fig. 5). In addition, ANSA binds to the MCP and to oxidized GS and inhibits the MCP (results not shown). Since oxidized and HNE cross-linked forms of Glu-6-PDH act as macromolecular substrate or inhibitors of the MCP, respectively, an increase in the accessibility of hydrophobic regions may reflect a common feature of macromolecular ligand recognition by the proteinase. It should be noted that Glu-6-PDH can be inactivated by HNE under relatively mild reaction conditions. Under these conditions, the protein does not become cross-linked, does not bind the hydrophobic probe ANSA, and exhibits

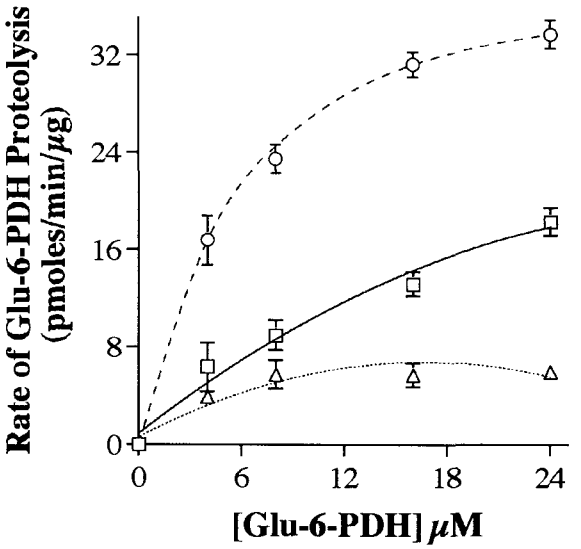

Fig. 2. Proteolysis of native, oxidized, and HNE-treated oxidized Glu-6-PDH by the MCP. Proteolysis of native and modified forms of Glu-6-PDH by the MCP (0.3 μM) was performed and monitored as described in Section 2. The rates of proteolysis for native (□), oxidized (○), and HNE-treated oxidized Glu-6-PDH (△) are presented as a function of protein concentration. Points represent the mean, and bars the standard deviation of the mean for three independent experiments.

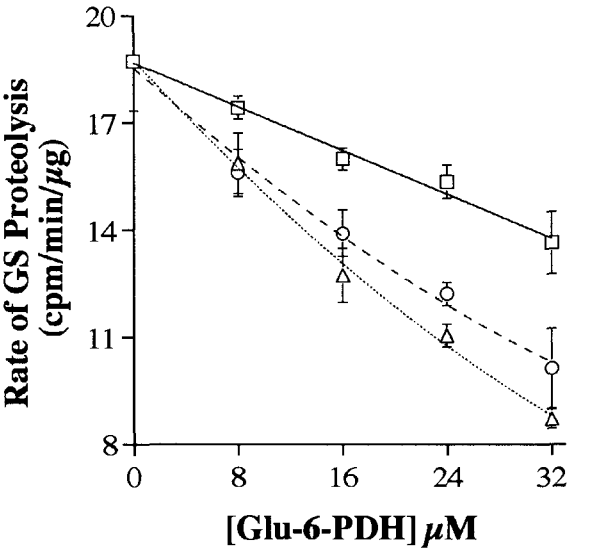

Fig. 3. Proteolysis of oxidized GS by the MCP in the presence of various forms of Glu-6-PDH. Proteolysis of radio-labeled oxidized GS (8 μM) by the MCP (0.3 μM) was determined in the presence of native and modified forms of Glu-6-PDH as described in Section 2. The rates of GS proteolysis are presented as a function of native (□), oxidized (○), and HNE-treated oxidized Glu-6-PDH (△). Points represent the mean, and bars the standard deviation of the mean for three independent experiments.

no change in proteolytic susceptibility [22]. These results support the conclusion that increased accessibility of hydrophobic regions on protein is a structural feature required for both substrate and inhibitor recognition by the protease.

4. Discussion

Modification of protein by lipid peroxidation products such as malondialdehyde and HNE has been implicated in various degenerative diseases associated with aging [25–28]. The present study was undertaken to determine if the fate of altered forms of protein obtained by reaction with HNE would be similar to that of oxidized protein. Oxidative modification of Glu-6-PDH, as reported for other enzymes, renders the protein more susceptible to degradation by the MCP [2–4]. Subsequent treatment of oxidatively modified Glu-6-PDH with HNE led to the formation of cross-linked protein which was resistant to proteolysis and served as an inhibitor of the MCP. In addition, oxidized Glu-6-PDH is more prone to cross-linking than the native enzyme, suggesting a potentially important mechanism by which oxidatively modified protein may accumulate with age and in various pathological conditions. The occurrence of such modified forms of protein may contribute to a reduced ability of the MCP to efficiently degrade damaged protein in vivo. As a noncompetitive inhibitor, HNE cross-linked protein would be a good candidate for promoting the accumulation of damaged protein since noncompetitive inhibitors are not sensitive to substrate concentration. It may be argued that the concentration of HNE used to cross-link Glu-6-PDH in these experiments is nonphysiological. This protein was used as a model to study interaction of HNE with amine residues on protein. It appears that similar cross-linking chemistry occurs in cellular organelles at low concentrations of HNE [14]. Arginase, a tetrameric protein, and carbonic anhydrase, a monomer, purified from bovine

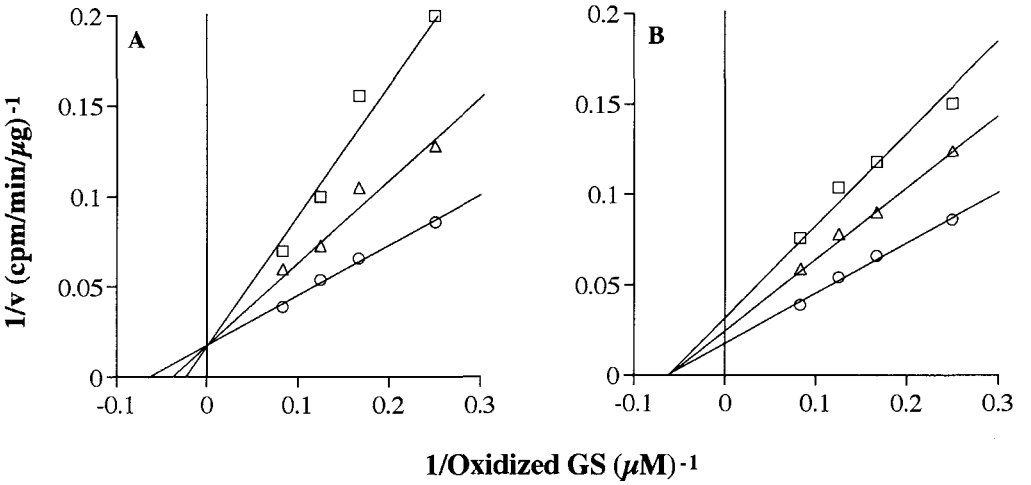

Fig. 4. Double reciprocal plot of the rate of GS proteolysis versus GS concentration in the absence and presence of oxidized or HNE-treated Glu-6-PDH. Proteolysis of radio-labeled oxidized GS (4, 6, 8 and 12 μM) by the MCP (0.3 μM) was monitored as described in Section 2 in the absence (○) and presence of 16 μM (△) and 32 μM (□) oxidized Glu-6-PDH (A) or HNE-treated oxidized Glu-6-PDH (B).

liver and erythrocytes, respectively, also become cross-linked upon exposure to HNE and resistant to proteolysis by the MCP (unpublished).

In an effort to determine the molecular mechanism whereby protein cross-linked by HNE inhibits the MCP, binding studies with the hydrophobic probe ANSA were performed. Unlike native enzyme, oxidized and HNE cross-linked forms of Glu-6-PDH bound ANSA efficiently, suggesting that a common structural feature of both macromolecular substrates and inhibitors of the MCP is an increased accessibility of hydrophobic regions on the protein. Indeed, exposure of hydrophobic regions in protein upon oxidative modification has been proposed to be a key event for substrate recognition by the MCP [4,22,29]. The noncompetitive inhibition observed with HNE cross-linked Glu-6-PDH could therefore be explained by a very slow off rate of the cross-linked Glu-6-PDH from a common hydrophobic site of recognition. This hypothesis is supported by the observation that ANSA binds to the MCP and inhibits the proteolysis of oxidized protein by the MCP. In addition, the MCP only releases short proteolytic peptide fragments [22,30,31]. Thus, the protein inhibitor may be retained at a hydrophobic recognition site of the MCP by virtue of its cross-linked structure.

These findings suggest a plausible mechanism for the observed accumulation of damaged protein during the progression of certain degenerative diseases associated with increased rates of free radical production. Oxidized protein is more prone to HNE cross-linking reactions leading to the formation of material which is not efficiently degraded and inhibits the MCP. Both effects appear to result from increases in the accessibility of hydrophobic regions on the protein. The fact that inhibition is noncompetitive underscores the potential for a buildup of damaged protein upon oxidative stress.

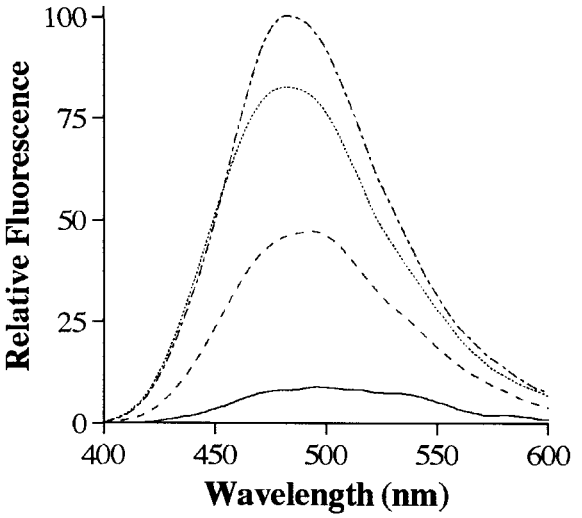

Fig. 5. Spectrofluorimetric analysis of ANSA binding to the various forms of Glu-6-PDH. Emission spectra (excitation 370 nm) of ANSA (100 μM) in the presence of the various forms of Glu-6-PDH (0.4 μM) were performed as described in Section 2. The increase in fluorescence intensity (λ_{max} = 490 nm) resulting from the binding of ANSA to the enzyme was determined by subtracting the emission spectrum of ANSA from that of ANSA in the presence of the different forms of the enzyme: native (——), oxidized (- - - -), HNE-treated (- · · · -), and HNE-treated oxidized (· · · ·) Glu-6-PDH.

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