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Determination of Site-Specific Modifications of Glucose-6-Phosphate Dehydrogenase by 4-Hydroxy-2-Nonenal Using Matrix Assisted Laser Desorption Time-of-Flight Mass Spectrometry

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Products of the reaction of 4-hydroxy-2-nonenal (4HNE) with native and heat-denatured *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (G6PDH) were analyzed to determine the structure and position of the protein modifications. Matrix assisted laser desorption time-of-flight mass spectrometry was used to measure molecular weights of the modified proteins and determine mass maps of peptides formed by digestion with cyanogen bromide. The molecular weight data show that one to two 4HNE molecules add to each subunit of native enzyme while approximately nineteen 4HNE molecules add to each subunit of heat-denatured enzyme. Peptides are observed in the cyanogen bromide mass map of modified native G6PDH that are consistent with selective modification of two segments of the amino acid sequence. One modified segment contains Lysine-182 that has been found to be part of the enzyme active site. Peptides are observed in the cyanogen bromide mass map of modified heat-denatured enzyme that are consistent with extensive modification of several segments of the amino acid sequence. The magnitude of the mass differences between modified and unmodified peptides were approximately 156 Da, consistent with a 1,4-addition of 4HNE. These results support the conclusion that 4HNE inactivates G6PDH by selectively modifying only two or three sites in the protein by a 1,4-addition reaction and that some aspect of the tertiary structure of the enzyme directs those modification reactions.

Key words: oxidative stress, 4-hydroxy-2-nonenal, protein modification, mass spectrometry, matrix assisted laser desorption

INTRODUCTION

A proposed molecular mechanism for oxidative damage to tissues is the oxidative modification of proteins.[1] Reactive aldehydes formed by oxidation of polyunsaturated fatty acids may also

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contribute to modifications of proteins associated with oxidative stresses through covalent modification reactions. For example, 4-hydroxy-2-nonenal (4HNE)-protein adducts have been detected by immunochemical methods associated with tissue damage in atherosclerotic vascular tissues, kidney tissue of animals treated with a renal carcinogen, and hydroxyl radical-oxidized hepatocytes.[2-4] Such data support speculation that aldehydic modification of proteins contributes to oxidative tissue damage.

One enzyme in which modification reactions are being studied is glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides.* This enzyme has a calculated subunit molecular weight of 54 316 Da and the amino acid sequence shares a 31% homoleogy to the human isotype.[5] The absence of cystine or cysteine in the primary structure and the ability to utilize either NAD^+ or $NADP^+$ as the hydrogen acceptor are unique features associated with this G6PDH.[6,7] Stadtman and co-workers have shown that *Leuconostoc mesenteroides* G6PDH is inactivated by both metal catalyzed oxidation reactions and by reaction with 4HNE.[8,9] These investigators used protection studies with glucose-6-phosphate to show that when the enzyme is inactivated by 4HNE, modification takes place at a lysine blocked by the substrate. Subsequent reaction of 4HNE-modified G6PDH with 2,4-dinitrophenylhydrazine or tritiated-sodium borohydride shows incorporation of 1 mole of 4HNE per mole of enzyme subunit and is consistent with 1,4-addition of the aldehyde to the amine.

The purpose of the experiments described in this report was to directly evaluate the extent, site and structure of 4HNE modification of G6PDH through mass spectral characterization of the products. Matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF) was used to measure molecular weights of the proteins and, following digestion with cyanogen bromide, molecular weights of peptides derived from those proteins (an experiment referred to as peptide mass mapping). Comparison of molecular weight and peptide mass mapping data obtained from modified native and heat denatured enzyme clearly show that 4HNE modification of native G6PDH is limited to specific portions of the protein and provides evidence that some unidentified aspect of the tertiary structure of the protein directs the sites of modification.

MATERIALS AND METHODS

Materials

Leuconostoc mesenteroides glucose-6-phosphate dehydrogenase, essentially fatty acid free bovine serum albumin, trifluoroacetic acid and 3-(N-morpholino)propanesulfonic acid (MOPS) were purchased from Sigma Chemical Company, St. Louis, MO. Cyanogen bromide, 3,5-dimethoxy-4-hydroxycinnamic acid, and α-cyano-4-hydroxycinnamic acid were purchased from Aldrich Chemical Company, Milwaukee, WI. 4HNE was purchased from Cayman Chemical Company, Ann Arbor, MI.

Assay of Glucose-6-Phosphate Dehydrogenase Activity

The activity of this enzyme was determined using a reagent kit purchased from Sigma Chemical Company. A 20 μL aliquot of an appropriate dilution of the enzyme solution was mixed with 0.5 mL of a reagent solution containing 1.5 mM $NADP^+$ and 12 mM maleimide. After a five min equilibration period, 1.0 mL of a substrate solution containing 1.05 mM glucose-6-phosphate and magnesium was added and the sample transferred to a 1-mL cuvette. Absorbance at 340 nm was recorded for 5 min and the rate of absorbance change calculated. One unit of enzyme activity is defined as the amount of G6PDH that will form one μmol of NADPH/min.

Modification reactions

The modification reactions were carried out in 1 mL of 100 mM potassium chloride, 10 mM 3-(N-

morpholino)propanesulfonic acid (MOPS) buffer, pH 7.4, containing 4.6 nmol of glucose-6-phosphate dehydrogenase, 460 nmol 4HNE, and 2 μmol $NaCNBH_3$. The reactions were carried out at 37°C for up to 50 h. In indicated experiments, G6PDH was denatured in boiling water for 15 min prior to the modification reaction. In studies of the native enzyme, aliquots were removed at selected time points to determine enzyme activity. At the completion of all modification reactions, the reagents were removed and samples desalted by size exclusion chromatography using a disposable Sephadex G-25 column (Pharmacia PD-10 desalting column) eluting the proteins with distilled water.

CNBr Digestion

Aliquots of desalted G6PDH reaction products containing 2 nmol of protein were placed in a polypropylene tube and lyophilized. The residue was reconstituted in 150 μL of 70% (v/v) aqueous trifluoroacetic acid containing approximately 5 mg cyanogen bromide and reacted overnight under nitrogen in the dark. The reagents were removed by repeated lyophilization and the resulting residue reconstituted in 100 μL of distilled water for mass spectrometric analysis.

Matrix assisted laser desorption time-of-flight mass spectrometry

All analyses were performed using a Finnigan-MAT LaserMat time-of-flight mass spectrometer. Molecular weights of the protein products were determined using 3,5-dimethoxy-4-hydroxycinnamic acid for the matrix with bovine serum albumin added to the samples as an internal mass calibrant assigned a molecular weight of 64 360 Daltons. Peptide mass maps were determined using α-cyano-4-hydroxycinnamic acid as the matrix and the masses calibrated by assigning ions at *m/z* 190.2 $(M + H)^+$ and 379.4 $(2M + H)^+$ in the matrix spectrum. Molecular weights of the peptides expected in the CNBr digestion were calculated based on the amino acid sequence of *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase obtained from the sequence database at the National Center of Biotechnology Information, National Library of Medicine, Bethesda, MD, accession number P11411.

RESULTS

Two 4HNE modification reactions were studied; the modification of native G6PDH and the modification of heat-denatured G6PDH. The reactions were carried out using conditions similar to those described by Stadtman *et al.* except for the addition of $NaCNBH_3$.[9] The $NaCNBH_3$ was added to stabilize any Schiff bases that may have been formed prior to subsequent structural investigations. This reducing agent selectively reduces Schiff bases without reducing free aldehydes and had no effect on enzyme activity with untreated controls retaining 95% to 97% of initial enzyme activity in all reactions.

Reaction of native G6PDH with 4HNE resulted in a loss of enzyme activity and the time course of the activity loss is shown in Figure 1. Modification

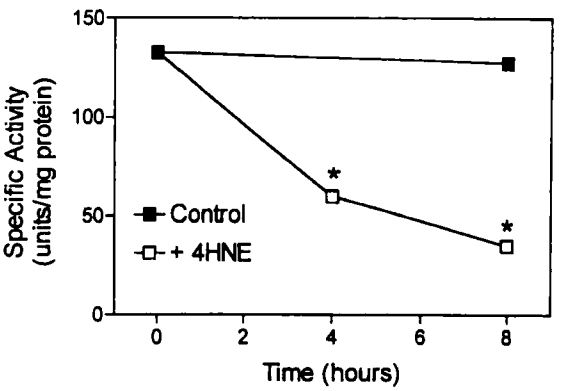

FIGURE 1 Time course of glucose-6-phosphate dehydrogenase inactivation by 4-hydroxy-2-nonenal. 4.6 nmol Glucose-6-phosphate dehydrogenase were reacted with 460 nmol 4-hydroxy-2-nonenal as described in the methods. Each point represent the mean ± standard deviation of triplicate measurements. Statistical comparisons were made by a one-way analysis of variance using a Tukey-Kramer multiple comparisons test. Statistically significant differences between a given time point and t = 0 ($p < 0.05$) are designated by a*.

TABLE 1 Sub-unit molecular weights of glucose-6-phosphate dehydrogenase measured using matrix assisted time-of-flight mass spectrometry.

Reaction Conditions	Molecular Weight (Da) (± 0.1% tolerance)[1]	Mass Difference[2] (Da)
Native G6PDH	54 403 ± 54[3]	
Native G6PDH + 4HNE	54 657 ± 55	254
Heat-Denatured G6PDH + 4HNE	57 367 ± 57	2 964

[1]Tolerance reflects uncertainty of MALDI-TOF mass measurements.
[2]Calculated as the difference between the measured molecular weight of the 4HNE modified forms and the native form of G6PDH.
[3]Sub-unit molecular weight calculated from the amino acid sequence is 54 310 Daltons.

of the native form of the enzyme by reaction with 4HNE reduced enzyme activity to 50% of control in approximately 4 h and less than 10% of control in approximately 8 h. Exhaustive dialysis of the reaction products in selected experiments did not restore enzyme activity indicative of a covalent modification. Masses of the native enzyme and the modification reaction products determined by MALDI-TOF analysis are listed in Table 1. The sub-unit molecular weight increases that were

TABLE 2 Molecular weights and corresponding mass shifts observed for peptides in the cyanogen bromide digests of glucose-6-phosphate dehydrogenase modified by 4HNE.

CNBr Fragment	Amino Acids Included	Measured Molecular Weight ($M + H^+$, Da) Control	Measured Molecular Weight ($M + H^+$, Da) 4HNE-modified	Mass Difference (4HNE-modified versus control) (Da)
Modification of the native enzyme				
CNBr-10	466–488	2 075	2 075	0
CNBr-2	117–145	3 185	3 185	0
			3 337	152
CNBr-9	424–465	4 477	4 477	0
CNBr-3	146–184	4 519	4 519	0
			4 669	150
			4 825	150 + 156
CNBr-4	185–236	5 738	5 738	0
CNBr-1	1–116	13 210	n.d.[1]	
CNBr-7	251–418	18 924	n.d.	
Modification of the heat denatured enzyme				
CNBr-10	466–488	2 055	2 215	160
CNBr-2	117–145	3 185	3 185	0
			3 341	156
			3 499	156 + 158
CNBr-9	424–465	4 483	n.d.	
CNBr-3	146–184	4 520	n.d.	
			4 675	155
			4 830	155 + 155
			4 992	155 + 155 + 162
CNBr-4	185–236	5 751	5 751	0
CNBr-1	1–116	n.d.	n.d.	
CNBr-7	251–418	n.d.	n.d.	

[1]Not detected.

observed correspond to average additions of one to two 4HNE molecules per subunit of the native form of the enzyme and approximately nineteen 4HNE molecules per subunit of the heat-denatured form of the enzyme, based on the 156 Da molecular weight of 4HNE.

Peptide mass mapping was then used to further localize the modification sites on G6PDH in both reactions. Desalted products of each reaction was digested with cyanogen bromide and the digests analyzed by MALDI-TOF; these data are summarized in Table 2. Details of the peptide mass mapping spectra of modified native and modified heat-dentured G6PDH are shown in Figure 2. In the modification of native G6PDH, peptides were detected at *m/z* 3337, *m/z* 4669, and *m/z* 4825 that correspond to modification of CNBr-2 by addition of one 4HNE molecule and modification of CNBr-3 by one and two 4HNE molecules, respectively. CNBr-3 contains Lysine-182 which has been identified as part of the active site of the *Leuconostoc mesenteroides* form of G6PDH based on homoleogy to an active site region in the human erythrocyte form of G6PDH.[10,11] No other peptides indicative of additional protein modifications were detected in the cyanogen bromide digests of modified native enzyme. More extensive modification is apparent in the peptide mass map of modified heat-denatured G6PDH. For example, peptides are seen at *m/z* 3341 and *m/z* 3499 that correspond to additions of up to two molecules of 4HNE to CNBr-2 (*m/z* 3185). Peptides indicative of modifications to CNBr-10 and CNBr-3 were also seen. The number of modified peptides detected in the cyanogen bromide digest of modified heat-denatured enzyme confirms the large increase in molecular weight seen for this modified protein in Table 1.

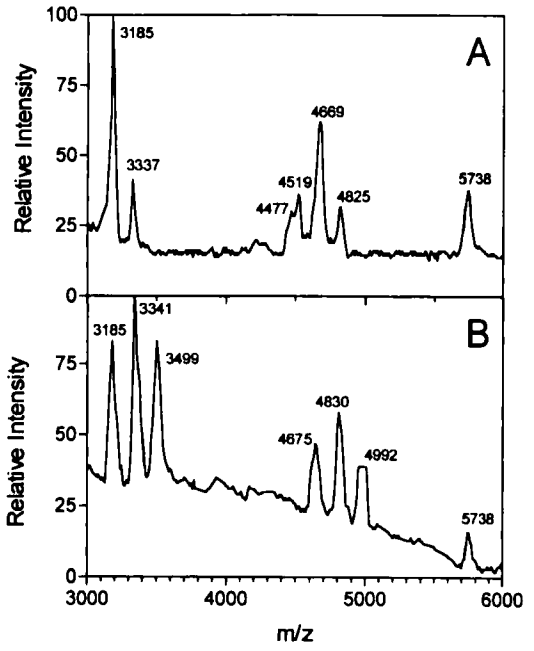

FIGURE 2 Details of the peptide mass maps of cyanogen bromide digests of A) 4HNE-modified, native G6PDH and B) 4HNE-modified, heat denatured G6PDH. MALDI-TOF analyses of the digests were performed as described in the methods. The measured *m/z* of the peptides are noted in the figure.

Table 2 also summarizes the magnitude of the mass shifts for the molecular weights measured for modified peptides relative to those measured for unmodified peptides. It is important to note that the magnitude of the mass increases are calculated, with the exception of the first modification of CNBr-3 in heat-denatured protein, from molecular weights measured in the same spectrum. As a result, these differences are not significantly affected by any variations in the accuracy of the mass assignments in a given spectrum since such variations would affect all assignments in that spectrum to the same degree. For the modification of native enzyme, the mass shifts were found to be 152 Da, 150 Da and 306 (noted in the table as 150 + 156) Da. These mass differences are consistent with addition of 4HNE molecules via a 1,4-addition reaction that is calculated to add 156 Da. For the modification of heat-denatured enzyme, the mass shifts were found to range from 155 Da to 162 Da, which are also consistent with a 1,4-addition reaction.

DISCUSSION

The reaction of 4HNE with native and heat-denatured G6PDH demonstrates two aspects of

G6PDH modification not previously described. The first aspect of this modification is the further localization of a limited number of addition sites in the native enzyme. The sub-unit molecular weight data presented in Table 1 indicate that 4HNE modification of native G6PDH is limited to approximately two 4HNE molecules per sub-unit of the enzyme. The corresponding peptide mass map data presented in Table 2 confirm that a limited degree of modification occurs and show that two peptides, one of which contains an active-site lysine residue, are modified. It is intriguing to speculate, based on the fact that 4HNE-modification inactivates the enzyme, that modification takes place at an active site lysine although histidine modifications are also possible. Considering that the primary sequence of G6PDH contains 36 lysine residues and 6 histidine residues, these data support a site specific modification reaction. The second aspect of this modification reaction is the extensive modification of heat-denatured enzyme seen in both the sub-unit molecular weight data presented in Table 1 and peptide mass map data presented in Table 2. Limited, specific modification of native enzyme contrasted with extensive modification of heat-denatured enzyme indicates that the tertiary structure of the native protein directs modification reactions in a manner that allows reaction at selected sites while preventing reaction at other sites. One would speculate based speculate based on the hydrophilicity of lysine residues that this direction is not due to simple accessibility of different lysines although the exact nature of this control is undetermined.

While these reactions have utilized higher concentrations of 4HNE and G6PDH than would be found in cellular systems, the results illustrate the ability of mass spectrometric analyses to perform detailed determinations of structural changes in proteins exposed to products of oxidative stress. As seen in the data presented in this report, MALDI-TOF analysis of proteins and peptides is characterized by excellent accuracy of the molecular weight determinations. In general, one can expect molecular weight measurements by MALDI-TOF to be within 0.1% of the true value. Accurate molecular weight determinations then allow post-translational modifications to be detected and localized in peptide mass maps.[12,13] Additional analyses are underway in our laboratory to more precisely determine the modification sites and structures using mass spectrometric peptide sequencing experiments. One could envision that, as different protein modifications associated with different oxidative stresses are determined in model systems, mass spectrometry based analyses could be used to detect those modifications as characteristic molecular markers of distinct types of oxidative stresses in more complex and physiologically relevant systems.

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