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IDENTIFICATION OF AN N-ACETYLATED MICROSOMAL GLUTATHIONE *S*-TRANSFERASE BY MASS SPECTROMETRY

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Abstract—Microsomal glutathione *S*-transferase (mGST) was purified to homogeneity from male Sprague–Dawley rat liver, as determined by SDS–PAGE. Removal of Triton X-100 and further separation by reversed phase HPLC revealed two proteins, mGST 1 and mGST 2, in a 1:3 ratio. Analysis of mGST 1 and mGST 2 by electrospray ionization mass spectrometry determined their molecular weights to be 17,354.2 ± 6.6 and 17,397.9 ± 6.6, respectively. mGST 1 was in close agreement with the calculated molecular weight of 17,348, as predicted by the previously reported cDNA sequence. Cyanogen bromide digestion and peptide mapping by fast atom bombardment mass spectrometry (FAB-MS) localized the mass increase to the N-terminal peptide, 1–7. FAB-tandem mass spectrometry of this peptide in conjunction with Edman reactions on the intact protein demonstrated the N-terminal alanine to be acetylated.

Key words: glutathione *S*-transferase; protein heterogeneity; enzyme induction; protein acylation; mass spectrometry; microsomes

The glutathione *S*-transferases are a family of enzymes that catalyze the conjugation of glutathione with a wide variety of electrophilic compounds [1–3]. The enzymes are found largely in the cytosol as homodimers or heterodimers composed from at least seven subunits [1, 2]. An mGST‖ has also been identified [3, 4] and purified from the rat [5, 6], mouse [7], and human [8, 9]. This enzyme accounts for as much as 3% of microsomal protein [10] and is thought to function as a trimer of identical subunits [5, 11, 12]. It is unique in that it is activated by sulfhydryl reagents [13] and proteolysis [14], although this activation process appears to occur only with the hepatic form [10].

DeJong *et al.* [15] isolated cDNA clones to rat and human liver mGST; 83% sequence homology was found between them, but only limited homology (seven amino acids) to cytosolic GST. Southern blot analyses of rat and human genomic DNA suggested that this protein is encoded by a single or low-copy gene in both species [5, 15, 16]. The expression of a separate isozyme, therefore, was not considered an explanation for the lack of activation of this protein in extrahepatic tissues of the rat.

We present here the identification of an N-acetylated form of mGST isolated from rat liver. In two separate purifications, the ratio of the nonacetylated form to the acetylated form was found to be 1:3. This post-translational modification may play a role in the ability of the protein to be activated by sulfhydryl reagents. This report also shows the great utility of mass spectrometry in not only the characterization of post-translational modifications of proteins, but also in their initial discovery.

MATERIALS AND METHODS

Glutathione, EDTA, Triton X-100, CM-Sepharose, Sephadex G-25, and CDNB were purchased from the Sigma Chemical Co. (St. Louis, MO). Hydroxyapatite was obtained from Bio-Rad (Richmond, CA). BCA Protein Assay Reagents were purchased from Pierce (Rockford, IL). Other chemicals were obtained from various sources at the highest quality available.

Male Sprague–Dawley rats weighing 100–150 g, purchased from Harlan Sprague–Dawley, Inc. (Madison, WI), were starved 24 hr prior to being killed. Microsomal glutathione *S*-transferase was purified according to the method of Morgenstern *et al.* [5], excluding the *N*-ethylmaleimide activation of the hepatic microsomes. Glutathione *S*-transferase activity was assayed according to the method of Habig *et al.* [17], using CDNB as the substrate. Purified protein was stored at −85° until further separation by HPLC.

Aliquots of the purified protein underwent further

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‖ Abbreviations: mGST, microsomal glutathione *S*-transferase; FAB-MS, fast atom bombardment mass spectrometry; MS/MS, tandem mass spectrometry; CDNB, 1-chloro-2,4-dinitrobenzene; CNBr, cyanogen bromide; ESI-MS, electrospray ionization mass spectrometry; and TFA, trifluoroacetic acid.

purification by HPLC to remove Triton X-100 and to separate the two proteins in preparation for the structural studies by mass spectrometry. Samples were purified using a Shimadzu gradient-controlled HPLC system (model LC-600 pumps; Japan) equipped with an Aquapore RP-300 7 mm analytical column (4.6 × 250 mm; Foster City, CA) and a Shimadzu SPD-6A spectrophotometric detector with UV detection at 215 nm. HPLC solvents were 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The gradient program was as follows at 1.0 mL/min: 5 min 10% B; 10 min 40% B; 75 min 70% B; 85 min 100% B; followed by reequilibration to 10% B. The proteins were collected, lyophilized, and stored at −85° until analysis by mass spectrometry. Peaks at retention times 41 and 43 min were identified as protein by comparison to chromatograms of 0.1% Triton X-100 in water. Proteins were identified as mGST by comparison of their molecular weights, determined by ESI-MS, to that deduced from the published cDNA sequence [15].

ESI-MS was performed with a Vestec electrospray source (Houston, TX) fitted to a Hewlett Packard 5988A quadrupole mass spectrometer (Palo Alto, CA). The proteins were dissolved in water:methanol:glacial acetic acid (47:50:3, by vol.) and introduced into the ESI source by direct infusion with a syringe pump (Harvard Apparatus, South Natick, MA) at a flow rate of 10 μL/min. Spectra were acquired using a Teknivent data system (Teknivent Corp., St. Louis, MO) and analyzed by standard deconvolution methodology [18].

Treatment of proteins with CNBr was carried out in 70% TFA in water for 20 hr at 25°. TFA, instead of formic acid, was used to prevent formylation of the peptides during the digestion procedure [19]. Peptides were either analyzed as a mixture or separated by HPLC, as described above, with the following gradient program: 2 min 5% B; 30 min 70% B; 35 min 70% B; 36 min 100% B; 40 min 100% B followed by reequilibration to 5% B. Collected peptides were lyophilized and stored at −85° until further analysis.

Cation FAB-MS was performed on a Jeol HX110/HX110 tandem mass spectrometer (Tokyo, Japan) equipped with a DA7000 data system at an accelerating voltage of 10 kV. Peptides were dissolved in 0.1% TFA in water and applied to the probe tip. The samples were desorbed from a thioglycerol matrix with a FAB gun operating at 6 kV with xenon gas. For collisional activation experiments, precursor ions were mass selected in MS-1 and collided in a 4-kV floated cell situated in the third field free region. Helium was introduced into the collision cell in order to produce a 40% attenuation of the main beam. Fragments were detected by B/E scans in MS-2. Resolution in MS-1 and MS-2 was 1000.

Automated N-terminal degradations were performed on HPLC purified proteins using a model 477A pulsed, liquid phase protein sequencer equipped with an on-line model phenylthiohydantoin analyzer (Applied Biosystems).

RESULTS

mGST is a membrane-bound protein and, therefore, was purified in the presence of the detergent Triton X-100. Desalting, pretreatment of the protein with Bio-beads [20], and extensive dialysis [21] all failed to remove enough of the detergent to allow its molecular weight to be determined by ESI-MS (data not shown). Presumably, Triton X-100 suppressed the protein signal.* High pressure liquid chromatography was found to be the most efficient method for removal of the detergent. Figure 1 shows a representative HPLC chromatogram of an aliquot of purified mGST. Protein peaks at 41 and 43 min, labeled mGST 1 and mGST 2, respectively, were consistently found in a 1:3 ratio. A second, separate purification of mGST produced the same ratio of the two proteins, suggesting that this was not an artifact of the preparation.

The two proteins were collected separately and analyzed by ESI-MS (Fig. 2). The molecular weight of mGST 1 was found to be 17,354.2 ± 6.6. This was in close agreement with the cDNA deduced protein sequence data reported by DeJong *et al.* [15] of 17,348. Of interest was the second protein, mGST 2. Its molecular weight was observed to be 17,397.9 ± 6.6, the mass being 44 Da greater than mGST 1.

To determine whether this mass increase was due to oxidation, a post-translational modification, or amino acid substitutions, the protein was mapped by CNBr digestion with and without further separation by HPLC. Comparison of the calculated molecular weights of each peptide deduced from the cDNA sequence to the observed molecular weights for mGST 1 and mGST 2 are shown in Table 1. The molecular weights of the peptides were determined by FAB-MS.

The CNBr digest of mGST 1 was analyzed as a mixture, without prior clean-up and separation by HPLC, and accounts for the presence of the natriated ion for peptide 8–13. The observed molecular weights were calculated from the protonated molecular ions for peptides 1–7, 14–26, and 144–154. All were in agreement with the expected molecular weights calculated from the published cDNA sequence. Peptide 28–143 was too large to be observed by FAB-MS, and the presence of residual Triton X-100 prevented its mass assignment by ESI-MS. To verify that the observed molecular weight for peptide 8–13 was indeed from a natriated ion, the intact protein was subjected to Edman sequencing. Thirty cycles of Edman degradation were carried out, and the sequence was found to be identical to the published sequence data.

Mapping of mGST 2 by CNBr digestion was followed by HPLC separation of the peptides. The observed molecular weights of peptides 8–13, 14–26, and 144–154 were in agreement with the published data and mGST 1 (Table 1). Peptide 1–7, however,

* Loo RRO, Dales N and Andrews PC, Electrospray ionization mass spectrometry of peptides and proteins in the presence of detergents. *Proceedings of the 41st ASMS Conference on Mass Spectrometry and Allied Topics* **581**, 1993.

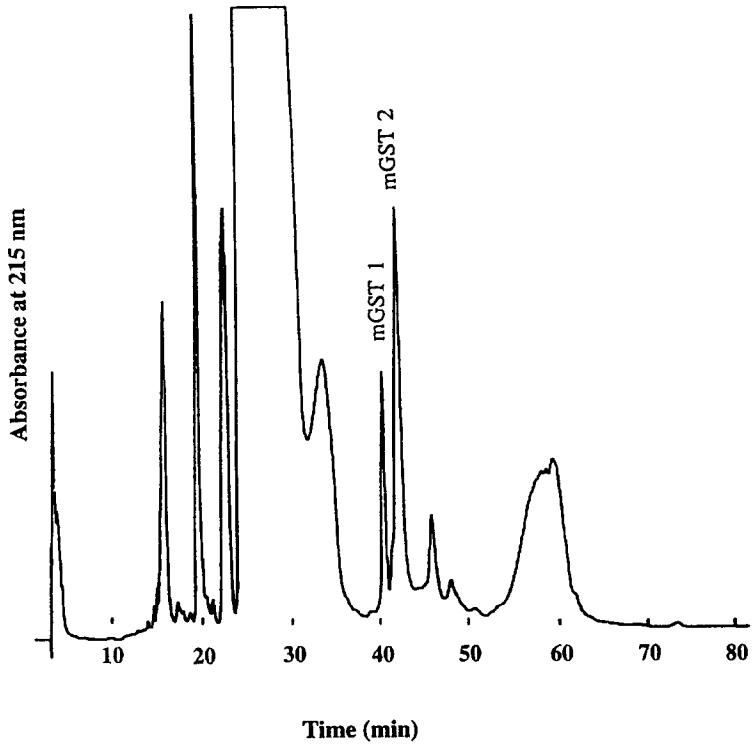

Fig. 1. Reversed phase HPLC chromatogram of "purified" mGST. Peaks at the retention times 41 and 43 min represent mGST 1 and mGST 2, respectively. All other peaks, except those at 46 and 48 min, presumably are different monomer populations of Triton X-100.

was observed to be 811.5 Da. The 41.5 Da increase was consistent with acetylation. To determine whether this was responsible for the mass increase, an MS/MS experiment was carried out by collisional activation of the molecular ion at m/z 812.5. The results of this are shown in Fig. 3, along with the deduced amino acid sequence derived by overlapping series of N- and C-terminal backbone product ions. The data demonstrated the N-terminal alanine to be acetylated. Further confirmation that the N-terminal was indeed blocked was provided through a negative result obtained by Edman degradation (not shown).

DISCUSSION

The results of the present study reveal the existence of an N-acetylated form of rat liver mGST. Previous structural studies of this protein were incomplete due to the limited resolving power of SDS–PAGE and the nature of the post-translational modification. Morgenstern *et al.* [3, 5] purified and characterized the enzyme in its activated and unactivated states to apparent homogeneity. Its primary structure was then determined by various proteolytic digests in conjunction with Edman degradation [21]. However, the existence of the N-acetylated form of mGST was missed due to its resistance to sequencing by Edman degradation and the lack of another change in structure elsewhere in the protein. Southern blot analysis of rat genomic DNA showed no evidence for heterogeneity [15], and thus it appeared that no isozymes existed. However, the identification in other tissues of a nonactivatable mGST [10] and a second inducible mGST activity [22] suggested the possibility of there being an additional form.

Removal of Triton X-100 from purified mGST by HPLC provided the first clue of an additional isoform (Fig. 1). ESI-MS demonstrated the molecular weight of mGST 1 to be in agreement with the previously described mGST (Fig. 2). The molecular mass of mGST 2 was observed to be 44 Da greater. The error associated with this determination allowed several interpretations: multiple oxidations of the protein, N-terminal acetylation, or the existence of another isoform with amino acid substitutions due to alternative splicing of mRNA. Presumably, oxidation could occur at the single cysteine or any of the five methionine residues.

CNBr digestion was chosen to map the proteins in order to generate a small N-terminal peptide, due to a reported methionine at residue seven, and also because of its inability to cleave at oxidized methionines [23]. As shown in Table 1, the identification of the expected peptides of mGST 2 ruled out methionine oxidation. The 44 Da mass increase observed with mGST 2 by ESI-MS was localized to the N-terminal peptide 1–7 (811.5 − 770.0 = 41.5 Da). This was consistent with N-terminal acetylation or substitution of the N-terminal alanine with either leucine or isoleucine.

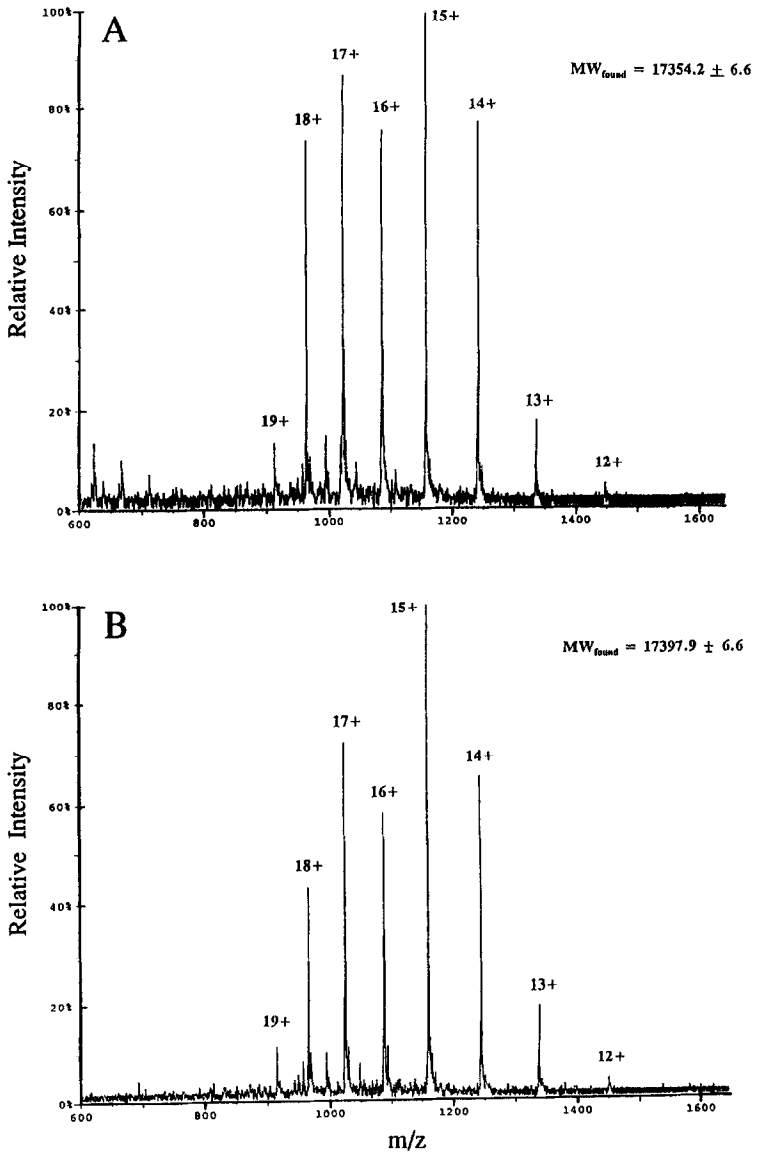

Fig. 2. ESI-MS spectra of mGST 1 (A) and mGST 2 (B). See Fig. 1 for HPLC elution profile. Each peak is a molecular ion with the indicated charge state.

Table 1. Comparison of expected and observed molecular weights of peptides produced by CNBr digestion

Peptide	MW_{calc} from cDNA sequence*	$MW_{observed}$ mGST 1	mGST 2
1–7	770.4	770.0	811.5
8–13	672.3	673.0†	673.0
14–26	1381.8	1381.2	1381.2
28–143	12880.8	ND‡	ND
144–End	1422.8	1423.2	1423.2

* Calculated for homoserine lactone terminus (except for peptide 144–End).
† Calculated from the natriated molecular ion.
‡ Not determined.

Fast atom bombardment tandem mass spectrometry sequence analysis of peptide 1–7 demonstrated that residues 2–6 were identical with those of mGST 1. The abundance of the b_1^+ ion (m/z 113.9) in this peptide was indicative of an N-terminal acyl group [24, 25]. N-terminal blocking of mGST 2 was confirmed by its resistance to Edman degradation.

Despite the lack of mass assignment of peptide 28–143 in both proteins, the difference between the two proteins appeared only to be N-terminal acetylation. A similar finding had been observed with actins of the cellular slime mold *Dictyostelium* [26]. The nonacetylated protein was thought to be a precursor to the acetylated form and to be present in a small quantity due to rapid turnover. Roesijadi *et al.* [27] described the presence of both blocked and unblocked forms of a metallothionein from

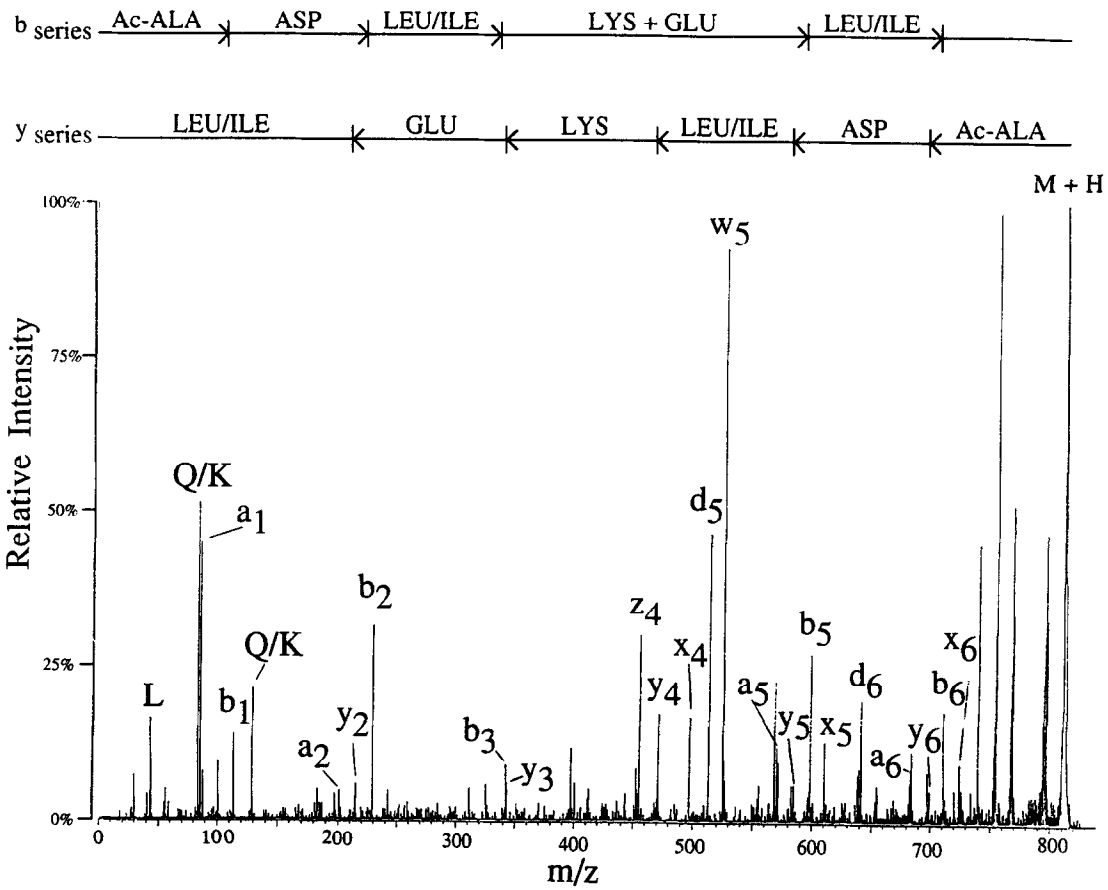

Fig. 3. FAB-MS/MS spectra of mGST 2 peptide 1–7. The b_n series defines the amino acid sequence of residues 1–6 sequentially from the N-terminus. Similarly, the y_n series ions from residues 1–6 enables the sequence to be read sequentially from the C-terminus. The sequence was determined by using both of these product ions.

Crassostrea virginica. The unblocked form was found to occur in greater quantity upon induction [28], presumably due to the inability of *N*-acetyltransferase to acetylate all of the protein molecules during synthesis.

Whether mGST 1 is a precursor for mGST 2 has yet to be established. However, the observed heterogeneity does suggest that the protein may not be a trimer of identical subunits as had been proposed [5, 11, 12]. It is conceivable that the ratio of the nonacetylated form to the acetylated dictates whether the protein will be activated, e.g. by *N*-ethylmaleimide, and whether modification of this ratio accounts for the reported increase in enzyme activity associated with specific inducers [4, 29]. These effects could be due to changes in the acetylated/nonacetylated ratio leading to changes in the quaternary structure ultimately manifesting itself in enzyme activity. Modulation of activity by acetylation has been reported for hemoglobin. Bunn and Briehl [30] reported that N-terminal blockage of hemoglobins led to lower oxygen affinities. Similarly, Moo-Penn *et al.* [31] reported that substitution of the N-terminal valine of the hemoglobin b_1 chain with acetylalanine, as observed in the human hemoglobin variant Hb Raleigh, led to a decreased affinity for oxygen and insensitivity to interactions with organic phosphate cofactors. Simple Northern blot analysis of the mRNA in various tissues, or after induction, cannot address these possibilities. Direct protein analysis by an HPLC method, as described in this study, may provide insight to these issues.

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