Microsomal glutathione transferase-1 (MGST1) is a membrane-bound enzyme involved in the deoxygenation of xenobiotics and the protection of cells against oxidative stress. The proposed active form of the enzyme is a noncovalently associated homotrimer that binds one substrate glutathione molecule/trimer. In this study, this complex has been directly observed by electrospray mass spectrometry analysis of active rat liver MGST1 reconstituted in a minimum amount of detergent. The measured mass of the homotrimer is 53 kDa, allowing for the mass of three MGST molecules in complex with one glutathione molecule. Collision-induced dissociation of the trimer complex resulted in the formation of monomer and homodimer ion species. Two distinct species of homodimer were observed, one unliganded and one identified as a homodimer-glutathione complex. Activation of the enzyme by N-ethylmaleimide through modification of Cys\(^{49}\) (Svensson, R., Rinaldi, R., Swedmark, S., and Morgenstern, R. (2000) Biochemistry 39, 15144–15149) was monitored by the observation of an appropriate increase in mass in both the denatured monomeric and native trimeric forms of MGST1. Together, the data correspond well with the proposed functional organization of MGST1. These results also represent the first example of direct electrospray mass spectrometry analysis of a detergent-solubilized multimeric membrane protein complex in its native state.

Membrane proteins are estimated to constitute about one-third of the total proteome (1) and have important and diverse roles in biology, including functions as cell-surface receptors, ion channels, and transporters and in cell adhesion. However, because of the difficulty in producing crystals of detergent-solubilized proteins for x-ray crystallography, only ~1% of the unique protein structures so far solved at high resolution are of membrane proteins (2). Integral membrane proteins thus pose an important challenge in structural biology.

Microsomal glutathione transferase-1 (MGST1)\(^{3}\) is a member of the MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) superfamily. At present, other mammalian members of the MAPEG family are two glutathione transferases/peroxidases (MGST2 and MGST3), 5-lipoxygenase-activating protein, leukotriene C\(_4\) synthase, and microsomal prostaglandin E\(_2\) synthase (the closest relative to MGST1) (3). MGST1 functions both as a glutathione transferase and as a peroxidase, thus potentially protecting cells from reactive compounds and oxidative stress (3, 4). The quaternary structure of MGST1 has previously been investigated by electron crystallography (5, 6), radiation inactivation (7), cross-linking (8), and hydrodynamic (9) studies and has been suggested to be a homotrimer both in the microsomal membrane and in the purified form. Electrospray (ES) mass spectrometry has shown that the enzyme is partly N-acetylated (10). Interestingly, equilibrium dialysis (11) and stopped-flow active-site titrations (12) have indicated that the MGST1 homotrimer binds only one molecule of the substrate GSH, making enzyme-substrate interaction an important study parameter. Thus, MGST1 constitutes a well studied, but incompletely defined model protein to probe the limits of mass spectrometry in the analysis of the native characteristics of membrane-bound proteins.

In recent years, ES mass spectrometry has developed from being a method to determine the molecular mass of proteins (13) to being a tool for observing noncovalent multimeric protein assemblies (for reviews, see Refs. 14–16). ES analyses of several biologically important soluble macromolecular complexes and their stoichiometric organization have been reported (17–20). Furthermore, secondary structure elements and structural dynamics can also be studied by mass spectrometry in hydrogen/deuterium exchange experiments, in which the exchange kinetics of amide protons are measured (21–23), making ES mass spectrometry an attractive tool for studies of protein structure and organization. However, the study of detergent-solubilized proteins by ES mass spectrometry is challenging because of protein ion signal suppression due to the presence of excess detergent (24). Consequently, analysis of integral membrane proteins has usually relied on the removal of detergent prior to sample analysis by, for example, organic solvent extraction (25) or reversed-phase (26) or size-exclusion (27, 28) chromatography. Recently, Heck and co-workers reported the analysis of short transmembrane peptides embed-
We have sought to extend the direct ES analysis of detergent-solubilized proteins to the analysis of noncovalent macromolecular assemblies. Purified rat liver MGST1 protein was solubilized in a minimum amount of detergent compatible with enzyme activity. The ES interface conditions were optimized for the preservation of noncovalent protein complexes while allowing desolvation from the MGST1 protein complex. This permitted the stoichiometry of the protein complex to be investigated. Additionally, "in-source" collision-induced dissociation of the complex was performed. This allowed the noncovalent binding of GSH to the complex to be investigated. Furthermore, to illustrate the ability of ES mass spectrometry to monitor functional changes, i.e. covalent modifications in intact membrane proteins, MGST1 was analyzed after enzyme modification by N-ethylmaleimide (NEM; relative molecular weight of M, 125,000) (35). As MGST1 contains one cysteine residue/subunit, this mass labeling was additionally beneficial in the identification of homotrimer peaks in the ES mass spectra. To the best of our knowledge, the results presented here represent the first application of ES mass spectrometry to the direct analysis of a biologically important noncovalently bound membrane protein complex.

MATERIALS AND METHODS

Reagents and Solutions—Horse heart myoglobin, GSH, yeast alcohol dehydrogenase, NEM, and Triton X-100 were obtained from Sigma (Stockholm, Sweden). The structures of Triton X-100, GSH, and NEM are shown in Scheme 1. Deionized water was used after further purification using a Milli-Q reagent-grade water system (Millipore S.A., Molsheim, France). Rat MGST1 was purified as described previously (36).

Mass Spectrometry—All data were acquired on a quadrupole time-of-flight mass spectrometer (QTOF1, Micromass, Manchester, United Kingdom) equipped with the standard Z-spray ES source. Sample solutions were pumped to the ES capillary at a flow rate of 10 μl/min using a Hamilton type 22 syringe pump. The source block temperature was maintained at either 60 or 80 °C. The nebulizer gas was set to maximum flow (20 liters/h), and the desolvation gas flow was set to 75 liters/h (both nitrogen). The declustering potential (cone voltage) was maintained at 180 V unless otherwise indicated, and the ES capillary voltage was +3.0 kV. To maintain noncovalent interactions during the transmission of ions into the gas phase and through the subsequent mass spectrometry analysis, the following instrumental parameters were optimized. Argon gas was admitted into the collision cell, raising the pressure reading on the nearby analyser Penning gauge from 7.6 × 10⁻⁶ to 4.0 × 10⁻⁸ millibars. The source rotary pump was "throttled," with the result that the pressure on the analyser gauge increased further to 5.1 × 10⁻⁵ millibars. The parameters controlling the transfer and collision cell hexapoles were set to allow maximum transmission of high m/z ions (>3000 Thomson). The time-of-flight pusher frequency was set manually at a pusher time of 200 μs.

Determination of Limiting Triton X-100 Concentrations for ES Mass Spectrometry—A preliminary experiment was performed to determine the detergent level compatible with ES analysis of multimeric protein complexes. Yeast alcohol dehydrogenase was used as a model complex, having a tetramer average relative molecular weight of M₄ ≈147,000. A stock solution of 100 pmol/μl alcohol dehydrogenase was prepared in 10 mM ammonium acetate and diluted in the same buffer to give a protein concentration of 10 pmol/μl. Triton X-100 was included in the buffer at 0, 0.02, 0.1, and 0.2% (v/v). Each sample was infused into the ES interface, and the ion intensity of the most intense tetramer charge state was normalized to that achieved in a detergent-free solution.

Buffer Exchange of MGST1—A solution of 2 mg/ml MGST1 in 10 mM potassium phosphate (pH 8), 1 mM GSH, 0.2% (v/v) Triton X-100, 0.1 mM EDTA, 0.2 mM KCl, and 20% glycerol was subjected to size-exclusion chromatography (Econo-Pac 10DG column, Bio-Rad) using 1 mM potassium phosphate (pH 8) and 0.1 mM GSH as a running buffer to obtain ~2 mg/ml MGST1 in the latter buffer and ~0.2% Triton X-100 (not removed in the size-exclusion procedure). The pooled sample was then diluted 5-fold with the same buffer to give 0.3–0.4 mg/ml MGST1 in 1 mM potassium phosphate (pH 8), 0.1 mM GSH, and ~0.04% Triton X-100.

Activation of MGST1 was accomplished by adding 1 mM NEM to an aliquot of 2 mg/ml MGST1 in 1 mM potassium phosphate (pH 8), 0.1 mM GSH, and 0.2% Triton X-100 on ice. The reaction was terminated by the addition of 1 mM GSH, after which the sample was diluted as described above. The concentration of the GSH-NEM conjugate was estimated to 0.2 mM in the final solution. This conjugate is a potential, but poor inhibitor of MGST1; and because of the higher affinity of the enzyme for GSH (0.2 mM; Kₐ = 20 μM), the primary enzyme species in the experimental buffer will be the E-GSSH (enzyme-glutathione anion) complex.

RESULTS

Electrospray Tolerance of Triton X-100—The maximum detergent concentration compatible with the ES analysis of a multiprotein complex was determined taking tetrameric alcohol dehydrogenase as an example. With 0.1% Triton X-100 in the sample buffer, the tetramer signal was reduced to 31% of its value when electrosprayed from a detergent-free solution (data not shown). Accordingly, in our experiments, a 0.1% detergent level was regarded as the maximum acceptable level of detergent for ES analysis.

Enzyme Activity at Limiting Detergent Concentrations—We have previously determined that MGST1 binds ~1.5 g of Triton X-100/g of protein (9). By varying the ratio of protein to detergent in this region, it was possible to find conditions...
that yielded fully active protein and no visible aggregation. Gel filtration was utilized for buffer exchange (to lower the ionic strength and to remove glycerol) because it was observed that, upon dialysis, the protein tended to aggregate. A solution of 0.5 mg/ml MGST1 in 0.05% Triton X-100 was fully active as determined by an enzyme activity assay (data not shown). It was important to include GSH in the buffer, which is in accordance with the known stabilizing effect of this substrate.

**Mass Measurement of MGST1**—Detergent-solubilized MGST1 (0.3–0.4 mg/ml, ∼20 pmol/μl) with ∼0.04% Triton X-100 was infused into the ES source at a flow rate of 10 μl/min. The resulting mass spectrum was dominated below m/z 2000 by detergent cluster ions, which were singly charged and separated by 44 Thomson (Figs. 1A and 2A). Triton X-100 is a polymer with the molecular formula C_{14}H_{21}O(CH_2CH_2O)_nH, where n has an average value of 9.5 (molecular mass of C_{14}H_{20}O = 44 Da); and the average mass of a Triton X-100 molecule is 625 Da. In the first few scans after sample infusion was started, a peak envelope was resolved in the m/z 1450–3000 range (Fig. 1A). Deconvolution of this ion series revealed the presence of two components of 17,340 and 17,382 Da, which correspond to the mass of acetylated MGST1 (10). In subsequent scans, the monomeric protein peaks were obscured by detergent ions.

Examination of the mass spectrum in the m/z 5000–9300 range revealed a second envelope of peaks (Figs. 1A and 2A). A declustering potential of 180 V was used because lower potentials failed to clearly resolve the m/z 5000–9000 peak envelope from the background. Manual interpretation of the data led to the charge state designation as shown in Fig. 1C. From the data in Fig. 1A, the mass of the neutral species was calculated to fall in the range of 53,033–53,277 Da (Fig. 1C). It should be noted that the peaks in the high m/z 5000–9300 range were broad (full-width at half-maximum: 10.7, 16.3, 22.1, 23.7, and 28.1 Thomson for the 10+ to 6+ peaks, respectively), and mass values were determined from the peak top m/z values. The expected mass of the MGST1 homotrimer falls in the range of 52,020 Da (3 × 17,340) to 52,146 Da (3 × 17,382) depending on the degree of acetylation. Experimentally, the measured mass was higher than these values, and the excess was found to depend on which charge state was used in the mass determination (Fig. 1C). The excess mass can be explained by the incomplete removal of solvent and other noncovalently bound adducts from the protein complex. The excess mass was greatest for lower charge state complexes and lowest for the higher charge state complexes. This can be explained by considering the desolvation (noncovalent adduct removal) process within the ES interface, where charged species are accelerated through a potential difference (cone voltage) and undergo collisions with residual gas molecules. Ions with a higher charge state are accelerated to a greater translational energy than their lower charge state equivalents and thus undergo more energetic desolvating collisions with the residual gas molecules. Therefore, noncovalently bound adducts and solvent molecules are more efficiently removed from the higher charge state ions. The additional mass observed allows for the addition of one GSH molecule (307 Da) in combination with Triton X-100 (e.g. C_{14}H_{21}O(CH_2CH_2O)_nH, 603 Da), water, phosphate, or potassium adducts.

**Collisionally Activated Dissociation of the Homotrimer Complex**—To confirm the composition of the observed homotrimer complex, the declustering potential (cone voltage) was increased to induce dissociation of the assembled complex. Increasing the cone voltage from 180 V (Fig. 2A) to 195 V (Fig. 2B) resulted in the appearance of a series of monomer peaks in the m/z 1700–2500 range (Fig. 2B). At a cone voltage of 195 V, a novel series of peaks appeared in the m/z 2600–5000 range (Fig. 2, B and C). The new peaks at m/z 2675, 3161, 3864, and 4968 were interspersed among the high m/z monomer peaks (m/z 2898 (6+), 3477 (5+), and 4347 (4+)) and were designated to correspond to odd-numbered charge states (13+ to 7+) of MGST1 homodimers that were formed by in-source collision-induced dissociation of the homotrimer complex. In Fig. 2C, the 195 V spectrum is shown on an expanded scale in the m/z range covering the 13+ to 7+ charge states of the dimer. The apparent discrepancy in the peak intensities of the 12+ and 10+ charge states is due to the overlap of monomer peaks onto homodimer peaks. Deconvolution of the 11+ to 9+ charge states on the true mass scale gives a mass for the major component of 34,764.5 Da (Fig. 2D). This is in good agreement with the mass of a homodimer of two acetylated MGST1 subunits (theoretical mass of 34,764.0 Da). Identification of the homodimer species was aided by the fact that MGST1 is only partially acetylated, thereby giving a triplet of peaks for each dimer charge state. This is shown in Fig. 2E for the 10+ charge state of the dimer, where homodimers of non/non-, non/mono-, and mono/mono-acetylated forms of MGST1 gave peaks at 3469.0, 3473.3, and 3477.4 Da, respectively. The respective forms of acetylated homodimers are also evident in the deconvoluted mass spectrum shown in Fig. 2D, giving observed masses of 34,679, 34,722.5, and 34,764.5 Da. The presence of...
enhanced MGST1 monomer and dimer series in the high cone voltage experiment and the concurrent disappearance of the MGST1 trimer indicate that the MGST1 trimer fragments under these conditions to a dimer and monomer.

Glutathione Binding to the MGST1 Homodimer—To stabilize the native protein, the enzyme substrate, i.e. reduced GSH, was included in the sample buffer at 0.1 mM in all experiments. In the ES mass spectra of MGST1 (Figs. 1A and 2A), the peaks corresponding to the MGST1 trimer were of low intensity and were broad due to the presence of undissociated Triton X-100, GSH, and water molecules and/or buffer salts adducted to the trimer. When the cone voltage was increased from 180 V (Figs. 1A and 2A) to 195 V (Fig. 2, B and C), the trimer dissociated to a monomer and dimer. The mass spectrum obtained at a cone voltage of 195 V showed a series of peaks corresponding to the MGST1 homodimer accompanied by a satellite series of peaks of higher mass (indicated by arrows in Fig. 2, C–E). These satellite peaks were not observed for the monomer charge states. For the 10+ charge state, the m/z increment of the adduct peak relative to the MGST1 homodimer was 30.5 ± 0.5 Thomson (Fig. 2E), translating to the addition of one GSH molecule (307 Da) to the complex. An additional series of adducts was also observed that corresponded to a complex ~40 Da heavier than the GSH complex (Fig. 2, D and E). This was probably due to the additional presence of a potassium adduct. As the origin of the homodimer is the homotrimer, the observation of the GSH adduct on the homodimer, but not the monomer, indicates that the parent homotrimer contains just one GSH molecule.

Enzyme Mass Labeling by NEM—MGST1 activity is increased up to 30-fold through covalent modification (+125 Da) of Cys49 by NEM (35). Incorporation of NEM was essentially complete (>85%) after 5 min of incubation as indicated by ES mass spectrometry. Upon incubation of MGST1 with NEM, enzyme activation was accompanied by a mass shift of the monomer corresponding to the addition of one NEM molecule/monomer (Fig. 3, A and B). The observed molecular mass of the most abundant species of monomer (i.e. acetylated) increased from 17,382 to 17,507 Da after NEM modification (Fig. 3). NEM labeling also shifted the peaks corresponding to MGST1 trimers to higher m/z values. The 9+ charge state of the MGST1 trimer was shifted from m/z 5898.6 to m/z 5940.5 (Fig. 3, C and D). This shift corresponds to a mass increase of 377 Da, in good agreement with the addition of 125 Da/monomer. These data also substantiate the identification of the envelope of ions in the m/z 5000–9000 range as corresponding to MGST1 homotrimers.
We have studied the multimeric assembly of an integral membrane protein, MGST1, using direct ES mass spectrometry measurements of the native detergent-solubilized enzyme. Detergent-solubilized proteins are difficult to analyze by ES mass spectrometry due to the competitive ionization of excess detergent. In a preliminary study, different classes of detergents (MEGA-10, Thesit, Zwittergent 3–12) were tested (data not shown), but Triton X-100 was found to be the most compatible with the ES analysis of proteins. Therefore, we initially determined the level of Triton X-100 compatible with the ES analysis of noncovalent protein complexes using the tetramer of yeast alcohol dehydrogenase as a model noncovalent protein complex. Unsurprisingly, there was a decreased response for protein multimer ions with increasing amounts of detergent under fixed desolvation conditions (data not shown). As the detergent is surface active, and in excess, it will compete with protein ions in the desorption process from the ES-generated droplets and, furthermore, will form adducts with protein ions. By recording ES spectra at a relatively high cone voltage (180 V), it was established that the energetic interface conditions improved the alcohol dehydrogenase tetramer peak shape and signal intensity. This preliminary study showed that, although the presence of detergent is deleterious to ES analysis, low levels of detergent (i.e. <0.1%) can be tolerated.

By reconstituting MGST1 in a minimum amount of Triton X-100 in the presence of GSH, the enzyme could be stabilized in its active form. Enzyme activity in the low detergent buffer was measured, demonstrating correctly folded and fully functional MGST1 in the sample solutions. When MGST1 was infused into the mass spectrometer at a protein concentration of ~0.35 mg/ml, a peak envelope was observed in the m/z 5000–9000 range (Fig. 1A). The spectrum was manually interpreted, and the molecular mass calculated from each charge state varied between 53,033 and 53,277 Da (average of 53,146 Da) (Fig. 1C). The mass of an unliganded adduct-free MGST1 homotrimer ranges from 52,020 to 52,146 Da depending on the degree of acetylation. These data suggest that we are observing a homotrimer species, the mass of which is elevated by the addition of noncovalently bound adducts. The excess mass may be accounted for by the incomplete removal of noncovalently bound solvent, buffer, and detergent as well as biological ligands, i.e. GSH. Besides the inherent influences of buffer and detergents on ES performance, a variable extent of adduct binding explains the increase in the observed mass of the trimer complex as the charge state of the complex is lowered (Fig. 1C). Ions of higher net charge are accelerated more by any given declustering potential than those of lower charge. Thus, removal of noncovalently bound adducts may be less efficient for ions of lower net charge; this can result in an observed mass increase for ions of lower charge state. The identification of a homotrimer species was further supported by the mass shift corresponding to the addition of 125 Da for each monomer after NEM labeling. The covalent modification of Cys48 has been shown to induce enzyme activity (35). Together, these results indicate that a homotrimer is indeed detected.

The structural features of the MGST1 multimer were obtained by dissociation of the complex. When the declustering potential was increased by as little as 15 V from 180 to 195 V, the trimer was found to dissociate to a monomer and dimer. There is also evidence of the noncovalent binding of one GSH molecule to the homotrimer complex. In the dimer species formed by in-source collision-induced dissociation, we detected an adduct of ~305 Da that was not present in MGST1 monomer ions. This indicates the presence of a noncovalent adduct only in the multimeric enzyme complex. Although the mass correlation is not perfect, it is tempting to identify this noncovalent ligand as the enzyme substrate GSH.

MGST1 has been the subject of extensive structural studies. The present consensus is that the enzyme is active in a homotrimeric conformation, binding one molecule of GSH/trimer (11, 40–42). The mass spectrometry data presented here are clearly in agreement with this consensus and further indicate the capability of a dimer to bind GSH. The binding of one GSH molecule to a homotrimer can, in principle, occur by two mechanisms. Either each subunit has a separate binding site, and negative cooperativity results in third-of-the-sites reactivity; or the trimer forms one binding site (which, because of symmetry, has to result in three overlapping, but mutually exclusive binding modes). The data favor the latter mechanism.

In this study, the MGST1 enzyme was analyzed directly from the detergent-containing solution by ES mass spectrometry. As enzyme activity was determined just prior to mass spectrometry analysis, this ensured that the protein was in its native and fully functional state and supports the view that the trimer observed is indeed the biologically relevant MGST1 species. In the case of other non-denaturing analysis methods (e.g. size-exclusion chromatography and dialysis) in which the stabilizing detergent is removed, the possibility exists of artificial aggregation effects due to, or in the process of, detergent removal.

In summary, our results show the presence of a noncovalent...
homotrimer of MGST1 in solution. These findings are in accordance with previously published reports on the functional complex for this membrane protein. Furthermore, they are indicative of the notion that, under optimized conditions, the subunit stoichiometry of membrane-embedded protein assemblies may be successfully analyzed by ES mass spectrometry.

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Observation of an Intact Noncovalent Homotrimer of Detergent-solubilized Rat Microsomal Glutathione Transferase-1 by Electrospray Mass Spectrometry

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