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Noncovalent Associations of Glutathione S-Transferase and Ligands: A Study Using Electrospray Quadrupole/Time-of-Flight Mass Spectrometry

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Human glutathione S-transferase A1-1 was observed predominantly as dimeric ions (51 kDa) during electrospray mass spectrometric analysis from aqueous solution at pH 7.4, in keeping with the known dimeric structure in solution. When analyses were performed on solutions of the enzyme containing glutathione (GSH), noncovalent adducts of protein dimer and one or two ligand molecules were observed; each mass increment, which exceeded the mass of GSH alone, was provisionally interpreted to indicate concomitant association of two water molecules per bound GSH. Noncovalent adducts of ligand and protein dimer were similarly observed for oxidized glutathione and for two glutathione inhibitors, both incorporating substituted thiol structures. In these instances, the mass increments exactly matched the ligand masses, suggesting that the apparent concomitant binding of water was associated with the presence in the ligand of a free thiol group. Collisionally activated decomposition during tandem mass spectrometry analyses of noncovalent adducts incorporating protein dimer and ligands yielded initially the denuded dimer; at higher collision energies the monomer and a protein fragment were formed. (J Am Soc Mass Spectrom 2000, 11, 606–614) © 2000 American Society for Mass Spectrometry

A large number of recent investigations have exploited the capability of the electrospray process to transfer to the gas-phase ions that appear to retain specific noncovalent associations that exist in the condensed phase (for reviews, see [1–5]). These associations include intramolecular interactions responsible, for example, for the secondary and tertiary structures of proteins [6, 7]. In addition, there is much evidence for the transfer to the gas phase of noncovalent adducts representing protein/protein interactions (yielding quaternary structure) [8–13] and protein/guest interactions (such as enzyme/substrate [14], enzyme/inhibitor [15], receptor/ligand [16], etc.). Interest in such mass spectrometric studies derives from the importance of noncovalent interactions in determining biomolecule function and the relative inconvenience of alternative analytical strategies.

The detection of noncovalently bound ion species during electrospray mass spectrometry (MS) requires judicious choice of analytical conditions, generally involving the introduction of aqueous solutions at near-neutral pH and the use of a low interface temperature. These conditions typically result in a reduced yield of ions for analysis, and lower charge states than observed under more conventional electrospray conditions using aqueous/organic solvent mixtures and reduced pH. Such investigations are therefore particularly suited to the use of mass spectrometers that achieve high sensitivities of detection and an extended mass/charge range. In the present work, studies of noncovalent interactions have been made using the combination of electrospray and a hybrid quadrupole/time-of-flight mass spectrometer. The preservation of noncovalent adducts has been aided by the use of low-flow electrospray with the associated reduced requirement for solvent removal in the interface as the initially formed droplets progress to fully desolvated species [4].

The glutathione S-transferases (GT) are a family of enzymes that catalyze the conjugation of glutathione (GSH; γ-glutamylcysteinylglycine) with a wide variety of electrophilic compounds [17–20]. Human GT A1-1 is the major isoenzyme found in human liver where it

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represents 2% of the total cytosolic protein [21, 22]. Structural and biochemical studies of GT A1-1 and of its interaction with ligands have been performed using X-ray crystallography [23, 24] and kinetic analysis [25–27]. GT A1-1 (with a monomer mass of 25 kDa) functions as a dimer that displays a high affinity for associated ligands [23, 24]. Here we describe a study of protein–protein and protein–ligand noncovalent interactions for GT A1-1, using low-flow electrospray mass spectrometry and MS/MS implemented on a hybrid quadrupole/time-of-flight instrument (Q-ToF).

Experimental

Materials and Reagents

Human recombinant glutathione S-transferase A1-1 (GT), produced from an overexpressing plasmid in *E. coli*, was purchased from Oxford Biochemical Research (Oxford, MI). GT was further purified and concentrated by ultrafiltration on Ultrafree NMWL:10,000 (Millipore, Bedford, MA). A reduced form (GSH) and an oxidized form (GSSG) of glutathione were obtained from Sigma (St. Louis, MO). S-(4-azido-2-nitrophenyl)-glutathione was prepared from GSH and 4-fluoro-3-nitrophenyl azide (Sigma) according to the method of Cooke et al. [28]. S-trityl glutathione (S-triphenylmethyl glutathione) was kindly provided by Professor K. Douglas (University of Manchester, Manchester, UK). All other chemicals were of the highest purity available.

Sample Preparation

Glutathione transferase (GT) was purified by ultracentrifugation in 10 mM ammonium acetate (pH 7.4) prior to electrospray mass spectrometry, in order to remove stabilizers (such as dithiothreitol, EDTA, and glycerol added to the enzyme to promote stability during storage) and buffer salts that might interfere with analysis. For the purpose of the determination of the molecular mass of the GT monomer, sample solutions were prepared to a concentration of approximately 2 pmol/μL in acetonitrile-water (1:1, v/v) with 0.1% formic acid. For analyses of the native state, solutions of GT were prepared to a final concentration of 5 pmol/μL in 10 mM aqueous ammonium acetate and adjusted to pH 7.4 with aqueous ammonia. Studies of protein/ligand interactions were performed by adding to the GT solution appropriate amounts of GSH solutions (in 10 mM ammonium acetate, pH 7.4) and inhibitors (in 10 mM ammonium acetate, pH 7.4, containing less than 1% dimethylsulfoxide).

Mass Spectrometry

The analyses were carried out on a Q-ToF mass spectrometer (Micromass, Manchester, UK) fitted with a dual orthogonal nanoelectrospray ion source. For the recording of conventional mass spectra, time-of-flight data were acquired over the range *m/z* 200–5000 (for detection of the denatured enzyme monomer) or *m/z* 2000–8000 (for detection of noncovalently associated species) with data accumulation of 3 s per spectrum. A portion (1–2 μL) of the protein solution was introduced into a metal-coated nanoelectrospray needle (Micromass) which was then mounted in the ion source. The sample flow rate was estimated to be 20–30 nL/min, allowing the recording of multiple spectra in more than one acquisition mode. In order to be able to maintain noncovalent complexes in the gas phase, an elevated instrument pressure was maintained by reducing analyzer pumping and introducing gas into the hexapole collision cell to achieve an analyzer pressure of approximately 1.0×10^{-4} mbar. Maximum entropy processing of conventional electrospray mass spectra of protein samples was performed using the MaxEnt program [29] incorporated in the Micromass MassLynx software.

For MS/MS analyses, the resolution of the mass-analyzing quadrupole was set to transmit the full isotopic envelope corresponding to the selected precursor ion. Argon was used as the collision gas. The collision offset potential was 55–100 V.

Results and Discussion

Analyses of Glutathione Transferase A1-1 (GT) Alone

The mass spectra of glutathione transferase A1-1 (GT) obtained using two different sets of electrospray conditions are shown in Figure 1. The spectrum (Figure 1A) obtained using "conventional" conditions (50% acetonitrile in water with 0.1% formic acid and a source temperature of 80 °C) showed only ions derived from the monomeric protein. The experimentally determined molecular mass was 25,498.4 $\pm$ 1.2 Da (n = 5), which is in good agreement with the figure of 25,499.8 Da calculated from the known amino acid sequence of GT [30]. No other prominent ion series was observed under these conditions of electrospray analysis; in contrast, previous studies [3] have reported the observation of covalent adducts involving mixed disulfides of GT and GSH.

Figure 1B shows the spectrum recorded in the absence of organic solvent and at higher pH, and with a reduced source temperature. Two prominent ion series are apparent, of which the more abundant corresponds to a mass of 50,998.1 $\pm$ 2.6 Da (n = 15), compared with a calculated value of 50,999.6 Da for the GT dimer. The less abundant ion series in Figure 1B corresponds to the GT monomer at much lower charge states than those observed for the analysis shown in Figure 1A. The observation of the prevalence of the gas-phase dimeric species is in accord with condensed-phase studies; X-ray crystallography of GT [23, 24] showed a preponderance of dimer.

The observation of the dimer during electrospray mass spectrometry analysis was found to be sensitive to the electrospray capillary voltage, capillary position relative to sample cone, and interface temperature. These parameters have previously been reported as

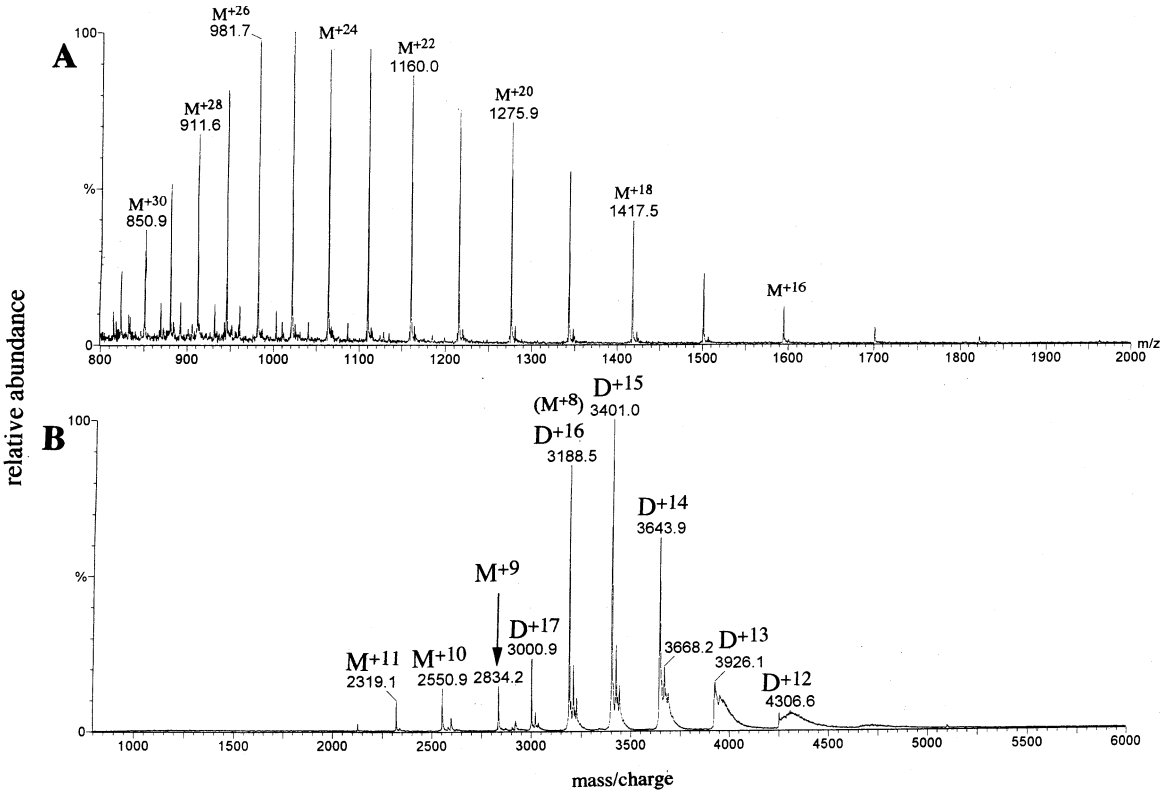

Figure 1. Electrospray mass spectra of glutathione transferase in (**A**) acetonitrile-water (1:1) including 0.1% formic acid, pH 3, and (**B**) 10 mM aqueous ammonium acetate, pH 7.4. The peaks are annotated to indicate their origin from monomeric (M) or dimeric (D) forms of the protein.

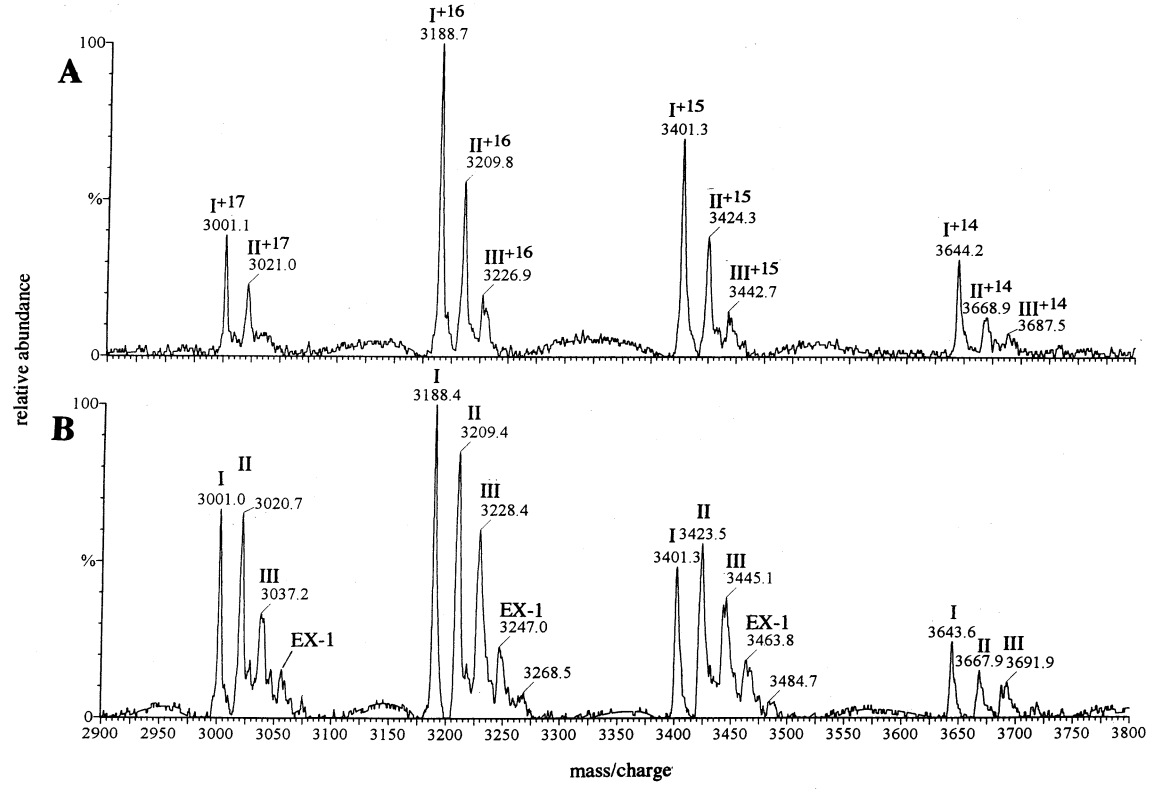

Figure 2. Partial electrospray mass spectra of glutathione transferase in 10 mM aqueous ammonium acetate, pH 7.4, with (**A**) equimolar amounts of glutathione (GSH) and (**B**) a fivefold molar excess of GSH. The origins of the various ion series are discussed in the text and summarized in Figure 3.

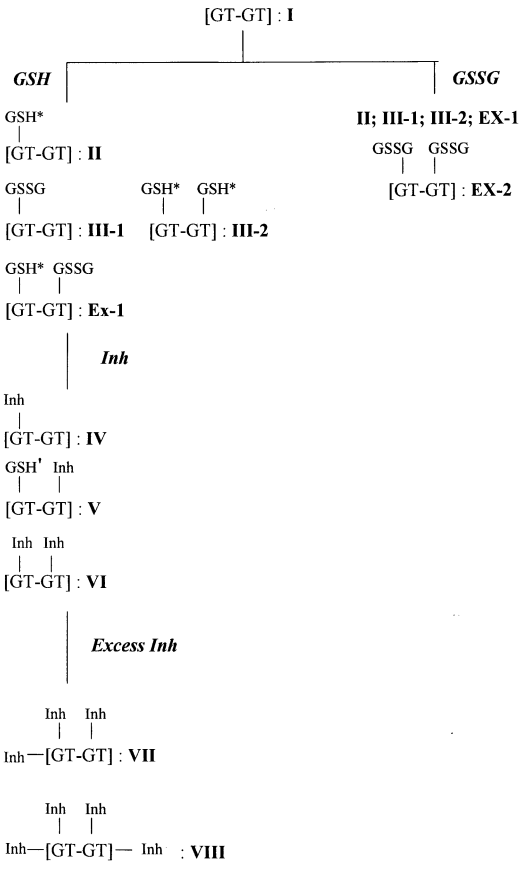

Figure 3. Summary of ion species detected during electrospray mass spectrometry analyses of glutathione transferase in the presence of glutathione (GSH), oxidized glutathione dimer (GSSG), and an enzyme inhibitor, S-(4-azido-2-nitrophenyl)-glutathione. GSH* represents an adducted moiety of approximately 341 Da observed following incubation with GSH.

critical in studies of noncovalent adducts by electrospray mass spectrometry [1, 3–5].

Detection of GT/GSH Adducts

When a mixture of GT and GSH (1:1, molar ratio) was analyzed by electrospray mass spectrometry under conditions designed to favor the retention of noncovalent associations, three series of ions were observed (Figure 2A). The most abundant ions (labeled I) corresponded to unmodified GT dimer but two additional series (II and III) were attributable to noncovalent adducts. (For convenience, the proposed assignments of all protein complex ions observed in this study are summarized in Figure 3.) The mass of the component giving rise to the series II peaks was calculated to be 51,338.8 ± 2.4 Da (n = 15), with the mass difference between the series I and II components therefore determined to be 340.7 ± 2.0 Da (n = 15). This mass increment compares with that of 307.3 Da expected for noncovalent addition of glutathione. Bearing in mind the imprecision of mass assignment, there are several possible explanations for this discrepancy. In principle, oxidation of the bound glutathione to the sulfone may have occurred, but this seems an unlikely explanation based both on the *exclusive* observation of the higher mass ligand and on the greater likelihood of the sulfonic acid as the oxidation product. Alternatively, the additional mass increment may be attributable to concomitant addition of a single molecule of oxygen or two of water; the accuracy and precision of mass assignment with the instrument used here does not allow discrimination between these possibilities. For convenience, we refer below to the adducted moiety of 340.7 Da as GSH*.

A sample analyzed under conditions which yielded evidence for the [2GST + GSH*] complex (Figure 2A) was subsequently analyzed under electrospray conditions which led to destruction of noncovalent associations (as in the analysis shown in Figure 1A). The measured mass of the glutathione monomer confirmed that no covalent modification of the enzyme had occurred; an attempt to determine the mass of the displaced ligand, however, was frustrated by an excessively high background in the low m/z region.

Series II ions were also observed at low relative abundance in the absence of explicitly added GSH (Figure 1B), presumably reflecting co-isolation of the enzyme and its native ligand.

The spectrum in Figure 2A shows a third ion series (III), the abundance of which is significantly enhanced when the molar ratio of GSH:GT is increased to 5:1 (Figure 2B). The appearance of the spectrum in Figure 2B suggests that ion series III may be heterogeneous; the mass increments are consistent with a dual origin from adduction of oxidized glutathione dimer (GSSG; 612.6 Da) or of 2 × GSH*. The possible origin of the minor ion series EX-1 (Figure 2b) is discussed below.

When GSSG was added explicitly to a solution of GT, electrospray mass spectrometry analysis (Figure 4) of the mixture showed at least five discernible ion series (I, II, III, Ex-1, and Ex-2) with the following proposed assignments:

I: [2GT]
II: [2GT + GSH*]
III: [2GT + 2GSH*] and [2GT + GSSG]
EX-1: [2GT + GSH* + GSSG]
EX-2: [2GT + 2GSSG]

The observation of adducts incorporating GSH following addition of GSSG is presumably due to the reduction of GSSG to GSH in solution.

Interaction of GT and Inhibitors

The interaction between GT and a recognized inhibitor, S-(4-azido-2-nitrophenyl)-glutathione (Inh-1) [28] has also been studied. Figure 5A shows the electrospray spectrum (recorded under conditions designed to favor the detection of noncovalent associations) for an equimolar mixture of GT, GSH, and Inh-1. Three new

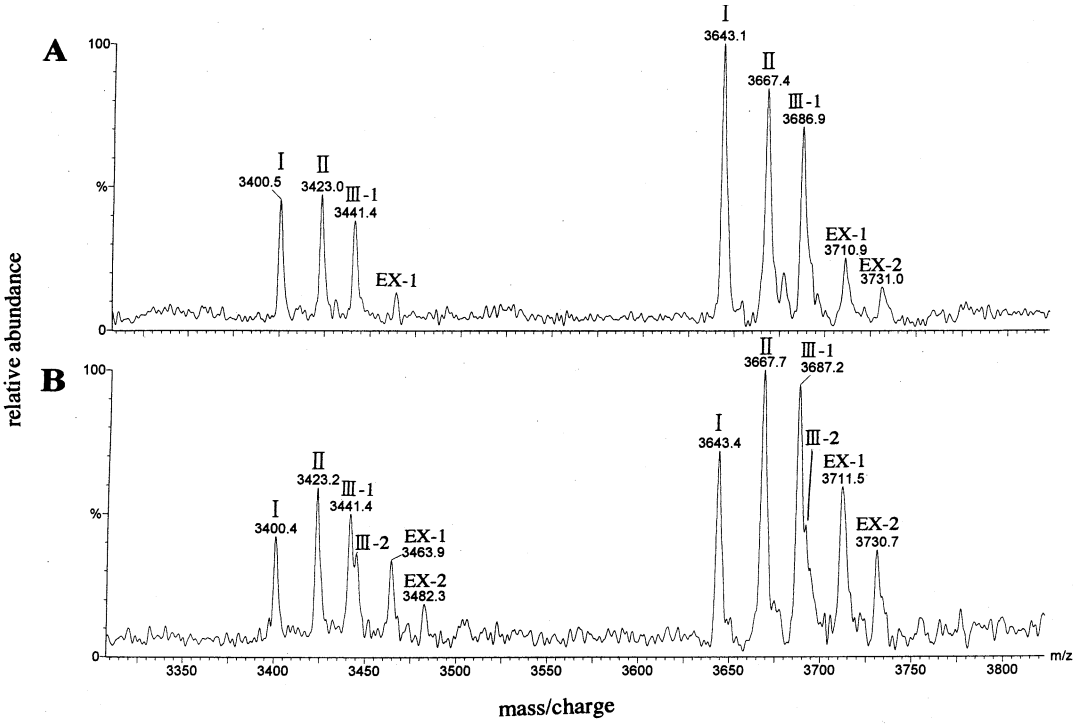

Figure 4. Partial electrospray mass spectra of glutathione transferase in 10 mM aqueous ammonium acetate, pH 7.4, with (**A**) equimolar amounts of oxidized glutathione dimer (GSSG) and (**B**) a fivefold molar excess of GSSG. The origins of the various ion series are discussed in the text and summarized in Figure 3.

series of peaks (IV, V, VI) are observed, as well as the series (I, II, III) discussed above. The mass of the complex giving rise to series IV ions was determined to be 51,470.6 Da, which is in good agreement with the sum of the masses of one molecule of Inh-1 (469.5 Da) and the GT dimer. The mass (51,936.4 Da) associated with series VI corresponds to a complex of two molecules of Inh-1 per GT–GT dimer, whereas ion series V is attributable to a complex incorporating one molecule of Inh-1, GSH*, and the GT dimer.

Additional analyses were performed after incubation of GT, GSH, and Inh-1 in the molar proportions 1:1:10. The electrospray spectrum (Figure 5B) indicated a marked reduction in the relative abundance of ion series I (2GT); ion series II ([2GT + GSH*]) and III ([2GT + 2GSH*] and/or [2GT + GSSG]) were not observed. Ion series VI ([2GT + 2Inh-1]) appeared at substantially increased abundance. This is consistent with (though does not prove) competition between GSH and Inh-1 for the same binding site. When the molar excess of Inh-1 was increased to 100-fold, the gas-phase species observed by electrospray mass spectrometry included ion series VII and VIII (Figure 5C) corresponding to incorporation of three and four molecules of inhibitor, respectively. These additional species may result from nonspecific binding of the inhibitor to GT under conditions of high molar excess of the ligand.

It is noteworthy that the mass increment associated with binding of Inh-1 to GT is exactly that expected for the ligand alone, paralleling the behavior of GSSG but

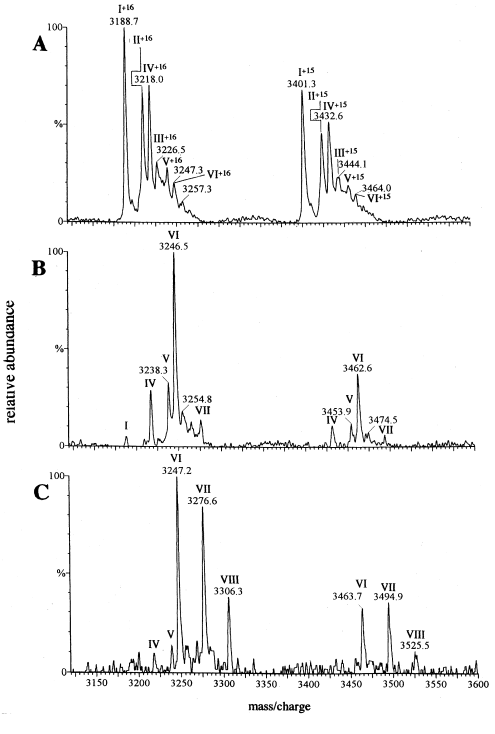

Figure 5. Partial electrospray mass spectra recorded in 10 mM aqueous ammonium acetate, pH 7.4. (**A**) Equimolar proportions of glutathione transferase (GT), glutathione (GSH), and S-(4-azido-2-nitrophenyl)-glutathione (Inh-1). (**B**) GT/GSH/Inh-1 in molar proportions, 1/1/10. (**C**) GT/GSH/Inh-1 in molar proportions, 1/1/100. The origins of the various ion series are discussed in the text and summarized in Figure 3.

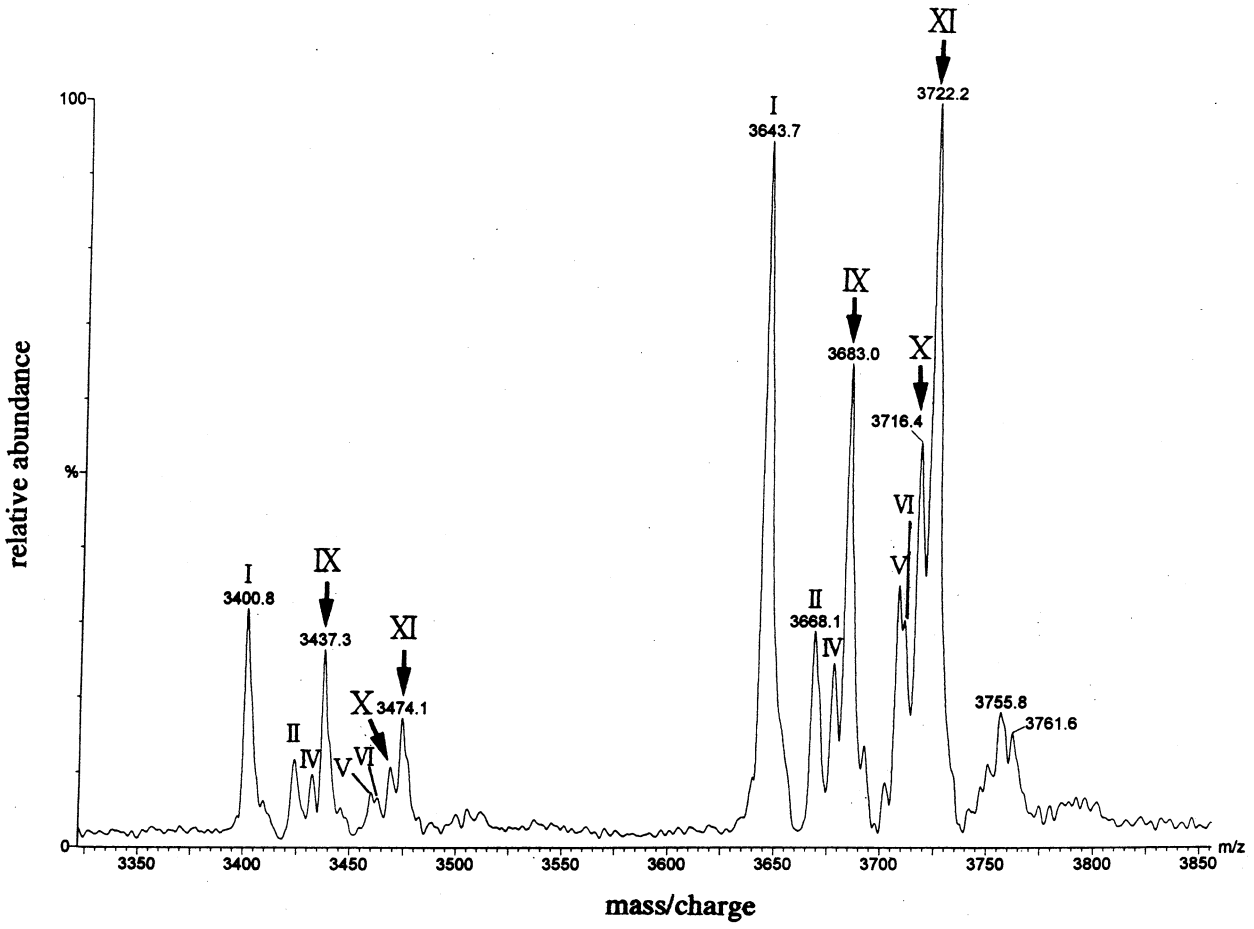

Figure 6. Partial electrospray mass spectrum, recorded in 10 mM aqueous ammonium acetate, pH 7.4, of an equimolar mixture of glutathione transferase, glutathione, and S-triphenylmethyl glutathione (Inh-2). The origins of the various ion series are discussed in the text and summarized in Figure 3.

differing from GSH where the evidence may suggest associated small molecules. This might indicate that the observations for the GT/GSH adduct are attributable to the presence in the ligand of a free thiol group. X-ray crystallographic studies of the GT/GSH complex have suggested that the hydrogen-bonding network in the glutathione/glutathione transferase complex involves one or two water molecules [24, 31, 32]. The associated water has been demonstrated to play an important role in the transferase activity [26, 33, 34]. On the basis of prior literature, we therefore favor the hypothesis that the discrepancy between the masses of the transferase/glutathione complexes and those expected for the association of one or two molecules of GSH to the GT dimer arises from concomitant and specific binding of two water molecules per GSH. The logical experiment to assess this hypothesis is to perform the incubations in $H_2\ ^{18}O$; the resolution and mass accuracy required to enable definitive conclusions from such an experiment are not, however, accessible on the instrument used for the present work. Recent reports from other laboratories [35, 36] have described the use of electrospray mass spectrometry to detect water molecules strongly bound to protein complexes.

We have also investigated the binding to glutathione transferase of a second inhibitor, S-triphenylmethyl glutathione (Inh-2), which is reported [28] to bind with a higher affinity than S-(4-azido-2-nitrophenyl)-glutathione. (Competitive inhibition constants K_i are 1.37×10^{-7} and 8.68×10^{-6} M, respectively [28].) Figure 6 shows the electrospray mass spectrometry analyses following incubation of the inhibitor with equimolar proportions of GSH and GT. Ion series IX, X, and XI yielded masses in good agreement with those expected for [2GT + Inh-2], [2GT + GSH* + Inh-2], and [2GT + 2Inh-2], respectively. The abundances of these ions substantially exceeded those of equivalent ions involving Inh-1 formed under similar conditions, consistent with the expected higher affinity of binding of Inh-2.

Tandem Mass Spectrometry of Noncovalent Adducts

Tandem mass spectrometry with collisionally activated decomposition (CAD) was used to dissociate the noncovalent complexes and identify the resulting fragments. Figure 7 shows the spectra of product ions

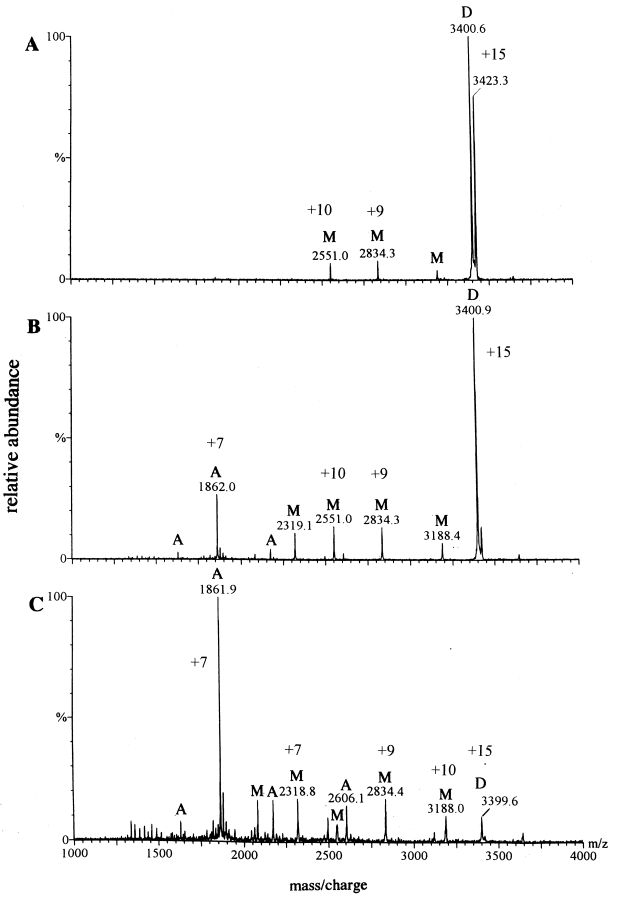

Figure 7. Product ion spectra recorded during MS/MS with collisional activation of the +15 charge-state ions of the noncovalent adduct involving dimeric glutathione transferase and glutathione. The collision offset potentials were (**A**) 55 eV, (**B**) 85 eV, and (**C**) 100 eV. Ions annotated as D, M, and A are attributable to the protein dimer and monomer (both without associated ligand), and a protein fragment, respectively.

derived from CAD (at three collision energies) of *m/z* 3423.6, corresponding to the 15+ ion of [2GT + GSH*]. The spectrum obtained at 55 eV (Figure 7A) showed a prominent peak (*m/z* 3400.9) corresponding to the 15+ ion of the dimer (50,998.4 Da) denuded of ligand. No signal was observed for the displaced ligand, consistent with its expulsion as a neutral. The data provide no evidence of elimination of water prior to loss of glutathione. This observation does not, however, enable firm conclusions concerning the nature of the bound species; if the complex does indeed incorporate bound water, the MS/MS data may simply suggest insufficient discrimination between the binding energy of the water and of the GSH, or that the binding affinity of GSH is substantially reduced by removal of the water. The facile loss of GSH*, with retention of the dimeric status of the protein, suggests less avid binding of the ligand(s) than between monomer units. The product ion spectrum obtained at a collision energy of 85 eV (Figure 7B) includes an envelope of multiply charged peaks (labeled M) corresponding to a mass of 25,500.3 Da, consistent with the protein monomer. In addition, a second envelope of multiply charged peaks (labeled A) was observed corresponding to a mass of 13,026.4 Da. When the collision energy was increased to 100 eV (Figure 7C), ions corresponding to the intact dimer were of very low abundance; the most abundant ions were associated with the fragment of 13,026.4 Da. In none of these MS/MS experiments was a product ion detected corresponding to the expelled ligand.

With regard to the origin of the fragment with an estimated mass of 13,026.4 Da, three portions of GT A1-1 may be considered, corresponding to residues 13–126 (13,026.3 Da), 86–199 (13,027.5 Da), and 109–221 (13,026.3 Da). The last of these represents the C-terminal fragment derived from cleavage adjacent to Pro-109. Low energy CAD of proteins has previously been shown to result in preferential cleavage N-terminal to proline residues [37, 38]; the tendency may be attributable to the enhanced gas-phase basicity of the proline nitrogen, favoring charge localization at this site. This part of the protein sequence is involved in the GSH binding site and Leu-108 is part of a hydrophobic pocket that has shown to interact with the benzyl group in the inhibitor S-benzyl glutathione [23].

Figure 8 shows the product ion spectra obtained following collisional activation of 15+ ions of the products of incubation of GT, GSH, and Inhibitor-1. The denuded dimer represented the principal product of CAD of both *m/z* 3454 (the 15+ ion of [2GT + GSH* + Inh-1], Figure 8A) and *m/z* 3463 (the 15+ ion of [2GT + 2Inh-1], Figure 8B). CAD of the [2GT + 2Inh-1] ion also yielded a product of *m/z* 3432, representing loss of a single Inh-1 moiety (Figure 8B). Interestingly, CAD of the [2GT + GSH* + Inh-1] ion (Figure 8A) gave a product of *m/z* 3423 [2GT + GSH*]; there was no evidence for the alternative initial loss of GSH*. This suggests a less avid association in the gas phase between the GT dimer and the inhibitor than between the dimer and GSH*; substantial further work is needed, however, before quantitative comparisons can be made, and extrapolation of this finding to the condensed phase may not be justified.

Conclusions

This study has exploited the capability of the electrospray process to transfer to the gas phase certain noncovalent complexes of biological molecules. Specifically, electrospray conditions designed to minimize the dissociation of noncovalent complexes have enabled the detection of dimeric glutathione transferase, consistent with the known prevalence of the dimer in nondenaturing solution [23, 24]. In addition, it has been demonstrated here that dimer/ligand complexes can be detected as gas-phase species. The GT dimer/GSH complex is of particular interest in that the observed mass is consistent with the concomitant association of two molecules of water; definitive identification of this complex, however, is dependent on additional analyses

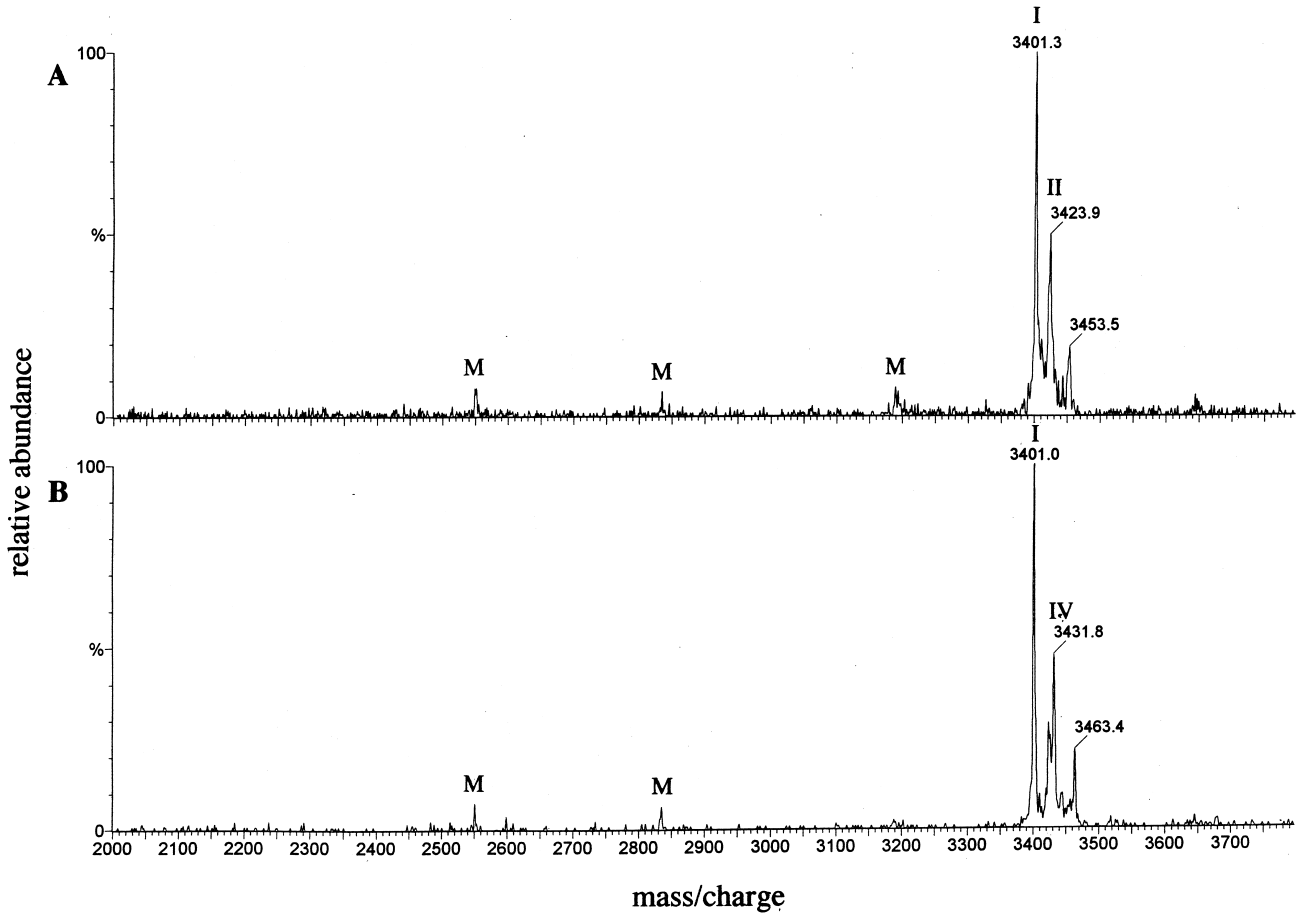

Figure 8. Product ion spectra recorded during MS/MS with collisional activation of the +15 charge-state ions of the noncovalent adducts involving (**A**) dimeric glutathione transferase, glutathione, and S-(4-azido-2-nitrophenyl) glutathione (Inh-1) and (**B**) the protein dimer and two molecules of the inhibitor. The assignments of the product ions are discussed in the text and summarized in Figure 3.

on an instrument of superior resolution. The masses of complexes between GT dimer and alternative ligands [oxidized glutathione (GSSG), and two GT inhibitors] were in agreement with structures that lacked associated water, suggesting that the incorporation of water (if such is the correct explanation) is specific to the presence in the ligand of a free thiol group.

The noncovalent adducts detected in the present study are summarized in Figure 3. The observations are consistent with the expected competitive binding to GT of GSH and the alternative ligands examined. In several cases the assignment of noncovalent adducts has been substantiated by performing collisional activation under mild conditions to yield the dimer without associated ligand. Such analyses involving a GT dimer with a single glutathione and a single inhibitor ligand suggested preferential retention of the glutathione. In summary, this study has substantiated the utility of careful application of electrospray mass spectrometry for the characterization of noncovalent complexes of biological significance. Current work seeks to extend these findings to the characterization of glutathione dimers incorporating substrates and products of the transferase activity.

Acknowledgments

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