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Intrinsic Neutral and Anionic Structures of Glutathione

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Gas-phase intrinsic structures of intact neutral and anionic glutathione (GSH) have been determined by means of a combination of negative ion photo-electron spectroscopy and quantum chemistry calculations. The inferred structures of the neutral parents of those peptide anions are canonical (non-zwitterionic). These intrinsic structures are compared to those already known in aqueous solution or determined by crystallography in binding sites of enzymes.

1. Introduction

Biomolecular structures are investigated under a wide variety of experimental conditions, ranging from the gas phase to living cells. Intrinsic properties of biomolecules deduced from gas-phase studies can be different from those resulting from the biological cellular environment.[1] Some of the experimental methods used for gas-phase structural studies impose restrictions upon the scrutinized species. For example, the powerful resonance-enhanced multiphoton ionization (REMPI) method coupled to infrared spectroscopy requires the presence of UV chromophores that are only present in three of the twenty natural amino acids.[2–4] Meanwhile, other methods are utilized that are more universal, although they still present difficulties. For example, microwave spectroscopy faces interpretation problems when the molecular size increases and is today mostly limited to systems such as amino acids[5] or dipeptides.[6] Infrared multiphoton dissociation (IRMPD) spectroscopy and negative-ion photo-electron spectroscopy (NIPES) are in principle applicable to any stable anionic species. With regard to NIPES, the crucial problem is getting intact (parent) anions of fragile neutral molecules. In particular, getting intact peptide anions is rather challenging and requires the use of carefully designed sources.

Glutathione is a widely distributed peptide in biological cells. It is synthesized in its reduced form (GSH) in cell cytosols from the precursor aminoacids, glutamate, cysteine and glycine. It is involved in many phenomena, such as differentiation, proliferation and apoptosis and plays a crucial role in protecting cells against oxidative damage. For that purpose, GSH performs detoxification of free radicals, metals and other electrophilic compounds through reaction of those chemicals with the –S–H group of cysteine and the subsequent export of the resulting GSH S-conjugates into the extra-cellular space.[7] In many enzymes' active sites and transcription factor binding sites, cysteine residues are present and exist in the thiolate anion form at physiological pH.[8] The mass-spectrometric simultaneous determination of GSH and its non-reduced form glutathione disulfide (GSSG) provides a clinical measurement of oxidative stress.[9] In the case of cancer, glutathione has both protective and pathogenic roles.[10]

Glutathione is an unusual tripeptide (γ-glutamyl–cysteinyl–glycine) with a peptide bond between the amine group of cysteine and the carboxylic group of the glutamic acid acid sidechain. It is a flexible molecule and its conformational energy landscape is strongly influenced by the environment. In particular, it can exist in its neutral form either as a canonical or a zwitterionic species. The latter form predominates in the condensed phase. The lowest-energy zwitterionic structures of GSH have been calculated at the semi-empirical AM1 and PM3 levels[11,12] and compared to the experimental crystal structure.[13,14] The GSH infrared spectrum has been recorded in solution[15] but the structure of isolated glutathione has not yet been experimentally determined.

2. Results and Discussion

Direct access to neutral gas-phase GSH structures is not offered by conventional IR or UV methods. Indirect information on GSH intrinsic structures can be obtained by a combination of NIPES experiments on cold anions and computations. The possible observation of intact GSH^- anions is not obvious, since experimental and computational studies on cysteine and its de-protonated form show that, among aminoacid groups, the thiol gas-phase acidity is even greater than that of carboxylic groups.[16] Nevertheless, herein our experimentally recorded mass spectrum provided as Supporting Information displays a large peak at the intact glutathione mass and another slight-

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ly less intense peak corresponding to the loss of S–H. The photoelectron spectrum of the intact glutathione anion displays an onset at ~2.5 eV and a peak maximum at 3.5 eV. The former can only provide an approximate value of the adiabatic electron affinity (EA_{ad}) [assuming minimal hot bands] if the anion and the neutral structures are similar. Otherwise, the Franck–Condon overlap between the anion and its neutral parent is too poor and one then only obtains an upper value of EA_{ad}. The EA_{ad} is the energy difference between the ground vibronic state of the neutral and that of its corresponding anion. The peak maximum is a measurement of the vertical detachment energy (VDE) value of the glutathione anion (the VDE is the energy difference between the anion and its neutral counterpart at the equilibrium geometry of the anion). Comparison between the experimentally determined vertical detachment energy (VDE, Figure 1) and predicted VDEs of the different possible anionic conformers provides information related to both neutral and ionic structures.

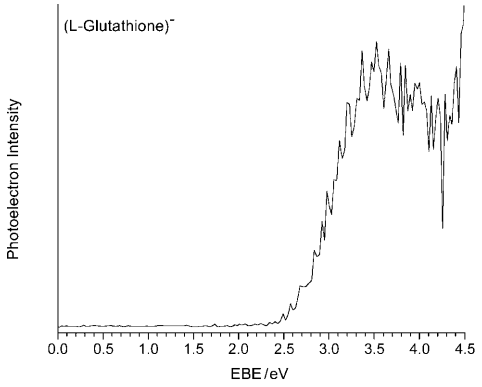

Figure 1. Negative-ion photo-electron spectrum of intact glutathione anions.

Herein, intrinsic canonical and zwitterionic forms of glutathione are investigated in the case of neutral parents and their corresponding daughter anions. Their most stable gas-phase global minima are further respectively labelled N1, N2, and ZW1, ZW2. The neutral structures are displayed in Figure 2 and the corresponding hydrogen bond patterns are provided in Figure 3. As already shown in ref. [12] in the case of crystal zwitterionic forms, we also observe that intrinsic folded structures are considerably more stable than unfolded ones, even in the case of canonical forms.

The respective relative energies, adiabatic electron affinities of the neutrals and vertical detachment energies of the daughter anion conformers calculated at the B3LYP/6-31+G(d) level are given in Table 1.

At this level of theory, the two neutral lowest-energy canonical conformers N1 and N2 are separated by 17 meV (1.64 kJ mol^{-1}), while the zwitterionic conformers lie considerably higher. From these results, we can predict that only canonical N1 and N2 conformers, and not the zwitterionic conformers, should be experimentally observed in the gas phase. In the corresponding anions, excess electrons are located on the glycil group for N1 and the γ-glutamyl group for N2 (Figure 4).

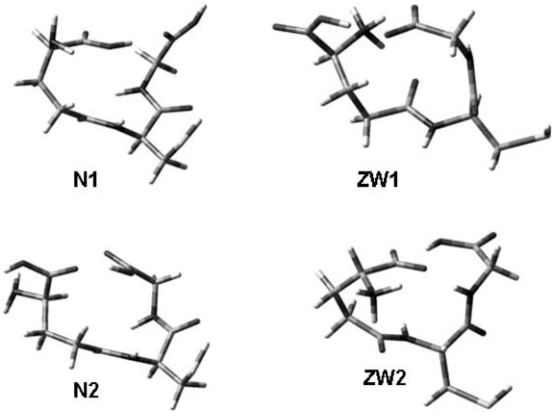

Figure 2. Lowest-energy structures of canonical (N1 and N2) and zwitterionic (ZW1 and ZW2) gas-phase neutral glutathione calculated at the B3LYP/6-31+G(d) level.

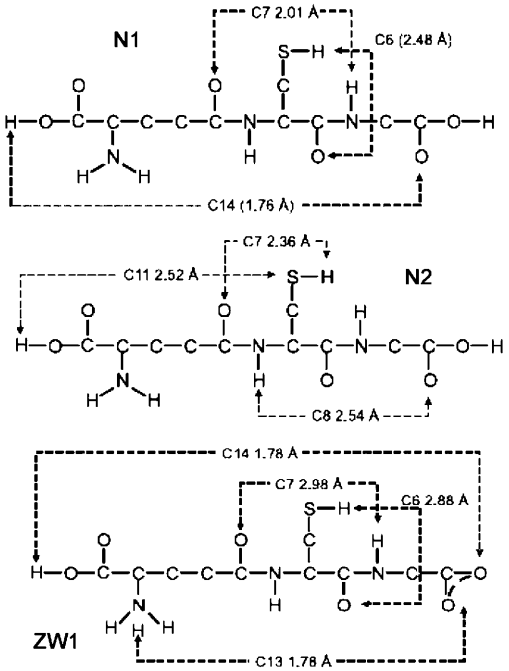

Figure 3. Hydrogen-bond patterns in the lowest-energy gas-phase conformers of neutral glutathione.

Table 1. Respective relative energies (RE) and adiabatic electron affinities (EA) of lowest-energy conformers of neutral gas-phase glutathione predicted at the B3LYP/6-31+G(d) level. The vertical detachment energies (VDE) of the corresponding anionic conformers are to be compared to the experimental VDE value equal to 3.5 eV.

Conformer	N1	N2	ZW1	ZW2
RE [meV]	0	17	169	216
EA_{ad} [eV]	0.345	0.696	0.870	0.467
VDE ([eV]	3.51	3.495	2.89	2.89

At the B3LYP/6-31+G(d) level, which can be considered as reliable as far as vertical detachment energies are concerned, the N1 and N2 anionic conformers possess nearly identical predicted VDEs that are in very fair agreement with the experimental

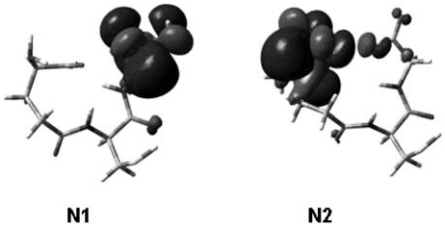

Figure 4. Excess electron orbitals in the gas-phase N1 and N2 gluthatione anionic conformers.

value. Energetically, anionic conformer N2 is predicted as much more stable than anionic conformer N1, due to the larger electron affinity of the neutral conformer N2.

It has been observed in previous NIPES experiments that the anions produced in a very cold supersonic expansion usually correspond to the most stable species. The B3LYP/6-31+G(d) level of theory generally overestimates adiabatic electron affinities. A much more reliable calculation conducted at the RI-MP2/TZVP level, including zero-point energy (ZPE) corrections (Table 2), provides an energy separation between N1 and N2

Table 2. Respective relative energies (RE), including ZPE correction, and electron affinities of the lowest-energy neutral canonical and zwitterionic conformers of gas-phase glutathione predicted at the RI-MP2/TZVP level. The vertical detachment energies (VDE) of the corresponding anionic conformers can be compared to the experimental VDE value equal to 3.5 eV.

Conformer	N1	N2	ZW1
RE [meV]	0	49	358
EA_{ad} [eV]	0.089	0.328	0.415
VDE ([eV]	3.306	3.315	2.67

equal to 49 meV (4.7 kJ mol^{-1}). The N2 anionic conformer remains the most stable (−279 meV with respect to neutral N1 as compared to −89 meV for the N1 anion). We can conclude that the experimentally determined VDE corresponds to anions in the N2 structure.

The most stable gas-phase intrinsic canonical structures observed here are folded due to strong intramolecular interactions between the terminal groups and between amide groups. The internuclear distances d_{C-C} between the carbon atoms of the two carboxylic groups lie between 4.07 and 4.42 Å. In addition, an interaction between the thiol hydrogen and the cysteine amide is present in gas phase. The S–H···O distance is comprised in between 2.45–2.48 Å in neutral forms and 2.56–2.88 Å in zwitterionic forms. The role of the thiol group is fundamental for the bioactivity of GSH. NMR studies suggest that the S–H···O interaction is still present in solution.[17] A continuous model SCRF calculation at the B3LYP/6-31+G(d) level in water also shows us that, as expected, the zwitterionic forms ZW1 and ZW2 become considerably more stable (respectively 259 and 343 meV with respect to N1) than the canonical forms. We have also conducted molecular dynamics simulations with explicit water molecules at the Amber level. Those simulations predict the persistence of a thiol–cysteine amide interaction in the lowest-energy conformers. This is indeed confirmed (Supporting Information) by the simulation of the experimental infrared spectrum of glutathione.[12]

3. Conclusions

It seems interesting to compare glutathione structures deduced from gas-phase or aqueous-phase experiments and simulations to structures determined in biologically relevant situations. When GSH is bound to enzymes such as glutathione S-transferase (pdb structures 1m0u and 1vf1), glutathione synthetase (1gsa) or prostaglandine D-transferase (1pd2), the internuclear distances d_{c-c} are comprised in between 8.51 and 10.6 Å. Glutathione then adopts unfolded S-shaped conformations with more intramolecular interactions but rather with the establishment of several non-covalent interactions with amino acid side chains (Supporting information).

These findings show that the intramolecular interactions responsible for the intrinsic structures of isolated GSH determined herein vanish to the benefit of intermolecular forces responsible for the binding of GSH to its target proteins.

Experimental Section

Low-power infrared laser pulses (1.17 eV/photon) from a Nd:YAG laser were used to desorb neutral biomolecules from a slowly moving graphite rod which was thinly coated with sample. Almost simultaneously, electrons were generated by visible laser pulses (another Nd:YAG laser operated at 532 nm, 2.33 eV/photon) striking a rotating yttrium oxide disk. Since yttrium's work function of ~2 eV is slightly below the photon energy of the visible laser, low-energy electrons were produced, and this process is critical to the formation of intact biomolecular ions. At the same time a pulsed gas valve provided a collisionally cooled jet of helium to carry away excess energy and stabilize the resulting parent anions. The photoelectron spectrum of the intact glutathione anion was recorded by crossing a mass-selected beam of glutathione parent anions with a fixed-frequency photon beam (third Nd:YAG laser operated at 266 nm, 4.66 eV/photon). The resultant photo-detached electrons were energy-analyzed using a magnetic bottle energy analyzer with a resolution of 35 meV at EKE = 1 eV. Photo-detachment of electrons is governed by the energy-conserving relationship, $h\nu = EBE + EKE$, where EBE is the electron binding (transition) energy, EKE is the measured electron kinetic energy, and $h\nu$ is the photon energy.

The gas-phase glutathione conformational space was systematically explored with both the MMFF force-field and the AM1 method for the canonical and zwitterionic forms of the neutral parent and its anion. In each case, the 25 lowest-energy conformers were further optimized at the B3LYP/6-31+G(d) level. The investigation of the GSH structure in solution was conducted by means of the replica-exchange method.[18] Molecular dynamics simulations starting from the ZW1 gas-phase structure surrounded by 155 water molecules were conducted with the Amber force field at 30 temperatures in between 100 and 1000 K during 1 ns.

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