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Evidence for Gliotoxin–Glutathione Conjugate Adducts

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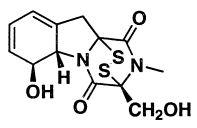
Abstract—The equilibrium constant for the gliotoxin/glutathione pair was found to be $1200\pm100\ M^{-1}$ at pH 7.0 at 25 °C. Under conditions where the reaction was quenched rapidly with the addition of acid, gliotoxin–glutathione conjugate adducts were detected. © 2001 Elsevier Science Ltd. All rights reserved.

Gliotoxin (**1**) is a biologically active cyclic disulfide that displays a wide range of biological effects including antiviral, antibacterial, and immunosuppressive properties.[1] Implicated in the mechanism of action of gliotoxin is the interaction of the disulfide linkage with sulfur nucleophiles in a thiol-disulfide exchange mechanism.[2] In view of the abundance of intracellular glutathione (GSH), it is likely that mixed disulfides of glutathione with gliotoxin are potential intermediates in cell systems treated with gliotoxin. Despite this, no evidence for the formation of glutathione–gliotoxin adducts has been reported. In this communication, we report our attempts to isolate and characterize these adducts using a combination of HPLC, NMR, and MS techniques.



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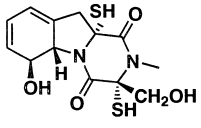

2

Our preliminary studies show that the rate at which equilibrium between gliotoxin and glutathione is established is dependent on a number of factors including the concentrations of glutathione and pH of the media. For example, at high concentrations of glutathione, the equilibrium is rapidly established (typically within 30 min). Similarly equilibration is more rapid at high pH values of the media. In these cases, there was no evidence for the formation of a gliotoxin–glutathione adduct by HPLC. For a typical measurement of equilibrium constants, a mixture of gliotoxin and a redox buffer (glutathione GSH/oxidized glutathione GSSG) was equilibrated under strictly degassed conditions. Samples were quenched with acid (to pH 2) prior to analysis by reverse-phase HPLC analysis. In the typical solvent system used to monitor the progress of reaction (see Experimental), the retention times of gliotoxin (**1**) (17 min) and reduced gliotoxin (**2**) (15.8 min) were sufficiently different to enable accurate integration of the areas. To ensure that the position of equilibrium had been reached, the reaction mixture was monitored after both 1 and 5 h and in all cases, the values obtained were constant. In addition, perturbation of equilibrium by the addition of the corresponding disulfide, GSSG, gave similar K_{eq} values. The equilibrium constant at pH 7.0 was measured as $1200\pm100\ M^{-1}$ for the gliotoxin/glutathione pair, giving rise to an E_o value of -0.17 V for the reduction of gliotoxin to reduced gliotoxin. As a comparison, similar equilibrium studies were carried out using gliotoxin and cysteine and the measured K_{eq} value was $400\pm4\ M^{-1}$. From these two K_{eq} values, the K_{eq} value for glutathione with cystine is calculated to be 3.0, which is in good agreement with the literature values.[3]

To optimize the observation of intermediate species, samples of gliotoxin and glutathione were incubated at pH 7.0 and the aliquots of the reaction were quenched at various time intervals with trifluoroacetic acid (TFA)/water. Analysis of the samples by HPLC showed that at fast quenching times (less than 30 s), new peaks at 10.8, 11.8, and 13.6 min were observed. These peaks were greatly diminished at higher quenching times, and provide evidence for the transient lifetime of these species.

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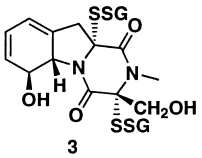
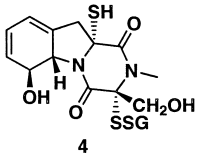
In order to determine the identities of the intermediate species, rapidly quenched samples were analyzed by HPLC and fractions corresponding to the new peaks were collected, lyophilized and re-analyzed. The peak at 10.8 min corresponded to a stable compound with a molecular formula of 938 Da as determined by ES-MS analysis.[4] This is consistent with the formulation of a double mixed disulfide (DMD) species with the structure **3**.



3

NMR analysis in D_2O in the presence of 0.1% deuterated TFA is complicated by a number of overlapping signals but resonances attributed to the gliotoxin and glutathione portion of the structure are clearly evident from 1-D and 2-D NMR analysis.

In contrast, the peaks corresponding to the retention times 11.8 and 13.6 min are much less stable. In the HPLC analysis of the collected, lyophilized fraction at 11.8 min, interconversion to the species at 13.6 min as well as to gliotoxin (17 min) is observed. This provides indirect evidence that the species at 11.8 and 13.6 min are mono mixed disulfides **4** and **5**. The mono mixed disulfide corresponding to the retention time 13.6 min is evidently the more stable of the two mono mixed disulfides. The fraction corresponding to this was collected, lyophilized and when analyzed by ES-MS, a molecular ion at 665 was observed.[5] This is consistent with the formulation of $C_{23}H_{31}N_5O_{12}S_3$, that is, the molecular ion of the mono mixed disulfide + O_2,[6] presumably arising from aerial oxidation in the isolation process.



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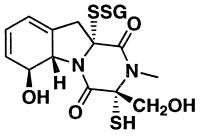

5

These studies show that the remaining thiol group in each of the mono mixed disulfides is clearly very reactive and is prone to intramolecular S_N2 reactions or to aerial oxidation. Attempts were made to derivatize the remaining thiol group of **4** and **5** via oxidation with hydrogen peroxide and by alkylation with iodoacetic acid. The former method gave a complex mixture of compounds whereas alkylation at pH 7.0 was slow and does not compete effectively with intramolecular reactions.[7]

The studies above show that glutathione interacts with the cyclic disulfide moiety of gliotoxin via a thiol disulfide exchange mechanism (Fig. 1). Due to the unsymmetrical nature of the disulfide, two mono mixed disulfides are formed. The two mono mixed disulfides can in principle undergo intramolecular thiol disulfide exchange as well as intramolecular cyclization to gliotoxin. From our current studies, it appears that one mono mixed disulfide species (MD 2, 11.8 min fraction) is more reactive towards inter- and intramolecular thiol-disulfide exchange reactions than the other (MD 1, 13.6 min fraction). The reason for this is not clear and may be related to increased steric congestion of the disulfide linkage in one mono mixed disulfide (presumably **5**) as compared to the other. Thus intramolecular thiol disulfide exchange reaction to form **4** and intramolecular cyclization to form gliotoxin will relieve some of the steric strain involved. It has also been established that the rate constant of thiol-disulfide exchange reactions correlate with the fraction of reactive thiolate ions present in solution. This in turn depends on the pK_a of the reacting thiol as well as the pH of the solution.[8] Thus it is also conceivable that the propensity of MD2 to convert to MD1 may be related to the relative acidities of the thiol groups.

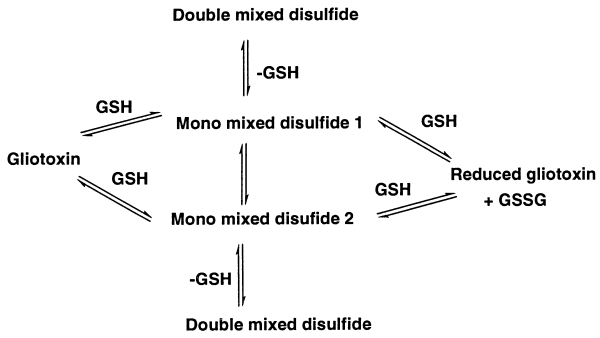

Figure 1. Possible routes for the interaction of gliotoxin with glutathione.

The double mixed disulfide species isolated can be envisaged as arising from the mono mixed disulfides via aerial oxidation with glutathione or via interaction of the thiol group of mono mixed disulfides with oxidized glutathione. As the double mixed disulfide species is not observed when quenching of the gliotoxin/glutathione reaction mixture is slow, this species must be reduced to the dithiol in the presence of excess glutathione. The equilibrium constant of the gliotoxin/glutathione pair indicates that reduction of the disulfide linkage of gliotoxin is strongly favoured and this implies that at physiological pH, the toxin is capable of depleting cellular glutathione. Related to this, reduced gliotoxin has been observed in treated cells.[9]

Gliotoxin, glutathione (GSH), and oxidized glutathione (GSSG) were purchased from Sigma. HPLC analyses were carried out using a Beckmann instrument consisting of a 126 Dual Pump Solvent Delivery System fitted with a UV detector. A wavelength of 260 nM was used unless otherwise specified. A 0.46 cm × 15 cm Beckman Ultrasphere ODS 5 micron reverse-phase column was used. Studies were carried out using 5% acetonitrile for the first 5 min, followed by a 5–40% gradient of acetonitrile in water over 15 min in the presence of 0.1% TFA and with argon bubbling through the solvent reservoirs at all times. For the preparation of the mixed disulfides, samples were isolated using an auto-fraction collector and were kept on ice or dry-ice before analysis. Electrospray MS was recorded on a Fisons VG Quattro II spectrometer equipped with a Hewlett Packard 1090 LCS.

Standards of gliotoxin and reduced gliotoxin as well as reduced and oxidized glutathione were sampled on the HPLC in order to determine their respective retention times. Concentrations of the glutathione solution were confirmed by the addition of Ellman's Reagent followed by UV–vis measurements. Standard curves were obtained independently for gliotoxin and reduced gliotoxin on the HPLC

A typical procedure for the determination of equilibrium constants

To 2.8 mL of phosphate buffer at pH 7 in a Reacti-vial were added 100 μL of a 30 mM solution of GSH and 100 μL of a 30 mM solution of GSSG. This GSH/GSSG redox buffer was mixed by gentle inversion. To initiate the reaction, 100 μL of a 3 mM solution of gliotoxin was added and the resulting solution was mixed by inversion. At 1 and 5 h later, a 300 μL aliquot was removed from the reaction mixture under nitrogen and placed in a HPLC vial containing 1 μL trifluoroacetic acid (TFA). The vial was then sealed under a nitrogen atmosphere and the aliquot was immediately analyzed by HPLC.

Gliotoxin and reduced gliotoxin concentrations were determined by comparison of peak areas to standard curves. Concentrations of GSH and GSSG were treated as constant since both compounds were in large excess, and the systematic errors were within 5%.

Similar procedures were used with different concentrations of GSH/GSSG, as well as at different pH's.

Typical procedure for the preparation of mixed disulfides

Five-hundred microlitres of a 12.9 mM gliotoxin/acetonitrile solution were added to 5 mL of degassed phosphate buffer (0.05 M, pH 7.0) solution under argon at 30 °C. The solution was degassed by bubbling the reaction mixture with Argon for 15 min. This was then followed by the addition of 500 μL of a 193.5 mM solution of glutathione in degassed buffer. The reaction was quenched almost immediately (10–30 s) by the addition of 600 μL of a 10% TFA/H_2O solution. The quenched mixture was then lyophilized, then redissolved in 300 μL of 1.67% TFA/H_2O solution, followed by HPLC separation.

Acknowledgements

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References and Notes

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4. $C_{33}H_{46}N_8O_{16}S_4$ (ES^+, 50 V, *m*/*z*) 940.9 (10%), 939.8 (15%), 938.8 (35%), 633.9 (7%), 470 (70%), 338.8 (67%), 308 (81%), 262.9 (56%), 86 (100%)
5. $C_{23}H_{31}N_5O_{12}S_3$ (ES^+, 50 V, *m*/*z*) 668 (22%), 666.9 (32%), 665.9 (100%), 633.9 (26%), 585.9 (15%), 584.9 (25%), 583.9 (90%).
6. The oxidation of a thiol group to the sulfinic acid is not common though this has been observed previously in alkaline media: Berger, H. *Rec. Trav. Chim.* **1963**, *82*, 773.
7. For successful *S*-alkylation, the thiolate ion must first be generated. Our model studies with thiol piperazinediones in organic solvents have shown that *S*-alkylation is difficult in the absence of base.
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