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GLIOTOXIN INACTIVATES ALCOHOL DEHYDROGENASE BY EITHER COVALENT MODIFICATION OR FREE RADICAL DAMAGE MEDIATED BY REDOX CYCLING

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Abstract—The fungal metabolite gliotoxin shows selective toxicity to cells of the immune system and has been implicated in the aetiology of invasive aspergillosis. The related toxin sporidesmin is the causative agent of facial eczema in sheep. The toxicity of these compounds has been related to their ability to redox cycle intracellularly and thus produce damaging free radicals. These toxins are also potentially capable of forming mixed disulphides with thiol groups on proteins by virtue of their bridged disulphide structure. We show here that gliotoxin can inactivate horse liver alcohol dehydrogenase by either oxidative damage or covalent modification of thiol groups on the enzyme. Either Cys-281 or Cys-282 is selectively modified. Neither of these residues are at the active site. Covalent modification occurs in the absence of reducing agents such as dithiothreitol. In the presence of dithiothreitol no protection is observed and the rate of inactivation is enhanced although as expected no covalent modification occurs. Gliotoxin can therefore inhibit alcohol dehydrogenase by either pathway and this will depend on the availability of reducing agents such as glutathione and/or how readily the reactive oxygen species generated are removed.

Key words: free radical damage; covalent modification; gliotoxin; alcohol dehydrogenase

Gliotoxin is a member of the ETP† family of fungal metabolites [1, 2]. It has been shown to possess antimicrobial and antifungal properties although its systemic toxicity make it unsuitable for clinical use [3, 4]. It also displays *in vitro* and *in vivo* immunosuppressive properties [5–7], the former allowing successful bone marrow transplantation in rodents following pretreatment of donor tissue with the toxin to selectively remove mature aggressive lymphocytes [8]. For these reasons we are interested in the mechanism(s) of toxicity of gliotoxin and related toxins. We have shown that gliotoxin can induce apoptosis or programmed cell death in cells of the immune system [9–11] and this property may give rise to its apparent selectivity for cells of the haematopoietic system. We have yet to elucidate the precise mechanism of induction of apoptosis by gliotoxin. It is clear that the bridged disulphide ring of ETP compounds is required for biological activity [2, 7]. Gliotoxin has been shown to inhibit RNA polymerase and farnesyl transferase [12, 13] although details of the mechanisms involved are sparse. Either the oxidized (disulphide) form or the reduced (dithiol) form have been shown to be the active moiety depending on the enzyme studied. We chose to examine the interaction of gliotoxin with equine ADH (EC 1.1.1.1; ADH). This enzyme is a zinc requiring enzyme with a total of 14 cysteine residues per subunit. Cys-46, Cys-174 and His-67 act as ligands for the zinc atom. We assumed that interaction of the gliotoxin with protein thiol groups via mixed disulphide formation would be a likely reaction and we were interested in investigating this possibility with ADH. Our results show that gliotoxin did inhibit activity of ADH by interaction with a single thiol group but that addition of dithiothreitol or glutathione, rather than provide expected protection, exacerbated inactivation due to initiation of redox cycling and hydrogen peroxide formation via the reduced form of the toxin.

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† Abbreviations: ETP, epipolythiodioxopiperazine; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography, TFA, trifluoracetic acid; DTT, dithiothreitol; BSA, bovine serum albumin.

MATERIALS AND METHODS

Chemicals. Gliotoxin was prepared as previously described [14] or purchased from Sigma. Radiolabelled gliotoxin was prepared using [^{35}S]sulphate in the growth media of the producing fungus *Penicillium terlikowskii* and purified as described [15]. ADH, trypsin, catalase and NADPH were purchased from Sigma and ADH activity was measured as described [16]. All other reagents were the purest available.

Electrophoresis. PAGE was performed as described [17] and autoradiography was performed using Amersham Hyperfilm MP.

Purification of labelled peptide. ADH (5 mg) was incubated with radiolabelled toxin at 100 μM in 10 mL HEPES buffer at pH 8 for 5 hr. After this time the inactive protein formed a precipitate. The precipitate was washed and suspended in 5 mL of 50 mM ammonium bicarbonate buffer pH 8 with

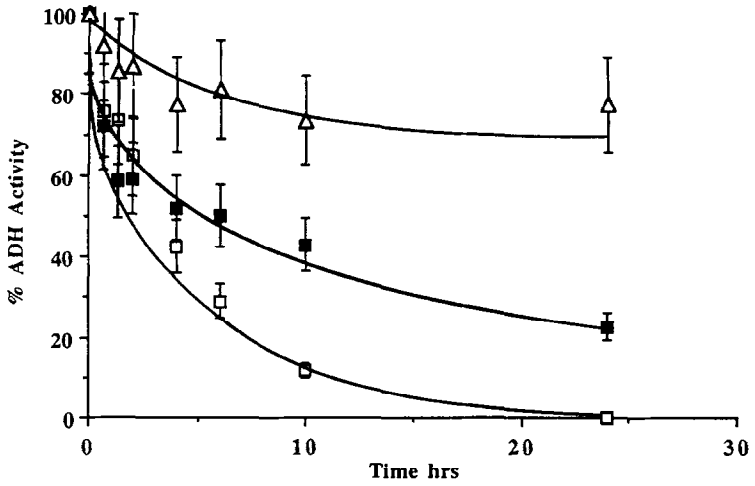


Fig. 1. Time dependent inactivation of ADH by 100 μM gliotoxin at (■) pH 6.5, (□) pH 9.0, and (△) untreated at 20°.

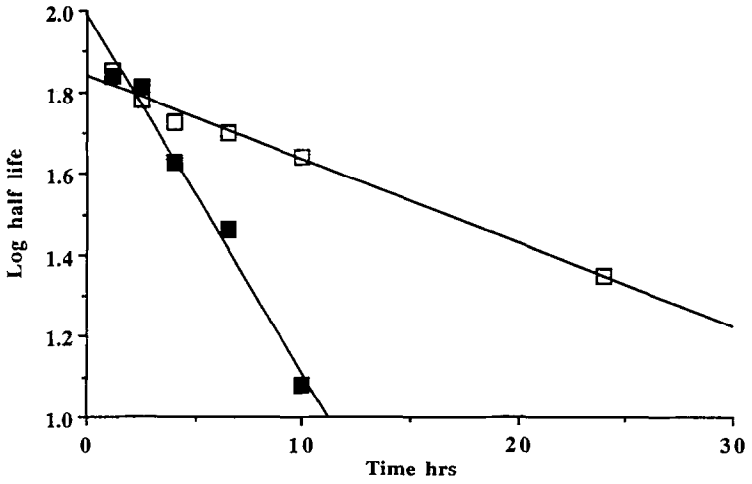


Fig. 2. Pseudo-first-order kinetics of inactivation of ADH at (■) pH 9.0, and (□) pH 6.5.

1 mg of trypsin and incubated for 3 hr at 37°. A further 1 mg of trypsin was added and further incubated for 10 hr. After this time the homogeneous solution was lyophilized, the residue dissolved in HEPES buffer (0.5–1 mL) pH 8 and filtered through a centricon 3000 filter. The filtrate was applied to a 15 × 2 cm CM Sephadex C-25 ion exchange column equilibrated with ammonium bicarbonate buffer and the column washed with 300 mL of this buffer. The eluent was changed to ammonium bicarbonate/0.25 M NaCl and 1.5 mL fractions collected at a flow rate of 20 mL/hr. Radioactive fractions were pooled and concentrated. The material from the ion exchange column (1 mL) was then applied to a 2 × 70 cm Biorad Bio-Gel P-2 column also equilibrated with ammonium bicarbonate buffer, eluted at a flow rate of 20 mL/hr and 1.5 mL fractions collected. Fractions with radioactivity were pooled, concentrated and finally purified using HPLC.

HPLC analysis and sequencing. Beckman system gold equipment was used with the on line model 171 radioactive detector and 166 UV detector tuned to 210 nm. A 0.46 cm × 15 cm Beckman Ultrasphere ODS 5 μm reverse-phase column was used for analytical and preparative separations. The column was equilibrated with 5% acetonitrile in water containing 0.1% TFA. Peptides were eluted using a 5–60% acetonitrile gradient with 0.1% TFA over 60 min at a flow rate of 1 mL/min. For preparative work, 200 μL of the concentrated labelled eluent from the Bio-Gel column was applied to the HPLC column and 100 μL fractions collected using the same elution profile. Pooled radioactive fractions were re-analysed to check purity. Only a single peak containing all the radioactive label was obtained at this stage. The peptide was also homogeneous as indicated by a single peak in the UV channel following re-chromatography. This peptide was

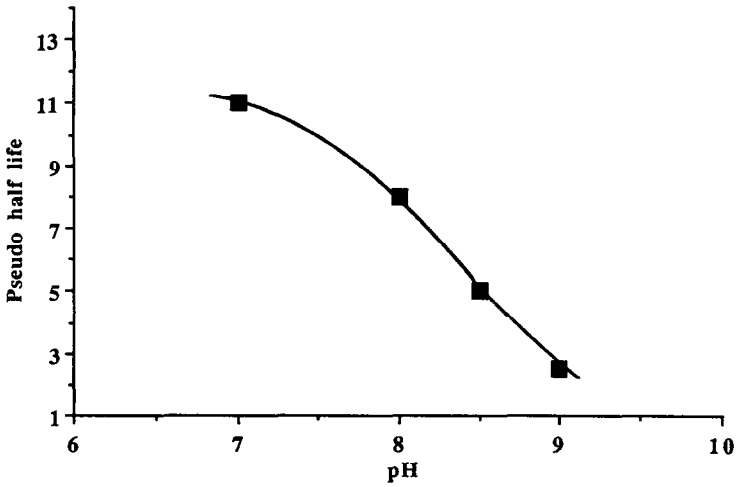


Fig. 3. pH dependence of inactivation of ADH by gliotoxin at 20°.

sequenced using an Applied Biosystems 477A protein sequencer with an initial yield of 88.9 pmol and a mean yield of 52.6 pmol over all sequencing cycles.

RESULTS

Time-dependent inactivation of ADH

Gliotoxin shows biological effects at 0.1–3 μM but we have shown that the toxin is rapidly accumulated by treated cells [15] which must give rise to at least a 100-fold increase in average intracellular concentration. Up to 80% of the toxin is found to be cell associated after 10–30 min [15]. For this reason we chose to study the interaction of gliotoxin with ADH using 100 μM toxin. When gliotoxin was incubated with ADH alone except for cofactors we observed a time and pH dependent irreversible inactivation which showed pseudo-first-order kinetics of inactivation (Figs 1 and 2). A plot of pseudo half life of inactivation against pH indicates interaction with a residue with a pK_a about 8.0 (Fig. 3).

Covalent interaction of gliotoxin with ADH and lack of protection by dithiothreitol or glutathione

When ^{35}S-radiolabelled gliotoxin was incubated with ADH, radiolabelled protein corresponding to ADH could be detected following dialysis and electrophoresis. Addition of either dithiothreitol or glutathione resulted in significant diminution of this labelled band (Fig. 4). Estimation of the amount of radiolabelled toxin bound per subunit (mol. wt. 40,000) of protein gave a ratio of bound toxin : ADH 0.90 ± 0.04 (N = 3). HPLC analysis of the tryptic digest of labelled ADH revealed a major labelled residue at 18.6 min. A second peak was seen at 22.1 min (Fig. 5). The counts seen at 1.6 min are in the void volume and were identified as labelled inorganic sulphate which was always formed following digestion with trypsin [15]. Surprisingly, when we examined activity in the presence of DTT or glutathione, inactivation was more rapid although

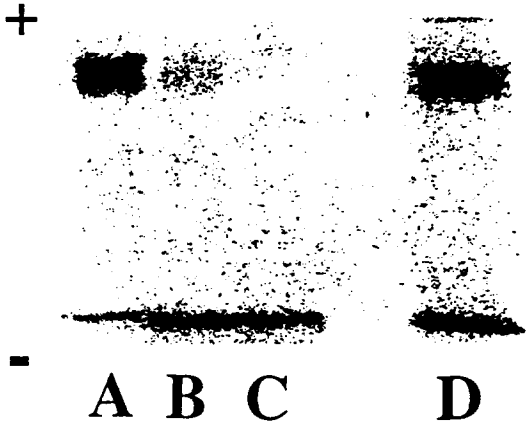


Fig. 4. Autoradiography of polyacrylamide gel of ADH treatment with ^{35}S-labelled gliotoxin. Lane A: enzyme treated with toxin for 5 hr; B and C: as for A but in the presence of 1 mM glutathione or 1 mM dithiothreitol, respectively; D: as for A in the presence of 1 mM dithiothreitol and 5000 units of catalase.

clearly no covalent interaction was occurring (Figs 4 and 6). When 5000 units of catalase was added in the presence of DTT, the rate of inactivation was restored to that in the absence of the reductant (Fig. 6), and furthermore covalently modified protein could now be detected (Fig. 4). Other proteins such as BSA at the same concentrations had no effect (data not shown) showing that inhibition was specifically due to removal of hydrogen peroxide. Glutathione at 1 mM had qualitatively the same effect as 1 mM DTT (Table 1).

Identity of the peptide residue modified

Analysis of the purified labelled peptide which eluted at 18.6 min gave SerCysCysGlnGluAlaTyr as the sequence. This corresponds to residues 280–286

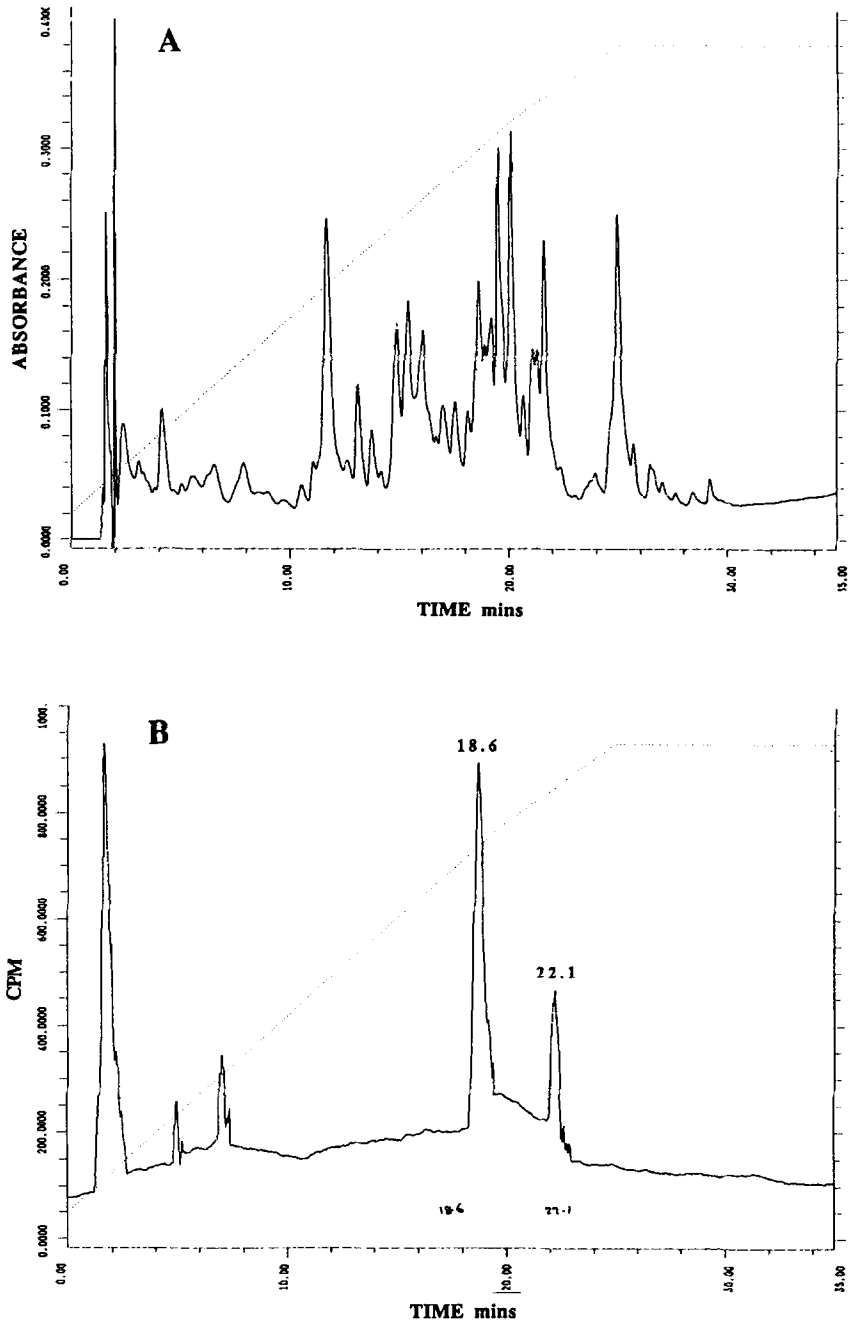


Fig. 5. HPLC analysis of tryptic peptides from ADH treated with ^{35}S-labelled gliotoxin for 5 hr. A shows the UV channel at 210 nm and B is the corresponding radiolabelled peptides. The major radiolabelled peptide elutes at 18.6 min.

of equine ADH. Mixed disulphide formation must be occurring at either Cys-281 or Cys-282. The formation of this fragment is consistent with contaminating chymotrypsin cleavage between Leu279–Ser280 and Tyr286–Gly287 not tryptic cleavage. This is fortunate since purely tryptic digestion would have yielded a larger fragment between Leu-272 and Arg-312. It is possible that the peak at 22.1 min is this tryptic peptide which is subsequently further cleaved. Under the conditions used for sequencing, we were unable to determine unequivocally which of the two adjacent cysteines were modified.

DISCUSSION

The data in Figs 1 and 2 are consistent with reversible interaction of the toxin with ADH followed

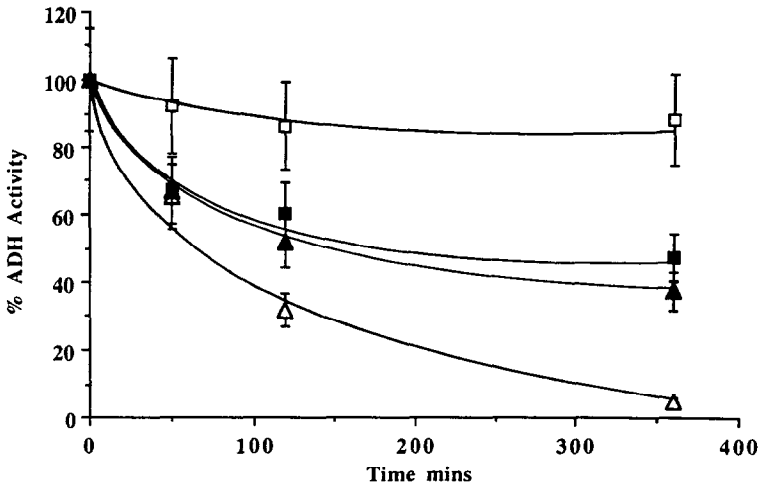


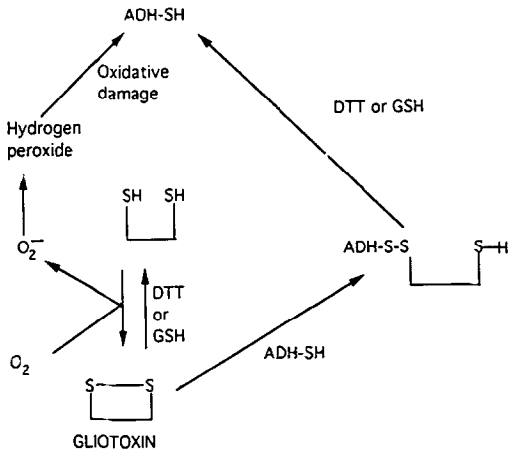
Fig. 6. Time-dependent inactivation of ADH by 100 μM gliotoxin at pH 8: (□) untreated; (■) toxin alone; (△) toxin plus 1 mM DTT; (▲) toxin plus 1 mM DTT plus 5000 units catalase.

Table 1. Effect of 1 mM glutathione on the activity of ADH in the presence of 100 μM gliotoxin after 6 hr

Treatment	% Activity
Control	91.0 ± 7
Toxin	42.5 ± 1.5
Toxin + GSH	0
Toxin + GSH + catalase	42.7 ± 3
GSH	94.1 ± 2
DTT	92.3 ± 3

by irreversible covalent interaction [18]. This is supported by PAGE analysis of inactivated protein showing bound radiolabelled toxin after denaturation. Gliotoxin appears to be binding to a thiol group of ADH based on its removal by DTT and the pH profile of inactivation. Figure 5 and the stoichiometry indicates that predominantly only a single thiol group is modified. ADH is known to possess a total of 14 free cysteine residues with Cys-46 and Cys-174 at the active site known to be important for activity and which have been shown to be capable of covalent modification by thiol specific agents [19, 20]. The cysteine residue modified by gliotoxin (either 281 or 282) is not at the active site but modification is sufficient to inactivate the enzyme possibly due to conformational changes induced in the enzyme following toxin binding. Cys-281 and Cys-282 are in a part of the enzyme which forms the pocket for nucleotide binding. They are also close to the surface of the ADH molecule as shown by molecular modelling using BioSym software on Silicon Graphics hardware. Modelling in fact showed that Cys-281 was more exposed than Cys-282 and we feel the exposed residue is more likely to be the one modified. Modification of Cys-281 may compromise binding of the nucleotide cofactor resulting in inactivation of the enzyme. Surprisingly the presence of DTT or glutathione results in enhanced inactivation which is clearly due to redox cycling between reduced and oxidized forms since this extra inactivation is inhibited by catalase. This is in contrast to the observed protective effect of low molecular weight thiols such as DTT on the toxic effects of ETP compounds [21, 22]. The presence of catalase restores inactivation to the previous rate in the absence of reducing agent and restores covalent binding of toxin to the protein. We would suggest that this is because oxidative damage by hydrogen peroxide is faster than covalent modification and dominates. Catalase would remove hydrogen peroxide but due to rapid oxidation of the dithiol back to the oxidized form of the toxin covalent modification could now occur. Oxidation of the dithiol form is rapid compared with modification of the ADH and so the latter would be the rate determining step hence restoring the rate of inactivation to that in the presence of toxin alone. These two mechanisms appear therefore to be mutually exclusive with indirect oxidative damage apparently occurring faster than covalent modification. Scheme 1 represents the competing reactions involved.

Members of the ETP family of fungal toxins have attracted considerable attention because of their observed antibacterial, antifungal and antiviral properties. In addition, sporidesmin is the causative agent of facial eczema in sheep [23] and gliotoxin may play a role in the aetiology of aspergillosis [24]. Much attention has been paid to the generation of free radicals by sporidesmin via redox cycling and this is clearly a possible mechanism of toxicity [25]. However, we have been unable to block the ability of gliotoxin to induce apoptosis with radical scavengers (Waring, unpublished data) indicating no gross oxidative stress at concentrations causing apoptosis although low level oxidative damage to specific proteins cannot be ruled out. A number of



Scheme 1.

different enzymes have been shown to be inhibited by gliotoxin and either the oxidized [12] or the reduced form [13] of the toxin has been shown to be the active form. Gliotoxin has also been shown to be capable of causing single and double stranded damage to isolated DNA by a process mediated by hydrogen peroxide formation during redox cycling of oxidized and reduced toxin [26]. This process requires the presence of reductants such as glutathione. We have also detected the reduced form of gliotoxin in activated T-cells treated with the toxin indicating that reduction does occur intracellularly [15]. Gliotoxin is capable of covalently modifying many cellular proteins although how this may relate to toxicity is not yet clear [15]. The fact remains that the toxicity of gliotoxin has been related to either reactive oxygen production or mixed disulphide formation. In this paper using ADH as a model enzyme we have shown that gliotoxin can inactivate this enzyme by either route depending on the balance and availability of reductants, e.g. glutathione, or agents protecting against oxidative damage, e.g. catalase with covalent modification occurring uniquely at one of two cysteines—281 or 282. This dichotomy needs to be kept in mind when the mechanism of toxicity of gliotoxin is under investigation. Treatment of cells with free radical inhibitors may simply switch damage from one involving the generation of reactive oxygen species to one involving mixed disulphide formation with macromolecules.

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