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Transannular Disulfide Formation in Gliotoxin Biosynthesis and Its Role in Self-Resistance of the Human Pathogen *Aspergillus fumigatus*

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Abstract: Gliotoxin (**1**), the infamous representative of the group of epipolythiodioxopiperazines (ETPs), is a virulence factor of the human pathogenic fungus *Aspergillus fumigatus*. The unique redox-sensitive transannular disulfide bridge is critical for deleterious effects caused by redox cycling and protein conjugation in the host. Through a combination of genetic, biochemical, and chemical analyses, we found that **1** results from GliT-mediated oxidation of the corresponding dithiol. *In vitro* studies using purified GliT demonstrate that the FAD-dependent, homodimeric enzyme utilizes molecular oxygen as terminal electron acceptor with concomitant formation of H₂O₂. In analogy to the thiol–disulfide oxidoreductase superfamily, a model for dithiol–disulfide exchange involving the conserved CxxC motif is proposed. Notably, while all studied disulfide oxidases invariably form intra- or interchain disulfide bonds in peptides, GliT is the first studied enzyme producing an epidithio bond. Furthermore, through sensitivity assays using wild type, Δ gliT mutant, and complemented strain, we found that GliT confers resistance to the producing organism. A phylogenetic study revealed that GliT falls into a clade of yet fully uncharacterized fungal gene products deduced from putative ETP biosynthesis gene loci. GliT thus not only represents the prototype of ETP-forming enzymes in eukaryotes but also delineates a novel mechanism for self-resistance.

Introduction

Pathogenic fungi have developed various chemical strategies to distress, weaken, or even kill their plant or animal hosts.^{1–3} In invasive aspergillosis,^{4,5} the leading cause of death in immunocompromised patients, gliotoxin (**1**, Figure 1A) plays a critical role in virulence.⁶ Gliotoxin is the prototype of a small family of epipolythiodioxopiperazines (ETPs) featuring unique transannular di- or polysulfide bridges.⁷ Extensive molecular studies have revealed that this rare structural motif is indispensable for the deleterious effects of gliotoxin.⁶ Two individual routes rationalize gliotoxin-mediated virulence (Figure 1). First, through redox cycling, reactive oxygen species (ROS) may be formed, which can severely damage the host cell. Second, gliotoxin can target proteins that are essential for vital functions. After passage through the membrane, the toxin enters the reductive milieu of the cytosol and is in equilibrium with its reduced dithiol form **2**,

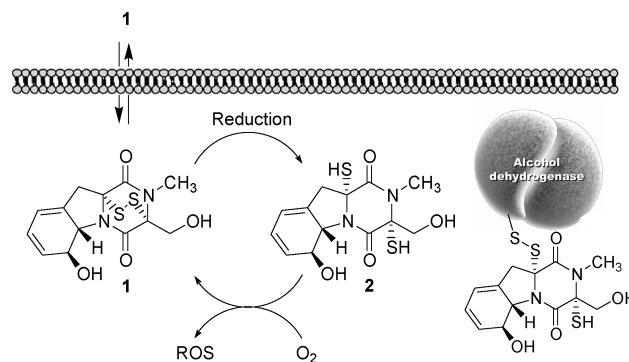


Figure 1. Gliotoxin (**1**) action inside the host cell accounting for *A. fumigatus* virulence: redox cycling resulting in formation of reactive oxygen species (ROS), and protein conjugation.

readily forming thioconjugates with susceptible thiols of target proteins, such as alcohol dehydrogenase.^{6,7} Due to the high importance of this group of ETP toxins,⁸ much effort has been devoted to the elucidation of their genetic basis.⁹ Several work groups have independently demonstrated that a mutant with a disruption of a nonribosomal peptide synthetase (NRPS) gene (*gliP*, Figure 2A) stalls the production of **1** in *Aspergillus fumigatus*.^{10–13} Yet, apart from the finding that the diketopiperazine core of

[‡] These authors contributed equally.

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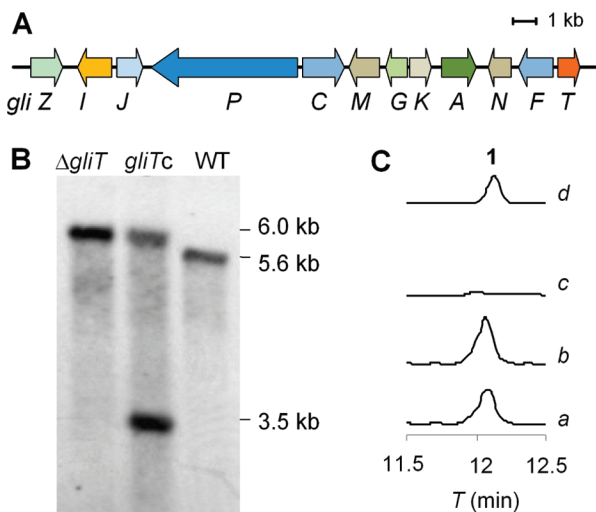


Figure 2. Functional analysis of *gliT*. (A) Organization of the gliotoxin biosynthesis gene cluster. The predicted function of the encoded proteins is as follows: zinc finger transcription factor, GliZ; aminocyclopropane carboxylate synthase, GliI; dipeptidase, GliJ; nonribosomal peptide synthetase, GliP; cytochrome P450 mono-oxygenases, GliC and GliF; methyl transferases, GliM and GliN; glutathione S-transferase, GliG; major facilitator superfamily transporter, GliA; hypothetical protein, GliK; and gliotoxin sulphydryl oxidase, GliT. (B) Southern blot analysis of *A. fumigatus* wild type (WT), the Δ gliT mutant, and the *gliT*-complemented mutant strain (*gliTc*). (C) Metabolic profiling of *A. fumigatus* strains by HPLC: WT (a), *gliTc* (b), Δ gliT mutant strain (c), and standard of **1** (d).

gliotoxin is assembled by a bimodular NRPS thiotemplate,¹⁴ the enzymology of the downstream reactions including dithiol formation has remained fully enigmatic.

Here we report that gliotoxin is generated from the corresponding dithiol by a novel FAD-dependent dithiol oxidase, GliT, and reveal that this enzyme also confers resistance against the toxin.

Results and Discussion

GliT Plays a Key Role in Gliotoxin Biosynthesis. Analysis of the gliotoxin biosynthesis gene cluster from *A. fumigatus*¹⁵ revealed a candidate gene (*gliT*) that might code for an oxidoreductase (Figure 2A). The deduced gene product, GliT, shares amino acid sequence similarities with thioredoxin-like oxidoreductases. To evaluate the phylogenetic relationship of GliT, we constructed a phylogenetic tree (neighbor-joining) using homologous amino acid sequences and found that GliT falls into a clade (III) of not yet fully characterized fungal gene products deduced from putative ETP biosynthesis gene loci (Figure 3, and Figure S2, Supporting Information). Surprisingly, clade III is rather distant from clade I, comprising putative fungal thioredoxin reductases, and more closely related to clade II, which comprises gene products implicated as oxidizing enzymes

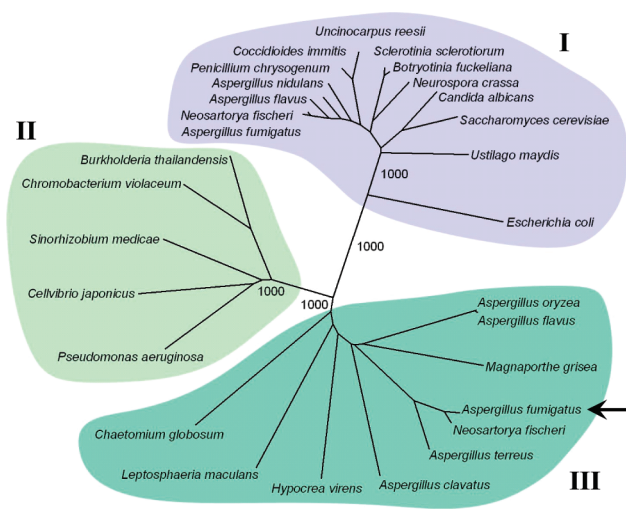


Figure 3. Phylogenetic analysis of GliT, its homologues from other fungal ETP clusters (clade III), (putative) bacterial natural product oxidizing proteins (clade II), and thioredoxin reductase-like proteins from various fungi (clade I). Homologue from *E. coli* as outgroup. Amino acid sequences were obtained from GenBank database and aligned with ClustalW.

in bacterial pathways. We thus postulated that GliT could function as an oxidase in the gliotoxin pathway. To investigate the role of *gliT* in gliotoxin biosynthesis, we first constructed a targeted *A. fumigatus* mutant lacking *gliT* (Δ gliT) and verified the successful gene deletion by polymerase chain reaction (PCR) (data not shown) and Southern blot analysis (Figure 2B). After digesting chromosomal DNA with *Nde*I, we detected a specific signal for *gliT* at 5.6 kb in the wild type (WT). The band at 4.0 kb indicates the partial deletion of *gliT* in the Δ gliT mutant.

According to high-performance liquid chromatography (HPLC) analyses of the mutant broth, the loss of *gliT* results in a complete abrogation of gliotoxin production (Figure 2C). To exclude the possibility of polar effects in the mutant, we monitored gene expression by reverse transcription (RT)-PCR and found that the absence of *gliT* does not have any impact on the expression of other genes from the *gli* locus (Figure S1, Supporting Information). Furthermore, we successfully complemented the Δ gliT mutant through ectopic integration of a *gliT* expression cassette and yielded a strain (*gliTc*) producing **1** at the wild-type level (WT, 4.5 mg L⁻¹; *gliTc*, 5.5 mg L⁻¹). We confirmed the complementation at the genetic level by detecting the 3.5 kb band characteristic of a DNA fragment comprising the *gliT* gene with flanking promoter and terminator sequences in the Southern blot (Figure 2B). While these experiments clearly show that GliT plays a crucial role in gliotoxin biosynthesis, HPLC-MS profiles of the Δ gliT mutant broth did not provide any clue about potential late-pathway intermediates. Thus, in order to corroborate the function of GliT, *in vitro* experiments were needed.

GliT Is a Homodimeric, FAD-Dependent Oxidase Employing Molecular Oxygen as Terminal Electron Acceptor. To investigate the function of GliT *in vitro*, we amplified the *gliT* ORF (1002 bp) and cloned the generated PCR fragment into an *Escherichia coli* expression vector. The resulting plasmid was propagated in *E. coli* DH5 α cells, and after sequencing of the insert, the vector was introduced into *E. coli* BL21(DE3) cells for protein overproduction. After the cells were harvested, His₆-tagged GliT was purified using a NiSepharose column (Figure 4A). Size exclusion chromatography revealed the homodimeric status of GliT (Figure 4B).

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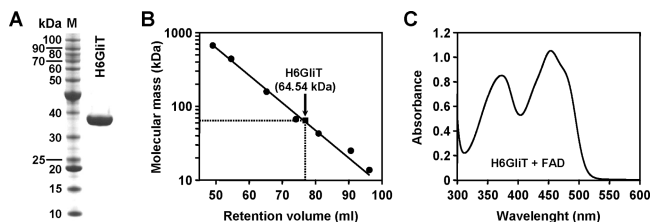


Figure 4. Purification and biochemical characterization of heterologously produced GliT. (A) Molecular mass of His₆-tagged GliT analyzed by SDS–PAGE. (B) Native molecular mass determination by gel filtration. (C) UV–visible absorbance spectra of FAD-saturated GliT.

To identify the cofactor(s) of the putative oxidoreductase, we searched for consensus motifs in the amino acid alignment and identified a conserved FAD binding motif within the GliT sequence (Figure S3, Supporting Information). This was confirmed by the spectrophotometric characterization of GliT (Figure 4C), which indicated a 1:1 stoichiometry of GliT–FAD. Notably, GliT lacks a canonical NADPH binding domain, which is typically found in thioredoxin reductases.¹⁶ Nevertheless, we found a conserved CxxC motif in the amino acid sequence of GliT and homologous proteins (Figure S3). This motif is a hallmark for various oxidoreductases, such as the quiescin-sulfhydryl oxidase family of flavoenzymes.¹⁷

Our next goal was to elucidate whether GliT functions as an oxidase or a reductase by various methods. First, we chemically reduced **1** to **2** using tris(2-carboxyethyl)phosphine hydrochloride (TCEP) to provide the reference and appropriate substrate for the enzymatic assays (Figure 5C, i). The potential reductase function was probed by standardized thioredoxin reductase activity assays.¹⁸ However, in a series of tested conditions, GliT did not show any reductase activity, neither with gliotoxin (**1**) nor with other substrates, e.g. oxidized glutathione. In contrast, we observed that **2** (0.36 μ mol, 1.8 mM) is readily converted into **1** in the presence of GliT (9.6 pmol, 0.32 μ mol). The time course of the reaction was monitored by HPLC (Figure 5A, a–d). Notably, no transformation of **2** could be observed with heat-inactivated GliT or with O₂ alone (Figure 5A, e, f). Gliotoxin oxidase activity was further corroborated by measuring the decrease of $c(\text{O}_2)$ from an assay after adding reduced gliotoxin (**2**) using an oxygen monitor (Figure 5B). Furthermore, oxygen depletion could be reversed by the addition of catalase (data not shown), which implied the formation of hydrogen peroxide as a side product of gliotoxin oxidation.

In sum, several lines of evidence support a model according to which GliT functions as a sulfhydryl oxidase using FAD as cofactor and oxygen as terminal electron acceptor (Figure 5C). On the basis of our biochemical data and analogies to well-studied flavoproteins¹⁹ and sulfhydryl oxidases,¹⁷ we propose a model for electron/proton flow in gliotoxin biosynthesis (Figure 5C): As in thioredoxin reductases, two redox centers are present, the flavin and the disulfide–dithiol (CxxC) redox rheostat. Accordingly, the sulfhydryl hydrogens of **2** are first transferred through the CxxC redox switch to the oxidized form of GliT (disulfide), yielding the reduced form of GliT (dithiol). Subsequently, FAD_{ox} receives the hydrogens from the active site of GliT with concomitant reduction. FAD_{red} is then

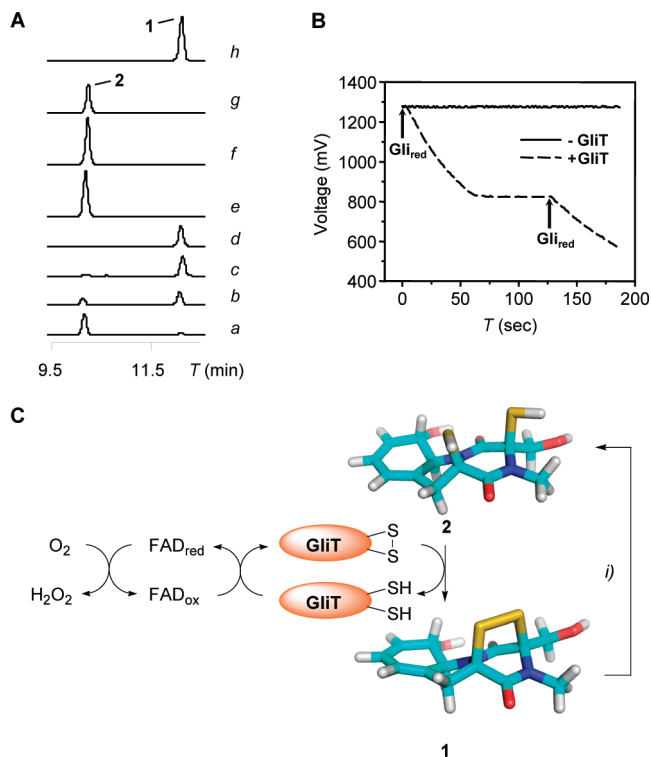


Figure 5. *In vitro* analysis of GliT action. (A) HPLC profile of GliT-catalyzed transformation of **2** to **1** after (a) 5, (b) 20, (c) 30, and (d) 40 min; (e) control with heat-inactivated GliT; (f) control with O₂-saturated buffer; (g) reference of **2**; and (h) reference of **1**. (B) Monitoring O₂ depletion in GliT assay. (C) Model for GliT-mediated transformation of **2** to **1** and preparation of **2** through chemical reduction of **1**: (i) 1.5 equiv of TCEP, MeOH/buffer, rt, pH = 7.0, 3 h.

reoxidized by molecular oxygen with formation of H₂O₂, as shown by the use of O₂ electrodes in our O₂ depletion assay, respectively. As has been demonstrated for thiol–disulfide oxidoreductase model systems, there are several possible scenarios for the exact mechanisms of electron/hydrogen transfer and thiol–disulfide exchange,^{20–22} and in particular oxygen reactivity of flavoenzymes is still a matter of intense research.²³

GliT Confers Gliotoxin Resistance to the Producing Organism. Our *in vitro* studies unequivocally show that GliT catalyzes disulfide formation in gliotoxin biosynthesis. Accordingly, one may conclude that the dithiol **2** is the immediate biosynthetic precursor of **1**. In light of this, the baffling phenotype of the *gliT* knockout mutant appears more plausible: In the absence of GliT, **2** would accumulate in the fungal cytosol, hardly detectable because of protein conjugation. This assumption is supported by some important further observations. Using higher doses of **1**, we were able to detect the dithiol in the cell lysate, supporting the model for cytosolic disulfide reduction (see Supporting Information). Furthermore, we noted that growth of the Δ *gliT* mutant is dramatically impaired compared to that of the WT upon addition of **1**. To quantify this effect, we probed the sensitivity of *A. fumigatus* strains toward **1** in an inhibition zone assay (Figure 6).

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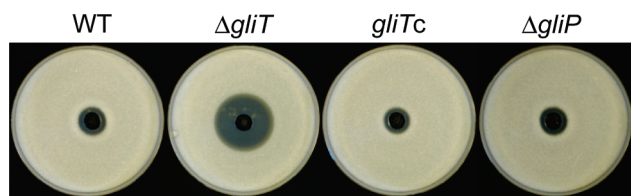


Figure 6. Sensitivity of *A. fumigatus* strains toward gliotoxin (**1**) ($c = 3$ mM) determined in an inhibition zone assay.

While the WT is insensitive toward **1**, we observed a clear inhibition zone (32.8 mm) on the $\Delta gliT$ mutant lawn. Notably, the *gliT*-complemented mutant fully restored resistance. To exclude any secondary effects, we employed a gliotoxin-negative strain lacking the NRPS gene *gliP* ($\Delta gliP$) as control. The finding that the presence of the *gliT* gene directly correlates with gliotoxin tolerance provides strong evidence that GliT confers host self-resistance toward gliotoxin. In light of our biochemical analysis, we reason that the activity of GliT would prevent unwanted redox cycling as well as conjugation of gliotoxin with susceptible proteins. Since reduced gliotoxin is not found in the broth of the $\Delta gliT$ mutant, one may also speculate that formation of the internal sulfur bridge is a requirement for the export of gliotoxin. To the best of our knowledge, enzyme-catalyzed epidisulfide formation represents an unprecedented antibiotic self-resistance mechanism.

GliT Is the Prototype of Fungal Enzymes Forming Epidithio Bridges. Enzyme-mediated disulfide formation is a universal reaction found in all kingdoms of life. However, to date virtually all investigated disulfide oxidoreductases are involved in protein folding and cross-linking of ribosomally synthesized peptide strands.^{24–26} In contrast to the ubiquitous occurrence of disulfides in proteins, disulfide-containing secondary metabolites are scarce, and there is only very limited knowledge on disulfide formation in such small molecules. Although various gene clusters coding for the biosynthesis of di- and trisulfide-containing secondary metabolites have been cloned and sequenced,^{9,27–31} requisite biochemical functions have remained elusive. Only recently, an oxidase (DepH) has been identified and characterized that mediates the formation of a disulfide bridge of a bacterial depsipeptide.³² To date, all studied disulfide oxidases invariably form intra- or interchain disulfide bonds in peptides. In stark contrast, GliT is the first studied enzyme producing an epidithio bond. While GliT shares the FAD binding motif and the CxxC box with thiol–disulfide oxidoreductases such as thioredoxin reductases, GliT exhibits no

classical NADP⁺/NADPH binding site. Instead of NADP⁺, GliT employs molecular oxygen as terminal electron acceptor. Another novelty reported in this study is the proven role of a disulfide-forming enzyme for self-resistance. In this context it is intriguing that our phylogenetic analysis revealed that GliT clusters with a number of yet unidentified putative gene products likely involved in fungal ETP pathways. Since GliT represents the prototype of this novel enzyme clade, we hypothesize that these enzymes may also confer resistance to unwanted ETP redox cycling in the requisite producer strains. Finally, considering the crucial role of GliT for self-resistance and virulence, one may consider an evaluation of this key enzyme as a novel target for antifungal therapy.

Experimental Section

General Analytical Procedures. Samples were measured on a JASCO HPLC with DAD and on an LC-Q electrospray MS from Thermo Electron, respectively, using a C18 Nucleosil column (250 × 4.6 mm, 5 μ m) from JASCO. HRESIMS were recorded on a Finnigan TSQ Quantum Ultra AM instrument from Thermo Electron. IR spectra were measured on a Bruker FT-IR (IFS 55) spectrometer using ATR technique. All solvents used were spectral grade or distilled prior to use. For HPLC measurements, 20 μ L of the concentrated sample was injected. Column, Nucleosil 100 (250 × 4.6 mm, C18, 5 μ m); eluent, 1 mL min^{−1} flow; gradient A, H₂O, 0.1% TFA, B, acetonitrile, start 20% B, in 20 min 65% B, after 28 min 100% B for 10 min. For LC-MS measurements, 25 μ L of the concentrated sample was injected. Column, Nucleosil 100 (250 × 4.6 mm, C18, 5 μ m); eluent, 0.6 mL min^{−1} flow; gradient A, H₂O, 0.1% HCOOH, B, acetonitrile, start 20% B, in 18 min 65% B, after 28 min 95% B for 10 min. Gliotoxin standard elutes on HPLC system after 12.1 min and from the LC-MS system after 17.3 min. Reduced gliotoxin elutes on HPLC system after 10.2 min and from the LC-MS system after 14.8 min.

Fungal and Bacterial Strains, Media, and Growth Conditions.

A. fumigatus strain CEA17 $\Delta akuB$ ³³ was used as wild type and for generation of *gliT* mutant strains. Strains were grown in *Aspergillus* minimal medium (AMM) as described previously.³⁴ AMM agar was prepared by addition of 1.6% (w/v) Select Agar (Invitrogen, Germany). For transformation of *Escherichia coli*, TOP10F⁺ cells (Invitrogen, Germany) were used. *E. coli* cells were grown at 37 °C in LB medium supplemented with ampicillin (100 μ g mL^{−1}). For production of *A. fumigatus* conidia, the fungus was cultivated for 5 days on AMM agar plates. The spores were harvested in 0.9% (w/v) NaCl/0.1% (v/v) Tween 80 and counted using a CASY cell counter (model TT, Innovatis AG, Germany). For isolation of DNA, the fungus was grown in liquid AMM for 24 h.

Isolation and Manipulation of Nucleic Acids. Standard techniques for manipulation of DNA were carried out using standard procedures.³⁵ Chromosomal DNA of *A. fumigatus* was prepared using the Master Pure Yeast DNA purification kit (Epicentre Biotechnologies, USA). For Southern blot analysis, DNA fragments were separated on an agarose gel and blotted onto Hybond N⁺ nylon membranes (GE Healthcare Bio-Sciences, Germany). Labeling of DNA probes, hybridization, and detection of DNA–DNA hybrids were performed as described previously.³⁶ For RNA isolation, *A. fumigatus* was cultivated under gliotoxin-producing conditions for 48 h. For RNA extraction, 100 mg of mycelium was used employing

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the RNeasy Plant Mini Kit (Qiagen, Germany). After DNase treatment with the TURBO DNA-free Kit (Ambion, Applied Biosystems, Germany), 10 μ g of total RNA was used for first-strand cDNA synthesis employing SuperScript III reverse transcriptase and anchored oligo(dT)20 primers (Invitrogen, Germany). Reverse transcription was conducted at 50 °C for 3 h.

RT-PCR. Reverse transcription PCR analysis was used to determine RNA transcription levels. Specific oligonucleotide combinations (Table S1, Supporting Information) allowed the amplification of short fragments of selected genes of the gliotoxin biosynthesis gene cluster. As control, mRNA steady-state levels of the constitutively transcribed *actin* gene were determined. Transcript amplification was carried out using GoTaq DNA polymerase (Promega, Germany) with 5 ng of *A. fumigatus* cDNA as template.

Generation of *A. fumigatus* Mutant Strains and Complementation. Partial deletion of *gliT* was done by using a PCR-based strategy. Upstream and downstream flanking regions of gene *gliT* (AFUA_6G09740) were amplified by PCR using primer pairs *gliT*5-for and *gliT*-ptrA-rev and *gliT*3-rev and *gliT*-ptrA-for, respectively. By this reaction, overlapping ends of the pyrithiamine resistance cassette³⁷ were introduced at the 3'-end of the upstream flanking region and at the 5'-end of the downstream flanking region of the *gliT* gene. The *ptrA* resistance cassette was amplified from plasmid pSK275 (kind gift from Sven Krappmann) with primers *ptrA*-for and *ptrA*-rev. All PCR fragments were purified by gel extraction. The final deletion construct was generated by a three-fragment PCR employing primers *gliT*-for and *gliT*-rev. All PCRs were performed with Phusion High-Fidelity DNA polymerase (Finnzymes) according to the manufacturer's recommendations. The resulting 3.7 kb PCR product was purified and used for transformation of *A. fumigatus* wild-type protoplasts as described previously.³⁴ Pyrithiamine (Sigma-Aldrich, Germany)-resistant transformants were analyzed for partial deletion of *gliT* by Southern blot analysis. One positive transformant, designated Δ *gliT*, was chosen for further analysis and for generation of a *gliT*-complemented strain.

To re-introduce the *gliT* gene in the Δ *gliT* mutant, i.e. to generate a *gliT*-complemented strain, the *gliT* gene was amplified from wild-type genomic DNA by PCR using Phusion High-Fidelity DNA polymerase and primers *gliT*-HindIII-F and *gliT*-XhoI-R. The obtained 1.7 kb PCR fragment comprised the intergenic region between *gliF* (AFUA_6G09730) and *gliT* and 0.3 kb of *gliT* terminator sequences. This fragment was inserted via *XhoI* and *HindIII* restriction into plasmid pUChph,³⁸ mediating hygromycin resistance. The resulting plasmid, pUChph-*gliT*, was used for transformation of strain Δ *gliT*. Transformants were selected for resistance against hygromycin (Roche Applied Science, Germany) and further analyzed by PCR and Southern blot.

Cultivation of *A. fumigatus* Wild-Type and Mutant Strains. For formation of conidia, *A. fumigatus* strains CEA17 Δ *akuB*, Δ *gliT*, and *gliT* complemented were grown on *Aspergillus* minimal medium at 37 °C for 5 days. In the case of Δ *gliT* mutant cultivation, pyrithiamine (1 mg mL⁻¹) was added to the media as selection marker. *A. fumigatus* strains CEA17 Δ *akuB*, Δ *gliT*, and *gliT* complemented were cultivated at 28 °C in 250 mL of Czapek Dox medium at 160 rpm for 6 days. The cultures were extracted three times with 100 mL of ethyl acetate. The combined organic phases were dried over sodium sulfate and concentrated to dryness. For gliotoxin determination, the residues were dissolved in 3 mL of MeOH and measured with HPLC. For quantification of gliotoxin, a calibration curve was obtained from 16 μ g to 1 mg of gliotoxin standard (Sigma-Aldrich). Gliotoxin eluted after 12.1 min as the standard.

Detection of Reduced Gliotoxin in Δ *gliT* Mutant. *A. fumigatus* strains were cultivated at 28 °C in 250 mL of Czapek Dox medium

at 160 rpm for 6 days. After 2 days, 5 mg of gliotoxin, dissolved in 1 mL of DMSO, was added. After 4 days, 10 g of Amberlites XAD-4 (SERVA), suspended in 50 mL of water, was added to the culture. The mycelium and amberlites were filtered off and washed with 200 mL of degassed MeOH. The solvent was removed under reduced pressure. The residue was dissolved in 500 μ L of degassed water and measured immediately by LC-MS. Reduced gliotoxin eluted after 14.8 min and could be detected only in the Δ *gliT* mutant when gliotoxin was added.

Chemical Reduction of Gliotoxin. Gliotoxin (**1**) was isolated from *A. fumigatus* and purified on silica gel (cyclohexane/EtOAc, 1:1), Sephadex LH-20 (MeOH), and RP-18 preparative HPLC. The physicochemical properties were identical with reported data.³⁹ Gliotoxin was reduced using tris(2-carboxyethyl)phosphine hydrochloride (TCEP) as reducing agent. To a solution of 2.4 mg (7.4×10^{-3} mmol) of gliotoxin, dissolved in 1 mL of degassed MeOH, 3.2 mg (0.01 mmol) of TCEP was added, dissolved in 1 mL of phosphate buffer (pH 7.0, 0.1 M). The reaction was complete after stirring for 3 h under an argon atmosphere. The solvent was removed under vacuum and the residue was dissolved in 4 mL of phosphate buffer (pH 7.0, 0.1 M). Reduced gliotoxin eluted after 10.2 min, and gliotoxin eluted as the standard after 12.1 min. Reduced gliotoxin was purified by preparative HPLC (column, Nucleosil Macherey-Nagel; eluent, 10 mL min⁻¹ flow; gradient A, H₂O, B, acetonitrile, start 20% B, in 20 min 65% B, after 28 min 100% B for 10 min) and analyzed by mass spectrometry. The physicochemical properties were identical with reported data.⁴⁰

Heterologous Production and Purification of GliT. The *gliT* ORF (1002 bp) was amplified by PCR using primer *gliT* forward and *gliT* reverse. The generated PCR fragment was cloned into the *Bam*HI/*Hind*III sites of pET43.1H6HapX to create pET43.1H6GliT.⁴¹ The resulting plasmid was propagated in *E. coli* DH5 α cells. The DNA sequence was verified by sequencing, and the vector was introduced into *E. coli* BL21(DE3) cells for protein overproduction. Recombinant GliT was produced by auto-induction in Overnight Express Instant TB medium (Novagen). Cells were harvested after 24 h incubation at 30 °C. His6-GliT-containing cleared cellular extracts in 50 mM NaH₂PO₄, 300 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM PMSF, pH 8.0, were applied to a 25 mL NiSepharose 6 FF column (all columns purchased from GE Healthcare Bio-Sciences, Freiburg, Germany). GliT was eluted with 200 mM imidazole and incubated with an excess of FAD at 4 °C for 2 h. GliT was transferred to storage buffer (100 mM KH₂PO₄, pH 7.0) using a HiPrep desalting column. GliT-containing fraction was identified by SDS-PAGE and stored in 50% glycerol at -20 °C. Protein concentrations were determined according to Bradford using the Coomassie Plus protein assay reagent (Pierce).

Determination of Physical and Biochemical Properties of GliT. The oligomeric status of GliT was determined by size exclusion chromatography using a HiLoad 16/60 Superdex 200 pg column. Running buffer used was 20 mM Tris/HCl (pH 7.5) and 150 mM NaCl, flow rate 1 mL min⁻¹. UV/vis spectra of GliT were obtained with a spectrophotometer (V-630, Jasco). The stoichiometry of GliT-FAD was determined in duplicate as follows. The spectrum of FAD-saturated GliT was recorded from 250 to 600 nm. GliT was subsequently denatured by boiling for 5 min to release bound FAD, the sample was centrifuged for 5 min, and the spectrum of the supernatant from 250 to 600 nm was recorded. The protein concentration was determined from the absorbance at 280 nm before heat inactivation using a molar extinction coefficient of $A_{280} = 30.0$ mM⁻¹ cm⁻¹ and the absorbance after boiling as blank. The

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concentration of free FAD was determined from the absorbance at 450 nm using a molar extinction coefficient of $A_{450} = 11.0 \text{ mM}^{-1} \text{ cm}^{-1}$.

Enzyme Assay. Enzymatic activity was proven by conversion of reduced gliotoxin (**2**) with GliT into gliotoxin (**1**). To GliT solution (0.32 μmol , 9.6 pmol in 30 μL of phosphate buffer, pH 6.5, 0.1 M) reduced gliotoxin (**2**) was added (1.8 mM, in 0.2 mL of phosphate buffer, pH 6.5, 0.1 M). The reaction was stopped after 5, 20, 30, and 40 min with TFA (10 μL), filtered, and measured directly with HPLC. The reaction was complete after 40 min. As a blind value, the same experiment was examined with heat-inactivated GliT and stopped after 40 min. Reduced gliotoxin (**2**) eluted after 10.2 min, and gliotoxin (**1**) eluted as the standard after 12.1 min.

Gliotoxin Oxidase Activity. Determination of gliotoxin oxidase activity was based upon depletion of O_2 from assay containing reduced gliotoxin (**2**), as measured with an oxygen monitor (Yellow Springs Instruments 5300) equipped with polarographic Clark-type electrodes.

Inhibition Zone Assays for Gliotoxin Sensitivity. The sensitivity of various *A. fumigatus* strains to gliotoxin was determined in an inhibition zone assay. Of the test strains, 1.5×10^7 conidia were mixed with 15 mL of YAG agar (0.5 % (w/v) yeast extract, 2 % (w/v) agar, 2 % (w/v) glucose, trace elements). Next, 100 μL of 3 mM gliotoxin was added in a hole in the center of the plate, and the diameter of the inhibition zone was determined after 24 h incubation at 37 °C. The experiment was repeated in quadruplicate.

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Supporting Information Available: RT-PCR, biochemical assays, bioinformatics studies, chemical analyses. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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