Inactivation of Rabbit Muscle Creatine Kinase by Reversible Formation of an Internal Disulfide Bond Induced by the Fungal Toxin Gliotoxin

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Gliotoxin is a member of the epipolythiodioxopiperazine (ETP) class of biologically active fungal metabolites that is characterized by the presence of a bridged disulfide ring that is essential for activity (1, 2). The ETP toxins are produced by a number of fungi including Aspergillus fumigatus and have been shown to rapidly accumulate in cells (3). This is consistent with the perceived role of ETP toxins as fungal chemical defense agents. ETP toxins may contribute to the etiology of animal and human fungal diseases (4, 5); they are known to be selectively toxic to immune cells and to induce apoptosis (6) although the cellular target(s) have not been defined. Gliotoxin has been shown to inhibit the activity of alcohol dehydrogenase (7), reverse transcriptase (8), farnesyltransferase (9), and the transcription factor NFκB (10).

Creatine kinase is a ubiquitous enzyme that catalyzes the reversible formation of ATP from creatine phosphate and ADP (11). Creatine kinase has been shown to be coupled to the action of ATP-dependent membrane ion pumps functioning in the rapid regeneration of ATP from ADP (12, 13). Mitochondrial creatine kinase may form a component of the mitochondrial permeability pore (14). Inhibitors of creatine kinase have been shown to display activity against a number of tumor cell lines (15). Rabbit muscle creatine kinase is believed to function as a non-covalently bound dimer of two identical 42-kDa monomers (16). Because creatine kinase is sensitive to inactivation by reactive oxygen species and to thiol-specific agents, we examined the effect of gliotoxin on creatine kinase activity to gain information on intracellular targets for ETP toxins. In this study, we have shown the novel conversion of creatine kinase to a 37-kDa oxidized form consistent with intracellular disulfide formation and consequent loss of activity. The active 42-kDa native protein was regenerated on treatment with reducing agents. In addition, we propose that a single functioning reduced monomer in the creatine kinase dimer is sufficient for full activity whereas the second monomeric component in the oxidized form does not affect overall activity. Formation of the doubly oxidized dimer results in complete loss of activity. The intramolecular disulfide bond is most probably formed between the Cys-282 and Cys-73, which are physically in close proximity (17).

MATERIALS AND METHODS

Gliotoxin, dithiothreitol, and glutathione were purchased from Sigma. Pepsin from porcine gastric mucosa was obtained from Roche Diagnostics. Radiolabeled gliotoxin was prepared and purified using Pericillus terlikowskii fed with [35S]labeled inorganic sulfate (Amer-aetham Pharmacia Biotech) as described previously (3). Rabbit muscle creatine kinase was purchased from Sigma and Roche Molecular Bio-chemicals and used without further purification. The kinase displayed a single major band on SDS-polyacrylamide gel electrophoresis of 42 kDa in the presence of dithiothreitol. A single contaminant that was identified as phosphoglycerate mutase was found in the Sigma sample but was present at less than 10% of the total protein.

Activity was measured using either the enzyme-coupled method (18) or the thymol blue assay (19) typically in the absence of reducing agents with a Carey UV-visible spectrophotometer. Both assays gave comparable results. Creatine kinase at 1 mg/ml (25 μM based on the monomer of 42 kDa) was incubated in NaOH/glycine buffer, pH 9, at 37 °C in a total volume of 1 ml for different time intervals with gliotoxin. A 5-μl aliquot was removed to assess enzyme activity. In some experiments, reducing agents were added to the reaction mixture either at zero time or after complete inactivation of the enzyme. Samples (30 μl) were also taken for non-reducing SDS-polyacrylamide gel electrophoresis, which was carried out using published procedures in the absence of reducing agents.

The biological activity of gliotoxin is dependent on the presence of a strained disulfide bond that can react with accessible cysteine residues on proteins. Rabbit muscle creatine kinase contains 4 cysteines per 42-kDa subunit and is active in solution as a dimer. Only Cys-282 has been identified as essential for activity. Modification of this residue results in loss of activity of the enzyme. Treatment of creatine kinase with gliotoxin resulted in a time-dependent loss of activity abrogated in the presence of reducing agents. Activity was restored when the inactivated enzyme was treated with reducing agents. Inactivation of creatine kinase by gliotoxin was accompanied by the formation of a 37-kDa form of the enzyme. This oxidized form of creatine kinase was rapidly converted to the 42-kDa species by the addition of reducing agents concomitant with restoration of activity. A 1:1 mixture of the oxidized and reduced monomer forms of creatine kinase as shown on polyacrylamide gel electrophoresis was equivalent to the activity of the fully reduced form of the enzyme consistent with only one reduced monomer of the dimer necessary for complete activity. Conversion of the second monomeric species of the dimer to the oxidized form by gliotoxin correlated with loss of activity. Our data are consistent with gliotoxin inducing the formation of an internal disulfide bond in creatine kinase by initially binding and possibly activating a cysteine residue on the protein, followed by reaction with a second neighboring thiol. The recently published crystal structure of creatine kinase suggests the disulfide is formed between Cys-282 and Cys-73.

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‡ The abbreviation used is: ETP, epipolythiodioxopiperazine.
agent (20). Bands were visualized with Coomassie Blue. Autoradiography was carried out on dried gels using Kodak Biomax MR x-ray film with enhancing screens.

Creatine kinase treated with \(^{35}\text{S}\)-labeled gliotoxin (100 \(\mu\text{M}\)) for 6 h was washed through a Centriprep-10 filter to remove unbound gliotoxin and digested with pepsin at 1% w/w for 3 h at 37 °C in 10% formic acid. Peptides were purified by two-dimensional chromatography using a thin layer electrophoresis system (Hunter). The first dimension (electrophoresis at 1 kV and 45 mA for 40 min) used 5% butyl alcohol, 2.5% acetic acid, 2.5% pyridine, and 90% water, pH 4.7, and the second dimension (chromatography over 5–6 h) used 39% butyl alcohol, 30% pyridine, 6% acetic acid, and 25% water as the mobile phase. The plate was air-dried, and labeled peptides were visualized by autoradiography. The labeled spots on the cellulose were placed in an Eppendorf tube with 100 \(\mu\text{l}\) of distilled water and polyvinylidene difluoride membrane and were incubated overnight with complete transfer to the membrane. Sequencing was then carried out as described previously (6).

The stoichiometry of binding of gliotoxin to creatine kinase was determined by first generating a standard curve using graded concentrations of gliotoxin at known specific activity that were run on polyacrylamide gels, dried, and exposed to film, and the bands were quantified using an enhanced laser densitometer (Ultrascan XL). A known mass of \(^{35}\text{S}\)-gliotoxin-labeled creatine kinase was also run in gels and similarly quantified, thus enabling the mass of gliotoxin associated with a known mass of protein to be calculated. Gels were exposed to allow for only those signals that fell in the linear portion of the standard curve to avoid saturation effects. Prior to drying and autoradiography, gels were also lightly stained with Coomassie Blue to determine the ratio of the 42-kDa (reduced) to 37-kDa (oxidized) forms of the enzyme.

**RESULTS**

**Gliotoxin Inactivates Creatine Kinase in a Time-dependent Manner**—Incubation of creatine kinase with gliotoxin resulted in a concentration- and time-dependent loss of activity (Fig. 1). Greater than 80% inactivation occurred by 6 h with 100 \(\mu\text{M}\) toxin. Gliotoxin at 1 and 10 \(\mu\text{M}\) had little effect on activity whereas the effect of 50 \(\mu\text{M}\) toxin was equivalent to 100 \(\mu\text{M}\) toxin (data not shown). We detected little or no inactivation in the first 90–120 min of incubation of creatine kinase with gliotoxin at 100 \(\mu\text{M}\). The profile of inactivation is consistent with a time-dependent covalent interaction of gliotoxin with creatine kinase.

**The Inhibition Is Abrogated by Reducing Agents Both Pre- and Postaddition**—Creatine kinase activity at 6 h in the presence of 50 \(\mu\text{M}\) gliotoxin was 25 ± 1.3% of control. In the continuous presence of 1 \(\text{mM}\) dithiothreitol or glutathione and gliotoxin, kinase activity was 93 ± 3 and 73 ± 13%, respectively. A trivial explanation for the protection seen in pretreatment with reducing agents is that the gliotoxin is now in the reduced form at time 0 and cannot easily form mixed disulfides with cysteine residues in the enzyme. More significant is the restoration of activity by dithiothreitol or glutathione after inactivation. In a separate experiment, 50 \(\mu\text{M}\) gliotoxin resulted in 19.5 ± 1% of the control activity at 6 h. Addition of either 1 \(\text{mM}\) dithiothreitol or glutathione at the 6-h point and re-assay 1 h later resulted in restoration of activity to 73.5 ± 1 and 56.2 ± 3% of the control, respectively. These results are consistent with inactivation caused by covalent modification of protein thiol(s) by gliotoxin and reversed by reduction of a newly formed –S–S bond. The addition of reducing agents to ETP toxins has been shown to produce reactive oxygen species by inducing redox cycling of the dithiol to the disulfide form of the toxin (21) although this is not observed with creatine kinase.

**Gliotoxin Induces the Formation of Oxidized Creatine Kinase**—Preliminary examination of native creatine kinase on polyacrylamide gels often revealed a second band of an apparent mass of 37 kDa that was present in varying amounts (Fig. 2). This band was most apparent using longer and higher resolution gels in the absence of reducing agents. Microsequencing of this band showed this to be identical with native creatine kinase (data not shown). Reducing agents diminished or completely eliminated this band and correspondingly inactivated the 37-kDa band. We detected little or no inactivation in the first 90–120 min of incubation of creatine kinase with gliotoxin at 100 \(\mu\text{M}\). The profile of inactivation is consistent with a time-dependent covalent interaction of gliotoxin with creatine kinase.

**FIG. 1.** Time-dependent inactivation of creatine kinase (CK) by 100 \(\mu\text{M}\) gliotoxin. A time lag of almost 90 min occurs before any significant inactivation. The reaction occurs at a concentration of creatine kinase of 25 \(\mu\text{M}\) (monomer) at pH 9.0, 37 °C. □, untreated creatine kinase; ■, creatine kinase treated with 100 \(\mu\text{M}\) gliotoxin.

**FIG. 2.** A, native creatine kinase showing the presence of a small amount of the 37-kDa oxidized form (lane 1). Lane 2, native creatine kinase treated with 500 \(\mu\text{M}\) dithiothreitol for 60 min. B, native creatine kinase following treatment with 500 \(\mu\text{M}\) dithiothreitol and exhaustive dialysis (lane 1). Lane 2, the same sample of creatine kinase treated with 500 \(\mu\text{M}\) dithiothreitol and kept on ice. The concentration of creatine kinase is 25 \(\mu\text{M}\) (monomer). Values in parentheses are the relative activities (as determined under “Materials and Methods”) of the two samples normalized with the undialyzed sample. Volume changes during dialysis were estimated at lower than 5%.
band at the expense of the 42-kDa band (Fig. 3). Almost complete conversion occurs by 6 h concomitant with loss of greater than 80% of the enzyme activity. Fig. 3 shows the biphasic production of the 37-kDa oxidized form. Complete conversion to 50% of the oxidized 37-kDa form of creatine kinase occurs within minutes with a slower complete conversion over 6 h. Addition of 1 mM dithiothreitol resulted in conversion to the 42-kDa form (Fig. 4) with the restoration of enzymic activity. Gliotoxin at 10 μM, which has a negligible effect on enzyme activity (data not shown), also resulted in significant conversion to the oxidized form. As shown below, this new band cannot be attributed simply to the addition of molecule(s) of gliotoxin to creatine kinase.

Loss in Activity Does Not Correlate with Stoichiometric Formation of the Oxidized Form—We consistently observed that the oxidized form of creatine kinase is formed within minutes of treatment with gliotoxin (Fig. 3) and that significant amounts of the 37-kDa form were produced with no loss in activity. We measured both the relative proportion of the 37-kDa band and the activity of the enzyme in the same experiment. Fig. 5 shows the relationship between the amount of the 37-kDa form of creatine kinase and activity. There was no loss of activity until 50% of the protein had been converted to the 37-kDa form. The data in Fig. 5 are consistent with our proposal that it is the oxidation of the second monomer of the creatine kinase dimer that correlates with loss of activity. The solid lines in Fig. 5 were constructed as follows. All points corresponding to a proportion of ≥50% of the oxidized 37-kDa form were ignored, and a best fit line was constructed. Similarly, all points which corresponded to ≤50% of the oxidized form were then ignored to generate a second line of best fit.

The data are consistent with initial oxidation of one monomer in the dimer (with no loss in total activity) followed by oxidation of the second monomer, which then results in loss of activity. The dotted line in Fig. 5 is the theoretical line for this latter model shown in Scheme 1.

Stoichiometry of Gliotoxin Binding to Creatine Kinase—Autoradiography of polyacrylamide gels of creatine kinase treated with [35S]labeled gliotoxin clearly showed that both the 42- and 37-kDa forms could be covalently labeled (data not shown). A conventional approach of estimating the number of moles of toxin bound per mol of protein would give only an average stoichiometry and would therefore contain no information on the relative binding to the two forms. We used the alternative method described under “Materials and Methods” to determine stoichiometry. Table I shows the result of such an analysis. At 5 min, there was an almost exact 1:1 mixture of reduced/oxidized monomers of creatine kinase with only a fraction of either form labeled. This indicates that the conversion of re-


Reversible Oxidation of Creatine Kinase by Gliotoxin

Identification of Cysteine Residues Covalently Modified by Gliotoxin—Pepsin in formic acid was used for digests to minimize scrambling of the radiolabel. A number of radiolabeled peptides were detected in digests of creatine kinase labeled with [35S]gliotoxin. The labeled peptide fragments that were isolated showed significant labeling of only Cys-253 and Cys-282. No peptides corresponding to Cys-73 and only trace amounts corresponding to Cys-145 were detected.

A typical experiment revealed four radiolabeled spots with two-dimensional chromatography. Of these, sequencing of two showed peptide residues consistent with binding to only Cys-253 (Arg-Arg-Phe-Cys253-Val-Gly). Sequencing of the other two labeled spots showed peptide residues from Cys-253 and in lesser amounts Cys-282 (Val-Leu-Thr-Cys282-Pro-Ser-Asn-Leu). These results establish binding to Cys-253 at 6 h and possibly some lesser binding to Cys-282.

**DISCUSSION**

The reactive cysteine in creatine kinase has been shown to be exclusively Cys-282 (22, 23) whereas the other cysteine residues (Cys-253, Cys-145, and Cys-73) are only accessible when the enzyme is denatured. Only two thiol groups per dimer are accessible to p-hydroxymercuribenzoic acid during early denaturation, and these have been shown to be the two Cys-282 residues on each monomer (23). At SDS/protein ratios exceeding 200, Cys-145 and Cys-253 become accessible. Cys-73 cannot be modified by p-hydroxymercuribenzoic acid even after complete denaturation by SDS (23). Cys-253 can become exposed under pressure (24). Cys-145 is positioned near the monomer/monomer interface (17) and may become exposed upon dimer dissociation.

Rabbit muscle creatine kinase forms a dimer in solution (25), and it is proposed that this is the active form (26) although there is evidence that the monomer can be active (27, 28). A large body of evidence has demonstrated differential activity of the two Cys-282 residues on each monomer of the dimer. In the presence of Mg2+, ADP, creatine, and nitrate, which together form a “transition state analogue,” the reactive thiol groups react at significantly different rates (29). A detailed kinetic study using 5,5′-dithiobis(2-nitrobenzoic acid) and iodoacetic acid confirmed the biphasic nature of the reaction with Cys-282 (30). Some modifying groups such as S-cyanide may initially bind to essential thiols and subsequently migrate or is lost, thus restoring enzymatic activity (31). It has been shown that regenerated creatine kinase following inactivation by S-thio- methylation is active and has only a single reactive thiol per dimer (32). These authors suggested that the slower reacting Cys-282 pair of the dimer is required for activity with the faster reacting Cys-282 thiol, providing protection. The essential role of Cys-282 in catalysis has been the source of much investigation. Recent mutagenesis studies have shown that Cys-282 may play a role in substrate binding by maintaining the conformation of the active site but is not essential for catalysis (33). The recent crystal structure of rabbit muscle creatine kinase has shown that Cys-282 is close to the active site region of the enzyme (17).

A faster running form of creatine kinase monomer has been identified using denaturing polyacrylamide gel electrophoresis and was postulated as an oxidized non-reducible form of the enzyme that did not involve the essential Cys-282 (34). The formation of internal disulfides in proteins is sufficient to induce conformational changes producing detectable alterations in mobility on denaturing polyacrylamide gels (35). Our results provide evidence for a subtle relationship between the oxidized and reduced forms of the monomers of creatine kinase and enzymic activity. Formation of up to 50% of the 37-kDa form when assessed by a 1:1 distribution on polyacrylamide gel electrophoresis leaves the activity unchanged. This is consistent with the proposal (32) that only a single active thiol is required per dimer if in our results only one of the two monomers of the dimer is oxidized (Scheme 1). The crystal structure of creatine kinase (17) shows that an internal disulfide can only

![Scheme 1](image.png)

**Scheme 1.** The three possible compositions of the dimer of creatine kinase formed by internal disulfide bond formation between Cys-73 and Cys-282. R, reduced; O, oxidized.

**Table 1**

| Time† | Composition‡ | No. of gliotoxin bound* |
|-------|--------------|------------------------|
| 5 min | 51 Ox.       | 0.087                  |
| 5 min | 49 Red.      | <0.080                 |
| 3 h   | 56 Ox.       | 1.09                   |
| 3 h   | 44 Red.      | 1.47                   |
| 4.5 h | 68 Ox.       | 0.94                   |
| 4.5 h | 32 Red.      | 3.4                    |

* Time after treatment with 100 μM gliotoxin.
† Ratio of oxidized to reduced form of creatine kinase after treatment; untreated was 81% reduced (Red.) and 19% oxidized (Ox.).
‡ Number of gliotoxin molecules bound per subunit (either the oxidized or reduced form).

produced to oxidized creatine kinase can occur without any permanent covalent binding of the toxin to the monomer. By 3 h, there was 1 gliotoxin associated with the 37-kDa form and an average of 1.5 gliotoxin molecules associated with the 42-kDa form. At 4.5 h, there was 68% conversion with again only 1 mol of gliotoxin associated with the 37-kDa form. The remaining 32% of the 42-kDa form was associated with more than 3 mol of gliotoxin.

Treatment with 10 μM gliotoxin, which results in a significant conversion of reduced to oxidized monomer of creatine kinase, showed less than 8% binding to creatine kinase even at 6 h (data not shown). Formation of the 37-kDa species was not simply due to the effect of increased covalent binding to gliotoxin. Neither the covalently modified reduced or oxidized form was altered in mobility relative to unmodified protein at 6 h.
be formed between Cys-282 and Cys-73. These residues are about 7 Å apart, and thus some movement in the region containing Cys-282 would be required to bring them close enough for reaction. This movement may be induced by covalent binding of gliotoxin to Cys-282. Reformation of the 42-kDa species by dithiothreitol is consistent with formation of such an internal disulfide. Further oxidation of the second monomer would account for subsequent loss of activity because the essential Cys-282 would now be internally modified in both monomers. Dithiothreitol releases free Cys-282 in both monomers and restores activity. Initial binding to Cys-282 forming a covalently bound complex as shown in Scheme 2 is supported by our observation that peptides, albeit generally in minor amounts, corresponding to Cys-282 can be isolated from digests of modified creatine kinase. This activates Cys-282 to internal disulfide formation by Cys-73 with the subsequent loss of disulfide bonds by glutathione (36). This proposal is consistent with very little gliotoxin covalently associated with creatine kinase at 5 min even though half of the monomers have been converted to the 37-kDa form with no loss in activity. Monomer cross-linking does not occur because an 84-kDa species is not detected. The slower conversion of the second monomer to the 37-kDa form results in loss of all activity, enzyme denaturation, and modification of Cys-253 consistent with a single gliotoxin molecule associated with the oxidized form at 3 and 4.5 h. The increased binding to the minor 42-kDa form at 4.5 h may reflect further binding of gliotoxin to free thiol groups although labeled peptides corresponding to other residues were not detected. When gliotoxin forms a mixed disulfide, the second thiol of the toxin is exposed (Scheme 2). Thus it may be possible for this thiol to react with a second gliotoxin resulting in an increase in associated label. The inactivation profile in Fig. 5 clearly supports our proposal that conversion to the 37-kDa form results in inactivation only when the second monomer of the dimer is so affected.

We have identified a novel route to inactivation of rabbit muscle creatine kinase by the fungal toxin gliotoxin involving formation of an internal disulfide bond probably between the essential Cys-282 and Cys-73. This is the first direct evidence of the protective effect ascribed to modification of one monomer of the creatine kinase dimer via Cys-282 against a redox-active toxin.

Gliotoxin has been shown to induce apoptosis in cells, and one possible cellular target for gliotoxin may be the mitochondria that play an essential role in apoptotic death (37). It is significant that gliotoxin has been shown to release calcium from isolated mitochondria by modification of as yet unidentified neighboring thiol groups (38, 39). Mitochondrial creatine kinase may be a component of the multiprotein mitochondrial pore (14), and the possibility that gliotoxin is interacting with these organelles via creatine kinase is under investigation. Gliotoxin and its analogs can also induce intracellular calcium fluxes (40) possibly by allowing direct entry of calcium through the plasma membrane. Inhibition of creatine kinase that is associated with plasma membrane ATP-dependent calcium pumps would disable this route of calcium extrusion and allow uncontrolled calcium increases.

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