Copper-induced oxidative cleavage of glutathione transferase F1-1 from Zea mays

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Abstract

Study of the interaction of glutathione transferase F1-1 from Zea mays (ZmGSTF1-1) with Cu(II), in the presence of ascorbate showed that the enzyme was rapidly inactivated. The inactivation was time and Cu(II) concentration dependent. The rate of inactivation showed non-linear dependence on Cu(II) concentration, indicating that a reversible complex with the enzyme (Kd 84.5 ± 6.5 μM) was formed. The inhibitors S-nitrobenzyl-glutathione or S-methyl-glutathione competes with Cu(II), suggesting the specificity of the chemical modification reaction. SDS-PAGE analysis of the inactivated enzyme showed that the enzyme is fragmented and two new bands of 13 and 11 kDa are formed. This shows that ZmGSTF1-1 was specifically cleaved at a single site, by the locally generated free radicals, through a Fenton-type reaction. Sequencing of the fragments allowed the identification of the Cu(II) binding site on ZmGSTF1-1. The three-dimensional structure of ZmGSTF1-1 reveals that the Cu(II) binding site is localized within the glutathione-binding site (G-site) and His40 and Gln53 are most likely the residues that provide the coordination sites for the Cu(II) binding. These findings were confirmed by site-directed mutagenesis. This copper-induced oxidative cleavage reaction of ZmGSTF1-1 may function as a detoxification route for Cu(II) for protecting plant cells from copper-induced deleterious effects.

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made but several questions are still unresolved. In the present study, the site-specific cleavage of maize ZmGSTF1-1 by the Cu(II)/ascorbate system was investigated. We found that two fragments were generated and identified by N-terminal amino acid sequence. Using the high resolution crystal structure of ZmGSTF1-1 in combination with site-directed mutagenesis, the sites of cleavage and the Cu(II) binding site were localized.

2. Materials

Ascorbate, glutathione (99%), CDNB and all other and all other enzyme substrates and biochemical (inhibitors, antibiotics, chromatographic materials) were obtained from Sigma-Aldrich (USA). Cupric chloride was from Merck (Germany).

3. Methods

3.1. Cloning, expression, purification and site-directed mutagenesis of ZmGSTF1-1

Cloning of ZmGSTF1-1 into a pQE70 expression vector to yield the pQEGST expression plasmid was described previously [20]. Expression and purification of ZmGSTF1-1 were performed according to [21]. Site-directed mutagenesis was performed according to the unique site elimination method [22], as described in [21].

3.2. Assay of enzyme activity and protein

Enzyme assays were performed by monitoring (340 nm, ε = 9.6 mM⁻¹ cm⁻¹) the formation of the conjugate of CDNB (1 mM) with GSH (2.5 mM) in potassium phosphate buffer (0.1 M, pH 6.5) [21]. Protein concentration was determined by the method of Bradford [23] using bovine serum albumin (fraction V) as standard.

3.3. Inactivation and cleavage of ZmGSTF1-1 by the Cu(II)/ascorbate system

Inactivation of ZmGSTF1-1 was performed in incubation mixture containing in 1 mL total volume Tris/HCl buffer pH 7.0, 100 μM; cupric chloride, 5–40 μM; ascorbate 20 mM, and 1 unit ZmGSTF1-1. Inactivation studies of ZmGSTF1-1 by Cu(II)/ascorbate in the presence of S-methyl-glutathione and S-nitrobenzyl-glutathione were performed in a total volume of 1 mL and the reaction mixture contained: Tris/HCl buffer pH 7.0, 100 μM; cupric chloride, 5–40 μM; ascorbate 20 mM, 1 μM of S-methyl-glutathione or S-nitrobenzyl-glutathione; and 1 unit ZmGSTF1-1. The enzyme was incubated with S-methyl-glutathione or S-nitrobenzyl-glutathione for 5 min before the addition of Cu(II)/ascorbate. The rate of inactivation was monitored by periodically removing samples (50 μL) for assay of enzymatic activity. The inactivation rates were estimated from the log(%) remaining activity) versus time (min). Analysis was achieved using the SigmaPlot v12.0 computer program. K0 determinations were performed according to [24–26].

Inactivation of ZmGSTF1-1 was also examined under anaerobic conditions. N2 was bubbled through a solution of ZmGSTF1-1 in 100 mM Tris/HCl buffer pH 7.0 for 60 min. This was done to replace O2 from the solution. After addition of Cu(II)/ascorbate to the enzyme solution and mixing, the enzyme activity was assayed for 30 min.

3.4. Isolation and analysis of fragments from the purified ZmGSTF1-1 exposed to Cu(II)/ascorbate system

For the analysis of protein fragmentation, ZmGSTF1-1 was inactivated and the protein sample was subjected to SDS/PAGE. SDS-PAGE was performed according to Laemmli [27] on a Bio-Rad minilab gel apparatus. The intact protein and the cleaved fragments were separated on a 15% polyacrylamide gel. Protein bands were stained with Coomassie Blue R-250. After electrophoresis, protein bands were electrophoretically transferred to PVDF membranes. After transferring (transfer efficiency ~60%) the protein bands of interest were cut out, dried with Speed-Vac, and subjected to N-terminal amino acid sequence analysis.

3.5. Molecular modelling studies

3.5.1. Energy minimization

The Cu(II) atom was manually positioned in the proximity of the His40 residue. Subsequent energy minimizations were used to relax and equilibrate the complex by removing any residual geometrical strain. The Cu(II) atom was optimally coordinated and the system reached equilibrium using the Charmm27 forcefield as it is implemented into the Gromacs suite, version 4.5.5 [28,29]. An implicit Generalized Born (GB) solvation was chosen at this stage, to speed up the energy minimization process. Molecular systems were subjected to unrestrained Molecular Dynamics simulations (MDS) using the Gromacs suite, version 4.5.5 (Hess et al. [28]). MDS took place in a SPC watersolvated, periodic environment. Water molecules were added using the truncated octahedron box extending 7 Å from each atom. Molecular systems were neutralized with counter-ions as required. For the purposes of this study all MDS were performed using the NVT ensemble in a canonical environment, at 300 K, 1 atm and a step size equal to 2 femtoseconds for a total 100 ns simulation time. An NVT ensemble requires that the Number of atoms, Volume and Temperature remain constant throughout the simulation.

4. Results and discussion

4.1. Kinetics of the inactivation of ZmGSTF1-1 by the Cu(II)/ascorbate system

In the presence of ascorbate (20 mM), cupric chloride (20 μM) caused a rapid time-dependent inactivation of ZmGSTF1-1 at pH 7.0 and 25 °C (Fig. 1). In the absence of Cu(II)/ascorbate or Cu(II), the enzyme activity was remained constant when the mixture was incubated under identical conditions. The rate of ZmGSTF1-1 inactivation was dependent on Cu(II) concentration in a nonlinear fashion, as illustrated in Fig. 2. This observation supports that the inactivation reaction obeyed pseudo-first order saturation kinetics [24,25] and suggests that a
4.2. Effect of glutathione analogues on the inactivation of ZmGSTF1-1 by the Cu(II)/ascorbate system

The specificity of an enzyme inactivation by a chemical compound can be evaluated by the ability of native enzyme’s ligands (e.g. substrate, inhibitor) to influence (promote or inhibit) the rate of inactivation. In the present work, the ability of the substrate analogues (e.g. inhibitors, inhibitor) to influence the rate of enzyme inactivation was assessed [24–26,30]. This type of interaction is described by the following equation:

\[ \frac{1}{k_{\text{obs}}} = \frac{1}{k_3} + K_D / (k_3 [\text{Cu(II)}]) \]

where: \( k_{\text{obs}} \) is the observed rate of enzyme inactivation by Cu(II), \( k_3 \) is the maximal rate of inactivation (min\(^{-1}\)) and \( K_D \) is the apparent dissociation constant of the E:Cu(II) Michaelis binary complex. From the data shown in Fig. 2, a \( K_D \) of 84.5 ± 6.5 μM was determined. The apparent maximal rate constants (k₃) were measured 0.017 ± 0.002 min\(^{-1}\).

4.3. Selective cleavage of ZmGSTF1-1 by Cu(II)/ascorbate

Since the inactivation of a protein (oxidative modification) by Cu(II)/ascorbate is usually accompanied by polypeptide backbone cleavage, the inactivated ZmGSTF1-1 by Cu(II)/ascorbate was subjected to polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) (Fig. 3). This analysis allowed the examination of the possible cleavage of the polypeptide chain. Native ZmGSTF1-1 has a molecular mass of ~24 kDa [31]. The inactivated enzyme clearly shows the presence of three bands, one at about 24 kDa, corresponding to the undigested enzyme, and two new bands at 13 kDa (F1) and 11 kDa (F2) (Fig. 3). This suggests that ZmGSTF1-1 cleaved at a single site in the polypeptide chain.

The loss of ZmGSTF1-1 activity correlates well with the cleavage reaction. For example, in the absence of ascorbate or Cu(II) the enzyme remains undigested (Fig. 3A). In addition, the presence of S-methylglutathione or S-nitrobenzyl-glutathione enhance or reduce, respectively, the digestion of the enzyme by the Cu(II)/ascorbate system, in agreement with the results of the kinetics analysis (Fig. 1).
Inactivation of ZmGSTF1-1 was also examined under anaerobic conditions \( \left( N_2 \right) \). The results showed that no loss of enzyme activity was observed, suggesting that the endogenous oxygen dissolved in the buffer is required for the inactivation of ZmGSTF1-1.

The Cu(II)/ascorbate system is an oxidative modification system for proteins [15,31–33]. The role of ascorbate is to reduce the Cu(II) to Cu(I). The reactive species causing enzyme cleavage are possible the free radicals generated by a Fenton-type reaction. These species are generated by the reaction of Cu(I) with oxygen molecules dissolved in solution [15]. A presumed mechanism is shown with the following equations:

\[
\begin{align*}
\text{Cu(II)} & \rightarrow \text{Cu(I)} \\
\text{Cu(I)} + O_2 & \rightarrow \text{Cu(II)} + O_2^- \\
2O_2^- + 2H^+ & \rightarrow H_2O_2 + O_2 \\
\text{Cu(I)} + H_2O_2 & \rightarrow \text{Cu(II)} + OH^- + OH^-.
\end{align*}
\]

From the data presented in this paper we can propose that the Cu(II)/catalysed oxidation of ZmGSTF1-1 may proceeds in three steps. In the first step, Cu(II) forms a reversible binary complex with ZmGSTF1-1. Then, the Cu(II) is reduced to Cu(I) by ascorbate and free radicals are generated, which can promote the cleavage of a peptide bond in close proximity and therefore cause enzyme fragmentation (Fig. 3). Free radicals can attack functional groups at or near the metal binding site and therefore lead to the cleavage of the polypeptide backbone.

### 4.4. Sequence analysis of the cleavage products and mapping of the Cu(II) binding site

In order to map the Cu(II) binding site in ZmGSTF1-1, the polypeptide fragments generated by the inactivation reaction were subjected to N-terminal amino acid sequence analysis. The results showed (Fig. 3) that the band indicated as F1 (Fig. 3) has N-terminal sequence Ala-Pro-Met-Lys-Leu, which corresponds to the N-terminal of the native ZmGSTF1-1. The band indicated as F2 (Fig. 3) has N-terminal sequence Gln-Val-Leuille-Ser-Pro, which corresponds to a C-terminal fragment of the ZmGSTF1-1 in which the peptide bond Phe114-Gln115 was cleaved.

Analysis of the crystal structure of ZmGSTF1-1 allowed to place the results of the kinetics and sequence analysis in a structural context. ZmGSTF1-1 monomer has two domains, an \( \alpha/\beta \) domain that includes helices \( \alpha_1-\alpha_3 \) and forms the GSH binding site (G-site). The second \( \alpha \)-helical domain comprised of helices \( \alpha_4-\alpha_9 \) and contains the hydrophobic pocket (H-site) that the electrophile substrates bind. The Cu(II) ion was manually positioned in the proximity of the ZmGSTF1-1 substrate binding site and upon energy minimizations and exhaustive molecular dynamics simulations the complex system reached equilibrium and the Cu(II) ion was coordinated. It was shown that the Cu(II) atom establishes direct interactions with Gln53 and His40 with distances 2.4 and 2.55 Å, respectively (Fig. 4). The metal chelate ability of the side chains of Gln and His is well known in other metalloproteins [34,35]. A third key residue was found to be Lys41, which establishes strong hydrogen bonding to Pro50 at 1.83 Å, thus locking the local conformation of the enzyme structure. The exact interaction pattern of the Cu(II) atom is shown in Fig. 5A. The area that the Lys41-Pro50 interaction fixes in the conformational space of the ZmGSTF1-1 has been cubically calculated and is shown in green cloud representation in Fig. 5B. In silico mutation of Lys41Ala and repeat of the same experiment results in a rather unstructured local conformation as in this case Ala41 is now located 7.01 Å away from Pro50 and the adjacent loop has now moved away from the core of the ZmGSTF1-1. This results in a 150% increase in the volume of the conformational space were the Cu(II) atom was originally coordinated and leads to an overall destabilization. Consequently, this His40 interaction is lost and the Cu(II) atom is no longer able to be stabilized and coordinated (Fig. 5C). Therefore, Cu(II) coordination is required to optimally position the triad His40, Gln53 and Lys41. Further conformational analysis indicated that the distances between the proposed metal binding site and the C_{\beta} of Phe114 and Gln115 were 14.9 Å and 18.5 Å, respectively (Fig. 5D). These values are very close to that found (∼15 Å) in the nuclease-EDTA-Fe complexes [34] or to that (12 Å) in the case of ribulose 1,5-bisphosphate carboxylase [35]. The C_{\beta} H groups of Phe114-Gln115 are completely exposed to the bound Cu(II) (Fig. 5D) such that the attack on C_{\beta} by ROS generated at the metal-binding site could be quite favourable.

To provide experimental evidence to this suggestion, side-directed mutagenesis experiments were achieved. The amino acid residues His40, Lys41 and Gln53 were mutated to Ala. The side chain of Ala does not exhibit any metal chelate capability. The mutated forms of ZmGSTF1-1 (His40Ala, Lys41Ala and Gln53Ala) was subjected to treatment with the Cu(II)/ascorbate system. Fig. 6 shows that all mutants exhibit decreased susceptibility to inactivation by Cu(II)/ascorbate system even at higher concentrations of Cu(II), compared to the wild-type enzyme. These results confirm that His40, Lys41 and Gln53 contribute to Cu(II) binding or to the formation of the Cu(II) binding-site.
4.5. Physiological role of Cu(II) promoted oxidative cleavage of ZmGSTF1-1

Exposure of plant cells to unfavourable conditions can promote the generation of toxic reactive oxygen species (ROS) such as singlet oxygen (1O₂), superoxide radicals (O₂⁻•), hydrogen peroxide (H₂O₂) and hydroxyl radicals (•OH) [36]. It is well established that plant cells, to protect themselves against ROS, use defence systems that include isoenzymes from the GST family [2,36]. GSTs in general are overexpressed in plants exposed to stress [2]. The isoenzyme ZmGSTF1-1 from maize has initially characterised as being expressed constitutively [31]. In particular, ZmGSTF1-1 constitutes as high as 1.5% of the total soluble proteins in Zea mays root cells [37]. However, later experimental work, based on DNA microarrays analysis, showed that the enzyme is inducible and overexpressed in roots and under certain stress conditions including the exposure of the plant to heavy metals [37,38]. One role of the stress-inducible GSTs is their contribution through their function as hydroperoxidases [5]. With this function, GSTs are able to detoxify hydroperoxides through a GSH-dependent reaction. Hydroperoxides are reactive toxic compounds that are produced under oxidative stress [5]. They contribute to the mechanism known as propagative lipid peroxidation, a degenerative process that affects cell membranes, proteins and other lipid-containing structures.

ZmGSTF1-1 does not exhibit hydroperoxidase activity, as the vast majority of inducible GSTs [1,2,10], to cope with oxidative stress [39]. The finding that Cu(II) binds to ZmGSTF1-1 with specific manner and micromolar affinity, allows the assumption of an additional unidentified role for ZmGSTF1-1. Binding of Cu(II) to ZmGSTF1-1 may represent one of the available detoxification routes for Cu(II), contributing to the protection of plant cells from metal-induced deleterious effects.

Studies on other GSTs support this assumption. For example, the barley isoenzyme GST 13 reduces the accumulation of ROS in Arabidopsis plants by functioning as a scavenger for the active oxygen species [40]. In another work, octopus sigma class GST, has been shown, to be able to bind with Cu(II) [32]. This function was suggested to have important biological role to enable cephalopods to avoid copper-induced cellular toxicity [32].

5. Conclusions

In conclusion, in this work we have presented a combination of protein chemistry and protein engineering studies to characterise the interaction of ZmGSTF1-1 with Cu(II). The results of the present study may have practical significance since they can help the clarification at molecular level the biological mechanism of Cu(II)-ZmGSTF1-1 interaction. In addition, the present work forms the basis for the rational design of new engineered forms of ZmGSTF1-1 to enable Zea mays to cope with copper-induced cellular toxicity.

Conflict of interest

None.
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