Selective disulphide linkage of plant thionins with other proteins

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Abstract Thionins are shown to form disulphide linkages with other proteins. The reaction with bacterial enzymes β-glucuronidase and neomycin phosphotransferase II could be prevented and reversed with dithiothreitol and blocked with *N*-ethylmaleimide. Other cysteine-rich low-molecular-weight toxic peptides from plants (LTP-3 from barley and P19 from potato) did not react as the thionins. Certain cysteine-containing proteins, such bovine serum albumin, ovalbumin and cytochrome *c*, reacted with thionins, while others, including carbonic anhydrase, soybean trypsin inhibitor, bovine-lung trypsin inhibitor and phosphorylase B did not. Selectivity of the reaction with a periplasmic component of the phytopathogenic bacterium *Pseudomonas solanacearum* was also shown.

Key words: Thionin; Toxin; Neomycin phosphotransferase; β-Glucuronidase; Disulphide bridge; *Pseudomonas solanacearum*

1. Introduction

Thionins are low molecular mass polypeptides of about 5 kDa found in different tissues of many plant species (for reviews see [1-3]). These proteins have in vitro toxic or inhibitory activity against several bacterial and fungal plant pathogens; it has been proposed that they are have a role in plant defense. They are cysteine-rich proteins, having three or four disulfide bridges, depending on the structural type. We have previously reported that thionins are able to inhibit certain enzymes, and have presented indirect evidence indicating that the inhibition occurred by the attachment of the toxic peptide to the enzyme, possibly through the formation of disulphide links [4,5]. The enzymes β-glucuronidase (GUS) and neomycin phosphotransferase II (NPTII) were inactivated by thionins in a concentration and time-dependant manner, both in vitro and when expressed in plant protoplasts, and the inactivation was prevented and reversed by DTT [4,5]. Thionins inhibit certain bacterial and fungal plant pathogens both in vitro and when over-expressed in transgenic plants, while other pathogens are quite resistant to these peptides [6,7]. Covalent binding of the plant toxin to pathogen components could thus be relevant to its toxicity mechanism and/or to the resistance of the pathogen to it.

Here, we report direct evidence of the formation of disulphide linkages between thionins and other proteins, show that their reaction can occur at physiological pH, and investigate the selectivity of the reaction with purified proteins and with a periplasmic component of the plant bacterial pathogen *Pseudomonas solanacearum*.

2. Materials and methods

2.1. Reagents

A mixture of equimolecular amounts of α1, α2 and β-thionins from wheat was obtained by HPLC as previously described [7]. Barley lipid transfer protein LTP3 was kindly provided by Dr. A. Molina (Madrid, Spain) and protein P19 from potato was the gift of M. Moreno and A. Segura (Madrid, Spain). Purified neomycin phosphotransferase II (NPTII) was purchased from 5 Prime → 3 Prime, Inc. and β-glucuronidase (GUS) was supplied by Sigma. All other purified proteins used were from Serva. $Na^{125}I$ (100 mCi/ml) was from Amersham.

2.2. Covalent binding assays

Iodination of thionin was carried out by the chloramine T method, essentially as described [8], except that 0.3 mg/ml sodium metabisulphite and 10 mM KI (final concentrations) were used to stop the reaction. Sephadex G-25 fractions containing [^{125}I]thionin were pooled and stored at −20°C. The specific activity of the protein was 1×10^6 cpm/μg (5×10^6 cpm/nmol); 1×10^5 cpm/track were loaded on gels. Binding assays were carried out in 50 mM Tris, pH 7.0, for NPTII and in phosphate buffer (50 mM Na phosphate, pH 7, 10 mM EDTA) for all other proteins, with the additions indicated in each case. Previously described methods were used to determine NPTII [9] and GUS [10] activities. Proteins were separated by SDS-PAGE in 10–20% polyacrylamide minigels (Bio-Rad) under non-reducing conditions, and stained either for GUS activity or for protein (Coomassie blue). In experiments involving [^{125}I]thionin, gels were vacuum-dried prior to autoradiography.

Protein-glutathione mixed disulphide formation was monitored by the method of Fariss and Reed [11]. Briefly, protein was precipitated with 10% TCA, washed with cold ethanol, resuspended in 50 mM *N*-morpholino-propane-sulphonic acid, pH 8.0, 25 mM DTT, and incubated for 1 h at 37°C. The extract was precipitated with TCA and the supernatant derivatized and analyzed by HPLC as described [12].

2.3. Bacterial extracts

The bacterium *Pseudomonas solanacearum* K60 was grown in liquid King's B medium [13] at 28°C for 24 h. Isolation of periplasmic proteins was performed by the chloroform shock method [14]: 2 ml of saturated culture were centrifuged at $1100 \times g$ for 10 min; the pellet was washed twice in PBS; 20 μl of chloroform were added to the cell and left at room temperature for 15 min. Then, 100 μl of ice-cold 10 mM Tris-HCl pH 8.0 were added, mixed and centrifuged at $6000 \times g$ for 20 min. Supernatants containing periplasmic proteins were transfered to a new tube and quantified. Total bacterial protein extracts were obtained by sonication of 0.5 ml of saturated culture, followed by precipitation with 15% TCA.

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Abbreviations: DTT, dithiothreitol; EDTA, ethylendiaminetetraacetic acid; GSH/GSSG, reduced/oxidized glutathione; GUS, β-glucuronidase; HPLC, high-performance liquid chromatography; NEM, *N*-ethylmaleimide; NPTII, neomycin phosphotransferase; PBS, phosphate-buffered saline; SDS-PAGE, sodium-dodecyl-sulphate polyacrylamide-gel-electrophoresis; TCA, trichloroacetic acid.

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3. Results

3.1. Thionin reactivity

A purified GUS preparation was incubated with thionin and then subjected to gradient PAGE in the presence of 0.1% SDS and stained for activity (Fig. 1). The initial activity band was progressively converted into a ladder of activity bands, the most prominent of which had a mobility that would correspond to the addition of one thionin molecule per GUS molecule. This conclusion was confirmed in a similar experiment using [^{125}I]thionin at a lower concentration (Fig. 1). Longer incubation times, or higher thionin concentrations, led to complete enzyme inactivation and to the formation of aggregates that do not penetrate the gel upon electrophoresis, probably through the formation of protein polymers with thionins as bridge molecules. In both types of experiments, the effect of thionin could be reversed by incubation for 1 h with 10 mM DTT (Fig. 1). Similar results were obtained by treatment of NPTII with thionin (not shown). The incorporation of radioactive thionin was also prevented by preincubation of the enzyme in the presence of *N*-ethylmaleimide (NEM), a sulphydryl blocking agent, and by an excess of unlabelled thionin (Fig. 2A). Altogether, these results indicate the formation of thionin–enzyme disulphide link(s). The reaction could take place at a wide range of pH values, with a broad optimum around pH 9 (Fig. 2B). In this experiment, excess thionin migrated with the dye in SDS-PAGE, so it was separated from the enzyme after less than 5 min of being at the alkaline pH of the electrophoretic buffer. The reaction was basically not affected by salt concentration (Fig. 2C).

In contrast with the band retardation that occurred upon inactivation of GUS with oxidized thionin, GSSG inactivated GUS with formation of a faster-migrating band (Fig. 3), while no effect on enzyme activity was observed upon incubation with GSH (not shown). Again, both inactivation and the alteration in mobility could be reversed by DTT. This suggested that the formation of intramolecular disulphide bridges, rather than the formation of a GS-protein derivative, was responsible for the inactivation. Indeed, no traces of GS-protein mixed disulphides were detected in GSSG-treated GUS by the method of Fariss and Reed [11].

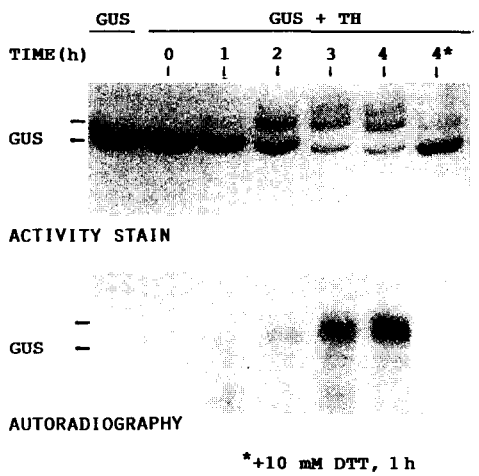

Fig. 1. Time course of thionin reaction with GUS. GUS (15 U/track) was incubated in the presence of 40 μM thionin, subjected to SDS-PAGE and stained for enzyme activity or with [^{125}I]thionin (1 μM), subjected to SDS-PAGE, and autoradiographed after vacuum-drying of the gel.

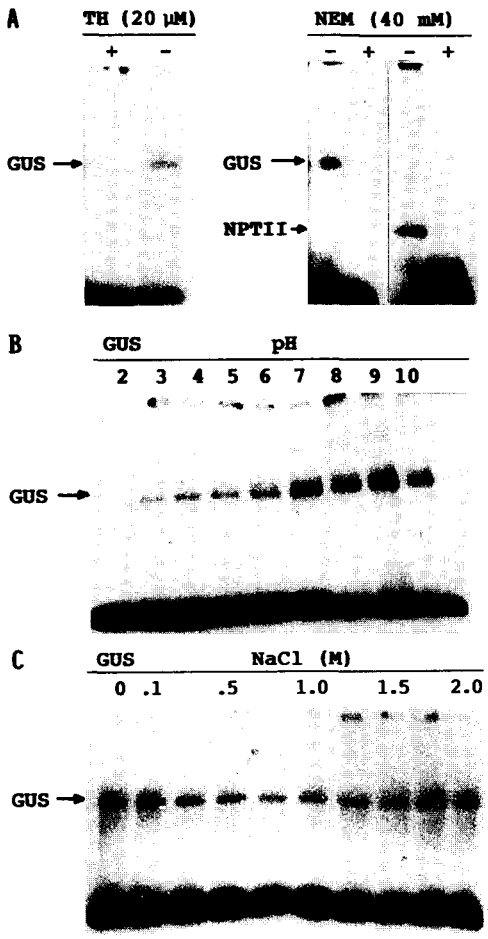

Fig. 2. Reactivity of thionins. (A) Effects of unlabelled thionin (TH) and NEM on [^{125}I]thionin incorporation. Treatment of GUS and NPTII with 40 mM NEM was for 1 h. Reactions were allowed to proceed for 3 h. (B) Effect of pH. (C) Effect of salt. (A,B,C) Horizontal arrows indicate the positions of the radioactive adducts GUS–thionin and NPTII–thionin.

3.2. Selectivity of thionin addition to proteins

The capacity of thionin to inhibit GUS and NPTII was compared with that of equimolar amounts of two other cysteine-rich, low-molecular-weight toxic polypeptides from plants, LTP3 from barley and P19 from potato, and no inhibitory activity was detected for them (Fig. 4A). Furthermore, the reactivity of [^{125}I]thionin was tested with seven additional cysteine-containing, purified proteins and only three of them (bovine serum albumin, ovalbumin, and cytochrome *c*) were able to form a radioactive derivative (Fig. 4B).

Total proteins from the bacterial pathogen *P. solanacearun* were incubated with [^{125}I]thionin and a simple pattern of radioactive bands was observed (Fig. 5). This pattern was essentially reproduced when only periplasmic proteins were incubated with [^{125}I]thionin. Formation of this particular pattern of radioactive bands was prevented by addition of excess unlabelled thionin or by DTT (Fig. 5). All the radioactive bands appeared upon mixing and were eliminated by adding 0.1% SDS to the

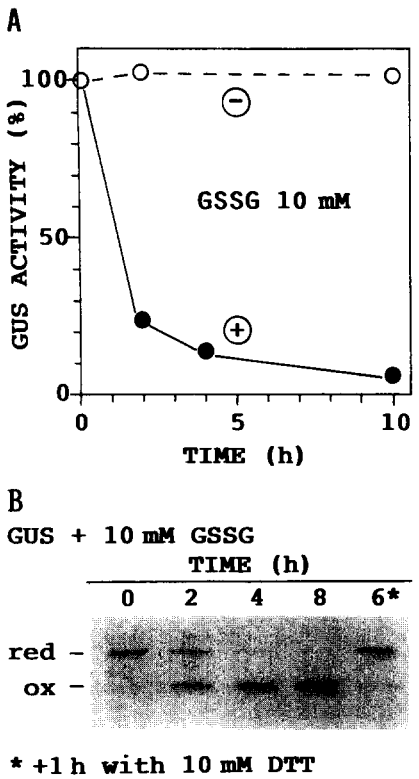

Fig. 3. Time course of GUS inactivation by 10 mM GSSG. (A) Effect on enzyme activity of 10 mM GSSG (●); control (○). (B) Effect on electrophoretic mobility. GUS was incubated in the presence of 10 mM GSSG during the indicated times, subjected to SDS-PAGE and stained for protein.

mixture, as well as by adding an excess of unlabelled thionin after 1 h of incubation with [^{125}I]thionin, except the most prominent one (arrowhead in Fig. 5). This main band was not affected under the latter conditions, but disappeared upon addition of DTT (Fig. 5), which indicated the covalent nature of the union.

4. Discussion

The formation of disulphide links between thionins and certain enzymes among those tested, in the absence of catalytic or redox coadjuvants, has been demonstrated by the incorporation of the radioactive peptide, as well as by the reversion of the reaction by DTT and its prevention by NEM. Also, the interaction has a basic pH optimum, which is in agreement with the nucleophilic nature of disulphide-exchange reactions. On short incubations with low concentrations of thionins, the 1:1 enzyme–thionin adduct predominates, whereas additional thionin molecules are probably incorporated at higher thionin concentrations or upon longer incubations. As judged from the GUS activity in gels, addition of one or two thionin molecules per molecule of GUS does not completely inactivate this enzyme, which is in agreement with the incomplete inhibition observed at intermediate thionin concentrations (10–20 μM). Both GUS and NPTII are cysteine-rich proteins (9 and 5 Cys, respectively) that are active in their reduced form, as befits their natural location in the reductive environment of bacterial cytoplasm. Therefore we predict their reaction with oxidized thionins would be of the following type:

ENZ–SH + TH–SS → ENZ–SS–TH–SH

This type of reaction does not take place between GUS and oxidized glutathione, in which case inactivation of the enzyme proceeds by the intramolecular formation of one or more disulphide bridges and the presumed generation of reduced glutathione. The reaction of thionins with proteins that have no free thiol groups, i.e. cytochrome *c*, probably involves a disulphide exchange in which 1 molecule of thionin forms 1 or 2 disulphide bridges with 1 molecule of cytochrome *c*.

The reactivity shown by thionins is not a general property of small oxidized peptides with high-cysteine content, as demonstrated by two other plant toxic peptides used as controls. The proteins LTP-3 from barley and P-19 from potato, which share these characteristics, but belong to different protein families, did not show enzyme inhibitory properties under the same conditions. Furthermore, not all cysteine-containing proteins react with thionins, and those that do react might have free -SH groups (e.g. GUS) or only -SS- bridges (e.g. oxidized cytochrome *c*). Therefore, the selectivity of the reaction probably depends on steric factors, as well as on the polarity and redox

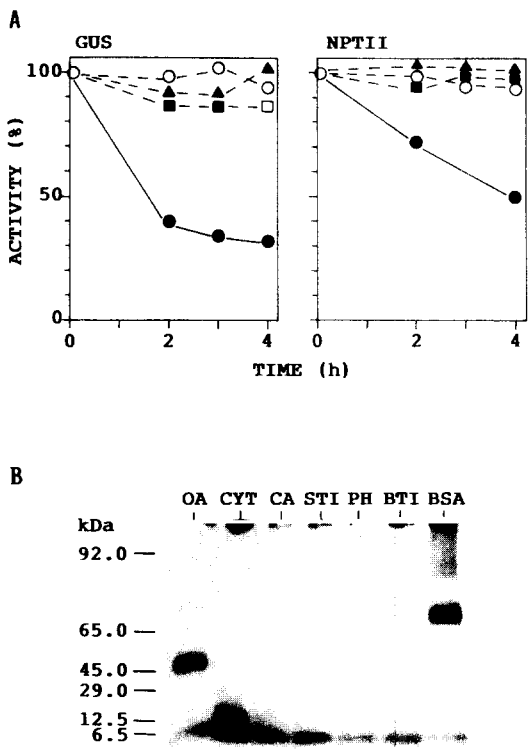

Fig. 4. Selectivity of the thionin reaction. (A) Time course of the inhibition by thionin (●), LTP3 (▲) and P19 (■) of GUS (left) and NPTII (right); untreated (○). Equimolar amounts of all three proteins were used (20 μM). (B) Differential binding of [^{125}I]thionin to the following cysteine-containing proteins: ovalbumin (OA), cytochrome *c* (CYT), carbonic anhydrase (CA), soybean trypsin inhibitor (STI), phosphorylase B (PH), bovine-lung trypsin inhibitor (BTI) and bovine serum albumin (BSA). Reactions were allowed to proceed for 3 h. Autoradiography was carried out on vaccuum-dried gels. No adduct bands were observed in the OA, CYT, and BSA tracks when DTT was added (not shown)

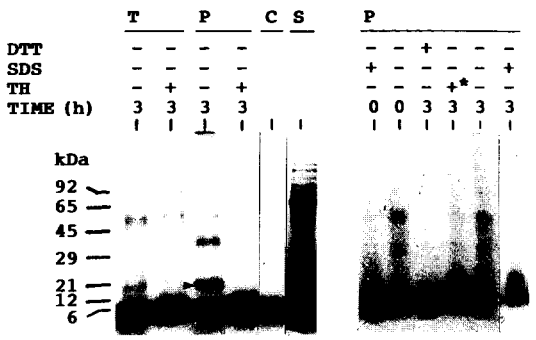

Fig. 5. Binding of [^{125}I]thionin to proteins of total (T) or periplasmic (P) extracts of *Pseudomonas solanacearum* K60, after the indicated times of incubation. Treatment (+) and control (−) are indicated. Periplasmic protein (P), 2.5 μg/track, and total protein (T), 5 μg/track, were loaded. Tracks corresponding to radioactive thionin (C) and total extract stained with Coomassie blue (S) are also shown. Additions of 2.5 mM DTT (DTT), 1% SDS (SDS), or 20 μM unlabelled thionin (TH) are indicated. (*) indicates addition of unlabelled thionin after 1 h of incubation with the radioactive protein.

properties of the microenvironment around the reactive cysteines.

The covalent binding of thionin to just one periplasmic component of the pathogenic bacterium *P. solanacearum* not only illustrates the selectivity of thionins when confronted with a natural, complex protein mixture, but also opens the possiblity that this interaction could be related to the toxicity mechanism or with resistance of the pathogen to the peptide. It should be pointed out that *P. solanacearum* K60 is very resistant to thionins, compared with other phytopathogenic bacteria (data not shown). A polypeptide transport system has been described in *Salmonella typhimurium* that is required for resistance to the antimicrobial peptides mellitin and protamine [15]. The *Salmonella* system includes a periplasmic component which seems to interact with the toxic peptides. The potential biological relevance of the interaction between thionin and the periplasmic component in *P. solanacearum* is being currently investigated through the isolation of appropriate mutants of the pathogen.

Another avenue of research is suggested by the vacuolar location of thionins ([16] and unpublished). Here it has been shown that they retain at least part of their reactivity at the acidic pH prevailing in these organelles, so the reaction could be relevant in vivo. The capacity of proteins to covalently associate with other proteins in vivo has not been extensively investigated, except for the case of the complex ubiquitin conjugation to various target proteins [17].

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