RESEARCH COMMUNICATION

Activation of oxidized cysteine proteinases by thioredoxin-mediated reduction *in vitro*

Andrew G. STEPHEN, Roy POWLS and Robert J. BEYNON*

Proteolysis Research Group, Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

Activity of the cysteine adducts of the cysteine proteinases papain and thaumatopain can be recovered by treatment with thioredoxin, thioredoxin reductase and NADPH. Recovery of proteinase activity did not occur if any of the components of the thioredoxin system were omitted, or if thioredoxin or thioredoxin reductase were heat-inactivated. Such an enzyme-mediated process may be of significance in the recovery of cysteine proteinases inactivated by oxidative attack.

INTRODUCTION

Cysteine proteinases (EC 3.4.22.X) are a group of enzymes that depend on a thiol group of a cysteine residue for catalytic activity (for example Cys-25 in papain). This cysteine residue is prone to oxidation such that, after tissue disruption, little activity can be measured unless low-molecular-mass thiol compounds are added. The nucleophilic cysteine residue of these proteinases may be oxidized by molecular oxygen, or could be attacked by other cellular oxidants to form a sulphenic (PrSOH), sulphinic ($PrSO_2H$) or sulphonic ($PrSO_3H$) acid derivative of the proteinase. Further, cysteine proteinases may be oxidized to mixed disulphide adducts of glutathione or cysteine (Klein and Kirsch, 1969). For example, the inactive form of Streptococcal proteinase is a mixed disulphide with methanesulphonic acid (Ferdinand et al., 1965; Lo et al., 1984). The oxidized cysteine residue can be recovered to the thiol form by reaction with excess thiol *in vitro* (Sluyterman, 1967). It is less clear, however, whether there is a mechanism that allows recovery of cysteine proteinases *in vivo*.

Accordingly, we have examined the possibility that redox active proteins, such as thioredoxin, may be able to re-activate cysteine proteinases. Thioredoxin is a small (12 kDa) redox active protein that contains the conserved active-site sequence Cys-Gly-Pro-Cys, which is found as either a reduced form or an oxidized form in which the cysteine residues form an intramolecular disulphide bond. Reduced thioredoxin has a number of roles *in vivo*, including hydrogen donor functions for ribonucleotide reductase, sulphate reductase and methionine sulphoxide reductase (Holmgren, 1985). Thioredoxin can also act as a general protein disulphide reductase and can catalyse the reversible reduction of exposed disulphides in proteins (Mannervik et al., 1983). In photosynthetic organisms the ferredoxin–thioredoxin system (FTR system) regulates the activity of some photosynthetic enzymes by thiol/disulphide exchange, reducing equivalents being supplied by light via ferredoxin and ferredoxin–thioredoxin reductase (Buchanan, 1986). Thioredoxin is maintained in the reduced form by the enzyme thioredoxin reductase, which transfers reducing equivalents to the protein from NADPH (Holmgren, 1985, 1989).

MATERIALS AND METHODS

Arils from ripe fruit of *Thaumatococcus daniellii*, a gift from Tate & Lyle Group Research and Development, were gathered in the Ivory Coast and shipped at −20 °C to the U.K. L-*trans*-Epoxysuccinyl-leucylamido(4-guanidino)butane (E64) and Z-Phe-Arg-7-amido-4-methylcoumarin (ZFRNHMec) were from Sigma, Poole, Dorset, U.K. Papain, 5,5′-dithiobis-(2-nitrobenzoate) (DTNB) and NADPH were from Boehringer Mannheim. Thioredoxin and thioredoxin reductase from *Escherichia coli* (both homogeneous by SDS/PAGE) were obtained from IMCO Ltd. AB, Hudiksvallsgatan 4B, S-113 30 Stockholm, Sweden. The specific activity of thioredoxin reductase was calculated as 8.6 μmol of DTNB reduced/min per mg of thioredoxin reductase (conditions of DTNB assay: 1.2 nmol of thioredoxin, 2 pmol of thioredoxin reductase, 0.3 μmol of NADPH and 0.2 μmol of DTNB in a final volume of 1 ml). All other chemicals were of analytical grade. Thaumatopain was prepared as described previously (Cusack et al., 1991).

The cysteine adduct of the cysteine proteinases was prepared according to the method of Sluyterman (1967). Papain (250 μg) or thaumatopain (120 μg) was incubated with 10 mM cysteine and 3 mM imidazole for 30 min in a final volume of 500 μl. To this was added $FeSO_4$ (1 mM final concentration), and the reaction was incubated at 4 °C for 40 h. This reaction was terminated by application of the mixture to a 10 ml Sephadex G25 column; proteins were eluted with 5 mM sodium phosphate buffer, pH 7.5, containing 10 mM EDTA. Fractions containing proteinase activity (measured after activation with dithiothreitol) were pooled, filtered through a 0.45 μm filter and dialysed overnight against 5 mM sodium phosphate buffer, pH 7.5, and 10 mM EDTA. The cysteine proteinases were quantified by active-site titration with E64 (Barrett et al., 1982).

Cysteine proteinase activity was assayed with the fluorogenic peptide derivative ZFRNHMec. Cysteine proteinases were activated with thioredoxin, thioredoxin reductase plus NADPH (NTR system) or dithiothreitol (1 mM), and aliquots were removed and added to 2.0 ml of 5 μM ZFRNHMec in 5 mM phosphate buffer/10 mM EDTA, pH 7.5. The formation of the

Abbreviations used: E64, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane; ZFRNHMec, Z-Phe-Arg-7-amido-4-methylcoumarin; DTNB, 5,5′-dithiobis-(2-nitrobenzoate); FTR, ferredoxin–thioredoxin system; NTR, NADPH–thioredoxin system.

*To whom correspondence should be addressed

product was monitored on a Perkin–Elmer 3000 recording fluorimeter (excitation 350 nm; emission 460 nm).

Thioredoxin was preincubated with thioredoxin reductase and NADPH in 5 mM NaH_2PO_4/10 mM EDTA, pH 7.5, for 10 min at ambient temperature. To this mixture was added either papain or thaumatopain in the same buffer. Samples were removed at various times and assayed for hydrolytic activity towards ZFRNHMec. Another sample of proteinase was incubated in the absence of the NTR system. At the end of each incubation, maximal proteolytic activity was determined by addition of dithiothreitol to a final concentration of 1 mM for 10 min before assay of activity towards ZFRNHMec. Special precautions were taken to exclude inactivation of low levels of active cysteine proteinases; the buffers were degassed and all incubations were in siliconized tubes overlaid with mineral oil.

RESULTS AND DISCUSSION

Commercial batches of papain displayed between 4 and 30% of the maximal activity in the absence of added chemical reducing agents. Papain purchased from Boehringer is prepared according to the method of Kimmel and Smith (1954), in which free cysteine is added to form the inactive mixed disulphide to protect the nucleophilic cysteine residue against oxidation and to minimize autolysis. For our studies, it was necessary to decrease the endogenous activity, and papain was treated further with cysteine to form the mixed disulphide. Papain thus treated was identical in activity to untreated papain after activation with 1 mM dithiothreitol (after 10 min: untreated papain, 4.6 μmol of NHMec released/min per mg of papain; cysteine adduct papain, 4.57 μmol of NHMec released/min per mg of papain).

Re-activation of oxidized papain was attained in a two-step incubation. In the first stage, thioredoxin (5 nmol) was preincubated with thioredoxin reductase (50 pmol) and NADPH (60 nmol) for 10 min. Papain (78 pmol) was added and samples were removed and assayed for activity towards ZFRNHMec. Replicate incubations were included from which each component of the thioredoxin system was omitted in turn (Figure 1). Activation of papain was only observed when the complete thioredoxin system was included. Thus thioredoxin reductase is apparently able to transfer reducing equivalents from NADPH to papain via thioredoxin.

The activity of the NTR system was assayed independently with DTNB (Holmgren, 1984). However, if thioredoxin or thioredoxin reductase were heated to 100 °C for 45 min or 2 min respectively, the ability to reduce DTNB was lost. Similarly, papain is no longer activated if either thioredoxin or thioredoxin reductase is heat-denatured (results not shown). Activation of papain by the NTR system is an enzymic process.

Thioredoxin reductase reacts very rapidly with thioredoxin (the catalytic centre activity is about 4000 min^{-1}; Williams, 1976). Under the conditions used here (10 pmol of thioredoxin reductase and 500 pmol of thioredoxin), all of the thioredoxin should be reduced within the first few seconds. The rate of papain activation is independent of the thioredoxin reductase concentration (with 500 pmol of thioredoxin, the rate of papain activation with 1 or 50 pmol of thioredoxin reductase was 16.2 and 17.9 pmol of papain activated/h respectively). The rate-limiting step is the reaction between thioredoxin and papain.

The rate of papain activation was also determined at different thioredoxin concentrations (Figure 2). The average rate of papain activation with 5, 0.5 and 0.05 nmol of thioredoxin was 17.5 (n = 3), 14.1 (n = 5) and 5.68 (n = 2) pmol of papain activated/h respectively. Activation of papain by thioredoxin is dependent on the concentration of thioredoxin, in an apparently saturable fashion (Figure 2, inset). At high thioredoxin/papain ratios, the rate of activation of papain is limited, possibly by a slow transfer of reducing equivalents from thioredoxin to papain or by the formation of a slowly decaying thioredoxin–papain complex.

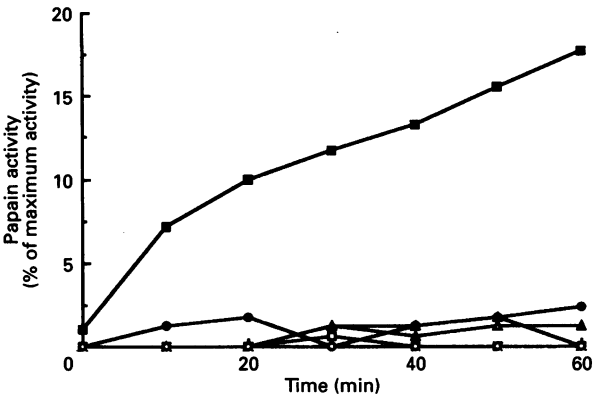

Figure 1 Activation of papain by thioredoxin-mediated reduction

Thioredoxin (5 nmol) was preincubated with thioredoxin reductase (50 pmol) and NADPH (60 pmol) in 5 mM NaH_2PO_4/10 mM EDTA, pH 7.5 (final volume 25 μl) for 10 min at ambient temperature (■). To this was added papain (78 pmol) in 5 mM NaH_2PO_4/10 mM EDTA, pH 7.5, making the final volume 200 μl. Aliquots of 10 μl (containing 3.9 pmol of papain) were removed and assayed for proteolytic activity with ZFRNHMec (5 μM in 5 mM NaH_2PO_4/10 mM EDTA, pH 7.5). Replicate incubations were prepared from which each component of the NTR system was omitted in turn, i.e. no thioredoxin (●), no NADPH (△), no thioredoxin reductase (▲), no papain (○) and no NTR system (□).

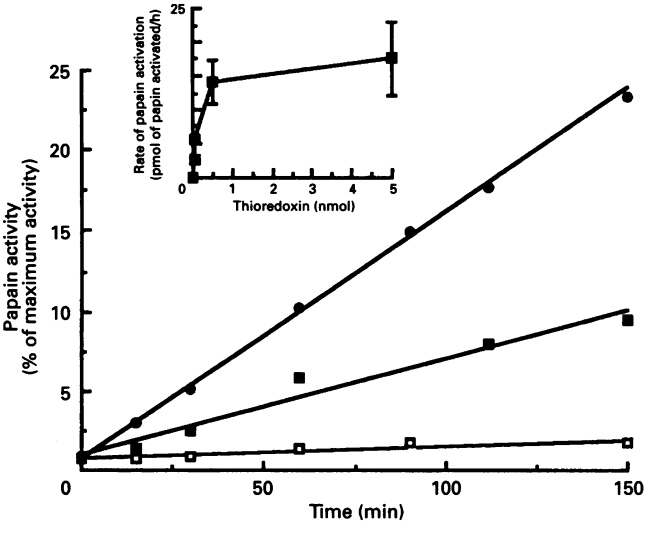

Figure 2. Dependence of papain activation on thioredoxin

Thioredoxin [500 (●) and 50 (■) pmol], thioredoxin reductase (10 pmol) and NADPH (60 nmol) were incubated in 5 mM NaH_2PO_4/10 mM EDTA, pH 7.5 (final volume 25 μl) for 10 min at ambient temperature. To this was added papain (156 pmol) in 5 mM NaH_2PO_4/10 mM EDTA, pH 7.5, making the final volume 200 μl. A control, in which papain was incubated in the absence of the NTR system, was included (□). Samples (10 μl, containing 7.8 pmol of papain) were assayed for proteolytic activity towards ZFRNHMec (5 μM in 5 mM NaH_2PO_4/10 mM EDTA, pH 7.5). The inset shows the relationship between the rate of papain activation and the thioredoxin concentration. The data points for 0.05 nmol of thioredoxin are single values; results for 0.5 nmol of thioredoxin and for 5 nmol of thioredoxin are presented as means ± S.D. (n = 5 and 3 respectively).

SDS/PAGE of thioredoxin labelled with fluorescent monobromobimane (Kobrehel et al. 1992) indicated that thioredoxin was not being proteolysed by papain. This reaction was also investigated with thaumatopain, the cysteine proteinase from the arils of the tropical plant *Thaumatococcus daniellii* (Cusack et al., 1991). The cysteine adduct of thaumatopain was activated by the NTR system: the initial rate of activation of thaumatopain by thioredoxin (500 pmol) was 5 pmol of thaumatopain activated/h (results not shown). Thus thaumatopain is activated at a similar rate to papain.

The storage proteins in many seeds are degraded by cysteine proteinases (Mikola, 1983). Many of these cysteine proteinases are synthesized *de novo* (Baumgartner and Chrispeels, 1977; Koehler and Ho, 1988, 1990). However, cysteine proteinases are also present in resting seeds, and these may require activation during the onset of germination (Mikola, 1983). Shutov and Vaintraub (1987) suggested that low-molecular-mass thiol compounds and protein disulphide reductases could maintain cysteine proteinases in the reduced active form. Protein disulphide reductases have been isolated from pea seeds (Hatch and Turner, 1960) and wheat grain (Gorpinchenko and Vakat, 1975). However, these groups did not consider the possibility of a thioredoxin-mediated process. Thioredoxin h in wheat seeds, activated by thioredoxin reductase and NADPH, can reduce disulphide bonds in storage proteins of the gliadin and glutenin classes (Kobrehel et al., 1992). Further, reduction of the gliadins and glutenins *in vivo* preceded their proteolytic degradation. Thioredoxin-mediated reduction could thus have two important roles in seed germination: to increase the susceptibility of storage proteins to degradation, and to recover oxidatively inactivated cysteine proteinases to effect that degradation. However, we recognize that the biological significance of these results depends on further experiments demonstrating similar systems for the reactivation of oxidized cysteine proteinases *in vivo*.

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REFERENCES

Barrett, A. J., Kembhavi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tamai, M. and Hanada, K. (1982) Biochem. J. **201**, 189–198
Baumgartner, B. and Chrispeels, M. J. (1977) Eur. J. Biochem. **77**, 223–233
Buchanan, B. B. (1986) in Thioredoxin and Glutaredoxin Systems: Structure and Function (Holmgren, A., Brändén, C.-I., Jörnvall, H. and Sjöberg, B.-M., eds.), pp. 233–242, Raven Press, New York
Cusack, M., Stephen, A. G., Powls, R. and Beynon, R. J. (1991) Biochem. J. **274**, 231–236
Ferdinand, W., Stein, W. H. and Moore, S. (1965) J. Biol. Chem. **240**, 1150–1155
Gorpinchenko, T. V. and Vakat, A. B. (1975) Biokhimiya **40**, 323–330
Hatch, M. D. and Turner, J. F. (1960) Biochem. J. **76**, 556–562
Holmgren, A. (1984) in Posttranslational Modifications Part B (Wold, F. and Moldave, K., eds.), pp. 295–304, Academic Press, New York
Holmgren, A. (1985) Annu. Rev. Biochem. **54**, 237–271
Holmgren, A. (1989) J. Biol. Chem. **264**, 13963–13966
Kimmel, J. R. and Smith, E. L. (1954) J. Biol. Chem. **207**, 515–531
Klein, I. B. and Kirsch, J. F. (1969) Biochem. Biophys. Res. Commun. **34**, 575–581
Kobrehel, K., Wong, J. H., Balogh, A., Kiss, F., Yee, B. C. and Buchanan, B. B. (1992) Plant Physiol. **99**, 919–924
Koehler, S. M. and Ho, T.-H. D. (1988) Plant Physiol. **87**, 95–103
Koehler, S. M. and Ho, T.-H. D. (1990) The Plant Cell **2**, 769–783
Lo, S.-S., Fraser, B. A. and Liu, T.-Y. (1984) J. Biol. Chem. **259**, 11041–11045
Mannervik, B., Axelsson, K., Sunewall, A.-C. and Holmgren, A. (1983) Biochem. J. **213**, 519–523
Mikola, J. (1983) in Seed Proteins (Daussant, J., Mosse, J. and Vaughan, J., eds.), pp. 35–51, Academic Press, New York
Shutov, A. D. and Vaintraub, I. A. (1987) Phytochemistry **26**, 1557–1566
Sluyterman, L. A. Æ. (1967) Biochim. Biophys. Acta **139**, 430–438
Williams, C. H., Jr. (1976) Enzymes 3rd Ed. **13**, 89–173