PUROTHIONIN: A SEED PROTEIN WITH THIOREDOXIN ACTIVITY

Keishiro WADA and Bob B. BUCHANAN+

Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan and +*Department of Plant and Soil Biology, University of California, Berkeley, CA 94720, USA*

Received 22 December 1980

1. Introduction

Purothionins are basic proteins of ~5000 M_r [1–3] that have 8 half-cystine residues bonded to form disulfide bridges [4–7]. Purothionins occur in the seed endosperm of cereals such as wheat, barley and rye [8,9], and show the curious property of being toxic to animals, when injected intraperitoneally or intravenously [10], and to certain yeasts [11] and cultured mammalian cells [12] when present in their growth media. The physiological function of purothionins has not been established.

Purothionins show a chemical resemblance to thioredoxins – a group of widely distributed multifunctional proteins of low M_r that undergo oxidation/reduction through the interconconversion of S–S/SH groups. Thioredoxins have been shown to function as regulatory proteins that alter enzyme activity through thiol redox changes [13]. Thioredoxins appear to play a particularly important regulatory role in photosynthesis through their ability to link light to the activity of several key chloroplast enzymes [14].

Because of the chemical similarity to thioredoxins, we considered it worthwhile to examine purothionin for thioredoxin activity. We now report that purothionin obtained from wheat endosperm (flour) can substitute for a specific thioredoxin from spinach chloroplasts in the activation of a photosynthetic enzyme. These findings provide a new in vitro assay for conveniently measuring purothionin activity and, in addition, constitute evidence that purothionins may function as regulatory proteins.

2. Materials and methods

Chemical compounds were purchased from Wako Pure Chemical Industries (Osaka), Nakarai Chemical Co. (Kyoto) and Sigma Chemical Co. (St Louis, MO). Wheat (*Triticum vulgare*) flour and spinach leaves were purchased from local markets. Corn (*Zea mays*) leaves were harvested from greenhouse-grown plants.

Purothionin I component was purified from wheat flour by the method described for barley purothionin homolog [15] except that a linear gradient (0.1–0.8 M) of NH_4HCO_3 buffer (pH 8.3) was used. Viscotoxin was prepared from European mistletoe leaves by this same procedure. Chloroplast thioredoxins *f* and *m*, fructose 1,6-bisphosphatase (FBPase), NADP–malate dehydrogenase (NADP–MDH) from spinach leaves and ferredoxin–thioredoxin reductase from corn leaves were purified as in [16]. Washed spinach chloroplast membranes were prepared in sucrose as in [17], except that the buffer solution consisted of 50 mM tricine (pH 7.6), 3 mM $MgCl_2$, 1 mM EDTA, and 2 mM sodium ascorbate. Assays for thioredoxins *f* and *m* were modifications of methods developed in [16].

3. Results and discussion

In the initial phase of this study, we tested purothionin in enzyme activation reactions that differentiate between the two types of chloroplast thioredoxins, thioredoxins *f* and *m*. These assay systems determine the ability of a thioredoxin (in the reduced state) to activate chloroplast FBPase and NADP–MDH; thioredoxins of the *f*-type preferentially activate FBPase while thioredoxins of the *m*-type activate NADP–MDH [16]. The thioredoxins analyzed in these assays are routinely reduced with the dithiol reagent, dithiothreitol (DTT).

As seen in table 1, purothionin showed significant thioredoxin *f* (FBPase activation) activity, but little, if any, thioredoxin *m* (NADP–MDH activation) activ-

Table 1
Effectiveness of purothionin in the activation of chloroplast FBPase and NADP–MDH with DTT as reductant

Treatment	FBPase activity (nmol P_i released/min)	NADP-MDH activity (nmol NADPH oxidized/min)
Control	3	0
Purothionin	38	0.3
Thioredoxin *f*	75	–
Thioredoxin *m*	–	7.7

The FBPase assay was carried out in air at 22°C in test tubes containing (in 0.45 ml) FBPase, 16 μg; thioredoxin *f*, 2 μg, or purothionin, 50 μg, as indicated, and the following (in μmol): tricine–NaOH buffer (pH 7.9), 50; $MgSO_4$, 0.5; DTT, 2.5. After the components were preincubated for 5 min, the reaction was initiated by adding 0.05 ml 60 mM fructose 1,6-bisphosphatate. The reaction was stopped after 20 min by the addition of 2.0 ml Fiske-SubbaRow P_i analysis mixture. For assaying NADP–MDH activity, partially purified spinach chloroplast NADP–MDH (32 μg) was preincubated at 22°C in 0.2 ml solution containing chloroplast thioredoxin *m* as needed, 20 μmol Tris–HCl buffer (pH 7.9), and 2 μmol DTT. After preincubation for 5 min, the mixture was injected into a 1 cm cuvette of 1 ml capacity that contained (μmol): Tris–HCl buffer (pH 7.9), 100; NADPH, 0.25. The reaction was started by the addition of 2.5 μmol oxalacetic acid (final vol. 1 ml). The ΔA_{340} was measured

ity. Under the standard assay conditions in table 1, the activation of FBPase by purothionin was ~2% that of authentic thioredoxin *f*. However, as seen below, it was possible to improve the activity of purothionin quite markedly by increasing the time of preincubation and the concentration of DTT.

Fig.1 shows that preincubation of FBPase with DTT and purothionin was necessary for optimal activity. Furthermore, the time required for full activation with the standard 5 mM DTT (2 hours) was ~5-times that required with chloroplast thioredoxin *f* [18]. Such a long preincubation time was, however, found unnecessary at elevated concentrations of DTT. As shown in fig.2, an increase in the DTT from 5–28 mM resulted in up to a 6-fold increase in the purothionin-mediated activation of the FBPase. When tested with 28 mM DTT, purothionin showed (on a mg basis) a maximal

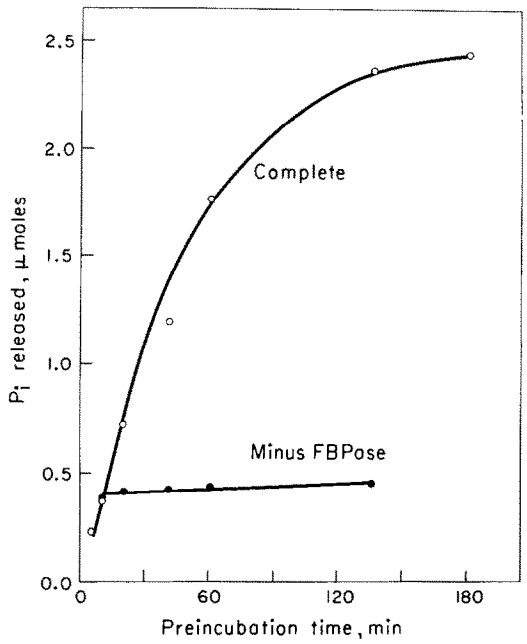


Fig.1. Effect of preincubation time on the purothionin-linked activation of chloroplast FBPase. Purothionin and DTT were preincubated either with FBPase (complete) or without FBPase (minus FBPase); except for using 16.5 μg purothionin and changing the time of preincubation; conditions were as in table 1.

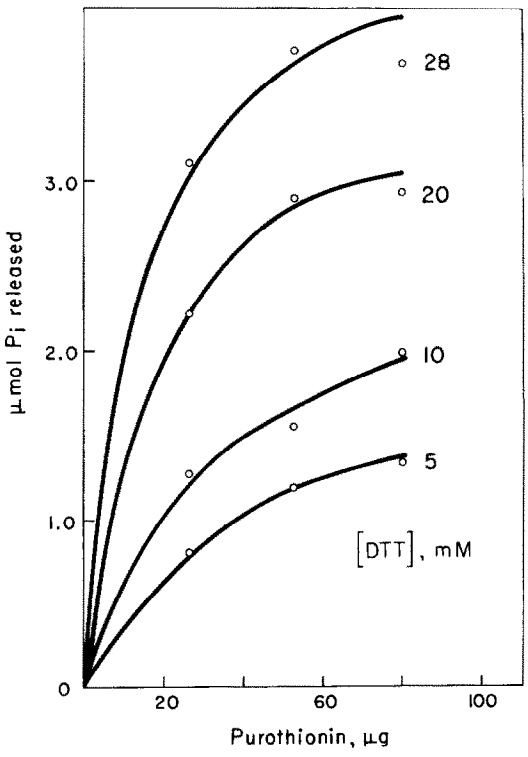


Fig.2. Effect of DTT and purothionin concentration on activation of chloroplast FBPase. FBPase (16 μg) was preincubated for 10 min with the indicated amounts of purothionin and DTT and then assayed as given in table 1, except that the reaction time was 15 min.

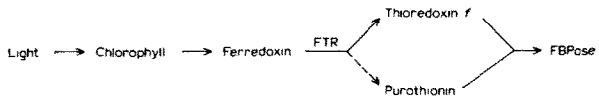
activity ~25% that of thioredoxin *f* (preincubation time, 10 min). It thus seems that purothionin can effectively promote the activation of FBPase, but that the rate of activation is low unless the DTT concentration is increased substantially over that normally employed. As is the case of thioredoxin *f* [19,20] neither reduced glutathione nor 2-mercaptoethanol could replace DTT in the activation of FBPase by purothionin. By contrast, reduced lipoic acid, a dithiol could be used in lieu of DTT in this reaction. It is noteworthy that, with DTT as reductant, disulfide-containing proteins related to purothionin also promoted the activation of chloroplast FBPase – i.e., barley seed purothionin homolog [15] and European mistletoe leaf viscotoxin [21].

The finding that purothionin activates FBPase in the presence of DTT raises the question whether purothionin can also promote photochemical enzyme activation in the presence of ferredoxin and ferredoxin–thioredoxin reductase. An answer to this question is provided by table 2 which shows that the purothionin-mediated activation of FBPase was marginal when purothionin was tested alone in the photochemical assay system. However, when purothionin was added together with thioredoxin *f*, a synergistic activation effect was observed – i.e., the activity with purothionin + thioredoxin *f* was appreciably greater than the sum of the activities with each of the proteins alone. Accordingly, it seems that purothionin is not effectively reduced by ferredoxin–thioredoxin reductase, but is reduced by thioredoxin *f*. Once reduced, purothionin can then participate in FBPase activation as indicated below. (The solid arrows indicate the main reactions; FTR = ferredoxin–thioredoxin reductase.)

Table 2
Synergistic effect of purothionin and thioredoxin *f* in the light activation of FBPase

Treatment	FBPase activity (nmol P_i released/min)
Control	7
+ Purothionin	13
+ Thioredoxin *f*	61
+ Purothionin + thioredoxin *f*	97

The reaction was carried out at 20°C in Warburg-Krippahl flasks containing (in the sidearm) 6 μmol sodium fructose 1,6-bisphosphate and (in the main compartment 24 μg spinach FBPase, twice-washed spinach chloroplast membrane fragments equiv. 0.1 mg chlorophyll, 55 μg spinach chloroplast ferredoxin, 15 μg corn ferredoxin–thioredoxin reductase, thioredoxin *f* (5 μg) and purothionin (25 μg) as indicated, and the following (μmol): tricine–NaOH buffer (pH 7.9); 100; sodium ascorbate, 10; 2,6-dichlorophenolindophenol, 0.1; $MgSO_4$, 1.0 (final vol. 1.5 ml). After equilibration for 5 min with N_2, vessels were preincubated for 5 min in the light (20 000 lux). The reaction was started by adding fructose 1,6-bisphosphate from the sidearm and was continued for 30 min under illumination. The reaction was stopped by the addition of 0.5 ml 10% trichloroacetic acid. The precipitate was centrifuged off, and 0.5 ml supernatant solution was analyzed for P_i



In view of the ability of purothionin to replace thioredoxin *f* in a specific assay, the question might be posed as to whether purothionin should be considered as a form of thioredoxin such as is known to be present in seeds [20,22,23]. While this question should be given further consideration when our knowledge of seed thioredoxins is more complete, it now seems that discrete differences exist between the two proteins. Accordingly, unlike the *f*-type thioredoxins that we have isolated from barley seeds [24], purothionin is stable to heat (3 min, 80°C) and toxic to mice (not shown). Thus, although purothionin resembles thioredoxins in several respects, it differs significantly in others and, pending further evidence, should retain its original name.

4. Concluding remarks

In demonstrating an association of thioredoxin *f* activity with purothionin, these experiments provide an in vitro assay that should be specific for purothionin once interfering thioredoxins are eliminated from seed extracts. The availability of the FBPase assay thus divorces purothionin investigations from the need for toxicity tests that had been required to monitor the protein and, thereby, opens the door for a search for nontoxic purothionins. It remains to be seen whether such forms of purothionin exist, and whether these forms (as well as the known toxic type) exercise a regulatory function in seeds as is suggested by these findings.

Acknowledgements

K. W. is grateful to Dr H. Yoshizumi of Suntory Institute for Biomedical Research (Suntory Ltd) for

interest and support and to Professor H. Matsubara for his encouragement in this work. The assistance of Ms Nancy Crawford is also gratefully acknowledged. This work was aided by a grant from the National Science Foundation to B. B. B.

References

[1] Balls, A. K. and Hale, W. S. (1940) Cereal Chem. 17, 243–245.
[2] Balls, A. K., Hale, W. S. and Harris, T. H. (1942) Cereal Chem. 19, 279–288.
[3] Balls, A. K., Hale, W. S. and Harris, T. H. (1942) Cereal Chem. 19, 840–844.
[4] Ohtani, S., Okada, T., Kagamiyama, H. and Yoshizumi, H. (1975) Agric. Biol. Chem. 39, 2269–2270.
[5] Ohtani, S., Okada, T., Yoshizumi, H. and Kagamiyama, H. (1977) J. Biochem. 82, 753–767.
[6] Jones, B. L. and Mak, A. S. (1977) Cereal Chem. 54, 511–523.
[7] Hase, T., Matsubara, H. and Yoshizumi, H. (1978) J. Biochem. 83, 1671–1678.
[8] Redman, D. G. and Fischer, N. (1969) J. Sci. Food Agric. 20, 427–432.
[9] Hernandes-Lucas, C., Carbonera, P. and Garcia-Olmedo, F. (1978) J. Agric. Food Chem. 26, 794–796.
[10] Coulson, E. J., Harris, T. H. and Axelrod, B. (1942) Cereal Chem. 19, 301–307.
[11] Okada, T., Yoshizumi, H. and Terashima, Y. (1970) Agric. Biol. Chem. 34, 1084–1088.
[12] Nakanishi, T., Yoshizumi, H., Tahara, S., Hakura, A. and Toyoshima, K. (1979) Gann 70, 323–326.
[13] Buchanan, B. B., Wolosiuk, R. A. and Schurmann, P. (1979) Trends Biochem. Sci. 4, 93–96.
[14] Buchanan, B. B. (1980) Annu Rev. Plant Physiol. 31, 341–374.
[15] Ozaki, Y., Wada, K., Hase, T., Matsubara, H., Nakanishi, T. and Yoshizumi, H. (1980) J. Biochem. 87, 549–555.
[16] Wolosiuk, R. A., Crawford, N. A., Yee, B. C. and Buchanan, B. B. (1979) J. Biol. Chem. 254, 1627–1632.
[17] Buchanan, B. B., Kalberer, P. P. and Arnon, D. I. (1967) Biochem. Biophys. Res. Commun. 29, 74–79.
[18] Schurmann, P. and Wolosiuk, R. A. (1978) Biochim. Biophys. Acta 522, 130–138.
[19] Buchanan, B. B., Schurmann, P. and Kalberer, P. P. (1971) J. Biol. Chem. 246, 5952–5959.
[20] Wolosiuk, R. A. and Buchanan, B. B. (1977) Nature 266, 565–567.
[21] Samuelsson, G. and Pettersson, B. M. (1971) Eur. J. Biochem. 21, 86–89.
[22] Buchanan, B. B. and Wolosiuk, R. A. (1976) Nature 264, 669–670.
[23] Suske, G., Wagner, W. and Follmann, H. (1979) Z. Naturforsch. 34c, 214–221.
[24] Crawford, N. A., Yee, B. C. and Buchanan, B. B. (1980) Plant Physiol. 65 (6S) 126.