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Research article

Identification of a 2-cys peroxiredoxin as a tetramethyl benzidine-hydrogen peroxide stained protein from the thylakoids of the extreme halophyte *Arthrocnemum macrostachyum* L.

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ABSTRACT

Tetramethylbenzidine-H_2O_2 staining of SDS-polyacrylamide gel is a widely used method for the specific detection of proteins with heme-dependent peroxidase activity. When this method was used with thylakoids from the halophytic plant *Arthrocnemum macrostachyum*, besides the cytochrome f and cytochrome b6 proteins usually found in higher plants and cyanobacteria, at least four additional bands were detected. One of them, a 46-kDa protein, was shown to be an extrinsic protein, and identified by mass spectrometry and immunoblotting as a 2-cys peroxiredoxin. Peroxidase activity was insensitive to oxidizing agents such as trans-4,4-diydroxy-1,2-dithiane or hydrogen peroxide, but was inhibited by treatment of thylakoids with reducing agents such as dithiothreitol or mercaptoethanol. By immunoblotting, it was shown that loss of peroxidase activity was paralleled by disappearance of the 46-kDa band, which was converted to a 23-kDa immunoreactive form. A dimer/monomer relationship between the two proteins is suggested, with the dimeric form likely being a heme-binding protein. This possibility was further supported by anionic exchange chromatography and *de novo* sequencing of tryptic fragments of the protein and sequence comparison, as most of the residues previously implicated in heme binding in 2-cys peroxiredoxin from *Rattus norvegicus* were conserved in *A. macrostachyum*. The amount of this protein was modulated by environmental conditions, and increased when salt concentration in the growth medium was higher or lower than the optimal one.

1. Introduction

Peroxiredoxins, the most recently discovered members of the peroxidase family, are a group of heme-independent, thiol-based redox proteins found in bacteria, animals and plants [1–3]. In Arabidopsis, peroxiredoxins are encoded by a gene family containing ten members [1,4] that belong, on the basis of their reaction mechanism and electron donor, to four different groups: 1-Cys Prx, 2-Cys Prx, type II Prx and PrxQ. In chloroplasts, the presence of three different types of peroxiredoxins has been reported: Prx Q, Prx II E, and two very similar forms of 2-Cys Prxs, 2-Cys Prx A and 2-Cys Prx B [1]. As oxygenic photosynthesis involves the transport of electrons in the presence of oxygen, it is almost unavoidable that reactive oxygen species are produced [5]. Thus, a role for peroxiredoxins in the protection from photo-oxidative stress has been put forward since their discovery in chloroplasts [6,7]. In addition to this function, peroxiredoxins have a role in signalling, as they contribute to the regulation of the level of reactive oxygen species [8,9] and can work in a highly oligomeric state, which depends on redox potential, as molecular chaperons [10].

In the reaction mechanism of peroxiredoxins, the peroxide molecule is attacked by a cysteine residue (the peroxidatic cysteine, Cys_P) which becomes transiently oxidized to sulphenic acid [11].

Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; nano-LC/Q-TOF-MS/MS, nano high pressure liquid chromatography coupled with electron spray ionization and quadrupole time-of-fly mass spectrometry; oxDTT, trans-4,4-diydroxy-1,2-dithiane; TMBZ, 3,3′,5,5′-tetramethyl benzidine.

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This intermediate is promptly reduced by a second cysteine residue (the resolving cysteine, Cys_R) which, in 2-cys peroxiredoxin, is located on a second, identical polypeptide. Water, or the corresponding alcohol, is then produced from the peroxide, and the thiol groups of the two cysteines are oxidized to form a disulphide bridge [3,11]. To enter in a new catalytic cycle, this disulphide bridge has to be reduced by an endogenous electron donor, the nature of which depends on the type of peroxiredoxin [11].

Some lines of evidence have suggested that typical 2-cys peroxiredoxin HPB23 from rat liver [12,13] is a heme-binding protein, as shown by binding of the protein to heme-agarose upon affinity chromatography; X-rays crystallographic studies have provided indications on possible aminoacidic residues involved in the heme binding [13]. More recently, another typical 2-cys peroxiredoxin, i.e. the AhpC from the bacterium *Streptococcus agalactiae* [14], was found to be a heme-binding protein, as it binds to heme-agarose resin and can be reconstituted with hemin. Given the high sequence similarity between these two heme-binding 2-cys peroxiredoxins and 2-cys peroxiredoxin from plants, as well as other similar structural features [15], we investigated the possibility that, at least under enhanced stress conditions, plant peroxiredoxins could also bind heme. With this aim in mind, the extreme halophyte *Arthrocnemum macrostachyum* was used as, when grown under different salt conditions, modifications of Photosystem II are induced, which resulted in enhanced light stress [16,17]. Tetramethylbenzidine–hydrogen peroxide staining was then used for the detection of anti-oxidant heme-containing proteins. A number of bands were detected in addition to the canonical cytochromes f and b6 and one of these, with an apparent molecular weight of 46 kDa, was shown to be a dimeric form of 23-kDa 2-cys peroxiredoxin. These findings provide new evidence that, at least in the dimeric form, also the plant peroxiredoxin(s) can bind heme.

2. Results

2.1. A 46-kDa extrinsic thylakoid peroxidase stained by TMBZ–H_2O_2 is tentatively identified a 2-cys peroxiredoxin

In Fig. 1A and B, a TMBZ–H_2O_2–stained and a Coomassie stained SDS gels containing *A. macrostachyum* and spinach thylakoids solubilised for 30 min at 0 °C in the absence of DTT are respectively shown. Upon TMBZ–H_2O_2 staining, two bands at 32 and 23 kDa were detected in spinach thylakoids (1A, spinach) which, according to a previous reports [18–20], can be ascribed to cytochrome f and cytochrome b6, respectively. In *A. macrostachyum* thylakoids, at variance, at least six bands were detected (1A, Arthrocnemum), that is a prominent band at 46 kDa (indicated by a black arrow on the left side of Fig. 1A), a quadruplet at 38, 37, 36 and 34 kDa, and a 23-kDa polypeptide (indicated on the right side of Fig. 1A). While the 23-kDa band was identified on the base of its apparent molecular weight as the cytochrome b6 protein (1A), the identification of cytochrome f was not obvious because of the presence of additional stained bands in the same region of the gel. As shown in Fig. 1C, upon immunoblotting with polyclonals to cytochrome f, a main 32-kDa band was recognised in spinach, whereas two closely migrating bands were immunodetected in the 36–38 kDa region in the *A. macrostachyum* sample, corresponding to the two slowest migrating bands of the quadruplet (i.e., bands at 38 and 37 kDa) detected by TMBZ–H_2O_2; the smaller band (i.e. the 37 kDa one) likely represented a degradation product of the 38-kDa cytochrome f polypeptide, as reported also in other species [21]. A diffuse background was also observed in the high molecular weight region of gel (Fig. 1A).

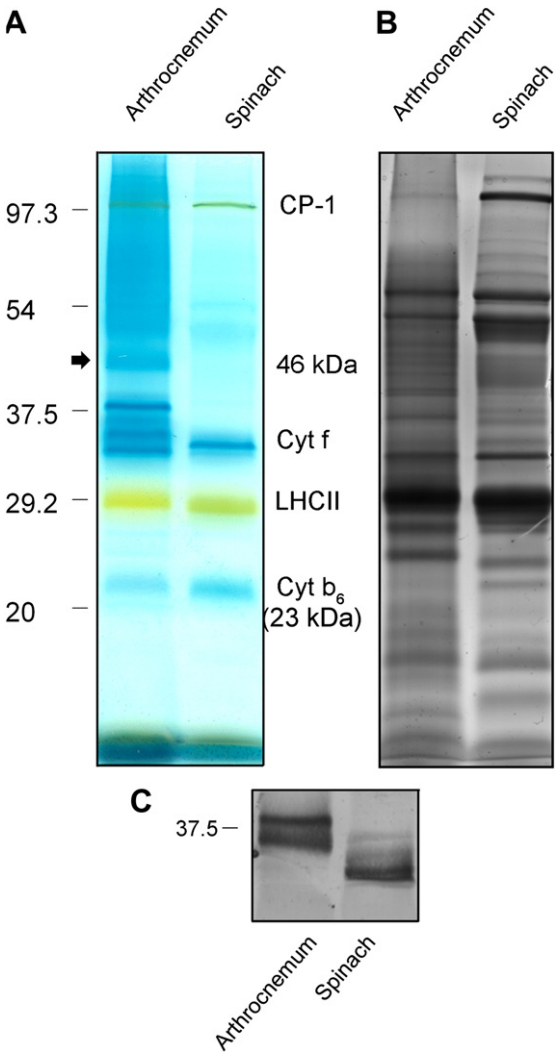

Fig. 1. A TMBZ–H_2O_2–stained gel (A), Coomassie-stained gel (B), and an immunoblot with anti-cytochrome f antibodies (C) of *A. macrostachyum* and spinach thylakoids. The 46 kDa band is indicated by a black arrow on the left side of panel A. The molecular weight of cytochrome b6 (23 kDa) is indicated too. On the left side of panel A and C, the position of molecular markers is indicated. A total of 4 μg chlorophyll was loaded on each gel lane.

Interaction of these additional TMBZ–H_2O_2–stained proteins with thylakoids was investigated by means of washing experiments. We found that when thylakoids (Fig. 2, lane 1) were treated with HEPES/EDTA at pH 7 or higher (a standard procedure for removal of proteins weakly bound to the stromal side of thylakoids), 46, 36 and 34 kDa bands were removed from the thylakoids (Fig. 2, lane 2); cytochromes, instead, were only slightly affected by washing; the 46-kDa protein was recovered in the supernatant and concentrated by using Centricon tubes (Fig. 2, lane 3) (it should be noted that this washing procedure also removed the 36 and 34-kDa proteins but their identity was not further investigated in this work). The 46-kDa protein, located by TMBZ–H_2O_2 staining (lane 3), was cut out of the gel and subjected to re-electrophoresis on a urea containing gel as a further purification step. Staining with Coomassie allowed the detection of a doublet around 46 kDa (Fig. 2, lane 4) which was subjected to in-gel trypsin digestion. The peptides were eluted, resolved by nano-LC, and analysed by tandem mass spectrometry obtaining, from both bands, the same peptides listed in Table 1. A ion MASCOT search indicated that their sequences matched significantly with those of an unknown gene product from *Vitis*