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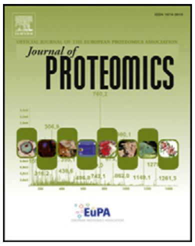

Functional and structural changes in plant mitochondrial PrxII F caused by NO

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ABSTRACT

Peroxiredoxins (Prxs) have emerged as important factors linking reactive oxygen species (ROS) metabolism to redox-dependent signaling events. Together with ROS, nitric oxide (NO) is a free radical product of the cell metabolism that is essential in the signal transduction. S-Nitrosylation is emerging as a fundamental protein modification for the transduction of NO bioactivity. Using recombinant pea mitochondrial PsPrxII F (PrxII F), the effect of S-nitrosoglutathione (GSNO) and sodium nitroprusside dehydrate (SNP), which are known to mediate protein S-nitrosylation processes, was studied. S-Nitrosylation of the PrxII F was demonstrated using the biotin switch method and LC ESI-QTOF tandem MS analysis. S-nitrosylated PrxII F decreased its peroxidase activity and acquired a new transnitrosylase activity, preventing the thermal aggregation of citrate synthase (CS). For the first time, we demonstrate the dual function for PrxII F as peroxidase and transnitrosylase. This switch was accompanied by a conformational change of the protein that could favor the protein–protein interaction CS-PrxII F. The observed *in vivo* S-nitrosylation of PrxII F could probably function as a protective mechanism under oxidative and nitrosative stress, such as occurs under salinity. We conclude that we are dealing with a novel regulatory mechanism for this protein by NO.

Biological significance
S-Nitrosylation is a post-translational modification that is increasingly viewed as fundamental for the signal transduction role of NO in plants. This study demonstrates that S-nitrosylation of the mitochondrial peroxiredoxin PrxII F induces a conformational change in the protein and provokes a reduction in its peroxidase activity, while acquiring a novel function as transnitrosylase. The implication of this mechanism will increase our understanding of the role of posttranslational modifications in the protein function in plants under stress situations such as salinity, in which NO could act as signaling molecule.

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1. Introduction

Nitric oxide (NO), together with reactive oxygen species (ROS), is the subject of increasing interest due to their involvement in the general metabolism of the cell in response to abiotic and biotic stresses, as well as in many physiological and pathological processes in plants and animals [1–4]. The NO signaling function depends on its reactivity with proteins and other non-enzymatic components. S-Nitrosylation, the reversible, covalent addition of NO moiety to the thiol side chain of cysteine, has been proposed as an important regulatory mechanism in nitric oxide-related signaling [5]. This post-translational modification affects the protein structure and conformation, modulating its function, stability and localisation [6,7].

The toxic effect of NO derives from its reaction with O_2^- to form the toxic peroxynitrite anion, $ONOO^-$, which provokes lipid peroxidation, protein oxidation and nitration [8]. At the crossroads of the ROS and NO signaling pathways, Prxs are very important proteins because they can reduce the hydrogen peroxide (H_2O_2), peroxynitrite and organic peroxides generated in the cell through their catalytic cysteine residues by using thiol-containing proteins such as thioredoxin (Trx), thioredoxin reductase (TrxR), glutaredoxin (Grx), glutathione, ascorbate or DTT as reductants [9–11]. In plants, *Arabidopsis* genome encodes 10 *prx* genes, one 1-Cys Prx and one PrxQ, two 2-Cys Prx (2-Cys Prx A and 2-Cys Prx B) and six Type-2 Prx (Prx A, Prx B, Prx C, Prx D, Prx E and Prx F) [9]. 1-Cys Prx is localized in cytosol and nucleus, 2-Cys Prx and Prx Q in chloroplasts and Type-2 Prx in chloroplast, cytosol and mitochondria [11–14].

Besides their well-known peroxidase function, it has been demonstrated that Prxs undergo multiple functions as molecular chaperone, enzyme activator, protein binding partner and redox sensor, observations that have led to the real function of Prxs being questioned. Prxs undergo a peroxidase-to-chaperone functional switch upon oxidative stress. Previous studies have shown that cytosolic yeast Prxs, cPrxI and II, with their diverse structural and apparent molecular weights, can act as peroxidase and molecular chaperone when exposed to oxidative stress and heat shock [15]. In this sense, it has been demonstrated that irreversible hyperoxidised Prx, Tsa1p-SO_3H, loses its peroxidase function but increases its chaperone activity 4-fold compared with the reduced form, Tsa1p-SH, suggesting that Tsa1p-SO_3H is a marker of cumulative oxidative stress in cells [16]. Other studies in Chinese cabbage have shown that 2-Cys Prx1 also behaves as a molecular chaperone under oxidative stress conditions [17]. In plants, Prx has been shown to bind to several protein targets, modulating their function; 2-Cys Prx activates the chloroplast 1,6-bisphosphatase (FBPase) enzyme involved in the Calvin cycle [18], this reductive activation being considered the main regulatory mechanism for this enzyme [19]. In pea plants, PsPrxII F has been shown to interact with the mitochondrial PsTrxo1 [20,21], and both proteins have been shown to protect mitochondria from oxidative stress in saline conditions [14]. In animals, the association of Prx6 with human cyclophilin-A has been demonstrated [22], and in plants, Bernier-Villamor et al. [23] reported the cyclophilin-dependent reduction of chloroplast 2-Cys Prx. Dimeric glutathione S-transferase pi (GST π) has been shown to reactivate oxidised 1-cysteine peroxiredoxin (1-Cys Prx) accompanied by the formation of a heterodimer complex between 1-Cys Prx with a C-terminal His_6 tag and GST π [24].

Several studies have shown that Prxs from animals and plants are targets of S-nitrosylation under both normal and stress conditions: *in vitro*, Prx2, the most abundant protein in mammalian neurons, is target of S-nitrosylation in human Parkinson's disease brains by reaction with nitric oxide on two critical Cys residues (C51 and C172), inhibiting its protective function against oxidative stress-induced neuronal cell death [25]. Prx is one of the 24 proteins reported to be S-nitrosylated in *Arabidopsis* plants [26], and has also been identified among the 49 protein targets of S-nitrosylation in response to salinity stress in citrus plants [27]. The S-nitrosylation of chloroplastic PrxII E inhibited its hydroperoxide-reducing peroxidase activity during the plant hypersensitive disease resistance response [28] and its $ONOO^-$ detoxification activity, causing a dramatic increase of $ONOO^-$-dependent nitrotyrosine residue formation [29]. Recently the mitochondrial PsPrxII F was identified as a target of S-nitrosylation in pea plants subjected to 14 days of salt stress [4]. However, no further information is available on the regulation of mitochondrial pea PsPrxII F by NO and its effect on protein function.

In the present study, we corroborate the *in vitro* S-nitrosylation of PsPrxII F and, using the mutated variants, C59S and C84S, we identify the Cys residues susceptible to S-nitrosylation. We also examine the effect of this post-translational modification on protein structure and on its peroxidatic function. Based on reports that Prxs might have a dual function as peroxidase and molecular chaperone, we study for the first time, the impact of S-nitrosylation on the function of recombinant PsPrxII F. The oligomerization status and S-nitrosylation of PsPrxII F in mitochondria under salinity stress conditions are studied it is demonstrated that oxidative and nitrosative stress is taking place [4,14].

2. Materials and methods

2.1. Expression and purification of recombinant PsPrxII F

PsPrxII F and its mutant C59S and C84S variants were produced using *Escherichia coli* strain BL21, which was transformed with the recombinant plasmids Pet-PsPrxII F and Pet-C59S. The expression and purification of the proteins were carried out according to Barranco-Medina et al. [30].

2.2. GSNO and SNP treatment of recombinant PsPrxII F

Recombinant PsPrxII F and its mutant C59S and C84S variants were previously reduced in 25 mM Tris–HCl buffer (pH 8) containing 50 mM DTT for 30 min at room temperature. The DTT excess was removed by Bio-Spin 6 gel filtration (BioRad, Spain). Reduced proteins (PsPrxII F, C59S and C84S) were incubated with 5 mM of the S-nitrosylating agent, S-nitrosoglutathione (GSNO, SIGMA), or 250 μM of the NO