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Arabidopsis glutathione-dependent formaldehyde dehydrogenase is an *S*-nitrosoglutathione reductase

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Abstract ***S*–Nitrosoglutathione (GSNO), an adduct of nitric oxide (NO) with glutathione, is known as a biological NO reservoir. Heterologous expression in *Escherichia coli* of a cDNA encoding a glutathione-dependent formaldehyde dehydrogenase of *Arabidopsis thaliana* showed that the recombinant protein reduces GSNO. The identity of the cDNA was further confirmed by functional complementation of the hypersensitivity to GSNO of a yeast mutant with impaired GSNO metabolism. This is the first demonstration of a plant GSNO reductase, suggesting that plants possess the enzymatic pathway that modulates the bioactivity and toxicity of NO.** © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Glutathione-dependent formaldehyde dehydrogenase; *S*–Nitrosoglutathione reductase; Nitrosative stress; Nitric oxide signaling; *Arabidopsis*

1. Introduction

It now appears that plants use nitric oxide (NO) as a signaling molecule that modulates the activation of antimicrobial defense responses, and may use NO to regulate other physiological and developmental processes [1]. In contrast to the accumulating knowledge of NO signaling in plant systems, much less is known about its metabolism. Although immunological and in vitro evidence indicates the existence of NO synthase-like proteins and other NO-generating enzymes [2–4], the origin of NO biogenesis is still not certain in plants, nor have any other plant enzymes unambiguously involved in NO metabolism been characterized. Hence, there is a need to clarify the metabolism of NO and related compounds in plants, not only to understand these signaling mechanisms, but also to unveil novel aspects of nitrogen metabolism.

Recent advances in understanding the mechanistic aspects of how microbial pathogens resist NO-derived antimicrobial products has begun to shed light on the enzymatic metabolism of NO-derived nitrogenous molecules, in particular those often referred to as reactive nitrogen intermediates (RNIs) [5]. Recently, flavohemoglobin and peroxiredoxin, which have totally unknown or only partly understood functions, have been shown to scavenge RNIs, thereby conferring protection against nitrosative stress [6,7]. More recent studies have examined glutathione-dependent formaldehyde dehydrogenase (GS-FDH; EC 1.2.1.1), a widespread enzyme also known as a class III alcohol dehydrogenase. GS-FDH reversibly catalyzes the NAD^+-dependent formation of *S*-formylglutathione from *S*-hydroxymethylglutathione that occurs through spontaneous interaction between formaldehyde and glutathione [8]. It has long been proposed that the primary function of this enzyme in biological systems is to detoxify endogenous and exogenous formaldehyde. Liu et al. [9] recently provided compelling evidence of the vital involvement of GS-FDH in the metabolism of *S*-nitrosothiols (SNOs), such as *S*-nitrosoglutathione (GSNO), a naturally occurring NO reservoir and also an RNI. The purification of substances with GSNO-metabolic activity from microbial and mammalian cells, and the characterization of the responsible enzyme have resulted in GS-FDH being assigned as GSNO reductase (GSNOR) [9]. Deletion of the gene for GS-FDH/GSNOR in mice and yeast has revealed the central importance of this enzyme in controlling the level of *S*-nitrosylation of proteins [9], which has recently been found to play a significant role as a prototypic redox-based signaling mechanism in regulating a broad spectrum of cellular physiology [10]. Moreover, a yeast mutant defective in the gene exhibited a phenotype that is hypersensitive to nitrosative challenge, demonstrating that GSNOR is indispensable for providing protection against nitrosative stress [9].

Its ubiquitous distribution in organisms and its newly discovered function strongly suggest that GS-FDH serves as a metabolic enzyme for SNOs in plant systems. GS-FDH from several plants has been characterized, including *Arabidopsis thaliana*, rice, maize, and pea [11–15]. Thus far, however, there is no evidence as to whether plant GS-FDH actually has GSNOR activity. Therefore, we examined (1) whether plant GS-FDH has the ability to metabolize GSNO, by using heterologous expression of the recombinant *Arabidopsis* enzyme in bacteria; and (2) the functional relevance of the plant enzyme in protection against nitrosative stress, by complementation of a yeast mutant that is hypersensitive to GSNO. This study identified GS-FDH as a GSNOR in plants.

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Abbreviations: GS-FDH, glutathione-dependent formaldehyde dehydrogenase; GSNO, *S*-nitrosoglutathione; GSNOR, GSNO reductase; IPTG, isopropyl 1-thio-β-D-galactoside; NO, nitric oxide; PCR, polymerase chain reaction; RNI, reactive nitrogen intermediate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SNO, *S*-nitrosothiol

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2. Materials and methods

2.1. Strains, plasmids, and media

Escherichia coli strains DH5α and BL21(DE3)pLysS were grown at 37°C in Luria-Bertani medium [16]. The plasmids used in this study were pGEM-T Easy (Promega, Madison, WI, USA) for cloning products of the polymerase chain reaction (PCR) and pET23d (Novagen, Madison, WI, USA) for expressing recombinant protein. *Saccharomyces cerevisiae* wild-type strain Y190 and the *sfa1* mutant, in which the gene encoding GSNOR is knocked out [9], were kindly provided by Dr. J.S. Stamler of Duke University Medical Center. The expression vector was pAUR123 (TaKaRa, Kyoto, Japan), which contains the *AUR-1C* gene for antibiotic selection and the promoter for the alcohol dehydrogenase I gene to drive gene expression. Yeast was grown at 30°C in rich medium (YPD) containing 1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, and 2% (w/v) glucose, pH 5.5.

2.2. Cloning and vector construction

Total RNA from shoots of *A. thaliana* (ecotype C24) was converted to single-stranded cDNA using oligo(dT)$_{20}$ primers and a recombinant reverse transcriptase (ReverTra Ace, Toyobo, Osaka, Japan). Based on the reported cDNA sequence [11], the coding sequence of *Arabidopsis* GS-FDH (approximately 1.2 kb) was amplified from the shoot cDNA by high fidelity PCR using *TaKaRa LA Taq*™ (TaKaRa). The forward primer contained an *Nco*I restriction site followed by 23 nucleotides after the start codon (5′-TTTCCATGGCGACTCAAGGTCAGGTTATCAC-3′). The reverse primer contained an *Xho*I restriction site followed by the 3′ coding sequence of 23 nucleotides excluding the termination codon (5′-AAACTCGAGTTTGCTGGTATCGAGGACACAAC-3′) in order to add a carboxyl-terminal hexahistidine tag. After ligation with pGEM-T Easy, the plasmid was introduced into *E. coli* DH5α. This intermediate plasmid was extracted and sequenced to verify that there was no misincorporation of deoxyribonucleotides during PCR. Following excision from the plasmid by cleavage with *Nco*I and *Xho*I, the GS-FDH cDNA was ligated to similarly digested pET23d downstream from an isopropyl 1-thio-β-D-galactoside (IPTG)-inducible promoter. The resultant plasmid, pAtGSFDH, was introduced into *E. coli* BL21(DE3)pLysS.

To construct a yeast expression vector, the coding sequence of GS-FDH was amplified by PCR as described above and subcloned into pGEM-T Easy. The forward primer introduced an *Xba*I restriction site immediately upstream from the initiation codon (5′-TTTTCTAGAAATGGCGACTCAAGGTCAGGTTATCAC-3′). The reverse primer introduced an *Sac*I restriction site immediately after the stop codon (5′-AAAGAGCTCTCATTTGCTGGTATCGAGGACACAAC-3′). Following digestion with *Xba*I, the linearized plasmid DNA was filled in with T_4 DNA polymerase (TaKaRa) and then cleaved by *Sac*I to release the GS-FDH cDNA. Subsequently, the GS-FDH sequence was ligated to the *Sma*I/*Sac*I-digested pAUR123, yielding pAtGSNOR. Transformation of a yeast *sfa1* mutant was performed with pAtGSNOR or pAUR123 in the presence of lithium acetate and polyethylene glycol 4000, and then stably transformed cells were selected for resistance to 0.5 μg/ml aureobasidin A (TaKaRa).

2.3. Recombinant protein expression

The recombinant *Arabidopsis* GS-FDH was overexpressed in *E. coli* BL21(DE3)pLysS that had been transformed with pAtGSFDH, after a 6-h induction with 1 mM IPTG in the presence of 5 μg/ml chloramphenicol and 40 μg/ml ampicillin. The soluble fraction was prepared using BugBuster™ Protein Extraction Reagent (Novagen) according to the manufacturer's instructions. 30 μg of protein from the soluble fraction were stained with Coomassie brilliant blue following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a denaturing 12% (w/v) polyacrylamide gel that contained 0.1% (w/v) SDS.

2.4. Enzyme assay

Enzyme activity was measured spectrophotometrically at 25°C by monitoring the formation or decomposition of NADH at 340 nm. Activities were calculated using the molar absorption coefficient (6220 M^{-1} cm^{-1}) of NADH at 340 nm [17]. Crude enzyme preparations were made from *E. coli* as above. For yeast, cells were grown until the OD_{600} of the culture was between 0.5 and 1.0. The cells were collected, mechanically broken in 50 mM Tris–HCl (pH 8.0) and 0.1% (v/v) Tween 20 in the presence of acid-washed glass beads, and centrifuged to remove the beads and insoluble material [16]. GS-FDH activity was determined by incubating the soluble fraction (equivalent to 10–240 μg protein) in 100 μl of 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM NAD^+, 2 mM reduced glutathione, and 1 mM formaldehyde [11]. The soluble fraction was also examined for GS-FDH activity in situ, after electrophoresis on a non-denaturing 8% (w/v) polyacrylamide gel, by incubating the gel at 25°C in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 mM NAD^+, 0.1 mM nitroblue tetrazolium, 0.1 mM phenazine methosulfate, 1 mM reduced glutathione, and 1 mM formaldehyde [12]. For GSNOR activity, the soluble fraction from *E. coli* (equivalent to 2–60 μg protein) or yeast (100–150 μg protein) was incubated in a 100-μl assay mix that contained 20 mM Tris–HCl (pH 8.0), 0.2 mM NADH and 0.5 mM EDTA. The reaction was started by adding GSNO to the mix at a final concentration of 400 μM [9]. GS-FDH and GSNOR activities were expressed as nmol NADH formed and consumed, respectively, per min per mg protein.

2.5. Functional complementation of the yeast sfa1 mutant

The ability of plasmids to suppress the GSNO-hypersensitive growth defect of the *sfa1* strain was evaluated by the growth in YPD supplemented with 5 mM GSNO, as described in [9]. Cells grown in mid-log phase ($OD_{600} = 0.5$) were diluted 20-fold in fresh YPD medium with or without GSNO, and then the culture was started again at 30°C in the dark. Cell density was recorded every 2 h by monitoring the OD_{600} of the culture.

3. Results

3.1. Arabidopsis GS-FDH possesses GSNO-metabolizing activity

In order to determine whether *Arabidopsis* GS-FDH has GSNOR activity, it was expressed in *E. coli* with a carboxyl-terminal hexahistidine tag. Fig. 1 shows the results of SDS–PAGE of soluble fractions from BL21(DE3)pLysS cells that had been transformed with pAtGSFDH and pET23d. Induction of the expression of the *Arabidopsis* cDNA in cells harboring pAtGSFDH by IPTG resulted in the appearance of a polypeptide with a relative molecular mass of 46.5 kDa (Fig. 1A, lane 2), which was in good agreement with that of the purified protein (45 kDa) [11]. The fusion tag might account for the slight discrepancy. There was no corresponding polypeptide in the fraction of cells harboring the empty vector (Fig. 1A, lane 1). Activity staining in situ following gel electrophoresis under the native condition demonstrated that the same fraction from pAtGSFDH transformants had robust GS-FDH activity that was clearly distinguished from the endogenous activity (Fig. 1B). The specific activity was 92.3 ± 11.3 and 0.5 ± 0.1 nmol/min/mg protein ($n = 3$) in extracts of cells expressing the *Arabidopsis* enzyme and in extracts of control cells, respectively. From these results, we concluded that the recombinant *Arabidopsis* GS-FDH was produced in *E. coli* in its active form.

GSNOR activity is determined by GSNO-specific oxidation of NADH that allows the reduction of GSNO to *S*-amino-L-glutathione via an *S*-hydroxylamine intermediate [9]. Therefore, we examined whether the recombinant expression of the *Arabidopsis* enzyme specified the oxidation of NADH that was dependent on the presence of GSNO by monitoring the decrease in the absorbance at 340 nm (Fig. 2A). Since *E. coli* has both GSNOR-specific and non-specific NADH oxidation activity [9], we first examined whether the endogenous activity was evident under the assay conditions (2 μg protein/100 μl reaction). No apparent oxidation of NADH was observed with soluble proteins from control cells in the presence or

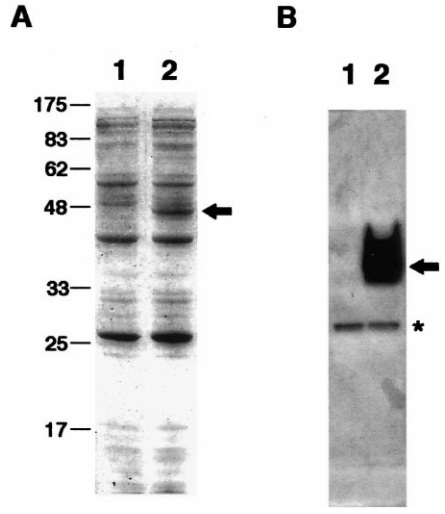

Fig. 1. Overexpression in *E. coli* of the recombinant GS-FDH of *Arabidopsis*. A: Protein staining after SDS–12% PAGE. B: Activity staining. The numbers to the left indicate the positions of molecular mass markers in kDa. Lane 1, soluble fraction of *E. coli* transformed with pET23d; lane 2, soluble fraction of *E. coli* transformed with pAtGSFDH. Arrows indicate the recombinant *Arabidopsis* GS-FDH. The asterisk refers to endogenous activity of *E. coli*.

absence of GSNO (Fig. 2A). Thus, endogenous NADH consumption activity was below the detectable level under the conditions used. Following the addition of GSNO to the reaction, the soluble fraction from cells expressing *Arabidopsis* GS-FDH rapidly decomposed NADH (Fig. 2A). The fraction showed a specific activity that was 117-fold higher than that of the control pET23d cells (Fig. 2B). The reaction appeared to be specific to NADH, because negligible GSNOR activity was detected when NADPH, instead of NADH, was used as an electron donor. The specific activity of GS-FDH (see above) in this fraction accounted for only 3% of the GSNOR activity. These results established that *Arabidopsis* GS-FDH has GSNOR activity.

3.2. *Arabidopsis GS-FDH complements the hypersensitivity to GSNO of the yeast sfa1 mutant*

In order to determine whether *Arabidopsis* GS-FDH functions as a GSNOR in vivo, the coding sequence was integrated into a yeast expression vector, and the vector was introduced into the *sfa1* mutant, a knock-out strain of the GS-FDH gene that lacks GSNOR activity and is consequently hypersensitive to GSNO [9]. Mutant cells transformed with the *Arabidopsis* GS-FDH construct showed GSNOR activity that was nearly twice as high as that of the wild-type strain, whereas both the mutant and the transformant with the empty vector showed much less enzyme activity than that of the wild-type strain (Table 1). More importantly, the expression of *Arabidopsis* GS-FDH complemented the growth defect of the mutant to GSNO (Fig. 3). No complementation was observed with the vector control (Fig. 3). These results showed that *Arabidopsis* GSNOR was functional in vivo and that it provided a defense against nitrosative stress in yeast.

Table 1
GSNOR activity is restored in the yeast *sfa1* mutant by a cDNA for *Arabidopsis* GSNOR

Yeast strain	GSNOR activity[a] (nmol NADH/min/mg protein)
Wild-type (Y190)	5.0 ± 0.3
sfa1	0.1 ± 0.1
sfa1/pAtGSNOR	9.3 ± 0.3
sfa1/pAUR123	0.0 ± 0.0

[a]Mean ± S.E.M. ($n = 3$).

4. Discussion

A growing body of evidence suggests that SNOs like GSNO play a key role in NO signaling, modulating activities of different classes of proteins through *S*-nitrosylation of mostly a single critical cysteine residue [10,18]. GSNO could act as a buffer for NO and thereby maintain the level of protein *S*-nitrosylation [9]. SNOs are cytotoxic, particularly when

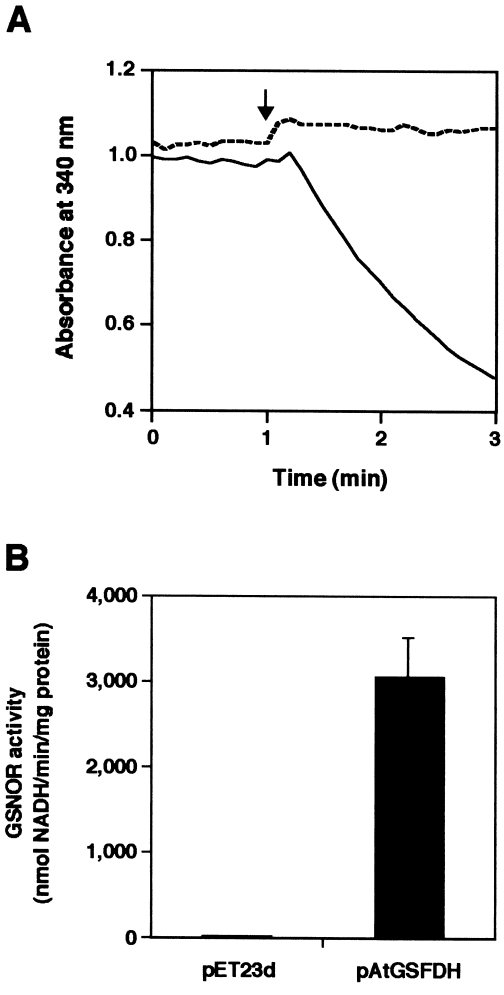

Fig. 2. GSNOR activity of the recombinant GS-FDH of *Arabidopsis*. A: GSNO-dependent oxidation of NADH by the soluble fraction from *E. coli* expressing *Arabidopsis* GS-FDH. A 100-μl reaction contained 2 μg protein. The addition of GSNO (final 400 μM) to the reaction is indicated by an arrow. Solid line, soluble fraction from *E. coli* transformed with pAtGSFDH; dashed line, soluble fraction from *E. coli* transformed with pET23d. The small rise in the absorbance after 1 min was due to the absorbance by GSNO. A typical case from five independent experiments is presented. B: GSNOR activity in the soluble fractions of *E. coli* transformed with pET23d or pAtGSFDH. The reaction was conducted with 0.6–60 μg soluble protein per 100 μl assay. Data are the means ± S.E.M. ($n = 3$).

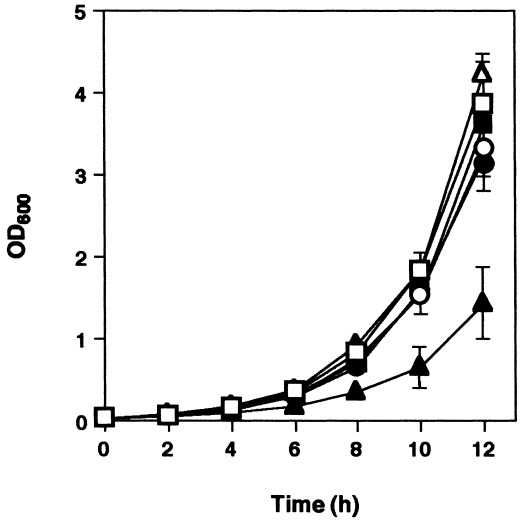

Fig. 3. Complementation of the hypersensitivity to GSNO of the *sfa1* mutant with the *Arabidopsis* GSNOR cDNA. Yeast strains were grown at 30°C in YPD in the presence (closed symbol) or absence (open symbol) of 5 mM GSNO. Strains were the wild-type (square), *sfa1* mutant transformed with pAtGSNOR (circle), and the mutant transformed with the empty vector (triangle). Data are the means ± S.E.M. ($n = 2$).

present in excess, as they may inhibit crucial protein functions by excessive *S*-nitrosylation [9,10] or mediate the formation of highly reactive peroxynitrite [19]. The toxic effect of SNOs is used as an antimicrobial strategy by mammalian hosts [8]. Hence, SNO metabolism has become of central importance in understanding how NO signaling controls numerous physiological functions. However, its enzymatic basis has only recently been explored in microbes and animals [9] and no such studies have been performed in plants. Our results demonstrate that *Arabidopsis* GS-FDH has GSNOR activity both in vitro and in vivo, as shown by heterologous expression in *E. coli* and yeast, respectively. This is the first identification of a plant enzyme that is potentially involved in NO and RNI metabolism. This finding strengthens the view that several aspects of NO signal transduction pathways are conserved in plants and animals [1].

The prevailing activity of GSNOR over that of GS-FDH, which has also been reported for the rat and mouse enzymes [9,20], is a good indication that GSNO is probably a preferred substrate for this enzyme in vivo. Although there is no direct evidence that GSNO occurs in plant tissues, several lines of circumstantial evidence indicate its presence. Exogenous GSNO induces activation of plant defense mechanisms against pathogens [21], suggesting that GSNO per se is an endogenous component of NO signal transduction. Elicitor treatments of epidermal tobacco cells result in NO bursts in several cellular compartments, including the cytosol and chloroplasts in plant cells [22], both of which possess the biosynthetic pathway to form glutathione and therefore contain a high level of this non-protein thiol [23]. As glutathione is a primary target of NO [24], these observations suggest that GSNO is formed in both the cytosol and chloroplasts. The known sequences of plant GS-FDH/GSNOR lack an obvious targeting sequence; therefore, it is presumably a cytosolic enzyme [11–15]. The *Arabidopsis* genome encodes GS-FDH on a single gene, which is mapped to chromosome 5 [13]. Whether functionally similar enzymes exist in the cytosol or other subcellular compartments, such as the chloroplast, remains to be investigated.

Although nothing is known of the presence of nitrosative stress in plants or its impact on various aspects of plant physiology, it seems likely that plants often suffer from nitrosative challenge. They not only produce RNIs, such as NO and nitrite, but they also take up nitrite from the soil and nitrogen oxides from air [25]. Heterologous complementation using the yeast mutant revealed the functional relevance of the *Arabidopsis* enzyme in protection against nitrosative damage, suggesting the evolutionary conservation of a metabolic pathway that copes with the possible threat of nitrosative stress in plants, animals, and microbes. As proposed in microbes and mammals [9], a plant GSNOR might play a dual role in turning off NO signaling that originates from GSNO and in preventing intracellular thiol compounds from *S*-nitrosylation to occur in excess. Expression of the GS-FDH/GSNOR gene is not induced by stressful conditions, and the mRNA level is relatively high in various *Arabidopsis* organs, including flowers, leaves, roots, and shoots [11,13]. The constitutive and high-level expression of GSNOR might meet the demand for the immediate decomposition of GSNO, thereby keeping it at non-hazardous levels under certain circumstances when NO production is stimulated. Transgenic approaches that produce changes in the level of GSNOR activity would provide a practical means of evaluating the importance of this enzyme in plants in NO signaling, defense against RNIs, and possibly other physiological phenomena.

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