Nitric Oxide 68 (2017) 68–76

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Characterization of S-nitrosoglutathione reductase from *Brassica* and *Lactuca* spp. and its modulation during plant development

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ARTICLE INFO

Article history:
Received 30 September 2016
Received in revised form
1 December 2016
Accepted 5 December 2016
Available online 8 December 2016

Keywords:
S-nitrosoglutathione
S-nitrosoglutathione reductase
S-nitrosothiols
Lactuca spp.
Brassica spp.
Plant development

ABSTRACT

Cellular homeostasis of S-nitrosoglutathione (GSNO), a major cache of nitric oxide bioactivity in plants, is controlled by the NADH-dependent S-nitrosoglutathione reductase (GSNOR) belonging to the family of class III alcohol dehydrogenases (EC 1.1.1.1). GSNOR is a key regulator of S-nitrosothiol metabolism and is involved in plant responses to abiotic and biotic stresses. This study was focused on GSNOR from two important crop plants, cauliflower (*Brassica oleracea var. botrytis*, BoGSNOR) and lettuce (*Lactuca sativa*, LsGSNOR). Both purified recombinant GSNORs were characterized *in vitro* and found to exists as dimers, exhibit high thermal stability and substrate preference towards GSNO, although both enzymes have dehydrogenase activity with a broad range of long-chain alcohols and ω-hydroxy fatty acids in presence of NAD^+. Data on enzyme affinities to their cofactors NADH and NAD^+ obtained by isothermal titration calorimetry suggest the high affinity to NADH might underline the GSNOR capacity to function in the intracellular environment. GSNOR activity and gene expression peak during early developmental stages of lettuce and cauliflower at 20 and 30 days after germination, respectively. GSNOR activity was also measured in four other *Lactuca* spp. genotypes with different degree of resistance to biotrophic pathogen *Bremia lactucae*. Higher GSNOR activities were found in non-infected plants of susceptible genotypes *L. sativa* UCDM2 and *L. serriola* as compared to resistant genotypes. GSNOR and GSNO were localized by confocal laser scanning microscopy in vascular bundles and in epidermal and parenchymal cells of leaf cross-sections. The presented results bring new insight in the role of GSNOR in the regulation of S-nitrosothiol levels in plant growth and development.

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1. Introduction

Similarly to other organisms, nitric oxide (NO) is known as an important gaseous signaling molecule involved in multiple developmental processes of plants and in their responses to various stress stimuli. S-nitrosylation of protein cysteine thiols has been established as one of the major routes of NO action on molecular targets. S-nitrosoglutathione reductase (GSNOR), considered as a key enzyme in the regulation of cellular S-nitrosothiols, belongs to the class III alcohol dehydrogenase family (ADH3, E.C. 1.1.1.1). In older literature, this enzyme was denoted as glutathione-dependent formaldehyde dehydrogenase (GD-FALDH, GS-FDH, E.C. 1.2.1.1). However, as it was elucidated that the true substrate of the enzyme was S-(hydroxymethyl) glutathione (HMGSH), an adduct of glutathione with formaldehyde, the enzyme was

Abbreviations used: ADH, alcohol dehydrogenase; BoGSNOR, S-nitrosoglutathione reductase from *Brassica oleracea*; EDTA, ethylenediaminetetraacetate; FALDH, glutathione-dependent formaldehyde dehydrogenase; GSH, glutathione; GSNO, S-nitrosoglutathione; hGSNOR, human S-nitrosoglutathione reductase; HMGSH, *S*-(hydroxymethyl)glutathione; ITC, isothermal titration calorimetry; LsGSNOR, S-nitrosoglutathione reductase from *Lactuca sativa*; NAD^+, β-nicotinamide adenine dinucleotide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SlGSNOR, S-nitrosoglutathione reductase from *Solanum lycopersicum*; SNOs, S-nitrosothiols; RNS, reactive nitrogen species; ROS, reactive oxygen species; 12-HDDA, 12-hydroxydodecanoic acid; 10-HDA, 10–hydroxydecanoic acid; 8-HOA, 8-hydroxyoctanoic acid.

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http://dx.doi.org/10.1016/j.niox.2016.12.002
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renamed to S-(hydroxymethyl) glutathione dehydrogenase (E.C. 1.1.1.284). Within the major formaldehyde detoxification pathway, it catalyzes the NAD^+-dependent oxidation of HMGSH to S-formylglutathione. However, the irreversible conversion of GSNO in the presence of NADH resulting in formation of glutathione disulphide (GSSG) and ammonia was later discovered as the physiologically more relevant reaction [3,27,32,39].

The crystal structures of plant class ADH3/GSNOR from *Arabidopsis thaliana* (AtGSNOR) and *Solanum lycopersicum* cv. Amateur (SlGSNOR) with bound substrates and cofactors revealed important differences between plant and human enzyme, namely within the substrate-binding pocket. GSNOR are unusually homodimeric cysteine-rich proteins, with each subunit consisting of a large catalytic domain with two zinc atoms and coenzyme binding domain [28,43,55].

The GSNOR activity has been conclusively demonstrated in many plant species including *Arabidopsis*, pea, pepper, sunflower, tomato or tobacco and found modulated upon various stress conditions including pathogenesis [1,2,4,8,19,21,28,29,31,54]. A single copy of the *GSNOR* gene has been identified in most plant genomes, whereas the enzyme has been found in the cytosol, chloroplasts, mitochondria and peroxisomes by immunogold electron microscopy [4,5,8,37,52]. In *Arabidopsis*, the GFP-tagged enzyme localized in cytosol and nucleus but not nucleolus. The highest expression of *GSNOR* was observed in shoot and root apical meristems, in anther filaments, ovary, stigma and petals [55].

GSNOR is involved in NO homeostasis, which is essential for numerous processes including proper auxin and abscisic acid (ABA) signaling. Mutations in *GSNOR* gene results in dwarfed plants, stem and trichomes branching defects and increased number of inflorescences [30,31], impaired auxin signaling and polar auxin transport [47,53] as well as ABA insensitivity in stomatal regulation. Thus, GSNOR seems necessary for normal plant development [33]. Recent study on six maize inbred lines [56] showed that two lines susceptible to drought display significantly higher GSNOR activities upon drought stress than more resistant lines. GSNOR also contributes to alkaline stress tolerance and its overexpression increases reactive oxygen species (ROS) scavenging efficiency [22].

Lettuce (*Lactuca sativa*) and cauliflower (*Brassica oleracea* var. *botrytis*) represent very important agricultural plants. We used these two model plant systems in previous studies focused on the role of NO and ROS in plant defense mechanisms during pathogenesis and oxidative stress [36,45,46]. The main goal of this work was to provide deeper insights into the regulation of NO homeostasis in these plants through the GSNOR activity. We performed a functional characterization of recombinant plant GSNORs *in vitro* and determined their molecular properties and kinetic parameters. We analyzed *GSNOR* gene expression and enzyme activity profiles during early developmental stages. Finally, we also performed immunolocalizations of GSNOR and GSNO in leaf sections of both plants.

2. Materials and methods

2.1. Plant material and chemicals

Plants of *Brassica oleracea* var. *botrytis* and five selected *Lactuca* spp. genotypes *L. sativa* L. (UCDM2), *L. sativa* L. (Mariska), *L. saligna* L. (CGN 05271), *L. virosa* L. (NVRS 10.001 602) and *L. serriola* (LSE/18) were used [46]. Plants were grown in a soil/peat (2:1, v/v) mixture in a growth chamber at 18/15 °C (day/night), 12 h photoperiod, irradiance of 100 μE m^{-2} s^{-1} provided by warm-white fluorescent lamps.

GSNO and HMGSH were prepared as described elsewhere [28]. Working solutions of GSNO, GSH, HMGSH and NADH were always prepared fresh just prior their use and kept on an ice bath protected from the light. GSNOR inhibitor N6022 was purchased from Axon Medchem (Groningen, Netherlands). All other substances were of analytical purity grade from Sigma-Aldrich (Steinheim, Germany).

2.2. BoGSNOR and LsGSNOR cloning and purification of recombinant enzymes

The total RNA from leaves of 6-week-old *Brassica oleracea* var. botrytis and *Lactuca sativa* UCDM2 was extracted using an RNeasy Plant Mini Kit (Macherey-Nagel, Germany). The cDNA was prepared using AMV transcriptase (2 units, Promega, USA) and oligo$(dT)_{15}$ primers. *BoGSNOR* open reading frame (1140 bp, GenBank accession number JX104828) and *LsGSNOR* open reading frame (1134 bp, GenBank accession number JX104827) were cloned into a pCDFDuet His-tag vector (Novagen, La Jolla, USA) using *Sac*I and *Xho*I endonucleases (New England BioLabs, USA) and following primer pairs (*BoGSNOR*, 5′-CCTGAGCTCGGCGACTCAAGGTCAGGTTA-3′ and 5′-AGGCTCGAGTCAATCGCTGGTACTGAGGACAC-3′; *LsGSNOR* 5′-CCTGAGCTCGGCTACTCAAGGCAAAGTAATC-3′ and 5′-GCGCTCGAGTCAAAGTGTAAGCACACAACG-3′). The constructs were then transformed into T7 express *E. coli* cells (New England Biolabs, Hitchin, UK) for expression as N-terminal 6xHis-tagged proteins. Expression, cell lysis and purification were performed as described previously [28].

2.3. Phylogenetic analysis of GSNOR protein sequences

Amino acid alignments were performed using MUSCLE v3.8 [17] followed by Gblocks [7]. A maximum likelihood phylogeny with bootstrap analysis was performed with PhyML v3.0 [25] using LG amino acid replacement matrix.

2.4. Molecular mass determination

The molecular mass of the native enzyme was determined by gel filtration chromatography on a Superdex 200 HR column (GE Healthcare) connected to a BioLogic Duo-Flow liquid chromatograph (Bio-Rad). The column was equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl at a flow rate of 0.7 ml min^{-1}. Calibration was done using commercial gel filtration standards (Bio-Rad, USA).

2.5. Isothermal titration calorimetry

The heat of NADH or NAD^+ binding to LsGSNOR was measured by isothermal titration calorimetry on a MicroCal Auto-ITC200 (Malvern, UK). Data analysis was performed using Origin 5.0 with the ITC plugin (MicroCal Software Inc.). In a single experiment, twenty 15 μl injections of 300 μM NADH or 800 μM NAD^+ were titrated into a 30 μM solution of LsGSNOR. Both protein and ligand solutions were prepared in 20 mM potassium phosphate buffer, pH 7.0. All solutions were degassed under the vacuum immediately prior to experiments. Solutions in the ITC titration cell were stirred throughout all experiments and the temperature was maintained at 25 °C. The reference cell of the microcalorimeter was filled with a solution of deionized water. Blank experiments were performed to measure the heat of ligand dilution, which was subtracted from the heat of protein-ligand interaction to obtain the true binding enthalpy. All measurements were done in duplicates.

2.6. Enzyme activity measurement

The activity was measured at 30 °C by monitoring spectral changes of NADH at 340 nm ($\varepsilon_{340} = 6220$ M^{-1} cm^{-1}) on a UV–Vis

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