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Research Article

S-nitrosoglutathione reductase activity of human and yeast glutathione-dependent formaldehyde dehydrogenase and its nuclear and cytoplasmic localisation

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Abstract. S-nitrosoglutathione (GSNO) formation represents a mechanism for storage and transport of nitric oxide. Analysis of human liver and *Saccharomyces cerevisiae* extracts has revealed the presence of only one enzyme able to significantly reduce GSNO, identified as glutathione-dependent formaldehyde dehydrogenase (FALDH). GSNO is the best substrate known for the human and yeast enzymes (kcat/Km = 444,400 and 350,000 $mM^{-1}min^{-1}$, respectively). Although NADH is the preferred cofactor, some activity with NADPH (Km = 460 μM) can be predicted in vivo. The subcellular localization demonstrates a cytosolic and nuclear distribution of FALDH in living yeast cells. This agrees with previous results in rat, and suggests a role in the regulation of GSNO levels in the cytoplasmic and nuclear compartments of the eukaryotic cell.

Key words. Formaldehyde dehydrogenase; alcohol dehydrogenase; nitrosoglutathione; ADH3; nuclear enzyme; nitrosative stress.

Nitric oxide (NO) is a cell-signalling molecule that has been shown to be involved in the regulation of numerous physiological functions in animals [1, 2] and plants [3]. However, it can also be involved in reactions producing cell damage through the generation of reactive nitrogen intermediates (RNIs) which can alter DNA, induce lipid peroxidation and deplete the antioxidant pool of the cell [4, 5]. One of the major endogenous metabolites of NO is S-nitrosoglutathione (GSNO), formed primarily by nitrosation of glutathione (GSH) with NO in the presence of oxygen [6]. GSNO has been found to be biologically active by itself as a vasodilator in animals, preventing platelet adhesion and aggregation [7, 8]. Nitrosylation of cysteine groups in proteins is also well established [9, 10]. The formation and decomposition of S-nitrosylated proteins and GSNO have been suggested to represent mechanisms for the storage and transport of NO in vivo, and for the prevention of its toxicity [6, 11].

Recent evidence strongly supports that glutathione-dependent formaldehyde dehydrogenase (FALDH), also known as class III alcohol dehydrogenase (ADH3) has an important role in GSNO metabolism [12, 13]. FALDH is a widely distributed and highly conserved medium-chain dehydrogenase/reductase that has been shown to be involved in formaldehyde detoxification, through the oxidation of the GSH-formaldehyde adduct, S-hydroxymethlyglutathione (HMGSH), to S-formylglutathione with the concomitant reduction of NAD to NADH [14]. In a recent report, rat FALDH displayed a high catalytic efficiency towards the reduction of GSNO [12] while experiments in *Escherichia coli*, *Saccharomyces cerevisiae* and mouse cell lines showed that FALDH is involved in the control of the intracellular levels of GSNO and S-nitrosylated proteins [13]. However, the kinetics of the hu-

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man and yeast enzymes with GSNO have not yet been investigated.

In contrast to other medium-chain alcohol dehydrogenases, of strict cytosolic localisation [15], FALDH was reported to be located not only in cytoplasm but also in the cell nucleus of rat hepatocytes and astrocytes [16]. No other studies have corroborated the presence of FALDH in the nucleus, where it could regulate the local levels of GSNO and play a role in protecting the genetic material from NO-induced damage.

In the present work, we investigated the GSNO reductase activity of human and yeast FALDH, and the subcellular distribution of the enzyme was studied in living yeast cells.

Material and methods

Human samples

Autopsy liver samples were obtained from donors at the Hospital Clínic i Provincial de Barcelona. The protocols were approved by the Ethical Committee of the hospital. Samples were kept at −80 °C. Prior to analysis, samples were thawed, homogenised in a Polytron and centrifuged at 12,000 g. Supernatants were used for activity and isoelectrofocusing analyses.

Electrophoresis and activity staining

Isoelectrofocusing of yeast (FY834 strain) and human liver extracts, and formaldehyde dehydrogenase staining were performed as previously described [17]. Native gel electrophoresis in 6% acrylamide was performed in Tris-boric-EDTA buffer, pH 8. Reductase activity staining was performed using a modification for GSNO of a reported general method [18]: gels were soaked in 0.1 M sodium phosphate, pH 7.4, containing 2 mM NADH, for 15 min in an ice-bath. Excess buffer was drained and gels were covered with filter paper strips soaked in freshly prepared 2 mM GSNO [19]. After 10 min, the filter paper was removed and gels were exposed to ultraviolet light and analysed for the disappearance of the NADH fluorescence, indicating reductase activity.

Expression and purification of FALDH

Yeast FALDH was expressed and purified as previously described [20]. For the expression and purification of human alcohol dehydrogenase, the coding part of *FALDH* cDNA [21] was amplified by PCR and inserted into the inducible plasmid pGEX-4T-2, fused to the glutathione S-transferase (GST) coding region. The GST-FALDH fusion protein was expressed in *E. coli* strain BL21. Cells were harvested, and disrupted in PBS, 1 mM DTT, by intermittent sonication followed by centrifugation at 20,000 g for 15 min. Extract was loaded on GSH-sepharose (2.5 ml) and GST-FALDH was eluted with 16 mM GSH. After treatment with thrombin, the preparation was loaded on Blue-sepharose (2 ml) equilibrated with 10 mM Tris-HCl, 0.5 mM DTT, pH 8.0. FALDH was eluted with 2 mM NADH, which was eliminated by a desalting PD10 column. From a 2-l culture, 0.480 µg of pure FALDH was obtained with a specific activity with formaldehyde/GSH of 4.1 U/mg. Protein concentration was determined colorimetrically [22]. Protein homogeneity was confirmed by SDS/polyacrylamide gel electrophoresis.

Kinetic studies

Enzyme activity was determined at 25 °C by monitoring the production of NADH at 340 nm (ε_{340} = 6.22 mM^{-1} cm^{-1}) for formaldehyde oxidation, or the consumption of NAD(P)H and GSNO (ε_{340} = 7.06 mM^{-1} cm^{-1}) for GSNO reduction in a Cary 400 Bio spectrophotometer. One unit (U) of activity corresponds to 1 µmol of coenzyme transformed per minute. The NAD-dependent formaldehyde dehydrogenase activity was measured in 0.1 M sodium phosphate, pH 8.0, with S-hydroxymethylglutathione (HMGSH; formed by mixing formaldehyde and glutathione) [14]. Formaldehyde was obtained and standardized as reported elsewhere [17]. GSNO reductase activity was measured in 0.1 M sodium phosphate, pH 7.5, with GSNO prepared immediately before the experiment [19]. Kinetic constants were calculated with the non-linear regression program Grafit 5.0 (Erithacus Software), and expressed as the mean ± SD of three independent determinations.

Construction and subcellular detection of FALDH-GFP

To determine the subcellular localisation of FALDH in the yeast living cell, a fusion of FALDH to green fluorescent protein (GFP) was performed according to an established method [23]. The yeast centromeric vector pRS416-GFP [24], kindly provided by Dr F. Posas, was used to express the FALDH-GFP construct. The yeast FALDH open reading frame (*SFA1*) was amplified by PCR from position −608 to the terminal codon using primers that replaced the termination codon with a *Xho*I site, and added an *EcoR*I site to the 5′ end. The PCR product was cloned into the *EcoR*I/*Xho*I sites of pRS416-GFP, resulting in a vector that would express the FALDH-GFP fusion protein under the control of the *SFA1* promoter. Junctions were verified by DNA sequencing. The construct was introduced into a FALDH knockout yeast strain (*sfa1Δ*::*HIS3*) [20], and the expression of the fusion protein was verified by FALDH enzyme activity and by Western blot analysis. A background fluorescence control was performed with the knockout strain transformed with the pRS416-GFP vector. Yeast cells at their logarithmic growth phase were observed using a Leica-DMRB microscope and a 100× PL Fluotar objective. GFP and DAPI signals were elicited and detected using a Leica 1.3 filter and a Leica A filter, respec-

tively. Images were captured with a Leica DC-200 camera driven by DC Viewer software.

Results

GSNO reductase activity in yeast and human liver extracts

While no GSNO reductase activity was detected in the FALDH knockout yeast strain, 0.087 U/mg protein was measured in the wild-type strain, grown under the same conditions. In human liver homogenate, the measured GSNO reductase activity was 0.075 U/mg. Isoelectrofocusing analysis of the yeast and human liver extracts, stained by activity with GSNO, revealed an activity band with the same mobility as that detected in the formaldehyde/GSH activity staining (fig. 1). Isoelectrofocusing analysis of the knockout strain, stained with either formaldehyde/GSH or GSNO, did not reveal any activity band (not shown).

To investigate the influence of nitrosative stress on the GSNO reductase activity, extracts of yeast cells grown in the presence of the NO donor DETA NONOate (3 mM) were analysed. The GSNO reductase activity increased by a factor of 1.5–1.8 with respect to that of yeast grown under normal conditions. By using native gel electrophoresis, only the band corresponding to FALDH was detected, indicating that no other enzymes with GSNO reductase activity were expressed under nitrosative stress in the yeast (fig. 2).

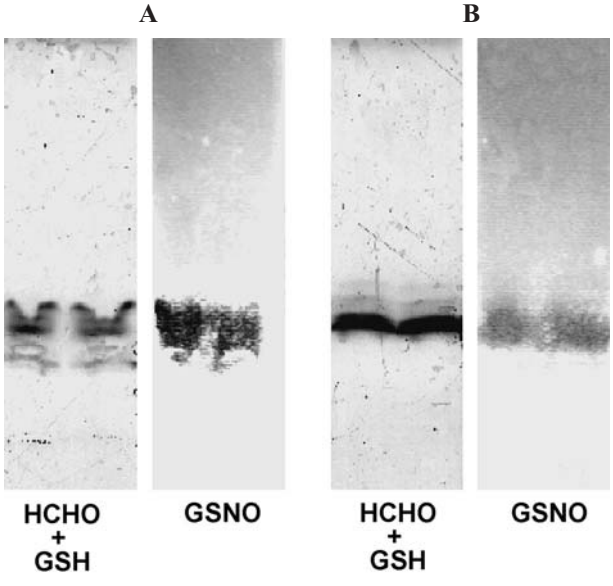

Figure 1. Isoeletrofocusing (pH 3–9) gel of human liver and yeast extracts, stained for formaldehyde dehydrogenase and GSNO reductase activities. Liver (130 µg protein) (*A*) and yeast (23 µg protein) (*B*) extracts were loaded in duplicate and incubated with formaldehyde, glutathione and NAD, or with GSNO and NADH.

Kinetic characterization of the GSNO reductase activities of human and yeast FALDH

Human FALDH was expressed and purified from *E. coli*. The kcat value for the recombinant enzyme with HMGSH (table 1) was essentially the same as that reported for the native human liver FALDH, but the Km value (1.4 µM) was lower than the one previously reported (4 µM, [25]), which resulted in a higher catalytic efficiency (kcat/Km) value. The catalytic efficiencies of the human and the yeast FALDH for GSNO reduction (in the presence of NADH) were higher than the corresponding constants for S-HMGSH oxidation in the presence of NAD, indicating that both enzymes are more active in GSNO reduction than in formaldehyde oxidation, at physiological pH (table 1). The yeast FALDH showed higher kcat and Km values than the human enzyme, although the kcat/Km was similar. The Km for GSNO reduction of human FALDH was similar to the reported for the rat FALDH [12], but the kcat for the human enzyme was approximately fourfold higher.

Although FALDH from human and yeast could also reduce GSNO in the presence of NADPH, the kcat/Km values measured were much lower than in the presence of NADH. The pH optima for the human GSNO reductase activity were 9.0 and 6.0 with NADH and NADPH, respectively, while they were 8.0 and 5.0 for the yeast enzyme. The specificity towards NADH was pronounced.

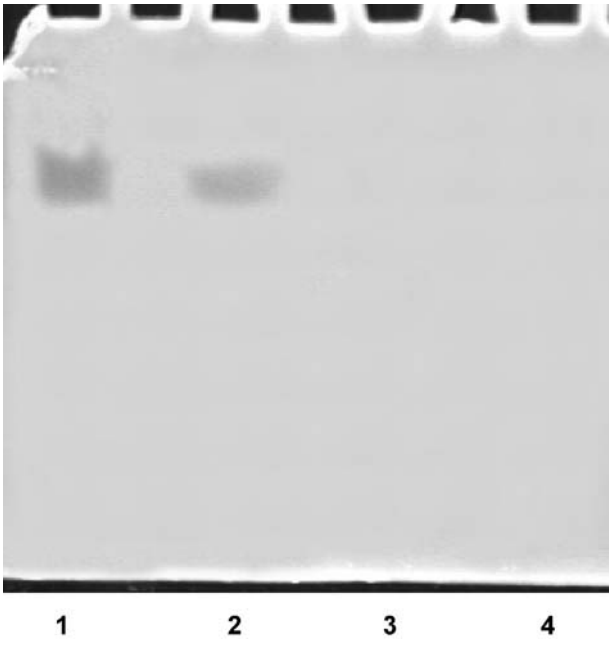

Figure 2. Native gel electrophoresis of yeast extracts stained for GSNO reductase activity. Yeast strains were grown in rich medium and 80 µg of total protein was loaded in each lane. 1, wild-type yeast grown in the presence of 3 mM DETA NONOate; 2, wild-type yeast grown without DETA NONOate; 3, knockout yeast (*sfa1Δ::HIS3*) grown in the presence of 3 mM DETA NONOate; 4, knockout yeast grown without DETA NONOate.

Table 1. Kinetic properties of human and yeast FALDH.

Substrate	Human FALDH			*S. cerevisiae* FALDH		
	Km (μM)	kcat (min^{-1})	kcat/Km (min^{-1} mM^{-1})	Km (μM)	kcat (min^{-1})	kcat/Km (min^{-1} mM^{-1})
HMGSH	1.4 ± 0.1	320 ± 5	229,000 ± 16,745	20 ± 9	3,100 ± 260	155,000 ± 70,545
NAD	7.0 ± 0.9	380 ± 13	54,300 ± 7,513	45 ± 15	3,620 ± 430	80,000 ± 28,350
GSNO	27 ± 11	12,000 ± 2,500	444,400 ± 200,100	150 ± 39	52,600 ± 6,900	350,000 ± 99,500
NADH	30 ± 5	10,200 ± 829	340,000 ± 68,699	130 ± 43	66,000 ± 13,600	508,000 ± 192,209
NADPH	460 ± 142	2,000 ± 624	4,350 ± 1,800	N.S	–	2,540 ± 91

HMGSH were determined in 0.1 M sodium phosphate, pH 8.0, and 2.4 mM NAD. Constants for NAD were determined in pH 8.0 buffer with 1 mM GSH, 1 mM formaldehyde. Constant for GSNO was determined in 0.1 mM sodium phosphate, pH 7.5, and 0.5 mM NADH. Constants for NADH and NADH were determined in pH 7.5 buffer with 0.3 mM GSNO. N.S., no saturation detected up to 0.8 mM NADPH.

Thus, at pH 7.5, the GSNO reductase specific activity in the presence of NADH was approximately eightfold higher than in the presence of NADPH.

Subcellular distribution of FALDH in the yeast living cell

To analyse the subcellular distribution of FALDH in yeast, we genetically fused the FALDH open reading frame (*SFA1*) to the N terminus of GFP. When expressed from its own promoter in a centromeric plasmid, FALDH-GFP was able to reconstitute the FALDH activity in a knockout strain (data not shown), demonstrating that the fusion protein was functional. Moreover, immunoblot analysis of an extract from yeast cells expressing the FALDH-GFP protein, performed with polyclonal antibodies against yeast FALDH, confirmed the correct size of the expressed fusion protein (data not shown). FALDH was absent from the prominent vacuole, and showed both cytoplasmic and nuclear distribution within the yeast cells (fig. 3).

Discussion

The analysis of the zymograms performed with human liver homogenates and yeast extracts (from cells grown in rich medium) detected only one enzymatic activity band for the reduction of GSNO (fig. 1). Bands corresponding to GSNO reductase and formaldehyde dehydrogenase activities exhibited the same mobility, demonstrating that FALDH is responsible for the two activities, and that no other GSNO reductase is detected under the present conditions in both human liver and yeast cells.

The high catalytic efficiencies of yeast and human FALDH towards GSNO are consistent with recent reports showing that deletion of FALDH makes yeast cells more sensitive to GSNO toxicity [13] and that the expression of *Arabidopsis thaliana* FALDH in yeast complements this hypersensitivity to GSNO [26]. Human and yeast FALDH are much more specific towards NADH than to NADPH in the GSNO reduction activity; however, the physiological NADH/NAD ratio is low. Reductases generally use NADPH, the concentration of which can reach 100 μM in some tissues [27]. The Km of the human enzyme for NADPH is 460 μM, which suggests that, although with low activity, the enzyme may use NADPH under cellular conditions.

Although a physiological role for NO has not yet been uncovered in *S. cerevisiae,* a putative NO synthase has been detected in this yeast using monoclonal antibodies [28]. Moreover, *Candida tropicalis* exhibits endogenous NO synthesis that suppressed the formation of pseudomycelia [29]. In addition, yeast and bacteria are protected from external NO by a variety of mechanisms, including flavohaemoglobins [30, 31]. From the induction of yeast FALDH under nitrosative stress and the high catalytic efficiency with GSNO, demonstrated here, and the fact that deletion of FALDH makes yeast hypersensitive to GSNO [13], one can conclude that this enzyme represents an additional and powerful system for RNI protection in microorganisms.

FALDH is detected not only in the cytoplasm, like other members of the medium-chain ADH family, but also in the nucleus of yeast cells. The presence of FALDH in the nucleus was previously reported in rat cells by immunodetection and enzymatic activity [16], but this is the first time its location has been studied in living cells. Presence in the nucleus seems, therefore, general for eukaryotic FALDH, where it can be involved in the metabolism of endogenous GSNO. In fact, several transcription factors and other nuclear proteins are regulated by NO/GSNO [32, 33]. Moreover, FALDH could protect the DNA from damage caused by RNIs (deamination and oxidation of bases, oxidation of deoxyribose, strand breaks, and several types of cross-link) [4, 34], as well as from formaldehyde toxicity [16, 35].

In conclusion, the results presented here demonstrate that FALDH is the major enzyme for GSNO reduction in humans and yeast and that its kinetic constants support a contribution of FALDH to the metabolism of GSNO and,

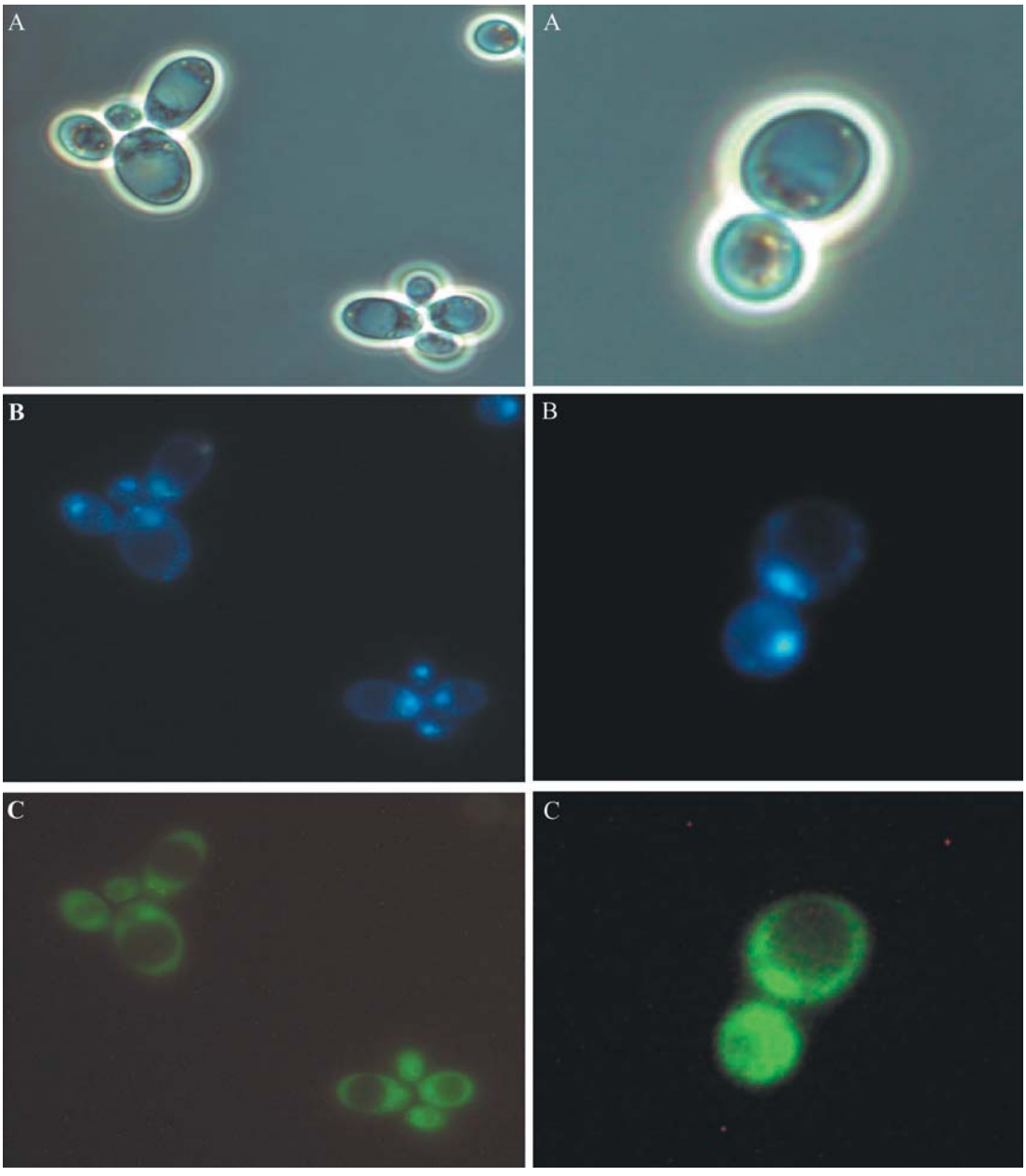

Figure 3. Intracellular distribution of yeast FALDH-GFP fusion protein. (*A*) Phase contrast image of yeast cells. The same field of cells was stained with DAPI to localise the nuclei (*B*) and observed for GFP fluorescence to localise the FALDH-GFP fusion protein (*C*).

therefore, to the regulation of NO biological action, in the cytoplasm and nucleus of eukaryotes.

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