Supporting Information

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SI Materials and Methods

Preparation of SNO-CoA and Purification of SNO-CoA Reductase from Yeast. SNO-CoA (50 mM stock solution) was freshly prepared for each experiment by combining equal amounts of CoA (0.1 M in 1 M HCl) and sodium nitrite (0.1 M in MilliQ water containing 100 μM EDTA and 100 μM diethylene triamine pentaacetic acid (DTPA)). Stoichiometric yield was verified spectrophotometrically (λ_{max} 340 nm, extinction coefficient 0.92 $mM^{-1}{\cdot}cm^{-1}$). This stock solution was stored on ice (SNO-CoA is stable at acidic pH), and aliquots were added directly to lysates; SNO-CoA–metabolizing activity was purified from 3 L of yeast cells harvested at A_{600nm} = 5.0. Cells were pelleted and resuspended in lysis buffer [20 mM bis-Tris propane, pH 7.0, 50 mM NaCl, 100 μM EDTA, 100 μM DTPA, 1 mM PMSF, and protease inhibitor mixture (Roche)]. The crude extract was prepared with a beadbeater (Biospec Products) by using glass beads of 0.5-mm diameter, with 15 × 1-min cycles of beating alternating with 1-min cooling intervals. After centrifugation at 60,000 × *g* for 1 h, the supernatant was taken as the starting material for assessment of enrichment of SNO-CoA metabolizing activity. At this and all subsequent stages, enzyme activity was assessed with 200 μM SNO-CoA and 100 μM NADPH in 20 mM bis-Tris propane (pH 7) containing 100 μM EDTA and 100 μM DTPA. The supernatant was precipitated with 30% (wt/vol) ammonium sulfate followed by centrifugation at 20,000 × *g*, and the resultant supernatant was reprecipitated with 70% (wt/vol) ammonium sulfate and pelleted at 20,000 × *g*. The pellet was resuspended and dialyzed against Tris buffer (pH 8.0) at 4 °C. The dialyzed extract was applied at 5 mL/min onto a HighPrep Q FastFlow 16 × 10-mm column equilibrated with 20 mM Tris buffer (pH 8.0) and eluted with a linear 0–0.3 M NaCl gradient in 20 mM Tris buffer (pH 8.0). Active fractions were pooled, and applied to a 2′–5′ ADP-Sepharose 26 × 120-mm column, equilibrated with Tris buffer (pH 8.0) at a flow rate of 2 mL/min, followed by elution with 10 mL of 1 mM $NADP^+$ in 20 mM Tris buffer (pH 8.0). Active fractions were dialyzed against 20 mM Tris buffer (pH 8.0). The sample was then applied to a MonoQ GL 5 × 50-mm column at a flow rate of 1 mL/min followed by elution with a linear 0–0.3 M NaCl gradient in 20 mM Tris buffer (pH 8.0). Active fractions were pooled, concentrated to <200 μL by ultrafiltration, and applied at a flow rate of 0.5 mL/min to a Superdex 200 10 × 300-mm column equilibrated with PBS. The protein was judged to be pure by SDS/PAGE.

Cloning, Expression, and Purification of Recombinant Adh6 and AKR1A1. Yeast *ADH6* was amplified from *S. cerevisiae* genomic DNA with the following primers: ADH6 F, 5′-GAGGCAGCTAGCTCTTATCCTGAGAAATTTGAAGGTATCGC-3′; and ADH6 R, 5′-GTGCGCCTCGAGGTCTGAAAATTCTTTGTCGTAGC-3′. ADH6 was cloned into pET21b vector by NheI and XhoI. Human *AKR1A1* was amplified from *AKR1A1*-encoding cDNA plasmid (Dharmacon; catalog no. MHS6278-202827721) by the following primers: AKR1A1, F-GCATGACATATGGCGGCTTCC TGTGTTCTACT; and AKR1A1 R, 5′-GCGCTACTCGAGGTACGGGTC ATTAAAGGGGT-3′. *AKR1A1* was cloned into pET21b vector by NdeI and XhoI. Both pET21b-ADH6 and pET21b-*AKR1A1* were expressed in Rosetta2(DE3)pLysS *E. coli* and induced by 100 μM isopropyl-β-D-1-thiogalactopyranoside at A_{600nm} = 0.5. The cells were subsequently grown for 4 h at room temperature. For purification of His-tagged proteins, cell pellets were lysed by sonication in 10 mL of lysis buffer [50 mM phosphate buffer, pH 8.0, 600 mM NaCl, 10 mM imidazole, 1 mM PMSF, and 1 protease inhibitor tablet (Roche)]. The lysates were clarified by centrifugation at 15,000 × *g* for 30 min, followed by incubation with Ni-NTA Agarose (Qiagen) for 1 h. The agarose beads were subsequently incubated with washing buffer (50 mM phosphate buffer, pH 8.0, 600 mM NaCl, and 30 mM imidazole) for 45 min, and the proteins were eluted by incubation of the beads in elution buffer (50 mM phosphate buffer, pH 8.0, 600 mM NaCl, and 250 mM imidazole) for 20 min. Purified proteins were then transferred into PBS by buffer exchange.

Products of Yeast and Mammalian SNO-CoA Reductases. Samples (1 mL) initially contained 20 mM ammonium bicarbonate buffer, 200 μM SNO-CoA, and 200 μM NADPH at 25 °C. Reactions were initiated by the addition of purified Adh6 or AKR1A1 and allowed to continue until absorbance at 340 nm indicated complete consumption of SNO-CoA (~1 h). Samples were then centrifuged through a 10-kDa cutoff ultrafiltration membrane, and the filtrate was stored at −80 °C until analyzed. For MS analysis, samples were diluted to a ratio of 1:2 in HPLC grade acetonitrile. Formic acid was added to the samples at a final concentration of 0.1% (vol/vol). Samples were injected into a Thermo LTQ Oribtrap XL at a flow rate of 1 uL/min. Mass spectra were acquired from 700 to 900 *m/z*. The ion at 799 *m/z* was isolated and fragmented by using collision-induced dissociation (CID) with a normalized collision energy (NCE) of 35 and an isolation width of 3.0.

Kinetic Parameters of the Yeast SNO-CoA Reductase. Kinetic analysis was carried out by using assay conditions as described above. For K_m determination, reactions (four or five replicates) were performed with a fixed amount of purified Adh6, 100 μM NADPH, and SNO-CoA concentrations of 16–375 μM. Initial rates were calculated from the absorbance decrease at 340 nm by using a combined extinction coefficient of 7.06 $mM^{-1}{\cdot}cm^{-1}$ for SNO and NADPH (1). The k_{cat} was determined at saturating substrate concentrations.

NADPH:SNO-CoA Stoichiometry of Yeast and Mammalian SNO-CoA Reductases. NADPH and SNO-CoA possess essentially identical λ_{max} (~340 nm), and the extinction coefficient of NADPH>>SNO-CoA. Therefore, reactions were performed by adding a limiting amount of NADPH (sequential additions of ~100 μM) to Adh6 or AKR1A1 (purified from yeast or kidney lysates) in the presence of excess SNO-CoA (~400 μM) and monitoring the reaction (NADPH consumption) by absorbance spectroscopy until all NADPH was consumed after each addition. The absorbance difference at 340 nm before and after each addition of NADPH was used to calculate the amount of SNO-CoA consumed in the presence of known amounts of NADPH.

SNO-CoA Reductase Activity in Yeast Lysates. Yeast cells were grown to A_{600nm} = 1.0 in yeast extract peptone dextrose (YPD) medium (Clontech). Cells were lysed as described above. For enzymatic assay, samples contained both 200 μM SNO-CoA or 100 μM SNO-CoA and 100 μM NADPH. Runs were initiated with the addition of lysate, and the absorbance decrease at 340 nm was followed.

Assay for Thiolase Activity. Acetoacetyl-CoA thiolase activity was assayed in the physiological forward direction (2 acetyl-CoA → acetoactyl-CoA + CoA-SH) by following the formation of CoA-SH with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) at 412 nm, as outlined recently for assay of HMG-CoA synthase activity (2).

This approach avoids background absorbance in lysates and interference from added SNO-CoA in the reverse-reaction assay of acetoacetyl-CoA consumption at 300 nm (acetoacetyl-CoA + CoA-SH → 2 acetyl-CoA) (3). The assay was performed in 20 mM Tris (pH 8.0), containing 0.1 mM EDTA and 0.1 mM DTPA. The reaction mixture (1 mL) contained yeast lysate and 100 μM acetyl-CoA. Reaction without the addition of acetyl-CoA was used as background control. In some experiments, various concentrations of GSNO or SNO-CoA were also added. The reaction was monitored after the addition of 0.5 mM DTNB for 3 min at 412 nm. Thiolase activity was determined by using rates between 1 and 3 min.

Assays for Protein S-Nitrosylation. For analysis in yeast, cells were grown to A_{600nm} = 1.0 and lysed by bead beating in lysis buffer [20 mM bis-Tris propane, pH 7.0, 50 mM NaCl, 100 mM EDTA, 100 μM DTPA, 1 mM PMSF, and protease inhibitor mixture (Roche)]. The lysate was centrifuged at 20,000 × *g* for 10 min, and the supernatant was adjusted to 1 mg/mL protein and retained for analysis. For analysis of SNO-proteins by mercury-coupled photolysis–chemiluminescence, samples were processed as described (4). For SNO-RAC, some samples (1 mg/mL) were left untreated or treated for 10 min with 60 μM SNO-CoA ± 100 μM NADPH. The reaction was stopped by adding 3 vol of ice-cold acetone, followed by precipitation at −20 °C for 20 min. Precipitates were dissolved in HEN buffer (100 mM Hepes, 1 mM EDTA, and 0.1 mM Neucuproine, pH 8.0) containing 0.2% S-methylmethanethiosulfonate and 2.5% SDS and incubated at 50 °C for 20 min with frequent vortexing. Proteins were then reprecipitated with 3 vol of acetone at −20 °C for 20 min, and precipitates were dissolved in 1 mL of HEN buffer containing 1% SDS. Proteins were again precipitated with 3 vol of ice-cold acetone at −20 °C for 20 min, and pellets were suspended in 1 mL of fresh HEN buffer/1% SDS. Samples were then incubated with freshly prepared ascorbate (30 mM) and thiopropyl-Sepharose for 4 h, and beads were collected and washed with HEN/1% SDS and 0.1× HEN/1% SDS. Beads were eluted in 0.1× HEN buffer containing 1% SDS and 10% β-meracaptoethanol. Eluates were analyzed by SDS/PAGE followed by Western blotting for Erg10 (see below), or, for MS analysis, individual lanes were excised from the gel and subjected to trypsin digest before processing for LC/MS-MS (see below).

For analysis by SNO-RAC of mammalian SNO-proteins, mouse liver or kidney was homogenized in 50 mM phosphate buffer (pH 7.0) containing 50 mM NaCl, 0.1 mM EDTA, 0.1 mM DTPA, 1 mM PMSF, and protease inhibitor mixture (Roche). The homogenate was centrifuged twice at 20,000 × *g* at 4 °C for 45 min. The supernatant was adjusted to 1 mg/mL total protein and left untreated or treated for 10 min with 60 μM SNO-CoA ± 100 μM NADPH. The reaction was stopped by adding 3 vol of ice-cold acetone, followed by precipitation at −20 °C for 20 min. SNO proteins were analyzed by the SNO-RAC method as described above. Eluted SNO-proteins were analyzed by SDS/PAGE followed by Coomassie staining or by Western blotting using an anti-GAPDH Ab (Abcam; ab9485).

Detection of SNO-Erg10. To evaluate S-nitrosylation of Erg10, we used a yeast strain that expresses the TAP-tagged version of Erg10 under the control of its native promoter (Thermo Scientific; YSC1178-202233637). SNO-proteins were pulled down by SNO-RAC from untreated or SNO-CoA–treated lysates by SNO-RAC, and levels of SNO-Erg10 were assessed by Western blotting with an anti-TAP Ab (Thermo Scientific; CAB1001) or by iTRAQ MS as described below.

Quantification of SNO Levels in Nitrite-Grown Yeast Cells. Yeast cultures were grown in minimal medium (Yeast Nitrogen Base; MP Biomedicals) containing 0.5% glucose and 5 g/L ammonium sulfate, supplemented with histidine, methionine, uracil (each 20 mg/L), and leucine (100 mg/L), pH 6.0. A starting culture was grown aerobically at 30 °C for 24 h in a shaker and transferred to an anaerobic glove box (85% N_2, 10% H_2, 5% CO_2). Fresh minimal medium containing 0, 100, or 500 μM sodium nitrite (equilibrated in the glove box) was inoculated with 0.5% of the starter cultures and grown for 24 h at ambient temperature (~25 °C). The cultures were removed from the glove box and washed twice in ice-cold water containing 0.1 mM EDTA. Cell pellets were stored at −80 °C until analyses. In time-course experiments, cells were grown in the presence of 100 μM nitrite, and samples were harvested after 6, 12, and 24 h.

Metabolic Profiling of CoA-Based Species.

Quantification of mevalonate levels. Yeast cells grown to midlog phase (A_{600nm} = 0.4–0.6) were left untreated or treated with EtCysNO (100 μM) for 2 h. Cells were harvested and washed with ice-cold H_2O containing 0.1 mM EDTA or DTPA and stored at −80 °C until further analysis. To the samples, 3 mL of MeOH: H_2O (8:2) was added followed by the addition of 100 μL of D4 (1 mM) citrate as an internal standard. Samples were homogenized for 1 min, and 1 mL of the subsequent homogenate was taken and centrifuged at 800 × *g* for 20 min. Supernatant was dried by nitrogen gas. Then, 60 μL of *N*,*O*-bistrifluoroacetamide was added to the dried residue and incubated at 70 °C for 30 min. Analyses were carried out on an Agilent 5973 mass spectrometer, linked to a 6890 gas chromatograph system equipped with an autosampler. An Agilent VF-5MS capillary column (60 m × 0.32 mm × 0.25 μm) was used for chromatographic separation. The carrier gas was helium (1 mL/min) with a pulse pressure of 20.1 psi. The injection was in splitless mode. The temperature for both inlet and transfer line was set at 300 °C. The ion source and quadrupole temperature were set at 230 and 150 °C. The GC temperature program was as follows: start at 80 °C, hold for 1 min, and increase by 10 °C/min to 300 °C, and it remained at 300 °C for 9 min. Mevalonate and internal standard signals were monitored at its nominal *m*/*z* with SIM mode. The *m*/*z* monitored for mevalonate and internal standard: 349 and 469, respectively. All of the masses were measured in Electron Impact Ion Source. The retention times of mevalonate and internal standard are 15.2 and 18.1 min, respectively.

Quantification of CoA and acyl-CoA. Yeast cells grown to midlog phase (A_{600nm} = 0.4–0.6) were left untreated or treated with EtCysNO (500 μM) for 2 h. Samples of yeast (~1 g) spiked with 0.5 nmol of [2,2,3,3,3-2H5]-propionyl-CoA were extracted for 2 min with 6 mL of methanol/water (1:1) containing 5% acetic acid using a Polytron homogenizer. CoA and acyl-CoAs were assayed as described (5).

Construction of adh7Δ and adh6Δadh7Δ Yeast Strains. The entire ORF of the *ADH7* gene was deleted from haploid WT (BY4741; Open Biosystems) or *adh6Δ* strain (Open Biosystems). We used primers ADH7F (5′-ATGCTTTACCCAGAAAAATTTCAGG-GCATCGGTATTTCCACGGATCCCCGGGTTAATTAA-3′) and ADH7R (5′-ATGCATTTTAAGAGATTCTGAAAAA-TATTACGTATATAGAGAATTCGAGCTCGTTTAAAC-3′) to amplify and add *ADH7* sequences to both ends of the NatMX cassette by PCR. Cells stably transfected with *NatMX* were selected by their resistance to nourseothricin antibiotic (100 $\mu g \cdot ml^{-1}$). Replacement of the *ADH7* gene by *NatMX4* in the yeast genome was confirmed by PCR using primers ADH7F-C (5′-GCAGAT-GTCACTAATTGA-3′) and NAT-C (5′-GGGCATGCTCATGT-AGAG-3′). Replacement of the *ADH6* gene by *KanMX* in the *adh6Δ* and *adh6Δadh7Δ* strains was confirmed with PCR reactions using primers *ADH6F-C* (5′-TTCACATCTGGAAGCGATAC-3′) and KAN-C (AAGTGAGAAATCACCATGAG-3′).

iTRAQ-Coupled SNO-RAC. Extracts of yeast cells were prepared and SNO-RAC (4 mg of protein per sample) was carried out as described above. SDS/PAGE gels were Coomassie-stained, and lanes were separated into eight segments top-to-bottom. Segments were incubated in a 1:1 mixture of acetonitrile and 100 mM ammonium bicarbonate solution for 2–8 h, followed by the addition of 10 mM Tris(2-carboxyethyl)phosphine 55 mM iodoacetamide. Gel segments were then dehydrated and rehydrated with acetonitrile and ammonium bicarbonate three times successively, followed by overnight digestion in 50 mM ammonium bicarbonate containing freshly prepared trypsin at a concentration of 10 ng/μL (Promega; sequencing grade). Peptides were extracted with 60% acetonitrile/0.1% trifluoroacetic acid, and extracts were dried under vacuum. For subsequent iTRAQ labeling, peptides were dissolved in 0.5 M triethylammonium bicarbonate (pH 8.5) followed by the addition of iTRAQ reagent in ethanol and incubation at room temperature for 2 h. Samples were then combined according to experimental design and dried under vacuum.

LC-MS/MS Analysis. Before analysis by LC-MS/MS, samples were passed through a C18 spin column (Thermo Pierce) and reconstituted in 0.1% formic acid. Separation of peptides via capillary liquid chromatography was performed by using the Waters nanoAquity system (Waters Corp.). Mobile phase A (aqueous) contained 0.1% formic acid in 5% acetonitrile, and mobile phase B (organic) contained 0.1% formic acid in 85% acetonitrile. Separation was achieved by using a C18 column (75 mm × 20 cm; Waters Corp.; Ethylene Bridged Hybrid column # BEH300) through a 150-min gradient of 6–45% mobile phase B at a flow rate of 0.30 mL/min. For peptide identification and quantification using iTRAQ reporter ions, MS analysis was performed by using a hybrid linear ion trap Orbitrap Velos mass spectrometer (LTQ-Orbitrap Velos; Thermo). Survey scans were operated at 60,000 resolution with an automatic gain control (AGC) target of 1E6. After three standard CID fragmentations, higher-energy collisional dissociation (HCD) was carried out for the top three precursor ion fragmentations with a 2-Da isolation window, 40% NCE, and a 5,000 signal count threshold. Tandem MS scans with HCD fragmentation were acquired in profile mode with 7,500 resolution and an AGC target of 2E5. Parent proteins were identified with a yeast database (*Saccharomyces* Genome Database, www.yeastgenome.org) containing 6,717 protein entries. A decoy database containing the reversed sequences of all proteins was appended to estimate false discovery rate (FDR) (6). Protein identification using Sequest (7) or ProLuCID (8) and DTASelect (9, 10) and quantification using Census (11) were performed through the Integrated Proteomics Pipeline (IP2; Integrated Proteomics Applications, Inc.) and checked with MassMatrix (12). Mass accuracy was limited to 10 ppm for precursor ions and 0.03 Da for product ions, with tryptic enzyme specificity and up to two missed cleavages. Static modifications included carbamidomethylation on cysteines (57 Da) and iTRAQ-4plex on lysines and N termini (144 Da). Variable modifications included iTRAQ-4plex on tyrosines (144 Da) and oxidation on methionines (16 Da). DTASelect (9, 10) was applied to generate search results of peptide-to-spectra matches with a maximum FDR of 5%, yielding a protein FDR of <2%. Protein quantification based on iTRAQ reporter ions was performed with Census (11), and ratios after median normalization from two or three biological replicates are presented. Peptides without ratios and those that were not replicated in at least two separate experiments were excluded from further analysis. We present fold change for proteins exhibiting enhanced S-nitrosylation in *adh6Δ* vs. WT yeast (>1.2) only when each of the individual experimental ratios were >1.0 in multiple replicates, with relative SD <35%. *P* values were calculated from the identified peptides.

Quantitative PCR Analysis of ERG10 Expression. WT and *adh6Δ* yeast, grown to $A_{600nm} = 0.6$, were left untreated or treated with EtCysNO (100 μM) for 2 h. Total RNA was isolated by using a Master Pure Yeast RNA Purification Kit (Epicentre) according to the manufacturer's directions. For reverse-transcriptase reactions, 1 μg of total RNA was transcribed to cDNA by using iScript Reverse Transcription Supermix for quantitative RT-PCR (RT-qPCR) (Bio-Rad). qPCR was performed with the TaqMan method (using the Roche Universal Probe Library System) on a Step One Plus Real-Time PCR System (Applied Biosystems). Relative expression was calculated by using the ΔΔCt method with normalization to *GAPDH*. To quantify *ERG10* expression, probe 86 (Roche) was used with the following primers: 5′-CCAGGGTTCTCTATCCTCCA-3′ and 5′-AGGCGCCTTTTAAAGCAAC-3′. To quantify *GAPDH* expression, probe 9 (Roche) was used with the following primers: 5′-CGAAGTTGTTGCTTTGAACG-3′ and 5′-TTGAACATGTAAGCAGCGTAGTC-3′.

Animals. Tissue was obtained after euthanasia of WT and $AKR1^{-/-}$ male mice at 8–12 wk of age. $AKR1A1^{+/-}$ mice were obtained from Deltagen Inc., and $AKR^{-/-}$ and WT breeding pairs were generated. Bovine tissues were obtained from Rockland Immunochemicals Inc. and were classified as waste (no IACUC approval required).

Homozygous AKR1A1 Deletion ($AKR1A1^{-/-}$ Mice). $AKR1A1^{+/-}$ mice were created by insertion of a Lac Z-Neo cassette. Genotyping of subsequently generated $AK1A1^{+/+}$ and $AKR1A1^{-/-}$ mice was performed according to the PCR protocol from Deltagen using the following primers: GS (E, T) 5′-GCAGAGATTCAACAAGTCTCCCCTC-3′ and NEO (T) 5′-GGGCCAGCTCATTCCTCCCACTCAT-3′ (expected band size of the targeted allele: 390 bp) and GS (E, T) and GS (E1) 5′-AGCTAAGGCTCCGAGCAGTGCTAAC-3′ (expected size of the endogenous allele: 190 bp) (see Fig. S7*C*).

Assay of NADPH-Dependent SNO-CoA Reductase Activity in Mouse. Tissues harvested from $AKR1A1^{+/+}$ and $AKR1A1^{-/-}$ mice were homogenized in lysis buffer [50 mM phosphate buffer, pH 7.0, 150 mM NaCl, 0.1 mM EDTA, 0.1 mM DTPA, 1 mM PMSF, and protease inhibitor mixture (Roche)]. Extracts were clarified by centrifugation (20,000 × *g*, 4 °C, 45 min, ×2), and protein concentration was determined by bicinchoninic acid assay. The NADPH-dependent SNO-CoA reductase activity was determined spectrophotometrically as described above for yeast. Briefly, the assays were performed in 50 mM phosphate buffer (pH 7.0; containing 0.1 mM EDTA and DTPA) with 0.2 mM SNO-CoA and 0.1 mM NADPH. Reactions were initiated by the addition of lysate and allowed to proceed for 1 min. All assays were performed in duplicate or triplicate.

Purification of Mammalian SNO-CoA Reductase Activity. Bovine kidney tissue (~50 g) was suspended in 100 mL of lysis buffer [50 mM phosphate buffer, pH 7.0, 150 mM NaCl, 1 mM PMSF, and protease inhibitor mixture (Roche)] and lysed in a blender, followed by homogenization with a dounce homogenizer (Wheaton). Following centrifugation × 2 at 60,000 × *g* for 45 min, the supernatant was collected. At all stages of purification, enzyme activity was assessed with 0.1 mM SNO-CoA and 0.1 mM NADPH in 50 mM phosphate buffer (pH 7) containing 0.1 mM EDTA and 0.1 mM DTPA. The initial supernatant was precipitated with 30% ammonium sulfate followed by centrifugation at 9,4000 × *g* for 30 min at 4 °C, and the resultant supernatant was reprecipitated with 60% ammonium sulfate and pelleted at 9,4000 *g*. The pellet was resuspended and dialyzed against Tris buffer (pH 8.0) at 4 °C. The dialyzed extract was applied at 2 mL/min onto a Q Fast Flow (High Prep) column equilibrated with 20 mM

Tris buffer (pH 8.0). NADPH-dependent SNO-CoA reductase activity was eluted with a linear 0–0.3 M NaCl gradient in 20 mM Tris buffer (pH 8.0). Active fractions were pooled, and ammonium sulfate was added to a final concentration of 1 M. The sample was then applied onto a phenyl superose (High Prep) column equilibrated with 20 mM Tris (pH 8.0) containing 1 M ammonium sulfate at a flow rate of 2 mL/min followed by elution using a linear gradient of 0.65–0.3 M ammonium sulfate in Tris buffer (pH 8.0). Active fractions were pooled and dialyzed overnight against 20 mM Tris buffer (pH 8.0). The dialyzed sample was applied to a MonoQ GL (GE Biosciences) column at a flow rate of 1 mL/min followed by elution with a linear 0–0.3 M NaCl gradient in 20 mM Tris buffer (pH 8.0). Active fractions were pooled and ammonium sulfate added to final concentration of 1 M. Sample was then applied onto a phenyl Sepharose (High Prep) column and preequilibrated with 20 mM Tris (pH 8.0) containing 1 M ammonium sulfate, at a flow rate of 0.5 mL/min. Active fractions were pooled, concentrated to <200 μL by ultrafiltration, and applied at a flow rate of 0.5 mL/min to a Superdex 200 column equilibrated with PBS. Purification was monitored by SDS/PAGE of fractions throughout the purification procedure. Cloning, expression, and purification of recombinant AKR1A1 is described above.

Kinetic Parameters of the Mammalian SNO-CoA Reductase. Kinetic analyses were carried out in 50 mM phosphate buffer (pH 7.0) containing 100 μM EDTA and DTPA. For K_m determination, reactions (two to four replicates) were performed with a fixed amount of purified AKR1A1, 100 μM NADPH, and 2–500 μM SNO-CoA. Initial rates were calculated from the absorbance decrease at 340 nm. The k_{cat} was determined at saturating substrate concentrations.

AKR1A1 Immunodepletion. Ten micrograms of mouse monoclonal anti-AKR1A1 Ab (Santa Cruz; clone 3B08) or control anti-IgG Ab were bound to 25 μL (packed volume) protein G Sepharose beads (Amersham) in dilution buffer (50 mM phosphate buffer, pH 7.0, 10 mM NaCl, 0.1 mM EDTA, and 0.1 mM DTPA), and the volume was brought to 1 mL. Coupling of Ab to beads was done for 1 h with rotation at 4 °C. Ab-bound beads were washed three times with dilution buffer to remove unbound antibodies and collected by centrifugation. Freshly prepared kidney extract (65 μg of total protein) from C57B/6 mice (Jackson) was added to the beads, and the volume was brought to 1 mL with dilution buffer, followed by rotation overnight at 4 °C. The sample was centrifuged at 1,000 × *g* for 2 min, and the resultant supernatant was retained. NADPH-dependent SNO-CoA reductase activity was assayed with 100-μL aliquots of supernatant. To assess immunodepletion of AKR1A1, 25 μL of supernatant was analyzed by Western blotting.

Statistical Analysis. Data are presented as means ± SDs except where noted. Statistical differences in discrete variables between two groups were tested with Student *t* test. ANOVA followed by post hoc testing was used to determine differences between multiple experimental groups. Results were considered significant at $P < 0.05$.

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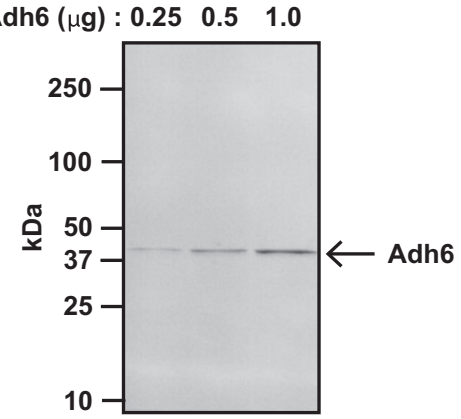
A



B

001 MSYPEK**FEGI AIQSHEDWKN PK**K**TKYDPKP FYDHDIDIKI EACGVCGSDI HCAAGHWGNM**
061 **KMPLVVGHEI VGKVVKLGPK SNSGLK**VGQR **VGVGAQVFSC LECDRCKNDN EPYCTKFVTT**
121 **YSQPYEDGYV SQGGYANYVR VHEHFVVPIP ENIPSHLAAP LLCGGLTVYS PLVR**NGCGPG
181 K**KVGIVGLGG IGSMGTLISK AMGAETYVIS R**SSRKREDAM K**MGADHYIAT LEEGDWGEKY**
241 **FDTFDLIVVC ASSLTDIDFN IMPK**AMKVGG R**IVSISIPEQ HEMLSLKPYG LKAVSISYSA**
301 **LGSIKELNQL LKLVSEKDIK IWVETLPVGE AGVHEAFER**M EKGDVRYR**FT LVGYDKEFSD**

Measured	Calculated	Mass error (ppm)	Residues
1559.7499	1559.7489	0.6	7 - 19
1898.9403	1898.9396	0.4	7 - 22
1995.9907	1994.9857	0.8	24 - 39
1262.6061	1262.6051	0.8	30 - 39
2430.0212*	2430.0205	0.3	40 - 61
1278.7240	1278.7239	0.1	62 - 73
1621.9546*	1620.9506	0.4	62 - 76
1000.5794	1000.5786	0.8	77 - 86
1697.7853*	1696.7782	2.2	91 - 105
1428.5897*	1428.5883	1	106 - 116
2765.2656	2764.2524	3.6	117 - 140
3734.027*	3732.0093	3	141 - 174
1788.0510	1787.0459	1	182 - 200
1659.9575	1658.9509	2	183 - 200
1197.5944	1197.5933	0.9	201 - 211
2022.8989	2021.8909	2.3	222 - 239
2925.419*	2924.4097	2	240 - 264
2399.3115*	2398.3050	1.3	272 - 292
1295.7228	1295.7206	1.7	293 - 305
857.5102	857.5091	1.3	306 - 312
3053.6252	3051.6150	1.2	313 - 339
2140.0918	2139.0870	0.7	321 - 339
942.4937	942.4931	0.6	349 - 356
1421.6683	1420.6631	1.3	349 - 360

Fig. S1. Adh6 is identified as a SNO-CoA reductase in yeast. (*A*) Assessment of the purity of the isolated SNO-CoA–metabolizing activity. Coomassie-stained SDS/PAGE gel illustrates serial dilutions of the purified protein. (*B*) Identification of the band, corresponding to *A*, by MS. Shown in *Upper* are peptide-mass matches for Adh6 (indicated in bold), which cover 88% of the protein sequence. *Cysteines are carbamidomethylated, and methionines are oxidized in these peptides. Isotopic C13 incorporated ions are automatically included.

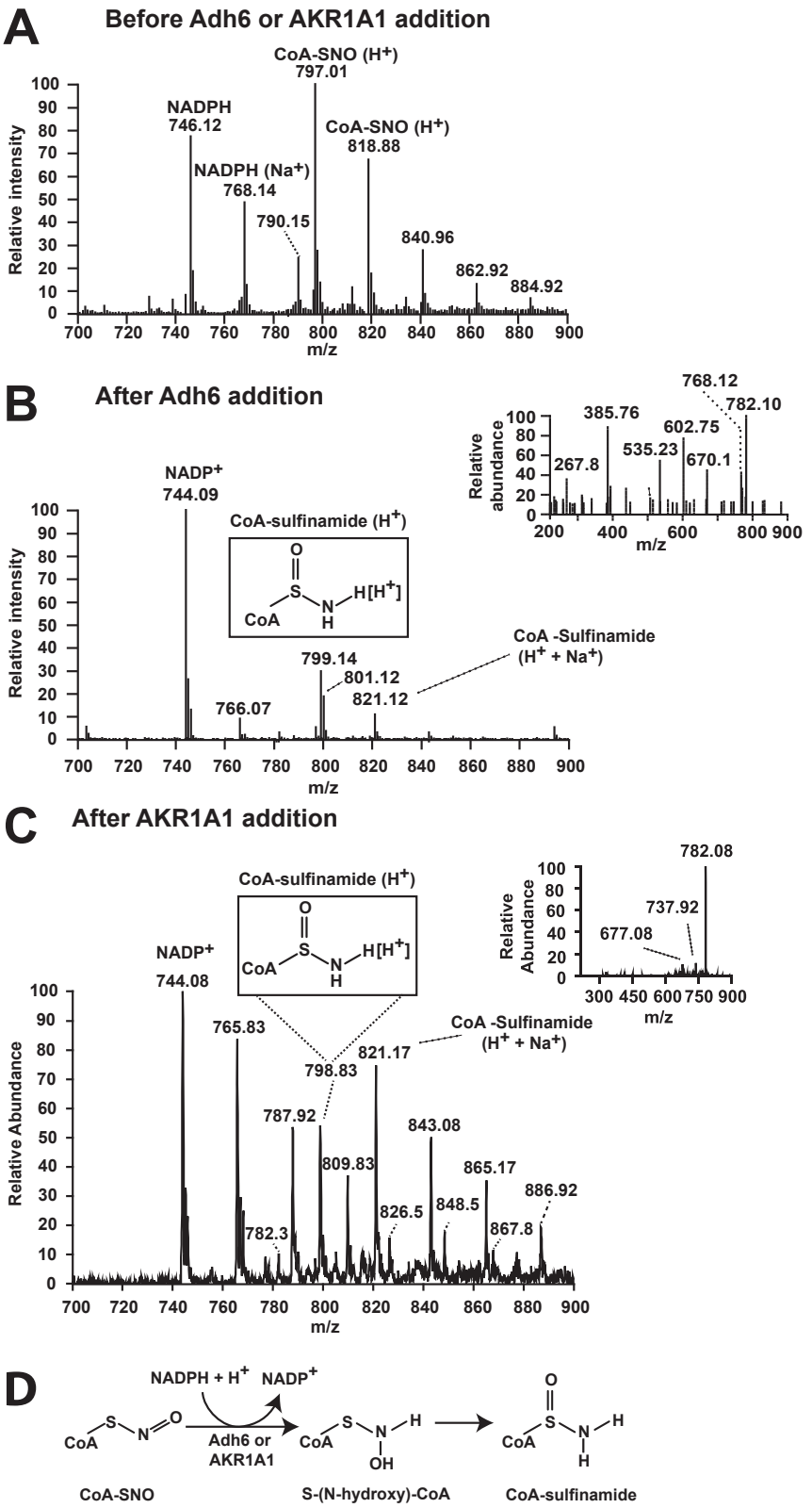


Fig. S2. Product analysis of SNO-CoA reduction by Adh6 and AKA1A1. (*A* and *B*) Electron-spray ionization mass spectra of the reactants (*A*) and products (*B*) of assay of Adh6 purified from yeast. In *A* and *B*, clusters of peaks at 22 atomic mass unit intervals represent sodiated species. In *B*, peaks at *m/z* 799 and 821 correspond to CoA-sulfinamide and its Na^+ adduct, respectively. *Inset* shows the MS/MS spectra of the peak at *m/z* 799. Presence of a peak at *m/z* 782 indicates a loss of NH_3^+, confirming CoA-sulfinamide as the major product. (*C*) Essentially identical results obtained by assay of AKR1A1 purified from bovine kidney. (*D*) Enzymatic reaction carried out by Adh6 and by AKR1A1.

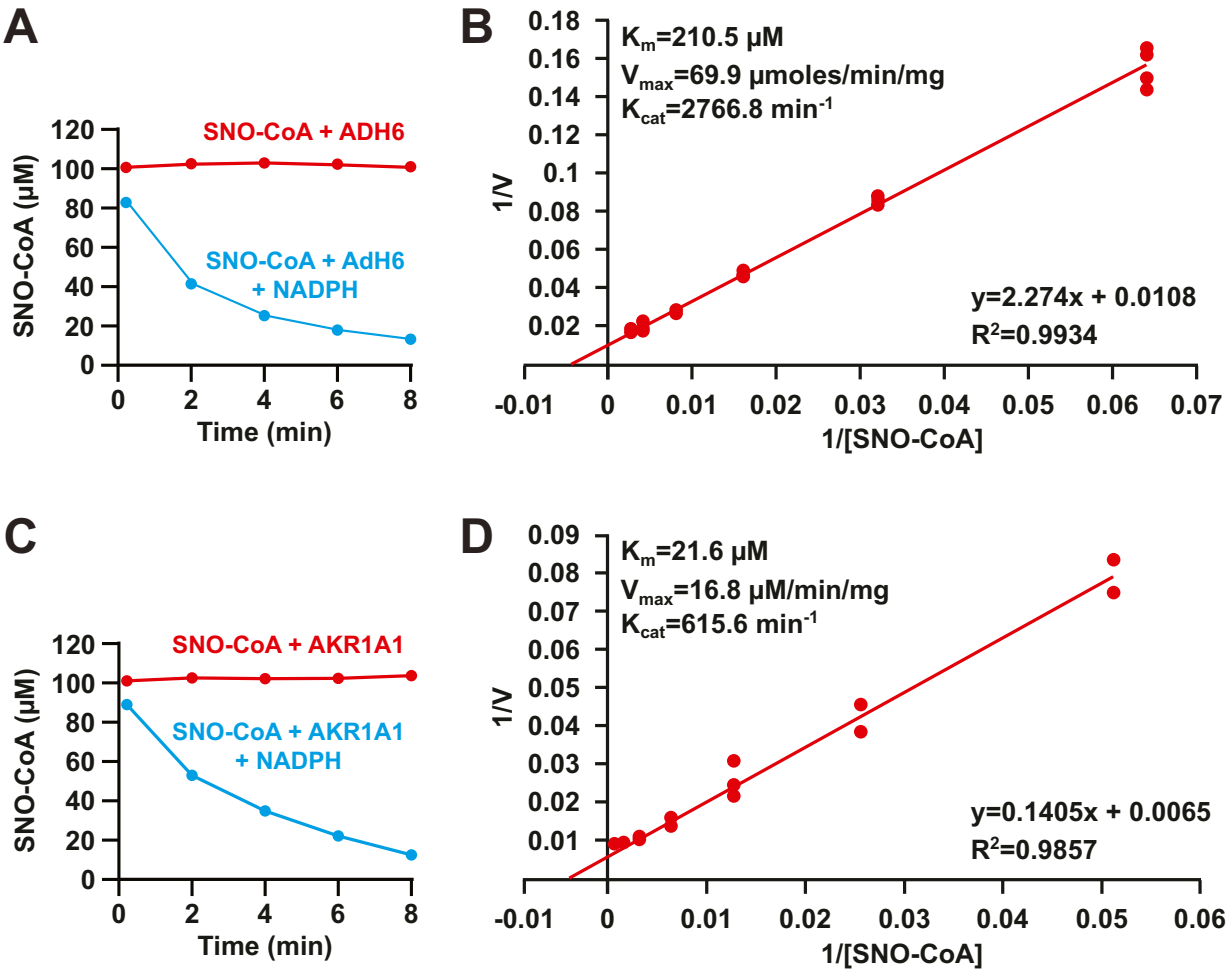


Fig. S3. Catabolism of SNO-CoA by Adh6 and by AKR1A1. (*A* and *C*) Recombinant Adh6 (100 nM; *A*) or (C) recombinant AKR1A1 (30 nM; *C*) (*SI Materials and Methods*) was incubated with SNO-CoA (100 μM), with or without NADPH (100 μM), and SNO-CoA was quantified directly by Saville assay (1) in aliquots withdrawn at 2-min intervals. (*B* and *D*) Lineweaver–Burk plots (derived from data shown in Figs. 2*B* and 5*B*) of SNO-CoA catabolism (NADPH consumption) by Adh6 purified from yeast extracts (*B*) and AKR1A1 purified from extracts of bovine kidney (*D*).

1. Saville B (1958) A scheme for colorimetric determination of microgram amounts of thiols. *Analyst (Lond)* 83:670–672.

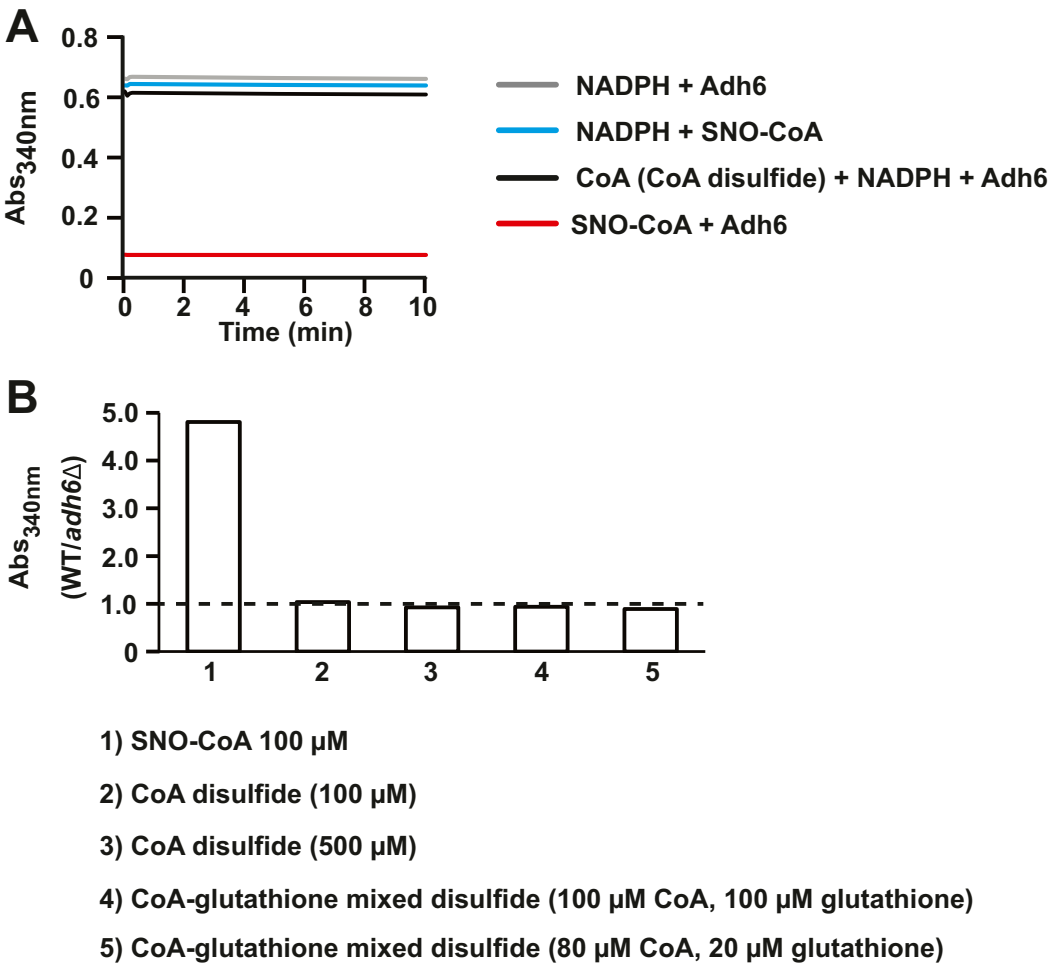


Fig. S4. Evidence for selective SNO-CoA reductase activity of Adh6. (*A*) Enzyme (purified Adh6), substrate (SNO-CoA) and cofactor (NADPH) are required for enzyme activity as assessed by decrease in absorbance at 340 nm. Note that untreated preparations of CoA contain ~30% oxidized CoA. (*B*) Analysis in cellular extracts from WT and *adh6Δ* yeast demonstrates that Adh6 reduces SNO-CoA, but not CoA disulfide or mixed CoA-glutathione disulfide. CoA-disulfide was purchased from Sigma, and CoA-glutathione mixed disulfide was prepared (1, 2) by treatment with equimolar H_2O_2 under conditions in which disulfide formation approaches unity (loss of free thiols confirmed by assay with dithionitrobenzoic acid). Data are presented as mean of two experiments.

1. Luo D, Smith SW, Anderson BD (2005) Kinetics and mechanism of the reaction of cysteine and hydrogen peroxide in aqueous solution. *J Pharm Sci* 94(2):304–316.
2. Abedinzadeh Z, Gardes-Albert M, Ferradini C (1989) Kinetic study of the oxidation mechanism of glutathione by hydrogen peroxide in neutral aqueous medium. *Can J Chem* 67: 1247–1255.

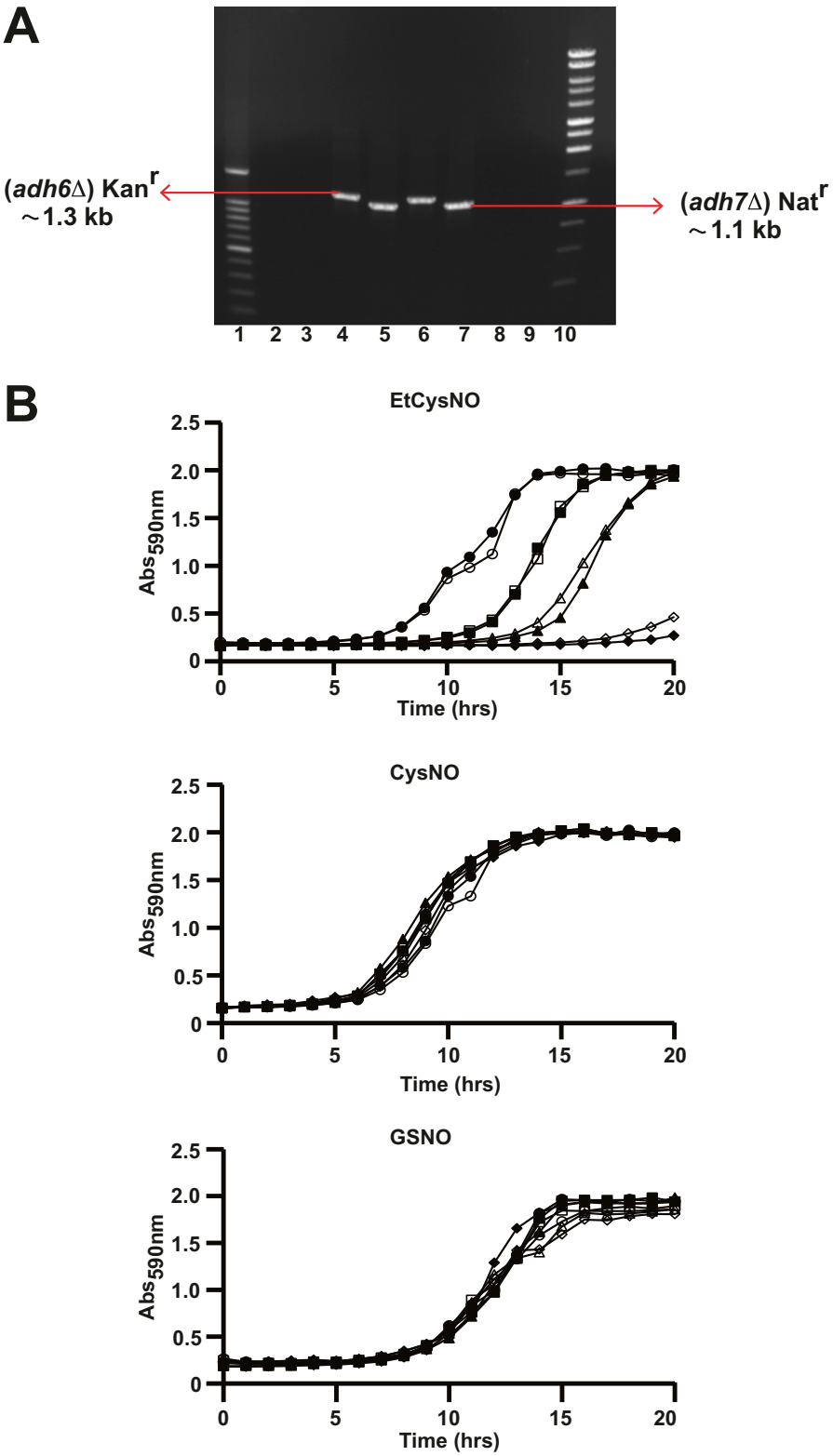


Fig. S5. Lack of protection by Adh6 against nitrosative stress. (*A*) Verification of deletion of *ADH6* and/or *ADH7* by PCR. The *adh6Δ* strain was obtained from Open Biosystems. The *adh7Δ* and *adh6Δadh7Δ* strains were constructed by replacement of the entire ORF of *ADH7* by NAT cassette in the haploid WT (BY4741; Open Biosystems) and *adh6Δ* backgrounds, respectively. To verify *ADH6* deletion by PCR, primer pairs ADH6F/KANR (lanes 2 and 4) were used to amplify WT and targeted fragments, respectively. To verify *ADH7* deletion, primer pairs ADH7F/NATR (lanes 3 and 5) were used to amplify WT and targeted fragments, respectively. To verify combined *ADH6*/*ADH7* deletion, primer pairs ADH6F/KANR and ADH7F/NATR (lanes 6 and 7) were used to amplify KAN and NAT fragments. Lanes 8 and 9 are no DNA controls showing amplification with primer pairs ADH6F/KANR and ADH7F/NATR, respectively. Lanes 1 and 10 show 100-bp and 1-kbp DNA size-ladders (Promega), respectively. (*B*) Sensitivity of WT and *adh6Δ* yeast to EtCysNO, CysNO, and GSNO. Midlog phase WT (filled symbols) or *adh6Δ* (open symbols) cells (A_{600nm} = 0.4–0.6 following overnight culture) were diluted (to A_{600nm} = 0.02) and cultured in YPD medium supplemented with 0 mM (circle), 0.25 mM (square), 0.5 mM (triangle), or 1 mM (diamond) EtCysNO, CysNO, or GSNO. Growth was monitored at 590 nm in 24-well microplates with continuous shaking. Data shown are representative of four similar experiments.

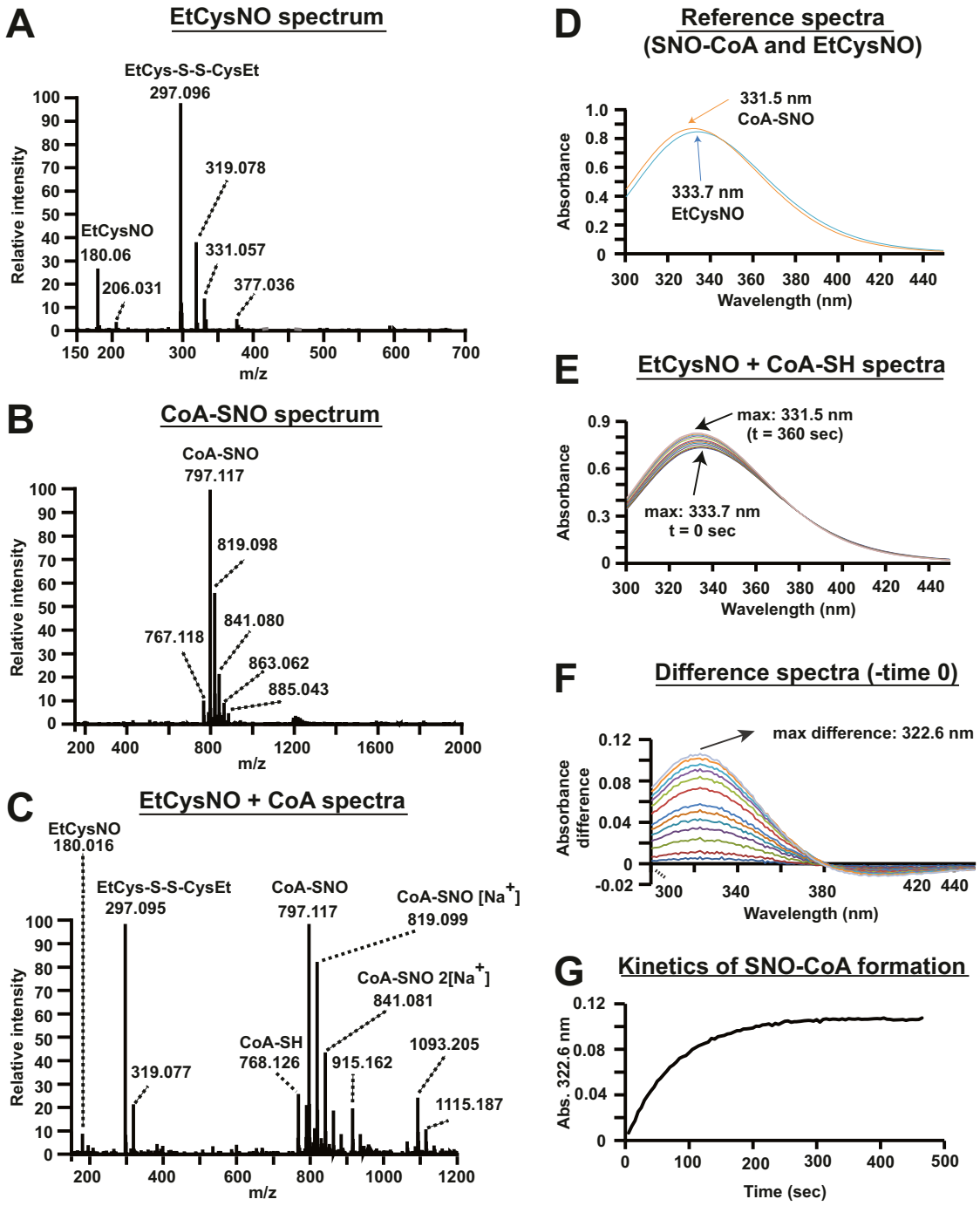


Fig. S6. The S-nitrosylating agent, EtCysNO, efficiently and rapidly transnitrosylates CoA forming SNO-CoA. (*A–C*) Electron-spray ionization spectra of EtCysNO alone (*A*), SNO-CoA alone (*B*), and the products formed from the reaction of equimolar (1 mM) EtCysNO and CoA (*C*). (*C*) Peaks at 797 and 819 correspond to SNO-CoA and its Na^+ adducts. (*D*) Absorbance spectra of pure EtCysNO and SNO-CoA (1 mM). (*E*) Absorbance spectra recorded during the reaction between equimolar (1 mM) EtCysNO and CoA (CoA-SH). Spectra were recorded at 5-s intervals. (*F*) Difference spectra obtained by subtracting the first spectrum obtained ($t = 0$) from all subsequent spectra shown in *E*. The maximum difference is observed at 322.6 nm, and an isosbestic point is at 382.7 nm. (*G*) Time course of SNO-CoA formation monitored at 322.6 nm.

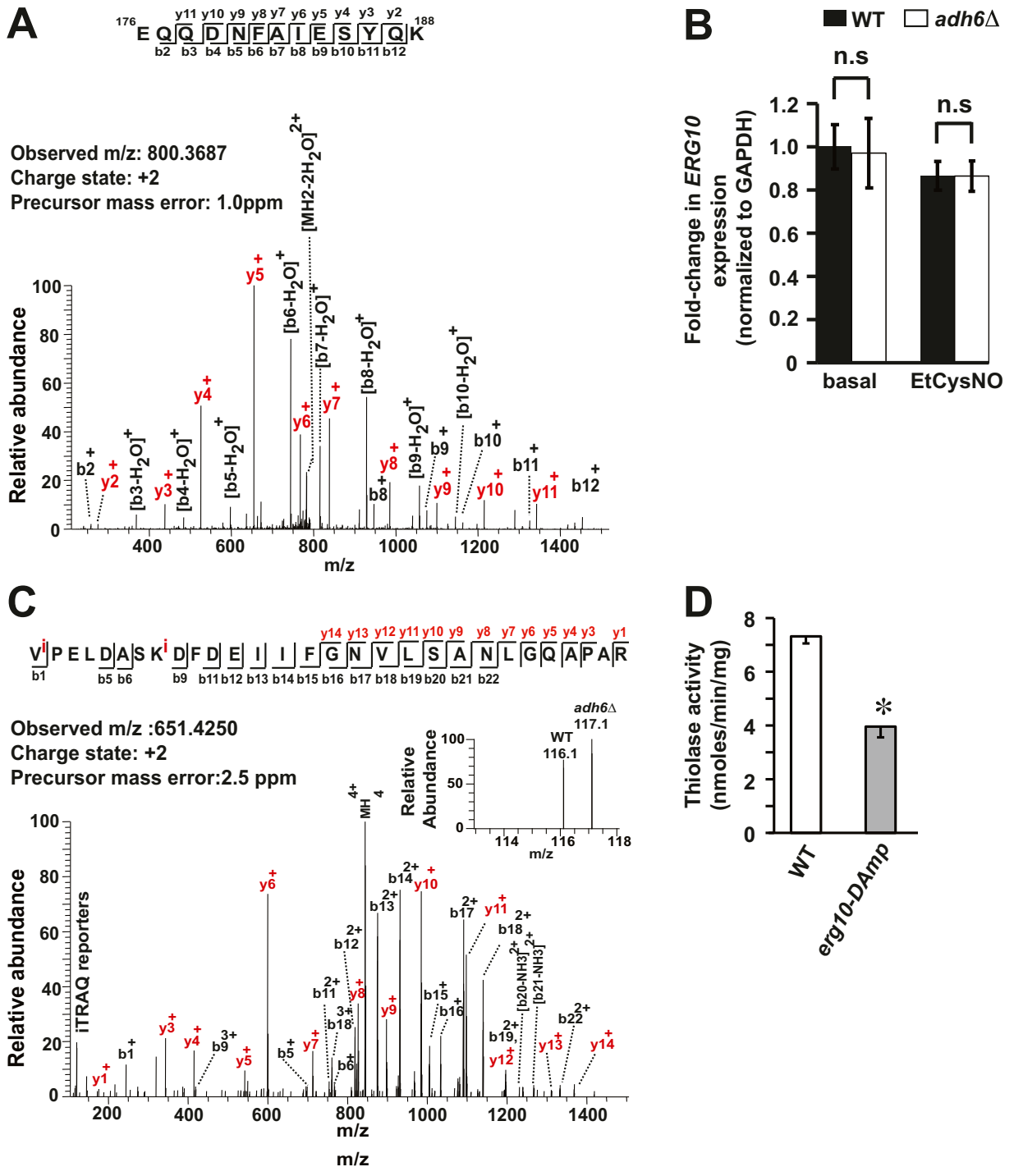
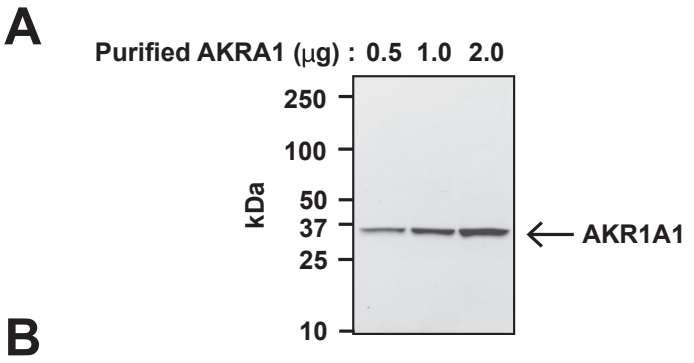
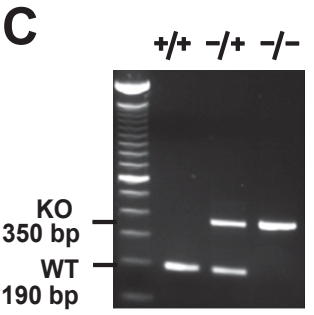


Fig. S7. Identification and quantification of SNO-Erg10 by SNO-RAC and LC-MS/MS. (*A*) S-nitrosylation of Erg10 by SNO-CoA. An annotated MS/MS spectra of a representative peptide derived from SNO-Erg10, S-nitrosylated by treatment of yeast lysate with SNO-CoA, obtained following SNO-RAC and trypsin digest. (*B*) Deletion of *ADH6* did not alter the abundance of Erg10 mRNA, assessed by qPCR, under basal conditions or after treatment (2 h) with EtCysNO (100 μM, 2h) ($n = 3$; $P > 0.05$ by Student t test). (*C*) Endogenous S-nitrosylation of Erg10 by SNO-CoA. Following SNO-RAC and trypsin digest, quantification by iTRAQ of endogenous SNO-Erg10 in WT vs. *adh6Δ* yeast demonstrates enhanced S-nitrosylation of Erg10 in the mutant strain. (*D*) Thiolase activity was assayed in extracts prepared from WT yeast and from the mutant strain *erg10-DAmp* (Thermofisher) ($n = 3$) *$P < 0.05$ by Student t test.

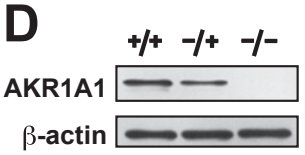


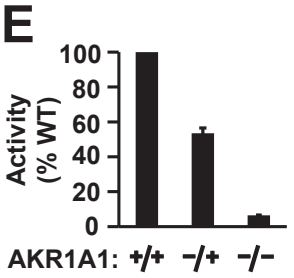


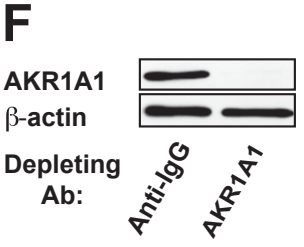
B

001 **MAASCILLHT GQKMPLIGLG TWKSDPGQVK AAIKYALSVG YRHIDCAAIY**
051 **GNETEIGEAL KENVGPGKLV PREELFVTSK LWNTKHHPED VEPALRK TLA**
101 **DLQLEYLDLY LMHWPYAFER GDSPFPKNAD GTIRYDSTHY KETWRALEAL**
151 **VAKGLVRALG LSNFNSRQID DVLSVASVR** P AVLQVECHPY LAQNELIAHC
201 QAR**NLEVTAY SPLGSSDRAW RDPEEPVLLK EPVVLALAEK HGRSPAQILL**
251 **RWQVQRKVSC IPK** SVTPSR **I LENIQVFDFT FSPEEMKQLD ALNKNLRFIV**
301 **PMLTVDGK** R **V PRDAGHPLYP FNDPY**

Measured	Calculated	Mass error (ppm)	Residues
1390.7018	1388.7024	-5.3	1 - 13
1115.6285	1115.6282	0.3	14 - 23
730.3727	730.3730	-0.4	24 - 30
1113.6249	1113.6262	-1.2	24 - 34
928.4881	928.4887	-0.5	35 - 42
2104.0041*	2104.0015	1.2	43 - 61
1165.6706	1165.6688	1.5	62 - 72
952.4993	952.4986	0.7	73 - 80
1942.9967	1941.9929	0.2	81 - 96
2916.4334*	2916.4277	2.0	98 - 120
747.3669	747.3672	-0.4	121 - 127
1640.7662	1640.7663	-0.1	128 - 141
913.4053	913.4050	0.3	135 - 141
1485.6762	1485.6757	0.3	135 - 145
814.5037	814.5033	0.5	146 - 153
1239.7799	1239.7783	1.3	146 - 157
1078.5644	1078.5640	0.4	158 - 167
1301.7057	1301.7060	-0.2	168 - 179
1608.7865	1608.7864	0.1	204 - 218
1452.785	1452.7845	0.3	219 - 230
1068.6303	1068.6299	0.4	231 - 240
1418.8125	1418.8114	0.7	231 - 243
897.5519	897.5516	0.3	244 - 251
716.3831	716.3838	-1.0	252 - 256
831.4771*	831.4757	1.7	257 - 263
2203.0643*	2203.0627	0.7	270 - 287
801.4472	801.4465	0.9	288 - 294
1184.6765	1184.6746	1.6	288 - 297
1235.6707*	1235.6704	0.2	298 - 308
1857.8906	1857.8919	-0.7	310 - 325
1505.67	1505.6696	0.3	313 - 325







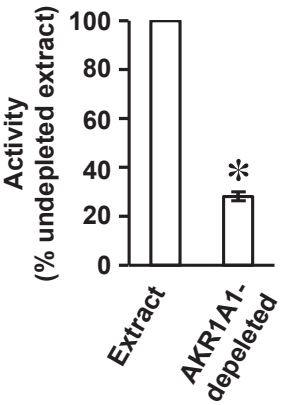


Fig. S8. AKR1A1 is identified as a SNO-CoA reductase in mammals. (*A*) Assessment of the purity of the isolated SNO-CoA–metabolizing activity from bovine kidney. Coomassie-stained SDS/PAGE gel illustrates serial dilutions of the purified protein. (*B*) Identification of the band indicated in *A* by MS. Shown above are peptide-mass matches for AKR1A1 (indicated in bold), which cover 90% of the protein sequence. *Cysteines are carbamidomethylated, and methionines are oxidized in these peptides. Isotopic C13 incorporated ions are automatically included. (*C–F*) Deletion or depletion of AKR1A1 in mice results in markedly diminished SNO-CoA reductase activity. (*C*) PCR amplification of the AKR1A1 gene with genomic DNA isolated from the tails of WT ($^{+/+}$), heterozygous ($^{+/-}$) and homozygous knockout ($^{-/-}$) mice. (*D*) Western blot analysis of AKR1A1 in kidney extracts from WT, heterozygous and homozygous knockout mice. Example shown is representative of three separate experiments. (*E*) NADPH-dependent SNO-CoA reductase activity was assayed in kidney extracts from the indicated strains ($n = 3$ per genotype). (*F*) Immunodepletion of AKR1A1 in WT mice kidney extract results in substantial reduction of the SNO-CoA reductase activity. Results are mean ± SD from three WT kidney extracts. *$P < 0.05$ by Student *t* test.

Table S1. Purification of SNO-CoA Reductases from Yeast and Mammals

Fractions	Volume, mL	Total protein, mg	Total activity, units	Specific activity, units/mg	Yield, %	Fold purification
Purification of an NADPH-dependent SNO-CoA reductase activity from yeast						
Cell free extract	35	825.65	14.245	0.01725	100	1
30–70% ammonium sulfate fractionation						
Fast flow Q-Sepharose	35	78.4	6.02	0.0767	42.26	4.44
2′–5′ ADP-Sepharose	10	0.705	8.91	12.63	62.5	732
Mono-Q	1	0.1004	3.97	39.54	27.86	2,292
Superdex-200	1.4	0.03756	1.8312	48.75	12.85	2,826
Purification of an NADPH-dependent SNO-CoA reductase activity from bovine kidney						
Cell free extract	78	2,008.34	48. 59	0.024	100	1
30–60% ammonium sulfate fractionation	43	964.9	32.63	0.033	67	1.4
Fast flow Q-sepahrose	13.4	59.6	18.29	0.306	37.6	13
Fast flow phenyl Sepharose	12	2.88	9.98	3.46	20.5	144
Mono-Q	1.8	0.88	6.28	7.14	12.9	298
Phenyl superose	0.25	0.698	2.74	15.7	5.63	654
G-200 Superdex	0.35	0.0826	1.51	18.32	3.11	763

Other Supporting Information Files

Dataset S1 (PDF)
Dataset S2 (PDF)