A Pathway for Repair of NAD(P)H in Plants*

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Background: Hydrated forms of NAD(P)H can impair metabolic networks and need to be recycled to functional forms.
Results: Two NAD(P)H repair enzymes are identified in mitochondria and plastids.
Conclusion: NAD(P)H repair enzymes exist in plants but are dispensable for normal plant growth.
Significance: This study paves the way to identify other metabolite recycling pathways in plants.

Unwanted enzyme side reactions and spontaneous decomposition of metabolites can lead to a build-up of compounds that compete with natural enzyme substrates and must be dealt with for efficient metabolism. It has recently been realized that there are enzymes that process such compounds, formulating the concept of metabolite repair. NADH and NADPH are vital cellular redox cofactors but can form non-functional hydrates (named NAD(P)HX) spontaneously or enzymatically that compete with enzymes dependent on NAD(P)H, impairing normal enzyme function. Here we report on the functional characterization of components of a potential NAD(P)H repair pathway in plants comprising a stereospecific dehydratase (NNRD) and an epimerase (NNRE), the latter being fused to a vitamin B₆ salvage enzyme. Through the use of the recombinant proteins, we show that the ATP-dependent NNRD and NNRE act concomitantly to restore NAD(P)HX to NAD(P)H. NNRD behaves as a tetramer and NNRE as a dimer, but the proteins do not physically interact. In vivo fluorescence analysis demonstrates that the proteins are localized to mitochondria and/or plastids, implicating these as the key organelles where this repair is required. Expression analysis indicates that whereas NNRE is present ubiquitously, NNRD is restricted to seeds but appears to be dispensable during the normal Arabidopsis life cycle.

Metabolites are prone to damage by unwanted and nonspecific enzymatic side reactions or chemical modifications that can impair metabolic networks and result in toxicity if not prevented. It is becoming increasingly apparent that there are recycling enzymes present in cells that can convert these “anti-metabolites” back into functional forms (i.e. metabolite repair), thereby maintaining the efficiency of metabolic processes (1, 2). Although proofreading is a well established phenomenon in the case of DNA metabolism and protein synthesis, metabolite repair (also known as metabolite proofreading) has only recently been recognized to be an important cellular occurrence (2). Metabolites that are repaired include highly reactive enzyme cofactors as well as damaged pathway intermediates, such as amino acids, nucleobases, and sugars (2). Many of these reactions are spontaneous as a result of the cellular milieu and may be further enhanced under stress conditions (1, 3). It is therefore surprising that the importance of enzymes involved in repair of such metabolites has been somewhat overlooked (1, 2).

Nicotinamide adenine dinucleotide (NADH) and its phosphorylated derivative, NADPH, are essential for all living organisms. They play a central role in metabolism as enzymatic cofactors in numerous redox reactions linked to the tricarboxylic acid cycle and oxidative phosphorylation as well as several other anabolic and catabolic reactions related to amino acid, sugar, and fatty acid metabolism. Enzymes that catalyze substrate oxidation mainly use NAD⁺, whereas enzymes that catalyze substrate reduction generally use NADPH. Both of these pyridine nucleotides also play roles during stress responses, including oxidative stress, salt stress, and abberant temperature responses (4). NADH also acts as the substrate for the addition of ADP-ribose moieties or the removal of acyl groups by transfer to ADP-ribose, which are major regulatory modifications of proteins and nucleic acids affecting signaling pathways (5). In addition, NAD(P)H pyridine nucleotides have been implicated in lifespan and cell death responses, calcium release, and control of the circadian clock (5, 6). This diversity of activity illustrates the fundamental role of NAD(P) and the importance of its homeostasis in cellular metabolism.

The biosynthesis of both NADH and NADPH has been extensively studied, but the importance of rectifying unfavorably modified adducts has been greatly overlooked for many years. One such modified adduct is the hydrated form of NAD(P)H referred to as NAD(P)HX, which is present in two epimeric states, (S)-NAD(P)HX or (R)-NAD(P)HX (Fig. 1). This adduct was first observed as a side product upon incubation of NADH with glyceraldehyde 3-phosphate dehydrogenase (7-9). In addition, NADHX (as well as NADPHX) can also be produced spontaneously when NAD(P)H is incubated in acidic conditions at moderately high temperatures (10). Because NAD(P)HX inhibits several dehydrogenases in vitro (11, 12), it is assumed to be toxic in vivo. Detoxification seems therefore to be crucial. Indeed, a dehydratase dependent on ATP was implicated already several years ago in this context (10) and is probably one of the earliest reports of what is now referred to as metabolite repair or proofreading. It is only recently that an NAD(P)HX dehydratase has been character-
ized and shown to be stereospecific, catalyzing the conversion of (S)-NAD(P)HX into NAD(P)H (3). Furthermore, an epimerase was identified in the same study that acts in conjunction with the dehydratase converting (R)-NAD(P)HX into (S)-NAD(P)H (3). Homologs of these enzymes were characterized from *Escherichia coli*, *Saccharomyces cerevisiae* (yeast), and *Mus musculus* (mouse). Interestingly, the two enzymes are part of a bifunctional fusion protein in *E. coli* but exist as separate entities in yeast and mouse (3) (Fig. 2A). Also, surprisingly, whereas the eukaryotic dehydratase homologs are dependent on ATP, the *E. coli* protein relies on ADP (3). Given the physiological importance of NAD(P)H homeostasis in plants in particular, not only as a signaling agent and as a cofactor for redox reactions of the metabolic pathways mentioned above but also as an electron donor/acceptor during photosynthesis in plants, it is imperative to study this pathway therein.

Here, we report on a potential NAD(P)H repair pathway in plants. In particular, we identified the *Arabidopsis thaliana* dehydratase (NNRD) and epimerase (NNRE) enzymes that constitute the pathway and demonstrate their activity as NAD(P)H repair enzymes. Gel filtration coupled to static light scattering demonstrates that NNRD is a tetramer, whereas NNRE is a dimer. The proteins do not physically interact, which is probably a function of the oligomeric state that is required for activity. Interestingly, we found that the enzymes localize to mitochondria and/or chloroplasts, indicating the importance of NAD(P)H elimination in these organelles. Expression data at the mRNA and the protein level indicate that whereas NNRE is present ubiquitously, NNRD is restricted to seeds but appears to be dispensable during normal growth.

**EXPERIMENTAL PROCEDURES**

*General Plant Material and Growth Conditions—* *A. thaliana* (Columbia ecotype) and the *nnrd-1* (GK-104E02) and *nnrd-2* (GK-173F11) mutant lines (obtained from the European Arabidopsis Stock Center) were grown under standard growth conditions (60% relative humidity, 100–150 μmol photons m⁻² s⁻¹, and 22 °C for 16 h, i.e. long day) unless specified otherwise, followed by 8 h of darkness at 18 °C). For *in vitro* culture, seeds were surface-sterilized with ethanol and sown on plates containing Murashige and Skoog medium (13) and 0.6% agar (Duchefa). Plant lines homozygous for *nnrd-1* and *nnrd-2* were verified by PCR analysis of genomic DNA using the following primer sequence pairs for the wild-type and T-DNA insertion allele, respectively: TGGAGGAAGACAGCCGATATC and TGCTGTCTTAACCACCAATG; ATATTGACCATCATGAGTTCC and ATGTCTTATCTTACTCAGCGAGAAG and CCGCTCGAGATGAGTTCC and GATGTGGAACTCATCATATGCTGTCTGATGATTGG). The absence of expression of NNRD in the respective lines was verified by quantitative real-time RT-PCR (qPCR) and immunocchemical analyses (see “Gene Expression Analyses”).

*Expression and Purification of Recombinant Proteins—* Arabidopsis PDX3 (At5g49970), NNRE, and NNRD (At5g19150) were amplified from cDNA from 10-day-old seedlings as for plant expression vectors using a proofreading polymerase and subcloned into the pCR2.1TOPO vector using the TOPO-TA cloning kit (Invitrogen) according to the manufacturers’ instructions. In all cases, the predicted transit peptide within residues 2–45 and 2–73 in NNRD and PDX3, respectively, was omitted (primer sequences for NNRE, CTACATATGTCTTATCTTACCTACGGGAAAG and CCGCTCGAGATTCTAACAACACATAGTGTC; PDX3, CTACATATGTCTTATCTTACCTACGGGAAAG and CCGCTCGAGTTGGCCTAACATCTATGATGATTTC; NNRD including transit peptide sequence, CTACATATGTTGGTGAAGCCCAGTATCATC and CCGCTCGAGATGAGTTCC; NNRE and NNRD were then cloned into the pET-24b and pET21a vectors (Novagen), respectively, for expression with a C-terminal hexahistidine tag. PDX3 was cloned into the pET-28a vector (Novagen) for expression with a N-terminal hexahistidine tag. Cloning in each case was facilitated through the use of the Ndel and Xhol restriction sites. Expression was carried out in the *E. coli* BL21 (DE3) strain using 0.1 mM isopropyl β-D-1-thiogalactopyranoside for induction when the cultures reached an optical density of 0.5 at 600 nm followed by 3 h of growth at 37 °C. After harvesting by centrifugation, the bacteria were resuspended in lysis buffer (25 mM Tris-HCl, pH 8.0, containing 300 mM sodium chloride, 10 mM imidazole, 0.1 mM PMSF, and protease inhibitor mixture (Roche Applied Science)) and lysed by lysozyme as well as sonication, and the extracted soluble protein was purified by nickel-nitritriacetic acid (Ni-NTA)² affinity chromatography using the same buffer as used for lysis but substituted with sequential rounds of 20 and 250 mM imidazole for washing and eluting the protein, respectively. The NNRE and NNRD purified proteins were buffer-exchanged into 25 mM Tris-HCl, pH 8.0, containing 100 mM potassium chloride and stored in aliquots at −80 °C or used directly in enzymatic assays. PDX3 was further purified by anionic exchange chromatography and size exclusion chromatography. Specifically, Ni-NTA-purified PDX3 was buffer-exchanged into 25 mM Tris-HCl, pH 8.0, containing 50 mM sodium chloride and loaded onto a MonoQ 5/50 GL column (GE Healthcare). After washing with Buffer A, the protein was eluted using a two-step gradient (21 and 50% 20 mM Tris, pH 8.0, containing 1 mM NaCl). The PDX3-containing fractions were pooled and concentrated, and the resulting sample was subjected to size exclusion chromatography on a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare) and eluted using 20 mM HEPES, pH 8.0, containing 150 mM KC1 and 5 mM β-mercaptoethanol. The purified protein was either used directly for enzyme assays or aliquoted and stored at −80 °C. For co-expression studies, stop codons were introduced between the respective coding sequence and the C-terminal affinity tag. The plasmids coding for either NNRE-His and NNRD native (or NNRE native and NNRD-His, respectively) were co-transformed into BL21 (DE3), and double transformants were selected by resistance to ampicillin and kanamycin. For co-expression of PDX3 and

²The abbreviations used are: Ni-NTA, nickel-nitritriacetic acid; qPCR, quantitative real-time RT-PCR; PMP, pyridoxamine 5'-phosphate; PNP, pyridoxine 5'-phosphate; Al-BP, apolipoprotein A-1-binding protein; IMAC, immobilized metal affinity chromatography.
NNRD, NNRD was first cloned into the pET-21a vector as described above. Proteins were purified by Ni-NTA chromatography exactly as described above but using buffers with reduced salt concentration (50 mM sodium chloride).

Production of NAD(P)HX and Enzyme Assays—The hydrated forms of NAD(P)H were produced as described by Achenes et al. (10) by dissolving 2 mg of NAD(P)H (Sigma) in 100 μl of 0.5 M sodium phosphate buffer, pH 6.0, and incubation at 35 °C for 30 min. The reaction was stopped by the addition of sodium hydroxide to reach pH 8.0. NAD(P)H solutions were prepared freshly for each use. The spectrophotometric assays were carried out essentially as described by Marbaix et al. (3) in 25 mM Tris-HCl, pH 8.0, containing 1 mM ATP, 0.1 mg/ml BSA, 2 mM MgCl₂, 5 mM KCl, and 0.4 μl of the generated NAD(P)HX mixture (corresponding to ~3.4 mM (S)-NADHX and 2.3 mM (R)-NADHX based on ε₂₉₀ = 13,500 M⁻¹ cm⁻¹ (7)) and in a total volume of 100 μl each containing a 0.125 μM concentration of the respective enzymes (NNRD, NNRE, or PDX3) at 25 °C in a 96-well plate reader (Biotek Synergy 2). NAD(P)H consumption and NAD(P)H production were measured simultaneously at 290 and 340 nm, respectively. After establishing a baseline, the reaction was started by the addition of NNRE followed by the respective enzymes (NNRD, NNRE, or PDX3) at 25 °C in a 2 ml of sample was injected, and separation was initiated using a methanol (MeOH) gradient under the following conditions: 0–30 min, 0–15.5% MeOH; 30–40 min, 15.5–0% MeOH; 40–41 min, 15.5–0% MeOH; 41–50 min, 0% MeOH; at a flow rate of 1 ml/min at 25 °C.

Size Exclusion Chromatography Coupled to Multiangle Light Scattering—One hundred μl of the respective purified proteins was separated by size exclusion chromatography on a Superdex 200 10/300 GL column (GE Healthcare) using 20 mM HEPES, pH 7.5, containing 50 mM potassium chloride and 0.01% sodium azide. A miniDAWN TREOS light scattering instrument (Wyatt Technologies) was connected immediately downstream of the separation media for light scattering analysis. The data were analyzed using the software Astra (Wyatt Technologies). The protein concentration required for weight average molecular mass (Mₐ) calculations was measured online using the generic UV detector of the AKTA purifier system (GE Healthcare) for NNRE and NNRD. Absorption coefficients used for the calculation of the Mₐ were 695 ml g⁻¹ cm⁻¹ for NNRE and 600 ml g⁻¹ cm⁻¹ for NNRD. For the calculation of the Mₐ of PDX3, the refractive index detector Optilab® T-Rex (Wyatt Technologies) was used as the concentration detector (the value used for the differential index of refraction (dn/dc) was 0.185).

Transgenic Fluorescent Plant Lines and Confocal Microscopy—Full-length PDX3 as well as the epimerase domain alone, including the transit peptide (TP-NNRE 1–927 bp) and NNRD were amplified from cDNA of 10-day-old seedlings using a proofreading polymerase (Strategene) and specific primer pairs (PDX3, CACCATGAGGAATGTGATAAGC and TGGGGC-CATCTATGAAATTTCC; NNRE, CACCATGAGGAATTGTGATAAGC and AATCCTAACACACATAGATGC-CCTG; NNRD, CACCATGTTGGTAGACCCAGAT-TCATC and AGATGCGGCAATATCCTCAAA). The amplified products were cloned into the pENTR-D/TOPO vector using the pENTR TOPO cloning kit (Invitrogen) according to the manufacturer’s instructions, sequenced, and subsequently cloned into Gateway® destination vectors by an LR reaction using LR clonase enzyme mix II (Invitrogen). Full-length PDX3 and TP-NNRE were cloned into pB7YWG2 (14), whereas NNRD was cloned into pUBC-YFP-DEST (15), all to be expressed as fusion proteins with YFP at the C terminus. The constructs and the empty vectors as a control were introduced into Agrobacterium tumefaciens strain C58 and used to transform wild-type (Col-0) Arabidopsis plants by the floral dip method (16). Because the respective constructs contain the BAR gene, transformants were selected by resistance to BASTA™. Resistant plants were allowed to self-fertilize, and homozygous lines were selected from the T3 generation according to their segregation ratio for BASTA™ resistance. Three-day-old transgenic seedlings were mounted in water in a flow chamber and subjected to analysis (cotyledon, hypocotyl, and root tissue) on an SP5 confocal laser-scanning microscope (Leica) equipped with a resonant scanner and a ×63 Oil, numerical aperture 1.4 PlanApo lens; the zoom was set so that the pixel size was between 80 and 120 nm. Arabidopsis mesophyll protoplasts were isolated from stable transformant lines according to previously described protocols, and microscopy analysis was performed in W5 solution (see below) (17, 18) in a flow chamber. Mitochondria of seedlings or protoplasts were stained by incubation in either a 500 or 50 nM concentration, respectively, of MitoTracker® Red CMXRos (Invitrogen; prepared according to the manufacturer’s instructions) for 30 min at room temperature in W5 solution (1.5 mM Mes-KOH, pH 5.6, containing 154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, and 5 mM glucose). MitoTracker staining was removed from protoplasts by centrifugation at 50 × g for 5 min in a swing-out rotor and resuspension in W5 solution. In the case of seedlings, the stain was removed by washing twice in W5 solution for 10 min. YFP, chlorophyll, and MitoTracker Red CMXRos were excited with the 514-, 470-, and 567-nm lines, respectively, of a white light laser, and their fluorescence was recorded between 519 and 570 nm, 650 and 800 nm, and 580 and 620 nm, respectively. Chlorophyll fluorescence was collected by a photomultiplier detector, whereas YFP and MitoTracker fluorescence signals were collected by HyD detectors set at a time gating between 2 and 10 ns and 2 and 11 ns, respectively, to minimize autofluorescence signals. Image analysis was performed using ImageJ (W. S. Rasband, National Institutes of Health). A Gaussian blur of radius 0.8 pixels was applied to all z slices, and maximum intensity projections were subsequently performed. For immunochemical analysis of mesophyll protoplasts, proteins were extracted by resuspending the protoplasts (pooled by centrifugation at 11,000 × g for 5 min) in 100 mM Tris-HCl, pH 6.8, containing 4% SDS and 20 mM EDTA and subsequent centrifugation (11,000 × g). The supernatant was decanted, and 15 μl was separated on a 10% SDS-polyacrylamide gel. Immunodetection was carried out as described under “Gene Expression
Analyses,” employing an antibody against GFP (Santa Cruz Biotechnology, Inc.) at 1:2000 dilution and the respective secondary antibody (peroxidase conjugated goat anti-rabbit, Bio-Rad) at 1:3000 dilution.

Gene Expression Analyses—Tissue samples were collected from soil-grown plants under long day conditions with the exception of roots and 10-day-old seedlings, which were collected from in vitro cultures on Murashige and Skoog medium (Duchefa) and imbibed seeds that were plated on wet filter paper. RNA was extracted using the RNA NucleoSpin plant kit (Machery-Nagel) according to the manufacturer’s instructions. DNA was removed by an on-column DNase digest during the RNA extraction. Reverse transcription was performed using up to 1 µg of total RNA as a template and Superscript II (Invitrogen) according to the instructions with the following modifications. Oligo(dT)20 primer stock concentration was 50 ng/µl, and 0.25 µl of SuperscriptII enzyme was used per reaction. qPCR was performed in 384-well plates in a 10-µl reaction volume on a 7900HT fast real-time PCR system (Applied Biosystems) using Power SYBR Green master mix (Applied Biosystems). Oligo(dT)20 primer stock concentration was 50 ng/µl, and 0.25 µl of SuperscriptII enzyme was used per reaction.

RESULTS

Identification of the Genes Involved in the NAD(P)HX Repair Pathway in Plants—As mentioned above, E. coli contains a fusion protein that harbors both the dehydratase and epimerase activities of the NAD(P)H repair pathway (annotated as Yjef), whereas yeast and mouse carry both activities as separate proteins (Fig. 2A). Given the involvement of the proteins in nicotinamide nucleotide repair, it has been proposed to annotate the corresponding NAD(P)X dehydratase and NAD(P)H epimerase as NnrD and NnrE, respectively (3) (Figs. 1 and 2). Homologs of NNRD and NNRE can be found in all sequenced plant genomes to date (see the Phytozome Web site). A search of the Arabidopsis genome reveals a single homolog each for NNRD and NNRE. Interestingly, the putative dehydratase, NNRD, is encoded as a single domain protein (At5g19150), whereas the putative epimerase, NNRE (At5g49970), is C-terminally fused to an oxidase involved in the salvage of vitamin B₆ (Fig. 2A). The latter protein has been demonstrated to oxidize pyridoxamine 5’-phosphate (PNP) to pyridoxine 5’-phosphate (PNP) to the cofactor form pyridoxal 5’-phosphate and is referred to as the POX domain (22). Intriguingly, NNRE is fused to this POX domain in algae, mosses, and lower and higher plants (i.e. the green lineage) (23).

The Arabidopsis NNRE (amino acids 1–327) is 29% identical with the E. coli (Yjef N-terminal domain), as well as with the yeast homolog (YNL200C), and 39% with the mouse homolog (apolipoprotein A-1-binding protein, AI-BP), respectively (Fig. 2B). The corresponding identity for Arabidopsis NNRD shows 32, 25, and 40% identity with the E. coli (Yjef C-terminal domain), Bacillus subtilis (YxKO), yeast (YKL151C), and mouse (annotated as Carkd) homologs, respectively (Fig. 2C). Surprisingly, there is no homolog of NNRE in B. subtilis based on amino acid sequence identity. A comparison of the amino acid sequence of Arabidopsis NNRE and NNRD with that of its characterized homologs reveals that the putative respective active sites are conserved (Fig. 2, B and C, respectively). The latter aspects are explored further under “Discussion.”

Arabidopsis NNRD and NNRE Function in NAD(P)H Repair—The homology between the Arabidopsis, bacterial, yeast, and
NAD(P)H Repair in Plants

![Scheme illustrating the repair of NADH hydrates.](image)

**FIGURE 1. Scheme illustrating the repair of NADH hydrates.** A water molecule can be added to NADH or its phosphorylated derivative, NADPH, to form hydrates called NAD(P)HX either as a side reaction of cellular dehydrogenases, spontaneously, or under certain stress conditions. The dehydratase, NNRD, is stereospecific for the (S)-NAD(P)HX epimer and regenerates NAD(P)H in an ATP-dependent manner (ADP in *E. coli*). The epimerase NNRE works in conjunction with NNRD and serves to provide more of the (S)-epimer from (R)-NADPHX.

Mouse NNRD and NNRE orthologs suggests that the NAD(P)HX repair is conserved in plants. In order to assess the corresponding activities, the putative dehydratase was amplified from seedling cDNA and cloned into a bacterial expression vector to be recombinantly expressed with a C-terminal hexahistidine tag, facilitating purification (NNRD-His). In the case of the epimerase, both the domain incorporating the epimerase (residues 74–298) and the full-length PDX3 gene were also amplified from seedling cDNA and cloned into a bacterial expression vector for recombinant protein expression with either a C-terminal or N-terminal hexahistidine tag, respectively (NNRE-His and His-PDX3). Notably, sufficient soluble functional expression of PDX3 could not be achieved when the hexahistidine tag was at the C terminus. In both cases, the putative organelle-targeting peptides (corresponding to residues 2–43 in NNRD and 2–63 in PDX3) were omitted for bacterial expression. Both NNRD and NNRE from *Arabidopsis* could be purified to near homogeneity using immobilized metal ion affinity chromatography (IMAC) (Fig. 3A). The yield of protein in each case was 8.3 and 9.3 mg/liter of bacterial culture. His-PDX3 was further purified using a combination of ion exchange and size exclusion chromatography to reach near homogeneity (Fig. 3A) with a yield of 0.2 mg/liter of bacterial culture. Electrospray ionization mass spectrometry confirmed the identity of the proteins: NNRD-His (expected mass, 35,702.9 Da; observed mass, 35,701.0 Da), NNRE-His (expected mass, 27,222.1 Da; observed mass, 27,090.0), and His-PDX3 (expected mass, 53,471.6 Da; observed mass, 53,477.5). Note that in the case of NNRE-His, the N-terminal methionine is processed.

In order to validate the anticipated activities, we utilized a spectrophotometric assay developed by Marbaix et al. (3). Briefly, the hydrated forms of NADH (or NADPH) can be produced by incubating the compounds in high concentrations of inorganic phosphate at slightly acidic pH (10). The result is a mixture of (S)-NAD(P)HX and (R)-NAD(P)HX as well as cyclic forms of NAD(P)HX. Each of these compounds is easily separated by HPLC, facilitating their identification (Fig. 3B). The addition of *Arabidopsis* NNRD (i.e. the dehydratase) and ATP to such an NADHX mix led to a decrease in absorbance at 290 nm, which is the wavelength where the absorbance difference is greatest between NADHX and NADH demonstrating NADHX consumption (Fig. 3C). Because the dehydratase is stereospecific and only expected to use (S)-NADHX, its epimer (R)-NADHX remains in solution. Therefore, the addition of the epimerase is expected to convert the remaining (R)-NADHX to (S)-NADHX, which can then be acted on by the dehydratase. Indeed, upon the addition of *Arabidopsis* NNRE, a second decrease in the absorbance at 290 nm was observed, validating the epimerase action of NNRE (Fig. 3C). Concomitant with NADHX consumption, an increase in the absorbance at 340 nm can be observed, demonstrating NADH production (Fig. 3D). Upon the addition of NNRD, a burst of NADH production was observed, consistent with the conversion of (S)-NADHX to NADH. The subsequent addition of NNRE and a second burst in NADH production demonstrate the epimerization of (R)-NADHX to (S)-NADHX and conversion of the latter to NADH by NNRD, already present in the mixture (Fig. 3D). These results were also corroborated by HPLC analysis, where the peak corresponding to (S)-NADHX cannot be observed in the presence of *Arabidopsis* NNRD (Fig. 3E, gray trace). When NNRD and either NNRE or PDX3 are both present, the peak due to (R)-NADHX is no longer observed (Fig. 3E, black traces). Neither enzyme acts on the cyclic NADH derivatives (Fig. 3E), as has also been observed by Marbaix et al. (3). It is noteworthy that similar profiles are observed with both the truncated epimerase domain (NNRE-His) and the full-length PDX3 (His-PDX3), indicating that the POX domain present in PDX3 does not interfere with the epimerase activity. The dehydratase and epimerase activities of NNRD and NNRE, respectively, were also performed and validated with NADPH, leading to very similar results (data not shown). These data are corroborated...
by an independent study that appeared while this manuscript was under review (24).

Arabidopsis NNRD and NNRE Function in Non-stoichiometric Oligomeric States—We next went on to characterize the biochemical properties of both enzymes in more detail. In the first instance, we employed size exclusion chromatography coupled to static light scattering to determine the oligomeric state of the respective proteins. In each case, the proteins eluted as monodisperse symmetric peaks, indicating homogeneity (Fig. 4A). The estimated size of the NNRE domain alone or the full-length PDX3 (52,280 ± 1052 and 104,000 ± 1052 Da, respectively) corresponded to a dimer (expected sizes of dimers, 54,440 and 107,200 Da, respectively). This is the same oligomeric state that has been reported for several crystallized NNRE homologs, such as mouse AI-BP and the human ortholog enhancer of decapping 3 (Edc3) (25, 26). Notably, all characterized PNP/PMP oxidases corresponding to the POX domain of PDX3 are also dimers either in solution (27–29) or as derived from crystal structures (30–33). Interestingly, in the case of the dehydratase NNRD, the estimated size (132,700 Da) is larger than expected for a monomer (expected size of monomer, 66,350 Da). This may suggest the presence of multimers, which are not resolved by the method of analysis used here. Further studies will be required to determine the exact oligomeric state of NNRD in solution. Notably, all characterized PNP/PMP oxidases corresponding to the POX domain of PDX3 are also dimers either in solution (27–29) or as derived from crystal structures (30–33). Interestingly, in the case of the dehydratase NNRD, the estimated size (132,700 ±

FIGURE 2. Conservation of NNR orthologs. A, domain organization of NNR orthologs from different organisms. In E. coli, the epimerase (NNRE) and dehydratase (NNRD) domains are fused in a single polypeptide chain, whereas in yeast and mammals, they exist as separate entities. In plants and algae, the NNRE domain is fused to a domain involved in vitamin B₆ biosynthesis (POX), whereas NNRD exists as a single protein. B and C, amino acid sequence alignment of NNRE and NNRD, respectively, from A. thaliana, M. musculus, S. cerevisiae, E. coli, and B. subtilis. Sequence identity is shaded black, whereas amino acids conserved in 60% of the sequences used are shaded gray. The black lines denote predicted target peptides (TargetP). Notably, there is no homolog of NNRE in B. subtilis. The amino acids marked with an asterisk correspond to annotated active site residues (34) as well as residues strictly conserved in the active site region across all species (Gly-135, Pro-136, Gly-137, and Asp-249 in NNRE; Ala-89, Glu-234, Lys-271, Gly-272, Gly-299, Gly-300, Gly-302, Asp-303, and Leu-304 in NNRD), with the exception of the FSF motif in NNRE and an asparagine in NNRD. The latter residues do not appear to be conserved in bacterial species. The methionine residue marked with a cross could represent an alternative translation start site.
664 Da) closely corresponded to a tetramer (expected size of a tetramer, 142,800 Da). NNRD homologs that have been characterized (34, 35) and for which the crystal structure has been determined classify them within the ribokinase superfamily (35). Members of this family vary in quaternary structure, and the implication of the oligomeric state observed here is further elaborated on under “Discussion.”

The possibility of an interaction between NNRD and NNRE as well as the interesting aspect of their non-stoichiometric oligomeric state prompted us to investigate if a stable complex...
Arabidopsis NNRD and NNRE Partially Overlap in Subcellular Localization but NNRD Has Multiple Locations—Although we did not have evidence for a physical interaction of NNRD and NNRE in vitro, we next wanted to test if these proteins are restricted to the same subcellular compartment. An in silico analysis using TargetP (36), Predotar (37), and the SUBA database (38) predicts N-terminal transit peptides for both PDX3 (found upstream of NNRE) and NNRD (Fig. 2, B and C), with a probability of mitochondrial and plastid localization for NNRD (TargetP, 0.439 versus 0.808. Predotar, 0.22 versus 0.56, for mitochondria and plastid, respectively) as well as for NNRE (TargetP, 0.363 versus 0.542; Predotar, 0.22 versus 0.00, for mitochondrial and plastid targeting, respectively). However, it has recently been reported that PDX3 is exclusively localized to the chloroplast based on epifluorescence data of transient expression in Arabidopsis protoplasts (23). In order to experimentally investigate the in vivo localization of NNRD and the NNRE domain of PDX3, we designed constructs resulting in the fusion of either protein to the N terminus of YFP (NNRD-YFP as well as both PDX3-YFP and NNRE-YFP, respectively) under control of the ubiquitin 10 or cauliflower mosaic virus 35S constitutive promoters, respectively (Fig. 5A). In the case of NNRE, we utilized both the full-length PDX3 protein construct and one incorporating the first 309 amino acids, which include the transit peptide and the NNRE domain. These constructs were transformed into Arabidopsis (Col-0) (16), and stable single insertion homozygous lines were selected. In the first instance, we examined protoplasts isolated from leaf mesophyll cells (Fig. 5B). Protoplasts isolated from the control YFP lines showed the expected fluorescence pattern indicative of a cytosolic and nuclear localization with no overlap on chlorophyll autofluorescence (Fig. 5B, top row). On the other hand, the pattern of NNRD-YFP fluorescence was indicative of multiple subcellular localizations (Fig. 5B, second row). There was clear overlap with the chlorophyll autofluorescence, indicative of a stromal localization (Fig. 5B, second row). Furthermore, co-staining with MitoTracker® Red CMXRos indicated that another fraction of the protein was localized to the mitochondria. The fluorescence pattern of a very small fraction of the fusion protein did not overlap with either chlorophyll autofluorescence or the MitoTracker® Red CMXRos fluorescence and could be indicative of cytosolic localization (Fig. 5B, see arrows in the overlap picture in row 2). Because fluorescent fusion proteins are well known to undergo cleavage resulting in the observation of fluorescence in the cytosol, we confirmed that the fusion protein was intact by immunochronal analysis of the Arabidopsis protoplasts used for fluorescence microscopy using a GFP antibody (Fig. 5B, right column). This suggests that NNRD is found predominantly in chloroplasts and mitochondria, whereas a small fraction may be retained in the cytosol. We also examined the cotyledons, hypocotyl, and roots of independent lines by confocal fluorescence microscopy. In all cases, the pattern of fluorescence was consistent with that observed in protoplasts. In both the guard cells and the hypocotyl, the NNRD-YFP fluorescence pattern partially overlapped with chlorophyll autofluorescence (Fig. 6A, top and middle panels). The additional fluorescence pattern is consistent with co-localization to mitochondria, but these tissues could not be pene-
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![Subcellular localization of NNRE and NNRD in Arabidopsis mesophyll protoplasts.](image)

A domain organization of transgenes expressed in Arabidopsis (Col-0). B, representative pictures of Arabidopsis mesophyll protoplasts isolated from stable transformants. The columns from left to right show YFP (yellow), chlorophyll (colored blue) and MitoTracker (red) and an overlay of fluorescence, respectively. YFP alone localizes to the cytosol and nucleus as expected and serves as a control. NNRD-YFP fluorescence overlaps with both the chlorophyll autofluorescence and the MitoTracker signal, indicating that this protein is dually targeted to both the plastids and the mitochondria. The small fraction that does not overlap with the other fluorescent signals appears to be cytosolic. NNRD-YFP fluorescence exclusively overlaps with the Mitotracker signal, suggesting that this protein is only localized to the mitochondria. The column on the right shows an immunochemical analysis employing an antibody recognizing YFP and protein extracts from the respective protoplast preparations.

NNRD Accumulates in Seeds—Because it became clear from the fluorescence microscopy data that both proteins are colocalized at least to the mitochondria, we next addressed the question of whether NNRD and NNRE (i.e. PDX3) have similar tissue expression profiles. We performed this analysis employing qPCR and antibodies specifically raised against the purified proteins (Arabidopsis NNRD and PDX3, respectively). Our qPCR data indicate that PDX3 is expressed throughout all developmental stages examined but is highest in green tissues and has a comparatively lower level of expression in roots and seeds, particularly dry seeds (Fig. 7A). The protein levels of PDX3 demonstrate a reasonable level of correlation with the transcript data, where the protein was detected in all tissues.

Leaf mesophyll protoplasts isolated from the NNRE-YFP lines (i.e. expressing the truncated epimerase domain alone by confocal fluorescence microscopy) were also analyzed. In all cases, the fluorescence pattern obtained overlapped exclusively with MitoTracker® Red CMXRos, and there was no overlap with the chlorophyll autofluorescence; therefore, it is indicative of a mitochondrial localization (Fig. 5B, bottom row). An immunochemical analysis of the Arabidopsis protoplasts used for fluorescence microscopy confirmed that the fusion protein was intact (Fig. 5B, right column). The analysis of cotyledons, hypocotyl, and roots of the independent lines by confocal fluorescence microscopy also corroborate our conclusion that NNRD is predominantly (if not exclusively) found in chloroplasts and mitochondria.

Mitochondrial targeting of PDX3—Because structural data of PNP/PMP oxidase from E. coli and humans show that the C terminus is important for dimerization (32, 39), we surmised that fusion of YFP to the C terminus of PDX3 might be problematic because structural data of PNP/PMP oxidase from E. coli and humans show that the C terminus is important for dimerization (32, 39). This region is conserved in PDX3; therefore, the addition of a tag may interfere with folding and, as a consequence, functionality. Indeed, as noted above, we observed that soluble recombinant expression of PDX3 in E. coli could not be achieved when the protein was expressed with a C-terminal tag but was resolved by placing the tag at the N terminus. The recent report of Niehaus et al. (24) is consistent with a multicompartamental localization for NNRD, although the latter study reported a higher proportion of the enzyme in the cytosol. In the same study, NNRE was observed in mitochondria, consistent with our data, but was also observed in the cytosol, and a small proportion was in plastids. These slight inconsistencies may be explained by the use of a heterologous system in the latter study (tobacco suspension cells), which may not accurately reflect the true situation in Arabidopsis.
FIGURE 6. Subcellular localization of NNRE and NNRD in Arabidopsis seedlings. A, representative pictures of guard cells, hypocotyl, and roots of 3-day-old Arabidopsis seedlings of the same lines and with the same fluorescence coloring as shown in Fig. 5. YFP (yellow) alone localizes to the cytosol and nucleus as expected. The pattern of NNRD-YFP overlaps with the chlorophyll autofluorescence (colored blue) in the guard cells of cotyledons and the hypocotyl as well as additional areas that are likely to represent the mitochondria. Notably, these tissues could not be penetrated with MitoTracker. On the other hand, NNRD-YFP fluorescence clearly overlaps with the MitoTracker signal in roots, suggesting localization to the mitochondria. NNRE-YFP fluorescence does not overlap with chlorophyll autofluorescence in guard cells or seedling hypocotyl but clearly overlaps with MitoTracker in roots. Scale bar, 5 µm. Additional independent lines expressing either NNRD-YFP or NNRE-YFP showed very similar fluorescence patterns (data not shown). B, representative pictures of the hypocotyl and guard cells of 3-day-old Arabidopsis seedlings expressing PDX3-YFP (scheme shown top left). The pattern of PDX3-YFP fluorescence (yellow) is typical of a mitochondrial localization showing no overlap with chlorophyll autofluorescence (colored blue). Scale bar, 5 µm.
examined, with the lowest level of expression being observed in dry seeds (Fig. 7B). On the other hand, the expression of NNRD in general is rather low relative to PDX3, but intriguingly and in stark contrast to PDX3, it is strongly expressed in seeds, in particular dry seeds, compared with any other tissue (Fig. 7A). The NNRD transcript data are corroborated by the immunochemical analysis of the same samples shown in A using antibodies raised against the recombinant Arabidopsis PDX3 and NNRD, respectively. R, roots; S, 10-day-old seedlings; IR, immature rosette (21 days old); MR, mature rosette (28 days old); St, stems; CL, cauline leaves; Fl, flowers; Sil, siliques; DS, dry seeds; 24, 24-h imbibed seeds; 48, 48-h imibed seeds; 72, 72-h imbibed seeds.

Arabidopsis lines. Notably, the linkage of NNRE with the POX domain within PDX3 (and therefore vitamin B6 biosynthesis) would make it more difficult to dissect effects specific to the epimerase activity, and in any case, pdx3 mutants have already been described in some detail (23, 40). Two independent T-DNA insertion lines (nnrd-1 and nnrd-2) from the GABI-KAT collection (41) were obtained for analysis (Fig. 8A). Analysis of the expression of NNRD at the transcript and protein level confirmed that both lines were knock-out lines because no significant expression could be detected in Arabidopsis seeds compared with wild type (Fig. 8, B and C). The nnrd mutant lines showed no phenotypic differences (example shown in Fig. 8D) compared with wild type when grown under standard growth conditions (either 8, 12, or 16 h of light at 100 μmol of photons·m⁻²·s⁻¹ and 22 °C/dark and at 18 °C). In particular, leaf morphology and development of reproductive organs as well as root growth were indistinguishable from the wild type. Because NNRD accumulates in dry seeds, we also searched for a seed-specific phenotype, but no significant differences in seed weight or size were observed between the different nnrd mutant lines and wild type. An in silico search of available transcript data (Genevestigator, Bio-Analytic Resource for Plant Biology, BAR, and ATgeneexpress visualization tool sites) did not reveal obvious conditions that could be tested for a disparate response from nnrd lines, because the only conditions where expression changed significantly were in germinating seedlings (as we have demonstrated above). Furthermore, seeds germinated as for wild type under our standard growth conditions...
either in culture or in Murashige and Skoog medium (13). Thus, we conclude that NNRD is dispensable for Arabidopsis growth, at least under the conditions used. This conclusion is consistent with that of Niehaus et al. (24). Notably, the latter study detected an accumulation of NAD(P)H hydrates in nnd mutants, confirming that NNRD is involved in preventing the build-up of these compounds in Arabidopsis (24).

**DISCUSSION**

Here we report on the presence of a pathway for NAD(P)H repair in plants. The pathway is defined by the spontaneous formation of hydrated forms of either NADH or its phosphorylated derivative NADPH (called NAD(P)HX) under conditions that are mildly acidic or at elevated temperatures. The hydrated compounds can also be formed as a nonspecific side reaction of cellular dehydrogenases and can cause their inhibition. The build-up of such potentially toxic intermediates of NAD(P)H is counteracted by the stereospecific dehydratase NNRD. We have characterized the pathway in Arabidopsis, where one copy of the dehydratase and epimerase, respectively, can be found. Intriguingly, the epimerase is fused to a protein involved in the salvage pathway of vitamin B6 in the green lineage, the reason for which is not currently known. The NAD(P)H repair pathway from Arabidopsis could be reconstituted in vitro, and the dehydratase and epimerase activities could be validated.

Although NNRD and NNRE work in conjunction with each other, we did not find evidence for their physical interaction at least in vitro. Interestingly, the oligomeric state of both proteins is disparate. NNRD forms a tetramer, whereas the NNRE domain of PDX3 either alone or as part of the full-length protein is a dimer. Although all known homologs of the POX domain of PDX3 form dimers (28, 29, 32, 33), it is noteworthy that the NNRE domain of PDX3 shows high homology with the previously determined structure of the dimeric mouse AI-BP (25), which is available with NADP⁺ bound (Protein Data Bank code 3RNO). An overlay of the predicted structure of the Arabidopsis NNRE domain illustrates strong conservation of the Rossmann-like fold as well as the NADP⁺ binding site (Fig. 9A). On the other hand, NNRD homologs are broadly classified within the ribokinase superfamily (35). Members of this family vary in quaternary structure based on the configuration of the active site and shielding of the substrate from solvent (35). In particular, certain members of the ribokinase family use oligomerization to form the active site, whereas others have an extra domain (a flap β-sheet) that forms a lid over the active site (42). Although the E. coli Yef orthologous fusion protein from Thermotoga maritima (Tm0922) has been reported to be dimeric (34), we noted that the close homolog of NNRD from B. subtilis (Fig. 2C) annotated as YxkO, which like Arabidopsis NNRE exists as a single domain protein, is also a tetramer (35).

**FIGURE 9. Homology models of the three-dimensional structures of NNRD and NNRE from Arabidopsis.** A, a ribbon representation of the NNRE homolog, AI-BP, from mice (Protein Data Bank code 3RNO) is shown in blue and is overlaid by a model of the NNRE domain of PDX3 (turquoise) from Arabidopsis (residues 65–327). Two of the subunits on the center are shown in ribbon representation, whereas the two subunits on the right are shown in surface representation (one of each in blue and gray, respectively). Each one of the YxkO ribbon-represented subunits is overlaid by a model of NNRD (magenta and salmon, respectively) from Arabidopsis. (S)-NADPHX bound at the interface of each monomer in the YxkO structure is colored yellow and according to chemical notation. The black box denotes the area shown in more detail in C. C, the respective monomer interfaces with conserved amino acids colored green. (S)-NADPHX (colored according to chemical notation (yellow, carbon; blue, nitrogen; red, oxygen; orange, phosphorus)), AMP (shown in the background, colored according to chemical notation (turquoise, carbon; blue, nitrogen; red, oxygen; orange, phosphorus), and a magnesium ion (dark gray sphere) bound at the interface of each YxkO monomer are also indicated. The respective Arabidopsis models were made using the program Phyre² (intensive mode) (46), and the illustrations were constructed in PyMOL (Schrodinger, LLC, New York).
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tion to form the active site. It is interesting to note that the architecture of B. subtilis YxkO is such that although two subunits are required to form the active site, four substrate molecules are bound in a highly efficient arrangement exemplifying the use of a tetramer. This may also be the case for NNRD from Arabidopsis permitting higher turnover. This arrangement could not be achieved if NNRD was fused to NNRE, which may provide insight into the evolutionary separation of these proteins.

The determined subcellular localization of NNRD and NNRE is intriguing. Although NNRD can be found predominantly in plastids and mitochondria, a small fraction was also observed in the cytosol, whereas NNRE is exclusively present in the mitochondria in our analyses. The latter result was unexpected for two reasons: 1) PDX3 has been previously reported to be exclusively found in the chloroplast (23), and 2) co-localization of NNRD and NNRE was anticipated because the proteins cooperate physiologically. However, we have noted that a recent proteomic study reports PDX3 to be localized to the mitochondria (47). The localization overlap in the mitochondria must be assumed to indicate that NAD(P)H repair involving both activities is most important in this organelle. It is not clear if the small fraction of NNRD observed in the cytosol in Arabidopsis protoplasts is of physiological significance, particularly because there is almost perfect overlap of NNRD-YFP fluorescence with that of the mitochondrial staining reagent MitoTracker® Red CMXRos in intact root cells (Fig. 6A), even after plasmolysis (data not shown). Nonetheless, we have noted that a second methionine is present in this protein that could be used as an alternative start codon to be expressed without a transit peptide (Fig. 2C). In vitro data from the study of Niehaus et al. (24), which appeared while this manuscript was under review, could support this statement. However, our in vivo data indicate that this would, at most, represent a very minor fraction of the protein. It is interesting that at the tissue level, NNRD accumulates strongly in seeds, in particular mature seeds, and degrades upon seed imbibition (Fig. 7), suggesting that NAD(P)H repair, or at least NNRE, is important for seed maturation and perhaps dormancy. Notably, this pattern of expression is maintained in other plants. Specifically, we examined microarray developmental expression data available for rice, tomato, potato, maize, rice, and barley (see the Bio-Analytic Resource (BAR) for Plant Biology Web site). NNRD is specifically highly expressed in seeds of rice, ripe tomato fruits, and potato tubers, whereas the highest level of expression is in the embryo in maize and barley, and it is degraded upon seed imbibition. This expression pattern may be inherent to the predicted presence of several absiccisic acid and gibberellic acid regulatory elements in the promoter region of NNRD. PDX3, on the other hand, appears to be more abundant and is ubiquitously expressed, with the noteworthy exception of dry seeds, which in contrast to NNRD display the lowest level of expression. However, it must be reiterated that PDX3 is a bifunctional protein that has PMP/PNP oxidase activity in addition to epimerization of NAD(P)HX. Therefore, the ubiquitous expression may be a reflection of its involvement in the salvage of vitamin B6. On the other hand, the compartmentation of NAD(P)H epimers is not known in vivo either at the cellular or tissue level. Therefore, the specific localization of NNRD to seed tissue and its compartmentation to the plastid (and cytosol) in addition to the mitochondria may be explained by the predominant presence of the (S)-epimer of NAD(P)HX in these areas, rendering NNRE dispensable. The contribution of NNRE in seed maturation and perhaps dormancy needs further exploration. Here, we have not noted a phenotype with the Columbia ecotype under standard growth conditions. It may be necessary to compare other Arabidopsis ecotypes and conditions to dissect the role NNRD plays in these processes.

It is being increasingly recognized that certain enzymes do not hold the rigid specificity that is often assumed and often act or accept true substrate derivatives or alternative substrates, albeit with less efficiency. The principle of metabolite repair is to increase the fidelity of the enzymatic process, similar to the proofreading activities of DNA polymerases and aminoacyl tRNA synthases in replication and translation. In addition to the pathway described here, other examples of metabolite repair include 1,2-hydroxy-glutarate dehydrogenase, which functions to remove the toxic product formed by 1,2-dehydrogenase when it acts on α-ketoglutarate instead of oxaloacetate (43), and GDP-δ-Glc phosphorylase, which compensates for the lack of specificity displayed by GDP-δ-Man pyrophosphorylase involved in the formation of glycoconjugates in mammals and worms (44). The importance of 1,2-hydroxyglutarate dehydrogenase is emphasized upon its deficiency in humans, which leads to the severe neurometabolic disease 1,2-hydroxyglutaric aciduria, increasing the susceptibility to develop brain tumors (45). NADH and NADPH are important cofactors regulating metabolic homeostasis as well as playing key roles in metabolic signaling. Thus, it is not surprising that nature has evolved several systems to maintain its availability. Although plants are able to perform de novo biosynthesis of NAD(P)H and have a salvage pathway in place to retrieve derivatives, the repair pathway described in this study would provide an additional mechanism to ensure that there is no deficit from the cellular NAD(P)H pool through deleterious enzyme side reactions or as a result of unfavorable conditions in the cellular milieu. As a corollary, the relevance of the NAD(P)H repair pathway may become more important in organisms that cannot perform de novo biosynthesis. We are confident that the stage has now been set to explore this area further, and several more examples of metabolite repair are likely to be revealed in the future.

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Addendum—While this manuscript was under review, an independent publication by Niehaus et al. (24) appeared in press on this topic.

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