Heterologous expression and biochemical characterization of an NAD(P)H:quinone oxidoreductase from the hemiparasitic plant Triphysaria versicolor

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Abstract

Quinones are widespread secondary metabolites that function as signal molecules between organisms in the rhizosphere. Quinones are particularly important in the exchange of chemical signals between plant roots, a phenomenon classically termed allelopathy. The bioactivity of quinones is due in large part to radical intermediates formed during redox cycling between quinone and hydroquinone states. In order to investigate the role of quinone oxidoreductases in processing quinone signals exchanged between plant roots, we characterized an NAD(P)H-dependent quinone reductase expressed in roots of the parasitic plant Triphysaria versicolor (TvQR2). The predicted amino acid sequence encoded by TvQR2 shares homology with quinone reductases from Archaea, Eubacteria and Eukaryota organisms. The complete TvQR2 cDNA was cloned into the fungus Pichia pastoris and the heterologous protein purified. The recombinant protein reduced a variety of quinones and naphthoquinones, including several of allelopathic significance, using either NADH or NADPH as electron donors. The protein had an absorption spectrum consistent with it being a flavoprotein and was inhibited by the quinone reductase inhibitor dicumarol. We propose that the TvQR2 protein functions as a quinone reductase in plant roots to mitigate the toxicity of exogenous quinones in the rhizosphere. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: Allelopathy; Parasitic plant; Rhizosphere signaling; Root organogenesis

1. Introduction

Plants release chemicals into the rhizosphere that can positively or negatively affect the growth and development of neighboring plants. Quinones and related phenols are the most common class of subterranean allelochemicals. The phytotoxicity of 5-hydroxy-1,4-naphthoquinone, an allelopathic compound released by walnut trees and commonly known as juglone, has been known for over half a century. The roots of walnut trees synthesize the relatively non-toxic hydrojuglone that transforms to the cytotoxic quinone upon exposure to oxygen in the soil. Another important allelochemical related to juglone is 6-methyl-1,3,8-trihydroxyanthraquinone (emodin), a phytotoxic quinone released by roots of the noxious weed Polygonum sachalinense (giant knotweed). The inhibition of neighboring plant growth by emodin is thought to contribute to the success of giant knotweed as an aggressively invasive colonizer.

Subterranean quinones also signal developmental events in the roots of some plants that enhance their growth. Probably the most remarkable is the development of haustoria on the roots of parasitic Scrophulariaceae in response to host root phenolics and quinones. Haustoria are invasive organs that connect the parasite and host roots and serve as a conduit through which the host is depleted of water and nutrients. 2,6-Dimethoxybenzoquinone (DMBQ) was identified as the haustorium inducing factor released from sorghum root exudates. Other small molecules also have haustorium inducing activity, at least when applied to parasite roots in vitro. The observations that haustorium inducing factors have similar redox potentials and that phenolic acids need to be oxidized in order to be active,
suggests that haustorium development is associated with redox signaling [28]. Furthermore, chemical inhibitors such as cyclopropyl-p-benzoquinone that specifically trap semiquinone radicals inhibit haustorium development [53]. This suggests that parasitic plants identify host roots and initiate heterotrophic growth programs through a redox signaling system involving host phenols and quinones. Other ecological interactions are mediated through oxidation–reduction transformations and redox signaling is likely to be a frequent event in the rhizosphere [3].

Quinones are widely used in medicine as anti-fungal, -malarial, and -cancer agents where their activity is due in large part to radical intermediates formed during redox cycling between quinone and hydroquinone states [33]. Cellular enzymes such as NADPH-cytochrome P450 reductase (EC 1.6.2.4) and NADH dehydrogenase (EC 1.6.99.3) catalyze one-electron quinone reductions that result in highly reactive, semiquinone intermediates [50]. The semiquinones readily donate electrons to molecular oxygen, producing superoxide radicals and other reactive oxygen intermediates (ROIs) that aggressively damage membranes, proteins, and nucleic acids. Biochemical pathways to protect cells from ROI damage evolved early in earth’s life history, presumably in response to the accumulation of atmospheric oxygen. One common mechanism for detoxifying electrophilic quinones involves their divergent reduction to the hydroquinone form followed by inactivation through conjugation reactions [130]. Enzymes that carry out this reaction belong to the NAD(P)H-dependent, quinone reductase family of the flavoprotein superfamily [132]. These flavoenzymes catalyze the direct hydride transfer from NAD(P)H to the enzyme bound flavin, either FAD or FMN. The two electrons from the reduced flavin are then transferred directly to the quinone in a ‘ping pong’ reaction mechanism that does not produce free radical semiquinone intermediates [132]. The human cytoplasmic enzyme DT-diaphorase (EC 1.6.99.2) is one well-studied member of this family because of its anti-cancer properties [103].

We are characterizing the activity of quinone reductases in roots of parasitic plants to determine their role in allelopathic signaling. Triphysaria is a small genus of parasites in the Scrophulariaceae family that grows as a common springtime annual in grassland stands and coastal bluffs throughout the Pacific Coast [13]. We isolated two distinct quinone reductase cDNAs from T. versicolor roots exposed in vitro to DMBQ [201]. Both of these are transcriptionally up-regulated within 30 min of exposure to DMBQ, juglone, and other allelopathic quinones. One of these, TvQR2, is up-regulated in both parasitic and non-parasitic species and is therefore hypothesized to function as a general plant response to allelopathic quinones [200]. We expressed the TvQR2 protein in a fungal expression system and characterized the purified protein. Spectrophotometric and biochemical analyses indicated that TvQR2p is an NAD(P)H-dependent flavoprotein that catalyzes the reduction of a broad spectrum of quinones, including several of allelopathic significance. We propose that this protein functions in plant roots to detoxify quinones and other xenobiotics in the rhizosphere.

2. Results

2.1. Heterologous expression of a parasitic plant quinone reductase

Four overlapping cDNAs were isolated from a subtractive library enriched for transcripts up-regulated in T. versicolor in response to exposure to DMBQ (GenBank accesses BE574746, BE574764, BE574790, and BE574791). These were assembled into a consensus sequence and its virtual translation searched by BLASTX against the public data bases [21]. A FASTA file of the cDNAs, the assembled sequence, and the BLAST reports, can be obtained from the Early DMBQ Induced Transcripts (EDIT) Supplemental Data Table available at http://veghome.ucdavis.edu/Faculty/Yoder/Lab/index.html.

The virtual translation of the assembled T. versicolor transcript, called TvQR2 (AAG53945), had significant homology to a family of proteins predicted in plants, yeasts, and bacteria [200]. While putative proteins homologous to the predicted T. versicolor protein have been identified in several plant sequencing projects, none of the plant proteins in this family have been biochemically characterized. The virtual translation that yields a protein most homologous to the T. versicolor protein comes from a cDNA (LED-3, GI:2564066) isolated from cell suspension cultures of Lithospermum erythrorhizon [32]. These cell cultures are of interest because they produce the secondary metabolite shikonin, a naphthoquinone with anti-microbial and anti-inflammatory properties [5].

The Triphysaria protein had homology to characterized proteins in some fungi. The homolog in Schizosaccharomyces pombe, encoded by the obr1 locus, confers resistance to the drug brefeldin [31]. The best biochemically characterized enzyme with homology to the T. versicolor protein is a quinone reductase from the lignin degrading fungus Phanerochaete chrysosporium (GI:4454993) [6,7]. As seen in Fig. 1, the P. chrysosporium quinone reductase shares 68% sequence similarity across the entire T. versicolor protein with 52% of the residues being identical. Based on these sequence comparisons, we hypothesized that the T. versicolor protein may encode a quinone reductase protein; this was tested by expressing the protein in yeast and characterizing the purified protein biochemically.

The protein-coding region for TvQR2 was cloned into the expression vector pPICZ B and transformed into Pichia pastoris. The vector contains a methanol inducible promoter that allows conditional expression of the protein once the yeast cultures have attained a high density. Western analysis using antiserum specific for the 6XHIS tag revealed that the
A recombinant protein (TvQR2p) was found mainly in the water soluble lysate which allowed for the purification of the native protein by nickel affinity chromatography (not shown). Fig. 2 shows that after the affinity chromatography the TvQR2 protein ran as a single band on a Coomassie-blue stained SDS-polyacrylamide gel. The molecular mass of the denatured protein was estimated from the gel to be about 25 kDa, which agrees with the molecular mass predicted from the deduced amino acid sequence of the protein plus the tag sequence (24.6 kDa).

The absorption spectrum of TvQR2p had peak absorbance near 375 and 450 nm (Fig. 3). When the protein was heat denatured and filtered, the filtrate, which contained no measurable protein, had an absorption spectrum similar to that of the native protein. These are typical absorption spectra for proteins containing flavin prosthetic groups.

2.2. TvQR2 is an NAD(P)H-dependent quinone reductase

The purified heterologously expressed TvQR2 protein was electrophoresed through a non-denaturing gel, which was then soaked in solution of menadione, the redox-sensitive, tetrazolium dye MTT, and either NADPH or NADH. As schematized in Fig. 4A, the enzymatic reduction of menadione to menadiol, coupled to the reduction of MTT, yields a blue formazan product. Fig. 4B shows that when all the components were included in the assay, a blue product formed at the same migration position as purified TvQR2p. The formation of this blue product was dependent on the presence of NADPH or NADH and when either was omitted there was no staining. The reaction also...
required menadione. Control reactions without TvQR2p or with heat denatured TvQR2p failed to produce the colored product (not shown). These results demonstrated that TvQR2p had a menadione reductase activity that utilized either NADPH or NADH as an electron donor.

The enzyme preference for electron donor was examined by measuring reaction rates at different concentrations of NADPH and NADH in solution (Fig. 5). The $V_{\text{max}}$ and the $K_m$ calculated from double reciprocal plots of MTT reduction versus the concentration of the NADH or NADPH at a fixed concentration of menadione are similar, indicating that TvQR2p utilized NADH and NADPH with near equal efficiency.

The TvQR2p-dependent oxidation of NADH was assayed with different electron acceptors (Fig. 6). Various quinones and naphthoquinones are effective electron accepting substrates, though some molecules were clearly preferred over others. Two quinones of biological significance in plant–plant signaling, juglone (3) and DMBQ (6), were both reduced by TvQR2p. Interestingly, DMBQ was a less effective electron acceptor that juglone, even though the transcript encoding TvQR2p was isolated from a DMBQ induced root tip [20].

There were no obvious correlations between substrate activity and either the number or type of substitution. For examples, the addition of a 5-methy to 3,5-dimethoxybenzoquinone (6) increased its activity 20-fold (7), while a similar substitution onto naphthoquinone (2) reduced its activity (1). As seen in Fig. 6, there was also no correlation between activity and the redox potentials as measured by Smith and colleagues [28]. The enzyme reduced DMBQ in a pH range 5.5–9.0 with an optimum of 6.5. There was no measurable activity ($<0.001 \text{OD}_{340} \text{min}^{-1} \mu g^{-1}$) without electron acceptor, electron donor, or enzyme.

We used the menadione reductase assay to test if TvQR2p was inhibited by the quinone reductase inhibitor dicumarol [10]. Fig. 7 shows double reciprocal plots of MTT reduction rates versus NADH concentration in the presence of various concentrations of dicumarol. Dicumarol had no effect on the $V_{\text{max}}$ of the reaction but did increase the $K_m$ with respect to the NADH. This is indicative of competitive inhibition with respect to NADH and a regression analysis of the replots of the slopes versus dicumarol concentration (Fig. 7, insert) yielded a $K_i$ value of 10.6 µM.

3. Discussion

Amino acid sequence similarity between TvQR2p and the quinone reductase from P. chrysosporium (PcQR2) suggested that TvQR2p might possess quinone reductase activity. Interestingly, TvQR2p and PcQR2, share little amino acid sequence similarity to DT-diaphorase [4]. The only stretch of sequence similarity (<40%) occurs in a 40 amino acid residue long region in the α2–β3–α3 region of DT-diaphorase (not shown). This region has been shown by X-ray diffraction studies to be part of the domain where the flavin moiety resides [13]. Recently, another cDNA encod
ing a flavin containing quinone reductase (AtNQR) was cloned from Arabidopsis [29]. The deduced amino acid sequence of this protein also shares little similarity to TvQR2p. Beside AtNQR, there are other putative genes in the Arabidopsis genome that share deduced amino acid similarity to TvQR2p. All the plant proteins in this family fall into a single clade distinct from fungal and bacterial homologs, suggesting similar functions may be served by the different plant homologs [20].

Besides sharing amino acid sequence similarity, TvQR2p also shared functional characteristics with PcQR2. Both had broad pH range with the optima being slightly acidic. Both enzymes utilized NADH and NADPH with equal efficiency with a $K_m$ in the 50–70 $\mu$M range [6]. This is in contrast to the Arabidopsis AtNQR quinone reductase, which favored NADPH over NADH by more than a factor of two [29]. Both the PcQR2 and TvQR2p were inhibited with dicumarol but the fungal enzyme was more sensitive; the $K_i$ for dicumarol was about five times greater with the plant than the fungal enzyme [7]. Spectral analysis of TvQR2p suggested that it contained a non-covalently bound flavin. Presumably this flavin moiety was obtained from Pichia since additional flavin was not needed for activity. Similarly, the Arabidopsis AtNQR expressed in E. coli did not require additional flavin for its activity [29].

Quinone reductases play important functions in xenobiotic detoxification [30]. They catalyze bivalent reductions of quinones and other small molecule toxins that preclude the
generation of radical semiquinones. Because semiquinones readily generate reactive oxygen intermediates in aerobic conditions, quinone reductases provide relief from oxidative stress. This is true for proteins investigated in fungi and humans\[7,10,31\]. The TvQR2p had a broad specificity for substrate quinones, a common feature for quinone reductase flavoenzymes and of xenobiotic detoxifying enzymes in general\[30\]. While the signaling of haustorium development requires the redox-cycling between the quinone and semiquinone form\[28\], several factors point to the conclusion that TvQR2 is functioning in quinone detoxification and not haustorium signaling. Juglone, a potent cytotoxin without haustoria inducing capacity, is more active as a substrate than the haustoria inducing factor DMBQ. Second, the TvQR2 transcript is up-regulated by exposure to DMBQ in both parasitic and non-parasitic plants\[20\]. It is similarly up-regulated in fungi\[11\]. The lack of correlation with haustorium formation is consistent with the protein serving a different function. Thirdly, homology comparisons suggest the reduction mechanisms catalyzed by TvQR2 are similar to those of the P. chrysosporium protein. These bivalent mechanisms are unlikely to generate the radical intermediates thought critical for haustorium initiation.

We therefore hypothesize that the TvQR2 protein is functioning in T. versicolor to detoxify quinones in the rhizosphere. If so, the induction of this enzyme by DMBQ will serve to degrade the haustoria inducing signal at the same time that a distinct, univalent quinone reductase generates the semiquinones needed for signaling. The coordinated action of univalent and bivalent quinone reductases might account for the transient nature of secondary haustorium development.

4. Methods

4.1. Materials

Menadione (2-methyl-1,4-naphthoquinone), plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), duroquinone (tetramethyl-p-benzoquinone), p-benzoquinone, 2,3-dimethoxy- and 5-methyl-1,4-benzoquinone were purchased from Sigma (St. Louis, MO). Phenyl-1,4-benzoquinone, 2,6-dimethyl-1,4-benzoquinone, methyl-1,4-benzoquinone, 5-hydroxy-1,4-naphthoquinone (juglone), and 5,8-dihydroxy-1,4-naphthoquinone were purchased from Aldrich (Milwaukee, WI). 1,4-Naphthoquinone was purchased from Fluka (Milwaukee, WI) and 2,6-dimethoxy-1,4-benzoquinone (DMBQ) from Pfaltz and Bauer (Waterbury, CT). All quinones, except DMBQ, were solubilized in 1,4-benzoquinone (DMBQ) from Pfaltz and Bauer (Waterbury, CT). All quinones, except DMBQ, were solubilized in water to 200 mM. Dicumarol, -3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), reduced β-nicotinamide adenine dinucleotide (NADH), reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), 2-N-morpholino-ethanesulfonic acid (MES), piperazine-N,N′-bis-2-ethanesulfonic acid (PIPES), Tris(hydroxymethyl) amino methane (Tris), and Cibacron blue 3GA were purchased from Sigma (St. Louis, MO).

4.2. Plasmid construction and Pichia transformation

Invitrogen’s Easy Select Pichia Expression Kit was used to express recombinant TvQR2 protein in Pichia pastoris (Invitrogen, Carlsbad, CA). The coding region of TvQR2 was PCR amplified from the cDNA 3K19 using the primers 3ExFox (5′CGTTAAAGCCGCCGCTTGTGTTGATC-TTC) and 5ExFox (5′ACGGCATGAATTCATTACATG-GCAACAGTC). The primers 3ExFox and 5ExFox were designed with Not I and EcoRI sites, respectively, within them. The amplified TvQR2 coding region was digested with Not I and EcoRI and cloned into expression vector pPICZ B as a translational fusion to the myc epitope and 6XHis tag. DNA sequence analysis determined that the chosen clone (2-ZB3) had the correct open reading frame. The plasmid p2-ZB3 was digested with BstXI and transformed into Pichia pastoris strain X33 via electroporation following Invitrogen’s protocol. The resulting transformed Pichia strain, L1-2, was Zeocin resistant and was able to grow on methanol as the sole carbon source (Mut+).

4.3. Pichia expression and purification

The expression of the recombinant TVQR2p was accomplished essentially as described in the Easy Select Pichia Expression Kit Manual (Invitrogen, Carlsbad, CA). The Pichia strain L1-2 was grown overnight at 30 °C in MGY media (1.34% (w/v) yeast nitrogen base without amino acids (YNB) (Gibco/BRL Products, Rockville, MD), 1% (v/v) glycerol, 4×10⁻⁵% (w/v) biotin, 4×10⁻⁵% (w/v) histidine). To induce expression of TVQR2p, the cells were harvested by 10 min centrifugation (2000 × g) and diluted to 1.0 OD₆₀₀ in MMH media (1.34% YNB, 0.5% (v/v) methanol, 4×10⁻⁵% (w/v) biotin, 4×10⁻³% (w/v) histidine). The cells were allowed to grow with vigorous shaking for 48 h at 30 °C in baffled flasks with an addition of 100% methanol to 0.5% (v/v) after 24 h. Cells were harvested as before and stored at −80 °C until used. Cell lysates were made by thawing the stored cells on ice and by resuspending them in Breaking Buffer (BB) (50 mM sodium phosphate pH 7.4, 1.0 mM EDTA, 5% (v/v) glycerol, 1.0 mM PMSF) and followed by centrifugation. The resulting cell pellet was resuspended in BB to an OD₆₀₀ of 50 and an equal volume of acid washed glass beads (0.5 mm) was added. The cells were broken by eight cycles of 30 s vortexing followed by 30 s on ice. The lysate was cleared by centrifugation for 10 min at 12,000 × g. The protein lysate was stored at −20 °C until use. Nickel affinity chromatography was done under native conditions according to the Xpress System Protein Purification Manual (Invitrogen, Carlsbad, CA). The bound protein was eluted by a step gradient of imidazole and
fractions were analyzed by SDS-PAGE. Fractions containing the protein of interest were pooled and dialyzed at 4 °C against 10 mM sodium phosphate buffer pH 7.0, 20% glycerol. Purified protein was stored at −20 °C. Protein concentrations were determined by the Bio-Rad Protein Assay (Hercules, CA) using solutions of purified bovine serum albumin as concentration standards. Protein preparations were analyzed by SDS-polyacrylamide gel electrophoresis.

4.4. Spectral analysis

One milliliter of purified TvQR2p (0.75 mg ml⁻¹) was dialyzed (12–14,000 molecular mass cut-off) two times in 1 l of 50 mM phosphate buffer (pH 7.0). The absorption spectrum of this protein under aerobic conditions was obtained with a Beckman DU-600 spectrophotometer. The protein was then denatured in boiling water for 4 min and subjected to ultrafiltration (3000 molecular mass cut-off) using a Microcon YM-3 device (Millipore, Bedford, MA). The absorption spectrum of the flow-through was determined as above. The Bio-Rad Protein Assay (Hercules, CA) measured protein concentrations.

4.5. Quinone reductase assays

The quinone reductase activity with different electron acceptors was measured by monitoring the oxidation of NADH and NADPH, and menadione as stated in the figure legends, and 1.0–3.0 µg of purified enzyme. Reaction mixtures contained 100 mM PIPES (pH 6.5), varying amounts of quinones and menadione as stated in the figure legends, 0.3 mg ml⁻¹ TvQR2p. Reactions were started with the addition of enzyme and the change in absorbance at 610 nm (ε₅₃₄ nm = 6.22 mM⁻¹ cm⁻¹) was followed for 1–5 min using a Beckman DU-600 spectrophotometer in kinetic mode. Reactions without enzyme were performed to determine the non-enzymatic NADH oxidation rates. Likewise, control reactions were run without quinones. To compare the efficiency of NADPH and NADH as electron donors, the enzymatic reduction of menadione to menadiol with either NADPH or NADH, and 30 M menadione was monitored by measuring the coupled reduction of MTT, 1.0 MTT, 1.0 mM Tris pH 7.5, 0.3 mg ml⁻¹ MTT, 1.0 NADPH or NADH, and 30 µM menadione with gentle swirling and protection from light until color developed (10–15 min). The reaction was stopped by transferring the gel slices to a 5% (v/v) solution of acetic acid. The enzyme was also assayed using the MTT-menadione assay in the presence of inhibitors at the various concentrations listed in the figure legend or text. A 10 mM stock of dicumarol was prepared in 0.1 N NaOH.

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References


