Cloning and heterologous expression of NAD(P)H:quinone reductase of *Arabidopsis thaliana*, a functional homologue of animal DT-diaphorase

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Abstract In higher plants, NAD(P)H:quinone reductase (NQR) is the only flavoreductase known to reduce quinone substrates directly to hydroquinones by a two-electron reaction mechanism. This enzymatic activity is believed to protect aerobic organisms from the oxidative action of semiquinones. For this reason plant NQR has recently been suggested to be related to animal DT-diaphorase. A cDNA clone for NQR of *Arabidopsis thaliana* was identified, expressed in *Escherichia coli*, purified and characterized. Its amino acid sequence was found related to a number of putative proteins, mostly from prokaryotes, with still undetermined function. Conversely, in spite of the functional homology, sequence similarity between plant NQR and animal DT-diaphorase was limited and essentially confined to the flavin binding site.

Key words: NAD(P)H:quinone reductase; DT-diaphorase; Flavoprotein; *Arabidopsis thaliana*

1. Introduction

NAD(P)H:quinone reductase (NQR) is a typical flavoprotein of higher plants endowed with a very efficient catalytic activity toward short chain quinones [1]. Albeit expressed at relatively low levels, NQR has been purified from several plant tissues and found to be constituted of subunits of apparently 24–27 kDa [1–4], which bind FMN and are arranged into catalytically active homotetramers [5]. A prominent catalytic feature of NQR consists in the two-site ping pong reaction mechanism, where NAD(P)H and quinone are the substrates, and $NAD(P)^+$ and hydroquinone, rather than semiquinone, are the reaction products [1,5]. This two-electron reduction mechanism of quinoid acceptors is reminiscent of animal DT-diaphorase (or NAD(P)H:quinone reductase; EC 1.6.99.2) [6–8], and contrasts with the one-electron reduction commonly performed by flavoreductases [9,10].

Quinones are chemically reactive compounds which can oxidize important biomolecules such as glutathione, protein thiols and flavins. Resulting semiquinones undergo rapid auto-oxidation, leading to detrimental production of superoxide [11]. On the other hand, hydroquinones are generally more stable, and in living plant tissues they are subjected to *O*-conjugation with loss of redox cycling properties [12]. Therefore one function of NQR may be to protect plant cells from oxidative damage. Similarly, a role of DT-diaphorase in animal cells is believed to be the detoxification of redox active compounds such as *o*- and *p*-quinones, produced by the metabolism of carcinogenic aromatic hydrocarbons [13,14]. Accordingly, DT-diaphorase expression is induced by a large number of redox active compounds [15], and both an antioxidant response element [16] and an overlapping xenobiotic response element [17] have been found in the promoter region of human DT-diaphorase.

In spite of the similar reaction mechanism, animal DT-diaphorase and plant NQR are structurally different. In fact DT-diaphorase is a dimer of 26 or 30 kDa subunits, each binding one molecule of FAD [18,19]. Also, DT-diaphorase is an A-stereospecific dehydrogenase [20], whereas NQR is B-stereospecific [5]. Proteins bearing clear structural relationships to DT-diaphorase have not been found in plants. Since quinones are heavily involved in the plant metabolism of lignin and other phenols, and in several allelopathic interactions [21,22], NQR may functionally replace DT-diaphorase in higher plants. An analogous flavoprotein was described in the ligninolytic fungus *Phanerochaete chrysosporium* [23].

The recent discovery that in animal systems DT-diaphorase might contribute to the reduced state of membrane quinones, such as ubiquinone and tocopheryl quinone [24,25], adds a further antioxidative function to this class of flavoenzymes. This might hold true for plant systems as well. In plants, NQR has been shown to interact with the plasma membrane [2,3,10] thereby assuming peculiar kinetic properties, including the sensitivity to diphenyleneiodonium and the capacity to interact with lipophilic ubiquinone homologs [4].

In this communication we report the first sequencing and recombinant expression of an NQR of a higher plant (AtNQR of *Arabidopsis thaliana*).

2. Materials and methods

2.1. Bacterial strains, plasmids and growth medium

The cDNA clone RAT7H10 of *A. thaliana* [26], kindly provided by Dr. D. Tremousaygue (Toulouse), was completely sequenced on both strands.

Competent *Escherichia coli* cells, strain HB101 (genotype: F^-, *thi*-1,

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Abbreviations: NQR, NAD(P)H:quinone reductase; AtNQR, *Arabidopsis thaliana* NAD(P)H:quinone reductase; DQ, duroquinone; NtNQR, *Nicotiana tabacum* NAD(P)H:quinone reductase; IPTG, isopropyl-1-thiol-β-D-galacto-pyranoside

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hsd S20, ($r_B^-m_B^-$), *sup*E44, *rec*A13, *ara*14, *leu*B6, *pro*A2, *lac*Y1, *gal*K2, *rps*L20(str^r), *xyl*-5, *mtl*-1, λ^-; Life Technologies) and strain BL21(DE3) (genotype: F^-, *omp*T, *hsd*S_B, ($r_B^-m_B^-$), *gal*, *dcm*, (DE3); Novagen), were transformed with the multicloning site vector pET-28a(+) (Kan^r, T7*lac* promoter; His-tag N-terminal leader, thrombin cleavage site; Novagen), according to the manufacturer's instructions. Cells were grown in LB medium at 37°C and 220 rpm. Selection was made with kanamycin (30 μg ml^{-1}) or ampicillin (100 μg ml^{-1}). Following 15–18 h growth, cells were collected and plasmid DNA was purified with a QIAprep Spin Miniprep Plasmid Kit (Qiagen).

2.2. Construction of expression plasmids and heterologous expression

Expression plasmids of AtNQR (pET($AtNQR_{196}$) and pET-($AtNQR_{191}$), see Fig. 1) were constructed by PCR using RAT7H10 as the template and two couples of primers (Up1-Down1 to construct pET($AtNQR_{196}$), and Up2-Down1 to construct pET($AtNQR_{191}$)) containing an *Nde*I site upstream and a *Xho*I site downstream the cDNA (Up1: 5′-CAT ATC CAT ACA TAT GGA AGC AGT A-3′; Up2: 5′-GGA AGC ACA TAT GGC GAT TAA GCC-3′; Down1: 5′-GAA TTA ATC CAC TCG AGT TAC TTA CC-3′). Standard PCR reactions were performed with the following temperature program: 94°C, 3 min (incubation without *Taq* DNA polymerase, Sigma); 72°C, 1 min (PCR was initiated by addition of 1.25 units of *Taq* DNA polymerase to each tube); 94°C, 1 min; 58°C, 1 min; 72°C, 2 min; 30 cycles; 72°C, 7 min. PCR products were separated on a low melting agarose gel (0.8%). Bands of about 600 bp were cut from the gel, purified by means of a QIAquick Gel Extraction Kit (Qiagen), digested with endonuclease *Xho*I and *Nde*I (Promega) and ligated into *Nde*I, *Xho*I predigested pET-28a(+).

The resulting recombinant plasmids were amplified into *E. coli* HB101 cells. Following sequencing, they were transformed for expression into BL21(DE3), spread on LB-kanamycin agar plates and incubated overnight at 37°C. A single colony from each transformation reaction was picked up, inoculated into 5 ml fresh medium, grown overnight, and finally transferred to 250 ml fresh LB-kanamycin medium. Following 3–4 h incubation at 37°C, expression was induced by adding 1 mM IPTG. Cells were collected 3 h later by centrifugation, resuspended in 50 mM Tris-Cl, pH 8.0, 2 mM EDTA, centrifuged again and the resulting pellets were stored at −70°C.

2.3. Protein purification

Both $AtNQR_{196}$ and $AtNQR_{191}$ were purified independently following the same protocol. Frozen cells were thawed on ice, resuspended in cold 50 mM Tris-Cl, pH 8.0 (10 ml of buffer to each g of cell fresh weight) and sonicated on ice for 4 min. Cell debris was discarded by centrifugation at 24 000 × *g* for 10 min, and the resulting supernatant was purified by metal affinity chromatography (His-Bind Resin, Novagen) according to the manufacturer's instructions. Immediately after elution, purified His-tagged proteins were concentrated by ultrafiltration (PM10 membrane, Amicon) and elution buffer was exchanged to 50 mM Tris-Cl, pH 7.5, 10% glycerol (v/v). The N-terminal His-tag was cut by thrombin protease (Novagen). Complete cleavage was achieved by overnight incubation at 20°C in 20 mM Tris-Cl, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$ at a protease to target protein ratio of 1:1000 (w/w). Excised His-tag was eliminated by metal affinity chromatography (His-Bind Resin, Novagen) and resulting pure proteins were stored at −70°C.

2.4. Sequencing of tobacco leaf NQR

NtNQR was purified from tobacco leaves as previously described [5]. Peptide separation and automated sequence determination were performed as in Sparla et al., 1998 [10].

2.5. Biochemical characterization of AtNQR

The native molecular weight of recombinant AtNQR was estimated by size-exclusion chromatography on a Superdex 200 column (HR 10/30) connected to a SMART System (Pharmacia) as described in [5]. The nature of the flavin cofactor was determined by fluorometry [27] using a Jasco FP770 fluorometer [5]. Photochemical reduction of purified AtNQR was according to Massey and Hemmerich, 1978 [28].

AtNQR activity was spectrophotometrically assayed (Uvikon 941 plus, Kontron) by following at 340 nm the decrease of absorbance of NAD(P)H (ε_{340} 6.23 mM^{-1}) in the presence of duroquinone (DQ). The assay mixture contained 40 mM MOPS, pH 7.0, 2 mM EDTA, 0.2 mM NAD(P)H, and 0.2 mM DQ. Initial velocity data were analyzed by non-linear regression according to [5].

3. Results and discussion

3.1. Nucleotide sequencing of the cDNA clone for A. thaliana NQR

The N-terminus of purified NtNQR (*Nicotiana tabacum* NQR) from tobacco leaves [5] and 12 internal peptides, obtained by enzymatic digestion, were analyzed into EST databases in order to find sequence similarity. We identified a single EST of *A. thaliana* (ACC No Z47060) showing a significant local homology with both the N-terminus and two internal peptides of NtNQR. The corresponding clone (RAT7H10, [26]) belonged to an organized cDNA library prepared from *Arabidopsis* suspension cells challenged by the phytopathogenic bacterium *Xanthomonas campestris*. Although the complete sequence and the nature of the corresponding protein were unknown, the clone RAT7H10 had been selected as a clone preferentially or specifically expressed during cell culture stationary growth [26]. By fully sequencing RAT7H10 (Fig. 1) we identified a 5′-untranslated region of 36 bp, followed by an open reading frame of 588 bp and a 3′-untranslated region of 156 bp containing a consensus polyadenylation site (AATAAA) and a poly(A)-tail (GenBank AF145234, [29]). Nine peptides of NtNQR could be aligned with the translated open reading frame of RAT7H10 with an overall identity of 52% (Fig. 1). Therefore RAT7H10 was a very good candidate to represent the cDNA clone of *A. thaliana* NQR (AtNQR). The predicted AtNQR protein contained 196 amino acids with a calculated MW of 21 527, in excellent

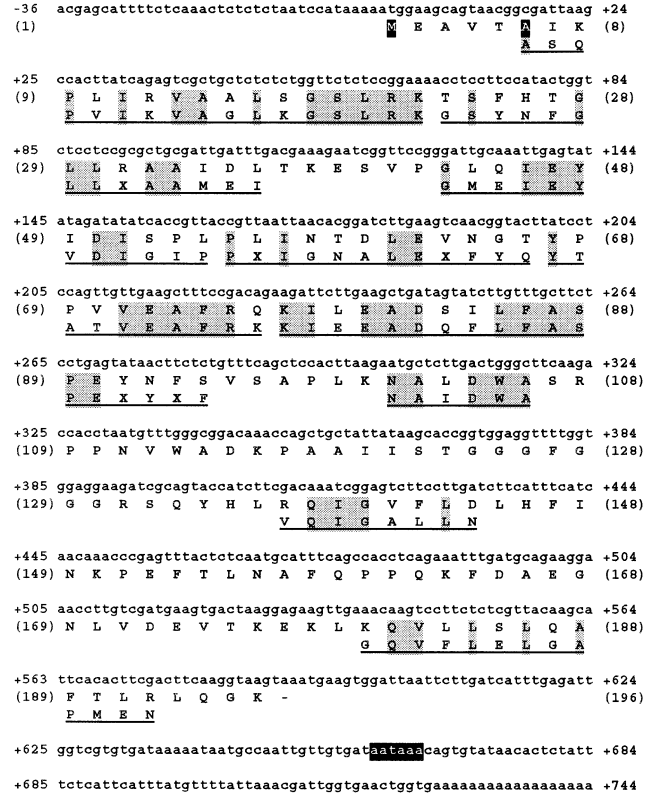

Fig. 1. Nucleotide and amino acid sequence of AtNQR, and alignment with NtNQR peptides. The polyadenylation consensus sequence and the first amino acids of $AtNQR_{196}$ (Met) and of $AtNQR_{191}$ (Ala) are black boxed. NtNQR peptides are underlined. Each aligned peptide was at least 30% identical to the corresponding *Arabidopsis* local sequence. Identical residues are shaded.

agreement with the MS-determination of 21 431 for NtNQR subunits [5]. However, alignment with the N-terminus of NtNQR, as determined by Edman degradation of the purified tobacco protein (Fig. 1), indicated that the first amino acid of the *Arabidopsis* mature protein might be the alanine five residues downstream the starting codon. In this view AtNQR would contain 191 amino acids corresponding to a slightly lower MW of 20 996. In any case, the possibility that mature AtNQR contained 196 amino acids by starting with methionine instead of alanine could not be excluded, and it was further investigated.

3.2. Heterologous expression and characterization of recombinant AtNQR

The conclusive demonstration that the open reading frame included in RAT7H10 actually coded for an NQR in *Arabidopsis* was obtained by heterologous expression in *E. coli*. Two different portions of RAT7H10, corresponding to either the 196 amino acids ($AtNQR_{196}$, starting with Met) or to the 191 amino acids form ($AtNQR_{191}$, starting with Ala), were independently cloned into the expression vector pET-28a(+) (Novagen). The recombinant vectors (pET($AtNQR_{196}$); pET($AtNQR_{191}$)), harboring an N-terminal His-tag and a thrombin cleavage site, were first introduced into HB101 *E. coli* cells for sequencing, and then expressed into BL21(DE3) (Novagen) by 1 mM IPTG. The two recombinant proteins were found as soluble His-tagged fusion proteins allowing purification on a nickel affinity column. Both fusion proteins exhibited NAD(P)H-dependent DQ reductase activity. Before any further characterization, the N-terminal His-tag was cleaved off by thrombin treatment. Resulting recombinant proteins were electrophoretically pure (not shown).

The polymerization state of recombinant AtNQR forms was investigated by size-exclusion chromatography on a Superdex 200 column. The elution of both native $AtNQR_{196}$ and $AtNQR_{191}$ was essentially identical to the elution of tetrameric NtNQR purified from tobacco leaves [5]. Consistently, the estimated molecular mass based on column calibration with marker proteins was around 90 kDa (not shown). It was concluded that recombinant AtNQRs were both homotetramers.

Recombinantly expressed AtNQRs showed typical flavoprotein absorption and fluorescence spectra with no differences between the two forms. In the visible light region, two absorption peaks at 378 and 448 nm were separated by a valley at 403 nm, and a shoulder was evident around 465 nm (Fig. 2). Fluorescence spectroscopy demonstrated that the flavin cofactor, which was non-covalently bound to the apoprotein, was FMN since the typical fluorescence response was much higher at neutral than acidic pH values [27]. As expected, the visible absorption spectrum of AtNQR was bleached upon reduction. In particular, gradual photoreduction under anaerobiosis demonstrated that flavin radicals were

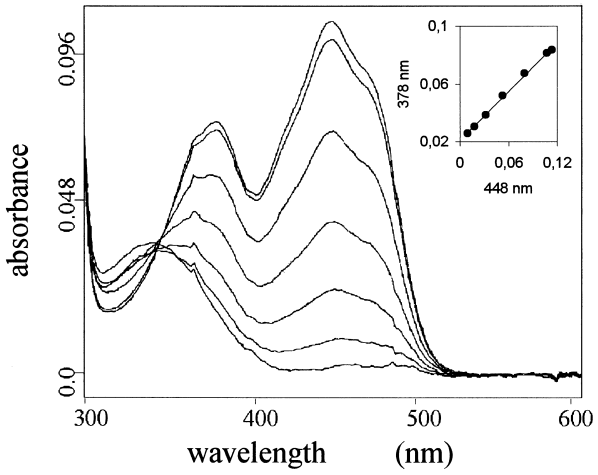

Fig. 2. Absorption spectra of $AtNQR_{191}$ following anaerobic photoreduction. $AtNQR_{191}$ in 50 mM HEPES, pH 8.0, 1 mM 5-deazaflavin-3-sulfonate, 5 mM benzylviologen, was irradiated under anaerobiosis at 25°C. Spectra (top to bottom) were recorded following irradiation steps of 5 s each. Inset: Plot of absorbance at 448 nm vs. absorbance at 378 nm at various times of irradiation.

intrinsically unstable, since no spectral evidence for either blue or red flavin semiquinones was apparent. The lack of detectable semiquinone forms [30] was indicated by the parallel decrease of absorbance of the two peaks at 378 and 448 (inset Fig. 2). This result is consistent with the two-electron reduction mechanism already proposed for plant NQR [1,5]. Also, when recombinant AtNQR was assayed with NAD(P)H as the donor, benzoquinone as the electron acceptor, and cyt *c* as a semibenzoquinone radical trap [1,9], no reduction of cyt *c* was detected (not shown). It was therefore confirmed that plant NQRs, including recombinant AtNQR, are FMN binding flavoenzymes which follow a two-electron reduction mechanism of quinones, with no apparent release of radical semiquinones.

3.3. Steady state kinetic analysis of recombinant AtNQR

Steady state kinetic analysis performed in the presence of DQ as electron acceptor resulted in parallel patterns in double reciprocal plots in the presence of either NADPH or NADH, suggesting a ping pong reaction mechanism (not shown). The general kinetic behavior of $AtNQR_{196}$ was similar to $AtNQR_{191}$. Both enzymes were not saturated by up to 0.25 mM NAD(P)H and 0.2 mM DQ. Higher substrate concentrations were not tested because of the limited water solubility of DQ, and of the excessive absorption of NAD(P)H at 340 nm. As for NQR of other plant tissues [1,5], no reliable estimations of the affinity constants and of the maximal catalytic activities could thus be obtained. On the other hand, the k_{cat}/K_m ratios could be precisely determined (Table 1). These were all in the order of magnitude of 10^7 M^{-1} s^{-1}, as expected

Table 1
Kinetic parameters of $AtNQR_{191}$ and $AtNQR_{196}$ as estimated by steady state kinetic analysis

(Electron donor):(electron acceptor)	Specificity constant	$AtNQR_{191}$ (10^6 M^{-1} s^{-1})	$AtNQR_{196}$ (10^6 M^{-1} s^{-1})
(NADPH):(DQ)	$k_{cat}/K_{m(NADPH)}$	21 ± 2	8.8 ± 0.7
(NADPH):(DQ)	$k_{cat}/K_{m(DQ)}$	12 ± 3	6.8 ± 0.6
(NADH):(DQ)	$k_{cat}/K_{m(NADH)}$	8.0 ± 1.5	5.4 ± 0.6
(NADH):(DQ)	$k_{cat}/K_{m(DQ)}$	16 ± 1	8.2 ± 1.1

Data are mean values ± S.D. of two to three independent experiments.

for a kinetically very efficient enzyme. The specificity constants of $AtNQR_{196}$ were lower than for $AtNQR_{191}$. In $AtNQR_{191}$ the specificity for NADPH was significantly higher than for NADH, but in $AtNQR_{196}$ the difference was less marked. In agreement with a two-site ping pong reaction mechanism, the specificity constants for DQ of either $AtNQR_{191}$ or $AtNQR_{196}$ did not change whether NADPH or NADH was the electron donor. We observe that the kinetic properties of AtNQR, including the values of the k_{cat}/K_m ratios, are fully consistent with those of NtNQR [5].

3.4. Sequence similarity studies

Using AtNQR amino acid sequence as a query, a FASTA search of non-redundant protein and nucleotide sequence databases led to the identification of ten nucleotide sequences, mostly from prokaryotes, showing significant similarity (FASTA3 $E() \leq 0.02$, [31]; Fig. 3). DT-diaphorase sequences were not on the list. Seven of these sequences are part of a protein family (PD 9603) in the Prodom Data Base (*Bacillus subtilis* O07529 and P74312; *Mycobacterium tuberculosis* P95105; *E. coli* P31465; *Yersinia enterocolitica* P74987; *Saccharomyces cerevisiae* Q07923; *Brevibacterium linens* O31130). The remaining three sequences are from *Streptomyces cyanogenus* (Q9ZGB0 and Q9ZGD0), and *Methanococcus jannaschii* (Q58483). All these sequences code for putative proteins, mostly identified in silico following complete sequencing of the respective genomes. Sequence sizes are relatively similar, ranging from 174 (*B. subtilis*) to 232 amino acids (*Yersinia*), and identity to AtNQR ranges from 20% (*Brevibacterium*) to 37% (*E. coli*). In no case was the function of these putative proteins demonstrated, therefore AtNQR is the only member of this group whose biochemical and catalytic properties have been determined.

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Strepto-Q9ZGB0   MSARAVVPASSPHLALLVGSTRSGGAGRTVARWFLARAEE-HGFR 44
Strepto-Q9ZGD0   ------MSQEPLRLALIIGSNREARFGEVVGDWFAGVAKR-RGDL 38
AtNQR            -MEAVTAIKPLIRVAALSGSLRKTSFHTGLLRAAIDLTKESVPGL 44
B. subtilis      -----------MNMLVINGTPRKHGRTRIAASYIAALYHT----- 29
M. tuberculosis  ----MSDTKSDIKILALVGSLRAASFNRQIAELAAKVAPD--GVT 39
E. coli          -----------MRVITLAGSPRFPSRSSSLLEYAREKLNG----L 30
                 ............**..*.**.*.......*......*........

Strepto-Q9ZGB0   DVDVIDLAAIELPVVTGAVTGEGPPPVVADLARRIDAAEAFVVVT 89
Strepto-Q9ZGD0   TVDVIDLADHELPTVLSQNPSPRVAAELEKVSPRLAEADAYVVVT 83
AtNQR            QIEYIDISPLPLINTDLEVNGTYPP-VVEAFRQKILEADSILFAS 88
B. subtilis      --DLIDLSEFVLPVFNGEAEQSELL-KVQELKQRVTKADAIVLLS 71
M. tuberculosis  VTMFEGLGDLPFYNEDIDTATEVPA-PVSALREAASDAHAALVVT 83
E. coli          DVEVYHWNLQNFAPEDLLYARFDSP-ALKTFTEQLQQADGLIVAT 74
                 .**.***....*..............*.....**..**..*..*

Strepto-Q9ZGB0   PEYNHSFPAPLKNAIDWFR-----HEWYAKPVSFVSY-GGISGGL 128
Strepto-Q9ZGD0   PEYNHSYPAALKNLIDWHH-----TQWQAKPVGFVSY-GGLSGGL 122
AtNQR            PEYNFSVSAPLKNALDWASRPP--NVWADKPAAIIST-GGGFGGG 130
B. subtilis      PEYHSGMSGALKNALDFLSS----EQFKYKPVALLAVAGGGKGGI 112
M. tuberculosis  PEYNGSIPAVIKNAIDWLSRPFGDGALKDKPLAVIGGSMGRYGGV 128
E. coli          PVYKAAYSGALKTLLDLLP-----ERALQGKVVLPLATGGTVAHL 114
                 ****.*....*******.........*..**..**...**..**.

Strepto-Q9ZGB0   RAVEQLR-------LVFGELHAVPIRDSVSFHRTREQ-FDEGGAP 165
Strepto-Q9ZGD0   RSVEHLR-------PVFAELHAVTVRDVVSLHMAWER-FGPDGAP 159
AtNQR            RSQYHLRQ-----IGVFLDLHFINKPEFTLNAFQPPQKFDAEGNL 170
B. subtilis      NALNNMR-------TVMRGVYANVIPKQLVLDPVH---IDVENAT 147
M. tuberculosis  WAHDETR--------KSFSIAGTRVVDAIKLSVP----FQTLGKS 161
E. coli          LAVDYALKPVLSALKAQEILHGVFADDSQVIDYHHRPQFTPNLQT 159
                 ......*........*...**.*...*...........*...*..

Strepto-Q9ZGB0   -KDPVGCGTAAKIM-LDQLSWWARSLTEARAKRPYTA 200
Strepto-Q9ZGD0   -KD-EGPAQAAKVL-LDQLVWWGNALREARAKTPYGI 193
AtNQR            -VD---EVTKEKLK-QVLLSLQAFTLRLQGK------ 196
B. subtilis      -VA---ENIKESIK-ELVEELSMFAKAGNPGV----- 174
M. tuberculosis  -VA-----DDAGLA-ANVRDAVGNLAAEVG------- 184
E. coli          RLDTALETFWQALHRRDVQVPDLLSLRGNAHA----- 191
                 .**.........*....*.......*...........
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Fig. 3. Multiple sequence alignments of AtNQR and related putative proteins identified with FASTA3. Only sequences with $E() < 10^{-5}$ are shown. Top to bottom: *S. cyanogenus* Q9ZGB0; *S. cyanogenus* Q9ZGD0; *A. thaliana* AF145234; *B. subtilis* O07529; *M. tuberculosis* P95105; *E. coli* P31465. Residues which are conserved in AtNQR and three out of five other sequences are shaded and corresponding positions are marked by an asterisk.

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DT-MOUSE     -AARRALIVLAHSEKTSFNYAMKEAAVEALKKRGWEVLESDLYAMNFNPI 49
DT-RAT       -AVRRALIVLAHAERTSFNYAMKEAAVEALKKKGWEVVESDLYAMNFNPL 49
DT-HUMAN(1)  MVGRRALIVLAHSERTSFNYAMKEAAAAALKKKGWEVVESDLYAMNFNPI 50
DT-HUMAN(2)  MAGKKVLIVYAHQEPKSFNGSLKNVAVDELSRQGCTVTVSDLYAMNFEPR 50
AtNQR        MEAVTAIKPLIRVAALSGSLRKTSFHTGLLRAAIDLTKESVPGLQIEYID 50
             .....**..*......*.............**.......**..........

DT-MOUSE     ISRNDITGELKDSKNFQYPSESSLAYKEGRLSPDIVAEHKKLEAADLVIF 99
DT-RAT       ISRNDITGEPKDSENFQYPVESSLAYKEGRLSPDIVAEQKKLEAADLVIF 99
DT-HUMAN(1)  ISRKDITGKLKDPANFQYPAESVLAYKEGHLSPDIVAEQKKLEAADLVIF 100
DT-HUMAN(2)  ATDKDITGTLSNPEVFNYGVETHEAYKQRSLASDITDEQKKVREADLVIF 100
AtNQR        ISPLPLINTDLEV-NGTYP-------------PVVEAFRQKILEADSILF 86
             **...*.....*..*..**.............*.*.*...**..**.***

DT-MOUSE     QFPLQWFGVPAILKGWFERVLVAGFAYTYAAMYDNGPFQNKKTLLSITTG 149
DT-RAT       QFPLYWFGVPAILKGWFPRVLVAGFAYTYATMYDKGPFQNKKTLLSITTG 149
DT-HUMAN(1)  QFPLQWFGVPAILKGWFERVFIGEFAYTYAAMYDKGPFRSKKAVLSITTG 150
DT-HUMAN(2)  QFPLYWFSVPAILKGWMDRVLCQGFAFDIPGFYDSGLLQCKLALLSVTTG 150
AtNQR        ASPEYNFSVSAPLKNALDWA-RPPNVW-----ADK-PAA----IIS-TGG 124
             ..*...*.*.*.**...*........*......*..*......***.*.*

DT-MOUSE     GSGSMYSLQGVHGDMNVILWPIQSGILRFCGFQVLEPQLVYSIGHTPPDA 199
DT-RAT       GSGSMYSLQGVHGDMNVILWPIQSGILRFCGFQVLEPQLVYSIGHTPPDA 199
DT-HUMAN(1)  GSGSMYSLQGIHGDMNVILWPIQSGILHFCGFQVLEPQLTYSIGHTPADA 200
DT-HUMAN(2)  GTAEMYTKTGVNGDSRYFLWPLQHGTLHFCGFKVLAPQISFAPEIASEEE 200
AtNQR        GFG------G--GRSQYHLR--QIGVFLDLHF-INKPEFTLNAFQPPQKF 163
             *.*......*..*..*..*...*.**.....*.*..*.........*...

DT-MOUSE     RMQILEGWKKRLETVWEETPLYFAPSSLFDLNFQAGFLMKKEVQEEQKKN 249
DT-RAT       RVQVLEGWKKRLETVWEESPLYFAPSSLFDLNFQAGFLLKKEVQEEQKKN 249
DT-HUMAN(1)  RIQILEGWKKRLENIWDETPLYFAPSSLFDLNFQAGFLMKKEVQDEEKNK 250
DT-HUMAN(2)  RKGMVAAWSQRLQTIWKEEPIPCTAHWHFGQ------------------- 231
AtNQR        DAEGNLVDEVTKEKLKQVLLSLQAFTLRLQGK------------------ 195
             ............*.*........*.........

DT-MOUSE     KFGLSVGHHLGKSIPADNQIKARK 273
DT-RAT       KFGLSVGHHLGKSIPADNQIKARK 273
DT-HUMAN(1)  KFGLSVGHHLGKSIPTDNQIKARK 274
DT-HUMAN(2)  -------------------------
AtNQR        -------------------------
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Fig. 4. Multiple sequence alignments of AtNQR and mammalian DT-diaphorases performed by Clustal W. Residues which are conserved in AtNQR and at least three out of four other proteins are shaded and corresponding positions are marked by an asterisk. The amino acids directly interacting with either isoalloxazine or ribitol in rat DT-diaphorase [8] are black boxed. Sequence entry names in the Swiss Prot Data Bank are as follows (top to bottom): DHQU_MOUSE; DHQU_RAT; DHQU_HUMAN; DHQV_HUMAN.

3.5. Relationships between plant NQR and animal DT-diaphorase

The reaction mechanism and the elevated kinetic efficiency, as well as the difficulties in experimentally saturating the enzyme with substrate are common features of both plant NQR and animal DT-diaphorase [7]. Major differences between plant NQR and animal DT-diaphorase refer to the polymerization state and the flavin coenzyme specificity. Indeed, DT-diaphorase is a dimer of 26 or 30 kDa subunits which bind FAD [15,18,19]. Moreover, sequence similarities between these proteins are limited. The best pairwise alignment was between AtNQR (or NtNQR partial sequence) and a human DT-diaphorase of 231 amino acids (DHQV_HUMAN, [19]), showing 18% identity. In a multiple alignment analysis (Fig. 4), conserved residues between AtNQR and mammalian DT-diaphorases were mainly located in the regions involved in the binding of the flavin coenzyme. Eight out of 11 residues which directly interact with either the isoalloxazine ring or the ribitol moiety of FAD in rat DT-diaphorase [8], are found conserved in AtNQR (Fig. 4). According to this finding, Li et al., 1995 [8] reported that the exact topology of the catalytic domain in rat liver DT-diaphorase resembles the topology of FMN-containing proteins without significant sequence identity. How-

ever, most crucial residues involved in pyridine nucleotide or quinone binding are not conserved between AtNQR and DT-diaphorases, except for the stretch of amino acids TTGGSGS (147–153, rat sequence) which is important for both flavin and NAD(P)H binding [8,32]. It is thus apparent that the functional homology between plant NQR and animal DT-diaphorase does not correspond to a strong structural similarity.

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