

Update of the NAD(P)H:Quinone oxidoreductase (NQO) gene family

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Abstract

The NAD(P)H:quinone acceptor oxidoreductase (NQO) gene family belongs to the flavoprotein clan and, in the human genome, consists of two genes (NQO1 and NQO2). These two genes encode cytosolic flavoenzymes that catalyse the beneficial two-electron reduction of quinones to hydroquinones. This reaction prevents the unwanted one-electron reduction of quinones by other quinone reductases; one-electron reduction results in the formation of reactive oxygen species, generated by redox cycling of semiquinones in the presence of molecular oxygen. Both the mammalian NQO1 and NQO2 genes are upregulated as a part of the oxidative stress response and are inexplicably overexpressed in particular types of tumours. A non-synonymous mutation in the NQO1 gene, leading to absence of enzyme activity, has been associated with an increased risk of myeloid leukaemia and other types of blood dyscrasia in workers exposed to benzene. NQO2 has a melatonin-binding site, which may explain the anti-oxidant role of melatonin. An ancient NQO3 subfamily exists in eubacteria and the authors suggest that there should be additional divisions of the NQO family to include the NQO4 subfamily in fungi and NQO5 subfamily in archaeobacteria. Interestingly, no NQO genes could be identified in the worm, fly, sea squirt or plants; because these taxa carry quinone reductases capable of one- and two-electron reductions, there has been either convergent evolution or redundancy to account for the appearance of these enzyme functions whenever they have been needed during evolution.

Keywords: NAD(P)H:quinone oxidoreductase gene family, diaphorase, cytochrome b5 reductase, quinone reductase, aminoazo dye reductase, flavoprotein clan, flavodoxins, nitric oxide synthases, ribonucleotide reductases, flavin reductases, melatonin

Introduction

Quinones comprise a large class of aromatic compounds, found widely in plants, in the general (benzoyl) form of C₆H₄O₂, but also found in all organisms as flavonoids, electron-carrying coenzymes and metabolic end-products of oxidation. Commercially, quinones are used in making dyes, tanning hides and in photography. Quinones are potentially dangerous, in that they can be involved in a one-electron reduction to form semiquinones that will elicit oxidative stress in any cell; benzoquinones and naphthoquinones are among the most reactive in this category. Quinones were undoubtedly present in the environment and in unicellular organisms between 2 and more than 3 billion years ago (at the time that partial pressure of ambient oxygen on the planet was rising). Hence, during this time it is very likely there was a strong selection to develop an enzyme that would reduce quinones to hydroquinones by a two-electron step, thereby avoiding free radical formation, which is highly destructive to the living cell.

Background and history

The NAD(P)H:quinone acceptor oxidoreductase (NQO) gene family is therefore believed to be ancient — probably between 2 and more than 3 billion years old. Cytosolic NQO flavoenzymes catalyse the beneficial two-electron reduction of quinones to hydroquinones. The two-electron reaction prevents the reduction of quinones by one-electron reductases, which would result in the formation of reactive oxygen species (ROS) generated by redox cycling of semiquinones in the presence of molecular oxygen.^{1,2}

In animals, in addition to the likely role in detoxication of many dietary quinones derived from plants, NQO enzymes have been shown to catalyse the reductive activation of chemotherapeutic quinones such as mitomycins and aziridinylbenzoquinones.^{3,4} The hydroquinones derived from these agents appear to be chemically more reactive, either because of rapid autoxidation or rearrangement — which, in either case, will produce reactive species that can kill malignant cells.^{5,6}

Discovery of DT diaphorase

Ernster and Navazio are credited with discovering (in the rat) the first member of the NQO family in the late 1950s.⁷⁻⁹ This enzyme is now officially named NQO1 (EC 1.6.99.2; previous names included DT diaphorase and aminoazo dye reductase, NQOR1, NMO1, NMOR1 and QR1). It was later concluded^{9,10} that this enzyme is probably identical to a vitamin K reductase that had been isolated by Martius and colleagues. The mammalian NQO1 enzyme is expressed constitutively in most tissues and is strikingly upregulated during oxidative or electrophilic stress; NQO1 has been shown to be a member of the [AHR] gene battery.¹¹ The crystal structure,¹² enzymic functions¹³ and polymorphisms¹⁴ in the *NQO1* gene have all recently been summarised.

The human *NQO1* gene on chromosome 16q22.1 comprises six exons, spanning approximately 18 kilobases (kb), encodes 274 amino acids and contains, to date, 93 single nucleotide polymorphisms (SNPs) within 10 kb 5'-ward of the first exon and 2 kb 3'-ward of the last exon (<http://SNPper.chip.org/>). Two additional variants, besides the consensus gene, were found in the Entrez Gene database (Figure 1). Compared with the human consensus (reference, 'wild-type') *NQO1*1* allele coding for normal NQO1 enzyme and activity, the *NQO1*2* allele encodes a non-synonymous mutation (Pro187Ser) that has negligible NQO1 enzyme activity. The *NQO1*2* allelic frequency ranges between 0.22 (Caucasian) and 0.45 (Asian) in various ethnic populations. A large epidemiological investigation of a benzene-exposed population¹⁵ has shown that *NQO1*2* homozygotes exhibit as much as a seven-fold greater risk of bone marrow toxicity, leading to diseases such as aplastic anaemia and leukaemia. The *NQO1*3* allele (Arg139Try) represents an additional *NQO1* polymorphism¹⁶ that has been characterised; allelic frequencies have been reported as high as 0.05 in some ethnic groups,¹⁷ and the *NQO1*3* allele has been linked to a higher rate of exon 4 deletion — an alternatively spliced form of NQO1 mRNA.¹⁴

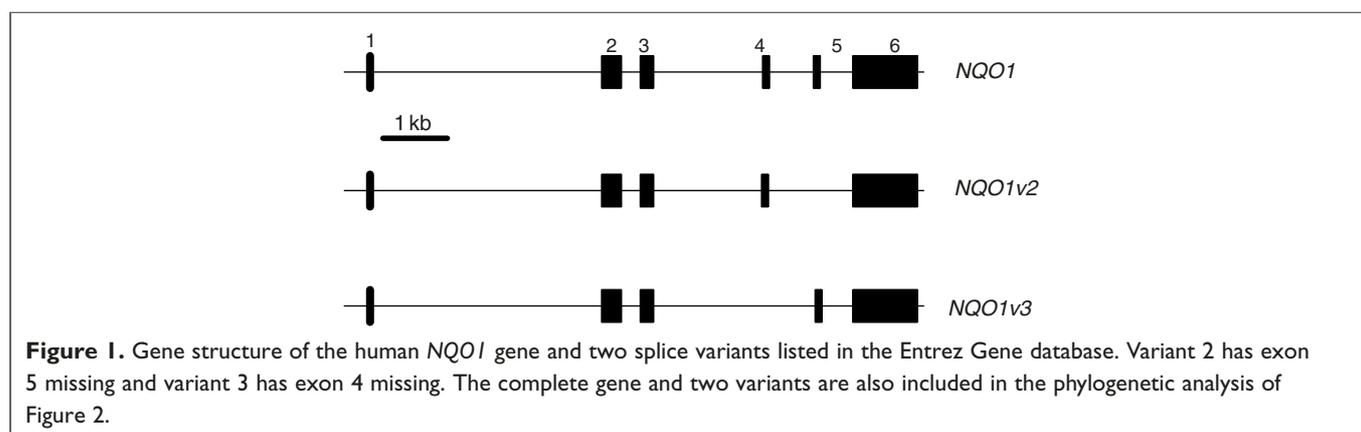
The other member^{18,19} of this mammalian gene family is *NQO2*, which has recently been demonstrated to be identical to a protein isolated from human kidney in 1962. *NQO2* is 43 residues shorter than *NQO1* at its C-terminus and shows 49 per cent sequence identity with *NQO1*. The human *NQO2* gene on chromosome 6p25.2 comprises seven exons (first exon non-coding) spanning 19.8 kb, encodes 231 amino acids and contains, to date, 254 SNPs within 10 kb 5'-ward of the first exon and 2 kb 3'-ward of the last exon (<http://SNPper.chip.org/>). Whereas earlier reports^{20,21} had suggested an association between both *NQO1* and *NQO2* polymorphisms and Parkinson disease, a more recent study²² showed no association between alleles of either *NQO1* or *NQO2* and Parkinson's disease.

NQO1 efficiently utilises NAD(P)H as the source of reducing equivalents, but — because the 43-amino acid deletion in *NQO2* contains a portion of the pyridine nucleotide binding site — *NQO2* has been shown to prefer dihydronicotinamide ribosyl, and other similar derivatives, as a cofactor.²³ Similar to *NQO1*, *NQO2* can also bioactivate certain anti-tumour agents, such as the CB1954 nitro compound.¹³ A crystal structure of human *NQO2* has been published,²³ but not many biological functions of this enzyme have so far been well characterised. Curiously, *NQO2* contains a melatonin-binding site,²⁴ which might be a reasonable explanation for the anti-oxidant role ascribed to melatonin.

Both *NQO1* and *NQO2* activities are observed at high levels in many human solid tumours, including lung, colon, liver and breast; this finding has encouraged oncologists to explore the possibility of *NQO1* and *NQO2* as drug targets for the development of novel anti-tumour agents that can be activated by these enzymes.¹³

Definition of 'Pfam' and 'clan'

Pfam is a means of identifying similar domain structures of all proteins that are summarised in UniProt (<http://www.ebi.uniprot.org/>). Both *NQO1* and *NQO2* were found to belong



to the flavodoxin-2 family (Pfam 02525) because they carry a domain having a flavodoxin-like fold.

The flavodoxin-2 family is a member of the flavoprotein clan. Based on the Pfam database definition:

*'A clan contains two or more Pfam families that have arisen from a single evolutionary origin. Evidence of their evolutionary relationship is usually determined by similar tertiary structures or, when structures are not available, by common sequence motifs.'*²⁵

The category of 'clans' is thus usually introduced in order to encompass two or more gene families that can be globally quite distant evolutionarily but have some segment of the gene products that show a high degree of similarity. Hence in a BLAST search, a sequence may significantly hit more than one member of another family of the clan — as well as all members within one family.

The flavoprotein clan

The flavoprotein clan includes four Pfam members: flavodoxin-1, flavodoxin-2, flavodoxin NdrI and FMN red (Table 1).

The flavodoxin-1 family includes flavodoxins and nitric oxide synthases. Members of this clan are composed of flavin mononucleotide (FMN)- or flavin adenine dinucleotide-binding redox proteins. Flavoproteins act in various electron transport systems as functional analogues of ferre-

doxins; they are characterised by an open twisted alpha/beta structure consisting of five parallel beta-sheets connected by alpha-helices which surround the sheet. Flavodoxins are electron-transfer proteins that function in various electron transport systems. They bind one FMN molecule, which serves as a redox-active prosthetic group, and are functionally interchangeable with ferredoxins. Flavodoxin genes are found in prokaryotes, cyanobacteria and some eukaryotic algae. Nitric oxide synthases (EC:1.14.13.39) produce nitric oxide from *L*-arginine and NADPH. Nitric oxide acts as a second messenger signalling molecule in the body. Five NOS genes have been found in the human genome: *NOS1* (neuronal); *NOS2A* (hepatocyte inducible); *NOS2B*; *NOS2C*; and *NOS3* (endothelial cell) (<http://www.gene.ucl.ac.uk/cgi-bin/nomenclature/>).

The flavodoxin-2 family includes bacterial and eukaryotic NAD(P)H:quinone reductases (as described above), as well as the bacterial acyl carrier protein (ACP), phosphodiesterase (EC:3.1.4.14). The acyl carrier enzyme converts the holo-ACP into apo-ACP by catalysing the hydrolysis of the phosphodiester linkage between Ser-36 of ACP and the 4'-phosphopantethein prosthetic group. ACP is a small (8.8 kDa) protein that plays a central role in the biosynthesis of fatty acids in bacteria, plant chloroplasts and other organisms. ACP phosphodiesterase is known to be encoded by the *acpD* gene in *Escherichia coli* and has been widely distributed in other bacteria, including *Azospirillum brasilense*, *Bacillus halodurans*, *B. stearothermophilus*, *B. subtilis*, *Haemophilus influenzae*, *Lactococcus lactis*, *Mesorhizobium loti*, *Mycoplasma pneumoniae*, *Pseudomonas aeruginosa* and *Streptomyces coelicolor*.

The flavodoxin_NdrI family contains ribonucleotide reductases, which are a family of complex enzymes that play an essential role in all organisms. These enzymes catalyse *de novo* synthesis of deoxyribonucleotides required for DNA replication and repair.²⁶

Finally, members of the FMN red family (flavin reductases) use flavins as substrates and are distinct from flavoenzymes, which have tightly bound flavins. The reduced flavin can serve to provide electrons for ferric ion complexes and iron proteins. In *E. coli*, reactivation of ribonucleotide reductase is achieved by the formation of reduced flavins by flavin reductase.

Table 1. Relationship of the NQO gene family to the flavoprotein clan.

Flavoprotein clan (four Pfam members)
Flavodoxin-1 family
Flavodoxins
Nitric oxide synthases
Flavodoxin-2 family
Bacterial NQO3 genes
Vertebrate NQO1, NQO2 genes
Fungal NQO4 genes
Archaeobacterial NQO5 genes
Bacterial acyl carrier protein phosphodiesterase
Flavodoxin NdrI family
Ribonucleotide reductases
flavin mononucleotide red family
Flavin reductases

Previous confusion about NQO genes and diaphorases

Much of the confusion that has arisen with the nomenclature of NQO1, NQO2 and related proteins can be traced back to the widespread use of the term 'diaphorase' in enzymatic studies more than 50 years ago. In fact, this terminology is still being carried forward.²⁷ The term 'diaphorase' has generally been used to describe a 'coenzyme' (often a flavoprotein) that can transfer electrons from reduced pyridine nucleotides to electron acceptors.^{9,28} In any biological system, therefore,

Table 2. Human *CYB5R* genes listed in the HUGO gene nomenclature committee database.

Approved gene symbol	Approved gene name	Location	Sequence accession IDs	Previous symbols	Aliases
<i>CYB5R1</i>	Cytochrome b5 reductase-1	1q32.1	AF169481 NM_016243	NQO3A2	humb5R2, I500005G05Rik
<i>CYB5R2</i>	Cytochrome b5 reductase-2	11p15.4	AF169802 NM_016229		
<i>CYB5R3</i>	Cytochrome b5 reductase-3	22q13.31-qter	M16461	DIA1	
<i>CYB5R4</i>	Cytochrome b5 reductase-4	6pter-q22.33	AF169803 NM_016230	NCB5OR	b5+b5R, dj676j13.1

there are many enzymes that can be characterised as 'diaphorases'.

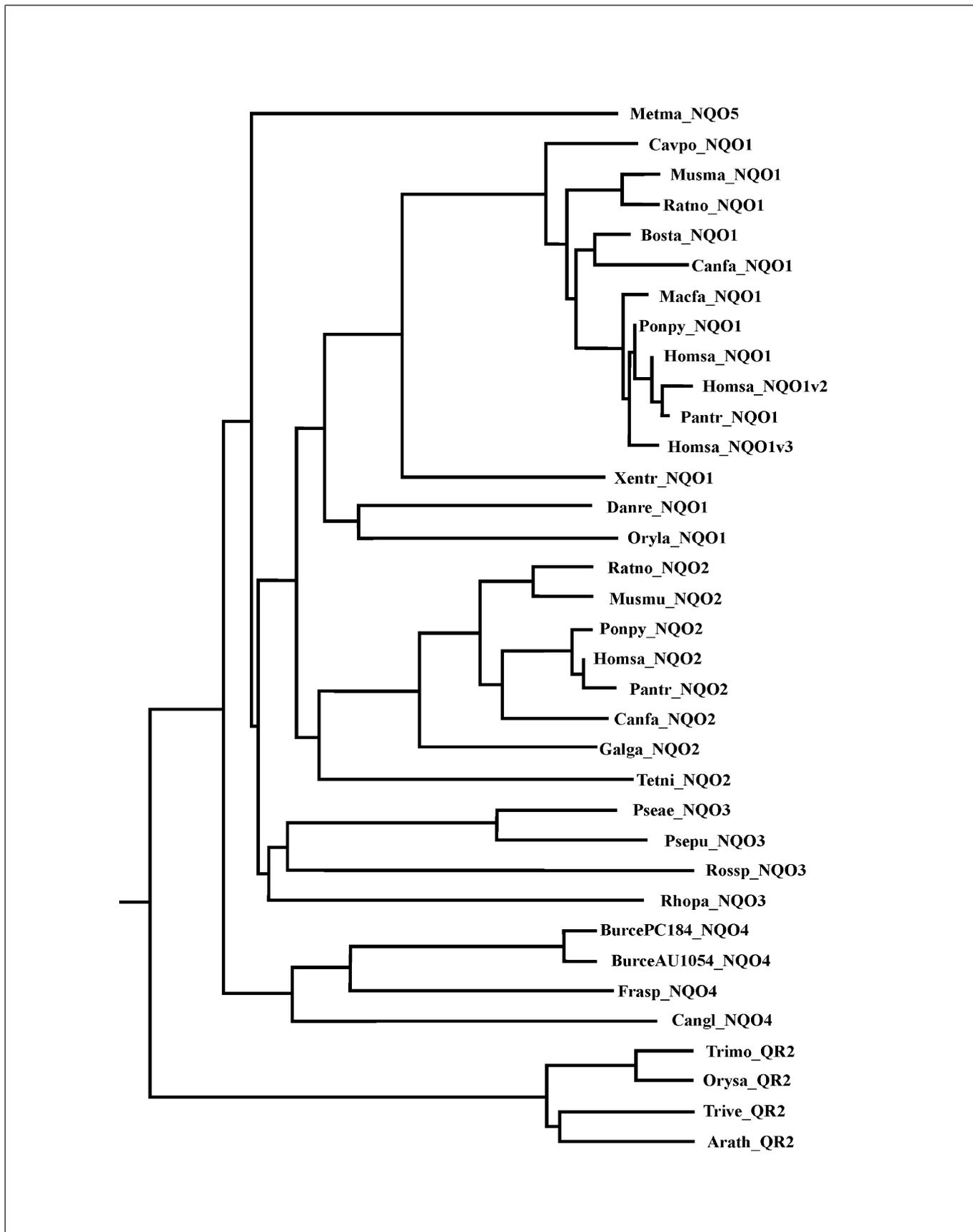
The term 'DT'-diaphorase was originally used by Ernster, to describe what is now known as NQO1, because the enzyme worked with equal facility, employing either DPNH (NADH) or TPNH (NADPH). Edwards *et al.*²⁹ discuss a series of 'diaphorases' defined in human tissues. Diaphorase-1 was NADH cytochrome b5 reductase-3 (Table 2).^{30,31} Diaphorase-2 was the major enzyme in erythrocytes catalysing the oxidation of NADPH. Diaphorase-3 was the principal source of oxidised coenzyme in sperm. Diaphorase-4 was characterised by Edwards *et al.*,²⁹ and this enzyme was found to be identical to the previously characterised DT-diaphorase.³² Interestingly, Edwards and colleagues also showed that diaphorase-4 activity was absent in approximately 4 per cent of the UK population, which is in agreement with more recent genetic studies of the prevalence of the null *NQO1*2* allele.¹⁴

All diaphorases discussed by Edwards²⁹ are distinct gene products but, because of their similarity based on enzymic function rather than evolutionary divergence, searches for

'NQO1' in some databases will still return descriptions of 'cytochrome b5 reductase'. These cytochrome b5 reductase 'diaphorases' are not evolutionarily related, are very different functionally and are encoded by separate gene loci (Table 2). A second source of confusion involves the use of the generic term 'quinone reductase', because a wide variety of enzymes in prokaryotes, plants and animals can reduce quinones via both one- and two-electron steps.

A search of the HUGO Gene Nomenclature Committee database using 'NQO' provided three hits—two *NQO* genes plus 'NQO3A2' (which actually encodes cytochrome b5 reductase-1)—the latter appears in the database because of its previous name as a diaphorase (see above). In fact, one of its aliases is 'NQO3A2', which has been listed in the <http://SNPper.chip.org/> database; the authors have pointed out this error to this organisation. A search of the SwissProt and TrEMBL databases using 'NQO' provided 168 hits (39 in SwissProt and 129 in TrEMBL), but not all of them belong to the flavodoxin-2 family. The term 'NQO' has therefore been used for other enzymes and care should be taken to avoid such confusion.

Figure 2. Phylogenetic organisation of selected *NQO* genes in bacteria and eukaryotes. Metma, *Methanosarcina mazei* (an archaeobacterium); Cavpo, *Cavia porcellus* (guinea pig); Musmu, *Mus musculus*; Ratno, *Rattus norvegicus*; Bosta, *Bos taurus*; Canfa, *Canis familiaris*; Macfa, *Macaca fascicularis* (macaque); Ponpy, *Pongo pygmaeus* (orangutan); Homsa, *Homo sapiens*; Pantr, *Pan troglodytes* (chimpanzee); Xentr, *Xenopus tropicalis*; Danre, *Danio rerio* (zebrafish); Oryla, *Oryzias latipes* (ray-finned fish); Galga, *Gallus gallus* (chicken); Tetni, *Tetraodon nigroviridis* (puffer fish); Pseae, *Pseudomonas aeruginosa*; Psepu, *Pseudomonas putida*; Rossp, *Roseovarius_sp_217*; Rhopa, *Rhodopseudomonas palustri*; BurcePC184, *Burkholderia cenocepacia PC184*; BurceAU1054, *Burkholderia Burkholderia cenocepacia AU 1054*; Frasp, *Frankia sp*; Cangl, *Candida glabrata*; Trimo, *Triticum monococcum*; Orysa, *Oryza sativa* (rice); Trive, *Triphysaria versicolor*; Arath, *Arabidopsis thaliana*; QR2, quinone reductase-2, which belongs to the flavodoxin-1 fold, and not to the flavodoxin-2 (*NQO*) fold. No *NQO* genes were found in *Caenorhabditis elegans*, *Drosophila melanogaster* or *Ciona savignyi* (sea squirt), nor in several plants whose genomic databases were searched.



Evolutionary divergence of the NQO genes

Figure 2 shows the evolutionary relatedness of a number of selected NQO gene products from mammals, birds, amphibians, fish, fungi and bacteria. Vertebrates carry the NQO1 and NQO2 genes while eubacteria carry the most ancient NQO3 genes. The authors suggest naming the fungal subfamily as NQO4 and the archaeobacterial subfamily as NQO5. Four plants (bottom of Figure 2) are complete outliers, being members of the flavodoxin-1 quinone reductase family. Curiously, it was found that frog, zebrafish and puffer fish have NQO1 but not NQO2; conversely, chicken and ray-finned fish have NQO2 but not NQO1. Whether the other homologous gene will be found in any of these genomes in the future remains to be determined. Undoubtedly, the NQO1 and NQO2 genes in vertebrates arose from the prokaryotic NQO3 ancestor. Fungal NQO4 genes also arose from bacterial NQO3, however it is impossible to say whether the eubacterial NQO3 genes or the archaeobacterial NQO5 genes represent the original ancestor. One prokaryotic taxon might have captured the gene from the other prokaryotic taxon by horizontal gene transfer.

The authors performed a series of BLAST searches — using NQO1, NQO2 or NQO3 sequences — and no homologous protein of significance in *Ciona intestinalis*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Anopheles gambiae*, *Oryza sativa* (rice) or *Arabidopsis thaliana* was detected. Wheat plants have been reported to show quinone reductase activity (both the one- and two-electron reductase forms), however, induced in response to powdery mildew infection;³³ infection and inflammation are known to be processes that induce oxidative stress.¹¹ Plants appear to utilise quinone reductase by way of evolutionarily unrelated classes of quinone oxidoreductases, carrying out both one- and two-electron reduction reactions,³⁴ and also alternative NAD(P)H dehydrogenases.³⁵ This finding might therefore be an example of convergent evolution: Mother Nature needed a particular enzyme in a particular location within the plant cell, did not have NQO enzymes in this phylum and therefore selected another type of quinone oxidoreductase or an alternative NAD(P)H dehydrogenase to carry out the enzymic function. Alternatively, this finding might be an example of redundancy, in which multiple systems have evolved independently to face a particular selective pressure.

Do plants suffer from oxidative stress? The absence of NQO genes in plants might reflect the fact that plants use carbon dioxide rather than molecular oxygen; therefore, plants may not experience oxidative stress in the way animals do. This is a fascinating bit of speculation that may be worthy of further study. The putative plant NQO equivalents (QR1, QR2) contain the flavodoxin-1 domain and belong to the flavodoxin-1 family. This domain is found in a number of proteins, including flavodoxin and nitric oxide synthase (as described above).

Putative role of NQO in oxidative stress

BCL2 is well known to be anti-apoptotic and therefore prevents apoptosis from proceeding.³⁶ Proposed functions of BCL2 include mediation of ROS levels and the redox status of the cell, prevention of nucleocytoplasmic trafficking of TRP53 and other cell cycle regulatory factors, neutralisation of BAX, BAD and other proteins that heterodimerise and promote apoptosis and deterrence of mitochondrial cytochrome c release.^{37–39} The *C. elegans* cell death-9 (*ced-9*) gene is the homologue of the mammalian BCL2 gene, *ced-9* is part of a polycistronic locus in *C. elegans* that includes the *cyt-1* gene encoding a protein similar to cytochrome b₅₆₀.³⁸ Cytochrome b₅₆₀ is an inner mitochondrial membrane protein involved in system II (succinate dehydrogenase), which transfers an electron from succinate to ubiquinone. Interestingly, this dehydrogenase has been described as a member of the NQO gene superfamily;⁴⁰ however, the authors found that cytochrome b₅₆₀ is instead a member of the more expansive flavodoxin-1 family (described above). They suggest that various members of the flavoprotein clan, including mammalian NQO1, probably participate in aiding the cell in preventing unwanted apoptosis.^{40,41}

Concluding remarks

Isolation and characterisation of a cytosolic enzyme in several rat tissues in the 1950s led to the description of a quinone reductase in at least two independent laboratories. A substantial number of publications, calling this enzyme 'DT-diaphorase', has led to confusion about the evolutionary relatedness of NAD(P)H:quinone oxidoreductases to one another. There are two NQO genes in vertebrates, a homologous NQO3 subfamily of genes in eubacteria, a NQO4 subfamily in fungi and a NQO5 gene subfamily in archaeobacteria. The fascinating functions of the human NQO1 enzyme include protection against myeloid leukaemia and other types of leukaemia in workers exposed to benzene and a possible anti-apoptotic role fundamental to the cell cycle. NQO2 appears to participate in the anti-oxidant properties of melatonin. Although many SNPs exist in the human NQO2 gene, little is known about any association with a physiological disorder or protection against environmental toxicants.

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