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Review

Flavin-dependent quinone reductases

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Abstract. Quinones are abundant cyclic organic compounds present in the environment as well as in pro- and eukaryotic cells. Several species have been shown to possess enzymes that afford the two-electron reduction to the hydroquinone form in an attempt to avoid the generation of one-electron reduced semiquinone known to cause oxidative stress. These enzymes utilize a flavin cofactor, either FMN or FAD, to transfer a hydride from an electron donor, such as NAD(P)H, to a quinone substrate. This family of flavin-dependent quinone reductases shares a flavodoxin-like structure and reaction mechanism pointing towards a common evolutionary origin. Recent studies of their physiological functions in eukaryotes suggest a role beyond detoxication of quinones and involvement in the oxygen stress response. Accordingly, mammalian quinone reductases emerge as central molecular switches that control the lifespan of transcription factors, such as p53, and hence participate in the development of apoptosis and cell transformation.

Keywords. Semiquinone, hydroquinone, redox cycling, reactive oxygen species, detoxication, flavodoxin-fold, transcription factor, proteasome.

Introduction

Quinones can be reduced by two- or one-electron reduction to the hydroquinone or semiquinone form (Scheme 1). Flavin-dependent quinone reductases (QRs) afford the strict two-electron reduction of the quinone to its hydroquinone form and avoid the generation of the semiquinone form which is prone to react with molecular dioxygen leading to the generation of superoxide radicals ('redox cycling'). This species is well known to cause oxidative stress in eukaryotic as well as prokaryotic organisms and hence QRs have a protective effect against quinone-related oxidative cell damage. In contrast to the strict two-electron reduction performed by flavin-dependent QRs, transfer of a single electron to quinones, leading to the generation of potentially harmful semiquinone species, is also known. The enzymes that catalyze these one-electron reductions appear to be a rather heterogeneous family of enzymes with either a flavin- or metal-dependent catalytic activity.

Since the handling of oxidative stress is important for both eukaryotic and prokaryotic organisms, flavin-dependent QRs have been identified in bacteria, fungi, plants and mammals. Originally, QRs were classified as 'DT-diaphorases' to express the fact that the enzyme utilizes both DPNH (NADH) or TPNH (NADPH) as a source of reducing equivalents [1]. At the time, the term 'diaphorase' was generally used to describe an enzyme (mostly a flavoprotein) that is capable of transferring electrons from reduced pyridine nucleotides to electron acceptors [2]. This nomenclature led to confusions concerning the iden-

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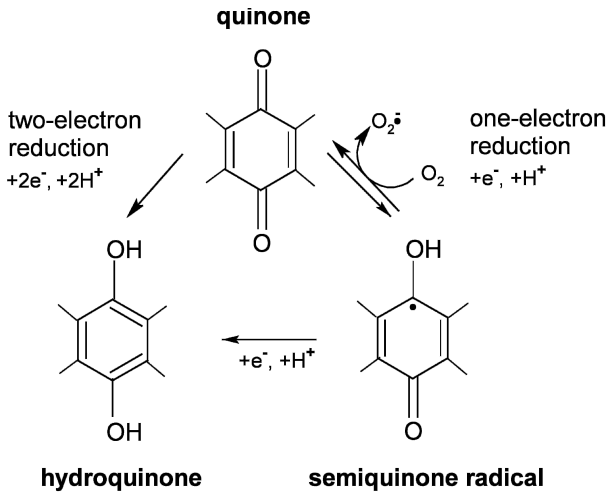


Scheme 1. Reduction mechanism of quinones. Direct formation of a hydroquinone is achieved by the two-electron reduction process, whereas the one-electron reduction of quinones supports the formation of a semiquinone radical intermediate.

tity of the enzyme activity as 'diaphorases' could be detected in numerous biological systems. The first 'DT-diaphorase' reported by Ernster and Navazio [3] is now known as mammalian NAD(P)H:quinone oxidoreductase (NQO1, isozyme 1). However, the acronym 'NQO' has been traditionally confined to QRs from mammalian sources. This review focuses on the family of flavin-dependent QRs sharing strong similarities with regard to sequence, structure and reaction mechanism with mammalian NQOs. First, we will discuss the common general features of QRs before we summarize the current understanding of their role in pro- and eukaryotes.

General properties

Primary sequence analysis

QRs can be classified according to their primary sequence into one of three classes [4]: (1) sequences which contain an NQO1-like C-terminal domain of ~40 amino acids; (2) sequences that lack this C-terminal domain (e.g. human NQO2 and QR from *Pseudomonas putida*), and (3) a subgroup of the shorter sequences that additionally have a deletion of about 18 residues between position 64 and 82 of NQO1 (these amino acids are also absent in bacterial flavodoxin, hence these QRs are considered more 'flavodoxin-like', e.g. *Escherichia coli* and *Saccharomyces cerevisiae*) (Fig. 1). Consequently, the sequence length varies between 174 (ca 19 kDa) and 334 amino acids (35 kDa).

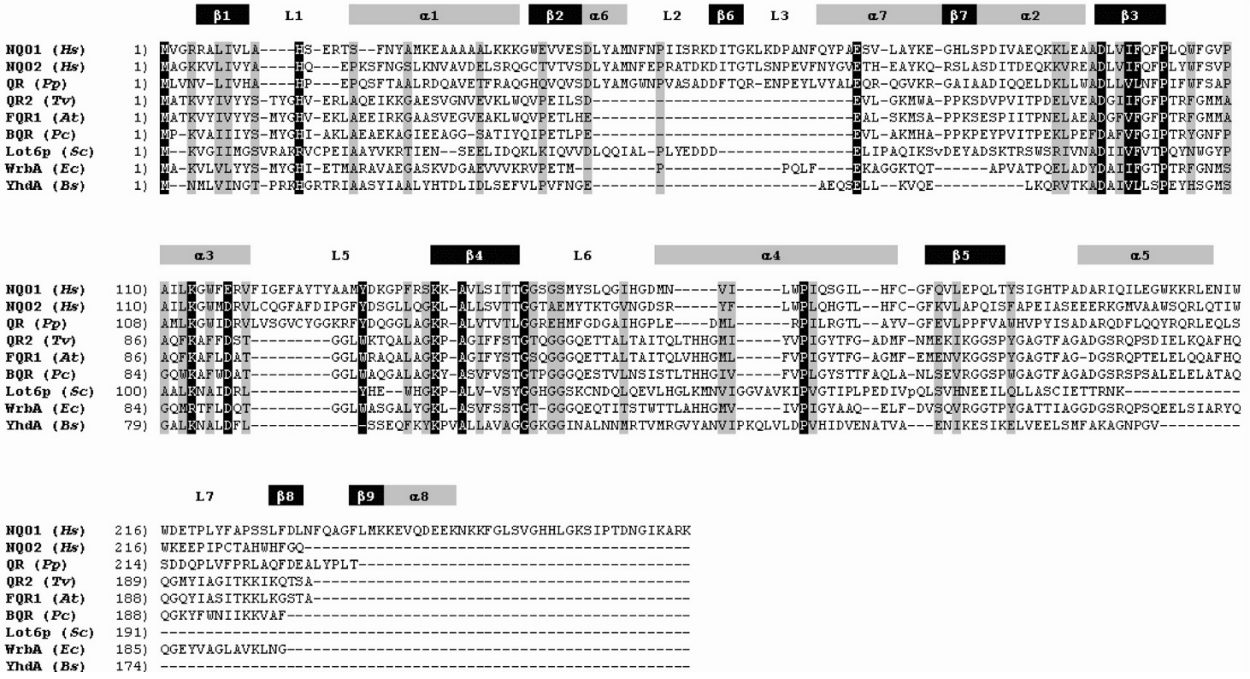


Figure 1. Sequence alignment of quinone reductases from *Homo sapiens* (*Hs*), *Pseudomonas putida* (*Pp*), *Triphysaria versicolor* (*Tv*), *Arabidopsis thaliana* (*At*), *Phanerochaete chrysosporium* (*Pc*), *Saccharomyces cerevisiae* (*Sc*), *Escherichia coli* (*Ec*) and *Bacillus subtilis* (*Bs*). Residues with high sequence similarity are highlighted with a black background, those with lower similarity with a gray background. The alignment was prepared using the program AliBee [117] and manually adjusted. Secondary-structure elements of QRs are indicated: α helices, black on gray background; β sheets, white on black background; loops, black on white background.

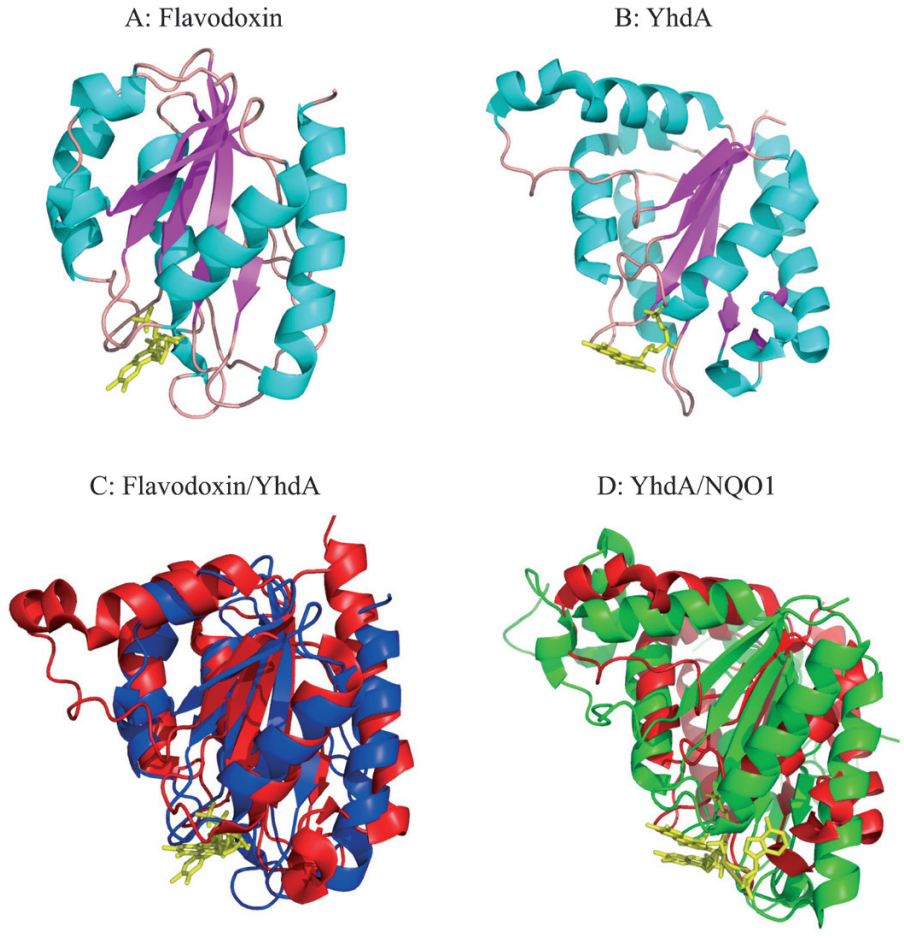


Figure 2. Ribbon representation of flavodoxin from *E. coli* (pdb code: 1ahn) (A), YhdA from *B. subtilis* (pdb code: 1nni) (B), superposition of flavodoxin (blue) and YhdA (red) (C) and superposition of YhdA (red) and NQO1 (green) from *H. sapiens* (pdb code: 2flo) (D). Only monomers are shown to facilitate comparison (see also the figure legend to Fig. 3).

Overall structure

The first successful crystallization of NAD(P)H:(quinone-acceptor) oxidoreductases was reported in the late 1980s, by Amzel et al. [5] and Ysern and Prochaska [5, 6]. However, the large unit cell of the enzyme isolated from mouse liver hampered the elucidation of the protein structure [5] and another couple of years were needed before Li and coworkers [7] eventually solved the X-ray structure of rat liver QR. The 2.1-Å crystal structure revealed that the fold of the N-terminal portion is similar to that of flavodoxin (Fig. 2), a bacterial FMN-containing enzyme. However, the structure of the C-terminal, non-catalytic domain bears no similarities to other known protein domains. This two-domain structure of the mammalian QR had been postulated by Chen and coworkers [8] based on proteolysis experiments revealing a compact N-terminal domain and a flexible C-terminal domain.

The biological unit of NQO1, as for most other QRs crystallized so far, is a dimer (Fig. 3), some bacterial and plant enzymes forming exceptions (Table 1). The overall fold of the N-terminal catalytic domain resembles that of flavodoxins which consists of a twisted central parallel β sheet surrounded by α helices on both sides (Fig. 2). Extensive contact between the two monomers confers high stability on the dimer. The FAD cofactor is non-covalently bound at the interface of the monomers (yellow model in Fig. 2). The redox-active isoalloxazine ring is positioned at one side of two equivalent crevices, forming two identical independent catalytic sites.

The determination of the X-ray structure of NQO2 confirmed the close structural relationship between NQO1 and NQO2 [9]. The NQO2 dimer also contains two identical active sites, located far from each other and at opposite ends of the dimer interface. Similar to NQO1, each catalytic site is a large cavity, lined by residues from both peptide chains with the isoalloxazine ring system of the cofactor forming the bottom [10]. In contrast to NQO1, NQO2 contains one metal-binding site per monomer. This site is tetracoordinated, with two histidine residues (His-173 and His-177) and one cysteine residue (Cys-222) implicated in coordination. Based on this finding, Foster et al. [9] proposed that NQO2 is a copper enzyme in which the metal may be involved in electron transfer rather than in structural stabilization, since the metal site may be

Table 1. Soluble flavoproteins with quinone reductase activity sharing the flavodoxin-like fold.

	Acronym	Species	Cofactor	Reducing agent	Reaction	Oligomerization	References
Mammalians	NQO1	*Homo sapiens*	FAD	NADH/NADPH	ping-pong, bi-bi	dimer	4, 83
	NQO2	*Homo sapiens*	FAD	N-ribosyl-, N-methyl derivates	ping-pong, bi-bi	dimer	73, 74
Plants		*Beta vulgaris*	FMN	NADPH	ping-pong, bi-bi	tetramer	101
		Nicotiana tabacum	FMN	NADH/NADPH	two-electron transfer	tetramer	100
		Hevea brasiliensis	FMN	NADH/NADPH	two-electron transfer	tetramer	103
	FQR1	*Arabidopsis thaliana*	FMN	NADH/NADPH	not reported	not reported	104
	NQR	*Arabidopsis thaliana*	FMN	NADH/NADPH	two-electron transfer	tetramer	99
Fungi	Lot6p	*Saccharomyces cerevisiae*	FMN	NADH/NADPH	ping-pong, bi-bi	dimer	36
		Planerochaete chrysosporium	FMN	NADH/NADPH	ping-pong, bi-bi	dimer	98
Bacteria	WrbA	*Escherichia coli*, *Archaeoglobus fulgidus*	FMN	NADH	two-electron transfer	monomer-dimer-tetramer equilibrium	18
	MdaB	*Helicobacter pylori*	FAD	NADPH	two-electron transfer	not reported	81, 82
	AzoA	*Enterococcus faecalis*	FMN	NADH	two-stage ping-pong	dimer	15, 41
	YhdA	*Bacillus subtilis* 168	FMN	NADPH	not reported	tetramer	28
		Bacillus sp. OY1-2	not reported	NADPH	not reported	monomer	16, 17
	AcpD	*Escherichia coli*	FMN	NADH	ping-pong, bi-bi	not reported	49
	AzoR	*Rhodobacter sphaeroides*	FMN	NADH	ping-pong	not reported	14
	Azo1	*Staphylococcus aureus*	FMN	NADPH	not reported	tetramer	29

linked to the active site by an electron transfer route similar to type I copper sites. However, the presence of the metal *in vivo* has not yet been established. Above all, it was recently shown that EDTA does not notably affect NQO2 activity, suggesting that the metal is not required for catalytic activity [11].

The crystal structure of Lot6p from baker's yeast is more similar to human NQO2 since it lacks the C-terminal non-catalytic domain found in NQO1 [12] (Fig. 3). However, the metal found in NQO2 is not present in the structure of the yeast quinone reductase Lot6p [12]. Although Lot6p adopts a similar flavodoxin-like fold and self-associates as a homodimer (Fig. 3), it binds one FMN per protomer instead of FAD as in the case of the two mammalian QRs.

Monomer structure

The NQO fold is a distinct fold within flavin-containing proteins. The N-terminal catalytic domain is similar to that of *Clostridium* flavodoxin but contains FAD instead of FMN. However, the flavin cofactor is bound in a very similar manner. The main part of the fold is a twisted five-stranded parallel β sheet encased by five α helices, which connect successive strands in an alternating α/β topology. In contrast to bacterial flavodoxins, there is an 18-amino-acid cross-over between β2 and α2 which participates in stabilization of the protein and is thought to prevent the active-site flavin from interacting with other proteins [13].

The NQO catalytic domain can be regarded as a modified Rossman fold in which the third α/β pair is either lost or included in the cross-over element between the two sections of the parallel β sheet [4]. This insertion forms an additional anti-parallel hairpin and an α helix that participates extensively in the dimer interface. In the yeast protein Lot6p, the interface is made up of hydrophobic interactions and further stabilized by 14 hydrogen bonds.

The bacterial QRs listed in Table 1 also adopt a flavodoxin-like fold consisting of 5 α-helical layers sandwiching a five-stranded parallel β sheet in the center. The QRs from *Bacillus subtilis* (strain OY1–2), *Enterococcus faecalis* (AzoA), *E. coli* (AcpD) and *Rhodobacter sphaeroides* (AzoR) possess an amino acid sequence typical for an NAD(P)H-binding motif [14–17]. Furthermore, members of the WrbA family share typical features of flavodoxins such as the PROSITE flavodoxin signature sequence (LIV)-(LIVFY)(FY)X(ST)(V)X(AGC)XT(P)-XXAXX(LIV), indicative of the N-terminal region that spans the FMN-binding site [18]. Unlike flavodoxins, WrbA proteins feature an α/β unit in addition

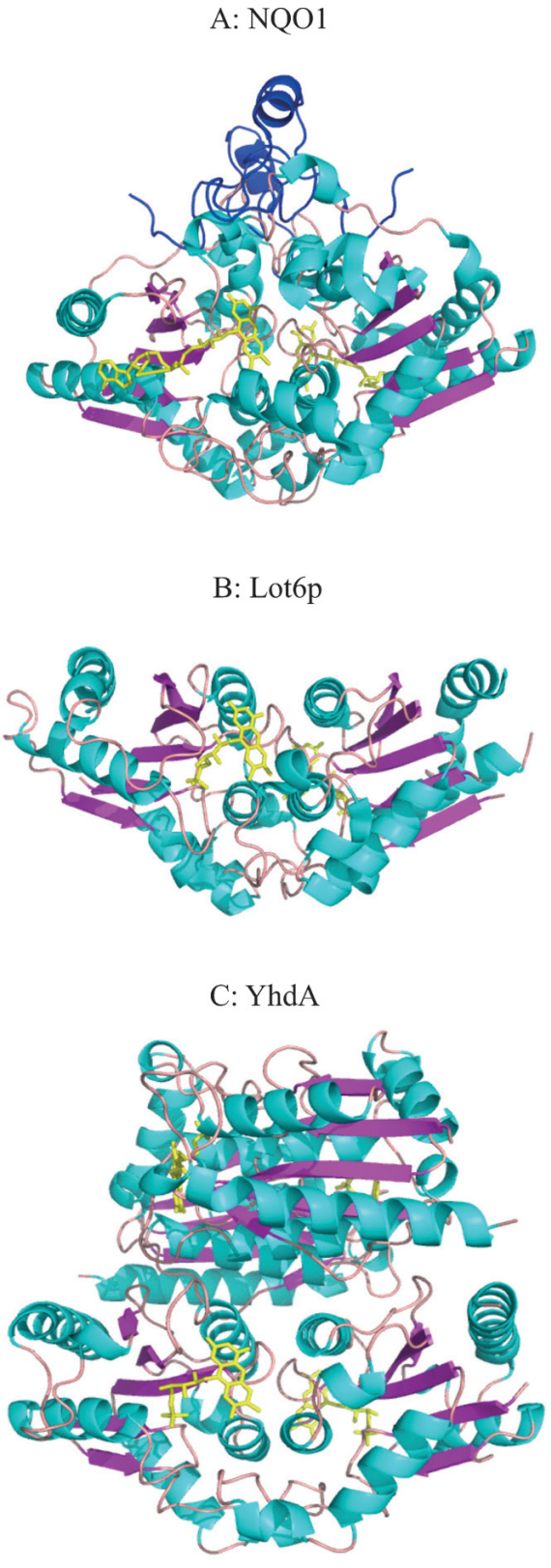


Figure 3. Ribbon representation of human NQO1 (the additional C-terminal structure is shown in blue) (pdb code: 2flo). (A), Lot6p from *S. cerevisiae* (pdb code: 1t0i) (B) and YhdA from *B. subtilis* (pdb code: 1nni) (C). NQO1 and Lot6p form dimers in solution whereas YhdA is a tetramer. The flavin cofactor is shown in yellow.

to the typical α/β twisted open-sheet fold unique to this family [19, 20]. Moreover, WrbA proteins contain a motif strictly conserved in the family of iron-sulfur flavoproteins, but the cysteine motif involved in 4Fe-4S cluster formation is not found in WrbA [18].

Several other bacterial proteins have been reported to possess QR activity (Table 2); however, it appears that this is, in some cases, an adventitious activity and quinones are not cognate substrates of these enzymes. Examples for this class of enzymes are FRase I and NfsB from *Vibrio fischeri* and *E. coli*, respectively [21]. These enzymes have an NAD(P)H:FMN oxidoreductase activity which provides reduced FMN for various other $FMNH_2$-dependent cellular reactions, such as the bioluminescence reaction in the marine bacterium *V. fischeri* [21, 22]. The structure of these proteins is also quite distinct from that of the flavodoxin-like QRs discussed above and consists of a three-layer α-β-α sandwich, a common fold in the NADH oxidase superfamily [23]. Another apparent member of this group of proteins is ChrR from *P. putida*, which was first described as a chromate reductase [24, 25]. As pointed out recently by Matin's group, quinones are physiologically relevant substrates for ChrR with an involvement of the enzyme in the oxygen stress response [26]. This clearly indicates that QR activity is not confined to enzymes with a flavodoxin-like fold and these two non-homologous and structurally unrelated protein families possibly cooperate in the event of oxygen stress.

Oligomerization

All mammalian QRs form stable dimers with the flavin-binding site near the dimer interface (Fig. 2) [27]. The yeast and bacterial QRs lack the C-terminal extension of the mammalian NQO1 (dark blue in Fig. 3) and hence the dimer exhibits a concave surface (Fig. 3). In the case of the *B. subtilis* QR YhdA and members of the WrbA family (e.g. WrbA from *Deinococcus radiodurans*), this concave surface is used to form a tetrameric protein in which one dimer is rotated 90° such that the two saddle-like surfaces assume complementarity (Fig. 3). This tetrameric assembly is further stabilized by hydrophobic packing and four additional salt bridges between lysine and aspartate residues in YhdA and hydrophobic, polar and hydrogen-bonding interactions in WrbA [19, 28]. Similarly, a QR from *Staphylococcus aureus* (Azo1) was found to be a tetramer in solution with four non-covalently bound FMN molecules, i.e. one FMN per subunit [29]. Removal of the FMN leads to the dissociation of the tetramer to its dimeric form, indicating that the cofactor plays a role in stabilization of the tetrameric state. Although both YhdA and Azo1 are from Gram-positive bacteria, the proteins

Table 2. Other soluble bacterial flavoproteins with reported quinone reductase activity.

Acronym	Species	Cofactor	Reducing agent	Reaction	Oligomerization	References
ChrR	*Pseudomonas putida*	FMN	NADH	two-electron transfer, ping-pong	dimer	26
FerA	*Paracoccus denitrificans*	addition of FMN	NADH	not reported	monomer	39
FerB	*Paracoccus denitrificans*	FAD	NADH/ NADPH	ping pong	dimer	39
FRase I	*Vibrio fischeri*	FMN	NADH/ NADPH	two-electron transfer, ping pong, bi bi	dimer	22, 23
NfsB	*Escherichia coli*	FMN	NADH/ NADPH	two-electron transfer, ping pong, bi bi	dimer	21

share only 32% sequence identity, and the latter protein also appears to lack the salt bridge involved in tetramer stabilization in YhdA. Obviously, shape and charge complementarity are sufficient for tetramerisation of Azo1, while additional stabilization of the tetramer has evolved in other Gram-positive bacteria. In the case of the bacterial WrbA, a monomer-dimer-tetramer equilibrium was reported [30]. As seen before for Azo1, FMN binding promotes WrbA association into tetramers, shifting the melting temperature by 40°C. Again, this finding emphasizes the critical role of flavin binding for the oligomerization state of QRs and can be correlated to its structural position between two polypeptide chains. It should be noted in this context that all flavodoxins are found as monomers in solution and that their flavin cofactor is less accessible than that of QRs (see Fig. 2) [31].

Catalytic site

The crystal structures of rat NQO1 complexed with either $NADP^+$ or duroquinone as the electron-accepting substrate identified the active site of the enzyme as a pocket at the dimer interface [4, 7]. The shape and size of the catalytic site accommodates a broad range of ring-containing substrates. The NQO1 and NQO2 active sites are very similar, with the exception of the three residues Tyr-126, Tyr-128 and Met-131 that are replaced by Phe-126, Ile-128 and Phe-131 in NQO2, thus making the NQO2 cavity slightly larger and more hydrophobic than that of NQO1. In the active sites of both enzymes, aromatic amino acids (Trp-105 and Phe-106) form the bottom of the pocket. The entrance to the catalytic site is limited by glycines 149 and 150 of loop L6, by His-194 and by Pro-68 of the N-terminal α7 helix of the second monomer. In NQO1, Tyr-128 gates the pocket and is thought to protect the reduced flavin from reoxidation through reaction with molecular dioxygen. In comparison to yeast QR Lot6p, NQO1 features a more apolar active site by containing several aromatic residues (Phe-106, Phe-178, Tyr-128, Trp-126, Trp-105). Most of those residues are replaced by non-aromatic or even polar amino acids in Lot6p. Conserved residues only comprise Tyr-126 and Gly-149 (Tyr-111 and Gly-126, respectively, in the case of Lot6p). Interestingly, the loop containing Tyr-128 thought to protect the cofactor against reoxidation by molecular oxygen is missing in Lot6p, which also resists reoxidation by molecular oxygen [Sollner et al., unpublished results]. Thus, the structural basis for oxygen reactivity of reduced QRs is currently not understood.

The NADPH phosphate-binding site of NQO1 is a cleft that ends in the catalytic site between the hairpin (β8-L9-β9) and L5. Three hydrogen bonds are expected to be missing when NADH instead of NADPH is bound to the enzyme. As judged from the NQO1-$NADP^+$ crystal structure [4, 7] , the hydride transfer is expected to take place from the 4-*pro*-S hydrogen (B side) of the reduced nicotinamide. In that conformation, Tyr-126 and Tyr-128 do not provide hydrogen bonds to the nicotinamide. In addition, the hydrogen bond involving Phe-232 is lost in the case of the unphosphorylated nucleotide. This lack of hydrogen bonds may explain the slight preference of NQO1 for NADPH over NADH [32]. However, the stereochemistry suggested by the crystal structure is in contrast to results reported much earlier, where NQO1 was proposed to be A stereospecific [33]. Further experimental evidence is needed to resolve this contradiction.

While FAD-protein interactions are well conserved between NQO1 and NQO2, several amino acid residues are replaced in the NADPH/NQO1 interaction and one is lost by truncation of the non-catalytic C-terminal domain of NQO1 [7, 9, 10]. This of course may explain the incapacity of NQO2 to use classical hydride donors such as NAD(P)H. In NQO1, aromatic stacking of the nicotinamide ring and the flavin isoalloxazine ring provides additional stabilization of NAD(P)H. This kind of interaction may also be responsible for the binding and stabilization of non-phosphorylated electron-donating cosubstrates in the NQO2 active site, as this enzyme is capable of using both polar and non-polar N-substituted dihydronicotinamide substrates [9].

Two NQO1 structures (human and rat) show the catalytic pocket occupied by duroquinone (2,3,4,6-tetramethyl-1,4-benzoquinone), a small quinone sub-

strate [7, 34]. Duroquinone occupies nearly the same position vacated by the nicotinamide ring of $NADP^+$. The quinone substrate is bound to the active site through a series of contacts involving both the flavin cofactor and several hydrophobic and hydrophilic residues. The substrate is sandwiched between the rings of Phe-178 and isoalloxazine rings A and B, with the quinoid carbonyl oxygens oriented roughly parallel to the length of the flavin ring. A similar binding was found for menadione (vitamin K3) bound to NQO2, with the quinone ring of the substrate on top of the benzene ring of the isoalloxazine system [9]. The quinone oxygen is 3.5 Å away from the flavin N(5), ideally suited for hydride transfer during the oxidative half-reaction.

Kinetic and docking studies using NQO1 have demonstrated that the NQO1 active site is able to accommodate quinones with one, two or three fused rings, and that, with an increasing number of fused rings, both binding affinity and maximal velocity decrease [35]. Similar results were obtained for Lot6p, the yeast ortholog [36]. The kinetic data suggest that the larger quinones rotate along the quinone carbonyl axis, relative to the duroquinone position in the X-ray structure, in order to provide optimal π stacking interactions with the isoalloxazine ring of the flavin cofactor [35].

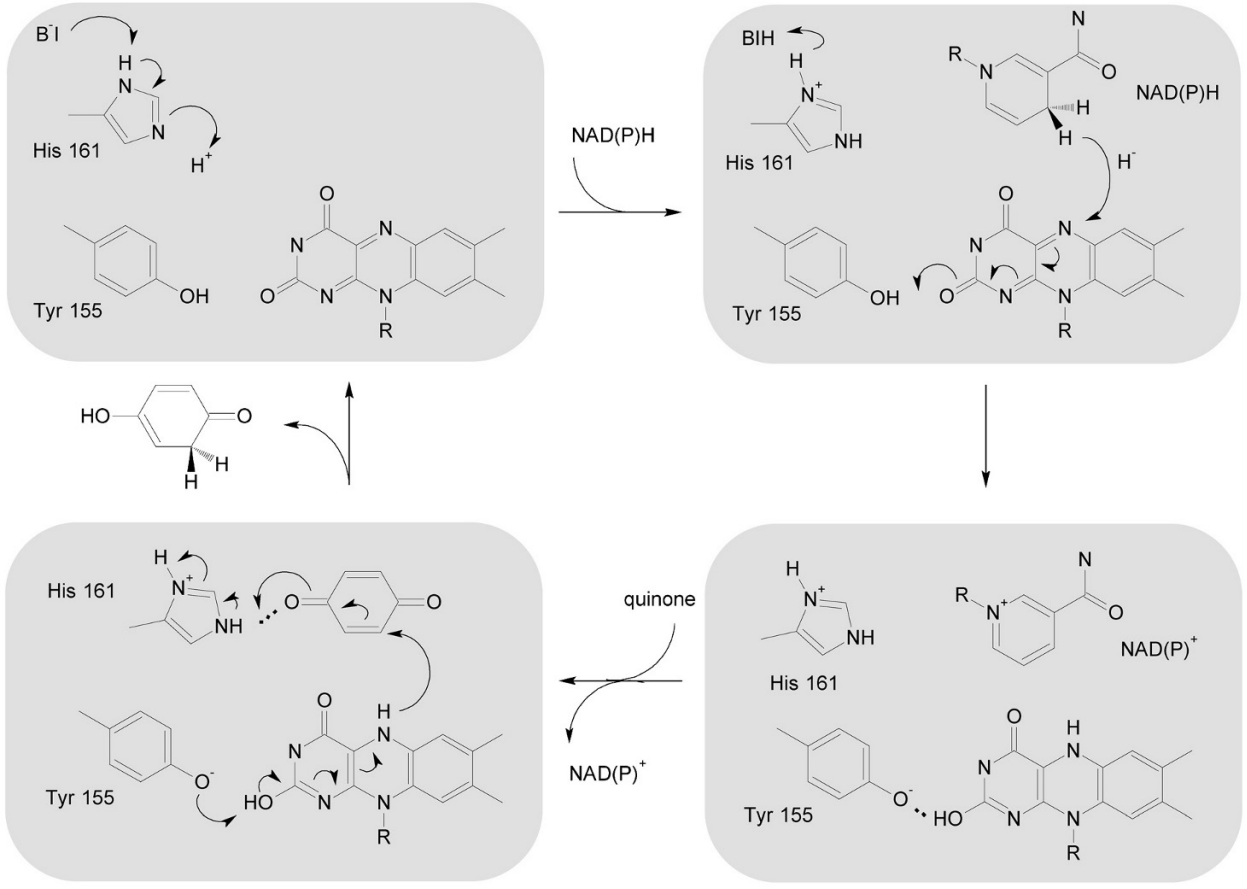
The observation that the binding sites for quinone substrates and $NADP^+$ are almost overlapping accounts for the ping-pong kinetics observed for all QRs so far: NQO1 with its bound FAD cofactor can only bind one or the other compound at one time, either electron donor [NAD(P)H] or electron acceptor (quinone). This type of reaction mechanism is more analogous to the reactions of electron carriers like flavodoxin than other FAD-containing enzymes which can bind both nicotinamide nucleotide and electron acceptor at the same time, consistent with the structural similarity between QRs and flavodoxin.

Reaction mechanism

The unique property of QR is its ability to transfer two electrons to a quinone, resulting in the formation of a two-electron reduced hydroquinone without generation of an one-electron reduced semiquinone as documented by electron spin resonance studies [37]. This finding suggests that the enzyme does not support the formation of a flavin semiquinone intermediate but promotes a simultaneous two-electron transfer to the substrate (Scheme 2). This mechanism was also shown for the yeast ortholog, Lot6p, pointing towards a conserved enzymatic mechanism involving the transfer of a hydride from the flavin N(5) position to the beta position of the carbon-carbon double bond (Scheme 2) [36]. When obligatory one-electron acceptors, such as potassium ferricyanide, are used, no flavin cofactor semiquinone could be detected, basically because the transfer of the second electron (to another substrate molecule) was much faster than the first-electron transfer [32]. Furthermore, it was observed that NQO1 reconstituted with 5-deaza-FAD, which can only be oxidized by a single two-electron step, supports the reduction of quinones [38]. Taken together, these results document a strict two-electron transfer mechanism catalyzed by NQO1 and its fungal and bacterial homologs. This enzymatic property also appears to be crucial for understanding the physiological role of this family of enzymes as a cellular detoxification mechanism that avoids the formation of semiquinones and hence the generation of harmful reactive oxygen species.

The outlined substrate reduction process has been proposed to occur via two direct hydride transfer steps: one from the electron donor (a reduced nicotine amide derivative) to the flavin cofactor (FAD or FMN) and a second from the reduced flavin to the substrate electron acceptor (e.g. a quinone). The X-ray structures of QRs with either electron donor or acceptor bound to the active site show that electron donor and acceptor occupy the same site with respect to the flavin cofactor [7]. Thus, binding of the electron acceptor cannot occur before the electron donor is released from the active site, thus providing a structure-based explanation for the observed ping-pong bi-bi mechanism, which appears to be in operation in all QRs (Scheme 2). Such a mechanism involves binding of the electron donor such that a hydride can be transferred directly to N(5) of the cofactor (Scheme 2). Indeed, structural studies have revealed binding of NAD(P)H to the active site with its 4-*pro*-S hydrogen (B side) in close vicinity (3.4 Å) to this nitrogen enabling hydride transfer to the flavin cofactor. Since the transfer of a hydride generates a negative charge on the flavin cofactor, stabilization by the enzyme is required especially upon dissociation of $NAD(P)^+$. A tyrosine residue (Tyr-155) near the N(1)–C(2)=O locus of the isoalloxazine ring system, where the negative charge is delocalized, appears to be a very likely candidate for the stabilization of the reduced flavin species, with a neighboring histidine (His-161) potentially involved in charge stabilization (Scheme 2) [4].

In the oxidative half-reaction, a quinone substrate binds to the active site near the reduced flavin and receives a hydrid in a process that involves the reversal of steps described above. Hydride transfer to the quinone gives rise to an ionized hydroquinone (hydroquinolate) and the enzyme may facilitate this reduction by providing a proton to the reduced substrate (Scheme 2). Therefore, the net result of the oxidative



Scheme 2. Reaction mechanism of human NQO1. The substrate reduction process has been proposed to occur via two direct hydride transfer steps: one from the electron donor (a reduced nicotine amide derivative) to the flavin cofactor (FAD or FMN) and a second from the reduced flavin to the substrate electron acceptor (a quinone), and thereby obeys a ping-pong bi-bi mechanism.

half-reaction may involve not only hydride transfer but also transfer of a proton from bulk water to the hydroquinolate [4].

Several QRs, especially from bacterial sources, were reported to accept other electron acceptors such as chromate, ferric-iron complexes, nitro-aromatic compounds and azodyes [16, 26, 39, 40]. The mechanism of substrate reduction seems to be conserved with these compounds; however, the reduction of azodyes requires the transfer of four electrons, i.e. two complete cycles have to be carried out instead of one. Moreover, it is not entirely clear whether reduction of these unphysiological substrates occurs in the active site or is mediated by a reduced species, for example free reduced flavin. These issues are further discussed in the next section.

Substrate specificity

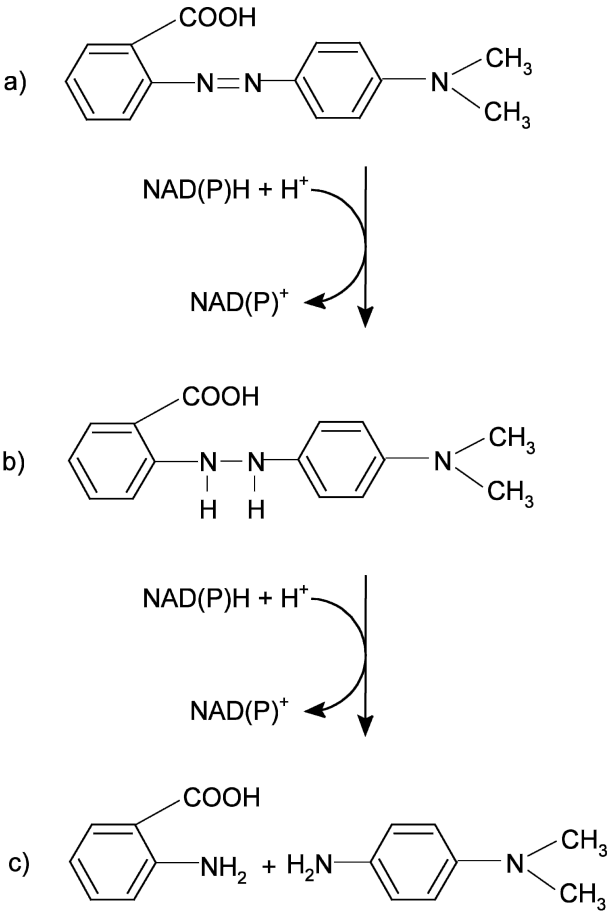
Some QRs utilize both NADH and NADPH as a source for electrons (e.g. NsfB from *E. coli* and Lot6p from *S. cerevisiae* [21, 36]) whereas others have developed a clear preference for either NADH (e.g. AzoA from *E. faecalis* [41]) or NADPH (e.g. YhdA from *B. subtilis* [28]). In contrast, NQO2 is unable to employ NADH or NADPH as a source of electrons and uses reduced *N*-ribosyl- and *N*-alkyl-dihydronicotinamide instead. Contradictory results were obtained with recombinant NQO2 expressed in COS cells, where NADH-dependent reduction of menadione was observed [42]. However, others have argued that this reaction could be due to an NQO1 contamination [10]. Therefore, the only confirmed reducing substrates for NQO2 are the *N*-ribosyl and *N*-methyl derivatives of dihydronicotinamide.

The issue of oxidizing substrates of QRs is far more complicated. Generally, it is assumed that enzymes involved in detoxification of xenobiotics do not possess endobiotic substrate(s) and have developed so that a broad range of chemical structures can be processed. In fact, the shape and size of the active sites of NQO1, NQO2 and Lot6p suggest that these enzymes have evolved to accept a wide range of ring-containing compounds. However, some naturally occurring quinones comprising vitamin K derivatives

(menaquinone and phylloquinone), coenzyme Q (ubiquinones) and dopaquinone, an orthoquinone, were also found to be substrates of mammalian QRs [10]. Kinetic data for quinones with one, two or three fused rings in the quinone nucleus indicate that, as the number of fused rings increases from one to three, both K_M values and maximal velocities decrease dramatically in the case of NQO1. Small alkyl substituents on the quinone nucleus were shown to have little or no effect on either K_m or k_{cat} values [35]. In addition, quinoneimines (e.g. *N*-acetyl-*p*-benzoquinoneimine, *N*,*N*-dimethylindoaniline) were shown to be substrates for NQO1, although substrate inhibition is reported at higher concentrations [43].

Consistent with the hypothesis that QRs are part of an enzymatic detoxification system, several other substrates, such as nitro-aromatic compounds [44], azodyes and ferric iron [45] have been identified (see above) [12, 46–48]. In fact many bacterial QRs are annotated as 'azoreductases' to emphasize their ability to carry out the reduction (and cleavage) of the azo bond to yield two aromatic amines (Scheme 3) [14, 28, 29, 41]. These enzymes have attracted some attention for their potential utilization in bioremediation processes, for example the treatment of waste water contaminated with azodyes [16, 17].

The QR from *E. coli*, termed AcpD, was originally classified as an acyl carrier protein phosphodiesterase, but recent investigations have shown that the protein does not convert the holo-acyl carrier protein into its apo form [49]. On the other hand, Nakanishi and coworkers [49] have found high azoreductase as well as quinone reductase activity, clearly demonstrating that AcpD is not an acyl carrier protein phosphodiesterase as postulated based on its sequence similarity to other known enzymes of that family. Several other bacterial QRs, such as AzoA from *E. faecalis* [41], AZR from *Bacillus* sp. OY1–2 [17], YhdA from *B. subtilis* [28] and AzoR from *R. sphaeroides* [14] were first described as azoreductases. More detailed investigations of the mechanism of azo bond cleavage revealed the existence of a partially reduced hydrazine intermediate, confirming the two-step reductive process shown in Scheme 3 [14].

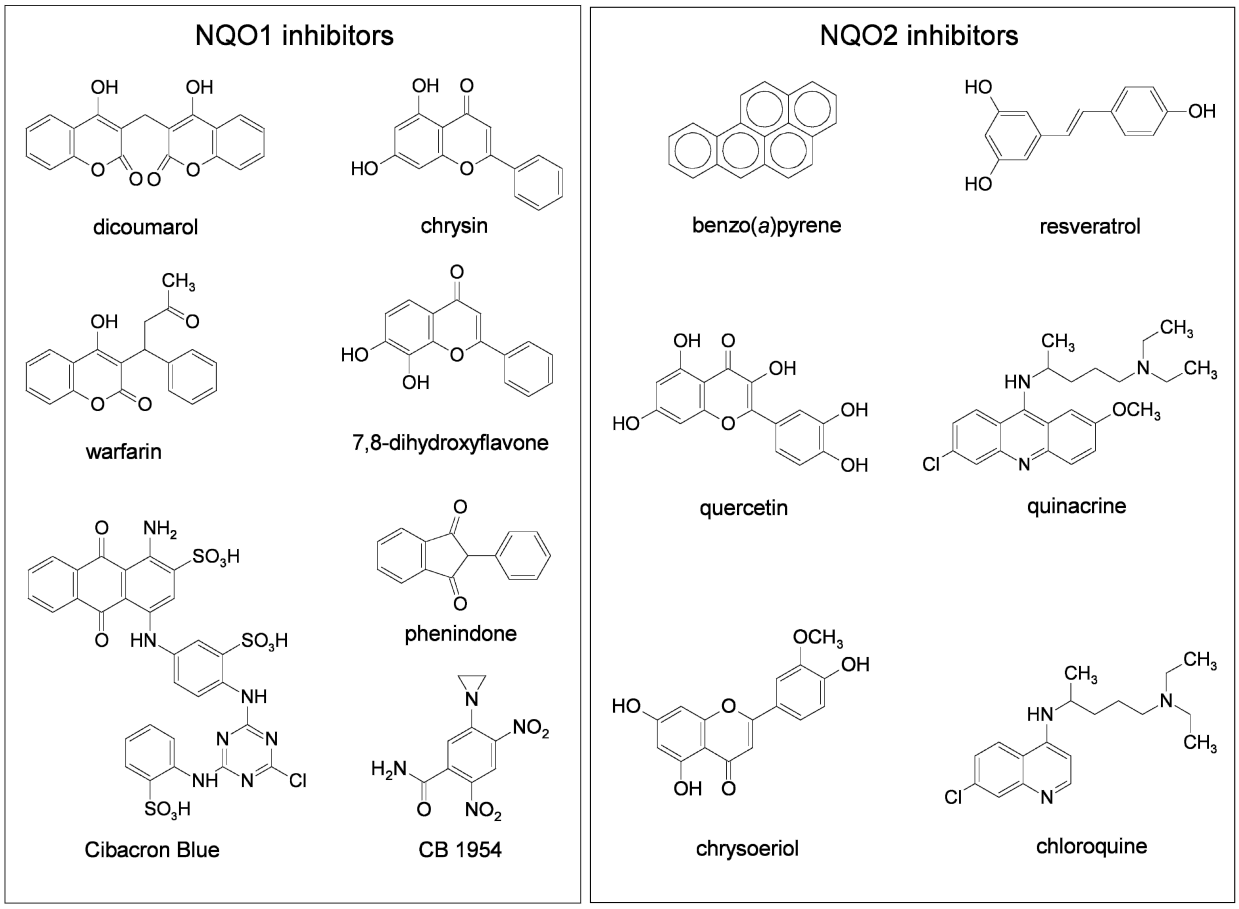


Scheme 3. Decolorization of Methyl Red. Flavin mediated electron transfer from NAD(P)H to the azodye is generally observed for oxidoreductases, such as quinone oxidoreductases, containing a flavin prosthetic group.

Inhibitors

Mammalian QRs, like human or rat NQO1, can function physiologically as one of several vitamin K reductases in the vitamin K cycling involved in the hepatic posttranslational modification of vitamin K hydroquinone-dependent blood coagulation factors [50]. Oral anticoagulants such as dicoumarol and warfarin have therefore been found to be potent competitive inhibitors with respect to nicotinamide coenzymes of QRs [51]. Results obtained from inhibition studies with dicoumarol indicate that the strong binding of dicoumarol ($K_I = 2$ nM and 0.5 nM for human and rat NQO1, respectively) can be explained by a π-π interaction of one ring with the isoalloxazine ring of the FAD cofactor and another π-π interaction of the second ring with the phenol ring of Tyr-128 [52]. These authors have also reported that Cibacron Blue (a triazine dye, $K_I = 500$ nM for human NQO1), chrysin ($K_I = 100$ nM), 7,8-dihydroxyflavone ($K_I = 30$ nM) and phenindone ($K_I = 500$ nM) are competitive inhibitors of NQO1 with respect to NAD(P)H [52]. Both dicoumarol and Cibacron Blue were also reported to be efficient inhibitors of yeast Lot6p, with K_I values of 40 nM and 16 μM, respectively [36].

NQO2, which is unable to use NAD(P)H as a source of electrons, is not inhibited by classical NQO1 inhibitors. NQO2 inhibitors reported in the literature comprise benzo(*a*)pyrene [53] and quercetin, a flavonoid once described as a tyrosine protein kinase inhibitor [54], both being competitive with respect to



Scheme 4. Inhibitors of quinone reductases. Structures of inhibitors of the NQO1 family are shown on the left, NQO2 inhibitors on the right.

the *N*-ribosyl derivative of dihydronicotinamide (NRH). Other flavone-like compounds (e.g. chrysoeriol) have been reported as inhibitors, though not with the same potency [55]. Moreover, the efficiency of resveratrol, a phytoalexin produced by several plants, was recently reported with a K_I of 35 nM [56]. Although this natural compound has been described as an inhibitor of several other enzymes as well, a nanomolar inhibition constant towards NQO2 seems to be unique. Finally, the anti-malaria drugs quinacrine and chloroquine were also reported as potent inhibitors of NQO2 in the 500 nM to 1 μM range [57]. Generally, it appears that inhibitors of the NQO1 family are cyclic ketones, whereas this is not a prerequisite for NQO2 inhibitors (Scheme 4). On the other hand, all of the inhibitor structures share an aromatic ring system that is obviously required for π stacking with the isoalloxazine ring moiety of the flavin cofactor.

Physiological role of quinone reductases

Quinones in nature

Quinones, particularly *p*-quinones, constitute an important class of ubiquitous and naturally occurring compounds. Quinones generated from polycyclic aromatic hydrocarbons are abundant in all burnt organic material, including urban air particles, automobile exhaust, cigarette smoke and many foodstuffs [58, 59]. Compounds containing a quinone nucleus are widely employed as anti-tumor agents. Quinones are highly reactive compounds that can undergo either one- or two-electron reductions. Enzymatic one-electron reduction, for example by cytochrome P450 reductases, generates extremely unstable semiquinone radicals which subsequently undergo redox cycling, leading to the production of highly reactive oxygen species (ROS) in the presence of molecular oxygen. These reactive compounds induce oxidative damage and, consequently, tissue degeneration, apoptotic cell death, premature aging, cellular transformation and neoplasia [60]. Strict two-electron reductions

of quinones and their derivatives by enzymes of the drug metabolism phase 2 prevent these deleterious effects. Instead, they produce more stable chemicals, hydroquinones, which can then be conjugated with glutathione or glucuronic acid and are rapidly excreted.

Putative role of QRs in detoxification

The functional importance of QRs has been a matter of debate since their discovery. The demonstration that a dicoumarol-inhibited vitamin K reductase described by Maerki and Martius [61] and the DT-diaphorase first described by Ernster and Navazio [3] is in fact the same enzyme only added to the speculations revolving around the functional role of QRs. Moreover, despite the demonstration that NQO1 is exquisitely sensitive to inhibition by dicoumarol and other anticoagulants, its participation in γ-glutamyl carboxylation reactions (that require the hydroquinone form of vitamin K which then undergoes oxidation to the quinone in the course of the reaction) involved in blood coagulation remains unclear. The view that mammalian QRs are primarily involved in xenobiotic metabolism and in averting toxicity and carcinogenicity of highly reactive compounds is a more recent development, last reviewed by Dinkova-Kostova and Talalay [62].

In the early 1960s, it was discovered that NQO1 was potently induced in the cytosol of rat hepatocytes as well as in other tissues after exposure to both azodyes and other polycyclic aromatic hydrocarbons [63]. This report described the close correlation between the potency of various azodyes in inducing NQO1 and their efficiency in protecting against the carcinogenicity and toxicity of hydrocarbons.

In contrast to potentially toxic azodyes and polycyclins, phenolic antioxidants that were already constituents of the human diet were also shown to substantially protect against a wide spectrum of chemical carcinogens, therefore suggesting a common underlying mechanism by which these antioxidants afford protection against toxicity and carcinogenicity [64]. Reports showing that the same phenolic antioxidants which blocked tumor formation also induced glutathione transferases and a number of other phase 2 enzymes in a range of animal tissues led to the suggestion that the coordinated induction of phase 2 enzymes was mainly responsible for the chemoprotective effects [65]. An explicit mechanism by which NQO1 might protect cells against quinone toxicity was provided by observations of Iyanagi and Yamazaki [37], who first distinguished between flavoenzymes which catalyze one-electron reductions and those, like NQO1, that catalyze strict two-electron reductions. Therefore, complete (two-electron) reduction of quinones can prevent (1) one-electron redox cycling that generates highly ROS, and (2) depletion of cellular glutathione by lowering the levels of quinones that easily react with thiol groups. Moreover, in contrast to semiquinone products generated by other flavoproteins that catalyze one-electron reductions, the hydroquinone products of the NQO1 reaction are not only more stable, but can also be further metabolized to glucuronide and sulfate conjugation products, thereby facilitating their excretion. Therefore, the possibility of forming very reactive semiquinone radicals, potential mediators of oxidative stress, is largely reduced [62].

Numerous observations in a variety of experimental systems have provided further support pointing towards the protective functions of NQO1. For example, menadione-induced superoxide production in hepatic postmitochondrial supernatant fractions was noticeably lower in mice fed a diet containing a QR inducer. Upon addition of dicoumarol and therefore inhibition of the enzyme, the protective effects of NQO1 were abolished [66]. Menadione treatment was also shown to lead to superoxide formation, depletion of intracellular glutathione and nicotinamide nucleotide pools, as well as alteration in Ca^{2+} homeostasis leading to cell surface disorganization [67].

Human erythrocytes, which do not express NQO1 in their mature form, were reported to be especially sensitive to quinone toxicity. Exposure to menadione leads to formation of methemoglobin. This effect can be largely reduced *in vitro* by introducing catalytically active NQO1 that is encapsulated into erythrocytes. *In vivo*, menadione administration is reported to cause hemolytic anemia and the accumulation of Heinz bodies (inclusions within red blood cells composed of denatured hemoglobin) [68].

Mice lacking expression of NQO1 and NQO2 protein were demonstrated to develop myelogenous hyperplasia of the bone marrow and to have an increased number of granulocytes in the peripheral blood. A decreased amount of apoptotic cells contributed to myelogenous hyperplasia. Studies on short-term exposure of NQO1–mice to benzene demonstrated substantially greater benzene-induced toxicity, compared to wild-type mice [69]. Recently, NQO1 was also found to be highly expressed in human adipose tissue, in particular in large adipocytes. Expression of NQO1 in adipose tissue was shown to be reduced during diet-induced weight loss and the expression levels correlate positively with adiposity, glucose tolerance and markers of liver dysfunction [70]. These findings would suggest an additional role for NQO1 in the development of human obesity.

In conclusion, these studies provide strong evidence that quinone metabolism is a rather complex process

and involves two major pathways: (1) one-electron reduction catalyzed by e.g. NADPH-cytochrome P450 reductase and (2) two-electron reductions catalyzed by flavin-dependent QRs such as NQO1. While reaction products of the first pathway are able to autooxidize readily and therefore participate in redox cycling, the hydroquinones of the latter pathway are further conjugated and excreted. Importantly, NQO1 inhibition by dicoumarol did not increase mutagenicity of quinone substrates, suggesting that the two-electron reduction is not involved in the mutagenic activation of quinones. Since the strict two-electron reduction mechanism of NQO1 leads to the formation of hydroquinones which are much more metabolically stable than semiquinones, QRs have been proposed to serve as a cellular control device against quinone toxicity [71].

Unlike the situation for NQO1, only a few studies have dealt with NQO2 gene expression induction, and these studies have arrived at contradictory conclusions. Because of the presence of an antioxidant response element in the NQO2 promoter region (similar to NQO1), it was suggested that NQO2 was coordinately induced with NQO1 and the other phase 2 enzymes. It has been shown that, like NQO1, NQO2 expression could be induced by β-naphthoflavone and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [72]. However, it was observed that TCDD treatment of human hepatoblastoma cells increased the level of mRNA of NQO1 but not NQO2, suggesting that NQO2 was not induced coordinately with NQO1 and other phase 2 detoxifying enzymes [73].

Several studies reported an association between 29-bp insertion/deletion polymorphisms of NQO2 with Parkinson's disease and schizophrenia [74, 75]. Depending on the DNA sequence of the promoter region, it was speculated that this polymorphism would result in decreased expression of the protein and may lead to an excess of the catecholamine-derived *o*-quinones in the brain. It has also been reported that the promoter containing the 29-bp insertion polymorphism exhibits lower NQO2 expression than that without. Indeed, the insertion was shown to introduce a binding site to the transcription factor Sp3, which in this case acts as a repressor [76]. As a consequence, the deletion associated with either Parkinson's disease or schizophrenia leads to enhanced expression of NQO2. This hypothesis has been confirmed using human fibroblasts from individuals with or without the deletion, showing that NQO2 activity was increased in fibroblasts with the deletion. It was concluded that higher NQO2 activity might make individuals more susceptible to Parkinson's disease. Similarly, NQO2 was reported to be down-regulated in hepatocellular and biliary tissues, while NQO1 was upregulated at the same time, suggesting not only a different regulation but also a different role in carcinogenesis [77].

Interestingly, the yeast QR Lot6p was also shown to play a role in managing oxidative stress by detoxifying cells from quinones. The oxidative stress resistance of wild-type, a *lot6* deletion and overexpressing strain was assayed by testing the ability of 1,4-naphthoquinone to reduce their growth rate. Deletion of Lot6p decreased the toxicity of 1,4-naphthoquinone considerably – only 53% of the cells were viable after 8 h, compared to 70% of the wild-type cells. Correspondingly, yeast cells overexpressing Lot6p were less susceptible to quinone stress, resulting in 84% relative viability after 8 h. The results demonstrated that the enzymatic activity of Lot6p is consistent with the phenotype of both *Δlot6* and Lot6p-overexpressing strains and that expression of Lot6p is undoubtedly important for managing oxidative stress caused by quinones [36].

Antioxidant functions of QRs

Red chemiluminescence (corresponding to the photoemission of singlet oxygen) can be detected when menadione is added to postmitochondrial mouse liver homogenates in the presence of both NADPH and oxygen. This chemiluminescence is thought to arise from the generation of ROS during the one-electron redox cycling reactions of menadione, and is considered to be an index of one of the major toxicities of menadione. Importantly, the intensity of chemiluminescence can be modulated (e.g. reduced upon treatment of the animals with NQO1 inducers, or intensified when dicoumarol was added). It was shown that addition of pure NQO1 to menadione-treated hepatic preparations reduced the low-level chemiluminescence in a dose-dependent manner [66]. Moreover, amounts of externally added NQO1 equal to the NQO1 levels present in the postmitochondrial fractions obtained from mice fed with NQO1 inducers resulted in identical chemiluminescence levels. Heat-inactivated enzyme did not show any effect, while dicoumarol increased the chemiluminescence. These experiments provide direct evidence that the light emission produced by oxidative cycling of menadione could be quantitatively quenched by the direct addition of pure crystalline NQO1.

There is another line of evidence that strongly indicates that in addition to the direct detoxification of quinones, NQO1 also plays a role in indirectly supporting the overall antioxidant functions of the cell. According to this proposal, NQO1 and similar enzymes could have evolved in order to act as coenzyme Q (ubiquinone) reductases, maintaining this naturally occurring quinone in its reduced state

and therefore protecting the membrane components of the cell from free radical damage [78]. It was shown that when NQO1 was incorporated into phospholipid vesicles, pure rat NQO1 was able to reduce coenzyme Q homologs of both short (e.g. CoQ_1, CoQ_3) and long (e.g. CoQ_9, CoQ_{10}) isoprenoid chain length, therefore affording protection against 2,2′-azobis(2,4-dimethylvaleronitrile)-induced lipid peroxidation. Additionally, the protection of intact hepatocytes against adriamycin-induced oxidative damage by reduced CoQ was abolished by addition of dicoumarol [79]. The demonstration that α-tocopherolquinone (which is a product of the oxidation of α-tocopherol) is a good substrate for human NQO1 to provide the powerful cellular antioxidant α-tocopherolhydroquinone [80] indicates a role for NQO1 in the metabolism of α-tocopherol and supports the involvement of this enzyme in reducing cellular oxidative stress. These experiments extend the protective function of NQO1 to the maintenance of two endogenous and powerful antioxidants, coenzyme Q and vitamin E, in their reduced and active forms.

A similar role of QRs has been reported for several prokaryotes, suggesting that these mechanisms have evolved very early and reflect a common strategy in handling oxidative stress. This conclusion is corroborated by the sequence similarity of QRs identified in various prokaryotic and eukaryotic species (see Fig. 1). Among the prokaryotic QRs, the WrbA family of proteins encompasses more than 100 members with detailed biochemical information being available for the enzymes from *E. coli* and the hyperthermophile *Archaeoglobus fulgidus*. Originally, WrbA was found to associate with the tryptophan repressor TrpR and therefore named accordingly [tryptophan(W)-repressor(r)-binding(b) protein A]. However, a specific role for WrbA in the TrpR-DNA interaction could not be demonstrated. On the other hand, *wrbA* transcription is controlled by the stress response gene *rpoS*, indicating a functional role in stress response. The protein is upregulated by various stressors, such as acids, salts, hydrogen peroxide and diauxie and repressed under anaerobic conditions. Thus, its physiological role can be seen in the oxidative stress response, although an additional function may involve cell signaling by reducing the cellular quinone pool which in turn leads to phosphorylation of ArcA, a signal redox sensor, and prompting a shift from respiratory to fermentative metabolism [18–20].

Other bacterial QRs have also been implicated in the oxidative stress response, like ChrR from *P. putida* [26] and MdaB from *Helicobacter pylori* [81, 82]. Since *H. pylori* adopts a microaerophilic lifestyle, QRs may play an essential role in coping with oxidative stress as compared to organisms that have adapted to an oxygen-rich environment. However, to ascertain the function and importance of bacterial QRs for distinct ecological niches, more biochemical and physiological studies are needed, before a firm conclusion can be drawn.

NQO1 as a component of the stress response: stabilization of p53

Studies with proteins typically considered as metabolic enzymes suggest that these proteins may play additional roles outside the range of their usual metabolic functions [83]. For example, glutathione-S-transferase was shown to associate with c-Jun N-terminal kinase leading to inhibition of kinase activity and modulation of signaling and cellular proliferation [84]. Similarly, recent studies have demonstrated that NQO1 may influence the stability of the tumor suppressor protein p53 by inhibiting its degradation [85].

The tumor suppressor gene *p53* encodes a labile protein, which accumulates in cells after different stress signals and can cause either growth arrest or apoptosis [for a review see ref. 86]. One of the p53 target genes, p53-inducible gene 3, encodes a protein with homology to oxidoreductases [87]. This finding has raised the possibility that oxidoreductases are regulated by p53.

In 2001, the relationship between NQO1 and p53 was first shown by experiments which provided evidence that inhibition of NQO1 activity by dicoumarol caused enhanced p53 proteasomal degradation which could be prevented by overexpression of NQO1 in human colon carcinoma cells [88]. The ability of NQO1 inhibition to enhance p53 degradation resulted in the reduction of p53 accumulation and suppression of p53-dependent apoptosis in γ-irradiated normal thymocytes and in myeloid leukemic cells that overexpress p53. The involvement of NQO1 in p53 accumulation suggests that redox reactions controlled by oxidoreductases such as NQO1 may therefore be an important factor in determining the p53 intracellular level, which of course may also have implications for tumor development and therapy. In this context, it is particularly interesting that NQO1 knock-out mice and a genetic polymorphism of NQO1 in humans that results in the loss of its oxidoreductase activity are associated with increased susceptibility to tumor development [89, 90].

Following this discovery, Asher and coworkers found that NQO1-mediated p53 stabilization was especially prominent under induction of oxidative stress [88]. Interestingly, only wild-type NQO1 but not the polymorphic NQO1 was shown to stabilize endogenous as well as transfected wild-type p53. NQO1 also partially inhibited p53 degradation mediated by the

human papilloma virus E6 protein but not when mediated by Mdm-2, the ubiquitin ligase protein that ubiquitinates p53 and targets it to the 26*S* proteasome. Experiments analyzing the mechanism of p53 degradation in the NQO1-regulated pathway indicate that NQO1 regulates degradation of p53 in the proteasomes by a mechanism that is independent of both Mdm-2 and ubiquitin [91].

NQO1 is also known to bind other proteins such as Hsp70 and Hsp40 [92]. Consequently, NQO1 was examined to see if it could interact with p53 via a protein-protein interaction [92]. It was demonstrated that ES936, a suicide inhibitor of NQO1 which irreversibly blocks the catalytic function of NQO1, has no effect on p53 stability. This result suggests that a redox mechanism of stabilization is unlikely. However, it was shown that NQO1 is indeed able to associate physically with p53 suggesting that a protein-protein interaction is responsible for the stabilization of p53 by NQO1, which might represent an additional mechanism that contributes to the chemoprotective activity of NQO1.

To further characterize this essential and highly regulated process, the role of NQO1 in proteasomal degradation was examined in more detail. Interestingly, it was found that the majority of NQO1 cofractionated with the 20*S* proteasomes (and not the 26*S* proteasomes which were excluded by previous ammonium sulfate precipitation), suggesting that the vast majority of cellular NQO1 is found in a large protein complex, which includes the 20*S* proteasome. Analysis of p53 distribution in gel filtration fractions showed that a portion of p53 cofractionated with 20*S* and NQO1, indicating the existence of a ternary complex of p53, NQO1 and the 20*S* proteasome. To examine the involvement of NQO1 in proteasomal degradation, *in vitro* degradation assays of p53 were performed in the absence or presence of *in vitro*-translated NQO1. Remarkably, p53 and p73α degradation by the 20*S* proteasome is inhibited in the presence of excess NQO1 and NADH, suggesting that NQO1 directly regulates the proteasomal degradation of these proteins. In contrast, 20*S* degradation of p73β, a p73 isoform lacking the C-terminal SAM domain region, was not affected by NQO1, indicating that this domain is required for direct binding to NQO1. Moreover, by performing *in vitro* binding assays in the presence of the NQO1 cofactors NAD^+, FAD or NADH, it could be shown that the binding of NQO1 to both p53 and p73α was increased in the presence of NADH in a dose-dependent manner. Tryptic digestion experiments suggested that NQO1 introduces a conformational change in p73α, which is augmented in the presence of NADH. However, the binding of p73α to NQO1 was decreased upon addition of dicoumarol, indicating that dicoumarol binding leads to dissociation of the preformed p73α-NQO1-complex. Taken together, these results provide evidence that NQO1 plays a role in p53 and p73α accumulation following ionizing radiation. Escaping Mdm2-mediated degradation may not be sufficient for efficient p53 stabilization following irradiation, since p53 is still susceptible to 20*S* proteasomal degradation. Therefore, it was assumed that in order to achieve efficient p53 accumulation following γ-irradiation, NQO1-p53 interaction is increased to eliminate p53 degradation by the 20*S* proteasome [93].

These findings shed new light on our understanding of p53 degradation and possibly protein degradation in general as reviewed recently [94]. A model has been proposed whereby some short-lived proteins (such as p53 and p73) are inherently unstable and are degraded 'by default' by the 20*S* proteasomes in cells, unless protected by a stabilizer such as NQO1, a mechanism distinct from the current 'modification to destabilization' mechanism that is mediated by poly-ubiquitination (Fig. 4).

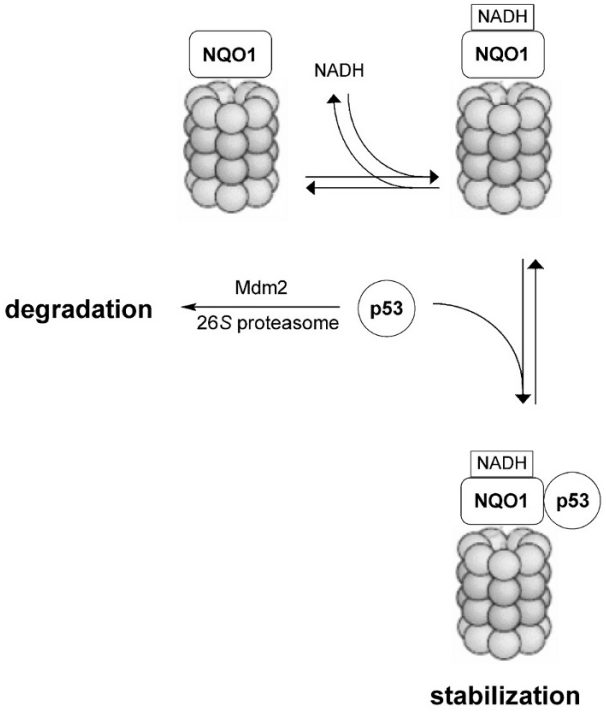


Figure 4. Proposed mechanism of p53 stabilization and degradation. The schematic representation shows both p53 accumulation and degradation by the 20*S* and 26*S* proteasome (see text for details). Adapted from Asher et al. [88] and Asher and Shaul [94].

Emerging role of QR in plants

Compared to the mammalian QRs, our current knowledge about plant QRs is very limited. In the 1990s, the occurrence of QR activity was reported

from the plasma membrane of various plant species [95–97]. These putative QRs were isolated from the root plasma membrane of maize (*Zea mays* L.) [95] and onion (*Allium cepa* L.) [96], designated NAD(P)H dehydrogenases I and II, and zucchini [97]. Although the enzymes isolated from onion root and zuccini plasma membrane exhibit some typical QR-like properties, such as utilizing quinones as electron acceptors, inhibition by dicoumarol and a molecular mass of around 27 kDa, their physiological role is unclear. Trost and coworkers [97] have suggested that the plasma membrane QRs may be involved in the two-electron reduction of ubiquinones thereby preventing oxidative damage of membrane components by ROS generated through oxidative semiquinone recycling.

A much clearer picture has emerged with regard to cytosolic QRs identified in the basidiomycete *Phanerochaete chrysosporium* [98] and several plant species [99–101]. The fungal enzyme is a dimer of 44 kDa, which binds one FMN per subunit. It has no preference for the electron-donating nicotine amide derivative and is oxidized by a range of quinones [98]. A kinetic analysis of the enzymatic reaction has revealed a typical ping-pong bi-bi mechanism to be in operation. In addition, dicoumarol and Cibacron Blue are competitive inhibitors of the nicotinamide nucleotide. With the exception of the coenzyme switch from FAD to FMN, this fungal enzyme possesses properties very similar to mammalian QRs. Owing to its life style as a wood-rotting fungus, it is postulated that the enzyme is involved in the reduction of lignin-, and possibly also pollutant-derived quinones and hence plays a role in lignin degradation [98].

On the other hand, the characterization of soluble plant QRs revealed some similarities as well as differences to the fungal enzyme. The plant enzymes from sugar beet [101], tobacco [100] and *Arabidopsis thaliana* [99] exist as tetramers in solution and are only weakly inhibited by dicoumarol. However, the plant enzymes share kinetic properties and the use of FMN rather than FAD with the fungal enzyme [99–101]. The physiological role of plant QRs appears to be the reduction of intracellular quinones followed by *O*-conjugation and removal of potentially harmful quinones in order to prevent their participation in redox reactions [99, 102]. In this context, an interesting role is proposed for a putative QR isolated from latex obtained from the rubber tree *Hevea brasiliensis* [103]. The isolated enzyme has an estimated molecular mass of 21 kDa and is a tetramer in solution, similar to the plant QRs mentioned above [97, 100, 101]. The function of this QR was suggested to be the two-electron reduction of quinones to the hydroquinones thereby avoiding the generation of ROS, which in turn contributes to the stabilization of latex [103].

Further insights into the role of QRs *in planta* were provided by analysis of differential display experiments using the model plant *A. thaliana* indicating that mRNA levels respond to auxin, a plant hormone [104]. The protein (termed FQR1) encoded by this auxin-responsive gene was described as an FMN-binding QR exhibiting sequence similarity to QR family members from plants, fungi and bacteria and, to a lesser degree, mammals. Although FQR1 was not subjected to any detailed biochemical and kinetic characterization, the enzyme contains FMN as its coenzyme, typical for a plant QR. Most interestingly, mRNA levels of FQR1 accumulate rapidly upon treatment with the plant hormone auxin and show one of the fastest responses observed for auxin-inducible genes [104]. The intriguing finding that plant QR activity is induced by auxin suggests that the enzyme is required to protect plant cells against the oxidative stress caused by the hormone, i.e. a similar role in protection against the toxic effects of redox cycling of semiquinones with the associated generation of ROS.

An entirely new role for plant QRs was recently discovered in the interaction of parasitic plants and their hosts during formation of haustoria, which enable the parasite to obtain access to host water and nutrient supplies. Quinones, such as 5-hydroxy-1,4-naphthoquinone (juglone) and 6-methyl-1,3,8-trihydroxyanthraquinone (emodin), are allelopathic compounds released by plants into the rhizosphere as a defensive measure against competitors for nutrients and light. Matvienko et al. [105] could demonstrate that 2,6-dimethoxybenzoquinone induces a quinone reductase in the parasite that converts the quinone to its semiquinone form by one-electron reduction leading to the generation of ROS which in turn are required for the development of the haustorium. The chemically induced assault on a potential host plant elicits the expression of a two-electron reducing QR that disables the generation of ROS through redox cycling of the semiquinone by reduction to the fully reduced hydroquinone. Interestingly, the expression of the one-electron reducing QR is accompanied by the expression of a two-electron reducing QR in the parasite, similar to the one expressed in the host plant [105]. Obviously, the parasite also needs to protect itself against the oxidative stress caused by the formation of semiquinones. In conclusion, exposure to allelopathic quinones leads to the induction of QRs in plants as part of a defensive strategy aiming at the detoxification of the rhizosphere. Further evidence for a role in pathogen defence is obtained from infection of wheat

(*Triticum monococcum*) by the fungus that causes powdery mildew infection [106]. In this case, infected cells expressed a protein homologous to the one found in the plant parasite host system described above. This 23-kDa protein shows sequence similarity to several other homologs of the plant QR family and is proposed to act as a detoxifying enzyme by removing intracellular quinones via two-electron reduction to the hydroquinone form which can then be removed from the noxious redox cycle by monoglycosylation, for example to their arbutin derivatives [102].

In summary, our general understanding of plant QRs is clearly unsatisfactory at the moment. At a molecular level, it appears that plant QRs utilize FMN and are organized in tetramers rather than dimers (for a comparison to bacterial, fungal and mammalian enzymes see Table 1). Despite these differences, the substantial sequence similarities suggest that plant QRs adopt a flavodoxin-like fold as found for mammalian, yeast and bacterial members. However, biochemical and structural studies are clearly needed to resolve these issues. Likewise, recent indications that plant QRs play major roles in plant defence are very promising but clearly need substantiation in order to understand the complex interspecies communication in plant-pathogen interactions.

Medical applications

Activation of prodrugs

The majority of the chemicals that are now used in cancer chemotherapy lack any intrinsic anti-tumor selectivity. Most of them act by an anti-proliferative mechanism, and their mechanism of action aims to disrupt stage-specific cellular processes occurring during cell division, rather than by a specific toxicity directed towards a particular type of cancer cell [107]. Thus, the therapeutic efficiency of most anti-cancer agents is limited by their toxicity to healthy host tissues that are the most rapidly dividing ones, such as bone marrow and the lymphatic system.

However, a higher selectivity might be obtained by the use of prodrugs, which are chemicals that are toxicologically and pharmacodynamically inert but are converted *in vivo* to active products. The conversion of the prodrug to the active molecule can be accomplished by several mechanisms, such as change of pH, oxygen tension, temperature or salt concentration, or by spontaneous decomposition of the drug and internal ring opening or cyclization [108]. The major approach in prodrug design for cancer therapy involves the synthesis of inert compounds, which are converted to an active drug by enzyme action. Therefore, in cancer chemotherapy, the inert prodrug is converted into a highly toxic compound *in vivo* by an enzyme present at high levels in cancer cells but not in other cells. For example, the enzyme β-glucuronidase was shown to selectively activate a relatively non-toxic alkylating agent to an extremely reactive and toxic metabolite in mouse models [109]. In this context, NQO1 appears to be a good target as a prodrug-activating enzyme since its activity is present in a variety of human tissues and cell lines. High NQO1 activity has been reported in human cell lines of breast, brain, colon, lung and liver origin [107]. There is a clear increase in the NQO1 level detected in solid human thyroid, adrenal, breast, ovarian, colon and cornea tumors [110–112]. Moreover, it was shown that NQO1 levels in bone marrow, a tissue highly sensitive to conventional cytotoxic chemotherapy, is low [113], directing toxicity away from tissues that are usually sensitive to convential chemotherapy. Therefore, NQO1 is being exploited as a target in the development of anti-cancer prodrugs.

Activation of nitro-compounds

NQO1 is also capable of reducing certain nitro-compounds aerobically, a property shared by other QRs from bacterial sources (see above). Although the nitroreductase activity of NQO1 is considerably weaker than its QR activity, it can be exploited for prodrug activation, increasing the potential of the enzyme as a prodrug activator. It is evident that the reduction of nitro-compounds can involve the formation of three reduction products corresponding to 2-, 4- or 6-electron reductions, thus resulting in nitroso, hydroxylamine or amine species. Nevertheless, there is one specific example in which aerobic nitroreduction by NQO1 leads to the formation of a very potent anti-tumor compound in rats, namely the dinitrobenzamide CB 1954 (5-[aziridin-1-yl]2,4-dinitrobenzamide; Scheme 4). Although structurally only a weak monofunctional alkylating agent toward nucleophiles, CB 1954 showed a dramatic and highly selective activity against the rat Walker 256 tumor and could actually cure it. Such selectivity was unprecedented for any monofunctional alkylating agent, and it was evident that this sensitivity of the Walker tumor towards CB 1954 points toward a unique biochemical feature [107]. The prospect that a human tumor could also be found that shared the sensitivity of the Walker tumor made the mechanism of action of CB 1954 the subject of ongoing interest for more than 30 years [114].

The reason why CB 1954 is so selective is that it is a prodrug that is enzymatically activated to form a bifunctional agent which is able to form DNA-DNA interstrand cross-links via its hydroxylamino groups. This nitroreductase activity occurs aerobically in the

presence of either NADH or NADPH and was shown to be executed by NQO1. The dose of CB 1954 needed to achieve the same degree of cytotoxicity was reported to be about 10000 times less in cells able to perform this conversion than in cells that cannot [44]. However, the human form of NQO1 metabolizes CB 1954 much less efficiently than rat NQO1. Thus, even cells that contain a high concentration of human NQO1 are insensitive to CB 1954 [44]. This catalytic difference between the two forms of NQO1 is mainly attributed to a single amino acid change at position 104 (tyrosine in the rat enzyme and glutamine in the human enzyme) [115]. Considering the proven success of CB 1954 in the rat system, it would be highly desirable to recreate its anti-tumor activity in humans. Aside from NQO1, there is an additional CB 1954-reducing activity in human tumor cells that is much greater than that attributable to NQO1, namely nitroreduction by NQO2 [116]. NQO2 in this context can be considered a human NRH-dependent nitroreductase, and NRH produces a dramatic increase in the cytotoxicity of CB 1954 in human tumor cell lines both *in vitro* and *in vivo*. NQO2 has been reported to be highly elevated in some human cancers, and this suggests that activation of CB 1954 could be achieved by administration of an NQO2 cosubstrate (i.e. NRH derivatives) in order to 'switch on' the enzyme. Although it may not be applicable to all tumor types, the activation of CB 1954 using endogenous NQO2 revives the concept of using CB 1954 as a simple chemotherapeutic agent.

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