Structure and mechanism of cytosolic quinone reductases

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Introduction

Quinones are ubiquitous dietary compounds. Reduction of quinones by one-electron reduction systems (cytochrome P450 reductases) results in the formation of reactive semiquinones that can be re-oxidized to quinones by molecular oxygen with the formation of superoxide radical ($O_2^{-\cdot}$). Superoxide can lead to the formation of other reactive oxygen species, including the highly mutagenic and carcinogenic hydroxyl radical. In addition, the presence of quinones and their one-electron reduction products can result in the depletion of reduced thiol species. Cytosolic quinone reductase (QR) 1 provides a pathway that obviates all these deleterious reactions. Reduction of quinones via the obligatory two-electron reduction carried out by QR results in the formation of hydroquinones that can be glucuronidated and readily excreted.

QR1 [NAD(P)H:(quinone acceptor) oxidoreductase; EC 1.6.99.2] is a widely distributed, predominantly cytosolic, FAD-containing flavoprotein that promotes the obligatory two-electron reduction of many quinones and quinoneimines. (The enzyme has also been referred to as menadione reductase, DT-diaphorase and vitamin-K reductase). It uses NADH or NADPH as reductants with equal facility and is potently inhibited by dicoumarol and similar anticoagulants. Furthermore, QR activity is elevated in many animal tissues and cell lines by addition of xenobiotics. The discovery, purification, molecular characteristics, induction and other properties of this enzyme have been presented previously [1,2].

The purification and properties of another mammalian cytosolic FAD-dependent flavoprotein was described in the early 1960's by Liao et al. [3]. This enzyme was able to catalyse the oxidation of reduced *N*-ribosyl- and *N*-alkylnicotinamides by menadione and other quinones. Recently, the determination of the sequence indicated that this enzyme is highly homologous with QR1 [4,5], leading to the conclusion that it is probably a quinone reductase, and was named QR2.

Both QR1 and QR2 are physiological dimers with two identical catalytic sites that operate independently. Although the monomer chain lengths of the two enzymes are significantly different (QR1, 274 residues; QR2, 231 residues), they share close to 50% amino acid sequence identity. Despite the high degree of sequence identity between QR1 and QR2, important differences are evident between the two enzymes. Notably, QR2 is unable to use the most common reductants NADH, NADPH and NMNH, but can use reduced *N*-ribosyl- and *N*-alkyl-nicotinamides as the source of reducing equivalents. In contrast, QR1 uses NADH and NADPH with almost identical kinetics.

In this article, we review the structures of QR1 and QR2, discuss their similarities and differences, and discuss the possible mechanism of the obligatory two-electron reduction reaction carried out by the enzymes.

Structure of QR1

Dimer structure

In the structure of QR1 (Figure 1), strong interactions are observed for monomers related by a crystallographic 2-fold axis: 265 interatomic distances are within 4 Å. These contacts include van der Waals interactions, hydrogen bonding and charge–charge interactions [6]. Among these interactions, 26 hydrogen bonds are observed with good geometry and distances shorter than 3.2 Å between donor and acceptor atoms. The main contacts between monomers occur between residues belonging to four main regions: several residues of loop L6 (residues 153–164) are in close contact with residues in the region 235–262 (α8 and the C-terminal loop); residues in loop L4 and α3 (104–113) of the two monomers contact each other, as do residues 42–52 (α6 and L2). The extensive contact between the monomers is in accordance with the high stability of the dimer. The catalytic and cofactor-binding sites occur close to the monomer–monomer interface.

Abbreviation used: QR, quinone reductase.
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Monomer structure

Each monomer contains two separate domains: a catalytic domain (residues 1–220) folded in a predominantly α/β structure, and a small 52-residue C-terminal domain (residues 221–273) [6]. The overall folding of the catalytic domain is reminiscent of that found in some flavoproteins: a twisted central parallel β-sheet surrounded on both sides by connecting helices. The catalytic domain comprises β-strands $\beta1$–$\beta7$, helices $\alpha1$–$\alpha7$ and connecting loops L1–L6. The active site is in a crevice outside the C-terminal ends of the β strands. This crevice is formed by two adjacent loop regions (L1 and L4) that connect strand 1 with helix 1 and strand 3 with helix 3 on opposite sides of the β sheet. The C-terminal domain, connected to the catalytic domain by loop L7, contains an antiparallel β-hairpin motif (strands $\beta8$ and $\beta9$) followed by one helix ($\alpha8$) and several loops. Surprisingly, the topology of the catalytic domain is not that found in other FAD-containing enzymes but resembles the topology of *Clostridium* flavodoxin [7], an FMN-containing protein. The similarity of the two folds exists in the absence of significant sequence identity. A likely explanation for this structural similarity can be found in the kinetics of QR1, which utilizes a Ping Pong mechanism, binding the flavin and only one other compound at the same time: either the NAD(P)H cofactor or the substrate. This more closely resembles the reactions of electron carriers like flavodoxin than those of flavoenzymes, which typically can bind both nicotinamide nucleotide and the electron acceptor at the same time. When the structures of QR1 and *Clostridium* flavodoxin are aligned, 80 α-carbons superimpose with an root mean square deviation of 1.8 Å between the two molecules. Within this portion of the structure, QR1 has an additional 40–45 residues comprising a β-α-β motif that connects strand 2 to helix 2, instead of the more direct connection found in flavodoxin. This additional region and the C-terminal domain play an important role in the formation of the dimer. In addition, these two domains participate in binding FAD, $NAD(P)^+$ and substrate.

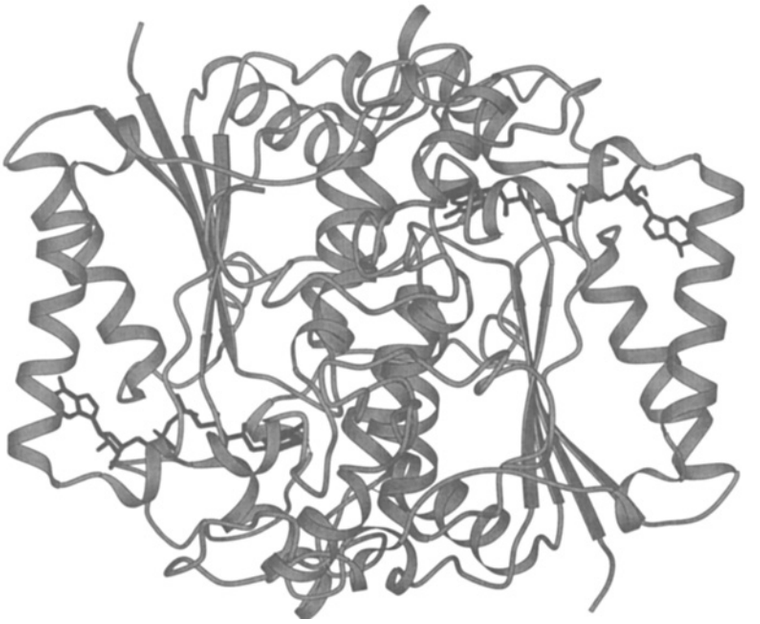
FAD-binding site

The isoalloxazine moiety interacts with residues in several loops: L1 and L4 of one monomer, and L3

Figure 1

Ribbon representation of the QR1 dimer viewed from the direction of the 2-fold axis

The two FAD, bound in the two binding sites at the dimer interface are shown (black structures).



of the other monomer. Aromatic residues Tyr^{104}, Trp^{105} and Phe^{106}, and the main chain and the side chain of Leu^{103} interact directly with the rings and appear to anchor the isoalloxazine moiety. The two oxygens of the flavin ring (O2F and O4F) form hydrogen bonds with the main-chain NH groups of Phe^{106} (O4) and Gly^{150}. The ring nitrogens also form hydrogen bonds with main-chain NH groups: N1F with Gly^{149} and N5F with Trp^{105}. In addition, Tyr^{155} makes two hydrogen bonds with the flavin: one with O2F, the other with N3F. Two residues from one monomer, Tyr^{104} and Trp^{105}, and four residues from the other monomer, Ile^{50}, Tyr^{67}, Pro^{68} and the main chain of Glu^{117}, form a pocket for the two methyl groups of the flavin. The ribitol interacts with residues of the loop connecting strand 3 and helix 3 and makes hydrogen bonds with the main-chain and side-chain groups. The FAD diphosphate is positioned at the N-terminus of helix 1 and in close proximity to the loop connecting strand 1 to helix 1. The two phosphates make several specific contacts with groups in the protein. The ribose is bound by residues from helix 1 and by the loop connecting strand 1 to helix 1. The adenine ring of the FAD lies between helix 5 and helix 1 in a hydrophobic pocket formed by the methylene groups of Arg^{200}, Phe^{16}, Ala^{20} and Leu^{204}.

NADP$^+$-binding site

$NADP^+$ has fewer interactions with the protein than does FAD. The nicotinamide ring is present in the complex in two alternative conformations related by a 180° rotation around the N1–C4 axis; that is, they differ only in the position of the carboxyamide moiety (occupancies of 60 and 40%). In both conformations, the nicotinamide ring and ring C of the dimethylisoalloxazine participate in a stacking interaction. In fact, stacking interactions in this region occur between three groups: the flavin ring, the nicotinamide ring of $NADP^+$ and the side chain of Phe^{178}. The average distance between the planes of two of the rings is approximately 3.4 Å. The binding site for $NADP^+$ involves not only residues of the same monomer that binds FAD, but also residues from the other subunit of the dimer. Facing the nicotinamide ring of $NADP^+$ are five aromatic residues from the other monomer (mostly from loop L5) either pointing at, or stacking with, the nicotinamide ring. (These include the side chain of Phe^{178} mentioned above). In addition, in the major conformation, the OH groups of Tyr^{126} and Tyr^{128} in loop L5 make two hydrogen bonds to the carbonyl group of the nicotinamide (O7N). Additional contacts and hydrogen bonds stabilize the position of the ribose and the phosphates. The AMP moiety interacts mainly with the hairpin loop formed by strands 8 and 9 of the other monomer: the ribose makes contacts with Phe^{232} and Phe^{236}, and the adenine moiety with the main chain of residues in the loop. The sequence TTGGSGS (147–153), which had been suggested to be part of the NADH-binding site [8–11], is packed very closely with both cofactors. As in other nucleotide-binding proteins containing this consensus sequence, the presence of the two glycines appears to be necessary to allow a close approach between main chain and cofactors.

Duroquinone and Cibacron Blue sites

The structure of the complex of QR1 with a substrate, 2,3,4,6-tetramethyl-1,4-benzoquinone (duroquinone), was determined in the presence of the potent QR1 inhibitor Cibacron Blue. In the complex, duroquinone (substrate) occupies a position very similar to that of the nicotinamide ring of $NADP^+$ in the nicotinamide complex. It stacks with the isoalloxazine ring and one of its oxygens forms hydrogen bonds with the OH groups of Tyr^{126} and Tyr^{128}. The Cibacron Blue overlaps the position occupied by the $NADP^+$ (the ribose, phosphate and adenosyl group) but does not interfere with quinone binding. The AMP moiety of $NADP^+$ and the Cibacron Blue interact very similarly with the enzyme, in agreement with the ability of the dye to bind to proteins with nucleotide-binding sites. Three of the ring systems of Cibacron Blue (B, C and D) play important roles in mimicking AMP binding. Overall, Cibacron Blue has stronger interactions with the protein than does $NADP^+$. However, no specific interactions exist between the protein and ring A that points in an opposite direction with respect to the nicotinamide ring of $NADP^+$. The observed binding provides a rationale for the pattern of inhibition of QR by Cibacron Blue: it is competitive with respect to NADH and non-competitive with respect to quinone [8,12].

Mechanisms of quinone reduction

The non-enzymic reduction of benzoquinones by NADH was studied by Carlson and Miller [13], who concluded that the reaction most probably occurs through a direct hydride transfer. Since the non-enzymic reduction appears to involve a direct hydride transfer, it is puzzling why QR absolutely

requires a flavin cofactor for catalytic activity [14]. In contrast to other flavoproteins capable of reducing quinones, QR does not show a detectable EPR signal when incubated in the presence of *p*-benzoquinone and NAD(P)H, indicating that in QR semiquinones are not released into the solution [15,16]. Furthermore, upon reduction of the QR FAD by NADPH under anaerobic conditions, the FAD absorbances at 370 and 450 nm disappear without the appearance of a red-shifted absorbance in the visible spectrum [8]. The absence of increased absorbance beyond 500 nm after FAD reduction argues against the presence of a stable semiquinone form of FAD; the existence of an FAD-disulphide redox-relay system like that observed in glutathione reductase and related enzymes [17] can also be completely ruled out by the absence of cysteine residues in the proximity of the FAD in the structure of QR1.

The structure determination revealed that the nicotinamide is bound in two alternative conformations of slightly different occupancies related by a rotation around the N1–C4 axis. In one position, the more occupied, the carboxyamide moiety is hydrogen-bonded to the OH groups of Tyr^{126} and Tyr^{128}. Both positions of the nicotinamide found in the complex are suited for a direct hydride transfer from NAD(P)H to FAD in the first half of the reaction cycle: in both, C4 of the nicotinamide is 3.8–4.0 Å from the N5 of flavin. Since it was shown [18,19] that the hydride transfer from NADH and NADPH by rat liver QR occurs with 4R (A-side) stereospecificity, the transfer must occur when the nicotinamide is in the alternative less-occupied conformation. As must certainly be the case in flavodoxin, in QR both the electron donor and the electron acceptor interact with the *si*-face of the flavin N5. This is consistent with the Ping Pong kinetics of QR but differs from the specificity observed in other flavoenzymes [20].

In the reaction pathway, the hydride transfer from the nicotinamide to the flavin involves a charge separation that creates two unfavourable situations: the generation of a positive charge in the nicotinamide and the generation of a negative charge in the flavin. Although these two charges can remain in close spatial proximity while the $NAD(P)^+$ remains bound, the negative charge on the reduced flavin will be uncompensated when the $NAD(P)^+$ leaves the binding site to allow binding of the quinone substrate. As mentioned above, the N1F of the FAD is hydrogen-bonded to the NH of Gly^{149}. In some flavin enzymes, it is assumed that this interaction is sufficient to stabilize a negative charge at N1F. In QR, this position occurs well buried in the protein and it is unlikely that the interaction with a main-chain NH would be enough to stabilize the charge. Since there are no other groups that can donate a proton to compensate a charge if it develops in N1F (as commonly proposed in other systems), the most likely tautomer for the reduced flavin in QR is the enolic form with the negative charge in O2F. Because O2F is already the acceptor atom of a hydrogen bond with Tyr^{155}, this tautomer can receive the proton from the OH of Tyr^{155}, which can, in turn, be stabilized by the positive charge of (or the transfer of a proton from) His^{161}. The imidazole of His^{161} is close to the nicotinamide, so the net effect of this step (in addition to the transfer of the hydride from the nicotinamide to the isoalloxazine) is the movement of a positive charge over a very short distance from the imidazole ring of His^{161} to the nicotinamide. The $NAD(P)^+$ can then leave the protein and the imidazole of His^{161}, which is exposed to the solvent, can reprotonate readily.

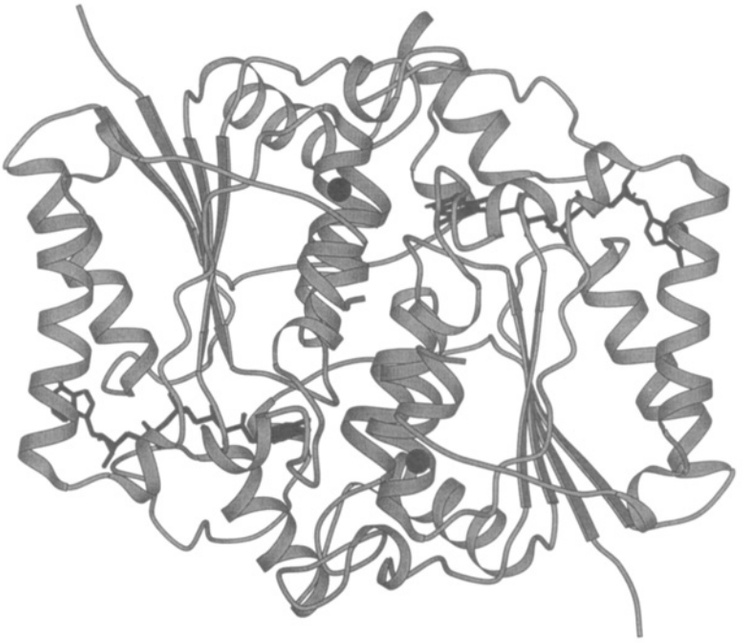
The reduction of a quinone by the reduced form of the enzyme can occur via a simple reversal of the steps just described for the reduction of the enzyme by NADH. This is made possible by the fact that the quinone and the nicotinamide ring share the same binding site. Also, binding of substrate cannot occur until $NAD(P)^+$ is released, providing a simple rationale for the Ping Pong mechanism of the enzyme. After nucleotide release, the quinone binds to the site freed by the nicotinamide in an orientation ideally suited to accept a hydride from the reduced $FADH_2$. Transfer of the hydride results in a singly ionized hydroquinone (hydroquinolate), and the quinoid form of the isoalloxazine. The proton on O2F is transferred back to the OH of Tyr^{155} and the imidazole ring of His^{161} becomes fully protonated again and can either transfer a proton to the hydroquinolate or simply stabilize its negative charge. In addition to the hydride transfer, the result of this second half of the reaction is the transfer of a proton from O2F of the flavin (accepted in the first half of the reaction) to the hydroquinolate.

Hydride transfer from $FADH_2$ to quinone can take the form of one of three alternative additions: a direct transfer to oxygen of the quinone, a Michael addition to a non-carbonyl carbon, or a transfer to the carbonyl carbon with a subsequent rearrangement. Discrimination be-

Figure 2

Ribbon representation of the QR2 dimer viewed from the direction of the 2-fold axis

The overall orientation is the same as that of QR1 in Figure 1. The two FAD, bound in the two binding sites at the dimer interface are shown (black structures). The position of the metal binding sites are also shown (black circles).



tween these different possibilities will require further experimentation.

Structure of QR2

Description of the structure and comparison with QR1

QR2 (230 residues) contains the complete N-terminal catalytic domain described in QR1 (up to residue 220), but lacks the C-terminal domain in its entirety (Figure 2 and [21]). The last 10 residues, 221–230, form a loop that has neither sequence nor structural homology with the corresponding residues in QR1. Unexpectedly, QR2 has a metal ion that is co-ordinated by four protein groups: Sγ and the carbonyl oxygen of Cys^{222}, and the Nδ atoms of His^{173} and His^{177}. The nature of the endogenous metal has not yet been determined, but the bacterially expressed recombinant enzyme contains Zn^{2+} at this site. We consider it possible that the metal is copper under physiological conditions. The location of the site on the protein surface makes it unlikely that the metal might have a structural role. Furthermore, the structure of the site is similar to those found in copper enzymes competent in electron-transfer reactions. It is intriguing that the metal site appears to be connected to the flavin by an efficient electron-transfer pathway.

The FAD-binding site and the catalytic site of QR2 are very similar to those of QR1. The majority of the residues involved in FAD binding as well as those involved in catalysis are conserved between the two enzymes. The major difference is present in the region of QR1 that binds the adenosine portion of NADH. This region involves residues of the C-terminal domain of QR1 that are not present in QR2. Thus QR2 has no NADH-binding site, in agreement with its reductant specificity. Regardless of this change in reductant specificity, the conservation of the other catalytic-site residues suggests that QR1 and QR2 operate with the same enzymic mechanism.

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