NQO1: a target for the treatment of cancer and neurological diseases, and a model to understand loss of function disease mechanisms

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Abstract

NAD(P)H quinone oxidoreductase 1 (NQO1) is a multi-functional protein that catalyses the reduction of quinones (and other molecules), thus playing roles in xenobiotic detoxification and redox balance, and also has roles in stabilising apoptosis regulators such as p53. The structure and enzymology of NQO1 is well-characterised, showing a substituted enzyme mechanism in which NAD(P)H binds first and reduces an FAD cofactor in the active site, assisted by a charge relay system involving Tyr-155 and His-161. Protein dynamics play important role in physio-pathological aspects of this protein. NQO1 is a good target to treat cancer due to its overexpression in cancer cells. A polymorphic form of NQO1 (p.P187S) is associated with increased cancer risk and certain neurological disorders (such as multiple sclerosis and Alzheimer´s disease), possibly due to its roles in the antioxidant defence. p.P187S has greatly reduced FAD affinity and stability, due to destabilization of the flavin binding site and the C-terminal domain, which leading to reduced activity and enhanced degradation. Suppressor mutations partially restore the activity of p.P187S by local stabilization of these regions, and showing long-range allosteric communication within the protein. Consequently, the correction of NQO1 misfolding by pharmacological chaperones is a viable strategy, which may be useful to treat cancer and some neurological conditions, targeting structural spots linked to specific disease-mechanisms. Thus, NQO1 emerges as a good model to investigate loss of function mechanisms in genetic diseases as well as to improve strategies to discriminate between neutral and pathogenic variants in genome-wide sequencing studies.

Keywords: quinone oxidoreductase; multiple sclerosis; Alzheimer's disease; protein misfolding; antioxidant enzyme; pharmacological chaperone

1. Introduction

Water soluble quinone oxidoreductases are widely distributed in all Kingdoms of life [1]. At least two are known to occur in humans – NAD(P)H quinone oxidoreductase 1 (NQO1; EC 1.6.5.2) and NRH quinone oxidoreductase 2 (NQO2; EC 1.10.5.1) [2]. The soluble, cytoplasmic quinone oxidoreductases are distinct from, and structurally different to, membrane bound enzymes which catalyse similar reactions. The best characterised group of the transmembrane quinone oxidoreductases participate in oxidative phosphorylation and other electron transport processes. These include mitochondrial complex I (NADH:quinone oxidoreductase; EC 7.1.1.2) and complex II ($FADH_2$:quinone oxidoreductase or succinate-conenzyme Q reductase; EC 1.3.5.1) [3, 4]. In addition, there is a mitochondrial, membrane-bound sulphide quinone oxidoreductase (EC 1.8.5.8) which is involved in hydrogen sulphide metabolism [5]. NQO1 catalyses the two-electron reduction of quinones to hydroquinones. This avoids the production of reactive semiquinone intermediates [6]. The precise *in vivo* role(s) of this reaction is/are unknown. Furthermore, NQO1 is a relatively promiscuous enzyme and is capable of catalysing the reduction of a wide range of compounds, including quinones and their derivatives, aromatic nitrogen compounds, iron (III) salts and superoxide radicals [7-13]. Likely *in vivo* substrates include coenzyme Q_{10} (ubiquinone) and the oxidised form of vitamin K [14-16]. However, the enzyme's role in the blood clotting cycle appears to be minor [17, 18]. Importantly, NQO1 is a stress-inducible protein by activation of the Nrf2 or Ah pathways, likely as a protective mechanism of the cell against stress [16, 19, 20]. The roles of NQO1 and the effects of its up- and down-regulation are summarised in Table 1.

2. Structure and Enzymology of NQO1

Human NQO1 is dimer of two identical 31 kDa subunits (Figure 1). The two active sites are located at the interface between the subunits and are formed from residues from both polypeptide chains [21]. Each dimer has two FAD molecules bound, one in each active site. It was generally considered that these cofactors were "tightly bound", although some early studies showed stoichiometries of less than 2 FAD:NQO1 dimer [22]. Recent work has

shown than FAD binding is slightly cooperative (*h*=0.84) meaning that, in effect while one cofactor molecule binds tightly, the second binds with lower affinity [23].

It is generally accepted that NQO1 has a substituted enzyme (or "ping-pong") mechanism. NADH or NADPH can both function as the reducing agent and do so with almost equal catalytic efficiency [24]. In the first stage of the reaction, NAD(P)H reduces an FAD molecule in one NQO1's two active sites. $NAD(P)^+$ then exits the active site, before the second substrate enters and is reduced by $FADH_2$. Thus, the two substrates never occupy the active site at the same time and never physically interact in the reaction cycle. Redox titrations demonstrated that there is a two electron transfer, and no semiquinone intermediate. The redox potential of the FAD/$FADH_2$ pair was approximately -160 mV at 25 °C and pH 7 [25]. Evidence for the substituted enzyme mechanism comes from detailed, classical enzyme kinetic studies. These demonstrated that NADPH alone could reduce the FAD cofactor in NQO1 (shown by disappearance of the characteristic yellow colour of the enzyme) [26]. The two half reactions both followed second order kinetics, with rate constants of approximately 10^9 $M^{-1}s^{-1}$ for the reduction of FAD by NAD(P)H and in the range 10^5 to 10^6 $M^{-1}s^{-1}$ for reduction of quinone substrates by $FADH_2$ [25].

Furthermore, Lineweaver-Burke plots of reciprocal rate against reciprocal concentration of NADPH at different concentrations of vitamin K_3 or pyrroloquinoline quinone were parallel, a characteristic diagnostic feature of this mechanism [25, 26]. The deviation from this at higher substrate concentrations of vitamin K_3 may be due to substrate inhibition (as has been observed in NQO2) [27].

The chemical mechanism of reduction by NQO1 has not been fully explored either experimentally or computationally. However, the availability of crystal structures with a variety of ligands bound, enable some rational speculation (Figure 2). It has been suggested that the first step in the reaction (reduction of FAD by NAD(P)H) occurs by direct hydride transfer between the two redox cofactors in the active site. The most likely tautomer of the resulting $FADH_2$ is the enolic form with the negative charge on O2F. This atom forms a hydrogen bond with the phenolic OH of Tyr-155, which can donate a proton to the $FADH_2$. The resulting negative charge on Tyr-155 is neutralised by transfer of a proton from His-161. Thus, there is a net transfer of one positive charge from His-161 to $NAD(P)^+$ [21]. In the second part of the reaction (reduction of the quinone), this process is reversed. The proton

on O2F is transferred back to the OH of Tyr-155 and the imidazole ring of His-161 becomes fully protonated again. The result of this is the transfer of the proton from O2F, promoting the transfer of a hydride from N5F of $FADH_2$ to the quinone. It is believed that the charge relay system formed by Tyr-155 and His-161 allows the reaction to take place without unfavourable charge separations [28]. Alteration of Tyr-155 to phenylalanine reduces activity to around 35% of wild-type [29, 30]. That this change does not abolish activity suggests that this residue is important in, but not critical to, catalysis and that other reaction pathways are possible. Similarly, alteration of His-161 to glutamine does not abolish activity [30]. The charge relay system assists catalysis, but it may be that direct reduction of the FAD cofactor and quinone substrate can occur without it, albeit less efficiently. Alternatively, it may be that other residues can assist with charge separation. It should be noted that there are two other tyrosine residues in the vicinity of the active site (Tyr-126 and Tyr-128) and it is possible that they could have a minor role in catalysis.

The best characterised inhibitor of NQO1 is the *bis*coumarin, dicoumarol (Figure 2). This compound was discovered in the 1940s after cattle eating partially fermented clover died as a result of haemorrhages. These were caused by the presence of dicoumarol in the feed which antagonised the blood clotting process [31, 32]. Dicoumarol is a competitive inhibitor which binds in the active sites of NQO1, directly blocking access by NAD(P)H [33]. Estimates for the inhibition constant (K_i) vary from 50 pM (from inhibition studies) to 120 nM (from isothermal titration) [26, 34]. Kinetically, it also has the key features of a competitive inhibitor, increasing the apparent Michaelis constant, with no effect on the maximum rate [22, 24]. Interestingly, dicoumarol causes a substantial decrease in the redox potential of the FAD/$FADH_2$ pair to approximately -230 mV at 25 °C and pH 7. This suggests that the inhibitor not only sterically hinders access t by NAD(P)H, but also affects the redox chemistry of the active site, potentially by altering its polarity [25, 35]. The widely used anticoagulant warfarin also inhibits NQO1 (K_i approximately 8 µM [36]), although vitamin K oxidoreductase is its pharmacologically important target (Figure 2) [18, 37]. Curcumin, a naturally occurring diarylheptanoid found in turmeric, also inhibits NQO1 (IC_{50} approximately 5 µM), although it is not clear how important this property in the compound's pharmacological roles (Figure 2) [38]. Early reports suggested that rat NQO1

exhibits negative cooperativity towards dicoumarol [22, 39]. Although this finding has not been verified in human NQO1, yeast Lot6p and human NQO2 demonstrate negative cooperativity towards the inhibitor resveratrol [40, 41]. The recent discovery of negative cooperativity of human NQO1 in FAD binding suggests a possible underlying cause given that these inhibitors bind by stacking over the isoalloxazine ring of FAD [23, 33].

3. NQO1 in cancer

The roles of NQO1 in cancer are well-established and well documented in the literature. It has been observed that many cancer cells have increased levels of NQO1 [42]. Thus, the inhibition of NQO1 has been proposed as a possible therapy for a wide range of different types of cancer. Experimentally, dicoumarol has been shown to kill pancreatic cancer cells in a mechanism linked to its inhibition of NQO1 and consequent rise in cellular levels of superoxide radicals [43, 44]. Dicoumarol is not an ideal anticancer agent since it has a number of other physiological effects, notably antagonism of the blood clotting cycle through inhibition of vitamin K oxidoreductase (VKOR, VKORC1, EC 1.1.4.1) and uncoupling of the mitochondrial proton gradient [32, 45-47]. Therefore, considerable efforts have been made to identify molecules which are effective NQO1 inhibitors, but lack these additional activities [48-57]. To date, no NQO1 inhibitors are used in anti-cancer therapy. A number of potential anticancer agents are activated by reduction catalysed by NQO1, including EO9 (apaziquone; 3-hydroxymethyl-5-aziridinyl-1-methyl-2-(*H*-indole-4,7-indione)-propenol; Figure 3) [58, 59]. This compound is converted to a highly reactive form *in vivo* which then damages DNA and other biomolecules [59]. The compound initially showed considerable promise as an anticancer agent, but failed in clinical trials due to poor pharmacokinetics when administered intravenously [60]. More recent work has shown that EO9 may be effective if administered directly to the affected tissues [61].

Interestingly, a lack of NQO1 activity is linked to an increased risk of cancer. A naturally occurring polymorphic form of the gene (*C609T*; NQO1*2; rs1800566) encodes a variant of the protein in which Pro-187 is substituted for serine (p.P187S; Figure 1). This single amino acid change reduces the FAD affinity and stability of the protein, resulting in a substantial reduction in affinity (see Section 5.1, below). This loss of activity has been linked to

increased lifetime probabilities of developing cancer in a number of tissues [62]. This polymorphism is common, with roughly one quarter of the human population having at least one *C609T* allele [63]. This is an unusually high rate for a deleterious polymorphism since these are typically selected against. The reason for its high incidence is not known. It can be speculated that the polymorphism confers some advantage in the heterozygous form (similar to the protection against malaria resulting from some mutations associated with sickle cell anaemia [64]). Alternatively, since the effects of the polymorphism will most likely be seen in later life, they are unlikely to be prevent individuals with them from having children. This would result in minimal selective pressure against the allele.

The increased risk is particularly high when combined with other risk factors such as smoking or exposure to benzene [65-68]. Loss of NQO1 activity is most likely linked to cancer risk due to reduced antioxidant activity in the cell and the increased likelihood of one electron reduction of quinones to semiqunones which can attack DNA, proteins and lipids [69]. The loss of functional, folded NQO1 protein is likely to have a second effect. NQO1 binds to, and stabilises, the tumour suppressor proteins p53 and p73 [70-73]. Loss of these interactions may prevent apoptosis occurring in damaged and potentially cancerous cells [74].

4. NQO1 in nervous system dysfunction

The role, function and mechanism of NQO1 in nervous system dysfunction or diseases has not been as well studied as its role in cancer. As a key anti-oxidative enzyme, NQO1 is likely to play a role in the brain, given the potential for oxidative stress in this organ. This results, in part, from the brain's high oxygen consumption, along with the presence of high levels of polyunsaturated fatty acids, transition metals and low levels of antioxidant enzymes [75]. In adult rats, NQO1 is located in a subset of oligodendrocytes and in the Bergman glia in the cerebellum [76]. It has also been found in rodent mesencephalon astroglial cells, and in rodent dopaminergic neurons, as well as in healthy human substantia nigra neurons that are under extensive oxidative stress [77-79]. NQO1 knockout mice display behavioural symptoms, including seizures [76].

Overexpression of NQO1 in human neuroblastoma cells (which are frequently used as a model of neurons) leads to increased resistance to cell death in response to 2-deoxyglucose, potassium cyanide, and lactacystin, but not hydrogen peroxide and serum withdrawal [80, 81]. However, NQO1 depletion by RNAi increased vulnerability of these cells to the high hydrogen peroxide concentrations (>150 μM) [81]. Overexpression of NQO1 in these types of cells has additionally been found to enhance mitochondrial activity and resistance to the mitochondrial toxins rotenone and antimycin A [82]. Caffeinated and decaffeinated coffee, and chlorogenic acid induced NQO1 in a primary neuronal cell culture, and was found to be at least partly involved in the neuroprotection against hydrogen peroxide exposure, in contrast to some other published data [83]. Sulforaphane induced NQO1 in a mouse immature hippocampal neuronal cell line and was found to play a part in protection against oxygen-glucose deprivation-induced cell death [84]. NQO1 levels are also increased by isoflavone exposure in rat primary astrocytes, aminochrome-induced toxicity in a human astrocytoma cell line and β-Lapachone in rat primary astrocytes. In each case, NQO1 reduced the build-up of ROS and prevented cell death. These effects could be reversed by dicoumarol or by knock-down of NQO1 [85, 86]. The anti-inflammatory effects β-Lapachone in microglia in response to LPS-stimulation may be mediated through induction of NQO1 [87].

NQO1 maintains dopamine metabolites in their reduced state, enabling their subsequent detoxification by sulphation or glucuronidation (Figure 4) [88]. NQO1 may also play a catabolic role for serotonin. In rats, NQO1 inhibition by dicoumarol led to an increase in 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA), and when administered in combination with a MAO inhibitor (L-deprenyl), led an increase in dopamine levels [89]. Thus, a picture emerges in which NQO1 is one of a number of key enzymes in neuronal cells which are responsible for controlling oxidative stress and combatting the effects of some toxins. Therefore, it seems reasonable to hypothesise that its levels may be increased in diseases which are associated with increased oxidative stress and that polymorphic forms which reduce its activity (e.g. p.P187S) may be associated with increased risks of these diseases.

4.1 Parkinson's Disease (PD)

Post-mortem tissue of humans with a diagnosis of PD have increased expression of NQO1 in astroglial and endothelial cells as well as dopaminergic neurons, especially in the substantia nigra pars compacta (SNpc) which is the area of the brain most implicated in the disease [90]. Genetic association studies with humans have revealed that the risk of PD was not significantly associated with the *C609T* NQO1 variant [91, 92]. Nevertheless, it appears that NQO1 plays a role in the neuroprotection of the dopaminergic system. Aminochrome only lead to neurotoxicity with the suppression of NQO1, manifesting itself with contralateral rotations and loss of dopaminergic neurons in rats [93]. Aminochrome toxicity was potentiated by NQO1 inhibition in a rat substantia nigra cell line. Similarly, aminochrome-induced disruption of actin and α- and β-tubulin was enhanced in the presence of dicoumarol [94, 95]. Dopaminergic cell lines with silenced expression of NQO1 show lysosomal dysfunction and increase in cell death in response to aminochrome [96, 97]. Interestingly, D-dopa, the main treatment for PD, is known to induce oxidative stress, and administration of this drug to cultured rat astroglia upregulated NQO1 levels [98].

In the manganese(III) PD model in rats, neurotoxicity was potentiated by dicoumarol [99]. A decrease in NQO1 expression in the cerebral cortex and striatum has been observed in the rotenone model of PD, which was reversed through the administration of sulforaphane [100]. A novel compound (KMS04014, a dimeric derivative of ferulic acid) was found to induce NQO1 gene expression and protect dopaminergic neuronal cells in a dopaminergic neuronal cell line against oxidative stress generated by MPP^+ (tetrahydrobiopterin, 1-methyl-4-phenylpyridinium) and hydrogen peroxide [101]. In 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, a prodrug for MPP^+) treated mice (another animal model of PD), KMS04014 reduced the loss of tyrosine hydroxylase-immunopositive dopaminergic neurons in the substantia nigra and reduced the degeneration of the nigral neurons and striatal fibres [101]. Human neuroblastoma cell lines transfected with NQO1 were more resistant to dopamine toxicity [102]. A chemoprotectant (3*H*-1,2-dithiole-3-thione; D3T) was found to induce NQO1 and increase resistance to oxidative and electrophilic neurotoxicity in response to dopamine, 4-hydroxy-2-nonenal (HNE; an unsaturated aldehyde involved in the pathogenesis of PD) and hydrogen peroxide [103]. β-Phenethylamine, believed to play a role in PD, inhibited NQO1, presumably increasing neurotoxicity [104, 105].

An overall picture emerges in which NQO1 plays a key role in mitigating some of the pathology of PD, particularly those mediated by oxidative stress. It is, therefore, surprising that the *C609T* polymorphism is not associated with increased risk of PD. Further studies in larger and/or more diverse population groups may be merited.

4.2 Multiple Sclerosis (MS)

Genetic association studies in a Greek population revealed higher frequency of the heterozygous and homozygous NQO1 *C609T* variant genotype in MS patients [106]. However, a similar study in a Spanish population did not show an association [107]. A combination of polymorphisms in the *GSTP* (encoding Glutathione *S*-transferase P; EC 2.5.1.18) and *NQO1* genes has been observed with increased frequency in MS patients, and individuals with this combination were more likely to be non-responders to Natalizumab, a key treatment for MS [108, 109]. Post-mortem lesions from MS patients indicate upregulation of NQO1, especially in hypertrophic astrocytes and foamy macrophages, and to a smaller extent in oligodendroglial-like cells [110].

4.3 Alzheimer's Disease (AD)

It has been proposed that Aβ protein toxicity may be due to the formation of reactive oxygen species (ROS) [111]. Association between the *C609T* polymorphism in NQO1 and sporadic AD has been found [112, 113]. However, later studies did not observe a significant association; nonetheless, a homozygous wild-type genotype was found reduce the chances of sporadic AD [114, 115]. Hippocampal tissue samples from AD patients demonstrated increased NQO1 localised in neurofibrillary tangles (an end-stage manifestation of AD), the cytoplasm of hippocampal neurons, pyramidal neurons in areas of tau-presence, neurons in the frontal cortex, and astrocytes in the occipital lobe surrounding senile plaques. This suggested that glial NQO1 upregulation is an early event and neuronal NQO1 is a later event [116, 117]. An increase in NQO1 was found in the hippocampus during the initial stages of the disease (2 months) in 3xTGAD mice which was then reduced by 6 months [118]. This suggests that in later stages antioxidant protection becomes insufficient to prevent

neuronal damage. Behaviourally, neophobia correlated with high expression of NQO1 in the initial stages of the disease [118]. Cyanidin-3-*O*-glucoside has been found to protect SH-SY5Y cells against Aβ(1-40)-induced oxidative stress via increased expression of NQO1 [119].

The co-chaperone STUB1/CHIP, which has been found to target phosphorylated tau protein for degradation, was found to downregulate NQO1 at posttranslational level, and knockdown of STUB1/CHIP resulted in increased NQO1 expression in mice [120, 121]. Postmortem hippocampal tissue analysis has shown reduced NQO1 levels in AD patients which correlated with the presence of the C609T polymorphism [121]. In the animal model, 3xTgAD mice, reduced levels and activity of NQO1 were observed in the hippocampus and cerebral cortex, areas associated with Aβ accumulation [122]. It is not clear if impaired NQO1 was reduced prior to neurodegenerative processes. Both the findings are in contrast to the previous studies which have reported increases in NQO1 [116, 117]. However, these studies looked at different stages in the pathological progression of AD.

4.4 Schizophrenia

A role for impaired antioxidant defence in schizophrenia has been proposed [123]. Adrenochrome, produced by the oxidation of adrenaline, has been found to be a neurotoxic substance, producing perception and thought disturbances [124]. NQO1 is known to be involved in the detoxification of adrenochrome [125]. However, no direct association has been found between the *C609T* NQO1 polymorphism and the development of schizophrenia [126]. However, the polymorphism was associated with susceptibility to tardive dyskinesia in patients with schizophrenia in some studies, but not others [126-128].

4.5 Mood Disorders

Major Depressive Disorder (MDD) has been associated with a reduction in antioxidant defences and an increase in proinflammatory cytokines [129, 130]. NQO1 levels have been found to decrease in the cortex and hippocampus (regions involved in mood disorders) in the glucocorticoid mouse model for depression and anxiety [131]. These levels were found to increase following administration of fluoxetine (a selective serotonin reuptake inhibitor)

[131]. No association has been found between the *C609T* NQO1 polymorphism and mood disorders in a Korean population [132]. However, this does represent a relatively small sample of a single ethnic group. Further studies over a range of ethnic groups may be useful.

4.6 Atherosclerotic stroke

Interestingly, the NQO1 *C609T* polymorphism was found to carry a reduced risk for ischemic stroke which may be due to these carriers having lower concentrations of blood coagulation factors [133, 134]. The effect is synergistic with non-smoking, low or zero alcohol consumption and polymorphisms in other the *VKORC1* and *GGCX* (encoding γ-glutamyl carboxylase) genes [133].

5. Disease-associated NQO1 loss-of-function (LOF) due to protein destabilization misfolding

To carry out their biological activity, most proteins must fold into a defined three-dimensional structure which lies in a free energy minimum of their conformational landscapes. Barriers that separate the native state from partially folded states are often small, and can be accessible through thermal fluctuations, with functional implications [135, 136](Figure 5A). Thus, *protein misfolding* can be simply defined as any perturbation (i.e. an inherited missense mutation occurring in the germline) that impairs proper folding and function [136, 137]. Obviously, this concept has widely different meanings for different proteins according to features such as the soluble/membrane bound state, intracellular location and function. Importantly, missense mutations often compromise protein folding and lead to protein misfolding [138, 139]. *In vivo*, this scenario in which a protein *decides* whether to fold or misfold depends upon its interaction with the *protein homeostasis network*, a vast array of highly interactive proteins evolved to preserve proper quality of the proteome (i.e. *protein homeostasis*)[140, 141]. However, random mutations in natural proteins are universally associated to protein destabilization, particularly those buried in the structure [142]. Thus, missense mutations are often the cause of genetic diseases in which protein function is compromised (loss-of-function diseases) through changes in the stability

of the native vs. non-native states (Figure 5B). Whether a mutation leads to a given loss-of-function molecular phenotype (aggregation, incorrect subcellular trafficking, enzyme inactivation, accelerated degradation) likely depends on the extent of mutational effects on protein stability, and how this propagates through the protein structure to promote the population of misfolding-associated partially folded states (Figure 5B; [143]).

Although the pathogenic manifestation of missense mutations may depend on many different factors [144, 145], it is likely that, generally, a certain degree of correlation exists between certain structural/energetic features of the mutation and the severity of disease phenotypes. These features may include magnitude of mutation-induced destabilization of the native state, the structural location of the mutated site and the propagation of stability effects to disease-associated protein states [143, 146-148]. The association of these features with pathogenicity is also particularly relevant to improve our capacity to predict the pathogenicity of mutations in genome-wide sequencing studies since it seems that a positive correlation exists between the pathogenicity of mutations found and their destabilizing effects [146]. Regarding NQO1, the Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org/) has compiled 73 missense variations in the NQO1 protein, with only three variants showing allele frequencies higher than 10^{-3}, and the two most common, p.P187S/c.559C>T (frequency of 0.245) and p.R139W/c.415C>T (frequency of 0.0382) have been characterized in detail (see below). In addition, 42 missense variants can be found in the COSMIC database (https://cancer.sanger.ac.uk/cosmic), of which only one, p.K240Q/c.718A>C, has been characterized experimentally. In this section, we summarize our current knowledge on the consequences on NQO1 function, stability and misfolding of these three missense variants, while a more general discussion on the potential effects of cancer-related mutations (COSMIC) and variants of unknown pathogenicity found in human population (ExAC) are discussed in section 7.2.

5.1. The p.P187S variant

The p.P187S variant is by the far the most extensively characterized missense variant in NQO1 (See Section 3, above). Early studies in cancer cell lines endogenously expressing this polymorphism revealed normal mRNA levels but virtually no NQO1 protein or activity,

suggesting folding and catalytic defects [149, 150]. The former effect was later determined to be caused by enhanced degradation of this polymorphism possibly due to ubiquitin-dependent and independent pathways [151-153]. The low catalytic activity arises from a 10-20-fold decrease in the binding affinity for FAD, thus easily explaining the isolation of this variant as an apo-protein [153, 154]. More recent biophysical and structural studies have revealed remarkable differences in the structure and dynamics between WT and p.P187S providing molecular insight into its low intracellular activity and stability [154-159].

Strikingly, determination of the structure of full-length WT and p.P187S in a ternary complex with FAD (holo-protein) and C-terminal domain (CTD) binding inhibitors (PDB: 4CF6 and 2F1O) showed virtually no difference at the protein structural level [155]. However, in the absence of inhibitors, p.P187S showed a highly dynamic (and possibly partially unfolded) CTD as supported by crystallographic, NMR spectroscopy and proteolysis studies (Figure 1; [155, 157]). The different stability and dynamics of the CTD in the p.P187S variant was shown to be critical for its enhanced ubiquitinylation and degradation of the protein even in the holo-state, while degradation of the WT enzyme seemed to simply operated through the levels of the apo-protein, and thus, the intracellular flavin levels [156, 157]. The thermodynamically unstable CTD in p.P187S might also act as initiation site for ubiquitin-independent proteasomal degradation [160]. Importantly, these results pointed to the CTD as a target for pharmacological correction of the intracellular stability of p.P187S, further confirmed by using ligands binding to and stabilizing this domain [156, 157].

Understanding the defective FAD binding to p.P187S has even more been challenging. Thermodynamic analyses showed that p.P187S interferes with the binding cooperativity of WT NQO1 and FAD [23] making comparative structure-thermodynamic analyses very difficult. Further, the apo-state is very challenging to characterize using high-resolution structural techniques and computational methods due to its intrinsic dynamics [158, 159], while characterization of their effects on the holo-state have revealed minimal changes [155]. Nevertheless, changes in structure and dynamics of the apo-state due to p.P187S have been demonstrated through molecular dynamics, mutational studies and thermodynamic analyses [157, 159, 161]. p.P187S seems to primarily affect the structure and dynamics of the binding site in the apo-state (particularly in the loop 57-66 and the region comprising Tyr127 and Tyr129; Figure 1), thus promoting the population of FAD

binding non-competent conformations in the apo-state ensemble and energetically penalizing binding [157, 159]. According to this view, an evolutionary-divergent mutation (p.H80R) and a phosphomimetic mutation at Ser82, were shown to increase and decrease the affinity of p.P187S for FAD acting on the population of binding competent states and the structure/dynamics of the loop 57-66 [159, 161, 162].

p.P187S also affects the intracellular stability of p53 and p73α as interactors of NQO1 [152, 163, 164], although whether this reflects structural alterations, protein:protein interactions (PPI) or just mirrors the low intracellular stability of the polymorphism has been controversial. An active NQO1 enzyme, particularly with a reduced FAD cofactor, has been proposed to be critical for these PPI based on *in vitro* immunoprecipitation studies [152, 163, 164]. However, biochemical and biophysical studies involving NQO1 species with virtually no activity (apo-P187S, C-terminal truncated NQO1, or NQO1 inhibited by dicoumarol or ES936) showed some capacity of these forms to interact with these oncosuppressors [158, 165]. Therefore, it seems that at least to some extent, destabilization of oncosuppressors by p.P187S reflects the low intracellular stability of this NQO1 variant [158].

It is remarkable that p.P187S affects functional sites of NQO1 located far away in the structure (at minimal distances of 9-17 Å) (Figure 1; [166]), including the FAD binding site, the dicoumarol binding site located in the CTD and the monomer:monomer interface (reducing the stability of the dimer) [154, 157, 158]. The fully buried location of Pro-187, and its non-conservative change to Ser, likely perturbs the protein packing and dynamics, and these effects propagate efficiently through the protein structure [143]. Interestingly, this propagation occurs in a non-trivial manner, as indicated by extensive mutagenesis studies [143, 158, 161]. Removal of the CTD essentially abolishes the effects of p.P187S on the remaining functional sites which show WT-like binding of FAD and thermal stability [158], consistent with the existence of allosteric communication between sites in NQO1 which is perturbed by the polymorphism [158]. This allosteric network likely communicates differently stability and dynamic alterations originated at the Pro-187 site, as shown by the mild effects of the entropy-promoting P187G mutant in the FAD binding site and CTD stability as well as their larger effects on the stability of the monomer:monomer interface [166]. In addition, stabilizing mutations also likely communicate their effects through this

network in a complex manner, with the p.H80R variant locally stabilizing the FAD binding site and the monomer:monomer interface, while the p.E247Q variant locally stabilizes the CTD and this effect is also transmitted the FAD binding site located at 24 Å and synergizes with p.H80R, located 40 Å away.

5.2. The p.R139W variant

A second, rarer, NQO1 polymorphism has also been associated with an increased risk of cancer. The p.R139W/c.415C>T polymorphism (rs1131341; NQO1*3) was isolated from cancer cell lines resistant to treatment with MMC [167-169]. This nucleotide change causes primarily two effects: i) altered normal splicing of RNA leading to skipping exon 4 [167], and thus, rendering a very unstable protein lacking part of the FAD binding site [157, 167, 170]; ii) the amino acid exchange p.R139W, affects a residue located in a solvent exposed loop not close to the FAD binding site (Figure 1). This mutation in the full-length protein causes only mild effects on thermal stability, FAD binding or catalytic activity [154, 157, 167, 170]. The conformation and dynamics of the p.R139W variant has been shown to be slightly affected, which has led to the proposition that the main pathogenic consequence of this polymorphism is the perturbation of proper RNA splicing [154, 157, 170].

5.3. The p.K240Q variant

The p.K240Q/c.718A>C mutation has been found in one sample of renal cancer and predicted to be deleterious in the COSMIC database. The Lys-240 residue is solvent-exposed and located in the CTD as part of a highly stabilizing electrostatic network, and structural analyses have suggested that the effects of mutations at this site are expected to act locally on the CTD (Figure 1; [143, 166]). In addition, Lys-240 is not close to the FAD or dicoumarol binding sites or the monomer interface, and predicted to be only mildly destabilizing (about 2 kcal·mol^{-1}; [166]). Thus, not surprisingly, p.K240Q does not affect NQO1 thermal stability and only mildly decreases FAD binding affinity, while the most prominent effect is to increase the dynamics of the CTD [166]. Whether this affects the intracellular degradation of NQO1 is still unknown. Interestingly, introduction of more perturbing mutations, causing

charge-reversal (p.K240E) or altering protein packing and conformational entropy (p.K240G), induced greater destabilization of the CTD that propagated to the distant FAD binding site [166]. Thus, these analyses further supported the proposal of complex communication of mutational effects through the NQO1 structural ensemble in the context of an allosteric network.

6. Potential for pharmacological intervention in misfolding of NQO1

Since protein destabilization is the core of protein misfolding, any approach to compensate mutation-induced destabilization should prevent it. Conceptually, we should search for ligands that selectively bind to the native state, thus decreasing the rate or extent of protein misfolding by the law of mass-action (i.e. increasing the height of the free energy barrier of misfolding in Figure 5B). This approach using native state ligands (called natural or pharmacological chaperones) has been developed for treating certain loss of function diseases over the last decades, either using natural compounds (e.g. vitamin/cofactor supplementation) or molecules identified upon high-throughput screening [171-178]. Another approach to correct misfolding includes proteostasis regulators which may boost globally or specifically certain nodes of the proteostasis network improving folding to the native state or preventing degradation of proteins [179, 180].

The pharmacological chaperone approach could be used to rescue the intracellular activity and stability of NQO1 polymorphisms and mutations. In the particular case of p.P187S, the most common and well-characterized NQO1 missense variant, the results gathered suggest that two different types of ligands would be necessary to boost its activity *in vivo*. First, riboflavin supplementation should increase the fraction of active holo-enzyme, although this treatment has mild positive effects on protein stability [157]. In principle, this treatment would cause negligible problems since it is efficiently used to treat many disorders associated with flavoproteins [181]. Finding the second type of ligand would be more challenging, since this would require binding to the CTD and increasing its stability [157]. Currently, the only well-characterized compound that increases the CTD stability in p.P187S and corrects its stability in cells is dicoumarol, a potent anti-coagulant and NQO1 inhibitor [32]. Although inhibitors can be used to correct misfolding, as long as their

inhibitory effect is counterbalanced by intracellular concentrations of substrates [182], the high affinity and toxicity of dicoumarol do not favour its use as a pharmacological chaperone. However, as long as the CTD is identified as the target, high-throughput screening could be carried out for similar ligands to identify ligands with high stabilizing and low inhibitory effects. A potentially interesting ligand would mimic the effect of the E247Q variant that restores the stability of the CTD despite being located far from the NADH/substrate binding site [161]. A particular interesting screening would be among chemical libraries of FDA-approved molecules [183] in order to speed up their potential translation to the clinical realm.

The perturbations induced by p.P187S in the conformation of the NTD also alters proper interactions with Hsp40/Hsp70 chaperones [184] possibly contributing to protein misfolding and enhanced degradation. These alterations are likely caused by the increased population of the apo-state in p.P187S that increase the flexibility of the N-terminus [157]. Thus, p.P187S function and stability might also be rescued by riboflavin supplementation (to form the holo-state) and with activators of the HSF-1/Nrf-2 pathways [179] to increase the availability of Hsp70 chaperones for proper folding and expression of the polymorphic variant [185]. In addition, proteasomal inhibition by the FDA-approved Bortezomib could also enhance p.P187S activity and stability [151, 153, 165] although proteasomal inhibition may have undesired non-specific effects in the stability of the proteome.

7. NQO1 as a model for loss of function disease.

The multi-functional nature of NQO1, the diversity of disease-causing mechanisms and the pleiotropic effects of single amino exchanges presented so far, make NQO1 an excellent model to further understand loss of function diseases. Some of these aspects are discussed in this section.

7.1. Structural and energetic determinants of proteasomal degradation in LOF diseases

It is often presumed, and only known in a few cases, that structural destabilization caused by missense mutations affect protein intracellular levels by acting on the

degradation rate of the variant proteins, and in this context, the ubiquitin-dependent proteasomal pathway plays a critical role [146, 147, 186]. Generally, regions with high flexibility and low local stability are associated with ubiquitin tagging and act as initiation sites for proteasomal degradation (so called degrons) [187, 188]. Therefore, how a missense mutation affects the degradation rate of a protein may depend on the direct *activation* of degrons or propagation of the destabilizing effect to them. Regarding the former factor, analyses have suggested at even mild mutational perturbations of the native state stability (in the range of 3 kcal·mol^{-1}) can significantly increase the degradation rates of variant proteins [146, 147, 186]. To the best of our knowledge, the latter factor has not been explored in detail. We propose that detailed mutational studies in NQO1 can provide novel insight into the interplay between local structural and energetic destabilization and proteasomal degradation, with implications to understand disease-mechanisms and molecular evolution. The factors determining the proteasomal degradation of WT NQO1 and the p.P187S variant are well established in structural terms and can be readily modulated by ligand binding [151, 154, 157]. Wild-type and p.P187S can be degraded in apo-state by either ubiquitin-dependent and independent proteasomal pathways, and the ubiquitin-dependent pathway seems to rely on the action of CHIP as ubiquitin E3 ligase [189, 190]. In principal, ubiquitylation of NQO1 does not require accessory proteins (such as molecular chaperones), although the interaction of NQO1 with Hsp70/Hsp40 may also play a role in the ubiquitin-dependent degradation of NQO1 [156, 191]. In addition, a variety of stabilizing and destabilizing mutations at different structural sites of NQO1 have been characterized *in vitro* [23, 143, 157-159, 161, 162, 166], which combined with studies of proteasomal degradation rates in cellular models and structural perturbation analyses can provide unprecedented insights into this interplay. NQO1 can also be used to gain insight into how evolutionary changes in local stability and due to post-translational modifications (PTMs) can translate into inter-species variations in proteasomal degradation rates as well as modulation of protein stability due to phosphorylation and other PTMs [161, 162, 192].

7.2. Prediction of the consequences of missense variations in the whole genome era

Advances in sequencing technologies are greatly increasing our knowledge of the protein sequence variability in the human proteome [193], and now, one of the main

challenges is to determine accurately and in a high-throughput manner the pathogenicity of missense variants [144, 146, 194-196]. Biophysical stability calculations and residue conservation analyses have emerged as potential tools to improve our prediction capacity of mutational effects on protein intracellular stability [146, 147, 186]. However, to improve this capacity we should consider an important aspect of mutational effects on proteins: the fluid-like properties of the protein interior that allow efficient propagation and dissipation of mutational effects to distant functional sites thus affecting multiple functional features (*pleiotropy*) [143, 197, 198]. Pleitropy could be a quite general (if not universal) phenomenon associated with missense mutations [143, 148, 166, 197, 199-213]. Recent structural and experimental perturbation analyses on NQO1 and other disease-associated proteins displaying the most common loss of function mechanisms have revealed that both the original stability perturbation at the mutated site and its propagation through the protein structure to promote different misfolding states to determine not only the severity of the phenotype, but also the phenotype itself [143].

Recent studies have systematically assessed the impact of disease-associated vs. neutral or common variants found in massive genome-sequencing initiatives [146, 147]. These studies have proposed that disease-causing variants may decrease protein intracellular stability due to a larger energetic perturbation of the native state stability, while variants found in the overall population typically show milder effects as they become more frequent. To compare with these studies, we have compiled NQO1 variants found in cancer samples (COSMIC; N=41) and in overall population (ExAC; N=69) and carried out structure-based stability calculations by using the FoldX force-field [214]. Overall, the two sets of variants are quite similar, with a median destabilizing of typically 2-3 kcal·mol^{-1} (Figure 6A). Although these values may appear mild, they are in the range shown to significantly decrease the intracellular stability of other disease-associated proteins [146, 147], and in the particular case of NQO1, mutations affecting the cancer-associated Lys-240 site destabilized it by 2-4 kcal·mol^{-1} and severely perturbed different functional features of the protein [143, 166]. The apparent bimodal distribution for the destabilizing effects (Figure 6A) likely reflects the larger destabilization caused by mutations affecting buried residues (Figure 6B), in agreement with previous large-scale and structure-based analyses [142] and also shown for a few disease-associated proteins [146, 147]. Importantly, the

effect of mutations affecting buried residues should percolate more efficiently through the structure and thus show stronger pleiotropic effects as recently found for p.P187S [143, 198, 215, 216]. As found recently for other disease-associated proteins (such as phenylalanine hydroxylase or the human mismatch repair protein, MSH2)[138,139][217]), it appears that the neutrality of ExAC mutation in NQO1 positively correlates with their frequency in human population (Figure 6C). Clearly, p.P187S may represent an exception, and this could be related with different factors. For instance, in contrast to human monogenic disorders, cancer is a multi-factorial in which many different factors (i.e. genetic or environmental factor) may determine the molecular phenotype. In addition, NQO1 expression is not required for normal development and physiology unless a heavy stress or burden is introduced [218], and counterintuitively, either NQO1 activity or inactivity may be associated with cancer development or progression due to its multiple roles in antioxidant defense, detoxification and interaction with tumour suppressor [185]. Still, we need further experimentation to understand how mutation-induced protein destabilization causes pleiotropic effects, and NQO1 seems to be an excellent model for that.

7.3. Loss of function diseases associated with human flavoproteins

Treatment of loss of function metabolic diseases with supplementation of cofactor precursors is a classic therapeutic approach [219]. NQO1 belongs to the set of human proteins constituting the flavoproteome, a set of a hundred proteins whose activity relies on FAD or FMN or related compounds [220]. A recent proteomic study has shown that most of human flavoproteins are very sensitive to starvation of the flavin cofactor, particularly NQO1 [156]. These effects seem to be mediated by specific recognition and degradation of apo-proteins by the ubiquitin-dependent proteasomal pathways [156]. Although it is appealing to apply this view to disease-associated mutation in human flavoproteins, we must also consider other factors. For instance, whether mutations alter the degradation pathway of the protein, as it has been shown for p.P187S which is efficiently degraded as the holo-protein [156, 157] or the cross-talk of ubiquitin-dependent degradation with other post-translational modifications [162]. Due to the rich diversity of post-translational modifications in NQO1 [192] and the availability of cancer-associated but uncharacterized NQO1 missense mutations, NQO1 emerges as an ideal case study to investigate further the

intracellular stability of human flavoproteins in health and disease. To end, recent work has also shown that changes in the chemical composition of the intracellular milieu, such as anion concentration and identity, could also affect flavin binding affinity and thus intracellular stability of human flavoproteins [221].

8. Conclusions

The extensive data which has now been gathered on NQO1 structure, dynamics, function and enzymology mean that there is a sound foundation upon which to build the search for lead molecules which act as pharmacological chaperones. Any drugs developed from this work would have potential utility in restoring NQO1 activity in individuals who are homozygous for the *C609T* allele and thus at high risk of developing cancer. They may also be useful in the treatment of cancers and some neurological conditions in people homozygous for the *C609T* allele. This would be particularly the case for those diseases where increased risk (or increased risk of more severe symptoms) has been linked to this allele, i.e. multiple sclerosis and Alzheimer's disease. These molecules would also be useful chemical biology probes for further studies on the dynamics of NQO1. Furthermore, the extensive knowledge base which has been established for NQO1 make it an excellent model for other loss of function diseases, particularly those involving flavoproteins. It is likely that many of the principles which are being established for NQO1 will also apply to less well-studied systems and diseases.

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Figure legends

Figure 1. Structure of human NQO1. Structural location of the naturally-occurring and cancer-associated p.R139W, p.P187S and p.K240Q variants and those regions in NQO1 associated with enhanced degradation (the CTD) and FAD binding. The figure was made using PDB: 2F1O [33].

Figure 2. Mechanism of NQO1. The scheme shows the generally accepted catalytic mechanism for NQO1. In the first stage NAD(P)H (only the reduced nicotinamide ring is shown here with the rest of the molecule represented here by R^2) reduces FAD, resulting in a negative charge on the isoalloxazine ring. This is then neutralised by transfer of a proton from Tyr-155, which is itself neutralised by His-161. Reduction of the quinone substrate occurs by reversal of the electron movements in $FADH_2$ and two protons are required from the solvent. Only the isoalloxazine ring of FAD is shown with the rest of the cofactor represented by R^1. Similarly, only part of the side chains for Tyr-155 and His-161 are shown with the rest of the protein shown by R^3 and R^4. NAD(P)H can be presumed to diffuse out of the active site once it has reduced the FAD. It has been omitted from subsequent stages of the reaction scheme.

Figure 3 A selection of compounds which interact with NQO1. The structures of three NQO1 inhibitors (dicoumarol, warfarin and curcumin). EO9, an anticancer agent which is activated by NQO1, is also shown.

Figure 4 NQO1 maintains dopamine metabolites in the reduced state. Dopamine can be oxidised to a quinone form which spontaneously rearranges to aminochrome. NQO1 can catalyse the reduction of the aminochrome to its corresponding hydroquinone, which is required for detoxicifying it by sulphation or glucuronidation [88].

Figure 5. Protein energy landscape, misfolding and disease. (A) Structure-based free energy surface for the NQO1 monomer. The two folding intermediates I_1 and I_2 represent states in which the NTD is essentially folded while the CTD is largely (I2) or scarcely (I1) folded. Calculations were kindly performed and provided by Dr. Athi Naganathan (Indian Institute of Technology Madras, India) using a WMSE structure-based statistical mechanical model at 298 K and using the structure PDB: 5EA2 [222] (see [197] for additional details); (B) Mutations can alter the kinetic and thermodynamic stability of the native state and

folding/unfolding intermediates leading to disease (i.e. degradation, aggregation, inactivation, etc)(adapted from [143]).

Figure 6. Structure-based energetic calculations of the effects on NQO1 stability by mutations found in COSMIC and ExAC databases. (A) Mutational effects on stability for mutations in COSMIC (N=41) and ExAC (N=69). In the left panel, boxes show the median and $25^{th}/75^{th}$ percentiles, vertical lines show data within 1.5 times the interquartile range (IQR) and dots show outliers (>1.5·IQR). In the right panel, Violin plots showing the distribution of stability effects as well as the mean $\pm$ s.d. for each data set (N=38 for COSMIC, N=63 for ExAC). Statistical analyses for these two populations provided a *p* value of 0.25. (B) Structural location and destabilization for COSMIC *plus* ExAC mutations showing no clustering for the magnitude of destabilizations as well as milder effects for surface mutations. Left and middle figures show the destabilizing effect mapped onto the structure (PDB 1D4A; [223]) following the colour code. The plot in the right shows average stability effects for COSMIC or ExAC mutations buried or exposed using a cut-off solvent exposure of 10% based on GetArea (http://curie.utmb.edu/getarea.html) calculations. Statistical analyses provided a *p* value of $1.2 \cdot 10^{-2}$ (COSMIC) and $4.4 \cdot 10^{-4}$ (ExAC). (C) ExAC mutations cause milder protein destabilization as their frequency is increased in human population. The arrow indicates p.P187S as an outlier. Data are provided as changes in folding free energy vs. WT NQO1 ($\Delta\Delta G$). Calculations were carried out by FoldX [214] using the dimeric structure of NQO1 (PDB 1D4A; [223]). Statistical analyses were carried out using a two-tailed non-parametric Mann Whitney U test.

Table 1: Major roles of NQO1 and effects of up/down regulation

Catalytic roles
Two electron reduction of quinones, avoiding semiquinone intermediates
Reduction of reactive oxygen species
Reduction of dopamine metabolites to maintain them in the correct form for detoxicifcation
Minor role in vitamin K cycling
Activation of potential anticancer agents, e.g. EO9
Roles depending on protein-protein interactions
Stabilisation of p53 and p73
Interactions with 20S proteasomal components control proteasomal activity
Effects of up-regulation
Increased survivability of cancer cells
Response to cellular stress; assists in reducing free radical load
Increased resistance of cells to some reagents which cause free radical stress
Effects of down-regulation or inhibition
Increased potential for semiquinone build-up and free radical damage; consequent increased cancer risk
Reduced viability of cancer cells
Abnormal behaviour and seizures in rats
Neuronal cells more sensitive to aminochrome toxicity

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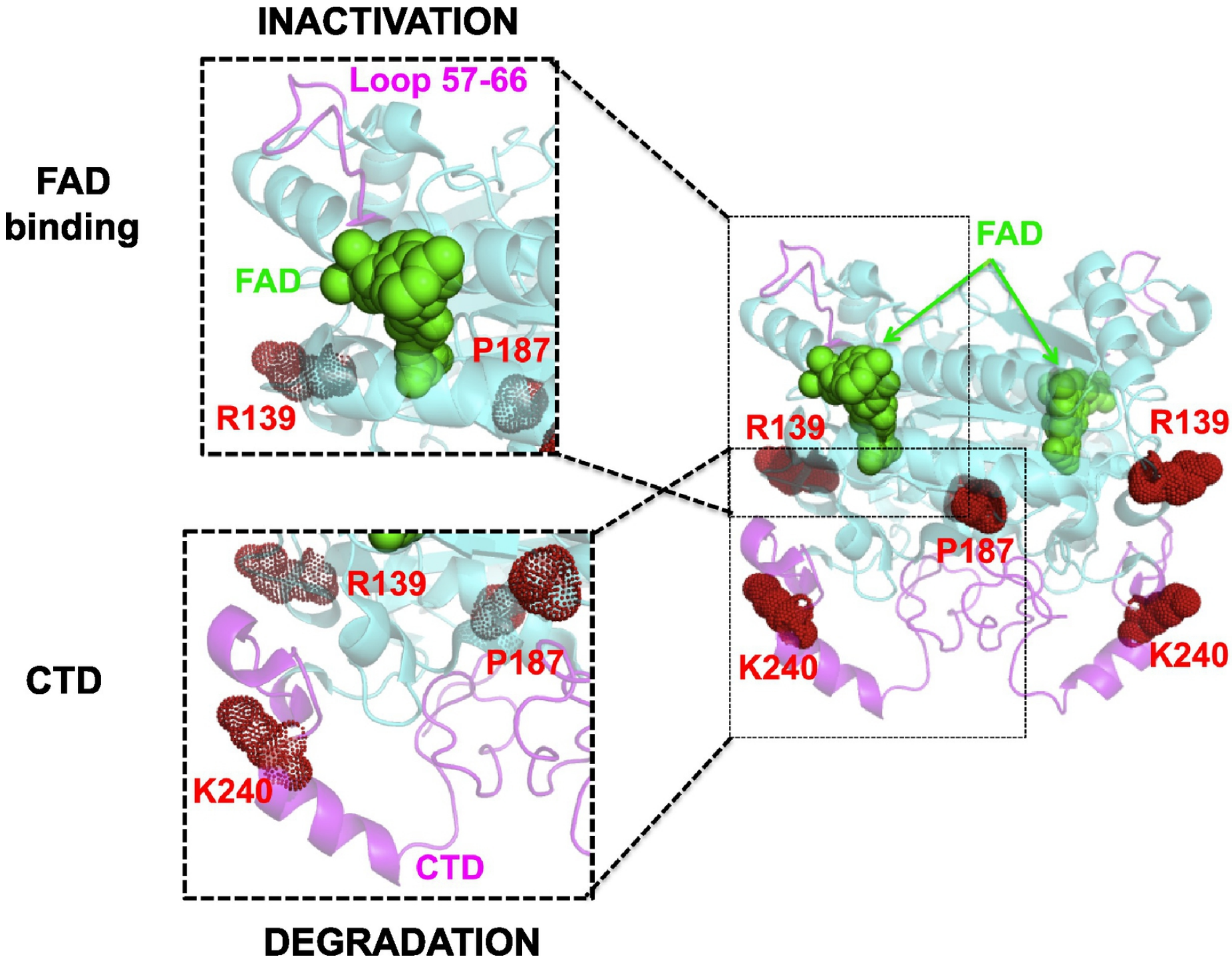
NQO1: a target for the treatment of cancer and neurological diseases, and a model to understand loss of function disease mechanisms

Conflict of interest statement:

The authors have no conflicts of interest to declare.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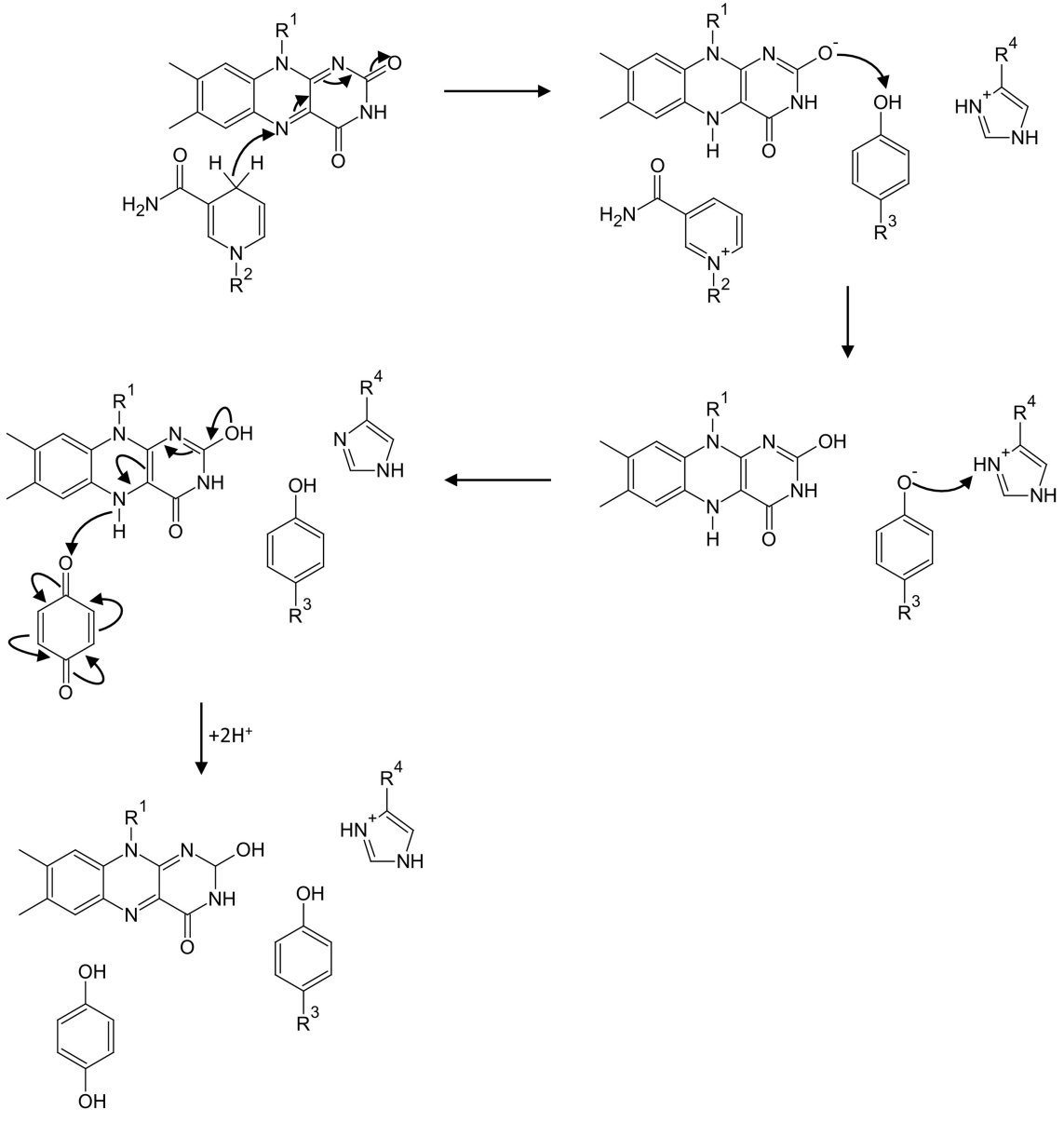
DEGRADATION

Figure 1



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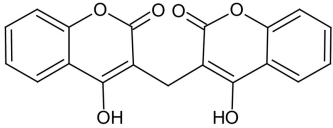

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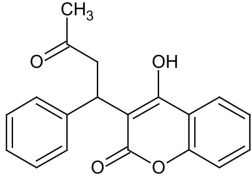

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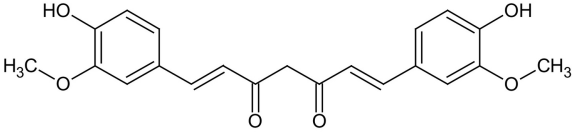

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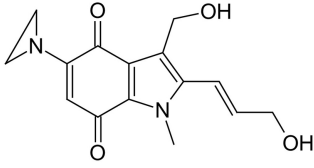

EO9 (Apaziquone)

Figure 3

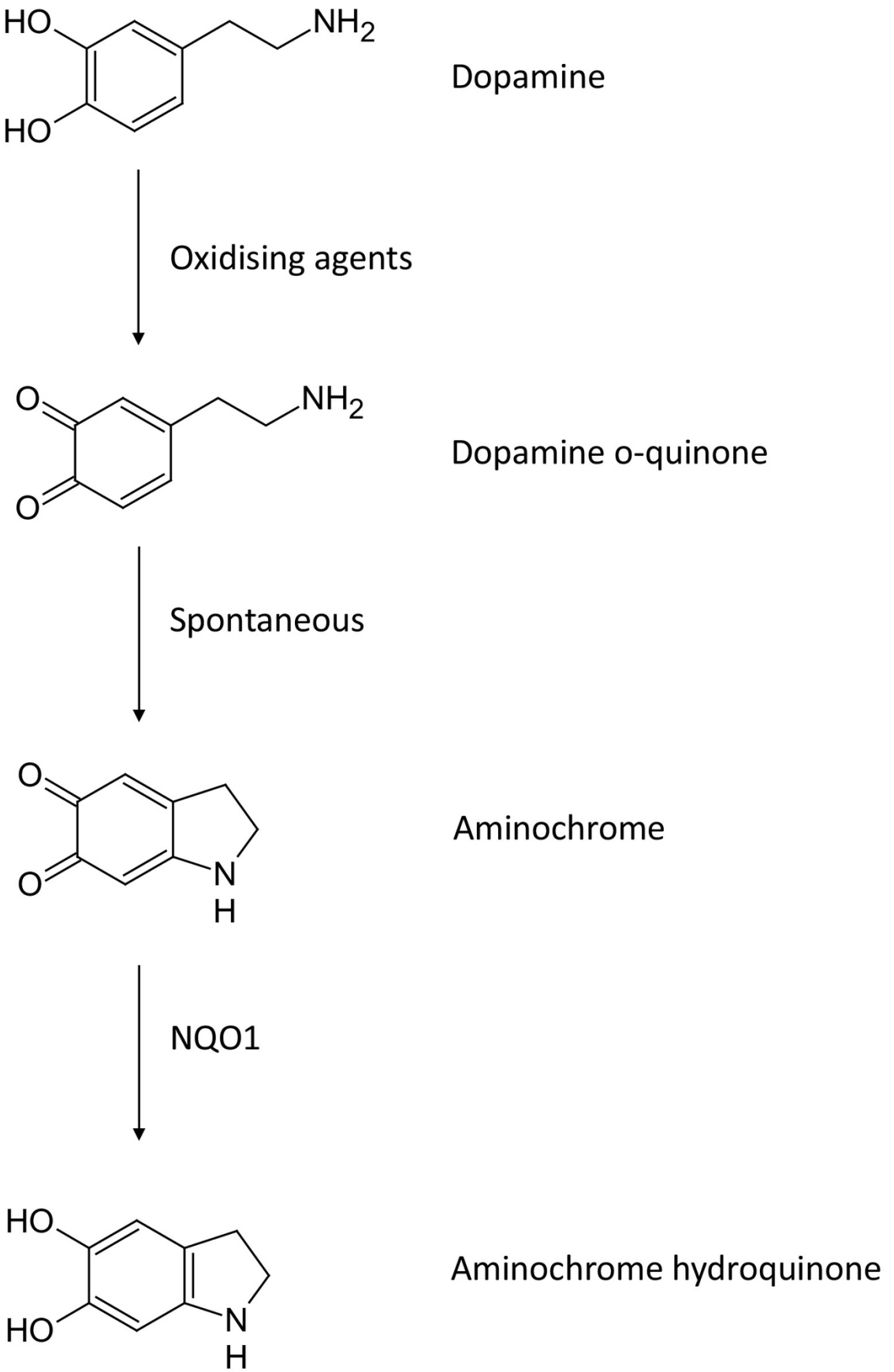
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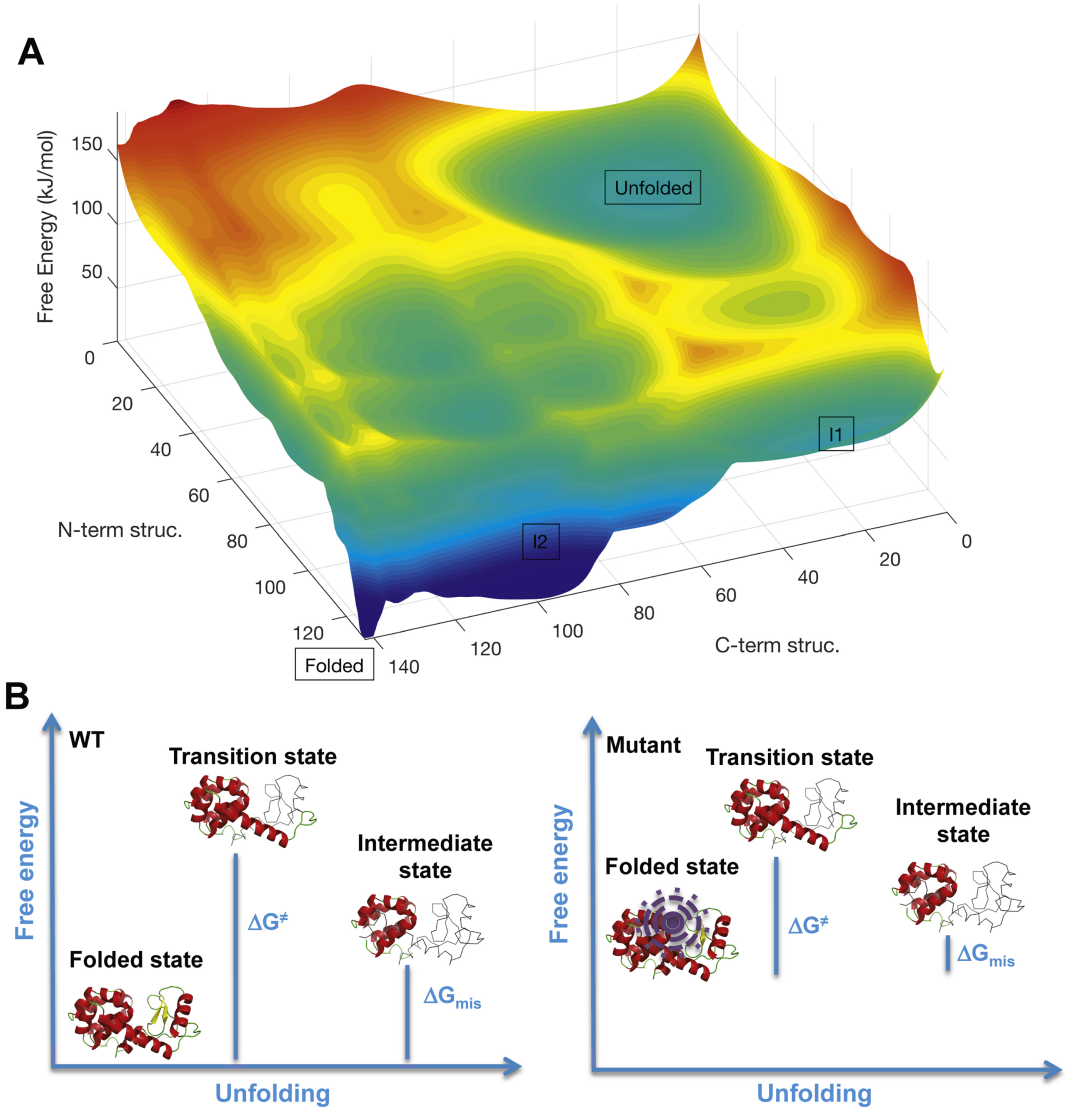
Figure 4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80
100
120
B
WT
Transition state
Intermediate state
Folded state
ΔG≠
ΔGmis
Free energy
Unfolding
Mutant
Transition state
Intermediate state
Folded state
ΔG≠
ΔGmis
Free energy
Unfolding

Figure 5

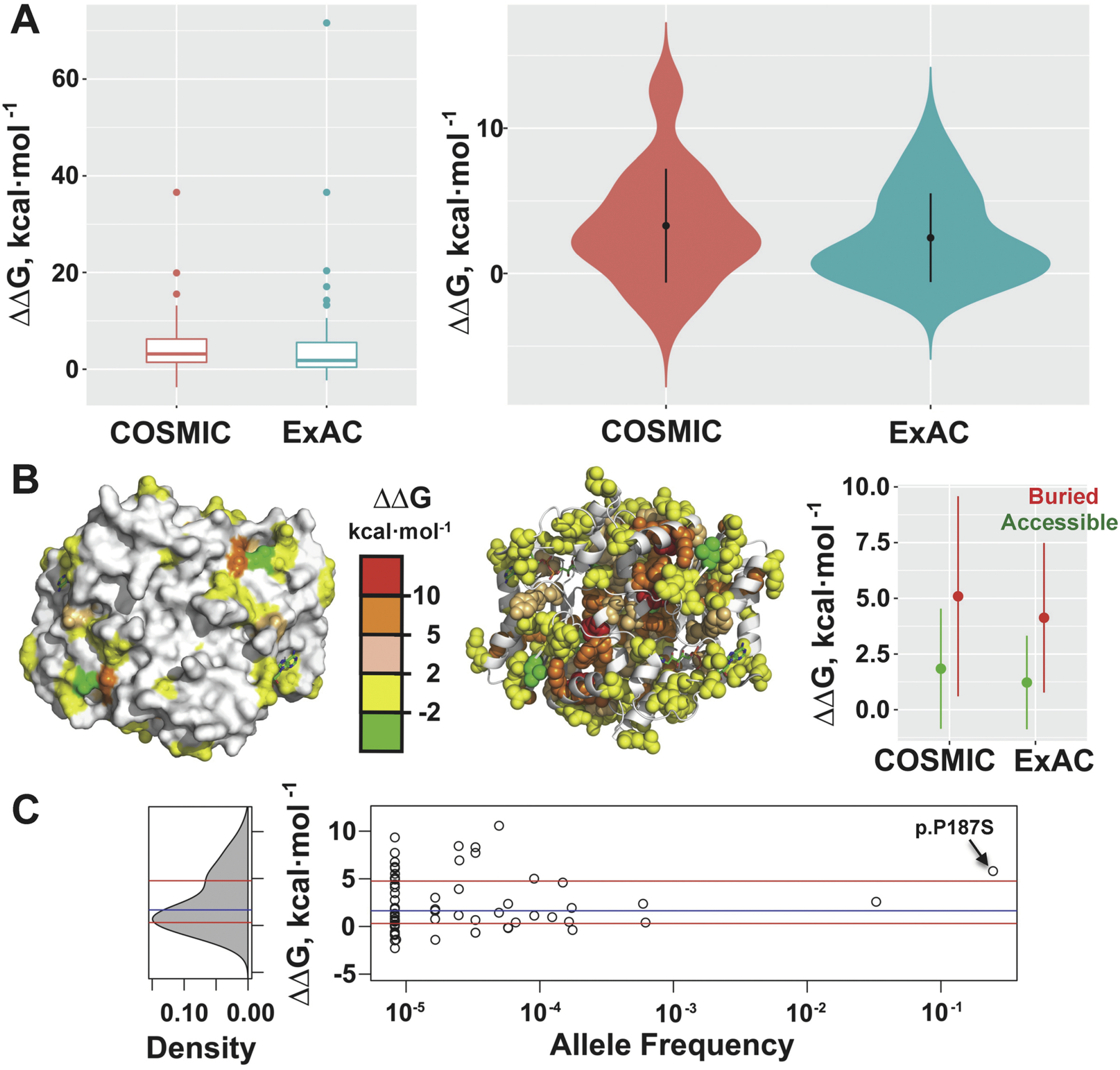
A
ΔΔG, kcal·mol-1
60
40
20
0
COSMIC
ExAC
ΔΔG, kcal·mol-1
10
0
COSMIC
ExAC
B
ΔΔG
kcal·mol-1
10
5
2
-2
ΔΔG, kcal·mol-1
10.0
7.5
5.0
2.5
0.0
Buried
Accessible
COSMIC
ExAC
C
0.10
0.00
Density
ΔΔG, kcal·mol-1
10
5
0
-5
p.P187S
10-5
10-4
10-3
10-2
10-1
Allele Frequency

Figure 6