



## Different phenotypic outcome due to site-specific phosphorylation in the cancer-associated NQO1 enzyme studied by phosphomimetic mutations

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### ABSTRACT

Protein phosphorylation is a common phenomenon in human flavoproteins although the functional consequences of this site-specific modification are largely unknown. Here, we evaluated the effects of site-specific phosphorylation (using phosphomimetic mutations at sites S40, S82 and T128) on multiple functional aspects as well as in the structural stability of the antioxidant and disease-associated human flavoprotein NQO1 using biophysical and biochemical methods. *In vitro* biophysical studies revealed effects of phosphorylation at different sites such as decreased binding affinity for FAD and structural stability of its binding site (S82), conformational stability (S40 and S82) and reduced catalytic efficiency and functional cooperativity (T128). Local stability measurements by H/D exchange in different ligation states provided structural insight into these effects. Transfection of eukaryotic cells showed that phosphorylation at sites S40 and S82 may reduce steady-levels of NQO1 protein by enhanced proteasome-induced degradation. We show that site-specific phosphorylation of human NQO1 may cause pleiotropic and counterintuitive effects on this multifunctional protein with potential implications for its relationships with human disease. Our approach allows to establish relationships between site-specific phosphorylation, functional and structural stability effects *in vitro* and inside cells paving the way for more detailed analyses of phosphorylation at the flavoproteome scale.

### 1. Introduction

Human NAD(P)H:quinone oxidoreductase 1 (NQO1) is a multifunctional protein whose dysregulation has been associated with developing cancer, Alzheimer's and Parkinson's disease [1–3]. It catalyzes the two-electron reduction of a wide variety of quinones, including maintenance of vitamins in their reduced state, superoxide scavenging, detoxification of xenobiotics and activation of cancer pro-drugs [1,3–5]. Additionally, NQO1 interacts with other proteins (PPI, e.g. p53 and HIF-1) and with RNA molecules [1,6–9].

NQO1 forms dimers with two active sites, each of them containing a tightly bound FAD molecule required for catalysis and also providing

intracellular stability [10–13]. The monomer contains two domains: an N-terminal domain (NTD; residues 1–225) containing the FAD binding site and most of the monomer:monomer interface (MMI), whereas the C-terminal domain (CTD, residues 226–274) completes the active sites [11–15].

NQO1 displays a remarkable functional chemistry. During its catalytic cycle, two half-reactions occur: i) in the reductive half-reaction the FAD molecules are reduced upon NAD(P)H binding and hydride transfer (HT), which constitutes the rate-limiting step of the catalytic cycle; ii) in the oxidative half-reaction, upon release of NAD(P)<sup>+</sup>, the substrate binds and is reduced by FADH<sub>2</sub> reconstituting the holo-enzyme [15,16]. Enzyme kinetic analyses have supported the existence of non-equivalent active sites that yield negative cooperativity to this catalytic cycle

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**Abbreviations**

BB	binding buffer
CTD	C-terminal domain
DBS	dicoumarol binding site;
Dic	dicoumarol
DT	deuteride-transfer
FAD	flavin-adenine dinucleotide;
FADH <sub>2</sub>	reduced flavin-adenine dinucleotide;
FBS	FAD binding site;
HDX	hydrogen-deuterium exchange
HT	hydride-transfer
IMAC	immobilized-metal affinity chromatography

KIE	kinetic isotope effect
LB	Luria-Bertani
MMI	monomer:monomer interface
NQO1	NAD(P)H:quinone oxidoreductase 1
NQO1 <sub>apo</sub>	ligand-free NQO1
NQO1 <sub>holo</sub>	NQO1 with FAD bound
NTD	N-terminal domain
PPI	protein:protein interactions
SEC	size-exclusion chromatography
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride;
TCS	thermolysin cleavage site.

[16–18].

In addition, ligands bound to NQO1 modulate its multiple functions. In the FAD-free state (NQO1<sub>apo</sub>), the enzyme is inactive, intracellularly unstable and displays altered PPI [10,19]. FAD binding renders a conformational state (NQO1<sub>holo</sub>), ready to initiate catalysis, intracellularly stable and with a different interactomic pattern than that of NQO1<sub>apo</sub> [10,19]. Upon NAD(P)H binding, the FAD is reduced to FADH<sub>2</sub> constituting a catalytically relevant state that also shuttles the proteins to interact with microtubules and enhances PPI [6,16,20]. Binding of dicoumarol (Dic), a competitive inhibitor of both NAD(P)H and the substrate [16], leads to an inactive state likely reflecting the ternary complex (NQO1<sub>holo</sub>:NAD(P)H) or transition state analogue, also abrogating PPI [6]. It is interesting to note that we have recently characterized the effects of FAD and Dic binding to WT NQO1, revealing that ligand binding sequentially stabilizes the FAD (FBS) and Dic (DBS) binding sites, and these stabilizing effects propagate far beyond these sites affecting the entire structure of the enzyme [21].

Phosphorylation is a common phenomenon in human flavoproteins, although the functional consequences of these events are largely unknown (<https://www.phosphosite.org/>). By July 30, 2022, there were 12 phosphorylation events identified in human NQO1 based on high-

throughput approaches (Fig. 1A). Most of them are localized in the NTD (Fig. 1A). Three of these sites (S40, S82 and T128) have been characterized in some detail at the molecular level by phosphomimetic mutations or by direct phosphorylation [2,18,22,23](Fig. 1B). The phosphomimetic mutation S82D affects a partially solvent exposed residue far from the FBS and the DBS (at minimal distances of 14 Å and 18.5 Å, respectively)(Fig. 1B). S82D causes local destabilization of the structure, and this effect propagates to the FBS and reduces by ~10-fold the affinity for the cofactor and thus its intracellular stability likely due to increased population of the NQO1<sub>apo</sub> state [22]. In addition, S82D decreases the catalytic efficiency for the slow FAD reduction pathway, thus exacerbating the functional negative cooperativity displayed by WT NQO1 [18]. The kinase responsible for phosphorylation of S82 is unknown. S40 is fully buried in the crystal structure and also locates far from the FBS and DBS (minimal distances of 14.5 Å and 20 Å, respectively). T128 is a solvent exposed residue that locates closer to the DBS (minimal distance of 6.8 Å) and at 12 Å from the FBS (Fig. 1B). Importantly, it is next to Y129, that undergoes important conformational rearrangements upon Dic binding [24]. S40 and T128 sites are both phosphorylated by the Akt kinase, although phosphorylation of the T128 site seems to be important to trigger phosphorylation at both sites [2]. Phosphorylation at S40 and T128 may facilitate ubiquitination and degradation of NQO1, with a more important role of phosphorylation at T128 [2]. This decrease in intracellular stability due to phosphorylation at S40/T128 may lower the antioxidant and cytoprotective capacity of NQO1 in cultured cells [2]. This study has also associated phosphorylation of T128 with increased propensity to develop Parkinson's disease [2].

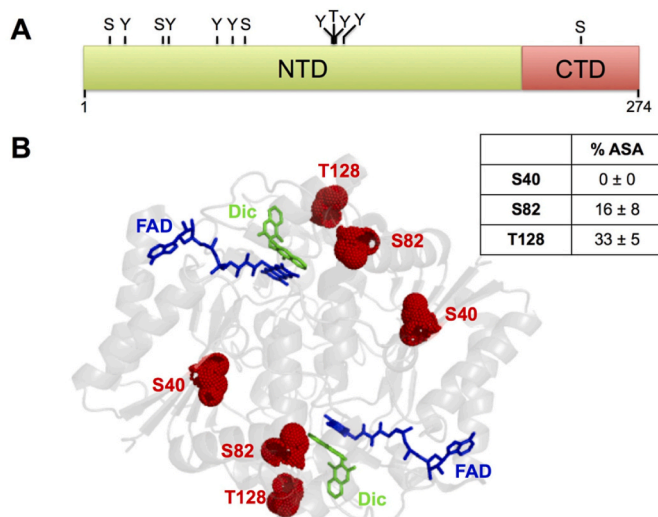
Overall, these studies suggest that phosphorylation of NQO1 at different sites (S40, S82 and T128) may impair the stability of the enzyme inside cells. However, the structural and functional consequences of these phosphorylation events individually on the multifunctional NQO1 are not well known. In this work, we have carried out a comparative and multidisciplinary structure-function analysis of these phosphorylation events using *phosphomimetic* mutations (S40D, S82D and T128D) and *unphosphorylatable* variants (S40A, S82A and T128A).

## 2. Materials and methods

### 2.1. Protein expression and purification

Mutations were introduced by site-directed mutagenesis on the wild-type (WT) NQO1 cDNA cloned into the pET-15b vector (pET-15b-NQO1) by GenScript (Leiden, The Netherlands). Codons were optimized for expression in *E. coli* and mutagenesis was confirmed by sequencing.

Typically, 40 mL of Luria-Bertani (LB) medium containing 0.1 mg mL<sup>-1</sup> ampicillin (Canvax Biotech, Córdoba, Spain) were inoculated with transformed cells and grown for 16 h at 37 °C. These cultures were diluted into 800 mL of LB containing 0.1 mg mL<sup>-1</sup> ampicillin, grown at



**Fig. 1. Phosphorylation sites in human NQO1.** A) Schematic representation of the different phosphorylation sites in the primary sequence of NQO1 (as described in Phosphosite Plus®; <https://www.phosphosite.org/>); B) Structural location of the phosphorylation sites at S40, S82 and T128 investigated in this work (PDB code 2F1O) [24]. The percentage of accessible surface area (% ASA) was determined using GetArea (<http://curie.utmb.edu/getarea.html>) [34] and it is reported as the average ± s.d. from eight monomers found in this crystallographic structure.

37 °C for 3 h to reach an optical density of about 1.0 and then these were transferred to 25 °C and induced with 0.5 mM IPTG (Isopropyl  $\beta$ -D-thiogalactopyranoside, Canvax Biotech). After 6 h, cells were harvested by centrifugation and frozen at -80 °C for 16 h. Cells were resuspended in binding buffer, BB (20 mM Na-phosphate, 300 mM NaCl, 50 mM imidazole, pH 7.4) with 1 mM PMSF (phenylmethylsulfonyl fluoride, Sigma-Aldrich, Madrid, Spain), sonicated in an ice bath, and the extracts were centrifuged (21000 g, 30 min, 4 °C). Supernatants were loaded into IMAC (immobilized-metal affinity chromatography) columns (Ni-Sepharose, Cytiva, Barcelona, Spain), washed with 30 vol of BB and eluted in this buffer containing 500 mM imidazole. These eluates were buffer exchanged using PD-10 columns (Cytiva, Barcelona, Spain) to 50 mM HEPES-KOH, pH 7.4 and stored at -80 °C. 5–10 mg of protein from IMAC were thawed, centrifuged for 10 min at 21000 g and 4 °C and loaded into a HiLoad® 16/60 Superdex® 200 pg (Cytiva, Barcelona, Spain). Size-exclusion chromatography (SEC) was carried out using 20 mM HEPES-NaOH, 200 mM NaCl, pH 7.4 at 20 °C and at a 1.5 mL min<sup>-1</sup> flow rate as described [16]. Fractions containing dimeric NQO1 were concentrated and exchanged to HEPES-KOH 50 mM, pH 7.4 using PD-10 columns. After centrifugation for 20 min at 21000 g at 4 °C, the amount of protein (in NQO1 subunit) and the FAD content was determined from the UV-visible spectrum using:  $\epsilon_{(280)} = 47900 \text{ M}^{-1} \text{ cm}^{-1}$  for NQO1 and  $\epsilon_{(450)} = 11300 \text{ M}^{-1} \text{ cm}^{-1}$  for FAD [25]. Spectra were collected in a Cary 50 spectrophotometer (Agilent Technologies, Waldbronn, Germany) using 0.3 cm path-length quartz cuvettes and ~30  $\mu\text{M}$  of NQO1 in protein subunit. Each NQO1 variant was expressed and analyzed at least three times to check for reproducibility. The purity of NQO1 proteins was evaluated by SDS-PAGE in 12% acrylamide gels (Fig. S1). To obtain apo-proteins, FAD was removed by treatment with 2 M KBr and 2 M urea, 1 mM  $\beta$ -mercapto-ethanol in BB and 1 mM PMSF as previously described [14,25]. Apo-proteins were buffer-exchanged to 50 mM HEPES-KOH, pH 7.4 and contained <3% of FAD bound based on UV-visible spectra. All purified proteins were stored at -80 °C upon flash-freezing in N<sub>2</sub>. For enzyme kinetic studies, purified proteins were mixed with a 1 mM FAD solution in 50 mM HEPES-KOH, pH 7.4 for 10 min at room temperature, and the excess of FAD was removed using PD-10 columns. In all cases, this procedure yielded NQO1 protein with more than 95% FAD bound per NQO1 monomer based on UV-visible spectroscopy.

## 2.2. Titrations with FAD

Fluorescence titrations were carried out at 25 °C using  $1 \times 0.3 \text{ cm}$  path-length cuvettes in a Cary Eclipse spectrofluorimeter (Agilent Technologies). Experiments were carried out in 20 mM sodium phosphate, pH 7.4. 20  $\mu\text{L}$  of a 12.5  $\mu\text{M}$  NQO1 stock solution (in NQO1 subunit) was mixed with 0–500  $\mu\text{L}$  of FAD 10  $\mu\text{M}$  and completed with buffer up to a 1 mL volume. Samples were incubated at 25 °C in the darkness for at least 10 min before measurements. Fluorescence spectra were acquired in the 340–360 nm range upon excitation at 280 nm (slits 5 nm) and spectra averaged over 10 scans using a scan rate of 200 nm min<sup>-1</sup>. Blanks were also measured similarly (containing only buffer) and subtracted. The intensity of the fluorescence at 350 nm ( $I$ ) vs. total concentration of FAD ( $[FAD]$ ) was used to determine the apparent dissociation constant  $K_{d(FAD)}$  using equation (1):

$$I = I_{apo} + (I_{holo} - I_{apo}) \cdot \left( \frac{[NQO1] + [FAD] + K_{d(FAD)} - \sqrt{([NQO1] + [FAD] + K_{d(FAD)})^2 - 4 \cdot [NQO1] \cdot [FAD]}}{2 \cdot [NQO1]} \right) \quad \text{Equation 1}$$

where  $I_{holo}$  and  $I_{apo}$  are the fluorescence intensities of NQO1<sub>holo</sub> and NQO1<sub>apo</sub>, respectively, and  $[NQO1]$  is the total protein concentration (250 nM). This model assumes that FAD binding sites in the NQO1 dimer are equivalent. Data from at least two independent titrations using different preparations of apo-proteins for each NQO1 were used for fittings. The apparent change in binding free energy,  $\Delta G_{(FAD)}$ , was determined using the following equation:  $\Delta G_{(FAD)} = R \cdot T \cdot \ln K_{d(FAD)}$ , where  $R$  is the ideal gas constant (1.987 cal mol<sup>-1</sup> K<sup>-1</sup>) and  $T$  is the absolute temperature (298.15 K) The associated error was determined by linear propagation from the errors of  $K_{d(FAD)}$ .

## 2.3. Partial proteolysis with thermolysin

Thermolysin from *Geobacillus stearothermophilus* (Sigma-Aldrich) was prepared in 50 mM HEPES-KOH, pH 7.4 with 100 mM CaCl<sub>2</sub>. The concentration of thermolysin stock solutions was determined spectrophotometrically using  $\epsilon_{280} = 66086 \text{ M}^{-1} \text{ cm}^{-1}$ . For proteolysis experiments, purified NQO1 proteins were prepared at ~15  $\mu\text{M}$  NQO1 with 100  $\mu\text{M}$  FAD or 100  $\mu\text{M}$  FAD + 100  $\mu\text{M}$  Dic in 50 mM HEPES-KOH, pH 7.4 (all concentrations were final concentrations). Solutions were incubated for 5 min at 25 °C, and reactions were initiated by addition of thermolysin (to 0.1–0.5  $\mu\text{M}$ , final concentration of 10 mM CaCl<sub>2</sub>). Aliquots were withdrawn at different times, mixed with 25 mM EDTA (ethylenediaminetetraacetic acid), pH 8.0 as final concentration, Laemmli's buffer was added in a 1:1 vol ratio and final solutions were denatured at 95 °C for 5 min. Controls without thermolysin were prepared similarly as samples for time zero. Samples were resolved in SDS-PAGE gels (12% in acrylamide), scanned and analyzed by densitometry using ImageJ (<http://rsb.info.nih.gov/ij/>). Time-dependent degradation of the full-length protein was fitted using a single exponential function to yield the first-order kinetic constant  $k_{obs}$ . The second-order rate constant  $k_{prot}$  was determined from  $k_{obs}/[Thermolysin]$ . Changes in the local stability (of native vs. cleavable state) upon mutation  $\Delta\Delta G_{prot(mut-WT)}$  were determined by equation (2) [26]:

$$\Delta\Delta G_{prot(WT-mut)} = R \cdot T \cdot \ln \frac{k_{prot(mut)}}{k_{prot(WT)}} \quad \text{Equation 2}$$

where  $R$  is the ideal gas constant,  $T$  is the absolute temperature (298.15 K) and  $k_{prot(mut)}$  and  $k_{prot(WT)}$  are the second-order rate constants for the mutant and the WT protein, respectively. Errors in  $\Delta\Delta G_{prot(WT-mut)}$  were determined by linear propagation of the fitting errors from the  $k_{prot}$  values.

## 2.4. Thermal denaturation of NQO1 enzymes

Purified NQO1 proteins were prepared at a 2  $\mu\text{M}$  final concentration in subunit in the presence 20  $\mu\text{M}$  of FAD in 50 mM HEPES-KOH, pH 7.4. Samples were loaded into  $3 \times 3 \text{ mm}$  path length quartz cuvettes. Thermal denaturation was carried out in a Cary Eclipse (Varian) spectrofluorimeter equipped with a Peltier element. Samples were equilibrated at 20 °C for 3 min and temperature was increased up to 70 °C at a 2 °C min<sup>-1</sup> scan rate. Fluorescence emission was recorded at 350 nm (slit 10 nm) upon excitation at 280 nm (slit 5 nm). Experimental curves were normalized using pre- and post-transition linear baselines to provide the apparent half-denaturation temperatures ( $T_m$ ). For each variant, 3–6 replicas were prepared and analyzed and  $T_m$  values were presented as

mean  $\pm$  s.d. for each variant.

## 2.5. Hydrogen-deuterium exchange (HDX) monitored by mass spectrometry

Backbone amide hydrogen/deuterium exchange (HDX) was monitored using mass spectrometry as NQO1<sub>apo</sub> and NQO1<sub>holo</sub> forms for WT, S40D, S82D and T128D NQO1 variants, and also as NQO1<sub>dic</sub> for WT and T128D. All procedures including sample preparation, sample measurement and data processing were performed almost identically as described previously [21]. Initial sample concentration was 20  $\mu$ M and in case of holo-proteins, FAD was included at 200  $\mu$ M concentration. Dic state was prepared from the holo-form by adding dicoumarol to final concentration of 200  $\mu$ M. After a 10 min incubation at 25 °C, H/D exchange reaction was initiated by a 10-fold dilution of the protein solution into D<sub>2</sub>O-based 50 mM HEPES-KOH, pD 7.4, 1 mM TCEP (tris (2-carboxyethyl)phosphine). Exchange was thus performed at 2  $\mu$ M protein concentration. Aliquots were taken at 10 s, 30 s, 2 min, 5 min, 20 min, 1 h and 3 h and time points 10 s, 5 m, 3 h were replicated. The reaction was quenched by the addition of 0.5 M Glycine-HCl, pH 2.3 in a 1:1 ratio and flash frozen in liquid N<sub>2</sub>. LC-MS analysis was started by rapid thawing of the sample and injection onto the custom-made coimmobilized nepenthesin-2 (Nep2) and pepsin column. Generated peptides were trapped and desalted on a SecurityGuard™ pre-column (ULTRA Cartridges UHPLC Fully Porous Polar C18, 2.1 mm, Phenomenex, Torrance, CA, USA). The digestion and desalting were driven by 0.4% formic acid (FA) in water pumped by 1260 Infinity II Quaternary pump (Agilent Technologies, Waldbronn, Germany) at a flow rate of 200  $\mu$ L min<sup>-1</sup>. Desalted peptides were then separated on an analytical column (LUNA® Omega Polar C18 Column, 100 Å, 1.6  $\mu$ m, 100 mm  $\times$  1.0 mm, Phenomenex, Torrance, CA, USA) using a linear gradient (10–45% B in 7 min) followed by a quick step to 99% B lasting 5 min, where the solvent A was 0.1% FA/2% acetonitrile (ACN) in water, B was 0.1% FA/98% ACN in water. The gradient was delivered by the 1290 Infinity II LC System (Agilent Technologies, Waldbronn, Germany) at a flow rate 40  $\mu$ L min<sup>-1</sup>. The analytical column was coupled to an ESI source of 15T FT-ICR mass spectrometer (Solarix XR, Bruker Daltonics, Bremen, Germany) operating in broad-band MS mode. Acquired data were exported using DataAnalysis v. 5.3 (Bruker Daltonics, Bremen, Germany), processed by in-house designed software called DeutEx and handled as described previously [21]. Peptide identification was performed using separate LC-MS/MS analyses employing the same UPLC system and settings but coupled to a timsTOF Pro PASEF instrument (Bruker Daltonics, Bremen, Germany). Data were searched by MASCOT (version 2.4, Matrix Science, London, United Kingdom) against a custom-built database containing sequences of the proteases, NQO1 variants and common contaminations as listed in the cRAP database. Decoy search was enabled, false-discovery ratio was set to 1% and ion score cut-off to 20. Fully deuterated proteins were prepared as described previously [21] and correction for back-exchange was applied according to Ref. [27]. HDX results are presented as  $\Delta\%D_{av}$ , a parameter that compares a given segment between two variants in a given ligation state (i.e. S40D<sub>apo</sub> vs. WT<sub>apo</sub>) as the average of the three time points that show maximal difference in percentage of incorporated deuterium (% D) along a time course [21].

The HDX-MS data have been deposited to the ProteomeXchange Consortium via the PRIDE [28] partner repository with the dataset identifier PXD034600.

## 2.6. Pre-steady state enzyme kinetic analysis

Hydride-(HT) or deuteride-(DT) transfer reactions from NADH/D to NQO1 were carried out under anaerobic conditions using a stopped-flow spectrophotometer (SX.18 MV, Applied Photophysics Ltd., Leatherhead, UK) interfaced with a photodiode array detector, essentially as described [16]. Reactions were performed in 20 mM HEPES-KOH, pH 7.4. The

reductive half-reaction was measured by mixing NQO1<sub>holo</sub> variants (7.5  $\mu$ M) with NADH ranging from 7.5 to 100  $\mu$ M. Multiple wavelength absorption data in the flavin absorption region were collected and processed as described [16]. Time-dependent spectral deconvolution was performed by global analysis and numerical integration methods using previously described procedures [16]. Deconvolution was carried out considering sequential and irreversible steps in the context of two (A $\rightarrow$ B $\rightarrow$ C) or three (A $\rightarrow$ B $\rightarrow$ C $\rightarrow$ D) step mechanisms, were A-D are spectral species, and allowed to determine observed rate constants ( $k_{obs}$ ) for these steps as well as spectroscopic properties of intermediate A to D species. According to a recent study, catalytically relevant processes involved steps A $\rightarrow$ B (*Fast*) and B $\rightarrow$ C (*Slow*) [16]. Hyperbolic dependences of  $k_{obs}$  vs. NADH concentrations were fitted using equation (3):

$$k_{obs} = \frac{k_{HT} \cdot [NADH]}{K_{d(NADH)} + [NADH]} \quad \text{Equation 3}$$

where  $k_{HT}$  is the limiting rate constant for HT and  $K_{d(NADH)}$  is the equilibrium dissociation constant to a given active site.

Primary KIEs in the HT process were estimated as previously reported [16] in samples containing equimolar mixtures (7.5  $\mu$ M of each component) by evaluating HT or DT  $k_{obs}$  ( $k_{obsHT}$  or  $k_{obsDT}$ ) from NADH and [4R-<sup>2</sup>H]-NADD to NQO1<sub>ox</sub> at different temperatures in the 6–20 °C range. Estimation of the activation parameters (frequency factor, A, and the activation energy, E<sub>a</sub>) were determined using the Arrhenius equation as described [16].

## 2.7. Expression analyses in eukaryotic cells

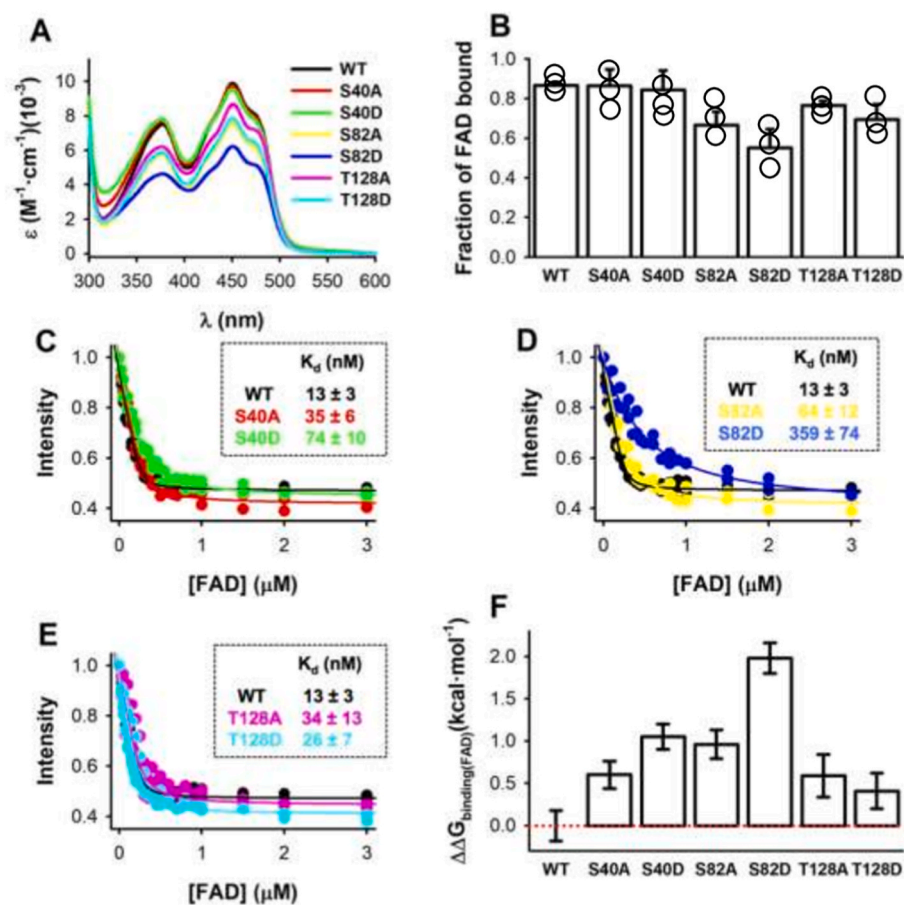
Site-directed mutagenesis to S40A, S40D, S82A, S82D, T128A or T128D was carried out on the wild-type (WT) NQO1 cDNA cloned into the pCI-NEO plasmid by GenScript (Leiden, The Netherlands). Mutagenesis was confirmed by sequencing the entire cDNA.

HAP1 NQO1 knockout cells (HAP1 NQO1-KO; Horizon, Waterbeach, UK) were cultured in Iscove's modified Dulbecco's medium (IMDM, Lonza, Barcelona, Spain) supplemented with 10% fetal bovine serum (HyClone, GE Healthcare, Barcelona, Spain), 100 U mL<sup>-1</sup> penicillin and 100  $\mu$ g mL<sup>-1</sup> streptomycin (Sigma Aldrich, Madrid, Spain) and cultured at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. Cells were transfected using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific, Madrid, Spain) and selected using 1 mg mL<sup>-1</sup> of G418 (Sigma Aldrich). For proteasomal inhibition studies, MG-132 (Calbiochem, Merck, Madrid, Spain) was added to the medium at 10  $\mu$ g mL<sup>-1</sup> for 6 h at 37 °C. After treatment with or without MG-132, cells were scrapped and lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, 0.1% sodium dodecyl sulphate, 1 mM sodium orthovanadate, 1 mM NaF, pH 8.0) with protease inhibitors (COMPLETE, from Roche, Spain). Soluble extracts were denatured using Laemmli's buffer, resolved by SDS-PAGE and transferred to Polyvinylidene difluoride (PVDF) membranes (GE Healthcare). Immunoblotting was carried out using primary monoclonal antibodies anti-NQO1 (sc-393736, Santa Cruz Biotechnology, Dallas, TX, USA) and  $\beta$ -actin (sc-47778, Santa Cruz Biotechnology) from mouse at 1:200 and 1:5000 dilutions, respectively. As a secondary antibody, we used anti-mouse IgG-HRP (sc-516102, Santa Cruz Biotechnology) at 1:2000 dilution. Samples were visualized using luminol-based enhanced chemiluminescence (from BioRad Laboratories, Hercules, CA, USA) and analyzed using Image Lab (from BioRad Laboratories). NQO1 levels were normalized using the levels of  $\beta$ -actin.

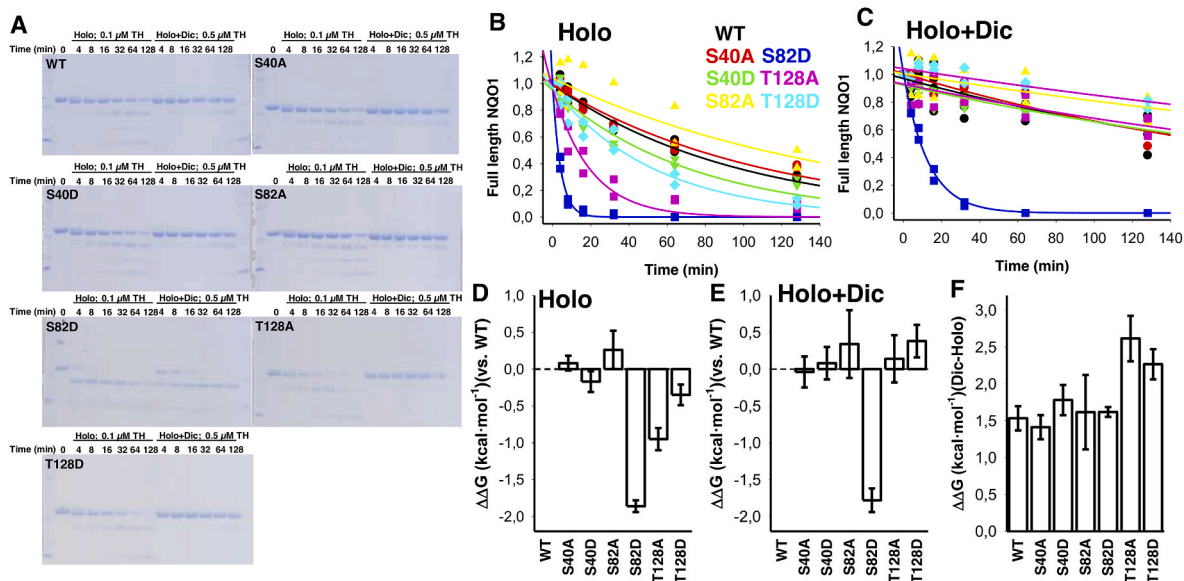
## 3. Results and discussion

### 3.1. Effect of phosphomimetic mutations on FAD binding

We produced and purified WT, S40A/D, S82A/D and T128A/D variants of human NQO1 (Fig. S1). All variants seemed well-folded, as



**Fig. 2. FAD content and binding affinity.** A) UV-vis spectra normalized per NQO1 monomer. B) The fraction of FAD bound determined from the spectra shown in A and considering  $\epsilon_{450} = 11300 \text{ M}^{-1} \text{ cm}^{-1}$  for NQO1 fully saturated with FAD. Data were the mean  $\pm$  s.d. from at least three independent purifications of each variant. Experiments in A-B were performed using  $\sim 30 \mu\text{M}$  NQO1 monomer in 50 mM HEPES-KOH pH 7.4. C-E) Titrations of apo-proteins with FAD.  $K_d$  values were estimated from simultaneous fitting of data from 2 to 4 independent titrations for each variant; Titrations were carried out using 250 nM NQO1 monomer in 20 mM sodium phosphate, pH 7.4 at 25 °C; F) Changes in FAD binding free energy (mutant-WT). Errors are those from linear propagation from those in  $K_d$  values.



**Fig. 3. Stability of the NTD evaluated by partial proteolysis.** A) SDS-PAGE analysis of proteolysis kinetics with thermolysin. B-C) Proteolysis kinetics of NQO1 proteins. Data are from at least 2 independent experiments for each NQO1 variant. D-E) Effect of different mutation on the thermodynamic stability of the cleavage site vs. WT ( $\Delta\Delta G = -R \cdot T \cdot \ln \frac{k_{mut}}{k_{WT}}$ ) as NQO1<sub>holo</sub> (D) and NQO1<sub>dic</sub> (E). F) Effect of Dic binding on the thermodynamic stability of the cleavage site for each variant ( $\Delta\Delta G = -R \cdot T \cdot \ln \frac{k_{dic}}{k_{holo}}$ ). Experiments were carried out using  $\sim 15 \mu\text{M}$  NQO1 with 100  $\mu\text{M}$  FAD or 100  $\mu\text{M}$  FAD +100  $\mu\text{M}$  Dic in 50 mM HEPES-KOH 10 mM CaCl<sub>2</sub>, pH 7.4 at 25 °C and the thermolysin concentrations indicated. Errors in panels D-F are those linealy propagated from those of the second-order rate constants  $k_{prot}$ .

indicated by size-exclusion chromatography (SEC) and circular dichroism (CD) spectroscopy (Fig. S1). We then carried out a detailed *in vitro* characterization of their functional properties. First, to test whether phosphomimetic mutations may affect FAD binding affinity, we measured the FAD content in NQO1 protein samples as purified, that qualitatively correlates with FAD binding affinity [25,29–31]. The most noticeable decrease in FAD content was found for S82D, whereas this decrease was milder in T128D (Fig. 2A and B). It must be noted that the mutated sites are not in close proximity to the FAD molecule: the minimal distance from S40 is of 14.5 Å, for S82 is 14 Å and for T128 is 12 Å (using the structure with PDB code 2F1O) [24] that supports long-range propagation of mutational effects to the FBS. To provide quantitative measurements on FAD binding affinity, we then prepared all variants in the NQO1<sub>apo</sub> state and carried out titrations with FAD monitored by fluorescence spectroscopy (Fig. 2C–F). The mutant S82D showed the largest effect, with a ~30-fold decrease in binding affinity (~2.0 kcal mol<sup>-1</sup> in binding free energy), whereas the effects of S40D and T128D were milder (6-fold and 2-fold, corresponding to changes in binding free energy of ~1.1 kcal mol<sup>-1</sup> and ~0.4 kcal mol<sup>-1</sup>, respectively). These results highlight that the proximity of the phosphorylation site to the FBS or the solvent accessibility of the phosphorylated site does not solely determine their impact on FAD binding affinity. Since FAD binding affinity and intracellular flavin availability may be important for NQO1 intracellular stability [10], these analyses suggest that different phosphomimetic mutations may cause different response to riboflavin supplementation in terms of intracellular stability and activity.

### 3.2. Effect of the phosphomimetic mutations on the local stability of the NTD

Proteolysis by thermolysin is a useful tool for evaluating the effect of mutations and ligand binding on the thermodynamic local stability of the NTD, in particular due to initial cleavage between S72–V73 (the thermolysin cleavage site, TCS) [13]. Among the phosphomimetic mutants investigated, the most noticeable effect was found for S82D that destabilized by 2 kcal mol<sup>-1</sup> both in the NQO1<sub>holo</sub> and NQO1<sub>dic</sub> states (Fig. 3). The effects of S40D and T128D were much weaker, and in the case of T128D, Dic binding essentially abolished the 0.5 kcal mol<sup>-1</sup> destabilization caused by this mutation in the TCS of the NQO1<sub>holo</sub> state. Thus, destabilization of the TCS correlated well with the effect of the phosphomimetic mutations on FAD binding affinity on the NQO1<sub>holo</sub> state. It is interesting to note that the control variant T128A displays larger sensitivity towards proteolysis than T128D in the NQO1<sub>holo</sub> state, suggesting that at this residue, a decrease in side-chain causes more significant perturbations on the local stability of the NTD than introduction of a negative charge.

### 3.3. Effects of phosphomimetic mutations on conformational stability

The thermal stability of NQO1 provides information on the stability of the monomers and the MMI in the dimer, since the NQO1 dimers undergoes unfolding and dissociation prior to the rate-limiting step of irreversible denaturation [30]. We thus evaluated the effects of phosphomimetic mutations on the conformational stability as NQO1<sub>holo</sub> proteins (Fig. 4). The largest effects were observed for the mutations S40D and S82D, that decreased by ~7 °C the thermal stability of the enzyme. It is interesting to note that denaturation of S82D showed a wider profile, possibly affecting the denaturation mechanism (e.g. this mutation may affect the *coupling* between domains in the irreversible denaturation process). Consequently, the S40D and S82D mutations may destabilize the NQO1 monomer and/or the MMI in the dimer.

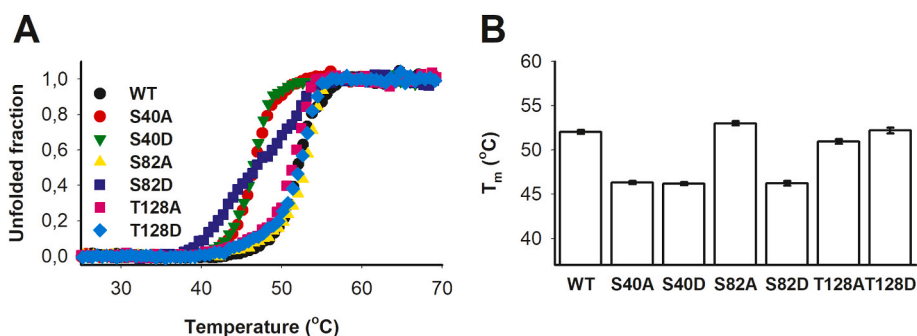
### 3.4. Effects of phosphomimetic mutations on the local stability from HDX

Hydrogen-deuterium exchange (HDX) has been proven to provide high-resolution information on the local stability effects on NQO1 due to mutations and ligand binding, including long-range propagation of these effects across the structure [18,21]. Since the EX1 regime was marginally detected (Fig. S2), it is reasonable to interpret our HDX data using a local thermodynamic stability approach (i.e. EX2) and, in this context, little information on protein dynamics can be inferred.

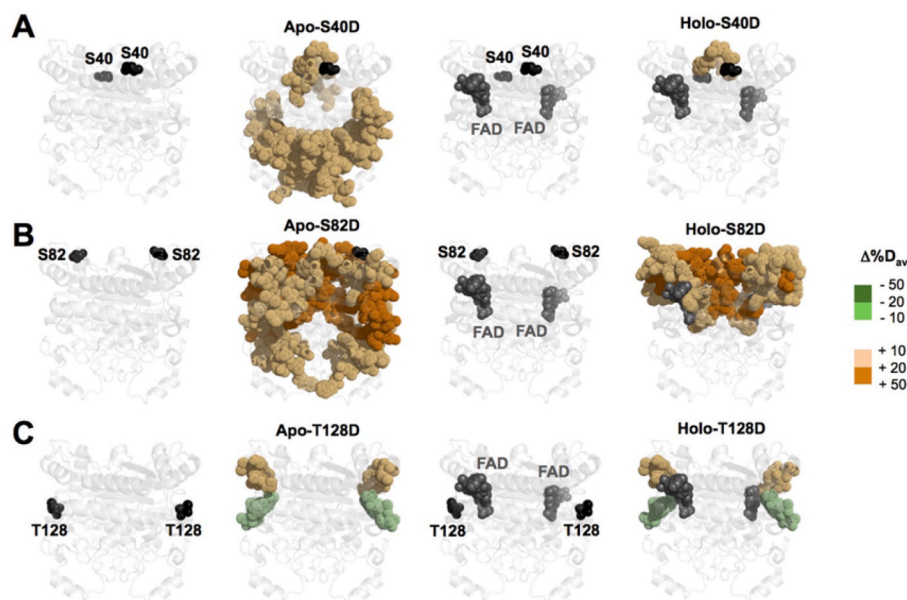
Despite their different solvent accessibilities (% ASA; S40 < S82 < T128), the impact of phosphomimetic mutations on the local stability of the protein, as determined by HDX, did not correlate with this feature and was strongly dependent on the ligation state (Fig. 5). The S40D mutant showed weak local destabilizing effects based on an EX2 mechanism, and also mildly affected residues of the CTD in the NQO1<sub>apo</sub> state (Figs. S2 and S3). HDX analyses also allow to suggest that, despite their solvent-accessibility in the crystal structure, these residues are exposed to the solvent (i.e. accessible for phosphorylation) due to local thermodynamic fluctuations (Fig. S2).

In the NQO1<sub>apo</sub> state, the destabilizing effects of S40D localized in the vicinity of the mutated residue and the CTD, whereas FAD binding restricted these effects to the local environment of S40 (Fig. 5A and Figs. S3–S4). The effects of S82D extended to almost the entire structure of NQO1<sub>apo</sub> and were of mild-to-moderate intensity (Fig. 5B and Figs. S3–S4). FAD binding restricted these destabilizing effects more locally around the mutated site. The effects of the T128D mutation were very local, destabilizing residues comprising 69–76, while stabilizing residues 126–131 in both the NQO1<sub>apo</sub> and NQO1<sub>holo</sub> states (Fig. 5C and Figs. S3–S4).

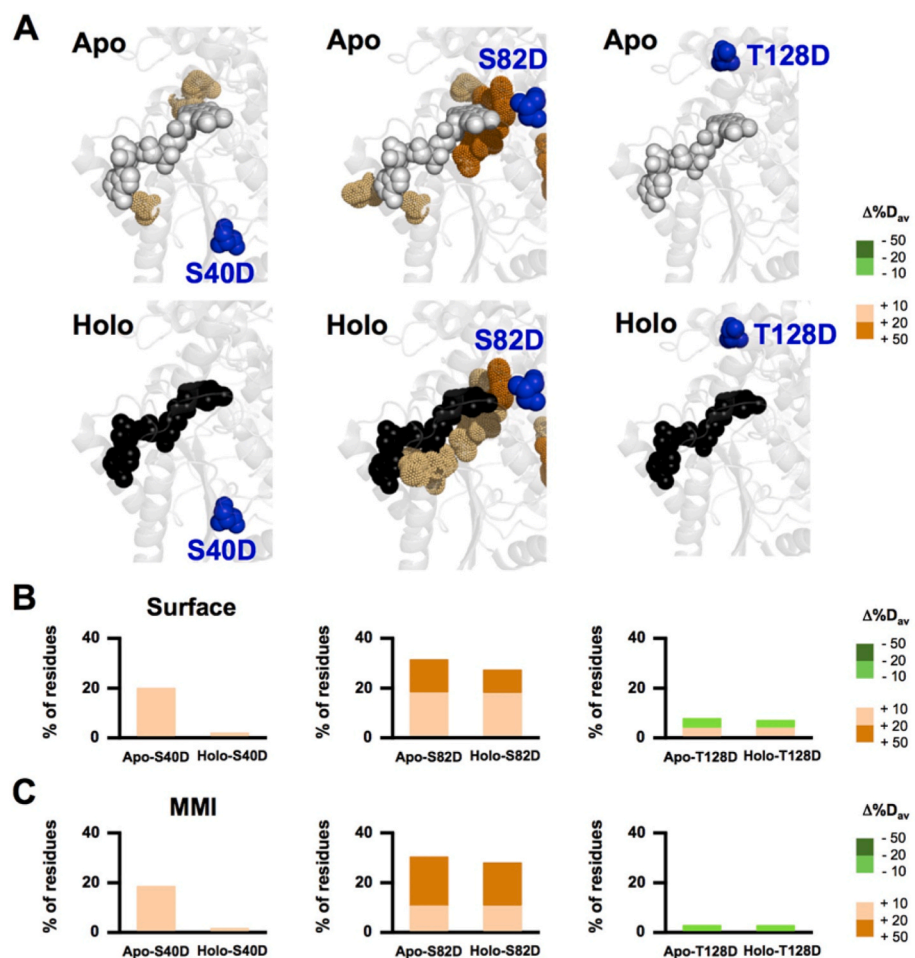
We then used our HDX studies to investigate different functional sites, namely the residues involved in FAD binding (i.e. the FBS, with implications for FAD binding affinity)(Fig. 6A), solvent-exposed residues (that could be involved in PPI)(Fig. 6B) and the MMI (whose stability is associated with the thermal stability of the enzyme)(Fig. 6C).



**Fig. 4. Thermal stability of NQO1 variants.** A) Thermal denaturation profiles of NQO1<sub>holo</sub> variants as monitored by fluorescence spectroscopy. B) Half-denaturation temperatures ( $T_m$ ) from at least three different technical replicas (mean  $\pm$  s.d.). Experiments were carried out using 2  $\mu$ M NQO1 in the presence 20  $\mu$ M of FAD in 50 mM HEPES-KOH, pH 7.4 and using a scan rate of 2 °C min<sup>-1</sup>.



**Fig. 5. Overall effects of phosphomimetic mutations on the local stability by HDXMS.** A-C) Effect of the mutations S40D (A), S82D (B) and T128D (C) on the local stability of the NQO1<sub>apo</sub> and NQO1<sub>holo</sub> states. Changes in stability (as  $\Delta\%D_{av}$  using the WT protein in a given ligation state as a reference) are colored according to the scale indicated in the right side of the figure (green tones indicate stabilization whereas orange tones indicate destabilization). Structural display was carried out using the structure with PDB code 2F1O [24]. Experiments were carried out using 20  $\mu$ M of apo-protein (NQO1<sub>apo</sub>) or purified protein with 200  $\mu$ M of FAD (NQO1<sub>holo</sub>) and the H/D exchange reaction was initiated by a 10-fold dilution of the protein solution into D<sub>2</sub>O-based 50 mM HEPES-KOH, pD 7.4, 1 mM TCEP at 25 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 6. Effect of phosphomimetic mutations on the local stability of the FBS, protein surface and the MMI.** A-C) Effect of the mutations S40D, S82D and T128D mutation on the stability of the FBS in the NQO1<sub>apo</sub> and NQO1<sub>holo</sub> states. B) Percentage of residues in the protein surface (considering those with at least 20% solvent accessibility the software GETAREA; <http://curie.utmb.edu/getarea.html>; (7)); C) Effect of phosphomimetic mutations on the stability of residues belonging to the MMI (considering those determined according to Ref. [21]). Changes in stability (as  $\Delta\%D_{av}$  using the WT protein as a reference) are colored according to the scale indicated in the right side of the figure (green tones indicate stabilization whereas orange tones indicate destabilization). Structural display and calculations were carried out using the structure with PDB code 2F1O [24]. Experiments were performed as indicated in the legend of Fig. 5. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Regarding the stability of the FBS, the mutation S82D caused large changes in stability of the FBS, both in the NQO1<sub>apo</sub> and NQO1<sub>holo</sub> states, thus explaining its large effect on FAD binding affinity (Figs. 2 and 6A). The mutation S40D only decreased the stability of small set of residues

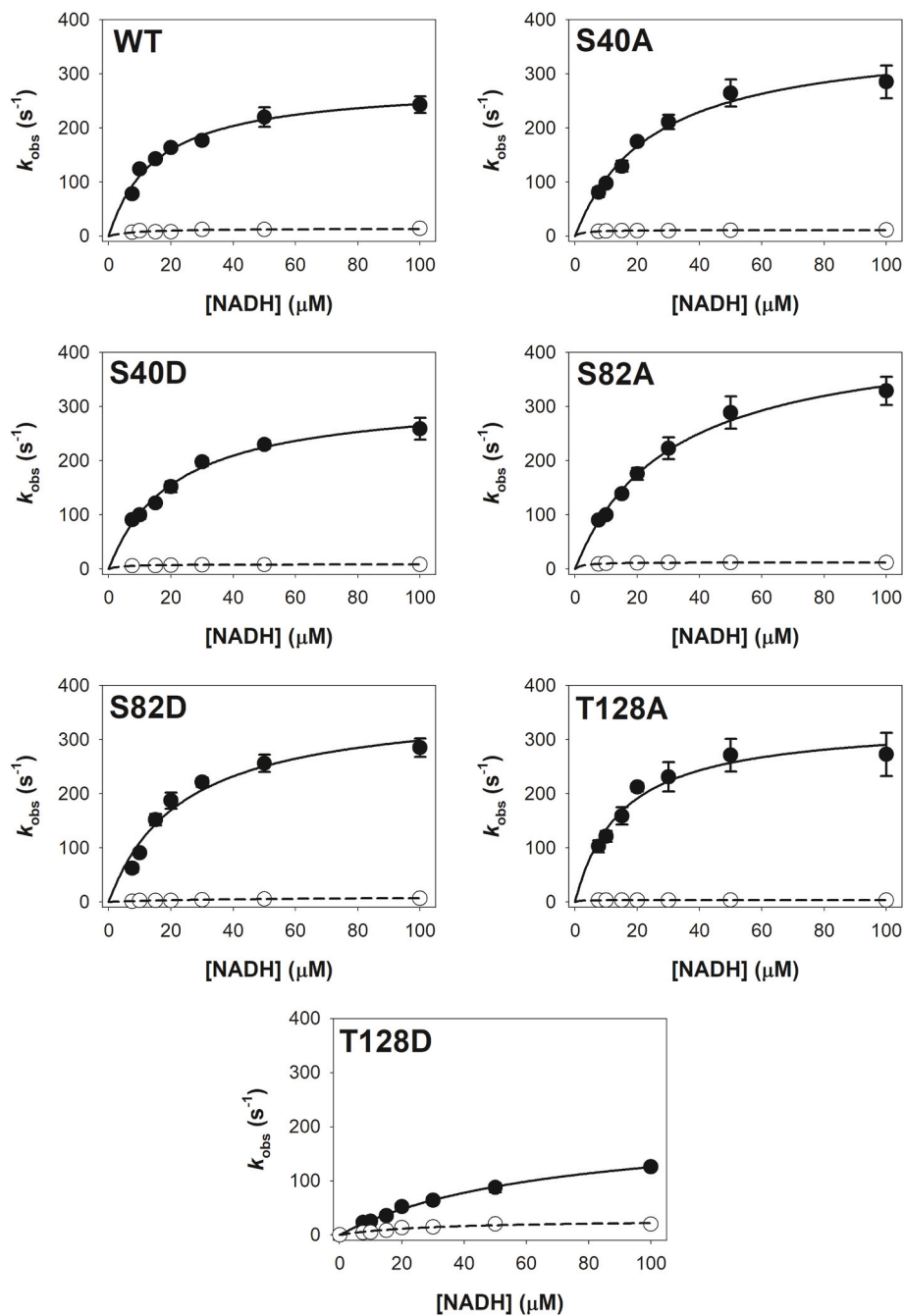
(A21, Y156 and H162) of the FBS in the NQO1<sub>apo</sub>, thus explaining its milder effect on FAD binding affinity (Figs. 2 and 6A). The lack of noticeable effects of the T128D on the stability of the FBS explains its marginal effect on FAD binding affinity vs. the WT protein (Figs. 2 and

6A).

Residues at the surface of NQO1 may be important for PPI. When we analyzed the effects of the phosphomimetic mutations S40D, S82D and T128D on the stability of solvent-exposed regions of the protein, we also observed different behaviors within the phosphomimetic mutants (Fig. 6B). In the NQO1<sub>apo</sub> state, the mutation S40D reduced the stability of about 20% of solvent-exposed residues, whereas upon FAD binding, this effect was virtually abolished (Fig. 6B). The mutation S82D affected the stability of about 30% solvent-exposed residues in both the NQO1<sub>apo</sub> and NQO1<sub>holo</sub> states (Fig. 6B). The impact of the T128D mutation on solvent-exposed residues was minimal, with mild stabilizing and destabilizing effects (Fig. 6B).

Thermal stability analyses on NQO1<sub>holo</sub> proteins revealed significant

destabilization due to S40D and S82D phosphomimetic mutations (Fig. 4). We then used our HDX studies to determine whether changes in the stability of the MMI correlated with thermal destabilization (Fig. 6C, S5-S6). In the case of S40D, a large destabilization of the MMI is observed in the NQO1<sub>apo</sub> state. For S82D, the MMI is largely destabilized in both the NQO1<sub>apo</sub> and NQO1<sub>holo</sub> states. Changes in the MMI stability were minimal for T128D, consistent with its thermal stability being close to that of the WT protein. Therefore, the large effects of S40D and S82D mutations on thermal stability correlated well with their effect on the stability of the MMI.



**Fig. 7.** Effect of phosphomimetic mutations on the reductive half-reaction of FAD using NADH. Data correspond to the fast reduction (closed circles) and slow reduction (open circles) steps and are displayed as mean  $\pm$  s.d. from three replicates. Lines are fits using equation (3). Fitting parameters are compiled in Table 1. Experiments were carried out using 7.5  $\mu$ M of NQO1<sub>holo</sub> in 50 mM HEPES-KOH, pH 7.4 and 6  $^{\circ}$ C.



**Table 1**

Kinetic parameters for the NADH-dependent reduction of FAD.

Variant	Fast reduction			Slow reduction		
	$k_{HT}$ ( $s^{-1}$ )	$K_d$ (NADH) ( $\mu M$ )	$k_{HT}/K_d(NADH)$ ( $s^{-1} \cdot \mu M^{-1}$ )	$k_{HT}$ ( $s^{-1}$ )	$K_d$ (NADH) ( $\mu M$ )	$k_{HT}/K_d(NADH)$ ( $s^{-1} \cdot \mu M^{-1}$ )
WT	281 $\pm 14$	15 $\pm$ 2	19 $\pm$ 3	14 $\pm$ 2	8.2 $\pm$ 3.6	1.7 $\pm$ 1.0
S40A	372 $\pm 22$	25 $\pm$ 4	15 $\pm$ 3	12 $\pm$ 1	2.3 $\pm$ 0.3	5.2 $\pm$ 1.1
S40D	320 $\pm 13$	21 $\pm$ 2	15 $\pm$ 2	8.6 $\pm$ 0.3	3.8 $\pm$ 0.5	2.3 $\pm$ 0.4
S82A	441 $\pm 19$	30 $\pm$ 3	15 $\pm$ 2	12 $\pm$ 1	2.3 $\pm$ 0.2	5.2 $\pm$ 0.5
S82D	368 $\pm 33$	23 $\pm$ 5	16 $\pm$ 5	10 $\pm$ 1	43 $\pm$ 8	0.23 $\pm$ 0.06
T128A	333 $\pm 21$	15 $\pm$ 3	22 $\pm$ 6	3.5 $\pm$ 0.1	1.1 $\pm$ 0.5	3.2 $\pm$ 1.5
T128D	210 $\pm 12$	67 $\pm$ 7	3.1 $\pm$ 0.5	28 $\pm$ 4	29 $\pm$ 10	1.0 $\pm$ 0.5

### 3.5. Effects of phosphomimetic mutations on the reductive half-reaction of NQO1

The reduction of the two flavin molecules within the NQO1 dimer occurs through pathways with very different rates (*fast* and *slow* steps) [16]. We have evaluated the effect of phosphomimetic mutations on these two pathways by evaluating rates for HT from NADH by stopped-flow measurements (Fig. 7 and Table 1). Regarding the *fast* pathway of FAD reduction, most of the mutants did not affect significantly the  $k_{HT}$  or  $K_d(NADH)$  values, although the mutation T128D reduced by 4.5-fold the affinity for NADH and by 6-fold the catalytic efficiency (Table 1). In the case of the *slow* reduction pathway, the mutation S82D reduced the affinity for NADH by 5-fold and the catalytic efficiency by 7-fold (Table 1). The mutation T128D increased both the  $k_{HT}$  and  $K_d(NADH)$  for the slow step leading to little changes in catalytic efficiency (Table 1). Therefore, the functional cooperativity observed for the WT enzyme is affected differently by the phosphomimetic mutations S82D and T128D (about 4-fold increase and 6-fold decrease, respectively, as the ratio of the catalytic efficiency of the *fast/slow* steps vs. that of WT) (Table 1). It is interesting to note that based on HDX data ( $\Delta\%D_{av}$ ), when we compare the NQO1<sub>holo</sub> and NQO1<sub>dic</sub> states of T128D and the WT proteins, some changes in the DBS (but not the FBS) are found for some residues, with different effects on these two states (Pro69 showed decreased stability whereas Tyr127, Tyr129 and Met132 were moderately stabilized)(Figs. S7–S9).

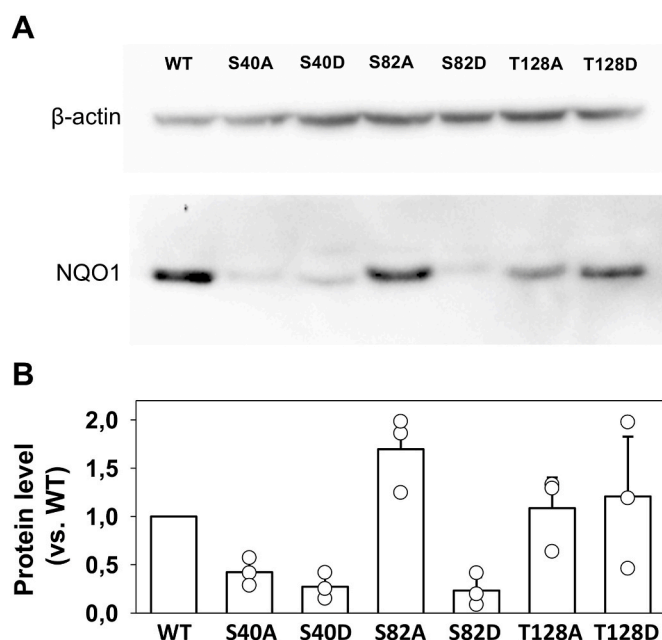
These effects of the mutations S82D and T128D on the enzyme catalytic efficiency for the fast and slow HT steps are likely associated with structural or energetic changes in the active site along the enzyme reaction coordinate. We have recently shown changes in the local stability of the active site (the FBS and DBS) in the NQO1<sub>holo</sub> and NQO1<sub>dic</sub> states due to the mutation S82D, particularly destabilization of these functional sites in the former state [18]. Here, we have carried out similar experiments with the mutant T128D (Figs. S7–S9). The mutation T128D affected locally and differently the NQO1<sub>holo</sub> and NQO1<sub>dic</sub> states. T128D mildly reduced the stability of a small set of residues in the vicinity of the NQO1<sub>holo</sub> active site (residues 69–74) whereas its effects were stabilizing, stronger and more extensive in the NQO1<sub>dic</sub> active site (affecting residues 69–74 and 122–132). A differential analysis of these effects on the local stability of the active site between NQO1<sub>dic</sub> and NQO1<sub>holo</sub> clearly showed that the overall effect of the T128D mutation on the active site stability is moderately stabilizing (Fig. S9). Considering that changes in the local stability of the active site between NQO1<sub>holo</sub> and NQO1<sub>dic</sub> states might reflect protein stability changes along the reaction coordinate for the HT process [16,18], our results suggest that the mechanisms associated with altered catalytic efficiency of S82D and T128D are different in structural and energetic terms. These results also

suggest that site-specific phosphorylation could affect the catalytic efficiency of NQO1 by altering the balance between structural, energetic and dynamic changes along the HT reaction.

To assess the impact of mutations on the dynamics in the active site of NQO1 along its catalytic function, we analyzed HT by using NADH and NADD at different temperatures (Figs. S10–S11). For all variants, *fast* and *slow* reduction steps were slowed down with NADD (Table S1), in general resulting, as reported for the WT, in kinetic isotope effects (KIEs) in the range 1.5–2.2 at the lower temperature evaluated. It is only worth noting a slight increase in KIE for the *fast* process of T128D and the lack of KIE for the *slow* process of T128A. KIEs for the *fast* process are essentially temperature-independent, as for the WT, with the value only slightly decreasing with temperature for S82A (Fig. S11, Table S1). Regarding the *slow* FAD reduction step, only the WT and S40D KIEs were temperature-independent. KIEs particularly decreased with temperature for S40A, S82A and S82D and slightly increased when mutating T128 (Fig. S11). This indicates that mutations hardly alter transitions under the energy barrier and tunnelling of both proton and deuterium in the *fast* HT step, but have a long-distance modulating effect in the *slow* step [16,18]. Analysis of data in the context of Arrhenius theory [activation energies ( $E_a$ ) and pre-exponential factors (A)] indicated that for the *fast* step of all variants *passive dynamics* is the main source to achieve tunnel ready conformations (Fig. S10). The largest differences were found for *slow* steps. Particularly in S40A, S82A, S82D and T128A that showed remarkable changes in A values (orders of magnitude) and in  $E_a$ , *gating* (donor-acceptor-distance sampling) possibly contributes to engage more favourable conformations for a possible tunneling. Thus, replacements at sites S40, S82 and T128 particularly modulate the overall packing and general dynamics of the *slow* active site of NQO1 during HT, and therefore, also contribute to modulate cooperativity in the reductive half-reactions of both active sites in the protein dimer.

### 3.6. Effects of phosphomimetic mutations on steady-state protein levels upon expression in eukaryotic cells

To evaluate the effect of phosphomimetic mutants on the



**Fig. 8.** Effect of phosphomimetic mutations on the NQO1 steady-state protein levels upon expression in stably-transfected HAP-1 KO cells. A) Representative Western-blot analyses; B) Protein levels expressed (considering WT as 1). Data are from three independent transfection experiments (mean  $\pm$  s. d.) and were normalized by the levels of  $\beta$ -actin.

intracellular protein levels, we transfected HAP-1 KO cells with plasmids encoding WT as well as mutant forms of NQO1 (Fig. 8 and Figs. S12–18). Under these conditions, we considered that steady-state protein levels were determined to some extent by proteasomal degradation of the NQO1 variants (Figs. S12–S15) in agreement with previous studies [2, 10, 18, 32]. The behaviors observed were clearly site-specific. At the S40 site, either S40A or S40D mutants showed lower protein levels (about 30–40% of WT levels), suggesting that even a small perturbation at this site (e.g. S → A) causes significant perturbation of NQO1 intracellular stability. This result suggests that non-conservative mutations S40L and S40W (very rare mutations identified in large-scale sequencing projects of the human genome; i.e. gnomAD database) have dramatic effects on NQO1 intracellular stability. At the S82 site, only the S82D mutant showed decreased protein levels (about 20% of WT), while mutations at the T128 site had little or no effect on protein levels under these conditions. Therefore, our results support that phosphorylation at S40 and S82 sites could lead to reduced protein levels due to accelerated NQO1 degradation.

#### 4. Conclusions

Protein phosphorylation is the most ubiquitous post-translational modification and serves to rapidly modify protein functionality [33]. In the particular case of human flavoproteins, our knowledge on the effects of site-specific phosphorylation is largely unknown [22]. In this work, we have carried out a comparative study of three phosphorylation sites (S40, S82 and T128) in human NQO1, a multifunctional flavoprotein whose dysregulation is associated with cancer and neurological disorders [1, 2]. To this end, we used phosphomimetic mutation (S40D, S82D and T128D). These phosphorylation sites display different solvent accessibilities in crystallographic structures, although these solvent accessibilities do not correlate with their impact on the structure, stability and function of NQO1 (Table 2). For instance, the phosphomimetic

mutation S40D affects a fully buried serine residue in the protein crystal structure, and causes noticeable effects on *in vitro* and intracellular stability but mild effects on FAD binding affinity. The mutation S82D, affecting a more solvent-exposed residue, affects the conformational and intracellular stabilities, FAD binding and enzyme catalysis of NQO1, and its destabilizing effects propagate more extensively through the protein structure than any of the other sites. The effects of T128D are quite subtle, only causing local stability effects (stabilizing and destabilizing) in the vicinity of the mutated residue that moderately affects enzyme catalysis and cooperativity without a clear effect for the overall *in vitro* or intracellular stability in HAP-1 cells. It is important to realize that our extensive structure-function analyses were carried out using *pseudo-phosphorylating* mutations to isolate the effects of a given individual site. At this point, we realize that a full view of phosphorylation dynamics must include the characterization of a more extended set of phosphorylation sites, the kinases/phosphatase involved, the potential interaction between sites and the parallel or sequential phosphorylation state of different sites [2, 22]. We anticipate that our experimental approach will help to improve our knowledge on this challenging task, and would be applied to investigate other human flavoproteins.

#### CRediT author statement

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**Table 2**

**Summary of the effects of phosphomimetic mutations.** For each protein variant and feature, these were clustered for semiquantitative comparison in four categories: (++++) mildly improved vs. WT; (++++) WT-like; (++) mildly-moderately impaired vs. WT; (+) largely impaired vs. WT.

Protein feature	NQO1 variant						
	WT	S40A	S40D	S82A	S82D	T128A	T128D
Conformation <sup>a</sup>	+++	+++	+++	+++	+++	+++	+++
FAD bound <sup>b</sup>	+++	+++	+++	++	++	+++	++
FAD binding affinity <sup>c</sup>	+++	+++	++	++	+	+++	+++
Proteolysis Holo <sup>d</sup>	+++	+++	+++	+++	+	++	+++
Proteolysis response to Dic <sup>e</sup>	+++	+++	+++	+++	+++	++++	++++
Thermal stability <sup>f</sup>	+++	+	+	+++	+	+++	+++
Global HDX Apo <sup>g</sup>	+++	N.Det.*	++	N.Det.*	+	N.Det.*	+++
Global HDX Holo <sup>h</sup>	+++	N.Det.*	+++	N.Det.*	+	N.Det.*	+++
FBS HDX <sup>i</sup>	+++	N.Det.*	+++	N.Det.*	+	N.Det.*	+++
MMI HDX <sup>j</sup>	+++	N.Det.*	++	N.Det.*	+	N.Det.*	+++
Fast FAD reduction <sup>k</sup>	+++	+++	+++	+++	+++	+++	+
Slow FAD reduction <sup>l</sup>	+++	++++	++	++++	++	++	++
Cooperativity <sup>m</sup>	+++	++++	+++	++++	+	+++	++
Intracellular abundance <sup>n</sup>	+++	+	+	+++	+	+++	+++

\* N. Det.: not determined.

<sup>a</sup> Based on SEC and CD spectroscopy (Fig. S1).

<sup>b</sup> Based on visible absorption spectroscopy (Fig. 2A and B).

<sup>c</sup> Based on FAD titrations (Fig. 2C–E).

<sup>d</sup> Based on proteolysis kinetics as holo-proteins (Fig. 3B and D).

<sup>e</sup> Based on the effects of Dic on proteolysis kinetics (Fig. 3D–F).

<sup>f</sup> Based on the effects on thermal stability (Fig. 4).

<sup>g</sup> Based on global HDX for the apo-state (Fig. 5A–C).

<sup>h</sup> Based on global HDX for the holo-state (Fig. 5A–C).

<sup>i</sup> Based on HDX results regarding the FBS (Fig. 6A).

<sup>j</sup> Based on HDX results regarding the MMI (Fig. 6C).

<sup>k</sup> Based on the results for the fast reduction pathway, including  $k_{HT}$ ,  $K_{d(NADH)}$  and  $k_{HT}/K_{d(NADH)}$  (Table 1).

<sup>l</sup> Based on the results for the slow reduction pathway, including  $k_{HT}$ ,  $K_{d(NADH)}$  and  $k_{HT}/K_{d(NADH)}$  (Table 1).

<sup>m</sup> Based on the results ( $k_{HT}/K_{d(NADH)}$ ) for the fast/slow reduction pathways (Table 1).

<sup>n</sup> Based on Western-blot analysis of transfected cells grown without MG-132 (Fig. 8).

curation, formal analysis, funding acquisition, methodology, resources, validation, visualization, software, supervision, writing – review & editing. **Milagros Medina.**- Data curation, formal analysis, funding acquisition, methodology, resources, validation, visualization, software, supervision, writing – review & editing. **Rogelio Palomino-Morales.**- Data curation, formal analysis, methodology, resources, validation, visualization, supervision, writing – review & editing. **Angel L. Pey.**- Conceptualization, Data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, validation, visualization, software, supervision, writing - original draft, writing – review & editing.

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## Declaration of competing interest

The authors declare no competing, personal financial or other competing interests. Funding or employment sources played no role in the design, presentation or discussion of the presented research.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.abb.2022.109392>.

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