Archives of Biochemistry and Biophysics 636 (2017) 17–27

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Thermodynamics of cooperative binding of FAD to human NQO1: Implications to understanding cofactor-dependent function and stability of the flavoproteome

Rafael Clavería-Gimeno[a,b,c], Adrian Velazquez-Campoy[a,c,d,e,f,*], Angel Luis Pey[g,**]

[a] Institute of Biocomputation and Physics of Complex Systems (BIFI), Joint Units: BIFI–IQFR (CSIC) and GBsC–CSIC, Universidad de Zaragoza, Spain
[b] Instituto Aragonés de Ciencias de la Salud (IACS), Zaragoza, Spain
[c] Aragon Health Research Institute (IIS Aragon), Universidad de Zaragoza, Spain
[d] Department of Biochemistry and Molecular and Cellular Biology, Universidad de Zaragoza, Spain
[e] Fundacion ARAID, Gobierno de Aragón, Spain
[f] Centro de Investigación Biomédica en Red en el Área Temática de Enfermedades Hepáticas y Digestivas (CIBERehd), ISCIII Madrid, Spain
[g] Department of Physical Chemistry, University of Granada, Granada, Spain

ARTICLE INFO

Keywords:
Flavin binding
Cooperativity
Thermodynamics
Oxidoreductase
Cancer
Mutation

ABSTRACT

The stability of human flavoproteins strongly depends on flavin levels, although the structural and energetic basis of this relationship is poorly understood. Here, we report an in-depth analysis on the thermodynamics of FAD binding to one of the most representative examples of such relationship, NAD(P)H:quinone oxidoreductase 1 (NQO1). NQO1 is a dimeric enzyme that tightly binds FAD, which triggers large structural changes upon binding. A common cancer-associated polymorphism (P187S) severely compromises FAD binding. We show that FAD binding is described well by a thermodynamic model explicitly incorporating binding cooperativity when applied to different sets of calorimetric analyses and NQO1 variants, thus providing insight on the effects *in vitro* and in cells of cancer-associated P187S, its suppressor mutation H80R and the role of NQO1 C-terminal domain to modulate binding cooperativity and energetics. Furthermore, we show that FAD binding to NQO1 is very sensitive to physiologically relevant environmental conditions, such as the presence of phosphate buffer and salts. Overall, our results contribute to understanding at the molecular level the link between NQO1 stability and fluctuations of FAD levels intracellularly, and supports the notion that FAD binding energetics and cooperativity are fundamentally linked with the dynamic nature of apo-NQO1 conformational ensemble.

1. Introduction

Beyond their essential role in catalysis and regulation, cofactors, coenzymes and allosteric regulators can be critical for the *in vivo* stability of many human proteins, with a particular relevance in human diseases [1–15]. Among them, flavin-dependent human proteins emerge as a specially sensitive case [12,13,16]. The human proteome contains roughly a hundred flavoproteins, many of them associated to genetic diseases [15]. The intracellular levels of human flavoproteins are strongly dependent on the availability of the flavin precursor, riboflavin, and in general, flavin starvation facilitates proteasome-mediated flavoprotein degradation [12]. As a result, a relationship likely exists between the *in vivo* sensitivity of flavoprotein levels/stability, the intrinsic conformational stability and dynamics of cofactor-free (apo-) proteins and the binding affinity for the flavin cofactor [5,12].

Among human flavoproteins, NAD(P)H:quinone oxidoreductase 1 (NQO1) shows one of the strongest dependences of *in vivo* protein levels with riboflavin bioavailability, due to the well-established relationship between its proteasomal degradation rates and the population of its highly dynamic apo-state [12,13,17,18]. In particular, the dynamics of the C-terminal domain (CTD), rather than the intrinsic conformational stability, seems to determine the degradation rates (and to a large extent the steady-state levels) of NQO1 [12,18–20]. NQO1 is a FAD-dependent, two-domain and dimeric protein with multiple functions [16,21,22]. It catalyzes two-electron reduction of quinones acting as a detoxifying and antioxidant enzyme and activating certain cancer prodrugs [16,21]. In addition, NQO1 interacts with transcription factors linked to cancer progression, such as p53, p73α and HIF-1α [23,24]. *In vitro* and cellular studies have shown that these interactions with NQO1 increase the protein levels of transcription factors by inhibiting their

* Corresponding author. Institute of Biocomputation and Physics of Complex Systems (BIFI), Joint Units: BIFI–IQFR (CSIC) and GBsC–CSIC, Universidad de Zaragoza, Spain.
** Corresponding author.
E-mail addresses: adrianvc@unizar.es (A. Velazquez-Campoy), angelpey@ugr.es (A.L. Pey).

http://dx.doi.org/10.1016/j.abb.2017.10.020
Received 19 September 2017; Received in revised form 25 October 2017; Accepted 27 October 2017
Available online 31 October 2017
0003-9861/ © 2017 Elsevier Inc. All rights reserved.

proteasomal degradation through different mechanisms [13,23,24]. Its role in cancer is further supported by its overexpression in certain types of tumours [25–27].

A single nucleotide polymorphism in NQO1 (rs1800566/c.C609T/p.P187S) was originally found in cancer cell lines resistant to the antitumour quinone mitomycin C [28]. This polymorphism shows a high frequency in human population and is significantly associated with increased cancer risk [29]. The P187S polymorphism is known to abolish NQO1 activity by strongly decreasing FAD binding affinity and accelerating protein turnover by the proteasome [5,13,17,18,20]. Intriguingly, the X-ray crystallographic structure of P187S in the FAD-bound (holo) state only showed subtle local rearrangements [22], which cannot explain the long-range communication of its effect to the N-terminal domain (NTD, where the FAD binding site is placed) or the CTD [18]. Accordingly, molecular dynamics (MD) simulations and proteolysis experiments have indicated that P187S affects the FAD binding affinity through local dynamic changes at its binding site in the apo-state, particularly in the 57–66 loop, while increased protein turnover is associated with a highly flexible CTD and only prevented upon binding of the inhibitor dicoumarol [18]. Indeed, Pro187 and FAD binding sites together with the CTD constitute an allosteric network that communicates dynamic information through long distances in the protein conformation ensemble [20]. Very recently, we have identified a set of consensus amino acids that have diverged along recent evolutionary history of NQO1, yielding the human protein more susceptible towards inactivation by P187S [19]. Experimental characterization of two back-to-consensus mutations (H80R and E247Q) has shown that they strongly protect the polymorphism towards inactivation through local stabilization of the mutated sites that in some cases propagates to distant functional sites [19]. In the case of H80R, crystallographic and small angle X-ray scattering analyses, combined with MD simulations and binding studies have revealed that Arg80 causes a structural switch in the apo-state of P187S that overcomes dynamic destabilization at the loop 57–66 neighbouring the FAD binding site thus stabilizing binding competent states [19,30].

Despite the prominent role of FAD binding to the stability and activity of NQO1, many details on the binding mechanism are unclear. Intriguingly, (direct) titrations of apo-NQO1 wild-type (WT) with FAD evidences cooperative behavior, while P187S shows much weaker non-cooperative binding [5,30]. Conversely, reverse titrations of WT and P187S into FAD have been described and interpreted as non-cooperative binding [31]. We report here a detailed characterization of FAD binding to WT and P187S NQO1 that explicitly and systematically describes the cooperativity of these interactions. Our analyses allow reconciling different (and apparently conflicting) recent works, demonstrating the role of the CTD and the H80R mutation on the affinity for FAD and its binding cooperativity and energetics. Furthermore, we show that the affinity and energetics of FAD:NQO1 interactions are strongly modulated by physiologically relevant environmental conditions.

2. Materials and methods

2.1. Protein expression and purification

NQO1 variants were expressed in and purified from BL21(DE3) *E. coli* cells transformed with pET46 Ek/LIC plasmid containing the corresponding DNA sequences [5,18,20]. His-tagged proteins were purified using a *standard procedure* [5,18,19] in which no steps particularly aimed at removing FAD bound to NQO1 proteins are included. To obtain apo-proteins, a *stripping procedure* which consisted in the incubation of purified proteins with 2 M KBr (and in WT and H80R, using 2 M urea) under reducing conditions (β-mercaptoethanol 5 mM) with protease inhibitors (EDTA-free, cocktail inhibitor from ROCHE) to release FAD bound, and subsequent isolation of apo-proteins was performed by metal affinity chromatography [5,22]. Apo-proteins were buffer exchanged to HEPES-KOH 50 mM pH 7.4 and stored at −80 °C upon flash-freezing in liquid nitrogen. In all cases, it has been verified that apo-proteins obtained by these procedures remain in a similarly folded, dimeric and stable state under the experimental conditions used [5,18–20,30].

2.2. Isothermal titration calorimetry (ITC)

Titrations were carried out in an ITC_{200} microcalorimeter (MicroCal-Malvern). Proteins and FAD were prepared in HEPES-KOH 50 mM pH 7.4. All the buffers were prepared and their pH adjusted at 25 °C. Therefore, small pH changes (lower than 0.15 units) may occur in experiments performed at the lowest temperatures (i.e. 15 °C). Nevertheless, these small changes in pH should not affect our thermodynamic analyses since control experiments in a wider pH range (7–8) show negligible effects on binding thermodynamic parameters (Table S1). FAD and apo-NQO1 concentrations were determined spectroscopically using $\varepsilon_{450nm} = 11300\ M^{-1}\ cm^{-1}$ and $\varepsilon_{280nm} = 47900\ M^{-1}\ cm^{-1}$, respectively. In *direct titrations*, NQO1 proteins were placed in the cell at 10–20 μM (full-length NQO1 variants) or 4–6 μM (Δ50 variants) in protein dimer, while FAD was placed in the titrating syringe at 0.3–0.4 mM (full-length NQO1 variants) or 0.15–0.2 mM (Δ50 variants). Titrations consisted of an initial 0.5 μL injection followed by 12–45 injections of 0.8–3 μL spaced 180–360 s. In *reverse titrations*, FAD was placed in the cell at 9–12 μM, and titrated using apo-NQO1 in the syringe (60–70 μM in protein dimer). In some cases, direct titrations were performed in K-phosphate 20 mM pH 7.4 (in the absence or presence of 50 mM salts, KCl or KF) or PBS buffer pH 7.4 (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCL).

2.3. Data analysis of ITC experiments

The binding equilibrium for a homodimeric protein with two ligand binding sites per dimer can be reduced to three energetically and structurally distinguishable states:

$$P + 2L \leftrightarrow PL + L \leftrightarrow PL_2 \quad (1)$$

This scheme may be represented in terms of the overall binding parameters:

$$\beta_1 = \frac{[PL]}{[P][L]} \quad \Delta H_1$$
$$\beta_2 = \frac{[PL_2]}{[P][L]^2} \quad \Delta H_2 \quad (2)$$

where β_i are the overall association constants, and ΔH_i are the overall binding enthalpies, or in terms of the site-specific binding parameters:

$$K = \frac{[PL]}{[P][L]} \quad \Delta H$$
$$K\alpha = \frac{[PL_2]}{[PL][L]} \quad \Delta H + \Delta h \quad (3)$$

where K and α are the site-specific association constant and the cooperativity constant, and ΔH and Δh are the site-specific binding enthalpy and cooperativity enthalpy. Importantly, in the expression for β_1 in eq. (2) [PL] represents the binding to any of the two binding sites, but in the expression for K in eq. (3) [PL] represents the binding to a specific binding site.

The two sets of equilibrium constants, $\{\beta_1, \beta_2\}$ and {K, α}, and the two sets of binding enthalpies, $\{\Delta H_1, \Delta H_2\}$ and {ΔH, Δh}, are related through these transformation equations [32]:

$$\beta_1 = 2K$$
$$\beta_2 = K^2\alpha$$
$$\Delta H_1 = \Delta H$$
$$\Delta H_2 = 2\Delta H + \Delta h \quad (4)$$

or, alternatively:

Download English Version:

https://daneshyari.com/en/article/8288860

Download Persian Version:

https://daneshyari.com/article/8288860

Daneshyari.com