International Journal of Mass Spectrometry 312 (2012) 87–96

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

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

Characterization of cofactors, substrates and inhibitor binding to flavoenzyme quinone reductase 2 by automated supramolecular nano-electrospray ionization mass spectrometry

Mathias Antoine[a], Estelle Marcheteau[a], Philippe Delagrange[b], Gilles Ferry[a], Jean A. Boutin[a,*]

[a] Biotechnologie, Pharmacologie Moléculaire et Cellulaire, France
[b] Département des Sciences Expérimentales, Institut de Recherches Servier, 125 Chemin de Ronde, 78290 Croissy-sur-Seine, France

ARTICLE INFO

Article history:
Received 14 March 2011
Received in revised form 1 July 2011
Accepted 11 July 2011
Available online 21 July 2011

Keywords:
Supramolecular mass spectrometry
Quinone reductase
Flavoprotein
Resveratrol
Melatonin

ABSTRACT

Quinone reductase 2 (QR2) is a cytosolic homodimeric enzyme implicated in the reduction of quinone in the presence of natural derivatives of NADH such as *N*-ribosyldihydronicotinamide. QR2 does not recognize NADH or NADPH as co-substrates, unlike quinone reductase 1 (QR1). This feature is not the only unusual one of this enzyme. Although it resembles quinone reductase 1, the well-described detoxifying enzyme, QR2 does not share many features with QR1. Particularly, it does not seem to have a similar detoxifying function in cells. Therefore, starting from basic knowledge on QR2 and adding up to previous works published on the enzyme, we wanted to rebuild its biochemical description because some of the recently described characteristics are surprising, and merit further explorations. For example, QR2 seems to be over-expressed in neurodegenerative diseases, and this over-expression seems to be linked to a worsening of the pathological conditions. Indeed, our specific inhibitors of QR2, tested *in vivo*, show outstanding properties impairing memory loss. These observations led us to further describe, at the molecular level, the relationship between QR2 and some of its inhibitors and co-substrates. In the present paper, we address this question using non-denaturing supramolecular nano-electrospray ionization mass spectrometry. This characterization helps understand the physical relationship between inhibitors such as resveratrol or melatonin and the enzyme.

1. Introduction

Quinone reductase 2 (QR2, E.C. 1.10.99.2) is a cytosolic enzyme with many enigmatic features [1]. Its main recognized activity would be the catalysis of a detoxification process for quinones. For at least two reasons, this activity is not validated. Firstly, QR2 is a close neighbour of QR1 (DT-diaphorase) that is, beyond any doubt, a detoxification enzyme. QR1 and QR2 are closely related [2], since they share 59% (cDNA) and 44% (protein) similarity in their sequences, with the interesting difference that QR1 has a 43 amino acid longer C-terminus than QR2 [3]. Due to this particular fact, QR2 does not recognize NADH and NADPH as co-substrates but rather natural compounds possibly derived from those, such as *N*-ribosyl- or *N*-methyl-dihydronicotinamide [4,5]. Secondly, two papers are absolutely crucial in understanding the role of QR1 and QR2 in animals. Those papers described the toxicity of menadione in wild type *versus* genetic knock out animals. For each of those enzyme genetic deletions, the net results are that menadione is toxic in wt animals, but less toxic in mice genetically deleted of the QR2 gene and more toxic in mice genetically deleted of the QR1 gene [6,7]. This observation (that was independently obtained in our own laboratory on our own, independently obtained, genetically deleted QR2 mice ([8], [Delagrange & Boutin, unpublished]) could be reasonably attributed to a harmful action of QR2 in the presence of menadione. Therefore, an unsolved question, so far, is the role of QR2 in physiology. In standard – and equivalent – situations, it seems that QR2 leads to the production of far more radical oxygen species (ROS) than QR1 [9]. The only difference between those two situations – aside from the sequence itself, obviously – is that, as stated before, QR1 uses standard hydride donors (NADH/NAD(P)H), while QR2 uses exotic ones, from either natural (*N*-ribosylnicotinamide or *N*-methylnicotinamide) or synthetic (*N*-benzylnicotinamide) origins. As of today, the abundance and role of the natural and putative co-substrates of QR2 are not known.

The molecular studies published on QR2 are rather scarce. Historical and key papers described its catalytic activity, and then its

Abbreviations: BSA, bovine serum albumin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; BNAH, *N*-benzyl dihydonicotinamide; QR2, quinone reductase 2; ESI-MS, electrospray ionization-mass spectrometry; SEC-MALS, size exclusion chromatography-multi angle light scattering; ITC, isothermal titration calorimetry.

* Corresponding author. Tel.: +33 1 55 72 27 48; fax: +33 1 55 72 28 10.
E-mail address: jean.boutin@fr.netgrs.com (J.A. Boutin).

1387-3806/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijms.2011.07.011

first crystallisation as well as some data on its enzymatic mechanisms [4,5,10]. Later on, stopped-flow experiments enhanced our knowledge of the mechanisms of action of the enzyme [11]. Finally, a handful of compounds (resveratrol, melatonin, catechol, 5-methoxycarbonylamino-*N*-acetyltryptamine (MCA-NAT), *N*-[2-(7-methylaminosulfonyl-1-naphthyl)ethyl]acetamide (S26695), *N*-[2-(2-methoxy-6H-dipyrido [2,3-*a*:3,2-*e*]pyrrolizin-11-yl)ethyl]-2-furamide (S29434) were co-crystallized with QR2 [12–15].

Our own studies of QR2 included the discovery of potent inhibitors such as S 26695 and S 29434 and others [16–18]. Some of these compounds have shown activities on the inhibition of memory loss [19], opening up a whole field of new investigations.

The basic knowledge on the relationship between QR2 and its potent inhibitors remains poor and sometimes complex to understand (see [17,18,20]). Therefore, we started a series of experiments using various biophysical approaches, including native mass spectrometry analyses and fluorescence spectroscopy, to complete our understanding of the molecular feature of human recombinant QR2.

In the present paper, we applied native ESI-MS and fluorescence spectroscopy to the study of the binding of FAD and inhibitors onto QR2. This is the first time native mass spectrometry was used to characterize the relationship between the enzyme and its inhibitors and its natural cofactors (whether zinc atom or FAD). These experiments demonstrate the strong potential of native mass spectrometry for deciphering the strength and the stoichiometry of enzyme-cosubstrate/inhibitor complexes.

2. Materials and methods

2.1. Reagents and chemicals

Acetonitrile, water, ammonium acetate and formic acid were mass-spectrometry grade, they were obtained from Sigma (St. Louis, MO, USA) as well as FAD, melatonin (*N*-acetyl-5-methoxytryptamine), menadione, resveratrol, horse heart myoglobin, and monomeric bovine serum albumin. *N*-benzyl dihydonicotinamide (BNAH) was from BioMol (Enzo Life Sciences Inc, Farmingdale, NY, USA). S26695 (*N*-[2-(7-methylaminosulfonyl-1-naphthyl)ethyl]acetamide) was synthesized in-house as described [17]. Melatonin, menadione, resveratrol and S26695 were solubilized to 10 mM in anhydrous EtOH. FAD was dissolved in water and quantified spectrophotometrically using an extinction coefficient of 11,300 M^{-1} cm^{-1} at 450 nm.

2.2. Cloning, expression and purification of human QR2

This work was outsourced to Protenia SA (Ifrane, Morocco). In brief, the human QR2 cDNA was obtained by PCR amplification from the pcDNA3.1(+)/hQR2 plasmid [21] and subcloned in the pFastBac1 vector (Invitrogen, Carlsbad, CA, USA). DH10Bac cells were transformed by pFastBac/hQR2 to generate the Bac-hQR2 bacmid, following the recommendations of the manufacturer. Sf9 cells in SFM medium were transfected by the Bac-hQR2 bacmid. After five days, the medium was centrifuged and the recombinant baculovirus contained in the supernatant were titrated and amplified to obtain a large stock of virus (5×10^6 pfu/mL). For the QR2 production, Sf9 cells grown in 1.5 L of PFM medium at 28 °C to 2×10^6 cells/mL were infected by 45 mL of recombinant baculovirus. Cells were harvested after 3 days. The pellet was suspended and lyzed in buffer A (25 mM Tris/HCl, 10 mM NaCl, 1 mM DTT) supplemented with 1 mM PMSF, 1 μm pepstatin A, 10 μM EDTA. After centrifugation (18,000 rpm, 15 min, 4 °C), the supernatant was applied to XK50/20 Fast Flow DEAE column equilibrated with buffer A and washed with a linear gradient of buffer B (Buffer A with 1 M NaCl). Fractions containing the enzyme (detected by SDS–PAGE) were pooled and concentrated. The concentrated enzyme solution was then diluted in buffer A and applied to an XK26-40 Source 15Q column equilibrated with buffer A. The enzyme was eluted with a linear gradient of buffer B. Elution was monitored by measuring the absorbance at 280 and 392 nm. Two peaks containing QR2 were resolved and pooled independently. Both pools were concentrated to 5–6 mg/mL and buffer-exchanged on Centricon devices (10 kDa cut-off) with buffer C (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10% glycerol). Concentrated QR2 solutions were aliquoted, flash-frozen and stored at −80 °C. QR2 was quantified by measuring absorbance at 280 nm using an extinction coefficient of 44,620 M^{-1} cm^{-1}, giving monomer concentration.

2.3. Size exclusion chromatography-multi angle light scattering

An AKTA Explorer (GE Healthcare, Buckinghamshire, England) fitted with a Superdex 200 5/150 column was connected to a DAWN EOS laser light scattering instrument (Wyatt Technology Corp., Santa Barbara, CA, USA) and an Optilab rEX differential refractometer (Wyatt Technology Corp., Santa Barbara, CA, USA). The column and following detectors were equilibrated overnight with 50 mM Tris–HCl, pH 8.0, 200 mM NaCl at 0.2 mL/min. The instrument was calibrated with monomeric BSA. Protein samples were centrifuged 15 min at 13,000 × *g* and 4 °C before injection on the column. 20 μL of 1 mg/mL QR2 were injected for analysis. Data were acquired and processed with the Astra software (Wyatt Technology Corp., Santa Barbara, CA, USA), using a dn/dc value of 0.185 for BSA and QR2.

2.4. QR2 activity assay

The QR2 enzymatic activity was measured at 25 °C using 100 μM menadione and serial dilutions of QR2, diluted in 50 mM Tris/HCl, pH 8.5, 1 mM *n*-octyl-B-D-glucopyranoside. Reactions were started by addition of 100 μM BNAH and followed by measuring the decrease of BNAH fluorescence at 440 nm with excitation at 340 nm, using a FluoStar Omega 96-well plate reader (BMG, Offenburg, Germany). Specific activity is expressed as nmole/min/mg of protein.

2.5. Nano-electrospray ionization mass spectrometry

Nano-ESI mass spectra were acquired on a time-of-flight mass spectrometer (LCT Premier XE, Waters, Milford, MA, USA) upgraded with the Non-Covalent Enhancement kit and fitted with an automated chip-based NanoESI system (Nanomate 200, Advion Biosciences, Ithaca, NY, USA). Standard nanospray parameters were used throughout the study: chip voltage was set at 1500 V and gas pressure was set at 0.3 psi. Purity and homogeneity of the QR2 samples were checked by mass spectrometry in denaturing conditions. Proteins were desalted by reversed-phase chromatography in acetonitrile/water/formic acid (50:50:1) with ZipTip C4 pipette tips following manufacturer instructions (Millipore, Billerica, MA, USA). Mass spectra were recorded in the positive ion V mode on the mass range *m/z* 500–2000, after calibration with 2 μM horse heart myoglobin dissolved in acetonitrile/water/formic acid (50:50:1, v/v). Before native ESI-MS measurements, QR2 samples were desalted in 200 mM ammonium acetate (pH 7.5) by three successive buffer-exchange steps with Zeba Spin Desalting Columns (7 kDa cut-off, Pierce, Rockford, IL, USA). ESI-MS measurements of QR2 samples in native conditions were performed in 200 mM ammonium acetate (pH 7.5). QR2 was diluted to 10 μM (monomer concentration). When compounds solubilized in EtOH were added, final EtOH concentration never exceeded 2.5%. The mass spectrometer was carefully tuned with gentle desolvation parameters to maintain