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## Chapter 24

### Measuring the NQO2:melatonin complex by native nano-electrospray ionization mass spectrometry.

**Jean A. Boutin<sup>1,\$,\*</sup>, Johann Stojko<sup>1</sup>, Gilles Ferry<sup>1</sup>, Sarah Cianferani<sup>2,3</sup>**

<sup>1</sup> Pole d'expertise Biotechnologie, Chimie & Biologie, Institut de Recherches Servier, Croissy-sur-Seine, France

<sup>2</sup> Laboratoire de Spectrométrie de Masse BioOrganique, Université de Strasbourg, CNRS, Strasbourg, France

<sup>3</sup> Infrastructure Nationale de Protéomique ProFI, FR2048 CNRS, Strasbourg, France

\$ Current address: PHARMADEV (Pharmacochimie et biologie pour le développement), Faculté de Pharmacie, 31000 Toulouse, France

Corresponding author: Jean A. Boutin [ja.boutin.pro@gmail.com](mailto:ja.boutin.pro@gmail.com)

**Running Head:** Mass spectrometry-based measure of ligand-protein complex

**Abstract.** Melatonin exerts its effects through a series of target proteins/receptors and enzymes. Its antioxidant capacity might be due to its capacity to inhibit a quinone reductase (NQO2) at high concentration (50  $\mu$ M). Demonstrating the existence of a complex between a compound and a protein is often not easy. It requires either that the compound is an inhibitor – and the complex translates by an inhibition of the catalytic activity – or the compound is radiolabeled – and the complex translates in standard binding approaches, such as in receptorology. Outside these two cases, the detection of the protein:small molecule complexes by mass spectrometry has recently been made possible, thanks to the development of so called native mass spectrometry. Using this approach one can measure masses corresponding to a intact noncovalent complex between a compound and its target, usually after titration or competition experiments. In the present chapter, we detail the characterization of NQO2:melatonin interaction using native mass spectrometry .

**Keywords:** native mass spectrometry; NQO2; melatonin; co-substrate; complex.

## 1. Introduction

Melatonin recognizes several protein targets to perform its key physiological role and beyond **(1)** [*e.g.* its antioxidant activities **(2)**]. Several of those proteins have been described, and the main ones are certainly, in mammals, the two G-protein-coupled receptors, MT1 and MT2. In the 1990s, reports revealed the existence of an extra one, called *MT3* **(3)**. A turning point occurred when Nosjean *et al* discovered *that MT3*, a putative melatonin binding site, was indeed the enzyme NQO2 **(4)**. NRH-dependent oxidoreductase (NQO2, E.C. 1.10.5.1) is a remarkable enzyme, the discovery of which spanned from early 60s **(5)** to 1997 **(6)**. We reviewed the information available on this enzyme and described some of its features **(7, 8)**. Amongst the many studies conducted to point at the identity of *MT3* and NQO2, three retain the attention as they are key in understanding at the molecular level the interaction between a protein and a small molecule such as melatonin. They are, in increase technical complexity (and novelty) order: calorimetry **(9)**, crystallography **(9, 10)** and native mass spectrometry **(11)**, even if the last two approaches are highly technical and require instruments that are not completely mundane: respectively a synchrotron and a mass spectrometer compatible with the native approach. Current biophysical approaches amenable to enzymes in general, and to NQO2 in particular, are used to measure the constants of association between two proteins or between a protein and one of its ligands.

Co-crystallization evidences the interaction between two entities it is in solid state. Visualization if the complex in liquid state can only be obtained so far in cryo-electronic microscopy (EM) but, in this domain, no report so far was published of EM complex with NQO2 [see discussion in Venien-Bryan *et al* **(12)**]. Native mass spectrometry (native MS) is the technique of choice to describe the stoichiometry (and in some cases the strength) of enzyme/cosubstrate or enzyme/inhibitor

complexes (**13, 14**). Basically, his approach enables to detect noncovalent complexes between NQO2 and its inhibitor(s).

## 2. Materials.

1. Mass spectrometer: Electrospray 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 (such as Nanomate 200, Advion Biosciences, Ithaca, NY, USA).
2. Purified NQO2. Cloning and purification of NQO2 has been reported numerous times (**4, 6, 9, 10**), including in **one chapter of the present book**. The enzyme preparation should be FAD-free (See **note 1**).
3. Prepare buffer A: 200 mM ammonium acetate, pH 7.5.
4. Prepare melatonin solution, 10 mM in anhydrous ethanol.
5. Prepare a BNAH (*N*-benzylidihydronicotinamide, NQO2 co-substrate) solution, 10  $\mu$ M in water.
6. Prepare a calibration solution of 2  $\mu$ M horse heart myoglobin in acetonitrile/water/formic acid (50:50:1, v/v).
7. Prepare a FAD buffer: 50 mM Tris, 250 mM NaCl, pH 8.0.
8. Prepare 4 solutions of increasing concentration of FAD in FAD buffer (0.5, 1.0, 2.0 and 2.5  $\mu$ M).

## 3. Methods

### 3.1 Sample preparation

1. Desalt the purified NQO2 by performing buffer exchange at least 3 times against buffer 1 with Zeba Spin Desalting Columns (7 kDa cut-off, Pierce, Rockford, IL, USA).
2. Split in two portions. The first portion is the deflavo-NQO2.
3. Saturate the second portion of the enzyme with FAD by gently stirring the enzyme with the increasing concentrated FAD solutions.
4. Measure the saturation in FAD by fluorescence ( $\lambda_{exc}$  280 nm;  $\lambda_{em}$  from 300 to 450 nm).
5. Finally, remove unbound FAD by 3 steps through desalting column.
6. Resuspend in buffer A. This is FAD-saturated NQO2.

### 3.2 Mass Spectrometry settings

1. Connect the automated chip-based nanoESI infusion source (Triversa Nanomate, Advion, Ithaca, USA) to the LCT Premier (Waters).
2. Mass spectrometer calibration: Native mass spectra were recorded in the positive V mode on the mass range  $m/z$  500–5000 after calibration with 2 mM horse heart myoglobin dissolved in acetonitrile/water/formic acid (50:50:1, v/v).
3. Mass spectrometer settings for native MS : The mass spectrometer was carefully tuned with gentle desolvation parameters to maintain non-covalent complexes intact during the ionization/desorption process. For this, in the Tune page, set the following parameters: Positive mode with a  $m/z$  range from 500 to 5 000. The cone, ion guide 1 and aperture 1 voltages were optimized to 50V, 50V and 10V, respectively. The pressure in the source region was increased to 2.4 mbar. Ions were detected with a multichannel plate (MCP) detector set at 2200V. Native mass spectra were recorded in the positive V

mode on the mass range  $m/z$  500–5000 after calibration with 2 mM horse heart myoglobin dissolved in acetonitrile/water/formic acid (50:50:1, v/v). Data were acquired and processed using MassLynx v4.1 (Millipore, Billerica, MA, USA). Spectra acquired for 1–2 min were combined and smoothed before centering

### 3.3 Native MS analysis of deflavo-NQO2

1. Dilute the deflavo-NQO2 preparation in buffer A to reach a concentration of 10  $\mu\text{M}$  of monomer (25864 Da). (See **note 2**).
2. Start a 2 min acquisition with a stable TIC. The mass spectrum is presented in **Figure 1**.
3. Data interpretation: In MassLynx 4.1, select your raw data file. Select a  $m/z$  range from 3,300 to 4,300, select 2 neighboring peaks and measure a mass of 51,856.8 Da, expected for a QR2 dimer containing 2 zinc atoms, each chelated by a deprotonated Cys ( $25,864 \times 2 + 65.4 \times 2 - 2$  Da).

### 3.4 Native MS analysis of FAD-saturated NQO2

1. Dilute the FAD-saturated NQO2 preparation in buffer A to reach a concentration of 10  $\mu\text{M}$  of monomer.
2. Start a 2 min acquisition with a stable TIC. The mass spectrum is presented in **Figure 1**.
3. Data interpretation: In MassLynx 4.1, select your raw data file. Select a  $m/z$  range from 3,300 to 4,300, select 2 neighboring peaks and measure a mass of 53,429 Da, corresponding to the QR2-Zn (51,857) dimer with two FAD bound ( $53,427$  Da =  $51,857 + (2 \times 785)$  Da).

### 3.5 Native MS analysis of melatonin binding to deflavo-NQO2

1. Dilute the deflavo NQO2 preparation in buffer A to reach a concentration of 10  $\mu\text{M}$  of monomer.
2. Incubate the deflavo NQO2 with 20  $\mu\text{M}$  (2 molar excess) melatonin for 2h (final EtOH concentration < 2.5% v/v)
3. Mass spectrometry analysis: Start a 2 min acquisition with a stable TIC. The mass spectrum is presented in **Figure 1**.
4. Data interpretation: the most intense peak correspond to a measured mass of 51,857 Da, corresponding to deflavo-NQO2. An additional minor ion series (on the right of the main peak) with a mass increase of +232 Da (52,090 Da: 51,857 +232 Da) corresponds to the binding of one melatonin molecule on a NQO2 dimer. Based on relative peak intensities, it could be deduced than no more than 5% of the species correspond to 2:1 NQO2:melatonin complex, suggesting a low affinity of melatonin for dimeric deflavo-NQO2.

### 3.6 Native MS analysis of melatonin binding to FAD-saturated NQO2

1. Incubate the FAD-saturated NQO2 preparation (10  $\mu\text{M}$  in buffer A) with 20  $\mu\text{M}$  melatonin for 2h.
2. Mass spectrometry analysis: Start a 2 min acquisition with a stable TIC. The mass spectrum is presented in **Figure 1** (See **note 4**).
3. Data interpretation: the most intense peak corresponds to a measured mass of 53,894 Da, corresponding to FAD-saturated-NQO2 dimer (one FAD/monomer) with two melatonin molecules (53,894 Da: 51,857 Da +

$2 \times 785 + 2 \times 232$ ). Based on relative peak intensities, it could be deduced that almost 95% of the species correspond to 2:2:2 NQO2:FAD:melatonin complex, suggesting a high affinity of melatonin for dimeric FAD-saturated-NQO2.

#### 4. Notes

1. It is important to obtain deflavo-NQO2 (FAD free-NQO2). To obtain such as sample, the final purification step of NQO2 is a ion-exchange chromatography step, as described in full details in Antoine et al (**11**). To ensure the absence of FAD in the enzyme [ so called the white sample, as opposed to the yellow sample, see (**11**) ] in the preparation, the method of Aliverti et al (**15**) has been used.
2. This measured mass for saturated-NQO2 (25864 Da) corresponds to the full length NQO2 (25952 Da) without the N-term Met (-131 Da) but with acetylation (+42 Da) at the N-terminus]. Of note, the initial mass could vary depending on the protein construct (for recombinant enzyme) and the various types of acylation occurring during the expression and purification processes. Furthermore, once saturated with FAD, the dimer mass is 53,429 Da, corresponding to the NQO2-Zn (See **note 3**) with two FADs.
3. Zhao et al (**6**) reported the presence of a metal atom associated with each monomers, a feature that is not found in NQO1 enzyme.
4. An example is given in **Figure 1**. The spectrum permits to determine all the masses present in the sample, as shown, and to determined, though,

the mass of each individual peaks (*e.g.* NQO<sub>2</sub> + 1 melatonin, NQO<sub>2</sub> + 2FAD, etc...). Ultimately, the analysis of the spectrum can also give a general idea of the concomitant presence of the amount of the various NQO<sub>2</sub>/Compound(s) complexes, in a relative way (qualitative rather than quantitative).

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## 6. Figure Caption

**Figure 1:** Analyses of the NQO2 complex with its mild inhibitor, melatonin, under various experimental conditions.

1: FAD-saturated NQO2. 2: FAD saturated NQO2 incubated with melatonin. 3: Various complexes formed by NQO2 and melatonin in the presence of Zn, FAD, and FMN. Green circles: Zn; blue squares: melatonin; yellow circles: FAD; red circles: FMN. The spectrum is from our work on NQO2/melatonin complex **(11)**.

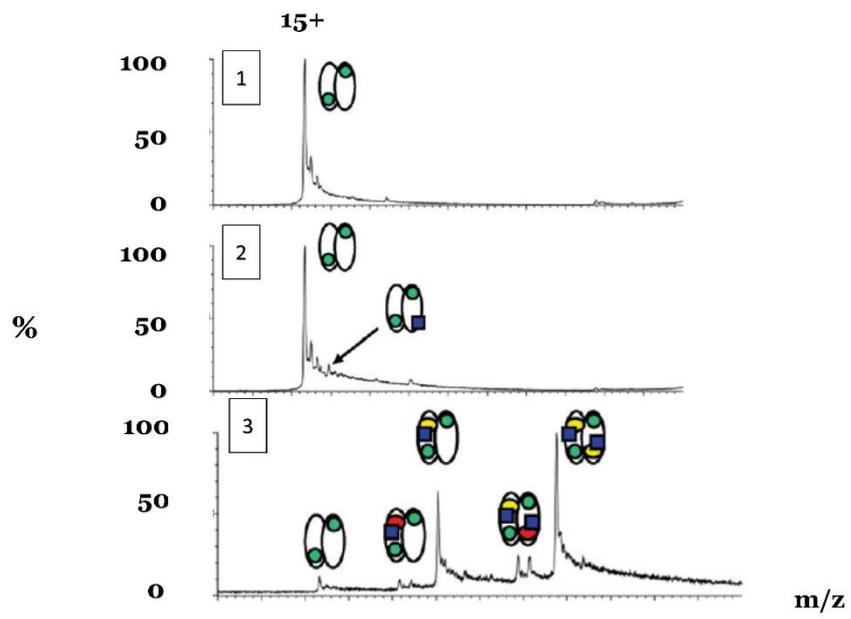


Figure 1