Detection of protein-drug complexes under native conditions in the low nM range using Magnetic Resonance Mass Spectrometry (MRMS)

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Introduction

Native mass spectrometric measurements of non-covalent protein-ligand binding have been reported for more than 25 years [1-3]. This technique combines electrospray ionization(ESI) and gentle desolvation. Magnetic resonance mass spectrometry (MRMS) is a technique to analyze intact biomolecular complexes at high mass resolution by native mass spectrometry (MS). It has been developed as a screening assay for drug discovery as both a primary and secondary screening tool. Native MS offers key advantages for detection of µM to low nM affinity complexes due to high sensitivity for proteins up to 100 kDa. This technique does not require modifications of the protein target and more importantly, it is the only biological assay which allows direct visualization of all molecules (target, substrates, products, byproducts, co-factors, isoforms) of interest in solution including the detection of non-specific interactions.

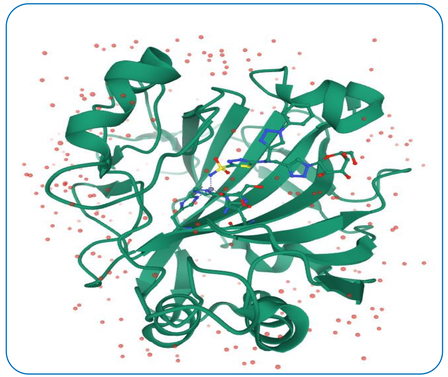

Figure 1: Non-covalent complex of Carbonic Anhydrase II (bovine) with the substrate acetazolamide.

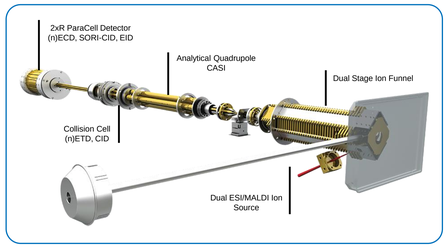

Figure 2: Schematic of the ion path of the scimaX® MRMS system used for the analysis of fragile protein-drug complexes under native conditions.

Methods

Sample preparation

CA II from bovine (CA II, C-3934) as well as the ligand acetazolamide (A6011) were purchased from Sigma-Aldrich. The ligand has a molecular formula of $C_4H_6N_4O_3S_2$ with monoisotopic molecular weight of 221.9881 Da. CA II was purified with 10 mM pH 7.0 ammonium acetate buffer using Amicon Ultra 10k cellulose membrane filters. 200 µl of the spray solution were prepared in 50 mM ammonium acetate for binding measurements using a protein concentration of 5 nM and 20 nM and ligand concentration ranging from 2 nM to 800 nM. The protein and ligand solution were mixed and then incubated for 5 min at room temperature to form the protein-ligand complex before mass spectrometric analysis.

Mass spectrometric analysis

CA II ligand complexes were measured with a scimaX® MRMS system (Figure 2) (Bruker Daltonics GmbH & Co. KG, Bremen, Germany). Sample solutions were at a flow rate of 4 µL/min using ESI in positive ion mode. Spectra were recorded in a mass range of m/z 300 – 4000 using resolving power of 70,000 at m/z 2645. 200 single scans were summed for the final mass spectrum.

Data processing

Mass spectra were mass deconvoluted. Charge states 10+, 11+ and 12+ were used for mass deconvolution. SNAP2 was used to generate monoisotopic masses of the native protein and protein-ligand complexes. K_D values (dissociation constant) were calculated with the software PRISM (GraphPad Software, San Diego, CA) by non-linear regression using specific binding.

Results

The deconvoluted mass spectra of the native CA II and the CA II-ligand complex at a protein concentration of only 5 nM and ligand concentration of 60 nM is shown in Figure 3. Both mass spectra are fully isotopically resolved. When adding acetazolamide to the protein solution, the CA II-ligand complex with acetazolamide is formed as well as small amounts of the CA II-ligand acetate complex due to high buffer concentration of 50 mM ammonium acetate.

The binding of carbonic anhydrase II to the studied ligand acetazolamide is very strong in the low nM range. Therefore, low protein concentration in the low nM range is needed to ensure correct measurement of the equilibrium dissociation constant K_D.

The formation of the protein-ligand complexes was measured at different ligand concentrations ranging from 2 nM to 800 nM using a protein concentration of only 5 and 20 nM. To calculate the equilibrium dissociation constant K_D of the protein-ligand complex, the ligand binding [PL]/([PL]+[P]) was plotted versus the ligand concentration for the protein concentration of 5 nM and 20 nM (Figure 3a and 3b). Regression factors of better than 0.99 were calculated for both protein concentrations using the non-linear regression for specific binding. 0

The K_D value for binding of acetazolamide to CA II were determined as 43 ± 7 nM and 52 ± 12 nM for a protein concentration of 5 nM and 20 nM, respectively.

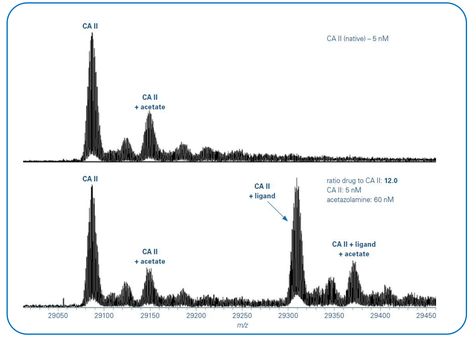

Figure 3: Isotopically resolved deconvoluted mass spectrum of CA II protein at 5 nM (top) and CA II-ligand complex formed at 5 nM CA II and 60 nM acetazolamide (bottom).

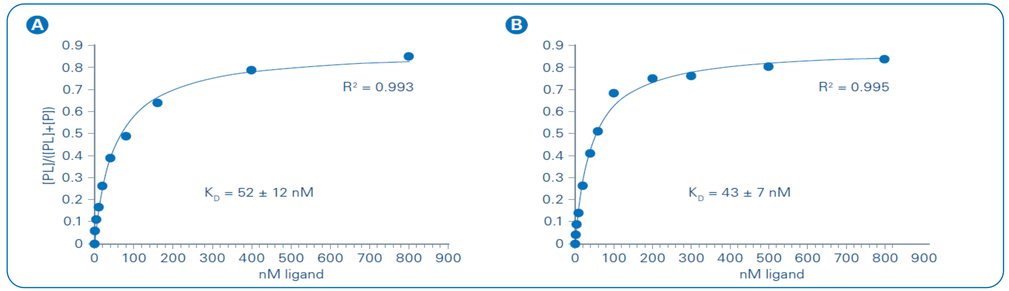

Figure 3: Plot of ligand binding [PL]/([PL]+[P]) versus the ligand concentration to determine K_D-values of the CA II-acetazolamide complex for protein concentrations of A) 5 nM and B) 20 nM. [PL]: intensity of protein-ligand complex; [P]: intensity of the free protein; ligand: acetazolamide.

These results are in good agreement with published K_D value of 20 ± 3 nM [4]. The monoisotopic masses of the protein and the protein-ligand complex were calculated with high mass accuracy using SNAP2. This algorithm calculates the monoisotopic mass based on the isotopic distribution using the repetitive building block for the molecular formula calculation of C 4.9384 N 1.3577 O 1.4773 S 0.0417 H 7.7583. The results for CA II at concentrations of 5 and 20 nM are shown in Table 1a and 1b, respectively. The calculated mass of the ligand (acetazolamide) could be determined very accurately by the mass difference of the free protein and the protein-ligand complex.

The ligand acetazolamide was measured with an average mass error of only 6.9 and 3.6 mDa for the protein concentration of 5 and 20 nM, respectively.

Table 1: Calculation of exact masses of CA II, CA II-ligand complex and the resulting mass of ligand (acetazolamide) based on SNAP2 for protein concentration of a) 5 nM and b) 20 nM.

a)

Protein-ligand ratio	Ligand conc.	Mass CA II [Da]	Mass CA II + ligand [Da]	Calc. Mass ligand [Da]	Mass error of ligand [mDa]
1:1	20 nM	29070.6129	29292.6005	221.9876	0.5
1:2	40 nM	29070.6410	29292.6273	221.9863	1.8
1:4	80 nM	29070.6269	29292.6105	221.9836	4.5
1:8	160 nM	29070.5975	29292.5779	221.9804	7.7

b)

Protein-ligand ratio	Ligand conc.	Mass CA II [Da]	Mass CA II + ligand [Da]	Calc. Mass ligand [Da]	Mass error of ligand [mDa]
1:12	60 nM	29070.6692	29292.6501	221.9809	7.2
1:20	100 nM	29070.6758	29292.6561	221.9803	7.8
1:40	200 nM	29070.6752	29292.6561	221.9809	7.2
1:60	300 nM	29070.6925	29292.6754	221.9829	5.2

Therefore, the formation of the protein-ligand complexes (CA II + acetazolamide) could be determined very accurately based on exact mass difference of the protein and the protein-complex which were detected with isotopic distribution.

Conclusions

- Isotopic distributions of CA II and their protein-ligand complexes were measured under native conditions in the low nM range.
- K_D value for the protein-ligand complex (CA II-acetazolamide) using a protein concentration of 5 nM was determined as 43 ± 7 nM, which is in good agreement with published data [4].
- MRMS is an excellent technique to perform protein binding analysis at low protein concentrations and high buffer concentrations.
- All measurements were performed with a standard Electrospray source.

MRMS Native MS

References
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