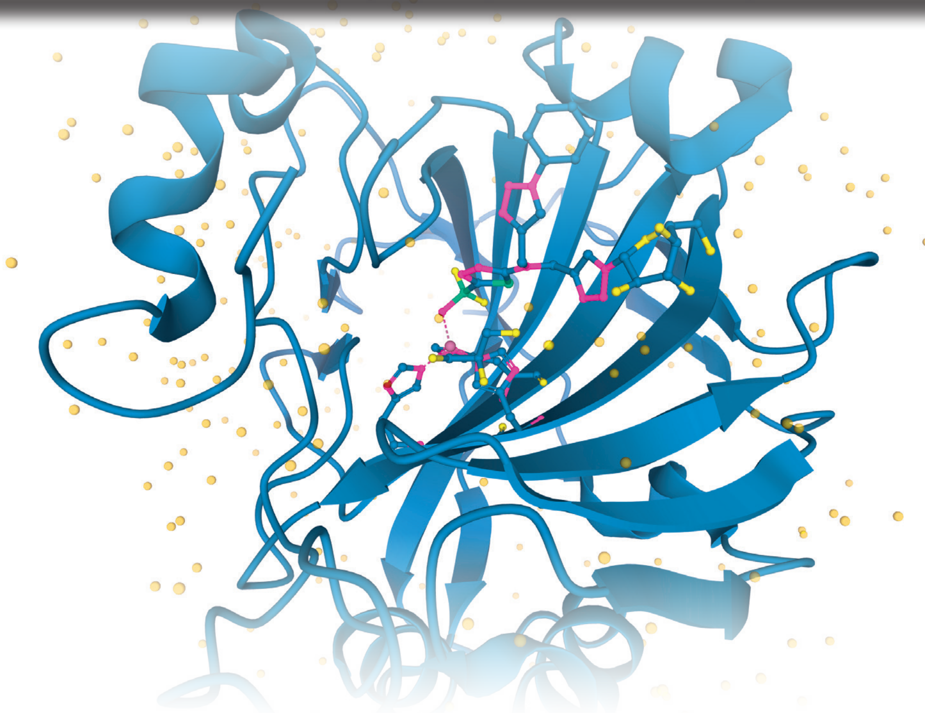

Studying protein-drug complexes under native conditions in the low nM range using MRMS

Mass spectrometry has been used as a tool to study a wide variety of protein-ligand and other biological interactions with great success [1]. Modeling solution phase physiological conditions is key to preserve native protein structure and interactions with substrates, ligands, and other proteins.

Abstract

Protein ligand complexes need to be preserved through the electrospray and detection process. This work describes high resolution native mass spectrometric measurements of the enzyme carbonic anhydrase II (Figure 1) and the non-covalent complex with the substrate acetazolamide (Figure 2) using a scimaX® magnetic resonance mass spectrometer (MRMS). The equilibrium dissociation constant K_D (strength of protein-ligand complex) was determined for the enzyme-drug complex. The high sensitivity of the scimaX® MRMS enables detection of protein-ligand binding in the low nanomolar concentration range, necessary for further drug design. Furthermore, studies where selectivity vs. multiple

Keywords:
scimaX, protein binding, protein-drug complex, carbonic anhydrase, molecular docking, drug discovery, MRMS, native mass spectrometry

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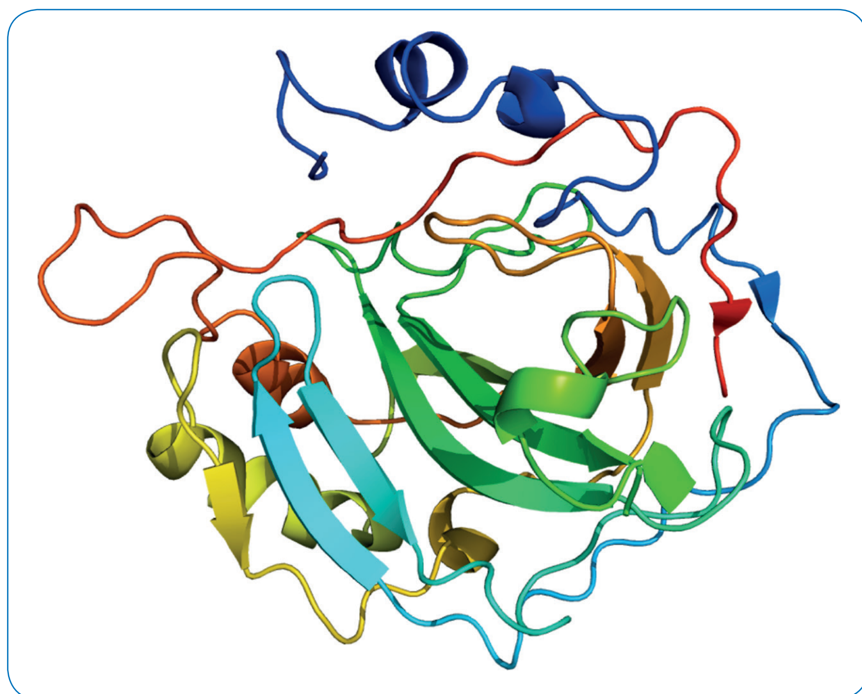

Figure 1: Structure of the protein Carbonic Anhydrase II.

isoforms must be determined to prevent toxicity or other adverse reactions can be simulated. The results show that MRMS has the right balance of desolvation and gentle ionization to enable measurement of native non-covalent complexes.

Carbonic anhydrase II and the protein-ligand complex have monoisotopic masses of 29,070 Da and 29,292 Da. These masses were obtained by mass deconvolution of isotopically resolved peaks from charge state 10+, 11+ and 12+. MRMS provided isotopically resolved mass spectra for the protein and protein-ligand complexes.

Introduction

Native mass spectrometric measurements of non-covalent protein-ligand binding have been reported for more than 25 years [2-4]. 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.

Methods

Sample preparation

The protein carbonic anhydrase 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. The protein CA II was purified five times with 10 mM pH 7.0 ammonium acetate buffer with an Amicon Ultra 10k cellulose membrane filter (Merck KGaA, Darmstadt, Germany) at a concentration of 33 µM. Stock solutions (50 mM) of acetazolamide ligand were prepared in water. 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

Carbonic anhydrase II ligand complexes were measured with a scimaX® MRMS system (Bruker Daltonik GmbH, Bremen, Germany). The mass spectra were externally calibrated using sodium trifluoroacetic acid cluster. The sample solutions were infused with the standard MRMS ESI source with a syringe (250 µL) at a flow rate of 4 µL/min using electrospray ionization in positive ion mode. Spectra were recorded in a mass range of m/z 300 – 4000 with 2M data size in magnitude mode resulting in a resolving power of 70,000 at m/z 2645. 200 single scans were summed for the final mass spectrum. An ion accumulation time of 7 s was used for data acquisition.

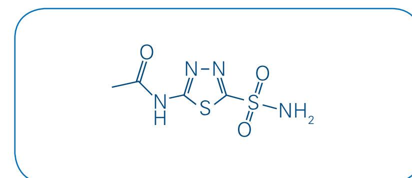

Figure 2: Structure of the ligand (drug) acetazolamide.

The ion source conditions were as follows: capillary voltage -4.5 kV, nebulizer gas 1.5 bar, drying gas 4 L/min, dry gas temperature 200°C, funnel 1 RF voltage of 150 V and skimmer 1 voltage of 70 V.

Data processing

Mass spectra were deconvoluted with MaxEnt in DataAnalysis 5.2 (Bruker Daltonik GmbH, Bremen, Germany). The charge states 10+, 11+ and 12+ were used for mass deconvolution. Additionally, spectra were mass deconvoluted with SNAP2 to generate monoisotopic masses of the native protein and protein-ligand complexes. The charge state 10+ was used to calculate the accurate masses and intensities of free protein and protein-ligand complexes. The 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 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. The published dissociations constants for acetazolamide with CA II (human) is 20 ± 3 nM. [5] 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

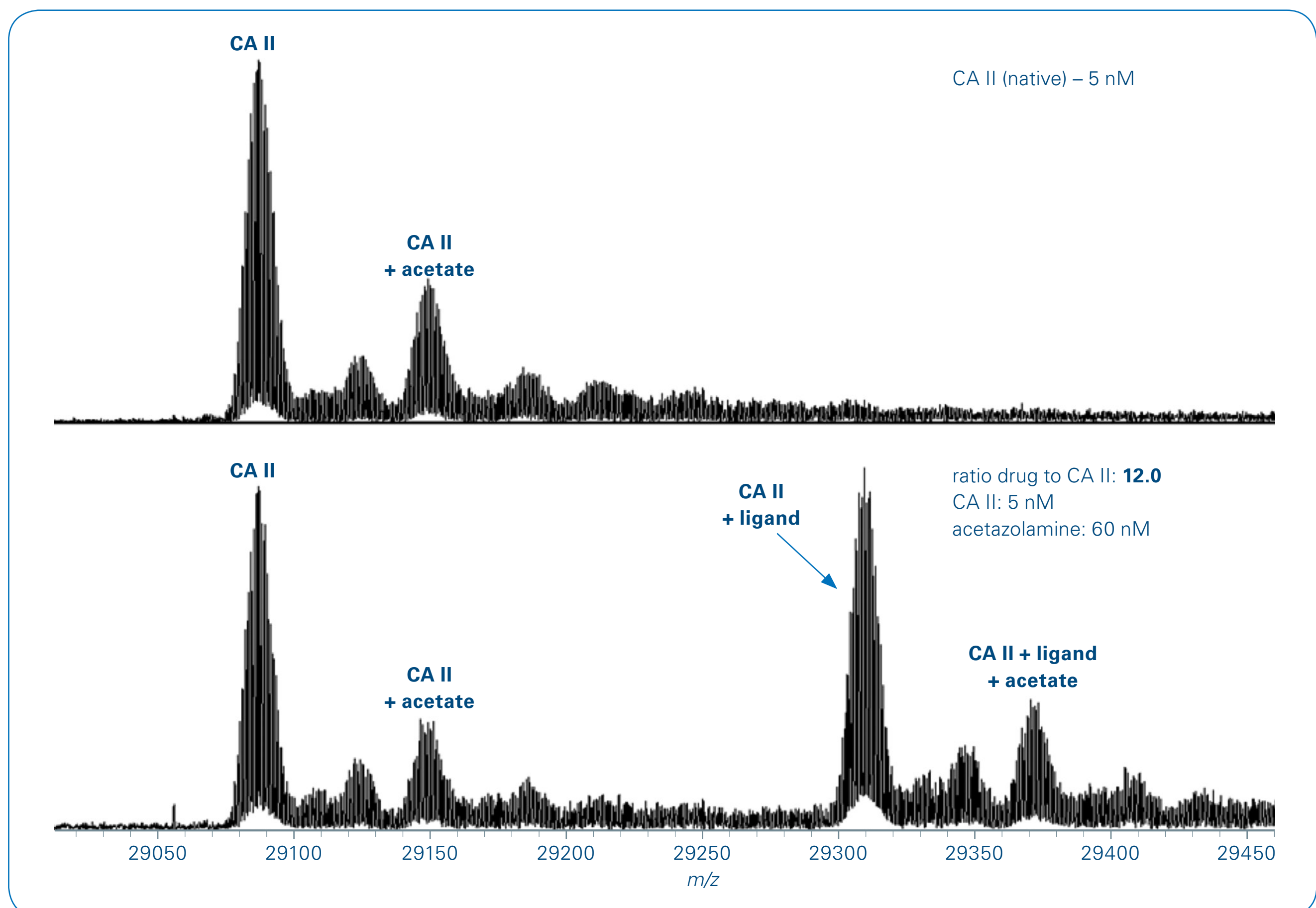

Figure 3: Isotopically resolved deconvoluted mass spectrum by MaxEnt of CA II protein at 5 nM (top) and CA II-ligand complex formed at 5 nM CA II and 60 nM acetazolamide (bottom).

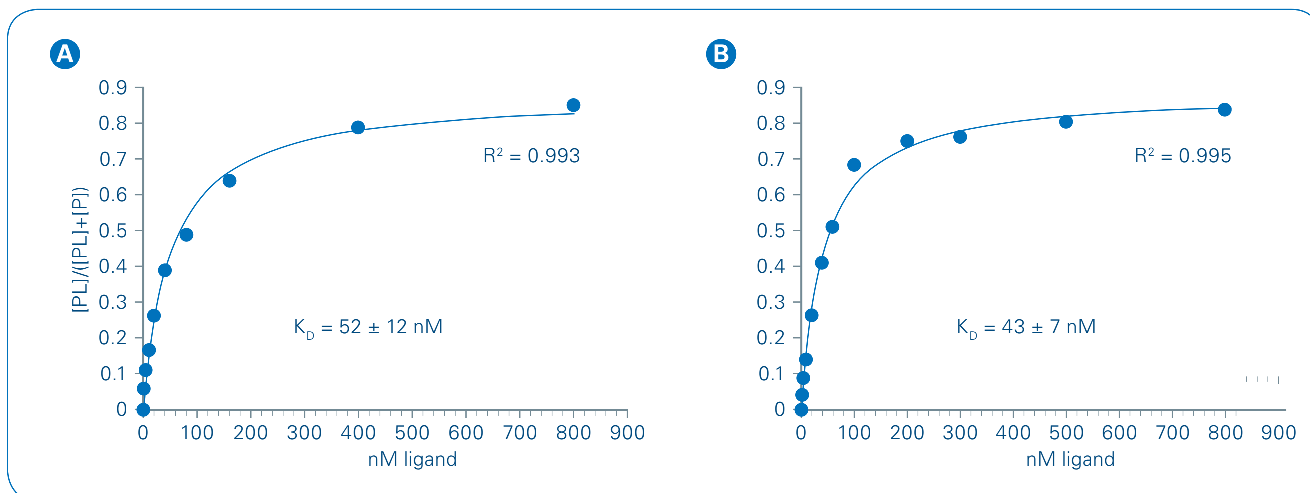

Figure 4: Plots of ligand binding [PL]/([PL]+[P]) versus the ligand concentration to determine K_D-values of the CA II-acetazolamide complex for protein concentrations of (A) 5 nM and (B) 20 nM. [PL]: intensity of protein-ligand complex; [P]: intensity of the free protein; ligand: acetazolamide.

Table 1a: 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 5 nM.

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

Table 1b: 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 20 nM.

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

20 nM (Figure 4a and 4b). Regression factors of better than 0.99 were calculated for both protein concentrations using the non-linear regression for specific binding. 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. These results are in good agreement with published K_D value of 20 ±3 nM [5].

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.

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. 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.

Conclusion

- The isotopic distributions of carbonic anhydrase II and their protein-ligand complexes were measured under native conditions in the low nM range using the scimaX® MRMS.
- The molecular mass (monoisotopic) and intensities of intact protein and protein-ligand complexes were obtained by mass deconvolution using SNAP2. The mass deviations of intact proteins and the protein-ligand complexes were within a few mDa over a wide range of ligand to protein ratios.
- The relative binding strength between protein and ligand could be assessed quickly and quantitatively from the relative peak intensities. 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.
- scimaX® MRMS is an excellent instrument to perform protein binding analysis at low protein concentrations and high buffer concentrations. As required for lead optimization by medicinal chemists, strong protein-ligand complexes with K_D values in the low nM range can be measured. Additionally, ligand specificity for protein isoforms can be directly observed, reducing need for multiple assays. With the scimaX® MRMS Bruker offers high sensitivity and versatility for molecular screening in drug discovery.

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