

# Label-free determination of protein–ligand binding constants using mass spectrometry and validation using surface plasmon resonance and isothermal titration calorimetry

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We performed a systematic comparison of three label-free methods for quantitative assessment of binding strengths of proteins interacting with small molecule ligands. The performance of (1) nanoelectrospray ionization mass spectrometry (nESI-MS), (2) surface plasmon resonance (SPR), and (3) isothermal titration calorimetry (ITC) was compared for the determination of dissociation constants ( $K_D$ ). The model system studied for this purpose was the human carbonic anhydrase I (hCAI) with eight known and well characterized sulfonamide inhibitors (Krishnamurthy *et al.*, *Chem. Rev.* 2008, 108: 946–1051). The binding affinities of the inhibitors chosen vary by more than four orders of magnitude e.g., the  $K_D$  value determined for ethoxzolamide by nESI-MS was  $5 \pm 1$  nM and the  $K_D$  value for sulfanilamide was  $145.7 \pm 10.0$   $\mu$ M. The agreement of the determined  $K_D$  values by the three methods investigated was excellent for ethoxzolamide and benzenesulfonamide (variation with experimental error), good for acetazolamide and 4-carboxybenzenesulfonamide (variation by  $\sim$  one order of magnitude), but poor for others e.g., sulpiride. The accuracies of the  $K_D$  values are determined, and advantages and drawbacks of the individual methods are discussed. Moreover, we critically evaluate the three examined methods in terms of ease of the measurement, sample consumption, time requirement, and discuss their limitations. Copyright © 2009 John Wiley & Sons, Ltd.

**Keywords:** nanoelectrospray mass spectrometry; noncovalent complexes; carbonic anhydrase; isothermal titration calorimetry; surface plasmon resonance

## INTRODUCTION

Noncovalent interactions are of great importance throughout nature. Noncovalent binding is not only critical in maintaining the three-dimensional structure of large biomolecules but also plays a fundamental role in molecular recognition in all biological processes from enzymatic catalysis to signal transduction. It is crucial in modern biological and medical research to have reliable methods at hand for quantitative determination of binding strengths. Many techniques to measure binding strengths (e.g., fluorescence polarization or radioassays) require labeling; difficult syntheses, radioactive waste, and incorrect results caused by the presence of a label can be problematic. Hence, label-free determination of the binding strength is desirable (Cooper, 2003). A common, truly label-free solution measurement is isothermal titration calorimetry (ITC), where the heat change associated with binding is the read-out. The ITC instrument is a heat-flux calorimeter and measures the amount of power ( $\mu$ cal/s) required to maintain a constant temperature difference between the sample/working and reference cell. The complex formation which takes place after each injection of the sample solution into the sample cell, is accompanied by a change in temperature (exothermic or endothermic reaction) between the two cells. ITC has rapidly become one of the most common methods for studying molecular recognition processes and is the method of

choice for determining the enthalpic and entropic contributions to the free energy of binding in a single experiment (Ladbury, 1998; Ladbury, 2004; Okhrimenko and Jelesarov, 2008). In fact, ITC has been termed the “gold standard” (Cooper, 2003) for characterizing biomolecular interactions, despite its main drawback, the fairly large amount of sample required for a measurement.

Surface plasmon resonance (SPR) is a widely accepted technique for the investigation of binding kinetics and binding strengths. Next to other biosensor methods used for studying biomolecular interactions such as quartz crystal microbalance, acoustic wave sensors, and refractometry, SPR is nowadays the

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most frequently used (Cooper, 2003). The principle of SPR measurements is relatively simple: a "bait" ligand is immobilized on the gold surface of the SPR chip. A solution containing the "prey" receptor is made to flow over the bait layer in a microfluidic system. The incident light of the instrument is electromagnetically "coupled" to a propagating surface plasmon of the gold layer. This surface plasmon is influenced by its proximate environment and will change, e.g., upon the noncovalent binding of the immobilized bait with the prey in the flowing solution. When the surface plasmon is affected by a binding event, the reflection angle of the incident light changes proportionally to the mass differences of the bait and the prey. This change of reflection angle as a function of time is the read-out of the SPR technique. Actually, SPR is not completely label-free, since one of the binding partners is immobilized onto a surface. It is generally assumed, however, that immobilization on thin hydrogel-like polymer layers such as dextran does not alter the protein conformation or activity (Löfås and McWhirter, 2006). The use of such semi-fluidic surfaces has become standard when studying biomolecular interaction with SPR.

Conventionally, SPR-based measurements are employed to determine binding strengths for macromolecular interactions (Myszka, 1999). However, SPR has gained more importance in recent years for the routine investigation of small molecules binding to macromolecular targets in drug discovery (Myszka *et al.*, 1996; Myszka, 1999; Rich and Myszka, 2008). Several groups have shown that biosensor experiments match solution-based methods (Day *et al.*, 2002; Bravman *et al.*, 2006; Navratilova *et al.*, 2007). Nonetheless, the reliability of binding constants determined from surface-based measurements is still debated. As shown by Myszka, SPR-based equilibrium constants do not necessarily match those obtained from solution-based methods for a variety of reasons, e.g., retaining an analyte on a surface could restrict its rotational freedom and diffusional properties, which alters the thermodynamics of the reaction and the binding kinetics (Myszka, 1997).

Quantitative determination of binding strengths by studying the noncovalent complexes with electrospray ionization mass spectrometry (ESI-MS) is an active field of research and has been reviewed several times (Henion *et al.*, 1993; Loo, 1997; Daniel *et al.*, 2002; Hofstadler and Sannes-Lowery, 2006). Nano-electrospray ionization mass spectrometry (nESI-MS) was introduced by Wilm and Mann (1996) and has been increasingly used to study noncovalent complexes (Zampronio *et al.*, 2004; Jecklin *et al.*, 2008). Automated MS measurements of noncovalent complexes and dissociation constants ( $K_D$ ) using an automated nESI robot was introduced by Zhang *et al.* (2003). The ligand titration method often used with ESI-MS involves detection of ions belonging to bound versus unbound proteins which are easily distinguished with MS by the characteristic mass shift corresponding to the noncovalently bound ligand molecule. The  $K_D$  value can be calculated from the ratio of the ion signal intensities for the bound and unbound protein as a function of added ligand assuming (1) that no dissociation takes place during the transmission through the mass spectrometer and (2) that the small molecule ligand bound to the protein does not alter the ionization efficiency of the noncovalent complex (Daniel *et al.*, 2002).

It is still debated, however, whether noncovalent complexes detected in the gas phase are specific and whether the relative peak intensities in ESI spectra reflect the concentration ratios in the solution (Cunniff and Vouros, 1995; Kitova *et al.*, 2002; Ashcroft, 2005; Hossain *et al.*, 2005; Iavarone and Parks, 2005;

Ruotolo *et al.*, 2005; Benesch and Robinson, 2006; Ruotolo and Robinson, 2006; Sharon and Robinson, 2007). For instance, there is some evidence that the stability of noncovalent protein-ligand complexes may not reflect their solution affinities for interactions with mixed hydrophobic and hydrophilic components (Yin *et al.*, 2008). It has also been shown that removal of water from a protein (dehydration) can have unexpected consequences on its structural properties and its interactions with ligands and other macromolecules (Patriksson *et al.*, 2007).

With the goal of evaluating how well the established methods for quantification of binding strengths match each other, we compared SPR (surface interaction), nESI-MS (gas phase interaction) with ITC (pure solution phase interaction) for the determination of the dissociation constant ( $K_D$ ). Such benchmarking studies of the ESI (or nESI) titration method against established methods are rare but necessary. A similar study was published by our group in 2007, where the interaction of calmodulin and melittin was compared for MALDI-SUPREX, circular dichroism, SPR, and ESI titration (Mathur *et al.*, 2007). The  $K_D$  values determined with the different methods were in the low nM range;  $K_D$  values of  $0.1 \pm 2.1$  nM (SUPREX), 5.7 nM (circular dichroism),  $6 \pm 23$  nM (SPR), and 52 nM (ESI titration) were determined, close to the lower limit of some of these methods. In the work presented here, we focus on the accessible  $K_D$  range of different methods. For this purpose we studied the binding of human carbonic anhydrase I (hCAI) with several sulfonamide inhibitors. The inhibitors chosen have very different binding affinities for the hCAI target and  $K_D$  values that cover a range greater than four orders of magnitude (e.g., ethoxzolamide  $K_D = 5 \pm 1$  nM; sulfanilamide  $K_D = 145.7 \pm 10.0$   $\mu$ M). It will be examined how well the ITC, SPR, and nESI-MS measurements are suited for measuring  $K_D$  values in different ranges. We also critically evaluate the three examined methods in terms of sample consumption, time requirement, and discuss their limitations.

## EXPERIMENTAL

### Materials

Carbonic anhydrase I (hCAI, EC 4.2.1.1, MW = 28.9 kDa) from human erythrocytes, all sulfonamide inhibitors (as listed in Table 1), caesium iodide, and buffer reagents were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Prior to analysis the protein concentration was determined using a NanoDrop (NanoDrop 1000, Thermo Scientific, Witec AG, Littau LU, Switzerland). For non-denaturing MS analysis, a volatile buffer has to be used to avoid ion suppression and adduct formation (Ashcroft, 2005; Sharon and Robinson, 2007). Therefore, a 50 mM ammonium acetate (AmAc) buffer at pH 7.4 was used for all the nESI-MS, ITC, and SPR measurements (unless indicated otherwise).

CM5 sensor chips, *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide (EDC), and ethanolamine HCl, as well as sampling vials, caps used for the SPR analysis were purchased from Biacore (Uppsala, Sweden).

### Nano-electrospray mass spectrometry (nESI-MS)

#### Instrument settings

Mass spectrometric analysis was performed with a hybrid quadrupole time-of-flight mass spectrometer (Q-ToF Ultima; Waters/Micromass Ltd., Manchester, UK) equipped with a Z-spray

**Table 1.** List of the sulfonamide inhibitors examined in this study

No.	Compound	MW	Concentrations nESI-MS	Concentrations SPR	Concentrations ITC
1	Acetazolamide	222	1, 2, 4, 6, 8, 10	0.016, 0.08, 0.4	500
2	Benzenesulfonamide	157	1, 2, 3, 4, 5, 6, 10	0.4, 2, 10	500
3	4-Carboxybenzene-sulfonamide	201	0.5, 1, 2, 3, 4, 6, 8	0.4, 2, 10	500
4	Ethoxzolamide	258	0.5, 1, 2, 3, 4, 5	0.016, 0.08, 0.4	50, 100
5	Furosemide	331	1, 2, 3, 4, 6, 8, 10	0.4, 2, 10	>100 μM, not soluble
6	Sulfanilamide	172	1, 5, 10, 20, 40	0.4, 2, 10	500
7	(±)-Sulpiride	341	No complex detected	2, 10, 50	500
8	Topiramate	339	0.5, 1, 2, 3, 4, 5	0.4, 2, 10	500

The concentrations (all given in μM) used for the different techniques are given in columns 4–6.

interface. The instrument was controlled via the MassLynx version 4.0 software. All measurements were performed in the positive ion mode. Argon was used as collision gas and a typical setting of 10 V was used for the collision energy parameter for optimized desolvation. The RF1 lens voltage, which is similar to the “declustering potential” (Yin *et al.*, 2008) in other instruments, was set to 35 V. All mass spectra for the titration experiments were recorded under nondenaturing conditions using the AmAc buffer. Mass spectra were accumulated during 2–3 min to obtain a good signal-to-noise ratio. Calibration of the instrument up to  $m/z$  6000 was performed using caesium iodide (CsI) clusters generated by spraying a solution of CsI in water/2-propanol (1/1, V/V) at a concentration of 2 μg/μl. The nESI-MS measurements were performed using an automated chip-based nESI robot (NanoMate Model 100, Advion Bioscience, Ithaca, NY, USA). It holds a 96-well sample plate, a rack of 96 disposable, conductive pipet tips, and a nanospray chip containing 20 × 20 nozzles of 5 μm diameter. A gentle backing pressure of 4–6 bar on the spray tip was used to assist the liquid sample flow.

#### Titration experiments and data fitting

Titration experiments were performed with a constant protein concentration (5 μM for hCAI) and varying ligand concentration as reported in Table 1. Mass spectra were first background subtracted and smoothed using the MassLynx software. Second, peak areas for free protein and the noncovalent complexes were integrated using JDPeakIntegrator—a software developed in our lab especially for this purpose. The model for the  $K_D$  calculation was adopted from Daniel *et al.* (2003). The ratio of the complex signal over the free protein signal was plotted against the concentration of ligand and titration curves obtained that way were fitted to the following equation:

$$\frac{I(P \cdot L)}{I(P)} = \frac{1}{2} \left( -1 - \frac{[P]_0 + [L]_0}{K_D} + \sqrt{4 \frac{[L]_0}{K_D} + \left( \frac{[L]_0 - [P]_0}{K_D} - 1 \right)^2} \right) \quad (1)$$

where  $I(P \cdot L)/I(P)$  stands for the intensity ratio of complex over free protein,  $[P]_0$  indicates the initial protein concentration,  $[L]_0$  the initial ligand concentration and  $K_D$  was the fitting parameter. We obtained different titration points by plotting the ratio of free protein over complex as a function of added ligand. It was assumed for all cases that the ionization efficiency is the same for complex and free protein, because the mass of the ligand is very

small compared to the proteins mass. Therefore, the ligand should not interfere greatly with the ionization processes. Furthermore, this assumption allowed us to use the intensity ratios of free protein over complex instead of their concentrations in solution; no response factor had to be introduced as described by Gabelica *et al.* (2003). The  $K_D$  calculation and titration curve fitting were performed using Origin v7.5 software (OriginLab Corporation, Northampton, MA, USA).

#### Surface plasmon resonance (SPR)

##### Instrumentation and reagents

SPR interaction analyses were performed using a Biacore T100 optical biosensor (Biacore Life Sciences/GE Healthcare, Uppsala, Sweden). Series S Sensor Chips CM5, NHS, EDC, and ethanolamine HCl, as well as sampling vials, and caps, were obtained from Biacore. Data were collected with the biosensor instrument thermostated to 25 °C. Either a solution of 0.02 M  $\text{Na}_2\text{HPO}_4$ - $\text{NaH}_2\text{PO}_4$ , 0.15 M NaCl, pH 7.4 (PBS) or 50 mM AmAc, pH 7.4 (AmAc) was used as running buffer.

##### Instrument cleaning and preparation

Our SPR biosensor is routinely cleaned to maintain the performance of the instrument using the “desorb” function in the Biacore Control software. The instrument was set to 25 °C and primed with water before inserting a maintenance chip. A maintenance chip is a sensor chip without the gold surface and is used to avoid introducing contaminants during the cleaning process. The standard desorb procedure was run twice and involved injecting 0.5% (w/v) SDS followed by 50 mM glycine, pH 9.5, through the system to clean the syringe pumps, integrated μ-fluidic cartridge (IFC), pneumatic valves in the IFC, needle, and instrument tubing.

##### Chip preconditioning

To prepare the sensor chip for interaction analysis, a series of solutions were injected over the chip surface. A new CM5 sensor chip was inserted into the instrument using the “dock” command and the instrument primed with running buffer. The conditioning solutions (100 mM HCl, 50 mM NaOH, 0.5% (w/v) SDS, and water) were used to hydrate and clean the dextran layer. Two aliquots of each conditioning solution were injected over all four flow cells (Fc) for 10 s at a flow rate of 100 μl/ml.

### Enzyme immobilization

A solution of 0.01 M sodium acetate, pH 5.0 was used as immobilization buffer. To minimize denaturation, hCAI was dissolved to a final concentration of 250  $\mu\text{g/ml}$  in the immobilization buffer only immediately before use. Using a flow rate of 10  $\mu\text{l/min}$ , the surface of flow cell 4 was activated for 7 min using a 1:1 mixture of 0.1 M NHS and 0.4 M EDC (both dissolved in water), 250  $\mu\text{g/ml}$  hCAI in immobilization buffer was injected for 7 min, and residual activated groups on the surface were blocked by a 7 min injection of 1 M ethanolamine, pH 8.5. A total of 16 800 response units (RU) of hCAI were immobilized. For this study, we chose to immobilize 10 100 RU BSA in flow cell 3 for using as a reference surface.

### Analyte injection method and order

An initial series of four buffer blanks was injected first before each analyte series to fully equilibrate the system. The analyte samples were analyzed first from the lowest to the highest concentration and then run in duplicate from the highest to the lowest concentration. During each binding cycle the analyte was injected for 1 min at a flow rate of 100  $\mu\text{l/min}$  and dissociation was monitored for 180 s.

### Data processing and kinetic analysis

Data collected on an SPR biosensor require processing to remove systematic artifacts stemming from nonspecific binding, signal drift, and bulk refractive index changes. Data sets were processed and analyzed using the Biacore T100 Evaluation Software. Double referenced (Myszka, 1999) association and dissociation phase data for the corresponding analyte were globally fit to a simple 1:1 interaction model ( $A + B = AB$ ).

## Isothermal titration calorimetry (ITC)

### Instrumental protocol

The ligands were diluted shortly before the analysis in the same buffer batch as the protein to minimize heat of dilution effects from a buffer mismatch. All experiments were performed with the ligand in the syringe (titrant) and protein in the cell (titrand). ITC measurements were performed using a Microcal VP-ITC instrument (MicroCal, Northampton, MA, USA). We used the following experimental parameters: 30 injections of compound, with an initial first injection of 2  $\mu\text{l}$  and subsequent 10  $\mu\text{l}$  injections of compounds into the protein with 180 s between each injection. The stirring speed was set to 270 rpm. All ITC measurements were performed at 25°C. Concentrations of the compounds used for the ITC measurements are listed in Table 1. The data obtained were evaluated with a single-site binding model using Origin software provided by MicroCal.

### Competitive experiments and data evaluation

In a classical ITC titration experiment (direct titration), the appropriate concentrations of reactants depend on (a) the sensitivity of the instrument (typically 0.1  $\mu\text{cal}$ , thus limiting the change of heat involved per injection to a minimum of 1  $\mu\text{cal}$ ), (b) the individual heat associated with the reaction of the binding partners ( $\Delta H$ ), and (c) the affinity of the interaction ( $K_D$ ). A dimensionless constant ( $c$ -value or Wiseman-constant) describes

the practical window for accurate determination of binding constants:

$$c = nK_A[P]_t \quad (2)$$

where  $K_A$  is the association constant,  $[P]_t$  is the total protein concentration in the working cell,  $n$  is the stoichiometry of the reaction. Typically, the concentrations of the reactants must be in the  $\mu\text{M}$  range because the sensitivity of the instrument is insufficient at lower concentrations (Ladbury, 1998; Ladbury, 2004; Velazquez-Campoy *et al.*, 2004). Data analysis, function fitting, and extraction of parameters (i.e.,  $K_D$ ,  $\Delta H$ ,  $\Delta S$ ) are thoroughly explained in the literature in very good detail (Ladbury, 1998; Ladbury, 2004; Velazquez-Campoy *et al.*, 2004).

Low affinity systems require soluble receptors and ligands at quite high concentrations to achieve a  $c$ -value of 10–100, if they ought to be studied in a classical direct titration experiment. However, the solubility of small organic molecules (like the sulfonamides examined here) at higher concentrations in aqueous buffers is sometimes very limited. Moreover, a large amount of protein sample is required for one low affinity titration experiment (to reach a measurable heat change upon binding). There are some interesting alternatives reported in the literature on how low affinity systems with low  $c$ -values can be made accessible to measurement by ITC e.g., via competitive experiments (displacement strategy) (Zhang and Zhang, 1998; Sigurskjold, 2000; Turnbull and Daranas, 2003; Turnbull, 2005). The beauty of such competitive experiments is that (a) only a small amount of protein sample is needed to perform the experiment and (b) the ligand concentration can be reduced compared to a direct titration experiment. However, the reference ligand has to be very well characterized and its parameters ( $K_D$ ,  $\Delta H$ , binding stoichiometry) have to be accurately known to minimize errors (Turnbull, 2005).

The  $K_D$  value (plus  $\Delta H$ ) of ethoxzolamide binding to hCAI was first determined using ITC via classical direct titration. The parameters obtained were  $\Delta H = -9.0 \pm 0.5$  kcal/mol,  $K_D = 7.3 \pm 2.0$  nM which are in excellent agreement with literature values ( $\Delta H = -8.9 \pm 0.3$  kcal/mol,  $K_D = 9.0 \pm 1.6$  nM (Conroy and Maren, 1995; Krishnamurthy *et al.*, 2008)). We could thus employ this compound as a reference competitor against other, weaker binders. To minimize sample consumption of protein and avoid solubility problems of the small organic inhibitors, the  $K_D$  values of the rest of the compounds were therefore evaluated using competitive experiments. This was achieved by preparing the samples for the working cell with 10  $\mu\text{M}$  hCAI in the presence of an excess of the ligand of interest (cf. Table 1 for the used concentrations for the individual compounds) and then performing a titration with the syringe solution containing 100  $\mu\text{M}$  of ethoxzolamide. The data evaluation and fitting procedure are described in the literature in detail (Zhang and Zhang, 1998; Velazquez-Campoy *et al.*, 2004).

## RESULTS

In soft ionization MS involving noncovalent complexes the specificity of compound binding can be an issue (Cuniff and Vouros, 1995; Kitova *et al.*, 2002; Hossain *et al.*, 2005; Iavarone and Parks, 2005; Ruotolo *et al.*, 2005; Benesch and Robinson, 2006; Ruotolo and Robinson, 2006; Yin *et al.*, 2008) and was therefore tested first. Denatured hCAI (5  $\mu\text{M}$ ) was incubated with an excess of the tightly binding ethoxzolamide (20  $\mu\text{M}$ ) (Krishnamurthy

*et al.*, 2008). No binding was observed, indicating that all protein activity has been lost (data not shown). In addition, we performed a negative control experiment, mixing hCAI (5  $\mu$ M) under nondenaturing conditions with 40  $\mu$ M adenosine-5'-diphosphate-diphosphate (ADP, a nonbinder). Again, no noncovalent complex was detected. In a next step, instrumental parameters (collision energy and RF1 lens) influencing the noncovalent complex stability of hCAI binding to ethoxzolamide were studied, as shown in Figure 1.

In 1999, Gao *et al.* used sustained off-resonance irradiation collision-induced dissociation (SORI-CID) experiments to determine the energetics of dissociation of CAII-sulfonamide complexes in the gas phase (Gao *et al.*, 1999). They concluded that the conformation of the binding pocket and activity of the carbonic anhydrase is retained in the gas phase which gives further indication that the signal ratio of free protein versus protein–ligand complexes detected by MS reflects the situation in solution.

Collision induced dissociation (CID) and RF1 lens breakdown curves experiments are important because additional information can be gained on the stability of noncovalent complexes in the gas phase and on their dissociation rate during the transmission through the mass spectrometer (Jecklin *et al.*, 2008). In the case of hCAI with ethoxzolamide, the noncovalent complexes are fully destroyed at an RF1 lens setting of 70 V and a collision energy setting of 30 V (cf. Figure 1). However, at an RF1 lens setting of 35 V and a collision energy setting of 10 V the noncovalent complexes are still intact. This result implies that when using the appropriate potentials (indicated by the dotted lines) the noncovalent complexes will remain intact. Lower settings lead to very broad signals (adduct formation with buffer components) and very low sensitivity, making it impossible to distinguish between free and complexed protein signals. The chosen setting is therefore a good compromise between desolvation of the protein ions where still no dissociation of the noncovalent complexes is observed.

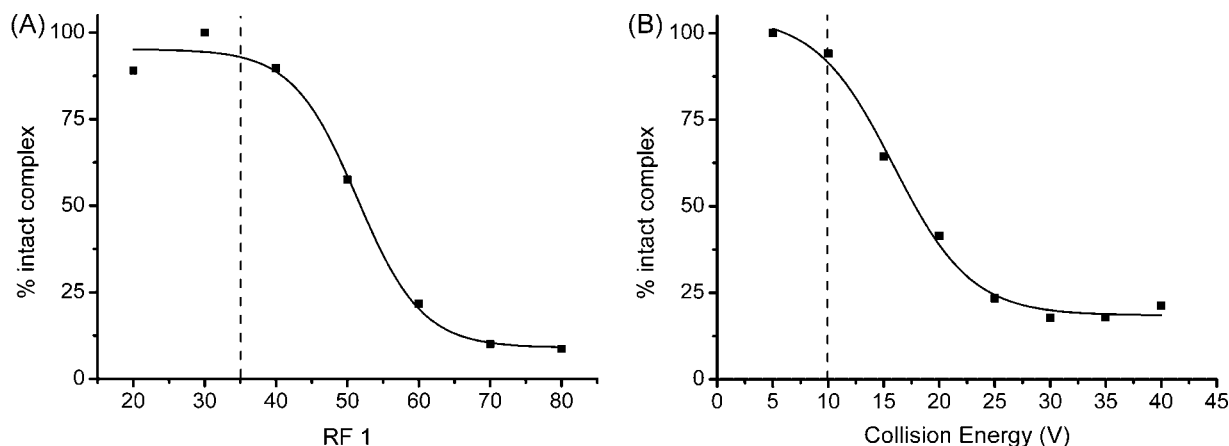
An example of an nESI-MS titration experiment of hCAI with ethoxzolamide is shown in Figure 2. The mass spectrum obtained for 5  $\mu$ M hCAI in the absence of ligand is given in Figure 2A. Only the charge states 11+, 10+, and 9+ were observed, characteristic for nondenaturing conditions (50 mM AmAc buffer pH 7.4). Figures 2B–D show mass spectra obtained for the noncovalent

hCAI-ethoxzolamide complexes using three different ligand concentrations. It is to note that the charge distribution observed does not change in the presence of ligand. The concentrations used for the full titration and  $K_D$  determination are listed in Table 2. As demonstrated in previous work, the ratio between the free protein signal and noncovalent complexes in nESI-MS can vary depending on the charge state (Jecklin *et al.*, 2008). This can also be reflected in the  $K_D$  value. In the present work, we determined the  $K_D$  value for each charge state and found a variation of only approximately 10% for all the ligands. All  $K_D$  values reported in this work for nESI-MS are averaged over the three observable charge states for hCAI.

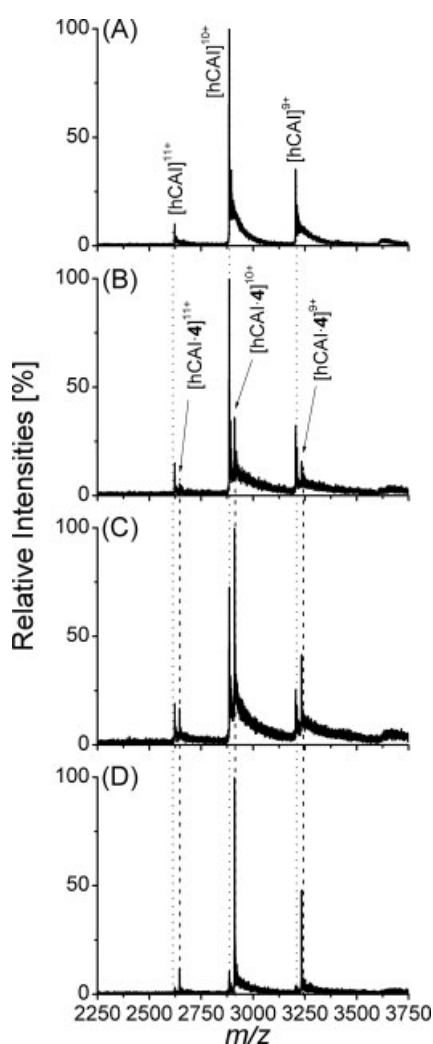
The different titration curves obtained for the nESI-MS approach are shown in Figure 3. The signal ratio of protein–inhibitor complex over the free protein signal is plotted as a function of total inhibitor concentration. The concentrations used for the individual titration experiments of the sulfonamide compounds are given in Table 1. The titration curves obtained were fitted using Equation 1, as described in the “Experimental” section (fits are depicted as dotted lines in Figure 3). The calculated  $K_D$  values for the different compounds are reported in Table 3. The slope of the titration curves reflects the affinity of the compounds for the hCAI protein target—the larger the slope (e.g., ethoxzolamide) the higher the affinity and *vice versa*.

The kinetic data obtained for the interaction of the sulfonamide compounds with hCAI immobilized on the SPR chip are shown in Figure 4. All sensograms obtained were of good quality, reasonable sensitivity, and no anomalous responses (e.g., problematic injections, spikes, baseline drifts, refractive index jumps are common) were found (Papalia *et al.*, 2006). The fairly low S/N ratio is typical for SPR experiments where the heavy binding partner (in this case, the protein) is immobilized, and the change in the SPR response is only due to a very small increase in the surface occupation due to the ligand. The raw data of the individual measurements are shown in gray, the fits obtained according to the 1:1 interaction model are shown in black. The rate and equilibrium constant determined from the sensograms are reported in Table 2.

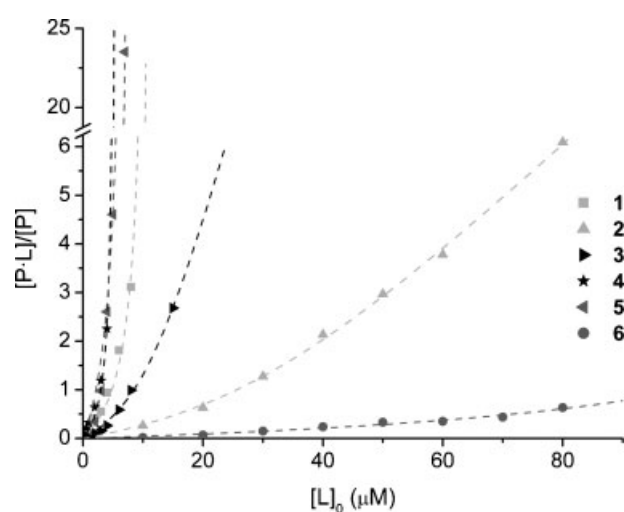
The interactions of the sulfonamide compounds with hCAI were also studied using ITC (pure solution interaction). Figure 5 shows the ITC analysis of ethoxzolamide in a direct titration



**Figure 1.** Breakdown curves are shown for the noncovalent complex between hCAI and ethoxzolamide monitoring the complex stability as a function of RF1 lens and collisional energy parameter. The noncovalent complexes are fully destroyed at an RF1 lens setting of 70 V and a collision energy setting of 30 V. However, at an RF1 lens setting of 35 V and a collision energy setting of 10 V, the noncovalent complexes are still intact. This study indicates that the values used for the titration experiments (shown as dotted lines) guarantee that the noncovalent complexes still remain intact.



**Figure 2.** NanoESI mass spectra of hCAI (5  $\mu\text{M}$  in 50 mM AmAc buffer, pH 7.4) in the presence of different concentrations of ethoxzolamide: (A) 0  $\mu\text{M}$ , (B) 1  $\mu\text{M}$ , (C) 2  $\mu\text{M}$ , and (D) 5  $\mu\text{M}$ . The free protein signal is indicated by the dotted line and the noncovalent complex between hCAI and ethoxzolamide is indicated by the dashed line. The signal for the noncovalent complex clearly increases with increasing amounts of ligand present in solution and at an equimolar concentration of 5  $\mu\text{M}$ , full complexation is reached.



**Figure 3.** Titration curves obtained for nESI-MS using 5  $\mu\text{M}$  of hCAI titrated with increasing amounts of ligand. The ratio of complex signal over free protein signal ( $[P-L]/[P]$ ) is plotted against the initial ligand concentration and the data were fitted with the equation described in the "Experimental" section (the dotted lines indicate the fits). The concentrations used for the individual compounds are listed in Table 1 and the determined  $K_D$  values are shown in Table 3.

experiment (A) and the competitive experiments with the other sulfonamide ligands (cf. Table 1) (B). The upper panel of Figure 5A shows the heat evolution when adding ethoxzolamide. The raw heat signals from this titration experiment were integrated to generate a plot of kcal/mol of injected ethoxzolamide versus the molar ratio of ligand and protein, as displayed in the lower panel. The plots of kcal/mol of the competitive experiments with the other ligands (cf. Table 1) are shown in Figure 5B. The molar ratios of all ligands are at 1 at the inflection point indicating a 1:1 stoichiometry for the interaction, which agrees very well with the literature (Krishnamurthy *et al.*, 2008). The  $K_D$  values obtained by ITC are reported in Table 3.

## DISCUSSION

### Accuracy of $K_D$ values determined by nESI-MS, SPR, and ITC

The  $K_D$  values obtained for nESI-MS titration, SPR, and ITC measurements are summarized in Table 3. A direct comparison of all three methods is only possible with five compounds, because two compounds (sulpiride and topiramate) could not be probed

**Table 2.** Kinetic rate constants determined with the SPR experiments for hCAI using the AmAc buffer

No.	Compound	$k_a$ ( $\text{M}^{-1} \text{s}^{-1}$ )	$k_d$ ( $\text{s}^{-1}$ )	$K_D$ ( $\mu\text{M}$ )
1	Acetazolamide	$2.38 \times 10^7$	0.3082	0.0130
2	Benzenesulfonamide	$9.18 \times 10^3$	0.01025	1.12
3	4-Carboxy-benzenesulfonamide	$2.58 \times 10^4$	0.075	2.91
4	Ethoxzolamide	$4.57 \times 10^6$	0.01084	0.0024
5	Furosemide	$2.12 \times 10^4$	0.05046	2.38
6	Sulfanilamide	6858	0.006337	0.924
7	( $\pm$ )-Sulpiride	400.4	0.01427	35.6
8	Topiramate	8647	0.01167	1.35

**Table 3.** Results for the  $K_D$  determination using three label-free quantification methods (all values in  $\mu\text{M}$ )

No.	Compound	nESI-MS	SPR	ITC	Literature <sup>a</sup>
1	Acetazolamide	$0.619 \pm 0.129$	0.0130	$0.48 \pm 0.02$ (*)	$K_i = 0.9$ , Winum <i>et al.</i> (2003) $K_i = 0.25$ , Winum <i>et al.</i> (2005) $K_D = 1.15$ , Ladbury (2004)
2	Benzenesulfonamide	$2.1 \pm 0.1$	1.12	$3.97 \pm 0.7$ (*)	$K_i = 2.1$ , Winum <i>et al.</i> (2003)
3	4-Carboxy-benzenesulfonamide	$4.6 \pm 0.34$	2.91	$15 \pm 5$ (*)	$K_D = 3.4$ , Winum <i>et al.</i> (2005)
4	Ethoxzolamide	$0.005 \pm 0.001$	0.0024	$0.0073 \pm 0.002$	$K_i = 0.025$ , Winum <i>et al.</i> (2003) and Winum <i>et al.</i> (2005) $K_D = 0.009$ , Conroy and Maren (1995)
5	Furosemide	$0.051 \pm 0.004$	2.38	Solubility problems	—
6	Sulfanilamide	$145.7 \pm 10.0$	0.924	$124.5 \pm 18.7$ (*)	$K_i = 28$ , Winum <i>et al.</i> (2005) $K_D = 151 \pm 140$ , Ladbury (2004) $K_D = 89.7$ , Conroy and Maren (1995)
7	(±)-Sulpiride	No complex	35.6	$4.83 \pm 1.61$ (*)	—
8	Topiramate	Non-spec clustering	1.35	$4.9$ (*)	$K_i = 0.25$ , Winum <i>et al.</i> (2003)

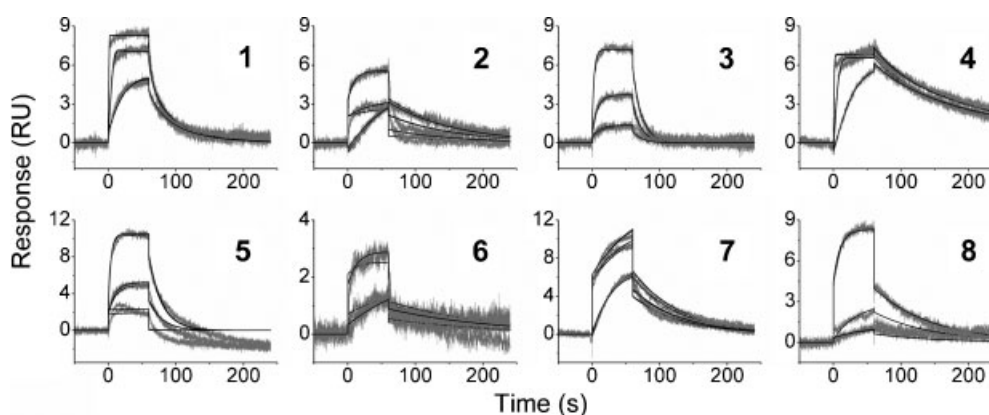
The compounds determined by competitive ITC experiments are marked with (\*). Literature values of the hCAI binding to the corresponding sulfonamide compounds are given in the last column.

<sup>a</sup>The inhibition constants ( $K_i$ ) were reported for the spectrophotometrically monitored inhibition of the hydrolysis of 4-nitrophenylacetate as substrate (Winum *et al.*, 2003).  $K_i$  values were determined according to the literature (Winum *et al.*, 2005; Khalifah, 1971).  $K_D$  values were determined with ITC,  $T = 37^\circ\text{C}$  (Ladbury, 2004).  $K_D$  values were determined with ITC,  $T = 37^\circ\text{C}$  (Conroy and Maren, 1995).

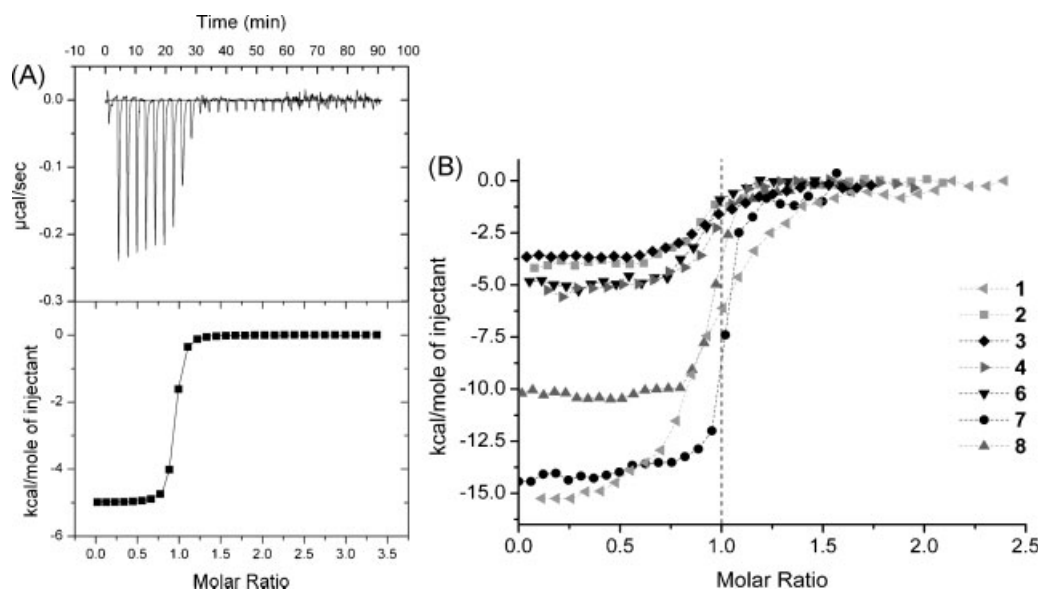
using nESI-MS and one compound posed problems for ITC (solubility). Table 3 also contains available literature values found for hCAI and the corresponding sulfonamide ligands. The inhibition constants ( $K_i$ ) were reported for the spectrophotometrically monitored inhibition of the hydrolysis of 4-nitrophenylacetate as substrate (Winum *et al.*, 2003).

Excellent agreement for all three methods was found for ethoxzolamide and benzenesulfonamide; the determined  $K_D$  values are all between 2.4 and 7.3 nM and 1.1 and 3.97  $\mu\text{M}$ , respectively, which is almost within experimental error. The  $K_D$  values determined using SPR are slightly lower than the values obtained by nESI-MS and ITC. Reasonable agreement between the methods was found for 4-carboxybenzenesulfonamide: the  $K_D$  values differ by a factor of 5.5 (maximum difference between SPR and ITC data) and all values are in the low  $\mu\text{M}$  range for this

compound. We note that the  $K_D$  value determined by SPR is again the lowest. For acetazolamide and sulfanilamide, a very good agreement was found between the nESI-MS and ITC values. A problem we faced with fitting the SPR data for acetazolamide was the fast association rates ( $k_{\text{on}}$  rates, cf. Table 2) which are influenced by mass transport, when the binding of the ligand to the protein on the sensor chip is faster than the diffusion of the ligand from the bulk solution to the protein at the surface (Myszka, 1997; Cooper, 2003). In this case, the information content of the curve shape is low and is prone to fitting errors. In the benchmarking study of Papalia *et al.* comparing different biacore models the same phenomenon was observed for acetazolamide binding to bovine carbonic anhydrase II (Papalia *et al.*, 2006). This can lead to a lower  $K_D$  value observed for SPR compared to the other techniques.



**Figure 4.** Data sets obtained using a Biacore T100 instrument. The raw data sensograms obtained are shown in gray and the individual fits obtained according to the 1:1 interaction model are shown in black.



**Figure 5.** ITC analysis of hCAI binding to ethoxzolamide (A) in a direct titration experiment, (B) shows the series of competition experiments performed with  $10\ \mu\text{M}$  hCAI incubated with  $500\ \mu\text{M}$  of the ligand of interest in the sample cell (cf. Table 1) titrated with  $100\ \mu\text{M}$  ethoxzolamide.

The signal-to-noise ratio was quite low for benzenesulfonamide and sulfanilamide with SPR, which is shown in Figure 4. The responses are quite low (e.g., only about three RUs for sulfanilamide) and the noise is quite large, leading to poor fits (two fits for the same concentrations of sulfanilamide do not overlap). Unfortunately, the mass of benzenesulfonamide and sulfonamide is  $<200\ \text{Da}$  (see Table 1), and since the response in SPR is proportional to the mass of the binding molecule, the signal is close to the limit of detection of the Biacore instrument for these ligands. As mentioned above, two compounds (sulpiride and topiramate) cannot be compared for all three methods because the nESI-MS data are missing. For sulpiride, no noncovalent complexes were detected. This might be due to a loss of noncovalent interaction between the protein and the organic compound in the gas phase.

In the case of topiramate, a different problem was found for the nESI-MS approach. For an equimolar mixture of hCAI with topiramate, several nonspecific complex signals  $(\text{hCAI} \cdot \text{topiramate})_{n=1-4}$  were detected. However, the crystal structures and available literature data clearly indicate a 1:1 stoichiometry for the binding of this compound. Nonspecific adduct formation is a known and well-studied phenomenon for soft-ionization MS involving noncovalent complexes. There are different ways to circumvent this problem and estimate the contribution of nonspecific interactions for  $K_D$  evaluation, e.g., by adding a reference protein (Sun *et al.*, 2006) or by modeling a statistical Poisson distribution (Daubenfeld *et al.*, 2006). However, such approaches present considerable amounts of additional work until optimal conditions are found. Moreover, it has to be assumed that the reference protein is affected by nonspecific interaction in exactly the same way than the protein of interest for the method described by Sun *et al.* (2006). For Poisson distribution modeling, many numerical parameters have to be assumed and adjusted (additional fitting parameters), and high quality spectra plus initial knowledge of the specific interactions are required (Daubenfeld *et al.*, 2006). This was beyond the scope of the present study.

Good agreement was found for the SPR and ITC data obtained for topiramate where the difference is only a factor of approxi-

mately 3 and the values are of the same order of magnitude. For sulpiride, the difference of the SPR and ITC values is a factor of approximately 7 which still represents a reasonable agreement. An affinity in the low  $\mu\text{M}$  range is indicated by the data.

Furosemide could also not be compared between the three techniques because this ligand was not soluble in our aqueous buffer at the concentrations required for either a direct ITC titration or a competitive experiment. The agreement of the  $K_D$  values obtained with nESI-MS and SPR, however, is quite poor. Since a third measurement is missing for this compound there could be a problem with the SPR or with the nESI-MS, as discussed above.

#### Comparing different buffers for SPR experiments

The  $K_D$  values determined with SPR using the AmAc buffer were in most cases lower than for the other two techniques (cf. Table 3). The SPR experiments were, therefore, repeated in PBS buffer and the values obtained are given in Table 4. The values between the two buffers agree well in most cases, the differences being less than one order of magnitude for the majority of them. This experiment suggests that the tonicity (the 50 mM AmAc buffer is quite hypotonic, the PBS buffer is isotonic) of the

**Table 4.** Results for the  $K_D$  determination using SPR for hCAI in AmAc and PBS buffer ( $K_{D\text{S}}$  in  $\mu\text{M}$ )

No.	Compound	$K_D$ (AmAc)	$K_D$ (PBS)
1	Acetazolamide	0.0130	0.0071
2	Benzenesulfonamide	1.12	0.962
3	4-Carboxybenzenesulfonamide	2.91	0.887
4	Ethoxzolamide	0.0024	0.0018
5	Furosemide	2.38	0.844
6	Sulfanilamide	0.924	2.7
7	( $\pm$ )-Sulpiride	35.6	18.4
8	Topiramate	1.35	0.349

aqueous buffer used does not greatly influence the interaction of hCAI with the examined sulfonamide compounds and the affinity of the corresponding compounds is very similar for both buffer systems.

### Comparison of method performance

Besides the accuracy of the binding constant, there are several other features that distinguish the three methods compared in this study. A comparison of all the methods used in this work in terms of analysis time, sample consumption, etc. is presented in Table 5. The nESI-MS titration is an interesting method for studying hCAI ligand complexes (a) because it allows direct examination of the stoichiometry of the interaction between the binding partners, (b) because the titration experiments are very easy to perform and can be automated using a chip-based nanoESI robot, and (c) because the sample consumption is relatively low (comparable to SPR). The accessible range for  $K_D$  determination is quite wide (low nM to hundreds of  $\mu\text{M}$ ). The best performance and greatest potential for the determination of  $K_D$  values of noncovalent protein–ligand complexes is probably in the range of tens of nM to tens of  $\mu\text{M}$ .

SPR and ITC provide a fully automated real-time analysis of noncovalent biomolecular interactions on a surface or in solution, respectively. The great advantage of SPR, in addition to its sensitivity, is the easy access to kinetic data of the noncovalent binding processes. A disadvantage is, however, that no stoichiometric information is available, and that the process of immobilization is sometimes delicate and often demands lengthy optimization. It is crucial to minimize or to completely avoid nonspecific surface–analyte interactions during binding experiments. ITC is still the method of choice when it comes to measurement of the thermodynamic properties of noncovalent binding processes. Moreover, compared to nESI-MS and SPR, ITC is a pure solution phase measurement and therefore there are no questions about the structural integrity of the protein or the specificity of the noncovalent complexes. The great disadvantages on the other hand are its large sample consumption and time requirement. The time needed to run a full titration experiment is at least 2.5 h and usually even more, since the thermal equilibration of the measuring cell after filling it and inserting the syringe can easily take an additional 30–60 min. As discussed above, the concentrations needed to achieve either a good  $c$ -value for a direct titration or the excess concentration needed to run a competitive experiment can lead to solubility issues for such small organic compounds (cf. furosemide).

Whereas the salt tolerance for ITC and SPR is usually not an issue (as long as buffer mismatches between the ligand and the protein solutions are avoided) one has to pay attention to the nature of the buffer when running nESI-MS experiments. The use of buffers for nESI-MS experiments involving nondenaturing protein conditions is limited to highly volatile aqueous buffers in order to avoid nonspecific clustering in the gas phase. Commonly, ammonium acetate or ammonium bicarbonate buffers are used (Ashcroft, 2005; Sharon and Robinson, 2007).

### Limitations and disadvantages of the three methods

#### ITC

The sensitivity of the instrument often is an issue—high concentrations needed for low affinity ligands—and proteins or small organic molecules are not always soluble (cf. furosemide). Moreover, the accessible  $K_D$  range is limited (see Equation 2, “Experimental” section). For high and low affinity ligands, competition experiments are often a better choice than direct titration (Zhang and Zhang, 1998; Sigurskjold, 2000; Turnbull and Daranas, 2003; Turnbull, 2005).

#### SPR

Problems with SPR measurements for protein–ligand interactions are described in a whole section of the review by Pattnaik (2005). Key issues are: (1) the sensitivity of an SPR instrument can be a problem since the angle of reflection is detected which is proportional to the mass of the analytes and can generate poor signals for small molecules (e.g., benzenesulfonamide and sulfanilamide in this work), (2) protein immobilization is dependent on the pl of the protein; there are different surface matrices and different immobilization chemistries available, (3) since in most cases the protein is immobilized on the surface, it is assumed that the protein conformation/activity remains unchanged, a typical assumption made for generally any labeling approach, (4) a homologous protein distribution is commonly assumed, and the use of high flow rates suppresses the diffusional properties of the ligand into (and out of) the activated chip surface (Myszka, 1997), (5) the majority of the published SPR data does not fit to the simple 1:1 model ( $A + B = AB$ ), which raises questions about the validity of biosensor analysis (Myszka, 1999), and (6) problems with fast  $k_{\text{on}}$  rates, leading to low  $K_D$  values (e.g., acetazolamide in this work) (Papalia *et al.*, 2006).

**Table 5.** Comparison of techniques with respect to time, ligand and protein amount needed for the  $K_D$  determination for one ligand binding to hCAI

Technique	Protein amount	Ligand amount	Time per data set (min)	Time for data evaluation (min)	Salt tolerance	Theor. accessible $K_D$ range
SPR	50–100 $\mu\text{g}^*$ (none after immobilization)	300 $\mu\text{L}$ of a 0.016–10 $\mu\text{M}$ solution	40–50	10	High	>1 nM–<100 $\mu\text{M}$
ITC	0.6–6.0 mg (10–100 $\mu\text{g}$ , 2 mL)	300 $\mu\text{L}$ of a 0.1–1 mM solution	140–150	15	High	10 nM–50 $\mu\text{M}$
nESI-MS	7.5 $\mu\text{g}$ (5 $\mu\text{M}$ , 10 titrations à 5 $\mu\text{L}$ )	1–20 $\mu\text{L}$ of a 40 $\mu\text{M}$ solution	30–40	20	Very low	10 nM–50 $\mu\text{M}$

\*The immobilized protein can be used for determination of whole sets of ligands.

## nESI-MS

As mentioned, there still is an ongoing debate whether the protein conformation in the gas phase is retained and whether the observed noncovalent complexes are specific. Nevertheless, an increasing number of examples of protein–ligand complexes studied by ESI-MS seem to be specific and the binding affinity measured by MS seems to reflect that in the solution (Henion *et al.*, 1993; Loo, 1997; Daniel *et al.*, 2002; Daniel *et al.*, 2003; Wendt *et al.*, 2003; Zhang *et al.*, 2003; Hofstadler and Sannes-Lowery, 2006; Mathur *et al.*, 2007; Jecklin *et al.*, 2008). An unfortunate drawback to this approach is the incompatibility with many commonly used aqueous buffers and the salt intolerance which limits the usable buffer compositions to a few volatile salts e.g., ammonium acetate and bicarbonate (Ashcroft, 2005; Sharon and Robinson, 2007). The instrument sensitivity can also be an issue since the limit of detection for folded proteins sprayed from a nondenaturing buffer is in the low  $\mu\text{M}$  range (usually 1–5  $\mu\text{M}$ ) and makes a differentiation and accurate determination of very high affinity ligands ( $K_D < 1 \text{ nM}$ ) impossible (Mathur *et al.*, 2007).

## CONCLUSIONS

Label-free methods for quantitative determination of binding strengths of protein–ligand systems are very important in life

sciences (Cooper, 2003). As demonstrated here for the model system hCAI with sulfonamide ligands, the overall agreement for the quantitative determination of binding affinities with nESI-MS, SPR, and ITC is good. The use of several independent methods is advantageous for validation in biomedical research. We can therefore conclude that nESI-MS can be a useful method for  $K_D$  evaluation of small molecule binders to proteins that should be employed to complement other  $K_D$  measurements. The speed of the nESI-MS method and the low sample consumption make it a powerful tool for examining the stoichiometry of a protein–ligand binding system and an easy to use technique for comparison with other established  $K_D$  determination methods.

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