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Impact of protein and ligand impurities on ITC-derived protein–ligand thermodynamics

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ABSTRACT

Background: The thermodynamic characterization of protein–ligand interactions by isothermal titration calorimetry (ITC) is a powerful tool in drug design, giving valuable insight into the interaction driving forces. ITC is thought to require protein and ligand solutions of high quality, meaning both the absence of contaminants as well as accurately determined concentrations.
Methods: Ligands synthesized to deviating purity and protein of different pureness were titrated by ITC. Data curation was attempted also considering information from analytical techniques to correct stoichiometry.
Results and conclusions: We used trypsin and tRNA-guanine transglycosylase (TGT), together with high affinity ligands to investigate the effect of errors in protein concentration as well as the impact of ligand impurities on the apparent thermodynamics. We found that errors in protein concentration did not change the thermodynamic properties obtained significantly. However, most ligand impurities led to pronounced changes in binding enthalpy. If protein binding of the respective impurity is not expected, the actual ligand concentration was corrected for and the thus revised data compared to thermodynamic properties obtained with the respective pure ligand. Even in these cases, we observed differences in binding enthalpy of about 4 kJ · mol^{-1}, which is considered significant.
General significance: Our results indicate that ligand purity is the critical parameter to monitor if accurate thermodynamic data of a protein–ligand complex are to be recorded. Furthermore, artificially changing fitting parameters to obtain a sound interaction stoichiometry in the presence of uncharacterized ligand impurities may lead to thermodynamic parameters significantly deviating from the accurate thermodynamic signature.
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1. Introduction

Thermodynamic characterization of protein–ligand interactions by isothermal titration calorimetry (ITC) has become a routinely used method in understanding interactions of biomolecules with naturally occurring binding partners as well as a powerful tool in drug design [1]. The obtained thermodynamic data of an interaction are most valuable to complement structural information resulting from X-ray crystallography or NMR spectroscopy methods in order to rationally improve a lead compound in structure-based drug design [2]. However, ITC experiments usually require substantial amounts of the interacting partners, both being of high purity. Successful production of high amounts and purity might be possible with well-selected model systems for which an efficient expression and purification protocol is available and ligands result from simple high yield synthesis. This becomes, however, increasingly difficult if real drug targets and ligands from multistep synthesis are considered. To investigate the impact of impurities present in both the protein and ligand solution focusing on relevant drug discovery cases, we characterized binding of several competitive inhibitors to *Bos taurus* trypsin and *Zymomonas mobilis* tRNA-guanine transglycosylase (TGT) by ITC, taking into account different amounts of protein impurities and using ligand preparations containing organic and inorganic impurities, respectively. Trypsin is a well-studied serine protease involved in digestive processes and serves frequently as a surrogate for actual drug targets in the design of protease inhibitors or to learn about specificity and selectivity discrimination [3–5]. However, being an extracellular mammalian protein, it is difficult to obtain it in high amounts in its active form by heterologous production in simple expression systems such as *Escherichia coli*. Similar issues arise for other proteins relevant to drug development such as thrombin [6]. For some of these proteins, natural sources are abundantly available but material might be contaminated with impurities and is usually supplied in lyophilized form which is

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known to contain hydrate water [7]. Protein concentration is then routinely determined by spectrophotometric measurements, which are often calibrated via an easily calculated extinction coefficient [8]. However, the provided extinction coefficient may differ from the actual one, thereby falsifying the protein concentration systematically. This difference is especially pronounced for trypsin's precursor trypsinogen and amounts to 11% [8]. Therefore, trypsin was an ideal candidate to investigate how errors in protein concentration and possible impurities affect the thermodynamic characterization of ligand binding determined by ITC.

The tRNA-modifying enzyme tRNA-guanine transglycosylase is known to play a key role in the pathogenicity of *Shigella*, the causative agent of Shigellosis [9]. It catalyzes a base exchange of guanine by a modified base in position 34 of the tRNA-anticodon loop [10]. This exchange is essential to produce virulence factors necessary for cell invasion. Thus, blocking the enzymatic activity of TGT prevents invasion of *Shigella* [11]. The expression protocol in *E. coli* is well established and yields, owing to favorable solubility features, protein of high purity as confirmed by mass spectrometry, SDS PAGE, and dynamic light scattering measurements [12,13]. However, the highly potent 6-aminoimidazo [4,5-g]quinazolin-8(7*H*)-ones (*lin*-benzoguanines) exhibit unfavorable solubility and polarity issues complicating the synthesis of compounds with high purity [14]. Hence, TGT was selected as a second real case example to study the influence of organic and inorganic impurities in ligand preparations on ITC measurements.

2. Material and methods

2.1. Synthesis

Ligand synthesis, their purification, and characterization can be found in the SI.

2.2. Protein preparation and quantification

Bovine pancreas trypsin from natural source was purchased as ethanol precipitate from Sigma (product number: T8003). Dry weight determination was performed in duplicate to estimate the amount of associated volatile substances, i.e. mainly hydrate water. The protein precipitate was dried at 378 K at normal atmospheric pressure and recurrently weighted after cooling in a desiccator until stable weight was reached [15]. Protein concentration was determined by two methods. Firstly, the micro-biuret method was used, measuring absorbance at 545 nm after trichloroacetic acid/desoxycholate precipitation and using bovine serum albumin as reference protein [16,17]. Secondly, UV spectrophotometry at 280 nm ($A_{280\ nm}$) was employed, using an experimental absorbance coefficient of 1.54 mg · mL^{-1} at 280 nm and a molecular weight of 23,305 Da as calculated by ProtParam for cationic bovine trypsin (UniProt-ID: P00760), giving $\varepsilon_{280} = 35,890\ M^{-1} \cdot cm^{-1}$ [18–20]. The extinction coefficient of trypsin at 280 nm was $\varepsilon_{280} = 37,650\ M^{-1} \cdot cm^{-1}$ as directly calculated by ProtParam. Percentage errors in protein concentration refer to the deviation from the concentration determined by the micro-biuret method.

Z. mobilis TGT was expressed and purified as described in detail elsewhere [12,13]. The protein concentration was determined firstly by UV spectrophotometry at 280 nm ($A_{280\ nm}$). An absorption of 0.778 (10 mm path) corresponds to 1 mg · mL^{-1} (23.4 μM) as suggested by ProtParam [20]. Secondly, a Bradford assay was applied on the basis of protein-dye binding using bovine serum albumin as a standard [21]. Both methods resulted in a closely similar protein concentration.

2.3. Isothermal titration calorimetry

ITC experiments were performed using an iTC200 microcalorimeter (GE Healthcare Europe GmbH, Freiburg, Germany). All experiments were performed at 25 °C using filtered solutions only. The reference cell contained demineralized water. The trypsin precipitate was dissolved in ITC buffer (50 mM Tris/HCl, 100 mM NaCl, 0.1% (w/v) polyethylene glycol 8000, pH 7.8) supplemented with 3% (v/v) DMSO and stored on ice. The protein solution was freshly prepared daily. No measurable trypsin autodigestion occurred as judged by the interaction stoichiometry remaining stable for at least 12 h under the applied protein concentrations. The final protein concentration in the sample cell was 15 μM based on weight, 12.3 μM based on dry weight measurements and 11.3 μM based on micro-biuret and spectrophotometric measurements. Ligand stock solutions of **1** and **2** of 10 mM were prepared by weight in 100% (v/v) DMSO, subsequently diluted to concentrations ranging from 250 to 400 μM with ITC buffer and adjusted to 3% (v/v) DMSO prior to the experiment. TGT was dissolved in the experimental buffer containing 50 mM Hepes, 200 mM NaCl, and 0.037% (v/v) Tween 20, pH 7.8 to a final concentration of 10 μM containing 3% (v/v) DMSO. Due to their low solubility, ligands **3** and **4** were first dissolved in 100% (v/v) DMSO and diluted with buffer solution to a final DMSO concentration of 3%. Ligand concentration in the syringe was adjusted to 200 to 245 μM with experimental buffer. The ligand solution containing syringe was stirred at 1000 rpm and injection started after a stable baseline had been established. A first injection of 0.3 μL was followed by 15 injections of 1.1 to 2.2 μL. All injections were spaced by 120 s to 180 s intervals. Raw data were collected as released heat per time. The collected data were analyzed using ORIGIN Software 7.0 (Microcal Inc.). The area under each peak was integrated, followed by correction for heats of dilution and mixing by subtracting the final baseline which consisted of small peaks of comparable size. The data point resulting from the first injection was deleted after integration as it is error prone [22]. A single-site-binding isotherm that yields the enthalpy of binding ΔH^0 and the dissociation constant K_d was fitted to the data [23]. Each experiment was performed at least in triplicate. For representative curves see Fig. 1.

2.4. Dynamic light scattering

Potential aggregation behavior of *Z. mobilis* TGT in absence and presence of an inhibitor was determined by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) equipped with a 10 mW HeNe laser at a wavelength of 633 nm at 25 °C. Scattered light was detected at a 173° angle with laser attenuation and measurement position adjusted automatically by the software. The same conditions as in ITC experiments were used (10 μM protein in ITC buffer, 3% DMSO, addition of 28 μM (=20 injections of 2 μl) of ligand **4**). The given data comprised three measurements of at least 10 runs.

3. Results and discussion

3.1. Errors in protein concentration did not affect the thermodynamic profiles of high affinity protein–ligand binding

To investigate the impact of impurities present in the protein preparation on the thermodynamic characterization of a protein–ligand interaction, binding of two competitive inhibitors (Fig. 2A) to trypsin was studied by ITC.

First, varying degrees of impurities present in the trypsin preparation were taken into account during modeling of thermodynamic data to study protein impurity-related deviations. The amount of hydrate water present in the protein precipitate was estimated by using the dry weight method. It revealed a hydrate water content of 18% (w/w). Therefore, the actual protein concentration would be overestimated by 22% if it was solely determined by weight. However, all thermodynamic parameters only showed subtle, if any, changes on considering hydrate water content in data modeling (Table 1). The only fitting parameter that changed was the interaction stoichiometry *n*. It increased to values closer to 1, the expected value for the interactions investigated. This supports the idea that considering the water content reveals the more correct model for the interaction, even in the absence of other changes in thermodynamic parameters. Hence, ignoring the water

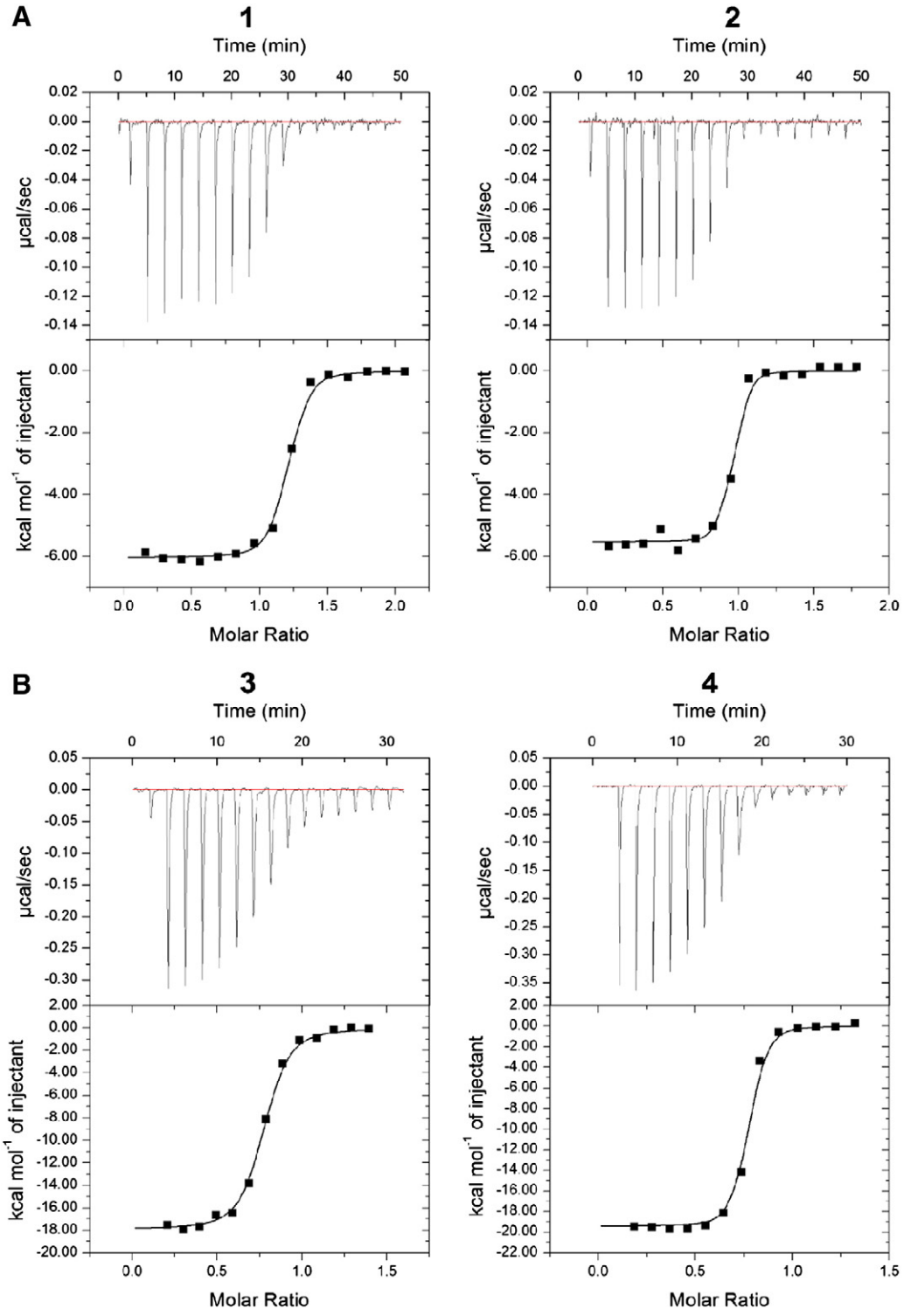

Fig. 1. Representative ITC thermograms for **1** and **2** directly titrated to trypsin (A) as well as **3** and **4** directly titrated to TGT (B). Shown are titrations of impure preparations, which were partially re-fitted for ligands **2**, **3**, and **4** to a more reasonable stoichiometry according to data from pure ligand **2** and to elementary analysis for **3** and **4**, respectively.

content of the used solid protein preparation did not influence the thermodynamic characterization of ligand binding. Next, the actual protein concentration was determined by a colorimetric and a spectrophotometric method, the micro-biuret and $A_{280\ nm}$-assay, respectively. Both techniques yielded very similar values if the experimentally determined extinction coefficient of trypsin was used for the $A_{280\ nm}$-assay. If the determination of protein concentration was based on weight, it deviated from the actual protein concentration by 33%. The difference between the calculated and previously experimentally determined extinction coefficient of trypsin resembled the already known difference for trypsinogen [8]. The difference in protein concentration to dry weight determination is probably due to low molecular weight substances such as salts. Nucleic acids were not present in the precipitate. Despite this rather large deviation of 33%, modeling based on the actual protein content also did not significantly change the values of thermodynamic parameters obtained by ITC experiments but interaction stoichiometries approximated much closer to 1, confirming that a better model was fitted (Table 1).

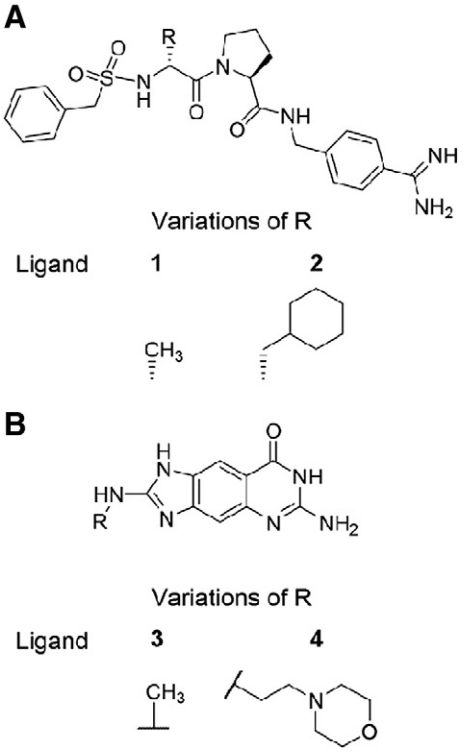

Fig. 2. Chemical structures of the studied ligands. A) Ligands binding to trypsin as competitive inhibitors. B) Ligands binding to TGT.

3.2. Organic ligand contaminations can affect thermodynamic profiles of protein–ligand binding significantly

In order to study how impurities in ligand preparations impact the obtained thermodynamic parameters, two preparations of each trypsin-binding ligand **1** and **2** of different purities were used, referred to as the "impure" and "pure" preparation, respectively. The impure preparation of ligand **1** contained a by-product of its synthesis with similar retention behavior in HPLC, constituting about 20% of the ligand preparation based on HPLC-coupled UV-spectrophotometry. Thermodynamic characterization revealed a high-affinity interaction with an interaction stoichiometry larger than one, indicating that less ligand than assumed was present (Table 2, Fig. SI-24). ITC experiments using a purified ligand preparation resulted in a slightly improved standard free binding energy as well as standard binding enthalpy ($\Delta\Delta G^0 = -1.9\ kJ \cdot mol^{-1}$, $\Delta\Delta H^0 = -2.3\ kJ \cdot mol^{-1}$) and an interaction stoichiometry n of reasonable value (1.0).

Ligand **2** was contaminated in its impure form with an organic compound. It was mass spectrometrically identified as 1,1′,1″-phosphinylidtrispyrrolidin (CAS-no. 6415-07-2), which is a reaction product of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), a peptide coupling agent used during synthesis. Characterizing ligand binding with the impure preparation showed a high-affinity interaction with trypsin ($K_d = 11$ nM, Table 2), which was apparently dominated by its entropic component, contributing almost 70% to the overall standard free binding energy. However, an abnormally high interaction stoichiometry of about 1.5 indicated considerable ligand purity issues. Further purification of the ligand and additional ITC experiments showed that the actual affinity of the pure ligand was slightly stronger ($K_d = 8$ nM). Therefore, the ligand purity did not strongly influence the measured binding affinity. In contrast, the obtained interaction stoichiometry approached values closer to the theoretically expected one (0.9). Furthermore, the standard binding enthalpy drastically improved ($\Delta\Delta H^0 = -5.8\ kJ \cdot mol^{-1}$). Consequently, the entropic contribution to binding decreased by a similar magnitude ($-T\Delta\Delta S^0 = 5.2\ kJ \cdot mol^{-1}$) and afforded then only about 55% of the total standard free binding energy. Re-fitting of thermograms obtained with impure ligand was done with a corrected ligand concentration giving similar interaction stoichiometries as the pure ligand ("impureM"). Correcting the ligand concentration yielded a concentration 40% less than originally assumed. Obtained thermodynamic data of these corrected fittings were compared to the data resulting from pure ligand preparation. Whereas the standard free binding energies were in close agreement ($\Delta\Delta G^0 = -0.7\ kJ \cdot mol^{-1}$), the enthalpic and entropic contributions still differed significantly by $-3.8\ kJ \cdot mol^{-1}$ and $3.1\ kJ \cdot mol^{-1}$, respectively [24]. Hence, the thermodynamic profile of the ligand resulting from the impure, newly modeled preparation did not reasonably agree with the profile obtained from a pure preparation.

Additionally, the impact of an organic impurity on the thermodynamic profile of TGT ligand **3** (Fig. 2B) was investigated by analyzing the ligand before and after HPLC purification. Unfortunately, the exact chemical composition of the impurity could not be identified. Elementary analysis revealed that the values for carbon and nitrogen are affected differently. Considering the determined carbon value, an actual purity of approximately 73% was estimated. In the case of the found value for nitrogen, purity appeared to be further lowered to around 66%. ITC measurements characterized **3** in its impure form as a potent inhibitor of TGT ($K_d =$ 86 nM) with a mainly enthalpic contribution to binding (Table 2). Accompanied by the described deviations found in elementary analysis, the interaction stoichiometry is increased to a value of 1.16. Re-fitting of the data for the impure ligand following the elementary analysis resulted in small changes of the standard free binding energy ($\Delta\Delta G^0 = -0.9\ kJ \cdot mol^{-1}$). However, the thermodynamic profile shows a more pronounced enthalpy term of $\Delta\Delta H^0 = -17.8$ to $-26.2\ kJ \cdot mol^{-1}$. The difference between the two newly fitted data sets is significantly increased, especially for ΔH^0 ($\Delta\Delta H^0 = 8.4\ kJ \cdot mol^{-1}$). As a consequence, the entropic term shifts to more unfavorable values. The interaction stoichiometry improved to reasonable values of 0.84 and 0.76, respectively, taking into account that expression and purification did not yield fully active protein. To gain information about the influence of the impurity, the values were compared to those of **3** in its pure state, after further purification

Table 1
Thermodynamic parameters of ligand binding as determined by ITC and modeling with different protein impurity levels.

Ligand	Correction[a]	n[b]	ΔG^0(kJ · mol^{-1})[b]	ΔH^0(kJ · mol^{-1})[b]	$-T\Delta S^0$(kJ · mol^{-1})[b]
1	None	0.75 ± 0.02	−45.9 ± 1.5	−27.5 ± 1.1	−18.4 ± 1.8
	Hydrate	0.92 ± 0.02	−45.9 ± 1.6	−27.5 ± 1.1	−18.4 ± 1.9
	Protein	1.00 ± 0.02	−46.0 ± 1.6	−27.5 ± 1.2	−18.4 ± 2.0
2	None	0.68 ± 0.01	−46.3 ± 0.9	−20.4 ± 0.7	−26.0 ± 1.1
	Hydrate	0.83 ± 0.01	−46.2 ± 0.7	−20.4 ± 0.7	−25.8 ± 1.0
	Protein	0.90 ± 0.01	−46.1 ± 0.7	−20.4 ± 0.7	−25.7 ± 1.0

[a] Correction states the method by which the protein concentration used for thermogram modeling was determined: none — concentration as calculated by protein weight (33% deviation); hydrate — hydrate water content was considered (22% deviation); protein-concentration as determined by micro-biuret/$A_{280\ nm}$. Deviations are relative to concentration measured by micro-biuret assay.
[b] Given errors are standard deviations of at least triplicates.

Table 2
Thermodynamic parameters of ligand binding and impact of ligand impurities.

Ligand	Purity	n	ΔG^0(kJ · mol^{-1})	K_d(nM)	ΔH^0(kJ · mol^{-1})	$-T\Delta S^0$(kJ · mol^{-1})
1	Pure[a]	1.00 ± 0.02	−46.0 ± 1.6	9 ± 6	−27.5 ± 1.2	−18.4 ± 2.0
	Impure	1.15 ± 0.03	−44.1 ± 1.0	19 ± 8	−25.2 ± 0.3	−18.9 ± 1.0
2	Pure[a]	0.90 ± 0.01	−46.1 ± 0.7	8 ± 2	−20.4 ± 0.7	−25.7 ± 1.0
	Impure	1.48 ± 0.05	−45.5 ± 1.9	11 ± 8	−14.6 ± 0.7	−30.9 ± 2.0
	ImpureM[b]	0.89 ± 0.02	−46.8 ± 1.8	6 ± 5	−24.2 ± 0.8	−22.6 ± 2.0
3	Pure	0.73 ± 0.05	−41.6 ± 0.1	52 ± 2	−74.8 ± 1.8	33.2 ± 1.8
	Impure	1.16 ± 0.01	−40.3 ± 0.3	86 ± 12	−48.3 ± 0.4	8.0 ± 0.5
	ImpureM-C[c]	0.84 ± 0.00	−41.2 ± 0.2	60 ± 8	−66.1 ± 0.5	24.9 ± 0.6
	ImpureM-N[d]	0.76 ± 0.00	−41.2 ± 0.4	61 ± 9	−74.5 ± 0.6	33.3 ± 0.7
4	Pure	0.68 ± 0.00	−42.2 ± 0.4	41 ± 6	−78.3 ± 1.2	36.1 ± 1.2
	Impure	2.23 ± 0.01	−42.4 ± 0.4	38 ± 5	−27.3 ± 0.1	−15.1 ± 0.4
	ImpureM-C[c]	0.73 ± 0.00	−45.6 ± 0.7	11 ± 3	−81.4 ± 0.7	35.8 ± 1.0

[a] Results from pure ligand preparations as in Table 1 shown for ease of comparability.
[b] Thermograms resulting from impure preparation were used and fitted to reach the same interaction stoichiometry n as thermograms obtained with pure preparation.
[c] Thermograms resulting from impure preparation were used and fitted based on the corrected ligand concentration deduced from the carbon value of elementary analysis.
[d] Thermograms resulting from impure preparation were used and fitted based on the corrected ligand concentration deduced from the nitrogen value of elementary analysis.

steps via HPLC. ΔG^0 remained the same within the range of error compared to the re-fitted value ($\Delta\Delta G^0 = -1.3$ kJ · mol^{-1}), similar to refitting data of the impure ligand. The enthalpic term drastically increased compared to the batch of **3** before HPLC purification ($\Delta\Delta H^0 = 26.5$ kJ · mol^{-1}). Contrary to that, the values are in excellent agreement with the re-fitted values for a purity of approximately 66% ($\Delta n = 0.03$, $\Delta\Delta G^0 = -0.4$ kJ · mol^{-1}, $\Delta\Delta H^0 = -0.3$ kJ · mol^{-1}, $-T\Delta\Delta S^0 = -0.1$ kJ · mol^{-1}).

3.3. Inorganic ligand contaminations can also affect thermodynamic profiles of protein-ligand binding

A second TGT inhibitor was included in our studies. Compound **4** shows low purity after synthesis without any further purification steps. According to elementary analysis, the powder contains the desired compound **4** to approximately one-third. Contrary to the results of **3**, the yield resulting from the ratio found to theoretical percentage of carbon and nitrogen, respectively, shows only subtle differences (Δyield = 0.9 %). Therefore, most likely an inorganic impurity originating from synthesis is present in the ligand preparation. The binding isotherm yielded a binding affinity of $K_d = 38$ nM for the impure sample of **4** and $K_d = 41$ nM for the pure one ($\Delta\Delta G^0 = 0.2$ kJ · mol^{-1}). Again, the largest changes between pure and impure preparations are noticeable in the thermodynamic partitioning: The enthalpic term increases remarkably from − 27.3 kJ · mol^{-1} to − 78.3 kJ · mol^{-1} ($\Delta\Delta H^0 = -51.0$ kJ · mol^{-1}), the entropic contribution decreases from − 15.1 kJ · mol^{-1} to an unfavorable term of 36.1 kJ · mol^{-1} ($-T\Delta\Delta S^0 = 51.2$ kJ · mol^{-1}). In addition, stoichiometry is diminished by a factor of about three from 2.23 to a more reasonable value for this inhibitor of 0.68. In the observed case, re-fitting of data could be performed easier since the results from elementary analysis indicated a purity level of approximately 33% due to an inorganic salt. The obtained values were in good agreement with the values derived from the pure ligand preparation, even if close to the significance level. Stoichiometry differed by 0.05, ΔH^0 by − 3.1 kJ · mol^{-1} and $-T\Delta S^0$ by − 0.3 kJ · mol^{-1}. Changes in ΔG^0 were slightly higher than for the other described compounds **1**–**3** ($\Delta\Delta G^0 = -3.4$ kJ · mol^{-1}, $K_d = 41$ nM vs. 11 nM).

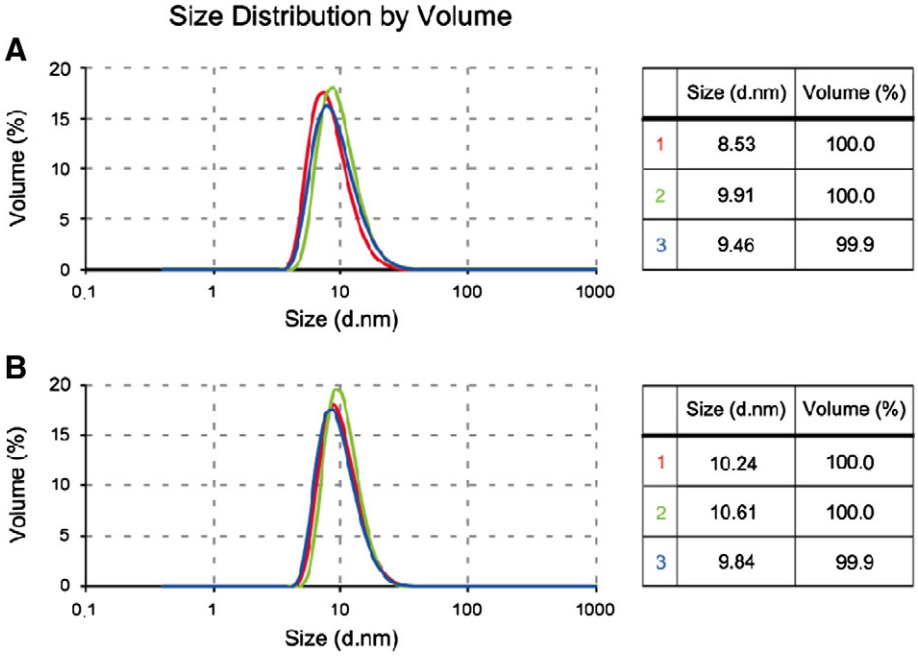

Fig. 3. Influence of **4** on the size distribution by volume of *Z. mobilis* TGT. In both cases a 10 μM protein solution was analyzed. A) A total of three DLS measurements of TGT in the absence of any ligand show a monomodal size distribution with an average diameter of 9.3 nm. The mean peak volume is 99.97%. B) A total of three DLS measurements of TGT in presence of **4** with a concentration of 28 μM (equivalent to 20 injections of 2 μl of a 200 μM inhibitor solution) show no influence on the monomodal size distribution. The average diameter of the protein is 10.2 nm with a mean peak volume of 99.97%.

It is known that salts can significantly influence the activity and stability of proteins in aqueous solution both in favorable and unfavorable means according to the Hofmeister series [25]. Indeed, TGT needs high salt conditions to be soluble in the long term. To determine possibly occurring protein or ligand aggregation due to an increased salt concentration, dynamic light scattering (DLS) measurements were performed additionally. While inactive aggregates formed by the protein are not expected to change the thermodynamic profile drastically as shown in our studies, aggregates formed by ligands are able to bind to the protein unspecifically leading to false positive signals [26]. Measurements were carried out in the absence and presence of **4** (Fig. 3) using the same conditions as in the ITC experiments. Neither the obtained mean peak size ($\Delta d = 0.9$) nor the average peak volume ($\Delta V = 0.0$) deviated significantly. Therefore, the difference in enthalpy of $\Delta\Delta H^0 = -3.1\ \text{kJ} \cdot \text{mol}^{-1}$ between re-fitted data originating from impure ligand preparation and data from repurified ligand preparation was unexpected.

3.4. Comparative discussion

In biophysical investigations, one is urged to work with protein and in the field of protein–ligand interactions also with ligands of high purity [27]. ITC is a key technique for thermodynamically characterizing chemical and biochemical binding processes. Before analyzing the interaction of interest, particular attention needs to be paid to carefully calibrate the microcalorimeter using well-known standard chemical reactions as well as to the achievable precision and systematic differences reported for various microcalorimeter models [28–30]. When it comes to analyzing the thermodynamics of protein–ligand interactions, ITC is a central method but frequently proteins and ligands are difficult to prepare at high purity. Therefore, we investigated the effects of errors in protein and ligand concentration and impurities on the obtained thermodynamic property values. We found that, for high-affinity interactions with c values over 100 [23], neither considering hydrate water nor further uncertainties in protein concentration resulted in differences of thermodynamic property values compared to values obtained with the simple protein weight concentration. Hence, ignoring a concentration error of up to 33% and thereby hydrate water and contaminants such as salts in the protein preparation did not affect the apparent thermodynamic data of a protein–ligand interaction. This finding is in agreement with a study of metal ions binding to crown ethers [31]. Errors in the receptor (crown ether) concentration of up to 15% lead neither to changes in the standard free binding energy nor the binding enthalpy [31]. The c value is defined as $c = n \cdot [\text{Prot}]_{total}/K_d$. Even a more than twice as large error of 33% was found to be acceptable in this study. The tolerance can be rationalized by the idea that for high affinity ligands, the amount of ligand binding to its receptor for each injection is almost solely dependent on the actual amount of ligand injected and therefore not sensitive to changes in receptor concentration and errors in its determination are compensated for during modeling by the stoichiometry parameter n [32,33]. Changes of the latter mainly lead to shifting the binding isotherm to different interaction stoichiometries accompanied by small changes in ΔG^0. Still it is important for experimental accuracy to determine the actual protein concentration as precisely as possible, especially if the interaction stoichiometry is unknown. We showed that routine use of a calculated extinction coefficient might not accurately reflect the actual absorption property of a protein as is the case for trypsin, hinting at differences in absorption profiles between its native and denatured form. In the absence of a known experimental extinction coefficient, the micro-biuret assay proved to be a valuable method safeguarding against inaccurately quantifying protein concentration.

Considering the ligand, a larger impact of concentration inaccuracies and impurities on thermodynamic properties is to be expected. The fitting process involves normalization of observed heat per injection by the amount of ligand added and the normalized heat is then plotted against the molar ratio of ligand per receptor. Supporting this reasoning, variability in thermodynamic parameters of a benchmarking protein–ligand interaction in an interlaboratory study was found to be mainly caused by varying errors in determining the ligand concentration [32, 34]. Indeed, impure ligand preparations of ligands **1**–**4** used in this study all showed a reduced apparent binding enthalpy compared to binding enthalpy of the pure ligand. Impurities present in a ligand preparation can be thought of as principally belonging to three major classes:

First, side products of synthesis, which contain fragments of the actual ligand and are capable of binding to the same protein, thereby acting as competitive ligand. The contaminant of ligand **1** showed a similar HPLC-retention and UV-absorption behavior as the ligand itself, pointing toward possible structural similarities to the ligand. Therefore, the contaminant itself might also exhibit protein-binding activity. A mixture of ligand and protein-binding by-product is difficult to model in ITC data, as quantification of the ligand content alone is not sufficient for accurate correction. In those cases, ligand repurification is inevitable. Due to the minor impurity content of ligand **1**, the deviations in thermodynamic parameters ΔG^0 and ΔH^0 were in the range of $2\ \text{kJ} \cdot \text{mol}^{-1}$.

The second class of impurities are organic compounds originating from the synthesis itself, such as coupling agent reaction products, or remaining from so called “leaking” columns used in purification. These compounds are unlikely to exhibit specific protein binding but might otherwise influence the protein–ligand interaction. The contaminants of ligands **2** and **3** belong to that class. In both cases, an impurity content of up to 40% led to a drastically decreased apparent binding enthalpy whereas ΔG^0 was much less affected. Thereby, a much more entropically driven ligand binding is suggested. As ligands **1** and **2** belong to the same congeneric ligand series where the latter contains a more hydrophobic substituent in position R, one could have expected a trend toward increased entropic contribution. However, characterization using the pure ligand preparation showed a less pronounced trend of such an entropic contribution. This case underlines the importance of considering the observed binding stoichiometry as a parameter for quality control. An interesting question arose after the pure ligand **2** was characterized: Are thermodynamics profiles of impure and pure ligand preparations of **2** comparable if corrected for the actual ligand concentration by fitting to the same interaction stoichiometry? Identical values indicate that solely adjusting ligand concentration during modeling might be sufficient. However, a difference in standard binding enthalpy of $3.8\ \text{kJ} \cdot \text{mol}^{-1}$ remained between re-fitted data and data of pure ligand, pointing toward the contaminant influencing the protein–ligand interaction. The underlying mechanism remained unclear. The magnitude of the observed difference is close to the threshold of $4\ \text{kJ} \cdot \text{mol}^{-1}$, above which a difference in binding enthalpy is considered to be significant [24].

In contrast, comparison of the re-fitted data of the contaminated ligand **3** and its pure form showed that it is possible to obtain the actual thermodynamic parameters under consideration of elementary analysis results. The data fitted to a purity of 66%, taking into account the ratio between the found and theoretical nitrogen value, give the same parameters like the pure compound does, within the error limits. However, the fit to a purity of 73%, as suggested by the ratio between found and theoretical values for carbon, failed to yield the same thermodynamic signature as for the pure ligand. Therefore, a prerequisite for the successful re-fitting is the knowledge of the composition of the impurity contained in the ligand preparation. Furthermore, even if no structural similarities are present, one has to reassure that the contamination does not interact with the protein or influence its stability or interaction with a ligand during the measurement period.

Hence, even when correcting for the actual ligand concentration, one has to be aware that the resulting apparent thermodynamics may not accurately reflect the actual thermodynamics. This deviation may go undetected if ligand concentration is adjusted during the fitting process in order to give a reasonable interaction stoichiometry and no revalidation is performed to what extent that adjustment is sufficient. Therefore, this manual adjustment is poor practice.

Thirdly, ligand preparation may contain a varying proportion of salts resulting from synthesis and/or purification, which may change the thermodynamic properties similarly like an organic impurity. As described for **2** and **3**, also **4** shows significant changes in its enthalpic and entropic properties while ΔG^0 remains largely unaffected. Refitting according to elementary analysis yielded improvements only toward the pure compound close to the significance limit. Influences of ions binding to the protein surface might be imaginable. Besides that, the solubility of compound and protein at high concentrations needed for ITC measurements might be negatively affected. The impact of ligand as well as protein aggregation can be easily monitored by Dynamic Light Scattering.

Within the error range of the method, elementary analysis gave the identical yield of **4** regarding analyzed values for carbon and nitrogen. As a consequence, it can be assumed that an inorganic salt resulting from synthesis as part of the ligand powder. When including the actual yield of **4** into the fitting procedure of the binding isotherm, the derived data showed a good approximation to the thermodynamic properties obtained with the further purified ligand. Despite this and similar to the modeled data of **1** and **2,** deviations close to the significance level could be observed ($\Delta\Delta G^0 = 3.4\ kJ \cdot mol^{-1}$, $\Delta\Delta H^0 = -3.1\ kJ \cdot mol^{-1}$). Also DLS measurements did not give any explanations for those deviations. Protein and ligand aggregation can be excluded according to the conducted experiments. This result was expected since the salt of the ligand powder increases the concentration of the salt contained in the experimental buffer by only 0.1% (w/v calculated as NaCl). However, also if no measurable protein or ligand aggregation can be observed in dynamic light scattering measurements, an influence of increased salt concentrations by other means cannot be totally excluded.

To further verify thermodynamic results, a reverse experimental setup can be devised. In such a reverse titration, protein solution is titrated into the ligand containing cell and is especially useful to check interaction stoichiometry [35]. For a simple 1:1 interaction, the results should be invariant to the normal experimental setup but may change if more complex interaction modes are investigated [36,37]. However, protein is required in higher amounts and concentrations in such experiments, making additional validation of the absence of possible protein aggregation necessary. The herein introduced DLS method is ideally suited for this purpose. Unfortunately, this setup is hardly feasible for our model proteins: The self-digestion of trypsin is increased at higher concentrations. Thus, no reliable results can be expected over the period of an ITC experiment. Similarly, TGT's solubility is drastically lowered at low salt concentrations applied during the titration. Therefore, precipitation of the protein occurred during a reversed experimental setup at the needed concentrations.

Additionally to systematic errors discussed above, statistical errors and their correct treatment should also be considered [38,39].

4. Conclusions

It can be concluded that impurities present in protein preparations used for thermodynamic characterization of a protein–ligand interaction by ITC do not necessarily translate into deviations from the actual thermodynamic data. Under special circumstances, they can even be ignored as was the case for the protein preparation in the study of high affinity interactions. More caution has to be paid to the preparation of high affinity ligands. Even contaminants without apparent protein-binding capabilities may distort the thermodynamic properties of a protein–ligand interaction, which goes undetected by sole fitting adjustments. Our studies dealing with high c value titrations demonstrated that it is possible to derive thermodynamic data from impure ligands, which are in good accordance to data obtained with the pure ligand. Basis for a successful fitting is knowledge about the composition of the contaminant and its potential influence on the analyzed system, which is unfortunately often difficult to estimate. Fitting the binding isotherm of an impure ligand following elementary analysis results yielded a good approximation to the actual value in one case, proving elementary analysis to be a well-suited complementary analysis to NMR and MS methods when it comes to ligand synthesis for ITC purposes. In another case, the same data as with a pure compound were obtained. Nonetheless, this procedure is limited since only a small data range is accepted before deviations get significant. For ligands showing large differences in their thermodynamic profile among each other, this might be negligible. However, the practice of sole fitting adjustments may mislead correlation to structural data of protein–ligand complexes or interpretation of thermodynamic trends observed in a congeneric series of ligands. The latter is especially deleterious as thermodynamic characteristics of such stepwise ligand modifications may not show big differences between the individual components of the series but are frequently used in conjunction with structural data to establish basic mechanisms underlying molecular interactions [40,41]. In summary, this study pointed out that thermodynamic characterization of high affinity protein–ligand interactions by ITC shows different robustness against possible experimental errors and highlights the experimental parameters which must be rigorously monitored to obtain accurate and reliable thermodynamic data. If especially ligand purity cannot be assured, the actual value might be at least approximated. However, accurateness of these assumptions and considered values may be difficult to assess. Therefore, the efforts should be rather put in reliable purification protocols, both for proteins as well as ligands, to provide the basis to obtain accurate data by ITC.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.04.018.

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