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# Ligand–Receptor Binding Affinities from Saturation Transfer Difference (STD) NMR Spectroscopy: The Binding Isotherm of STD Initial Growth Rates

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*Dedicated to Professor Jesús Jiménez-Barbero on the occasion of his award of the Whistler International Award in Carbohydrate Chemistry*

**Abstract:** The direct evaluation of dissociation constants ($K_D$) from the variation of saturation transfer difference (STD) NMR spectroscopy values with the receptor–ligand ratio is not feasible due to the complex dependence of STD intensities on the spectral properties of the observed signals. Indirect evaluation, by competition experiments, allows the determination of $K_D$, as long as a ligand of known affinity is available for the protein under study. Herein, we present a novel protocol based on STD NMR spectroscopy for the direct measurements of receptor–ligand dissociation constants ($K_D$) from single-ligand titration experiments. The influence of several experimental factors on STD values has been studied in detail, confirming the marked impact on standard determinations of protein–ligand affinities by STD NMR spectroscopy. These factors, namely, STD saturation time, ligand residence time in the complex, and the intensity of the signal, affect the accumulation of saturation in the free ligand by processes closely related to fast protein–ligand rebinding and longitudinal relaxation of the ligand signals. The proposed method avoids the dependence of the magnitudes of ligand STD signals at a given saturation time on spurious factors by constructing the binding isotherms using the initial growth rates of the STD amplification factors, in a similar way to the use of NOE growing rates to estimate cross relaxation rates for distance evaluations. Herein, it is demonstrated that the effects of these factors are cancelled out by analyzing the protein–ligand association curve using STD values at the limit of zero saturation time, when virtually no ligand rebinding or relaxation takes place. The approach is validated for two well-studied protein–ligand systems: the binding of the saccharides GlcNAc and GlcNAcβ1,4GlcNAc (chitobiose) to the wheat germ agglutinin (WGA) lectin, and the interaction of the amino acid L-tryptophan to bovine serum albumin (BSA). In all cases, the experimental $K_D$ measured under different experimental conditions converged to the thermodynamic values. The proposed protocol allows accurate determinations of protein–ligand dissociation constants, extending the applicability of the STD NMR spectroscopy for affinity measurements, which is of particular relevance for those proteins for which a ligand of known affinity is not available.

**Keywords:** binding constants · binding isotherms · dissociation constants · NMR spectroscopy · proteins

## Introduction

Nowadays, there is great interest in investigating the nature of the molecular recognition processes between biomolecules, since their specific interactions regulate essential processes of life. This interest reflects the relevance of their biomedical applications, particularly the major importance of molecular recognition processes of small ligands by biological receptors for the design of new active compounds in pharmaceutical research. Consequently, the structural and energetic characterization of these interactions at a molecu-

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lar level constitutes an indispensable facet of this activity. To this aim, NMR spectroscopy has been proven to be a powerful technique and a large amount has been published on NMR spectroscopy investigations of protein–ligand interactions.[1] An important number of NMR spectroscopy applications have also been widely used to characterize the equilibrium binding constant for small-molecule–biomolecule complexes by means of both, receptor- and/or ligand-observed methods.[2]

In particular, among the ligand-observed NMR spectroscopy methods, saturation transfer difference (STD) NMR spectroscopy experiments have proven to provide high sensitivity and robustness, requiring little amounts of unlabeled macromolecules, and being valid to study complexes involving receptors with high molecular weight.[3] Briefly, the method consists of applying a selective radiofrequency irradiation to saturate only the macromolecule NMR signals. Difference spectroscopy then detects the transfer of this saturation by intermolecular NOE to any small molecule that is bound to the macromolecule under conditions of fast chemical exchange. The magnitude of any transferred saturation is related to the proximity of the ligand proton to the protein surface, so of particular interest is its ability to get information at the atomic level about the ligand-binding epitope.[4] Recently, the scope of STD NMR spectroscopy experiments was extended to allow the quantitative analysis of bound ligand conformation within the protein binding pocket by full-matrix relaxation approaches (CORCEMA-ST and SICO procedures).[5]

From its basic principles, it can be inferred that the intensity of an STD signal ($\eta_{STD}$) corrected by the excess of ligand (STD amplification factor, STD-AF) gives indirect information about the concentrations of protein–ligand complexes in solution,[4,6] and therefore, STD NMR spectroscopy results from titration experiments might be employed to derive ligand–receptor binding affinities. However, direct approaches have failed to give correct values of equilibrium dissociation constants ($K_D$) from STD NMR spectroscopic titrations, since it has been demonstrated that the magnitudes of the determined constants depend on the particular STD signals of the ligand chosen to build the corresponding binding isotherms.[7] Thus, there is a relatively large uncertainty that is apparently inherent to the determination of $K_D$ by STD NMR spectroscopy which precludes the use of this technique for accurate measurements of protein–ligand affinities in solution. As a way of overcoming those difficulties, accurate $K_D$ values have been determined by competition studies,[4,8] in which the unknown $K_D$ of a ligand is determined by monitoring its displacement from the protein binding site while a reference ligand of known affinity is titrated over the same sample, using the Cheng–Prusoff equation.[9] The applicability and robustness of this com petitive approach have been broadly demonstrated,[4,8,10] and even novel modified pulse sequences using isotopically labeled ligands have been devised to avoid possible problems of signal overlap.[11] Nevertheless, the main drawback is the need for a reference competitive inhibitor for which the affinity must be previously known by other technique.

We are engaged in STD NMR spectroscopy quantitative studies of protein–ligand interactions, some of which involve multimodal binding of the same ligand on one binding site, in which STD intensities can be perceptively affected by ligand cross-rebinding processes.[12] We have shown that such multimodal systems can be quantitatively treated by an appropriate analysis of STD NMR experiments based on STD initial growth rates. Thus, the experimental system can be deconvoluted as a simple sum of the contributions from each mode.[12]

This approach is justified by considering the effects of fast ligand rebinding during the receptor saturation in the STD experiment. If there is a certain probability that a given ligand molecule reenters into the binding site after a preceding binding event (and this rebinding is fast enough related to the relaxation properties), then the ligand spin populations would be partially perturbed due to the previous transfer step, and hence, its capacity to receive more transfer of saturation from the receptor will be different than that from a fresh ligand molecule.[13] Consequently, the amount of magnetization transferred to the free ligand would be smaller. To the best of our knowledge, the effects of this rebinding process in STD have not been considered before, probably because typical experimental conditions for standard STD NMR spectroscopy (i.e., for binding detection, screening, and determination of ligand group epitopes) disfavor ligand rebinding because it uses high ligand-to-protein ratios.[1b] In contrast, protein–ligand titration experiments can involve large fractions of bound ligand, during the low ligand-to-protein ratio regions of the experimental isotherm, increasing then the likelihood of rebinding events. Indeed, we demonstrate herein that rebinding is one of the main causes of errors in the determination of affinities by STD NMR spectroscopic titration experiments.

Herein, we show that STD NMR spectroscopic titration experiments can deliver accurate equilibrium dissociation constants $K_D$ of protein–ligand interactions, if those factors that are affecting the accumulation of the ligand proton saturation in the bulk solution are removed by an appropriated experimental setup. This can be done by constructing the binding (Langmuir) isotherm as a function of the ligand concentration in the sample using the initial growth rates of the STD amplification factors ($STD\text{-}AF_0$), instead of the STD-AF factors at a given saturation time.[14] The initial growth rate corresponds to the STD-AF value at the limit of zero $t_{sat}$, when virtually no ligand turnover takes place, therefore, avoiding the potential effects of fast protein–ligand rebinding processes. They are analogous to the STD initial slopes used to remove relaxation biases in the determination of ligand epitopes.[15] The feasibility of the binding isotherm of STD initial growth rates approach for obtaining accurate experimental values of ligand–receptor dissociation constants is demonstrated for two well-studied protein–ligand systems, affinities of which have been reported in the literature: the binding of *N*-acetylglucosamine (GlcNAc)

and *N,N′*-diacetylglucosamine (GlcNAcβ1,4GlcNAc, chitobiose) to the plant lectin wheat germ agglutinin (WGA),[16] and the interaction of L-tryptophan to bovine serum albumin (BSA).[17]

## Results and Discussion

According to the dependence of STD intensities on the bound ligand concentration, STD-NMR might be used to estimate binding affinities. Unfortunately, there are several well-known factors intrinsic to the STD experiment that affect to STD signals and have precluded their use to derive dissociation constants from titration experiments.[7] The intensity of an STD signal (and hence the magnitude of STD-AF) reflects mainly two factors: the efficiency of saturation transfer from protein protons during the bound state (intermolecular protein–ligand NOEs) and the rate of accumulation of saturated ligand molecules in the free state during the saturation time. Whereas the first factor is intrinsic to the system under study, since it depends on the 3D geometry of the protein–ligand complex, the second is related to the kinetics of the system, and depends on saturation time, concentrations, protein to ligand ratio, temperature, and so forth.[1b,5d] The influence of these parameters on the accumulation of saturated ligand is the source of $K_D$ values bias. However, these factors are susceptible to optimization by an appropriate experimental setup. Therefore, it is of great interest to determine the impact of these parameters on the final outcome.

First, we analyzed the dependence of the $K_D$ measured from STD-AF on several factors: 1) the saturation time of the STD NMR experiment, 2) the amount of saturation transfer, and 3) the fraction of bound ligand (concentration of protein). We have reproduced the reported dependences of $K_D$ values on the monitored ligand proton STD signal[7] and describe the dependence of new factors, such as saturation time and the residence time in the bound state. We also discuss the key role of fast rebinding processes in the final outcome of the titration experiments. Finally, we have applied an alternative protocol using the STD-AF initial growth rates to build the binding isotherms, showing how all of the observed deviations are cancelled out, and the values from the different ligand protons converge towards one single value, which is the best approximation to the thermodynamic value of $K_D$ by STD NMR spectroscopy.

### Analysis of the factors affecting the determination of ligand–receptor dissociation constants by STD NMR titration experiments

*Effect of STD NMR saturation time ($t_{sat}$)*: Typical STD NMR spectroscopic titration experiments monitor the evolution of STD-AF[4] as a function of the total increasing concentration of ligand by recording series of STD experiments at different ligand-to-receptor ratios. A key experimental variable of the STD NMR spectroscopy experiment is the saturation time ($t_{sat}$), since it affects the intensities of the peaks and, therefore, the sensitivity of the experiments. In fact, for any given ligand concentration, STD intensities will grow with $t_{sat}$, up to a point at which a steady state is reached for long saturation times (e.g., $t_{sat} > 5$ s). Although $t_{sat}$ can be adjusted for each case, in most of the STD applications saturation times of 2 s are typically used.

We have studied the influence of the saturation time, $t_{sat}$, of the STD NMR spectroscopy experiment on the final binding isotherm and, consequently, in the final determination of the dissociation constant. To that aim, we carried out a titration of chitobiose in a sample containing 46 μM of the lectin WGA and constructed four different binding isotherms from the dependence of the STD-AF values with the ligand concentration, each one made from values obtained at different saturation times (1–4 s) in the corresponding STD NMR spectroscopy experiments (Figure 1).

The binding isotherms based on the STD-AF values ($t_{sat}$ = 1, 2, 3, 4 s) of the methyl signal (acetamide) of the reducing GlcNAc ring of chitobiose (Figure 1a) upon binding to WGA are shown in Figure 1b. For larger saturation times, higher values of STD-AF are obtained, which in turn leads to higher plateau values of the curves (the parameter $\alpha_{STD}$, see the Experimental Section), and better sensitivity of the experiments. The normalized STD-AF curves (Figure 1c) demonstrate the impact of saturation time on the binding isotherm, as an increase of $t_{sat}$ leads to a slower growth of STD-AF values with ligand concentration. This effect leads to an increase of the "apparent" $K_D$ value (Figure 1c, inset).

The magnitudes of apparent dissociation constants determined from fitting to a one-site Langmuir equation are gathered in Table 1. The apparent $K_D$ increases monotonically with $t_{sat}$, underestimating the protein–ligand affinity for long saturation times. Interestingly, the best agreement with the dissociation constant determined by isothermal titration calorimetry (200 μM)[16], corresponds to the apparent $K_D$ determined at the lowest saturation time (1 s, 300 μM). These results highlight the disadvantages of making use of large saturation times for achieving better signal-to-noise ratios, since the deviations in the determinations of $K_D$ values by STD NMR spectroscopy are, disappointingly, greater under these conditions.

*Effect of STD NMR signal intensity*: Because the best signal-to-noise ratio corresponds to the most intense STD signal, it would be advantageous to use these signals to monitor the binding isotherm for the determination of dissociation constants. However, if rebinding of a previously polarized ligand is fast in the relaxation ($T_1$) timescale, the most intense STD signals will be the most affected by interference caused by consecutive binding steps and therefore subject to larger errors in the determination of $K_D$. To analyze this issue, we have studied the binding of L-tryptophan to BSA at several saturation times by following the same methodology as before.

The STD-AF buildup with saturation time for four selected protons (Hα, Hδ1, Hε3, and Hζ2) of L-tryptophan

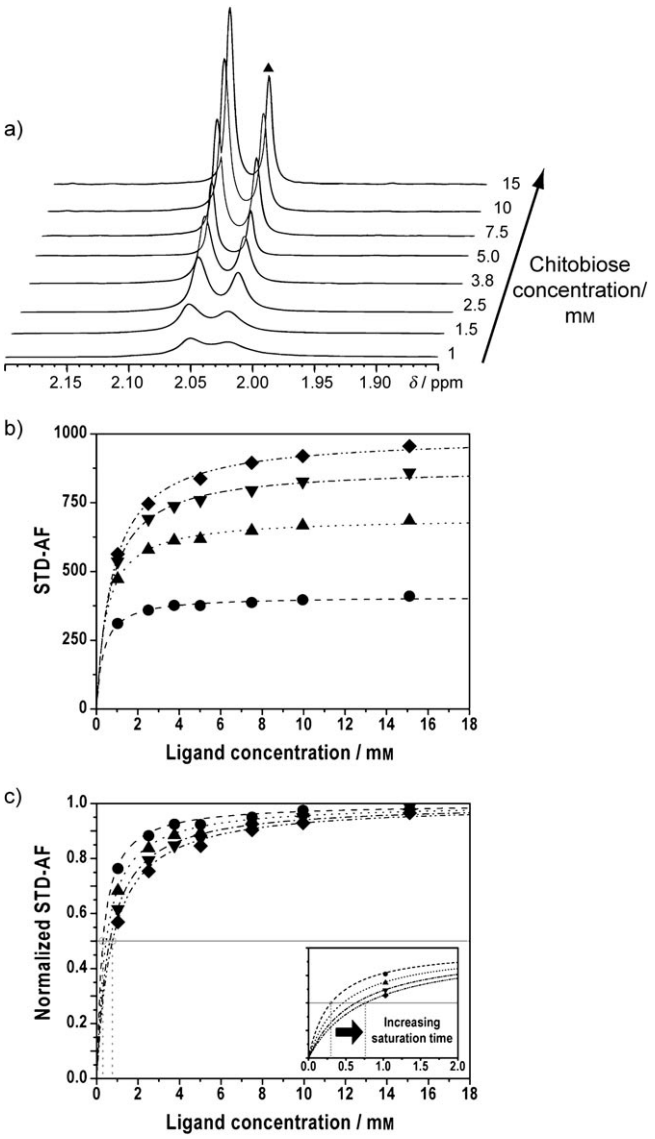



Figure 1. Effect of the saturation time of the STD NMR experiments on the determination of protein–ligand binding affinity. a) Stacked plot of the acetamide spectral region of 1D STD NMR spectra recorded at a single saturation time ($t_{sat}=2$ s, 15 °C) during the titration of a sample of 46 μM of WGA with chitobiose. Labeled with a black triangle is the signal monitored in the isotherms below. b) Binding isotherms of STD-AF values of the acetamide methyl proton of the reducing sugar ring of chitobiose obtained by using different saturation times (1 (●),2 (▲), 3 (▼), and 4 s (◆)). c) Isotherms normalized against their corresponding plateau values ($\alpha_{STD}$). Note that the ligand concentration for semisaturation of the protein binding site (STD-AF = 0.5) corresponds graphically to the $K_D$ value. The inset shows an expansion of the first part of the Langmuir curve and the range of variation of $K_D$ is graphically delimited by the vertical dotted lines. The dashed and dotted straight lines correspond to the mathematical fitting of the data and in all cases there was good fitting to a one-site Langmuir isotherm equation.

(1 mM) upon binding to BSA (20 μM) are shown in Figure 2. Hζ2 and Hδ1 show the largest saturation transfer, whereas Hα is the lowest one, depicting that the region of the ligand spanning Hζ2 and Hδ1 (Figure 2) is making the closest contacts with the protein binding site, whereas the polar amino acid region is more solvent exposed.

Table 1. Apparent equilibrium dissociation constant for the binding of chitobiose to WGA using STD-AF from STD NMR spectroscopic titrations, as a function of the saturation time ($t_{sat}$).[a]

| Saturation time [s] | Apparent $K_D$ [μM] |
|---|---|
| 1.0 | 300 |
| 2.0 | 460 |
| 3.0 | 630 |
| 4.0 | 730 |

[a] 15 °C, 46 μM WGA

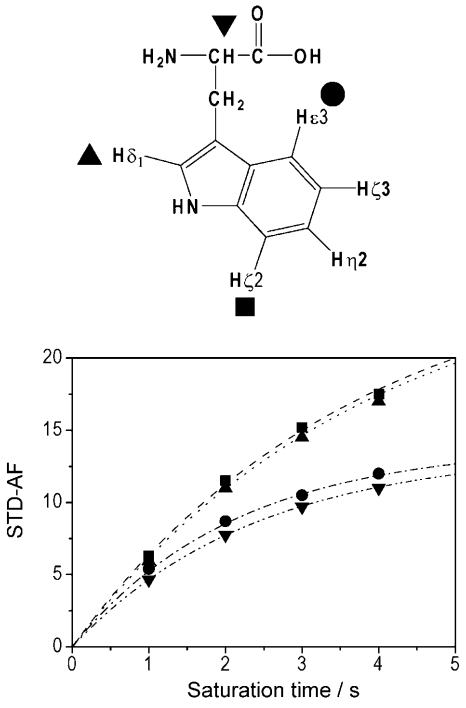



Figure 2. STD-AF values of selected protons of L-tryptophan (Hζ2 (■), Hδ1 (▲), Hε3 (●), Hα (▼)) as a function of the saturation time. The sample contained 1 mM of ligand and 20 μM BSA. Symbols represent the experimental values and the lines are the mathematical fit to a monoexponential asymptotic function.

To highlight the different behavior of the association curves as a function of the chosen proton, the isotherms of normalized STD-AF ($t_{sat}=3$ s) of four protons of L-tryptophan were constructed (Figure 3). The comparison of the results reveals variations in $K_D$ measurements depending on the observed signal of the L-tryptophan (see normalized STD-AF binding isotherms in Figure 3, and compare with Figure 2). A dependence of the growing rate of the isotherm for a given signal with the magnitude of the STD-AF can be observed. Those protons corresponding to the largest STD-AF (Hζ2 and Hδ1 see Figure 2) show binding curves with slower initial growing rates (Figure 3), giving rise to a larger apparent $K_D$; on the other hand, the isotherms of the least saturated protons (Hε3 and Hα) show the largest initial slopes and hence the lowest apparent $K_D$.

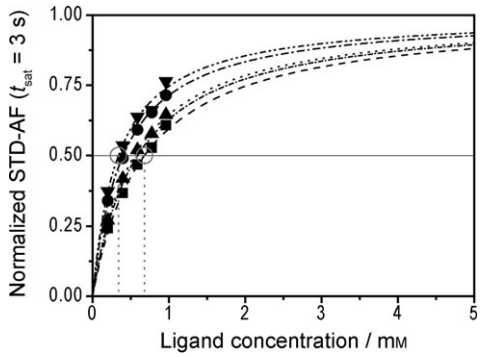



Figure 3. Effect of the individual magnitudes of saturation transfer (STD intensities) of different protons (Hζ2 (■), Hδ1 (▲), Hε3 (●), Hα (▼)) on the determination of L-tryptophan–BSA binding affinity. Binding isotherms of STD-AF values normalized to their corresponding maximum plateau value ($\alpha_{STD}$) of selected protons of L-tryptophan ($t_{sat}$ = 3 s). The sample contained 20 μM BSA. Vertical dotted lines graphically represent the range of variation in the determination of $K_D$.

Quantitative analysis of the curves yielded the apparent $K_D$ values shown in Table 2.[18] These results are also compatible with the mentioned influence of saturation time because, for all ligand protons, there is a monotonic decrease of apparent protein–ligand affinity with increasing times (see Table 2). In addition, the results also evidence the dependence of the measured $K_D$ on the degree of saturation of the considered proton (see Table 2). The signals of the protons receiving the largest amounts of saturation yield the largest apparent $K_D$ and vice versa for all the saturation times measured.

Table 2. Apparent equilibrium dissociation constant $K_D$ [μM] for the binding of L-Trp to BSA from STD NMR spectroscopic titrations, as a function of the saturation time ($t_{sat}$), following STD signals from different ligand protons.[a]

| | Apparent $K_D$ [μM] | | | |
|---|---|---|---|---|
| Saturation time [s] | Proton Hζ2 | Proton Hδ1 | Proton Hε3 | Proton Hα |
| 1.0 | 340 | 220 | 230 | 140 |
| 2.0 | 510 | 420 | 290 | 260 |
| 3.0 | 680 | 550 | 400 | 340 |
| 4.0 | 950 | 730 | 560 | 430 |
| STD-AF [4 s] | 17.5 | 17.0 | 12.0 | 11.0 |

[a] 25 °C, 20 μM BSA

Comparing the experimental data in Table 2 with the $K_D$ value reported in the literature for the interaction of L-tryptophan with BSA ($K_D$ = 125–230 μM)[17], it can be inferred that 1) the closest apparent $K_D$ measured from a given ligand proton STD is again the one obtained by using the lowest saturation time and 2) related to STD intensities, the isotherms yielding the best approximation to the reported values of $K_D$ are those constructed by using the least intense STD signal. Once more, unfortunately, our results show that the best conditions for better signal-to-noise ratio, and hence, for more accurate determination of $K_D$, that is, the most intense STD signals, give, in fact, the largest deviations in the determination of the dissociation constant.

*Effect of the fraction of bound ligand*: It can be inferred from previous results (Tables 1 and 2 and Figure 1c) that the bias of $K_D$ using STD-AF at a single $t_{sat}$ reflects an underestimation of STD-AF values at the initial points of the titration experiment. These points, in the region of low ligand-to-protein ratios, correspond to the largest fractions of bound ligand during the experiment. Remarkably, under these conditions, there is an increased likelihood of fast ligand rebinding taking place on the relaxation timescale (see the Supporting Information).

To test this issue, we carried out further titration experiments with the BSA-L-tryptophan system, modifying the fraction of bound ligand by increasing the receptor concentration. In this case, if ligand rebinding causes the bias in the determination of $K_D$, the effect should be stronger when using a higher receptor concentration. When the $K_D$ for L-tryptophan binding was measured at 60 μM of BSA, keeping the same experimental conditions and the same total concentrations of added ligand as before (20 μM of BSA), the values of apparent $K_D$ were larger (Tables 2 and 3). To highlight this issue, in Figure 4 (bottom) we compare the resulting isotherms of normalized STD-AF values from the same proton of L-tryptophan (Hζ2) for two STD NMR titrations carried out with 20 and 60 μM of BSA. Consistent with the expected influence of the fraction of bound ligand, the effects of saturation time and signal intensity on the values of $K_D$ are also amplified for larger concentrations of protein (compare Tables 2 and 3). This is also noted when comparing the dispersion of the apparent affinities among the different signals of L-tryptophan, which is wider for the experiments performed with the largest fraction of bound ligand during the whole titration ([BSA] = 60 μM, see Figures 3 and 4 (top)). Finally, the same effect on the apparent $K_D$ upon increasing the fraction of bound ligand was observed in STD NMR spectroscopic titration experiments carried out on the WGA–chitobiose sample when using two different protein concentrations (18 and 46 μM; see Supporting Information)

Table 3. Apparent equilibrium dissociation constant [μM] for the binding of L-Trp to BSA (concentrated sample) from STD NMR spectroscopic titrations, as a function of the saturation time ($t_{sat}$) following STD signals from different ligand protons.[a]

| | Apparent $K_D$ [μM] | | | |
|---|---|---|---|---|
| Saturation time (s) | Proton Hζ2 | Proton Hδ1 | Proton Hε3 | Proton Hα |
| 1.0 | 560 | 490 | 420 | 360 |
| 2.0 | 1580 | 1200 | 1010 | 710 |
| 3.0 | 2500 | 2100 | 1270 | 990 |
| 4.0 | 3300 | 2700 | 1600 | 1060 |

[a] 25 °C, 60 μM BSA

Because the fraction of bound ligand is also a function of the intrinsic affinity, different protein–ligand systems will have different sensitivities to the mentioned error sources.

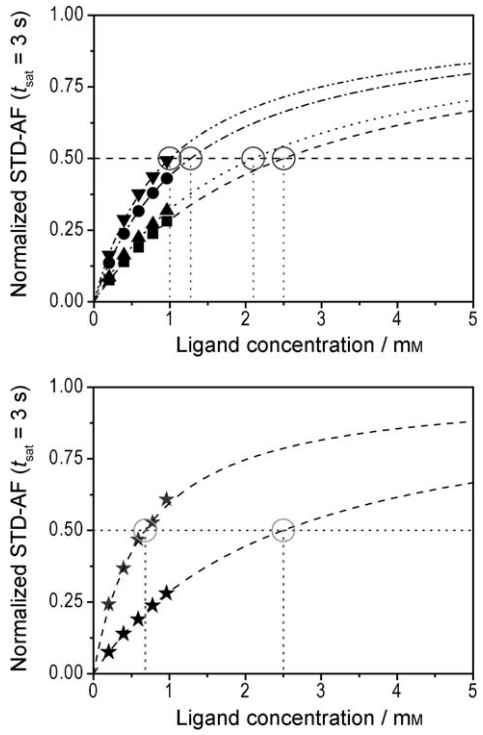



Figure 4. Effect of the fraction of bound ligand on the determination of L-tryptophan–BSA binding affinity. Top: binding isotherms of STD-AF values ($t_{sat}$ = 3 s) normalized to their corresponding maximum plateau value ($\alpha_{STD}$) of selected protons of L-tryptophan (Hζ2 (■), Hδ1 (▲), Hε3 (●), Hα (▼)). The sample contained 60 μM BSA. Bottom: comparison of the binding isotherms of STD-AF of proton Hζ2 of L-tryptophan ($t_{sat}$ = 3 s), for two different concentrations of BSA protein (20 (★), 60 μM (★)) all the remaining experimental conditions being the same. In both figures, the range of deviations in $K_D$ is graphically represented by the vertical dotted lines.

We have performed an additional study with the lectin WGA and N-acetylglucosamine (GlcNAc) to evaluate the binding of chitobiose, since they differ in affinity constants by an order of magnitude ($K_D$ 2.5 mM, and 0.2 mM, respectively)[16]. This comparison should determine whether the extent of the deviation of $K_D$ with the saturation time also depends on the magnitude of the protein–ligand affinity.

Table 4 shows the dissociation constants obtained from binding isotherms of STD-AF values of the methyl protons of the acetamide group at several saturation times. The variation of the calculated $K_D$ with saturation time follows the same trend as in the case of chitobiose: longer saturation times yield larger apparent dissociation constant. Once again, the closest value to the equilibrium dissociation constant determined by calorimetry corresponds to that obtained by using the shortest saturation time (1 s). Interestingly, the difference between the apparent dissociation constants at 1 and 4 s is smaller in the case of GlcNAc (1.25 times) than in the case of chitobiose (2.50 times). Thus, a comparison of the results for the interactions of chitobiose or GlcNAc with the lectin WGA indicates that the $t_{sat}$-dependent overestimation of $K_D$ values is proportional to protein–ligand affinities.

Table 4. Apparent equilibrium dissociation constant for the binding of GlcNAc to WGA from STD NMR spectroscopic titrations, as a function of the saturation time ($t_{sat}$).[a]

| Saturation time [s] | Apparent $K_D$ [mM] |
|---|---|
| 1.0 | 2.4 |
| 2.0 | 2.7 |
| 3.0 | 2.7 |
| 4.0 | 3.0 |

[a] 15 °C, 42 μM WGA.

## Discussion of the effects

All the results shown above demonstrate and experimentally characterize the high sensitivity of the measurements of affinity by STD NMR spectroscopic titration experiments to factors related to the accumulation of saturated ligand in solution. The identified factors introducing additional dependence on STD-AF that obscure the direct relationship between STD-AF values and the concentration of complex, precluding the accurate calculation of dissociation constants. In particular, it must be considered that for experimental conditions leading to high fractions of bound ligands the probability that a given ligand molecule binds the protein on two (or more) occasions during $t_{sat}$ cannot be neglected.

Rebinding will have an effect on the accumulation of saturated ligand in solution if its residence time in the free state ($\tau^F_{res}$) is shorter than the $T_1$ of the ligand proton considered (see the Supporting Information for estimates of the probability of fast ligand rebinding for a one-site bimolecular protein–ligand interaction). In this case, the extent of its influence will depend on the fraction of time that the ligand spend in the free state ($\tau^F_{res}$) in comparison to the characteristic time of the total rebinding cycle ($\tau^F_{res}+\tau^B_{res}$), in which $\tau^B_{res}$ is the residence time of the ligand in the bound state. This proportion ($\tau^F_{res}/\tau^F_{res}+\tau^B_{res}$) corresponds to the fraction of free ligand and, accordingly, low fractions of free ligand will favor rebinding and its influence on the accumulation of saturated ligand in the bulk. For experimental conditions typically found in STD NMR studies, $\tau^F_{res} < T_1$ and rebinding takes place fast on the relaxation ($T_1$) timescale (see the Supporting Information). Under these conditions, the delay between the binding events is not long enough to allow a complete relaxation of magnetizations, so that the protons of the rebound ligand have a lower capacity to receive saturation from the receptor.[13] Therefore, they contribute to the macroscopic intensities (STD-AF) with less saturation transfer than fresh ligands. In fact, all of the previously reported biases in $K_D$ determinations are in perfect agreement with the expected effects of fast rebinding processes.

According to this analysis, when fast protein–ligand rebinding is taking place, there will be a reduction in the STD-AF values of the ligand protons proportional to the degree of rebinding. This explains the increase in apparent $K_D$ observed when the total concentration of the protein (BSA) was increased (Tables 3 and 4 or Figure 4, bottom). Increasing the total protein concentration increases the free

protein concentration, enhancing the likelihood of fast rebinding processes (reduction of $\tau_{res}^{F}$, see the Supporting Information).

The effect of STD intensities on apparent $K_D$ values can also be explained by taking into account rebinding effects. Those ligand protons with larger saturation transfer will, after a first protein–ligand encounter, leave the protein binding site with stronger perturbed spin populations. Under fast ligand rebinding conditions, the ulterior binding events, characterized by the rebinding cycle time $\tau_{res}^{F}+\tau_{res}^{B}$, will add significantly less saturation to these ligand signals than in the previous binding events, leading to proportionally larger reductions in macroscopic STD-AF compared with those ligand protons with lower intensities. This was observed in the case of the binding of L-tryptophan to BSA (Figures 3 and 4) in both samples (20 and 60 μM BSA).

Fast rebinding processes will not have a measurable impact on the accumulation of saturation in solution until the number of saturated ligand molecules in the bulk increases enough to make probable the existence of "protein-saturated ligand" reencounters. This number is proportional to both the saturation time of the STD NMR spectroscopy experiment, and the intrinsic off rate ($k_{off}$) of the protein–ligand interaction under study. Increasing the saturation time will amplify the number of saturated ligand molecules in the bulk, enhancing the probability of fast rebinding processes involving previously saturated ligand molecules. This is the reason for the generalized underestimation of affinities upon increasing the saturation time, observed in all the protein–ligand systems studied herein.

Our study has demonstrated that $K_D$ values calculated from STD NMR spectroscopic titrations based on STD-AF are sensitive to saturation time, receptor concentration, and intensity of the monitored signal. Unfortunately, the optimization of these conditions to minimize the effects on $K_D$ (e.g., short saturation times and minimum fractions of bound ligand) would lead to a serious compromise of the signal-to-noise outcome of the experiment, transferring large errors to the affinity measurement.

**The binding isotherm of initial growth rates of STD-AF**

We have found a strong correlation between the saturation time and the effects of the above-mentioned factors (i.e., the fraction of bound ligand, the relaxation properties, or the magnitude of the saturation transfer to the ligand protons), since the estimated dissociation constants seem to converge and approach their thermodynamic value at short enough saturation times. This correlation strongly suggests that such deviations are related to fast ligand rebinding processes, which play a key role in the reported uncertainties of affinity determinations by STD NMR spectroscopic titrations. If these rebinding processes were the cause of the deviations, the effects of the described factors should be cancelled out in the limit of zero saturation time, since no ligand turnover would be possible. At this limit, all of the STD-AF isotherms would be essentially the same, independent of the experimental conditions, and the "true" dissociation constant, $K_D$, could be derived from mathematical analysis of any of them.

To check this hypothesis, we constructed the binding isotherms using the initial growth rates of the ligand STD-AF values (STD-$AF_0$) at each ligand concentration along the titration instead of the experimental STD-AF at a given saturation time, as done before. The determination of the initial slopes of the STD-AF buildup curves can be done by fitting the whole experimental curve to an appropriate mathematical equation and extrapolating it to the limit of zero saturation time. Matrix relaxation theory[5d] and experimental evidence[15] have demonstrated that the growth of STD-AF values with saturation time can be appropriately described by a monoexponential asymptotic equation: $\text{STD-AF}(t_{sat}) = \text{STD-AF}_{max}[1-\exp(-k_{sat}t_{sat})]$, in which $\text{STD-AF}_{max}$ represents the maximum STD-AF achievable for a given proton (i.e., for very long saturation times), and $k_{sat}$ is its saturation rate constant. After the fit, the initial slope (STD-$AF_0$) was easily obtained by the product $\text{STD-AF}_{max}k_{sat}$.

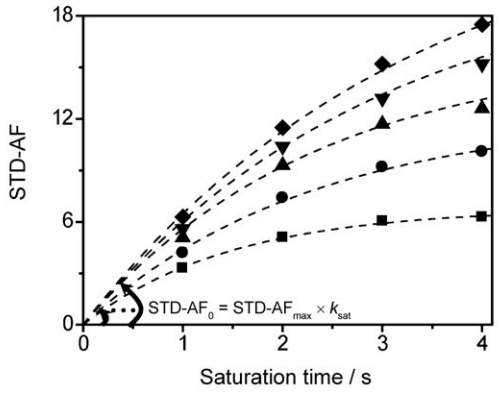
The protocol for protein–ligand affinity determinations by STD NMR spectroscopy is illustrated in Figure 5, for the L-tryptophan-BSA system. As an example, the STD-AF values of proton Hζ2 are monitored, but any other ligand proton can be used (see below). The growth of STD-$AF_0$ with ligand concentration leads to a hyperbolic Langmuir-like behavior which, after mathematical fit (Figure 5C) gives the best approximation to the thermodynamic $K_D$ (210 μM, see table 5 below). The number of data points for obtaining accurate STD-$AF_0$ values (build-up curves) at each ligand concentration will much depend on the intrinsic signal-to-noise ratio of the protein–ligand system under scrutiny, as it happens in distance determinations from cross relaxation rates. From our experience, even the minimum of three data points per ligand concentration is feasible, as long as the first and the last saturation time used are chosen to sample adequately the growth and the plateau regions of the build-up curve.

The analysis of the saturation rate, $k_{sat}$, provides additional evidence of the presence of fast rebinding. This parameter, that determines how fast the plateau of the STD-AF build-up curve is reached, is not constant through the titration, decreasing with ligand concentration (Figure 5). This means that a steady state is reached at shorter saturation times for low ligand concentrations, as a consequence of the decrease of maximum STD-AF caused by rebinding. This phenomenon is observed for all ligand protons of the studied systems and the effect is more dramatic upon increasing the protein concentration, since fast rebinding is favored (see the Supporting Information). In addition, for the weakest system studied (GlcNAc and WGA), in which rebinding is slower, there was essentially no variation of $k_{sat}$ with ligand concentration, which was reflected in smaller deviations of $K_D$ determined by standard STD NMR spectroscopic titrations (Table 4).

The accuracy of the proposed approach was demonstrated for the binding of chitobiose to WGA and the binding of L-

a)




b)

| Ligand concentration [mM] | STD-AF$_{max}$ | $k_{sat}$ [s$^{-1}$] | STD-AF$_0$ (STD-AF$_{max}$ × $k_{sat}$) |
|---|---|---|---|
| 0.2 | 6.8 (± 0.2) | 0.68 (± 0.04) | 4.6 (± 0.4) |
| 0.4 | 12.3 (± 0.7) | 0.44 (± 0.05) | 5.5 (± 0.9) |
| 0.6 | 16 (± 1.0) | 0.43 (± 0.07) | 7.0 (± 1.5) |
| 0.8 | 21 (± 1.0) | 0.34 (± 0.04) | 7.1 (± 1.2) |
| 1.0 | 25 (± 1.5) | 0.30 (± 0.03) | 7.2 (± 1.5) |

c)

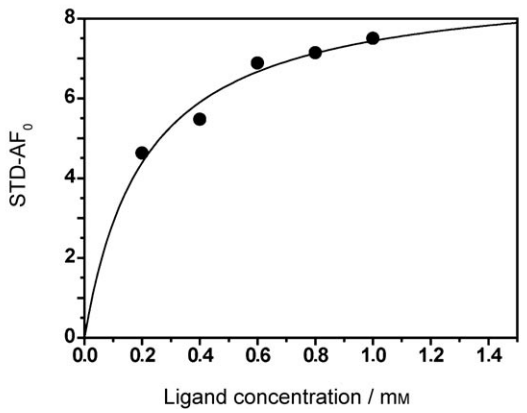



Figure 5. The binding isotherm of STD-AF initial growth rates approach. The new protocol for protein–ligand affinity measurements by STD NMR spectroscopy is illustrated for the L-tryptophan–BSA system (20 μM in protein) by taking the proton Hζ2 of the ligand as an example. a) For each ligand (L-Trp) concentration (0.2 (■), 0.4 (●), 0.6 (▲), 0.8 (▼), and 1.0 mM (◆)), STD-AF values (Hζ2 proton) are obtained at different saturation times (1,2, 3, and 4 s) and fit by using the equation STD-AF($t_{sat}$) = STD-AF$_{max}$[1−exp(−$k_{sat}t_{sat}$)] (-----). b) Each STD-AF buildup curve is fitted to the equation STD-AF($t_{sat}$) = STD-AF$_{max}$-[1−exp(−$k_{sat}t_{sat}$)], and the initial slopes, STD-AF$_0$, are obtained from STD-AF$_0$ = STD-AF$_{max}$$k_{sat}$; errors are given in brackets. c) The initial slopes are represented as a function of the ligand concentration, and the mathematical fit to a Langmuir isotherm ($y = B_{max}x/(K_D+x)$; $B_{max} = 9.0$ (±0.5)) delivers the thermodynamic $K_D$ value of 0.210 (±0.05) (cf. Table 5).

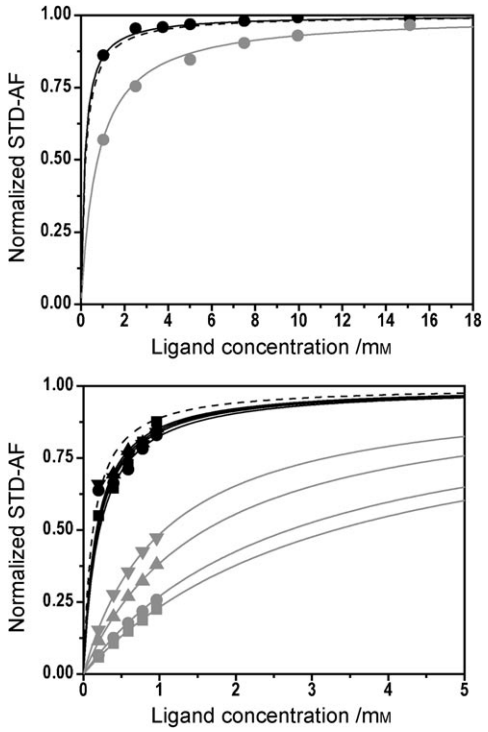



Figure 6. The binding isotherm of STD-AF initial growth rates approach. Black curves show the resulting binding isotherms by using the initial slopes of the STD-AF values of the methyl protons of chitobiose (●) upon binding to WGA (46 μM) (top) and different protons of L-tryptophan (Hζ2 (■), Hδ1 (▲), Hε3 (●), Hα (▼)) upon binding to BSA (60 μM) (bottom). Dashed curves are the mathematical isotherms obtained by using the Langmuir equation with the $K_D$ values reported in the literature. To highlight the benefits of the proposed approach, the binding isotherms of STD-AF values from classical STD NMR titration experiments using a saturation time of 4 s are also included (gray curves). Symbols represent experimental data; solid lines are the mathematical fits.

tryptophan to BSA (Figure 6). In both cases the isotherms built by the new protocol (black curves) converge to the isotherms corresponding to the $K_D$ values reported in the literature (dashed curves).[16–17] Moreover, all the signal-dependent deviations observed in classical STD NMR spectroscopic titrations (gray curves) are cancelled out, and the resulting isotherms converge, showing the same behavior (see the case of L-tryptophan Figure 6, bottom). Therefore, building the binding isotherm from the initial growth rates of STD-AF yields results that are independent of the considered proton or experimental conditions.

We have calculated the dissociation constants of all of the protein–ligand systems under study herein by following the proposed protocol (Table 5). As expected from the reasons discussed above, the values of dissociation constants calculated from STD-AF$_0$ (Table 5) correspond to the limit at zero saturation time of $K_D$ estimated from SDT-AF (Tables 1–4). Note that the $K_D$ values estimated by the protocol based on STD-AF$_0$ are comparable to the reference values (Table 5). In this way, for the binding of WGA ligands, the protocol allowed the determination of $K_D$ values that are in good agreement with the literature: 2.4 mM for GlcNAc and 170–200 μM for chitobiose. This means a significant enhancement in accuracy compared with a standard STD NMR spectroscopic titration setup, particularly for the strongest binder, chitobiose (cf. Table 1). The dissociation constant for the binding of L-tryptophan to BSA obtained

Table 5. Dissociation constants calculated by isotherms of initial growth rates of STD-AF values of the protein–ligand systems studied herein.

| Protein–ligand system | $K_D$ (from STD-AF initial slopes) [μM] | | | Reported $K_D$ [μM] |
|---|---|---|---|---|
| WGA + GlcNAc | | | 2400 (±0.3) | 2500 (±0.15)[a] |
| WGA + GlcNAcβ1,4GlcNAc | [WGA] 46 | | 170 (±20) | 200 (±10)[a] |
| | [WGA] 18 | | 200 (±10) | |
| BSA + L-Trp | [BSA] 60 | proton Hζ2 | 190 (±50) | 125 (±60)[b] |
| | | proton Hδ1 | 170 (±20) | 230 (±90)[b] |
| | | proton Hδ3 | 210 (±50) | |
| | | proton Hα | 180 (±60) | |
| | | average | 190 (±20) | |
| | [BSA] 20 | proton Hζ2 | 210 (±50) | |
| | | proton Hδ1 | 200 (±20) | |
| | | proton Hε3 | 220 (±50) | |
| | | proton Hα | 110 (±60) | |
| | | average | 185 (±20) | |

[a] From reference [16]. [b] From reference [17].

by the STD NMR spectroscopy protocol described herein is slightly larger than that reported from NMR line width measurements. However, it is close to the result on the same system by NMR spectroscopy using WaterLOGSY ($K_D$ = 230 μM). This uncertainty in the determination of $K_D$ for a BSA-ligand system seems to be a particular characteristic of the thermodynamic of the interactions of small molecules with this albumin that, additionally to the specific binding site, has a less specific Sudlow site I that can be accessible to the ligand at high concentrations (in fact, it was detected in our experiments above 1 mM ligand, see the Supporting Information).

In the cases in which the same system was measured by using signals from different protons, or by using several protein concentrations, the application of the isotherm of initial slopes approach led to convergence to a single value within the experimental error of the technique. Thus, for L-tryptophan binding to BSA, the $K_D$ values calculated for the four protons (Hα, Hδ1, Hε3, and Hζ2) are well clustered around 180–220 μM, meaning that the factors leading to the large differences in $K_D$ as a function of the monitored proton have been essentially cancelled out. Moreover, the effects of protein concentration can also be removed (see Table 5 entries at 60 and 20 μM BSA).

The accuracy of affinity determination by the new protocol is reflected in the standard deviations from the average (11% for the 60 μM BSA sample, average 190 μM, and 27% for the 20 μM BSA sample, average 185 μM). On the other hand, these deviation values give an indication of the intrinsic accuracy of the STD NMR spectroscopic technique for the determination of protein–ligand affinities.

## Conclusion

In the context of a wider study about the application of STD-NMR spectroscopy to estimate binding constants, we have performed a detailed experimental analysis of the factors that may influence the magnitude of STD-AF, masking its dependence on the concentration of the complex, concluding that they are proportional to the STD saturation time and hence could be cancelled out at zero time. These experimental factors, saturation time, intensity of STD, and fraction of bound ligand, lead to unacceptable deviations in the calculation of $K_D$. This dispersion is evident when $K_D$ values are calculated simultaneously from different protons of the same ligand. The impact is worse for ligands with very slowly relaxing protons, such as those present in most aromatic residues, which are common building blocks of hit and lead molecules for drug discovery. A likely optimization of the conditions would use a short saturation time, low complex concentration, and minimum signal intensity. Unfortunately, such experimental conditions also correspond to low signal-to-noise ratios, implying the use of long experimental times, and compromising the accuracy of the measurement.

The analysis of the effects of saturation time and other factors on the estimated $K_D$, together with the consideration of the kinetics and thermodynamics of the system, reveals that ligand rebinding cannot be discarded in titration conditions with high bound-ligand proportions. The macroscopic consequence of rebinding of previously saturated ligand molecules will be a decrease in the STD-AF magnitude and, as a result, an underestimation of $K_D$ proportional to the saturation time. Therefore, the extrapolation of STD-AF to zero time (STD-$AF_0$, initial growth rate), when there are no previously saturated molecules, affords a magnitude insensitive to rebinding effects and proportional to the fraction of complex, allowing the $K_D$ value to be calculated by means of the corresponding binding isotherm.

We have developed a suitable protocol for measuring dissociation constants of ligand–receptor interactions from titration experiments using STD NMR spectroscopy to monitor the variation of complex concentration at different ligand-to-protein ratios. In this method, the ligand–receptor binding isotherm is constructed by using the initial growth rates of STD amplification factors (STD-$AF_0$), instead of the amplification factors (STD-AF) themselves at a given saturation time. We have demonstrated that by using this approach those factors that cause large errors in titrations based on STD amplification factors are efficiently cancelled out. The present protocol increases the known applications of STD NMR spectroscopy, significantly improving its accuracy in the determination of dissociation constants from single ligand titrations, which will be of particular interest for those protein–ligand systems for which a competitive inhibitor of known affinity is not available. We also envisage the feasibility of this approach to obtain thermodynamic parameters of binding ($\Delta H$ and $\Delta S$) from variable temperature

STD NMR spectroscopic titrations and studies to explore this issue are currently underway.

## Experimental Section

**NMR spectroscopy**: All NMR spectroscopy experiments were performed on a Bruker Avance DRX 500 MHz spectrometer equipped with a 5 mm inverse triple-resonance probe head. NMR samples were prepared in 500 μL of 99.9% $D_2O$ buffer containing 50 mM sodium phosphate pH 7.4 (uncorrected for $D_2O$), for the BSA samples, or containing 50 mM sodium phosphate/50 mM KCl at pH 7.0, for the WGA samples. Pure protein samples and GlcNAc were obtained from Sigma–Aldrich, and chitobiose was from Dextra Laboratories.

For STD NMR experiments, a pseudo-2D version of the STD NMR sequence was used for the interleaved acquisition of on- and off-resonance spectra. For selective saturation, cascades of Gaussian pulses with a length of 49 ms and 50 dB of attenuation were employed, with an interpulse delay of 1 ms.[4] The on-resonance frequency was set to 0.86 ppm, for the BSA–L-tryptophan samples, and 7.35 ppm for the WGA–GlcNAc or WGA–chitobiose samples, whereas in all cases the off-resonance frequency was 40 ppm. In all cases appropriate blank experiments, in the absence of protein, were performed to test the lack of direct saturation to the ligand protons. Saturation times to obtain the STD buildup curves were 0.5, 0.75, 1, 1.5, 2, 2.5, 3, and 4 s. The total duration of each experiment was 6.5 s. Typically, the number of scans was 64, though for the least concentrated protein samples, an increase to a range of 256–512 was beneficial for signal integration.

**Binding isotherms from STD amplification factors**: The concentrations of protein were 18 or 46 μM of WGA, for the chitobiose titration; 42 μM of WGA for GlcNAc; and 20 or 60 μM of BSA for L-tryptophan binding. Ligands were titrated onto the corresponding protein samples from concentrated stock solutions to minimize dilution effects. In STD NMR spectroscopic titration experiments, at a given ligand concentration, the STD intensity ($\eta_{STD}$) of a given proton can be considered to be directly proportional to the fraction of bound ligand, $f_B^L=[PL]/[L]_0$. STD-AF, defined by Mayer and Meyer[4] as the product of $\eta_{STD}$ by the ligand excess ($\varepsilon=[L]_0/[P]_0$), makes $\eta_{STD}$ dependent on the fraction of bound protein, $f_B^P$ [Eq. (1)].

$$\text{STD-AF} = \varepsilon(I_0 - I_{sat})/I_0 = \varepsilon\eta_{STD} \quad (1)$$

Thus, plotting STD-AF values at increasing [L] gives rise to a Langmuir hyperbolic dose-response curve as described by Equation (2):[6]

$$\text{STD-AF}\ ([L]) = (\alpha_{STD}[L])/([L] + K_D) \quad (2)$$

Mathematical fitting yields both parameters: the equilibrium dissociation constant, $K_D$, and $\alpha_{STD}$ (a dimensionless scaling factor representing the maximum STD amplification for the monitored signal).

For each ligand concentration, STD-AF buildup curves were recorded. To determine $K_D$ values following the standard procedure, the STD-AF values (at a given saturation time) of a ligand proton were plotted as a function of the concentration of ligand, and the resulting curve was mathematically fitted to the dose-response equation described above, to yield the $K_D$. We named these values "apparent" $K_D$. After the fitting results, all of the curves were normalized by dividing all the STD-AF values by the parameter $\alpha_{STD}$. In this way, all of the isotherms grow between 0 and 1, independent of the chosen proton, making it possible to superimpose the curves to check graphically the different outcomes.

**Binding isotherms from initial slopes of STD amplification factors**: The binding isotherms were constructed from initial slopes of STD amplification factors (STD-$AF_0$) calculated at every ligand concentration along the titration. Each value of STD-$AF_0$ was obtained by fitting the STD-AF evolution with the saturation time to the equation STD-AF $(t)=a(1-\exp(-bt))$[15] as the product of the coefficients $ab$.[19] The STD-$AF_0$ values were then plotted as a function of the concentration of ligand, and the resulting isotherm of initial slopes was mathematically fitted to a Langmuir equation to obtain the dissociation constant.

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[19] Herein, we have used an exponential equation for the determination of the STD initial slopes, but the initial growth rates could also be obtained by linear regression analysis using short saturation times, if linearity is assured.

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