Ligand–Protein Complexes

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Dissociation Kinetics of a Binary Complex in Solution by Protein Displacement**

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Characterization of a ligand–protein complex is mostly done in terms of thermodynamic equilibrium properties. The notion of the equilibrium dissociation constant K_D thereby allows to calculate the precise amount of complex formed from the starting concentrations of both partners. The same variable can also be expressed as the ratio of the off-rate/on-rate, equilibrium being defined as equal numbers of complexes dissociating and forming per unit of time. Both kinetic rates are of fundamental interest, with the on-rate, for example, giving information on the precise mechanism of complex formation.[1] However, in the framework of in vivo drug–target interactions, where the ligand concentration is not constant over time, the off-rate has recently gained importance. It indeed is directly related to the lifetime of the complex in the open system that is the cell or even the body.[2–4] The residency time of the ligand on the target has been shown[2,4] to have equal importance as its availability at target site and its thermodynamic binding affinity towards the target for drug action.

Measurement of the kinetic parameters of a protein–ligand complex is mostly done by ligand displacement or by surface plasmon resonance (SPR) methods allowing for the time-dependent detection of complexes.[5] Whereas the former method requires the synthesis of a ligand incorporating a fluorescence or radio-activity probe,[6,7] one potential pitfall of the off-rates measurement by SPR is the possibility of recapture at the surface. Recognized early on,[8] alternative use of the method by competition experiments still allows to obtain accurate K_D values.[6] Ligand solubility is another potential problem, and is solved in many cases by the addition of co-solvents. Ideally, however, one would like to obtain the kinetic constants not at a surface, but directly in solution without the addition of any co-solvent. Dilution of the complex and monitoring of the evolution to the novel equilibrium is one possibility, but with high-affinity ligands, one needs to dilute to concentrations below the K_D value to have a significant shift, and detection becomes a problem.

Here, we present a novel approach based on the displacement of the protein rather than the ligand. The solubility issue thereby is transferred from the ligand to the protein. We demonstrate the method with liquid-state nuclear magnetic resonance (NMR) spectroscopy as the detection method to follow the exchange, and differential isotope labeling of the protein to distinguish initial apo and holo forms of the protein. An alternative detection method based on mass spectrometry, whereby distinguishing proteins by the absence or presence of a tag alleviates the requirement for isotope labeling, is equally demonstrated.

Our experimental system concerns the Cyclophilin A (CypA) peptidyl-prolyl cis–trans isomerase in its complex with Cyclosporin A (CsA) or its non-immunosuppressive analog Alisporivir (or Debio025). As the former is a clinically important immunosupressor, an intense research effort has been directed towards the molecular characterization of the CypA–CsA complex.[9–15] An important conclusion is that the solvent determines to a large extent the conformation of the free CsA molecule, with notable differences between the conformation of CsA in apolar solvents and its CypA-bound conformation.[16] Early SPR results have suggested that a rate-limiting step of the complex formation would be the CsA cis–trans isomerization of the bond between positions 9 and 10.[17] However, later work with a water-soluble analog did not reach the same conclusion.[18] Because kinetic parameters obtained by SPR for solubility reasons mostly start with a concentrated solution in an organic solvent,[19] the low solubility at the surface and the possible isomerization step both could modify, in a poorly understood manner, the resulting kinetic parameters.

We recently have used the differential NMR spectra of CypA in its apo form or in its complex with CsA and/or Alisporivir to obtain a relative K_D value for both ligands without addition of any co-solvent.[20] The latter molecule, currently in advanced clinical trials against the hepatitis C virus, is only subtly different from CsA (Figure S1 in the Supporting Information), but the few molecular changes lead to a better antiviral effect even in a cell model where the question of immunosuppression is without relevance.[21–23] The difference in equilibrium dissociation constant of both drugs towards CypA that we found thereby seems small to explain the enhanced in vivo efficacy of Alisporivir. Here, we

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describe the development of a novel method to measure the off-rate for both CypA–CsA and CypA–Alisporivir complexes in the absence of any co-solvent. Surprisingly, we find that the off-rate distinguishes both complexes significantly more than their equilibrium dissociation constants.

For both CsA and Alisporivir, ligand binding induces distinct chemical shifts that thereby act as indicators of both the apo and holo proteins.[20] The crystal structure of CsA-bound Cyclophilin A shows two lysine residues in or near the CsA binding site,[12] and we previously found that Alisporivir binds in a very similar manner.[24] We therefore produced for this study a selectively ^{15}N-Lys-labeled Cyclophilin sample, and found that both the Lys82 and Lys125 can be used to distinguish both protein forms (Figure S2 in the Supporting Information). Other labeling schemes are possible, and could include the ^{13}C methyl labeling of the Ile,[25] Val, Leu[26] or Ala[27] residues, thereby increasing the sensitivity of the NMR detection.

Starting from a solution of ^{15}N-labeled protein in a 1:1 complex with the ligand, we dilute this sample with the same protein in its ^{14}N apo form. The thermodynamic equilibrium shift can be analytically expressed (see section S3 in the Supporting Information), with the resulting time scale being mainly determined by the on-rate of the initial free ligand towards the incoming apo protein. Labeling of the protein is irrelevant for this process, which for physically relevant on-rates mostly occurs within the dead time of the mixing experiment. After this initial step, the ligand will be redistributed over the ^{15}N-labeled and unlabeled (^{14}N) protein molecules, and the relative concentrations of ^{15}N ligand bound and ^{15}N apo proteins can be followed by monitoring the NMR resonances that distinguish both forms.

Analytically, mixing of the ^{15}N-labeled protein–ligand complex with the ^{14}N apo form of the protein requires that we write separate equations describing the time evolution for both labeling forms of the protein. We consistently will indicate the ligand concentration as [D], whereas [CypA] and [^{15}N CypA] indicate the concentrations of total (irrespective of labeling) or ^{15}N-labeled Cyclophilin A. The concentration of the complex is indicated by [^{15}N CypA–D] in Equation (1).

$$\mathrm{d}[^{15}\mathrm{N\ CypA-D}]/\mathrm{d}t = -k_{\mathrm{off}}[^{15}\mathrm{N\ CypA-D}] + k_{\mathrm{on}}[^{15}\mathrm{N\ CypA}][\mathrm{D}] \quad (1)$$

Replacing [D] by its thermodynamic equilibrium value $[\mathrm{D}]_\infty$, and taking into account the high affinity of the complex, implying that all ligand will be bound when the protein is in excess at NMR-compatible concentrations (see section S4 in the Supporting Information), we can express the equilibrium dissociation constant K_D as [Eq. (2)].

$$\begin{aligned} K_\mathrm{D} &= k_{\mathrm{off}}/k_{\mathrm{on}} = [\mathrm{CypA}]_\infty\ [\mathrm{D}]_\infty/[\mathrm{CypA-D}]_\infty \\ &\approx [^{14}\mathrm{N\ CypA}]_0[\mathrm{D}]_\infty/[^{15}\mathrm{N\ CypA}]_0 \end{aligned} \quad (2)$$

Equation (1) therefore can be expressed in sole terms of [^{15}N CypA–D] as Equation (3).

$$\begin{aligned} \mathrm{d}[^{15}\mathrm{N\ CypA-D}]/\mathrm{d}t = &-k_{\mathrm{off}}(1 + [^{15}\mathrm{N\ CypA}]_0/[^{14}\mathrm{N\ CypA}]_0) \\ &\cdot[^{15}\mathrm{N\ CypA-D}] + k_{\mathrm{off}}[^{15}\mathrm{N\ CypA}]_{\mathrm{tot}} \\ &\cdot[^{15}\mathrm{N\ CypA}]_0[^{14}\mathrm{N\ CypA}]_0 \end{aligned} \quad (3)$$

The apparent time constant of the ^{15}N protein–ligand complex disappearance hence is given in Equation (4).

$$1/k_{\mathrm{off,app}} = 1/k_{\mathrm{off}}[^{14}\mathrm{N\ CypA}]_0/([^{14}\mathrm{N\ CypA}]_0 + [^{15}\mathrm{N\ CypA}]_0) \quad (4)$$

To get a more intuitive feeling for this result, we simulated the time-dependent redistribution of the ligand over the ^{15}N-labeled and (^{14}N) unlabeled species with a Monte Carlo simulation. Akin to the procedure we previously developed for simulating exchange broadening,[28] we use a simple model in which we challenge the ^{15}N protein–ligand complex with increasing amounts of ^{14}N apo protein (Figure 1). Defining

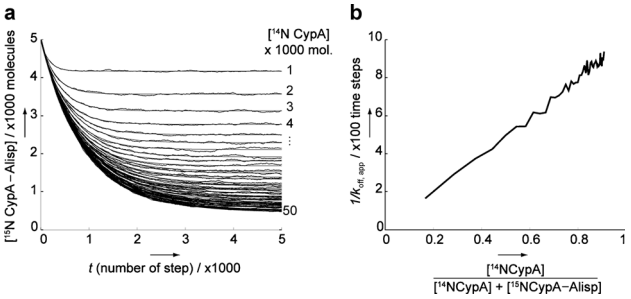

Figure 1. a) Results of a Monte Carlo simulation in which 5000 copies of ^{15}N protein–ligand complexes are challenged with increasing amounts of ^{14}N protein (the number of unlabeled proteins is indicated on the right). b) The fitted time constants for each individual curve obey the linear relationship predicted by Equation (4).

a probability of complex dissociation per time step, and starting with 5000 ^{15}N CypA–D complexes, only these have initially a probability to dissociate (see section S5 in the Supporting Information). As we require that the mixture is at thermodynamic equilibrium, all free ligand molecules resulting from a dissociation event re-associate in the same time step with a free CypA protein. Whether the latter is ^{15}N labeled or not, however, is a completely random process and hence will only be determined by the relative proportions of both species. The novel distribution is the starting point for the next time step. When we add increasing amounts of ^{14}N CypA to a fixed initial concentration of ^{15}N CypA–D, our simulation shows as expected a lower final concentration of ^{15}N protein–ligand complex, whereby the 5000 ligand molecules are evenly distributed over all CypA molecules. This increased amplitude leads to a redistribution of ligand between the ^{15}N-labeled and (^{14}N) unlabeled protein forms that takes more time. The resulting time constant indeed behaves as shown in Equation (4) (Figure 1).

In a typical experiment, we prepared the selectively ^{15}N-Lys-labeled CypA sample charged with Alisporivir, and a second sample containing ^{14}N CypA without any ligand. After careful calibration of both protein concentrations, we mixed both samples to reach the required ratio of

$[^{14}N\ CypA]_0/[^{15}N\ CypA–Alisporivir]_0$, and immediately recorded ^{15}N-edited 1D HSQC NMR spectra at 4 °C (HSQC = heteronuclear single quantum coherence). The dead time before the acquisition of the first spectrum was of the order of 3 minutes, but could clearly be improved upon by using a stopped-flow apparatus. The kinetic equilibrium change is monitored by following the decrease in intensity of the ^{15}N CypA–Alisporivir Lys82 signal and the increase in intensity of the ^{15}N apo CypA Lys82 resonance (Figure 2b).

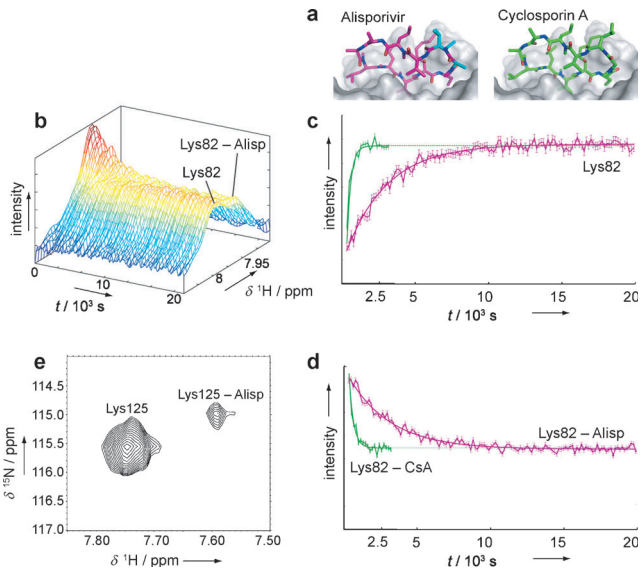

Figure 2. a) Molecular structures of alisporivir (magenta) and CsA (green) in the binding site of CypA (gray). b) Series of ^{15}N-edited 1D spectra of ^{15}N CypA taken immediately after mixing the ^{15}N CypA–Alisporivir sample with ^{14}N CypA, with a ratio of $[^{14}N\ CypA]_0/[^{15}N\ CypA–Alisporivir]_0 = 4.2$. c) Plot (magenta) of the intensity of the resonance of Lys82 in apo ^{15}N CypA derived from the experiment described in (b), and plot (green) from a $[^{14}N\ CypA]_0/[^{15}N\ CypA–CsA]_0 = 3.2$ mixing experiment. d) Idem for the peak of Lys82 in ligand bound ^{15}N CypA, using the same color coding. e) Zoom of the final 2D HSQC spectrum centered on Lys125 for the complex with Alisporivir. Integrals of these peaks determine the relative concentration of both species.

Fitting these curves to a mono-exponential for both signals (Figure 2c and d) led to a value of $k_{off,app} = 3.5 \times 10^{-4}\ s^{-1}$. Alternatively, the appearance of the isolated Lys125 signal was used and led to similar $k_{off,app}$ values (see section S7 in the Supporting Information). While this resonance was isolated, hence made its intensity analysis straightforward, it was not possible to analyze the reciprocal disappearance of Lys125–Alisp resonance due to overlap with other signals.

The experiment was repeated with various ratios of ^{14}N CypA and ^{15}N-labeled CypA charged with its ligand Alisporivir, as well as with the reciprocal situation in which ^{14}N CypA–Alisporivir was mixed with ^{15}N-labeled Cyclophilin (see section S6 in the Supporting Information). The observed apparent off-rates $k_{off,app}$ (Figure 3) as well as the calculated k_{off} using Equation (4) are summarized in Table 1, and the relation between $k_{off,app}$ and the relative amounts of ^{15}N CypA–Alisporovir and ^{14}N CypA is shown in Figure 4. The true k_{off} rate was determined by fitting the experimental

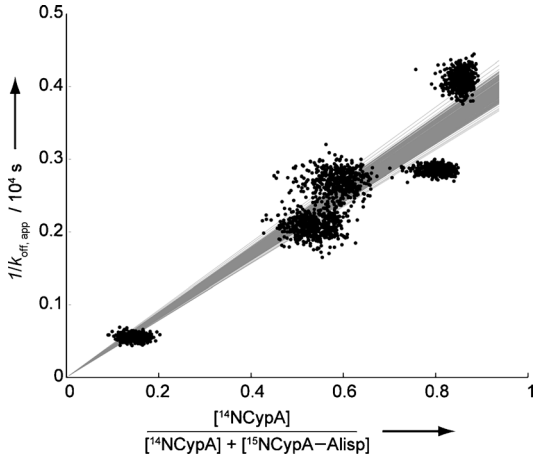

Figure 3. Plot of $1/k_{off,app}$ as a function of $[^{14}N\ CypA]/([^{14}N\ CypA] + [^{15}N\ CypA–Alisporivir]$, using Equation (4). The dots represent the experimental data distributed over their uncertainty, as explained in detail in section S6 in the Supporting Information. The gray lines represent the distribution of $k_{off,app}$ over its uncertainty.

Table 1: Apparent ($k_{off,app}$) and calculated (k_{off}) off-rates using Equation (4) for different relative concentrations of CypA over CypA–Alisp with different labeling schemes. The experimental data for each ratio is shown in detail in the Supporting Information in section S8–S12.

	$[^{15}NCypA]/[^{14}NCypA : Alisp]$		$[^{14}NCypA]/[^{15}NCypA : Alisp]$		
ratios	0.2 ± 0.02	1.4 ± 0.2	1.2 ± 0.2	4.2 ± 0.6	6.1 ± 0.8
$k_{off,app}$ [$10^{-4}\ s^{-1}$]	18.3 ± 1.3	3.7 ± 0.2	4.8 ± 0.3	3.5 ± 0.1	2.4 ± 0.1
k_{off} [$10^{-4}\ s^{-1}$]	2.7 ± 0.2	2.2 ± 0.1	2.6 ± 0.2	2.8 ± 0.05	2.1 ± 0.1

points to Equation (4), and a value of $2.4 \pm 0.1 \times 10^{-4}\ s^{-1}$ was found.

The same approach was applied to the complex of Cyclophilin A with the immunosuppressor CsA. However, for this complex, the equilibrium was reached almost consistently within the experimental dead time of our experimental set-up, and only with a ratio of $[^{14}N\ CypA]_0/[^{15}N\ CypA–CsA]_0 = 3.2 \pm 0.5$, we were able to obtain workable curves that could be fitted to an apparent off rate of $35.9 \pm 3.8 \times 10^{-4}\ s^{-1}$ (Figure 2c,d and section S13 in the Supporting Information). This corresponds to a k_{off} of $27 \pm 3 \times 10^{-4}\ s^{-1}$, which is an order of magnitude larger than the one determined for the complex between CypA and Alisporivir. A subsequent attempt to measure the off-rates by a SPR-based method without co-solvent but with limited ligand concentration confirmed this large difference in off-rate for both complexes (see section S14 in the Supporting Information).

Although the presented method does not require the synthesis of any competing ligand, can deal with low solubility without the addition of any co-solvent, and does not depend on an accurate measure of the absolute concentration of ligand, the detection by NMR spectroscopy requires the use of important quantities of isotope-labeled proteins, which might be a limit to its wider use. Moreover, the dead time of

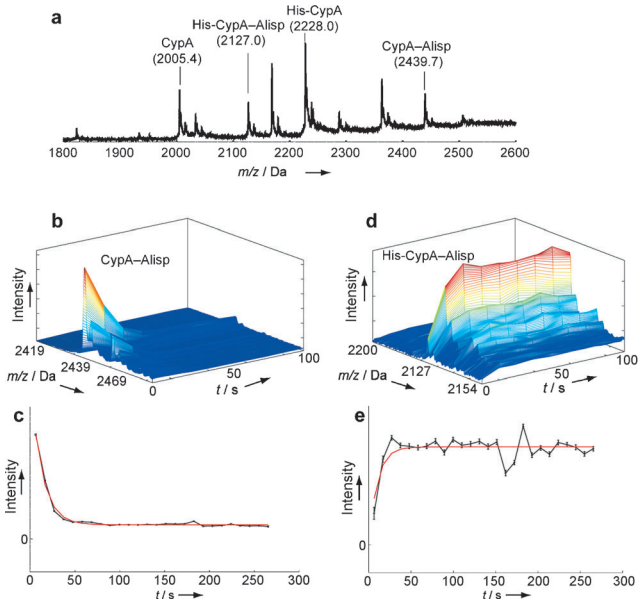

Figure 4. a) Native mass spectrometry detection of the four species formed by CypA/His-CypA and their complexes with Alisporivir. b) Series of mass spectra of CypA–Alisporivir (2439.7 Da, 8+) taken immediately after mixing the CypA–Alisporivir sample with His-CypA, with a ratio of $[\text{His-CypA}]_0/[\text{CypA–Alisporivir}]_0 = 3$, and c) fit of the corresponding intensity to a mono-exponential. d) Series of mass spectra of His-CypA–Alisporivir (2127.0 Da, 8+), and e) fit of the corresponding intensity to a mono-exponential.

the NMR experiment and subsequent requirement to acquire a 1D spectrum with reasonable signal/noise ratio limits it to complexes with an off-rate inferior to $(300\ \text{s})^{-1}$, that with a diffusion controlled on-rate correspond to 10–100 nanomolar dissociation constants (depending on the on-rate). We hence adapted the experiment to detection by native mass spectrometry to eliminate the requirement of labelled proteins. Using as the two distinguishable species a Cyclophilin A sample without and with a histidine tag (CypA and His-CypA, respectively), we could detect the four species at equilibrium (CypA, 18293.7 Da; CypA–Alisporivir, 19510.3 Da; His-CypA, 20044.6 Da; His-CypA–Alisporivir, 21261.2 Da) after mixing CypA–Alisporivir and His-CypA (Figure 4 and Supporting Information, S15).

For the kinetic experiment, we mixed CypA–Alisporivir and His-CypA, and directly injected the resulting mixture using a syringe that feeds the sample using a capillary in the mass spectrometer. The individual species could be detected when summing 10 individual 1 second mass spectra, and allowed to monitor the equilibration reaction both on the disappearing CypA–Alisporivir or His-CypA and on the appearing His-CypA–Alisporivir or CypA species (Figure 4 and section S15 in the Supporting Information). When challenging a 3.3 μM [CypA–Alisporivir] sample with a threefold excess of [His-CypA], we determined an apparent off-rate of $9.0 \pm 1.0 \times 10^{-2}\ \text{s}^{-1}$, corresponding to an effective $k_{\text{off}} = 8.0 \pm 0.8 \times 10^{-2}\ \text{s}^{-1}$. Considering that the ligand exchange in this experiment occurred at room temperature, and that previous SPR work on the CypA–CsA complex determined a hundredfold increase in off-rate when changing the temperature from 16 to 35 °C,[19] this value is reasonable. More importantly, it demonstrates that mass spectrometry, with its reduced dead time and shorter acquisition time than NMR spectroscopy, can significantly extend the range of the presented protein displacement experiment to weaker complexes.

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