

Analysis of the HIV-1 Genomic RNA Dimerization Initiation Site Binding to Aminoglycoside Antibiotics Using Isothermal Titration Calorimetry

Serena Bernacchi, Eric Ennifar

▶ To cite this version:

Serena Bernacchi, Eric Ennifar. Analysis of the HIV-1 Genomic RNA Dimerization Initiation Site Binding to Aminoglycoside Antibiotics Using Isothermal Titration Calorimetry. RNA Spectroscopy Methods and Protocols, 2113, pp.237-250, 2020, Methods in Molecular Biology, 10.1007/978-1-0716-0278-2_16. hal-02911038

HAL Id: hal-02911038 https://hal.science/hal-02911038

Submitted on 3 Aug 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Chapter 16

2

3

4

5

6

17

Analysis of the HIV-1 Genomic RNA Dimerization Initiation Site Binding to Aminoglycoside Antibiotics Using Isothermal Titration Calorimetry

Serena Bernacchi and Eric Ennifar

Abstract

Isothermal titration calorimetry (ITC) provides a sensitive, powerful, and accurate tool to suitably analyze 7 the thermodynamic of RNA binding events. This approach does not require any modification or labeling of 8 the system under analysis and is performed in solution. ITC is a very convenient technique that provides an 9 accurate determination of binding parameters, as well as a complete thermodynamic profile of the molecular 10 interactions. Here we show how this approach can be used to characterize the interactions between the 11 dimerization initiation site (DIS) RNA localized within the HIV-1 viral genome and aminoglycoside 12 antibiotics. Our ITC study showed that the 4,5-disubstituted 2-desoxystreptamine (2-DOS) aminoglyco-13 sides can bind the DIS with a nanomolar affinity and a high specificity.

Key words HIV-1, Viral RNA, Aminoglycosides, Dimerization initiation site, ITC, Thermodynam- 15 ics, RNA–drug interaction 16

1 Introduction

Interaction of biological macromolecules either with each other or 18 with small ligands constitutes the basis for molecular recognition. 19 The development of the approaches providing a detailed under-20 standing of these interactions at the molecular level is required to 21 facilitate the design and development of drugs. In this frame, the 22 isothermal titration calorimetry (ITC) is an equilibrium solution 23 technique that directly *characterizes* all parameters regulating the 24 molecular interactions [1–4]. In a typical experiment, aliquots of a 25 titrant in the syringe are injected into the cell containing the sam-26 ple. Upon each titration, the amount of heat either released or 27 absorbed during the binding event is measured, leading in one 28 single experiment the determination of all the binding parameters: 29 the equilibrium dissociation constant (K_d), the reaction stoichiom-30 etry (n), the binding enthalpy (ΔH), the binding entropy (ΔS), and 31 the Gibbs free energy change (ΔG). Moreover, the analysis at 32 several temperatures gives also access to the heat capacity change 33 (ΔCp) . Importantly, thermodynamic analysis can give access to 34 structural data and hence provides information on hydrogen bond-35 ing, hydrophobic interactions, and charge-charge interactions. A 36 serious advantage of ITC over other alternative approaches is that 37 ITC is a true solution technique which does not require any modi-38 fication, labelling, or immobilization of any partner (truly label-39 free) and that there is no restriction in the molecular weight of the 40 reagents or in the choice of the buffer composition. Since the 41 measured enthalpy results not only from the heat of the binding 42 events but also from other heat sources such as solvent effects 43 and/or heat of dilution, one has to pay attention to prevent any 44 buffer mismatch between the syringe and the sample cell (see Note 45 1). Importantly, the application field of ITC has been recently 46 extended to kinetics, in addition to thermodynamics, by the devel-47 opment of kinITC [5–9]. This method leads to the determination 48 of on- and off-rates close to those obtained by surface plasmon 49 resonance [10, 11]. 50

In this chapter, we provide a detailed protocol for the charac-51 terization of interactions between a fragment of viral RNA with 52 aminoglycoside drugs. ITC is a well-adapted technique for the 53 study of RNA/ligand interactions [12–17]. The RNA used in this 54 chapter is a synthetic 23-nucleotide containing the HIV-1 genomic 55 RNA dimerization initiation site (DIS). Dimerization of the geno-56 mic RNA is a crucial step of the HIV-1 retrovirus replication cycle. 57 The DIS is a highly conserved stem-loop sequence located in the 58 5'-untranslated region of the viral genome which is essential for 59 genome dimerization [18, 19]. A six-nucleotide palindromic 60 sequence within the DIS promotes genome dimerization by form-61 ing a loop-loop complex (or kissing-loop complex) [20-23]. This 62 initial kissing-loop complex is further stabilized in vitro into an 63 extended duplex form by extension of interstrand Watson-Crick 64 base pairs [24–28]. X-ray structures of the DIS as a loop-loop 65 complex [29, 30] and as an extended duplex [31, 32] revealed 66 striking resemblance with the bacterial 16 S ribosomal A site, 67 which is the natural target of aminoglycoside antibiotics, thus 68 opening the opportunity of targeting the HIV DIS using such 69 compounds [33, 34]. In this frame, ITC analysis showed that the 70 4,5-disubstituted 2-desoxystreptamine (2-DOS) aminoglycosides, 71 but not 4,6-disubstituted 2-DOS, can bind specifically DIS dimers. 72 Binding parameters revealed that the specific interaction between 73 aminoglycosides and DIS kissing loop is ensured by rings I, II, and 74 III, while rings IV and V enhanced affinity through unspecific 75 interaction with sugar phosphate backbone. The RNA determi-76 nants of this interaction were also investigated, and we could 77 show that DIS hairpin monomer, as well as the HIV-1 subtype-B 78 DIS sequence, is not specifically recognized by aminoglycosides. 79 This exhaustive ITC analysis of the binding and thermodynamic 80 parameters contributed to solve crystal structures of the DIS kissing 81 loop and extended duplex in complex with several 82 4,5-disubstituted 2-DOS aminoglycosides [35–39]. 83

2	Materials		84
2.1	Instrumentation	1. iTC ₂₀₀ , PEAQ-ITC (MicroCal, Malvern Panalytical, Malvern, UK) or equivalent highly sensitive isothermal titration calorimeter (<i>see</i> Note 2).	85 86 87
		2. Refrigerated centrifuge for a rotor adapted for 50-mL Falcon tubes.	88 89
		3. Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) or equivalent.	90 91
		4. Dry bath for 90 $^{\circ}$ C incubation of Eppendorf tubes.	92 93
2.2	Instrument	1. Hamilton glass loading syringes for calorimeter.	94
Acc	cessories	2. BD Luer-Lok 1-mL syringe.	95 96
2.3 Prej	Sample paration	1. 23-mer HIV-1 RNA (sequence 5- '-CUGCUUGAAGUGCACACAGCAAG3') was chemically synthetized at the 1 µmol scale and purified (>98%) as described previously [32].	97 98 99 100
		2. Lividomycin, neomycin, or paromomycin sulfate, powder. These aminoglycosides were purchased from Sigma-Aldrich and used without further purification. Paromomycin is shown in this example.	101 102 103 104
		3. Amicon Ultra-4 centrifugal filter unit 10,000 MWCO (Millipore, Billerica, MA, USA).	105 106
	~	 4. 10× ITC buffer: 250 mM potassium chloride, 20 mM magnesium chloride, 200 mM sodium cacodylate, pH 7.0 (<i>see</i> Note 3). Prepare 10 mL. 	107 108 109
	V.	5. 1× ITC buffer: 150 mM potassium chloride, 2 mM magne- sium chloride, 20 mM sodium cacodylate, pH 7.0. Prepare 10 mL.	110 111 112 113
3	Methods		114
3.1 Pre	Sample paration	For each experiment, 300 μL of RNA at 10–30 μM should be prepared.	115 116
		 RNA is aliquoted in 3 Eppendorf tubes, each tube containing 1 mL of RNA at 2 μM in pure water (<i>see</i> Note 4). 	117 118

2. Heat RNA sample for 5 min at 90 °C and snap cool in water on 119 ice at 0 °C for 10 min. 120

- 3. Add 111 μ L of 10× ITC buffer in each Eppendorf tube.
- 4. RNA sample is concentrated to a final volume of ~300 μL using 122 an Amicon Ultra-4 concentrator. Centrifuge for ~10 min at 4500 g at 10 °C (recommended speed in a swinging rotor). 124 Higher temperature should be avoided in order to prevent the conversion from the loop–loop complex RNA to the extended 126 duplex form [40]. 127

121

135

- 5. Check the absorbance at 260 nm using a spectrophotometer 128 (take a full spectrum and not a single value). Here a final 129 concentration of 16.8 μ M (in strand) is obtained. 130
- 6. Prepare 1 mL of aminoglycoside solution at a 400 μ M concentration in 1 × ITC buffer. This chapter shows an example with the paromomycin aminoglycoside. The optimal aminoglycoside concentration may be different for another compound. 131 132 133 134

During an ITC experiment, aliquots of ligand are injected into the 136 sample cell. The interaction between the ligand and the molecule in 137 the cell induces the release (exothermic interaction) or absorption 138 (endothermic interaction) of heat which is directly measured by the 139 calorimeter. The titration curve of kcal mol⁻¹ vs. molar ratio 140 (ligand/sample) is generated by the integration of each heat pulse 141 with time and normalized for concentration. After the fitting of the 142 resulting isotherm with a binding model, the affinity constant 143 $(K_{\rm a} = 1/K_{\rm d})$, the stoichiometry (n), and the enthalpy of interaction 144 (ΔH) are obtained. The Gibbs free energy change (ΔG) and the 145 entropy change (ΔS) are deduced via the thermodynamic 146 relationships: 147

$$\Delta G = -RT \ln K_{a} = RT \ln K_{d}$$
$$\Delta G = \Delta H - T\Delta S$$
¹⁴⁸

The determination of the appropriate concentration of the 150 component placed in the sample cell (*MT*) depends on the binding 151 affinity, the stoichiometry, and the heat of binding ΔH . If the 152 Wiseman coefficient ($c = n \cdot K_a \cdot MT$) calculated is in the 1–1000 153 range (preferably between 10 and 500) [3], a sigmoid is then 154 obtained, and this allows the determination of the accurate binding 155 constant and ΔH .

In general the required concentrations of sample in the cell are 157 ranging from 5 to $100 \,\mu$ M. Typical concentration for the ligand in 158 the injection syringe is normally 10-20 times higher (and even 159 higher in the case of low Wiseman c value experiments [41–43]). 160 These conditions will ensure that the cell sample will become 161 saturated at the end of the titration experiment. Therefore, the 162 sample requirement in order to perform an ITC experiment 163 under ideal conditions (c~100) might be significant for biological 164 low affinity systems. A crucial point to obtain high-quality ITC 165

3.2 Designing a Typical ITC Experiment

3.2.1 Experimental Considerations 3.2.2 Instrument

Parameters

experiments is to minimize the heat signal originated from mis- 166 matches between the solutions that are mixed (*see* **Note 1**). For this 167 reason, it is imperative to prepare the two samples (from the syringe 168 and the cell) rigorously in the same buffer. 169

On iTC₂₀₀ and PEAQ-ITC microcalorimeters, the glass titration 171 syringe holds up to 40 μ L. Typical injections are ranging from 0.25 172 to 2 μ L. The number of injections per experiment should be set in 173 order to saturate the RNA at the end of the titration (a final molar 174 ratio of 3–5 is acceptable in most situations). The injection speed 175 should be 0.5 μ L s⁻¹. The spacing between the injections is a 176 frequently overlooked parameter, leading to truncated peaks and 177 erroneous data if a complete return to the baseline is not achieved. 178 We have shown that the time required for equilibration is strongly 179 dependent on the rate constants $k_{\rm on}$ and $k_{\rm off}$ of each system [9]. The 180 spacing value can be set to 120 s by default on the iTC₂₀₀, but it 181 might be increased to 240 s or even longer in some situations 182 [5, 10]. The filter value on iTC₂₀₀ instrument corresponds to the 183 integration time that will be used to produce a single data point. 184 Usually the filter period is set to 5 by default; however, setting 1 or 185 2 for a better sampling of each peak in view of performing a kinetic 186 analysis of the thermodynamic data is also possible [6, 9]. This 187 value is fixed to 1 on the PEAQ-ITC instrument and cannot be 188 modified. 189

The feedback mode of the calorimeter setup should be set to 190 "high gain," yielding the fastest response time. The "reference 191 power" value corresponds to the baseline (in μ Cal s⁻¹) during the 192 experiment. In the high gain mode, the instrument's reference 193 power ranges from 0 to 12.25 μ Cal s⁻¹ for the iTC₂₀₀. In most 194 cases, the reference power can be fixed to 5 μ Cal s⁻¹. The stirring 195 speed depends on the syringe geometry. It should be set to 196 1000 rpm for straight-bladed syringes on iTC₂₀₀, but this value 197 can be reduced to 750 rpm for helical-bladed syringe available on 198 both iTC₂₀₀ and PEAQ-ITC (*see* **Note 5**). 199

Most experiments are performed between 4 and 37 °C (the 200 instrument's theoretical operating range is 2–80 °C, but extremes 201 are not usable in practice). The choice of the experiment tempera-202 ture is often overlooked, while it might be crucial to optimize 203 results in some situations. Indeed, most biomolecular interactions 204 are characterized by a temperature dependence of the ΔH due to a 205 negative heat capacity change (*see* **Note 6**). In addition, the van't 206 Hoff relationship 207

$$\partial \ln K_{\rm d} / \partial T = -\Delta H / (RT^2)$$

also shows that K_d is affected by temperature change. Changing the cell temperature is thus an easy way to play on the Wiseman *c* value. 209

209

3.3 Performing the ITC Experiment	Below is described the procedure for an iTC_{200} microcalorimeter. 2 The experiment will be performed at 25 °C. 2	211 212
	1. Fill the reference cell with ultrapure water using a 0.5-mL glass 2 Hamilton syringe. 2	213 214
	 In the instrument controls tab, set the temperature at 25 °C in 2 "Thermostat Control," and click "Set Jacket Temp." 	215 216
	3. Rinse the sample cell, two or three times, with $1 \times$ ITC buffer. 2	217
	4. Warm up the RNA sample at 25 °C before loading in the 2 ^r sample cell. 2 ^r	218 219
	5. Gently load the sample cell with 300 μ L of RNA sample. The 22 small excess should be removed. The final sample volume in the cell is ~280 μ L. Care must be taken to avoid bubble formation 22 in the sample cell. 22	20 21 222 223
	6. To load the titration syringe, place a microcentrifuge tube with 22 at least 70 μ L of aminoglycoside sample at 400 μ M. Place the 23 syringe titration above the microcentrifuge tube in order that 24 the tip of the syringe is immerged in ligand solution. Manual 24 loading of the syringe is recommended since it is faster and safer 25 than the semiautomatic procedure on the iTC ₂₀₀ , especially if 25 the sample is viscous (i.e., with a buffer containing some glycerol or with highly concentrated protein). Connect the BD 26 syringe to the titration syringe and load the sample manually 27 by aspiration into the titration syringe (before aspiration, make 26 sure that the plunger is in the open position). When the sample 27 gets to the plunger, click "Close Fill Port" in the instrument 26 controls tab; this will move the plunger tip down and block the 27 fill port. Perform the "purge/refill" procedure to remove small 27 air bubbles in the syringe. 29 stringe from the 29 stringe. 21 stringe stringe. 22 stringe stringe. 23 stringe stringe stringe. 24 stringe stringe stringe stringe. 25 stringe stringe stringe stringe stringe stringe stringe stringe. 25 stringe stringe stringe stringe stringe stringe stringe stringe. 26 stringe stringe stringe stringe stringe stringe stringe. 27 stringe string	24 25 26 27 28 29 30 31 32 33 23 23 23 23 23 23 23 23 23 23 23
	7. Place the titration syringe into the sample cell. 24	240
	8. Set the reference power to 5 μ Cal s ⁻¹ . 24	:41
	9. Set the initial delay to 100 s and the stirring speed to 1000 rpm or 750 rpm depending on the syringe geometry. 24	.42 243
	.0. Set the number of injections to 28, the sample cell concentration to 0.0168 mM, and the concentration in the syringe to24.0.4 mM. This ensures a final molar ratio of ~5, required in such24RNA/ligand experiments.24	244 245 246 247
	1. Set the titration parameters (the number and volume of the injections as well as the spacing between two injections) in the "Injection Parameters" window into the "Advanced Experimental Design" tab. Set the first injection volume to $0.3 \mu\text{L}$ (see Note 7) and the next injection volume to $1.5 \mu\text{L}$. Set the spacing between each injection to 150s .	248 249 250 251 252 253

- 12. Press the "Start" button to begin the experiment. The experi- 254 ment will start when both cells are thermostated at 25 °C. 255
- 13. A blank experiment should be also performed by injecting 256 aminoglycoside into $1 \times$ ITC buffer without RNA, in order 257 to subtract the heat of dilution from the reaction heat data. 258

259

3.4 ITC Data Analysis Various software are available for ITC data processing: MicroCal 260 Origin 7 (VP-ITC and iTC₂₀₀ from MicroCal); NanoAnalyze 261 (TA Instrument); AFFINImeter [8]; the NITPIC, SEDPHAT, 262 and GUSSI package [44, 45], or the MicroCal PEAQ-ITC analysis 263 software (Malvern PEAQ-ITC). ITC data analysis using the Micro-264 Cal Origin7 software was previously described in details in [46]. 265

> Here we will describe the procedure for data processing using 266 the MicroCal PEAQ-ITC analysis software provided with the 267 PEAQ-ITC instrument. 268

- Open the software and select both the RNA-ligand titration 269 and the blank experiment (use the "Ctrl" key to select both 270 files). They are quickly processed and integrated by the soft- 271 ware and integrated heats are displayed on the left tab (Fig. 1). 272 Binding is detected for the RNA/ligand titration ("Binding" is 273 displayed in green, Fig. 1a), whereas "No binding" is displayed 274 in orange for the blank experiment (Fig. 1b). 275
- On the right arrowhead of the blank experiment, change "No 276 binding" for "Control" (now displayed in blue).
- On the main "Fitting Model" window, change the fitting 278 model from "One Set of Sites" to "Two Sets of Sites" (see 279 Note 8).
- 4. On top of the main window, change from "Overview" to 281 "Adjust Baseline." This tab allows to manually correct integration heat for each data point (Fig. 2). 283
- 5. The "Time Factor" parameter on the bottom of the window 284 might be useful if the baseline is not stable (large oscillation of 285 the baseline due to air-conditioning, for instance), but 5 is 286 generally a good standard value.
- 6. The number of points per injection is set by default to 25, but 288 this can be changed if necessary. 289
- Baseline for the RNA/ligand experiment and the buffer/ 290 ligand control can be adjusted, if necessary. This is generally 291 not required in good experimental conditions: well-maintained 292 instrument, samples without aggregates and injections without 293 bubbles, and no heat perturbation around the instrument. 294
- On top of the main window, change from "Adjust Baseline" to 295 "Assign Control." On "Control Parameter," change from 296 "Fitted Offset" to "Single." The control experiment is then 297 automatically selected. The "Line" Method is generally 298



Fig. 1 Screenshot of the "Overview" tab after selection of the RNA/ligand (a) and the blank (b) experiment

preferable over the "Point-to-point" and "Mean" ones 299 (Fig. 3). In the absence of control experiment, it is possible 300 to use the heat of dilution from the last injections of the 301 experiment, provided the RNA oversaturated with ligand. It 302 is however highly recommended to perform a control experiment to obtain more accurate results. 304



Fig. 2 Screenshot of the "Adjust Baseline" tab. The blue area corresponds to the integrated heat. It is possible to adjust the baseline by moving individually each point and the markers. Individual points can be excluded (or included) on the top-right part of the window



Fig. 3 Screenshot of the "Assign Control" tab. On the left part are shown the integrated heats for the experiment. On the right part are shown the integrated heats for the blank experiment and the corrected integrated heats for the experiment with RNA as well



Fig. 4 Screenshot of the "Adjust Fit" tab

- 9. On top of the main window, change from "Assign Control" to 305 "Adjust Fit." Fitted parameters can be adjusted here (Fig. 4). 306 Here, N1 (which corresponds to the stoichiometry of the first 307 class of binding sites) is very close to 1.0 (as expected since the 308 RNA dimer binds two aminoglycoside ligands), showing that 309 concentrations of the RNA and ligand were correctly estimated 310 and that the RNA is properly folded. This binding site corre-311 sponds to the specific binding site and entails the highest 312 affinity ($K_{d1} = 112 \text{ nM}, \Delta H = -9.8 \text{ kcal mol}^{-1}$). The stoichi-313 ometry of the second set of binding sites, N2, is relevant of 314 unspecific binding due to electrostatic interactions with the 315 lowest affinity ($K_{d2} = 40 \ \mu M, \ \Delta H = -1.6 \ \text{kcal mol}^{-1}$). 316
- It is important to check errors on refined parameters! Indeed, 317 in this model, the high correlation between the different fitted 318 parameters could lead to multiple equivalent solutions. 319
- 11. On top of the main window, change from "Adjust Fit" to 320 "Result Table." To visualize correlation between fitted parameters, click on "Show Errors" in the bottom of the window. 322 The "dependency" value should be examined for all refined 323 parameters (closer to 0 is better; closer to 1.0 is worst). 324
- 12. Select the "Final Figure" tab to display a publication quality 325 figure (Fig. 5) which can be easily exported in various graphical 326 formats. 327



Fig. 5 Screenshot of the "Presentation" tab, showing the final figure ready to be exported for publication

4 Notes

- 1. Buffer mismatch is due to differences between the ligand and 330 the sample solution, as well as any difference in pH, salt, 331 glycerol concentration, buffer concentration, or additives. In 332 order to minimize these differences, interactants must be 333 prepared in identical buffers or dialyzed against the same 334 buffer. 335
- 2. Since microcalorimeter (especially iTC₂₀₀) is very sensitive to 336 temperature variations, it is highly recommended to place the 337 instrument far away from any airflow, especially 338 air-conditioning, and in a room exposed to minimal tempera-339 ture changes (avoid heaters, large windows, or other instru-340 ments generating lots of heat such as freezers). The instrument 341 should be regularly and meticulously cleaned. 342
- Low-salt conditions were chosen at this temperature to prevent 343 conversion of the loop–loop dimer into the extended duplex 344 dimer [40].
- 4. RNA concentration at this step should be less than $\sim 3 \mu M$ in 346 order to prevent formation of extended duplex dimers as 347 observed in [40]. 348
- Although the reduction of the stirring speed improves the 349 baseline stability, it might introduce artifacts due to insufficient 350 mixing of the injected ligand.
 351

6. Heat capacity change is defined by the relationship:

$$\Delta C_p = (\delta \Delta H / \delta T)$$
³⁵³

The heat capacity change is generally negative and asso-354 ciated to a local folding following binding [47]. It is a valuable 355 tool for optimizing the study of any binding event: for an 356 exothermic reaction, ΔH will be more negative at 35 °C than 357 25 °C and, consequently, the signal will be increased at 35 °C. 358 In some situations, no signal can be detected by ITC. This can 359 result from athermal reactions (ΔH is null in the conditions of 360 the experiment) where the interaction exists, but cannot be 361 detected by ITC (an example is provided in [48]). In such 362 situations, the change in temperature may allow the interaction 363 to be observed due to the heat capacity change of the system. 364

- 7. A first small injection $(0.3-0.5 \ \mu L)$ is performed at the beginning of the titration. It will be discarded during data analysis because it is generally affected by error due to sample dilution during the equilibration procedure and to the backlash of the screw mechanism of the syringe motor. 365
- Since the aminoglycoside ligand is positively charged, the 370 RNA/aminoglycoside interaction in this example is character-371 ized by a highly specific interaction coexisting with unspecific 372 interactions due to electrostatic interactions. These unspecific 373 interactions can be strongly reduced by increasing salt concen-374 trations (the specific interactionis also reduced, however). This 375 situation holds true for most of RNA/ligand interactions. 376

377 References

- Leavitt S, Freire E (2001) Direct measurement
 of protein binding energetics by isothermal
 titration calorimetry. Curr Opin Struct Biol
 11:560–566
- 2. Privalov PL, Dragan AI (2007) Microcalorimetry of biological macromolecules. Biophys
 Chem 126:16–24
- 386 3. Velazquez Campoy A, Freire E (2005) ITC in
 387 the post-genomic era...? Priceless. Biophys
 388 Chem 115:115–124
- 4. Wiseman T, Williston S, Brandts JF, Lin LN (1989) Rapid measurement of binding constants and heats of binding using a new titration calorimeter. Anal Biochem 179:131–137
- 5. Bec G, Meyer B, Gerard MA, Steger J, 393 Fauster K, Wolff P, Burnouf D, Micura R, 394 395 Dumas P, Ennifar E (2013) Thermodynamics of HIV-1 reverse transcriptase in action eluci-396 the mechanism of action 397 dates of non-nucleoside inhibitors. J Am Chem Soc 398 135:9743-9752 399

- 6. Dumas P, Ennifar E, Da Veiga C, Bec G, Palau W, Di Primo C, Pineiro A, Sabin J, Munoz E, Rial J (2016) Extending ITC to kinetics with kinITC. Methods Enzymol 567:157–180
 400 401 402 403 404
- Munoz E, Sabin J, Rial J, Perez D, Ennifar E, Dumas P, Pineiro A (2019) Thermodynamic and kinetic analysis of isothermal titration calorimetry experiments by using KinITC in AFFI-NImeter. Methods Mol Biol 1964:225–239
- Pineiro A, Munoz E, Sabin J, Costas M, Bastos M, Velazquez-Campoy A, Garrido PF, Dumas P, Ennifar E, Garcia-Rio L, Rial J, Perez D, Fraga P, Rodriguez A, Cotelo C (2019) AFFINImeter: a software to analyze molecular recognition processes from experimental data. Anal Biochem 577:117–134
- 9. Burnouf D, Ennifar E, Guedich S, Puffer B, Hoffmann G, Bec G, Disdier F, Baltzinger M, Dumas P (2012) kinITC: a new method for obtaining joint thermodynamic and kinetic
 420

405

406

407

408

409

410

411

412

413

414

415

- 421 data by isothermal titration calorimetry. J Am422 Chem Soc 134:559–565
- 10. Guedich S, Puffer-Enders B, Baltzinger M,
 Hoffmann G, Da Veiga C, Jossinet F,
 Thore S, Bec G, Ennifar E, Burnouf D,
 Dumas P (2016) Quantitative and predictive
 model of kinetic regulation by E. coli TPP
 riboswitches. RNA Biol 13:373–390
- 11. Zihlmann P, Silbermann M, Sharpe T, Jiang X, 429 430 Muhlethaler T, Jakob RP, Rabbani S, Sager CP, 431 Frei P, Pang L, Maier T, Ernst B (2018) KinITC-one method supports both thermody-432 433 namic and kinetic SARs as exemplified on FimH 434 antagonists. Chemistry 24:13049-13057 435
- 436 12. Barbieri CM, Srinivasan AR, Pilch DS (2004)
 437 Deciphering the origins of observed heat
 438 capacity changes for aminoglycoside binding
 439 to prokaryotic and eukaryotic ribosomal RNA
 440 a-sites: a calorimetric, computational, and
 441 osmotic stress study. J Am Chem Soc
 442 126:14380–14388
- 443 13. Feig AL (2007) Applications of isothermal
 444 titration calorimetry in RNA biochemistry and
 445 biophysics. Biopolymers 87:293–301
- 446 14. Feig AL (2009) Studying RNA-RNA and
 447 RNA-protein interactions by isothermal titra448 tion calorimetry. Methods Enzymol
 449 468:409–422
- 450 15. Kaul M, Pilch DS (2002) Thermodynamics of aminoglycoside-rRNA recognition: the binding of neomycin-class aminoglycosides to the A site of 16S rRNA. Biochemistry 454 41:7695–7706
- 455 16. Pilch DS, Kaul M, Barbieri CM, Kerrigan JE
 (2003) Thermodynamics of aminoglycosiderRNA recognition. Biopolymers 70:58–79
- I7. Salim NN, Feig AL (2009) Isothermal titration
 calorimetry of RNA. Methods 47:198–205
- 460 18. Haddrick M, Lear AL, Cann AJ, Heaphy S
 461 (1996) Evidence that a kissing loop structure
 462 facilitates genomic RNA dimerisation in
 463 HIV-1. J Mol Biol 259:58–68
- 464 19. Paillart J-C, Berthoux L, Ottmann M, Darlix
 465 J-L, Marquet R, Ehresmann C, Ehresmann B
 466 (1996) A dual role of the dimerization initia467 tion site of HIV-1 in genomic RNA packaging
 468 and proviral DNA synthesis. J Virol
 469 70:8348–8354
- 470 20. Laughrea M, Jetté L (1994) A 19-nucleotide
 471 sequence upstream of the 5' major splice donor
 472 site is part of the dimerization domain of
 473 human immunodeficiency virus 1 genomic
 474 RNA. Biochemistry 33:13464–13474
- 475 21. Muriaux D, Girard PM, Bonnet-Mathonière B,
 476 Paoletti J (1995) Dimerization of HIV-1lai

RNA at low ionic strength. J Biol Chem 270:8209–8216

- 22. Paillart JC, Skripkin E, Ehresmann B, Ehresmann C, Marquet R (1996) A loop-loop "kissing" complex is the essential part of the dimer linkage of genomic HIV-1 RNA. Proc Natl Acad Sci U S A 93:5572–5577
- 23. Skripkin E, Paillart JC, Marquet R, Ehresmann B, Ehresmann C (1994) Identification of the primary site of the human immunodeficiency virus type I RNA dimerization *in vitro*. Proc Natl Acad Sci U S A 91:4945–4949
- 24. Laughrea M, Jetté L (1996) Kissing-loop model of HIV-1 genome dimerization: HIV-1 RNA can assume alternative dimeric forms, and all sequences upstream or downstream of hairpin 248-271 are dispensable for dimer formation. Biochemistry 35:1589–1598
- 25. Muriaux D, Fossé P, Paoletti J (1996) A kissing complex together with a stable dimer is involved in the HIV-1_{Lai} RNA dimerization process *in vitro*. Biochemistry 35:5075–5082
- 26. Rist MJ, Marino JP (2002) Mechanism of nucleocapsid protein catalyzed structural isomerization of the dimerization initiation site of HIV-1. Biochemistry 41:14762–14770
- 27. Takahashi KI, Baba S, Chattopadhyay P, Koyanagi Y, Yamamoto N, Takaku H, Kawai G (2000) Structural requirement for the two-step dimerization of human immunodeficiency virus type 1 genome. RNA 6:96–102
- 28. Takahashi KI, Baba S, Koyanagi Y, Yamamoto N, Takaku H, Kawai G (2001) Two basic regions of NCp7 are sufficient for conformational conversion of HIV-1 dimerization initiation site from kissing-loop dimer to extended-duplex dimer. J Biol Chem 276:31274–31278
- Ennifar E, Dumas P (2006) Polymorphism of bulged-out residues in HIV-1 RNA DIS kissing complex and structure comparison with solution studies. J Mol Biol 356:771–782
- 30. Ennifar E, Walter P, Ehresmann B, Ehresmann C, Dumas P (2001) Crystal structures of coaxially stacked kissing complexes of the HIV-1 RNA dimerization initiation site. Nat Struct Biol 8:1064–1068
- Ennifar E, Walter P, Dumas P (2010) Cationdependent cleavage of the duplex form of the subtype-B HIV-1 RNA dimerization initiation site. Nucleic Acids Res 38:5807–5816
- 32. Ennifar E, Yusupov M, Walter P, Marquet R, Ehresmann B, Ehresmann C, Dumas P (1999) The crystal structure of the dimerization initiation site of genomic HIV-1 RNA reveals an

49

477

478

479

480

481

482

483

484

485

486

487

488

489

490

491

492

493

494

495

496

497

498

499

500

501

502

503

504

505

506

507

508

509

510

511

512

513

514

515

516

517

518

519

520

521

522

523

524

525

526

527

528

529

530

531

- extended duplex with two adenine bulges.Structure 7:1439–1449
- 33. Ennifar Ε, Paillart JC, Marquet 535 R, Ehresmann B, Ehresmann C, Dumas P, Walter 536 P (2003) HIV-1 RNA dimerization initiation 537 538 site is structurally similar to the ribosomal A site and binds aminoglycoside antibiotics. J 539 540 Biol Chem 278:2723-2730
- 34. Bernacchi S, Freisz S, Maechling C, Spiess B, Marquet R, Dumas P, Ennifar E (2007) Aminoglycoside binding to the HIV-1 RNA dimerization initiation site: thermodynamics and effect on the kissing-loop to duplex conversion. Nucleic Acids Res 35:7128
- 547 35. Ennifar E, Aslam MW, Strasser P, Hoffmann G,
 548 Dumas P, van Delft FL (2013) Structure549 guided discovery of a novel aminoglycoside
 550 conjugate targeting HIV-1 RNA viral genome.
 551 ACS Chem Biol 8:2509–2517
- 36. Ennifar E, Paillart JC, Bernacchi S, Walter P,
 Pale P, Decout JL, Marquet R, Dumas P
 (2007) A structure-based approach for targeting the HIV-1 genomic RNA dimerization initiation site. Biochimie 89:1195–1203
- 557 37. Ennifar E, Paillart JC, Bodlenner A, Walter P,
 558 Weibel JM, Aubertin AM, Pale P, Dumas P,
 559 Marquet R (2006) Targeting the dimerization
 560 initiation site of HIV-1 RNA with aminoglyco561 sides: from crystal to cell. Nucleic Acids Res
 562 34:2328–2339
- 38. Freisz S, Lang K, Micura R, Dumas P, Ennifar
 E (2008) Binding of aminoglycoside antibiotics to the duplex form of the HIV-1 genomic
 RNA dimerization initiation site. Angew Chem
 Int Ed Engl 47:4110–4113
- 39. Bodlenner A, Alix A, Weibel JM, Pale P,
 Ennifar E, Paillart JC, Walter P, Marquet R,
 Dumas P (2007) Synthesis of a neamine
 dimer targeting the dimerization initiation site
 of HIV-1 RNA. Org Lett 9:4415–4418

40. Bernacchi S, Ennifar E, Toth K, Walter P, Langowski J, Dumas P (2005) Mechanism of hairpin-duplex conversion for the HIV-1 dimerization initiation site. J Biol Chem 280:40112–40121

573

574

575

576

577

578

579

580

581

582

583

584

585

586

587

588

589

590

591

592

593

594

595

596

597

598

599

600

601

602

603

604

605

606

607

608

609

610

- Tellinghuisen J (2008) Isothermal titration calorimetry at very low c. Anal Biochem 373:395–397
- 42. Tellinghuisen J (2016) Analysis of multitemperature isothermal titration calorimetry data at very low c: global beats van't Hoff. Anal Biochem 513:43–46
- 43. Turnbull WB, Daranas AH (2003) On the value of c: can low affinity systems be studied by isothermal titration calorimetry? J Am Chem Soc 125:14859–14866
- 44. Brautigam CA, Zhao H, Vargas C, Keller S, Schuck P (2016) Integration and global analysis of isothermal titration calorimetry data for studying macromolecular interactions. Nat Protoc 11:882–894
- 45. Keller S, Vargas C, Zhao H, Piszczek G, Brautigam CA, Schuck P (2012) High-precision isothermal titration calorimetry with automated peak-shape analysis. Anal Chem 84:5066–5073
- 46. Da Veiga C, Mezher J, Dumas P, Ennifar E (2016) Isothermal titration calorimetry: assisted crystallization of RNA-ligand complexes. Methods Mol Biol 1320:127–143
- 47. Spolar RS, Record MT Jr (1994) Coupling of local folding to site-specific binding of proteins to DNA. Science 263:777–784
- Ramirez J, Recht R, Charbonnier S, Ennifar E, Atkinson RA, Trave G, Nomine Y, Kieffer B (2015) Disorder-to-order transition of MAGI-1 PDZ1 C-terminal extension upon peptide binding: thermodynamic and dynamic insights. Biochemistry 54:1327–1337