Thiazotropsin aggregation and its relationship to molecular recognition in the DNA minor groove

Marie-Virginie Salvia[a], Fiona Addison[a], Hasan Y. Alniss[b†], Niklaas J. Buurma[d], Abedawn I. Khalaf[a], Simon P. Mackay[b], Nahoum G. Anthony[b], Colin J. Suckling[a], Maxim P. Evstigneev[c], Adrián Hernandez Santiago[e], Roger D. Waigh[b] and John A. Parkinson*[a]*

SUPPORTING INFORMATION

1.0 Preparation of Solutions for ITC and NMR Measurements

1.1 Preparation of PIPES and ACES buffers for ITC measurements

Aquepis PIPES and ACES buffer solutions were prepared at pH = 6.8 with the following composition: 10 mM PIPES or ACES, 20 mM NaCl and 1 mM EDTA. For this purpose PIPES or ACES (1.5119 g), NaCl (0.5844 g) and EDTA (0.1462 g) were dissolved in distilled water (500 mL). In order to adjust the pH, NaOH and diluted HCl were added prior to adjusting the solution to the final volume of 500 mL in a volumetric flask.

1.2 Preparation of the solution of AIK-18/51 for ITC measurements

Solutions of AIK-18/51 in PIPES and ACES buffer were prepared at a concentration C = 5 mM. For this, AIK-18/51 (1.03 mg, 1.40 µmol) was dissolved in 2.807 mL of PIPES buffer. ACES buffer, (2.9709 mL) was also prepared from AIK-18/51 (1.08 mg, 1.47 µmol).

1.3 Preparation of solutions of AIK-18/51 for NMR studies

AIK-18/51 (MW = 733.8 g.mol^{-1}) was prepared at a concentration C = 1 mM in a solution of 90% H_2O/10% D_2O. For this, AIK-18/51 (0.43 mg, 0.58 µmol) was dissolved in the mixed solvent (550 µL). The sample was then admitted to a 5 mm ∅ NMR tube (528-PP, Wilmad Glass).

A solution was also prepared at a concentration C = 5 mM in 100 mM phosphate buffer solution (pH = 7.4) in D_2O. For this, AIK-18/51 (2.02 mg, 2.75 µmol) was dissolved (550 µL) in this solvent and the sample was then admitted to a 5 mm ∅ NMR tube (528-PP, Wilmad Glass). The self-association study began from this solution.

For the examination of its complex with DNA, AIK-18/51 was matched in quantity to its partner DNA (double stranded) such that $n_{AIK\text{-}18/51} = n_{DNA}$ = 1142975 picomoles. **3** (0.84 mg) was therefore measured out and dissolved in distilled water (50 µL).

1.4 Preparation of DNA solutions

For NMR studies d(CGACTAGTCG) was purified of volatile residues. For this the DNA was dissolved in 1 mL of distilled water, frozen and then freeze dried. Following this the whole DNA sample, 2285950 picomoles was dissolved in 1.1 mL of 90% H_2O/10% D_2O and half of this solution was admitted to a 5 mm ∅ NMR tube (528-PP, Wilmad Glass) together with 0.5 µL of a stock solution of trimethylsilylpropionic acid-d_4 sodium salt (TSP) solution which acted as an internal chemical shift reference standard. The remaining sample was freeze dried to remove the solvent and resuspended in 550 µL of D_2O. The solvent was removed once again from both samples by freeze drying and the DNA was finally dissolved in 100 mM phosphate buffer (pH = 7.4, 90% H_2O/10% D_2O for one half of the sample and 100% D_2O for the other half). Formation of the 2:1 ligand:DNA duplex complex was monitored by 1D ^{1}H NMR spectroscopy through titration of the prepared 50 µL solution of AIK-18/51 into the DNA solution.

2.0 ITC Measurements

A Vp Viewer ITC (2000) instrument was used for all ITC measurements with a sample cell volume of 1.4399 mL.

Ligand association titration experiments used a solution of AIK-18/51 at a concentration C = 5 mM in PIPES buffer which was placed into the delivery syringe. The cell was filled with buffer solution. Ligand was gradually injected into the cell using 25 injections of 10 µL volume each. A delay

of either 300 s or 600 s was inserted between each injection to allow for full equilibrium to become established (the delay of 600 s being used from injection 6 to injection 19 for reasons associated with complex studies with DNA not pertinent to this article). Measurements were made at three different temperatures namely T = 298 K, 308 K and 318 K. The experiment was repeated using ACES buffer instead of PIPES buffer but only at T = 298 K for comparison purposes.

The binding constant, K_{agg}, and the enthalpy of association, ΔH_{agg}, could not be obtained directly from the instrument and for this reason the software package IC-ITC was used for data analysis.

3.0 NMR Spectroscopy Measurements

NMR data were acquired on a Bruker AVANCE III 600 NMR spectrometer operating at a proton resonance frequency of 600.13 MHz (14.1 T magnetic field strength) using a TBI-z probehead and running under Topspin (version 2.1, Bruker Biospin, Karlsruhe). All data were accumulated under full automation using IconNMR. 1D ^{1}H NMR spectra were acquired using either a single pulse-acquire pulse program with solvent suppression carried out either by presaturation (for samples in 99% D_2O) or by excitation sculpting (for samples in 90% H_2O / 10% D_2O). Typically the proton 90° pulse was calibrated as p1 = 10.48 µs. Data were typically acquired over a ^{1}H frequency width equivalent to 16.0221 ppm centred at 4.692 ppm. Ligand signal assignments were made based on 2D [^{1}H, ^{1}H] NOESY, COSY and TOCSY NMR data.

3.1 Complex Formation with DNA

Titration of d(CGACTAGTCG)$_2$ with AIK-18/51 in 90% H_2O / 10% D_2O for the purposes of complex formation was monitored by 1D ^{1}H NMR spectroscopy with data acquired using both excitation sculpting and presaturation for solvent suppression. 128 transients were acquired for each data set with a relaxation delay of 2 s. Data were acquired during 0.664 s over a ^{1}H frequency width equivalent to 20.5547 ppm centred at 4.694 ppm (excitation sculpting) or during 1.363 s over a ^{1}H frequency width equivalent to 20.0276 ppm centred at 4.692 ppm (presaturation). Further details of the complete study of the DNA complex between AIK-18/51 and d(CGACTAGTCG)$_2$ will be the subject of a separate article (in preparation).

3.2 Variable Temperature and Concentration Dependent ^{1}H NMR study of AIK-18/51.

The evolution of ^{1}H chemical shifts for AIK-18/51 were measured as a function of temperature at fixed concentrations typically of C = 2 mM and C = 0.2 mM. For each concentration, the measurements were carried out in the temperature range from T = 278 K to T = 353 K with ΔT = 5 K and settling time to equilibrium at each rise in temperature of 10 minutes prior to sample reshimming and data acquisition. Subsequently corrected ^{1}H NMR chemical shifts were plotted as a function of temperature. The evolution of chemical shifts associated with AIK-18/51 were measured as a function of solute concentration at a fixed temperature of T = 298 K. Typcially measurements were made at concentrations of 5 mM, 4.5 mM, 4.0 mM, 3.8 mM, 3.2 mM, 2 mM, 1.6 mM, 0.8 mM, 0.4 mM, 0.2 mM, 0.1 mM and 0.05 mM by addition of specific volumes of stock buffer solution (100 mM phosphate buffer, pH = 7.4). Chemical shifts were measured with respect to the internal methyl singlet resonance of TSP whose concentration and temperature dependence was calibrated against the singlet resonance of tetramethylammonium chloride ($(CH_3)_4N^+Cl^-$, TMA, δ^1H = 3.178 ppm) whose resonance has no temperature or concentration dependence. Measured chemical shifts were corrected on the basis of the calibration curves. Chemical shift information was read off from each 1D ^{1}H NMR spectrum at different concentrations and the evolution of the ^{1}H chemical shift was plotted as a function of concentration.

3.3 Diffusion NMR data

Diffusion measurements were carried out using either a bipolar gradient routine (Bruker pulse program ledbpgppr2s) or a double stimulated echo routine (Bruker pulse program dstegppr3s) for which different numbers of transients (NS = 32, 64, 128 or 256) were used depending on the concentration of the sample being studied in order to optimize signal-to-noise in each data set. The diffusion results were processed within the T1/T2 analysis module of Topspin and fitted according to Eq. 5

$$I = I_0 e^{-D(2\pi\gamma G\delta)^2(\Delta - x)\times 10^4}$$

Eq. 5

where, γ is the gyromagnetic ratio (Hz/G, γ = 4257.7 Hz/G for proton), δ is the duration of the gradient (s), Δ is the time between the 2 spatially encoding gradients (s), I is the signal intensity, G is the amplitude of the pulsed field gradient (G/cm) and x is a pulse sequence dependent correction factor. Calibration of the spectrometer gradient strength for the purposes of diffusion measurements was carried out using a small amount of H_2O contained within a Shigemi NMR tube assembly. A literature value for the self-diffusion coefficient of H_2O at 298 K of 2.299×10^{-9} m^2/s measured by the diaphragm cell technique, was used as the calibration value[i]. The diffusion measurement was carried out using the pulse program stebpgp1s2d with 32 transients for each of 32 linear gradient increments. A plot of $\ln(\frac{I}{I_0})$ *vs* $(2\pi\gamma G\delta)^2(\Delta - \frac{\delta}{3} - \frac{t}{2})\times 10^4$ yielded a line with slope equal to the diffusion coefficient, D, with excellent linearity.

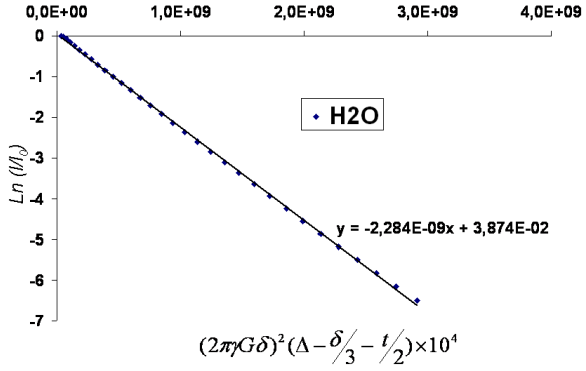

Figure S1. Diffusion data for H_2O (NS = 32) measured using restricted sample conditions at 298 K

A gradient calibration curve was established on the basis of Eq. 5 according to

$$G = \sqrt{\frac{\ln\frac{I_0}{I}}{D(2\pi\gamma G\delta)^2(\Delta - \frac{\delta}{3} - \frac{t}{2})\times 10^4}}$$

where D is the theoretical self-diffusion coefficient value of water at 298 K and the other variables were experimentally measured values or constants from which (calculated gradient) = 1.0072 × (Experimental Gradient).

4.0 Isothermal Titration Calorimetry - Data Fits

Fits of the isodesmic aggregation model (and the identical dimerization model) to the experimental data reproduced the data well (Figure S2).

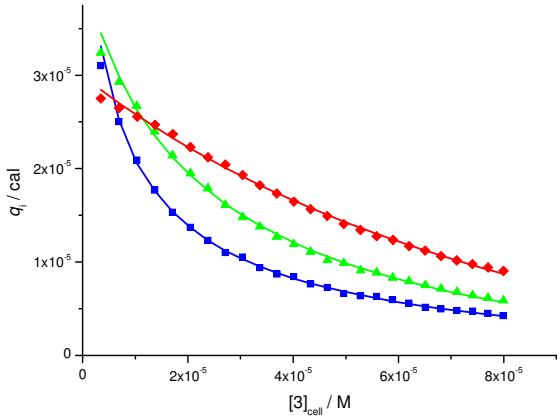

Figure S2. Dilution heat effects for AIK-18/51 in 10 mM PIPES, 20 mM NaCl, 1 mM EDTA, pH = 6.8 at 25 (blue squares), 35 (green triangles), and 45 (red diamonds) °C and corresponding fits to the data (solid lines).

4.1 Isothermal Titration Calorimetry - Evaluation of error margins for K_{agg} and ΔH_{agg}

The error margins for K_{agg} and ΔH_{agg} follow from the dependence of the normalised Σdev^2/d.o.f. (Figure S3).

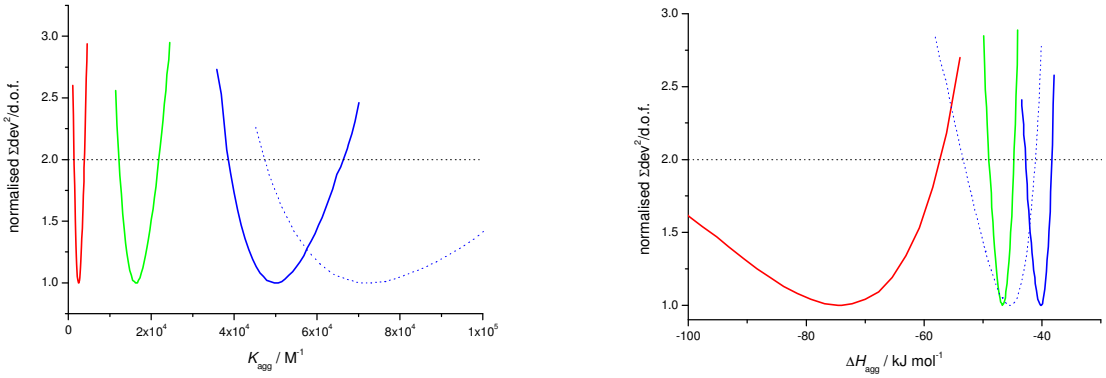

Figure S3. Normalised Σdev^2/d.o.f. as a function of the value of the optimisable parameters K_{agg} (left) and ΔH_{agg} (right). Solid blue, green and red lines represent data in 10 mM PIPES, 20 mM NaCl, 1 mM EDTA, pH = 6.8 at 25, 35 and 45 °C, respectively, the dotted blue line represents data in 10 mM ACES, 20 mM NaCl, 1mM EDTA, pH = 6.9 at 25 °C. Dotted horizontal lines indicate the condition normalised Σdev^2/d.o.f. < 2, corresponding to the error limits on the optimisable parameters.

4.2 Isothermal Titration Calorimetry - Heat Capacity

The heat capacity change ΔC_p for isodesmic self-aggregation was determined from the change in interaction enthalpies ΔH_{agg} with temperature (Figure S4).

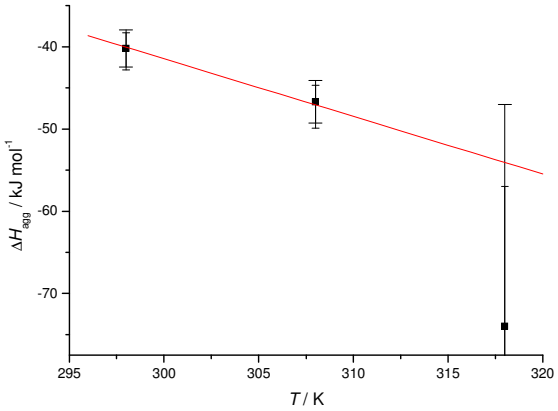

Figure S4. ΔH_{agg} as a function of temperature. Wide-capped error bars represent the average error for each datapoint, while narrow-capped error bars represent the asymmetric error margins as derived from plots of normalised Σdev^2/d.o.f. as a function of the value ΔH_{agg}. The line is a linear fit to the data, using the average errors for weighting factors.

The heat capacity change for isodesmic self aggregation, $\Delta C_{p,agg}$, is found to be -0.70 kJ mol^{-1} K^{-1}. Consequently, the heat capacity change assuming dimerisation, $\Delta C_{p,dim}$, is -1.40 kJ mol^{-1} K^{-1}.

4.3 Ligand aggregate size distributions

The distribution of aggregate sizes at selected concentrations during the ITC experiments was calculated (Figure S5).

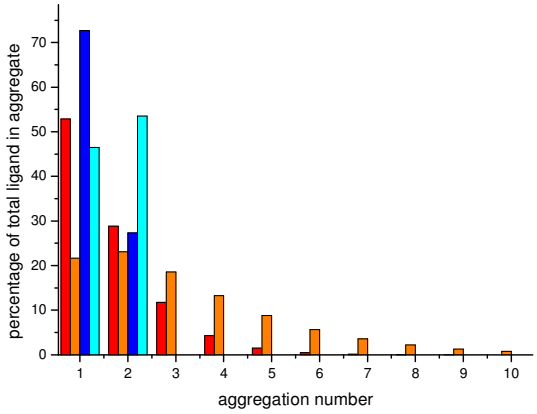

Figure S5. Aggregate size distributions in 10 mM PIPES, 20 mM NaCl, 1 mM EDTA, pH = 6.8 at 25 °C assuming an isodesmic aggregation model (red and orange bars) or a dimerization model (blue and cyan bars) at concentrations of AIK-18/51 of 10.3 µM (red and blue bars) and 49.6 µM (orange and cyan bars). Concentrations were selected from actual calorimeter cell concentrations during titrations.

Figure S5 show models predicting significant fractions of the ligand in its dimeric state.

[i] Mills, R. Self-Diffusion in Normal and Heavy Water in the Range 1-45°C, *J. Phys. Chem.* **1973**, *77*, 685-688.