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DNA Binding Properties of the Marine Sponge Pigment Fascaplysin

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Abstract—Association of fascaplysin with double-stranded calf thymus DNA was investigated by means of isothermal titration calorimetry, absorption spectroscopy, and circular dichroism. The UV spectroscopic data could be well interpreted in terms of a two-site model for the binding of fascaplysin to DNA revealing affinity constants of $K_1 = 2.5 \times 10^6\,M^{-1}$ and $K_2 = 7.5 \times 10^4\,M^{-1}$ (base pairs of DNA). Based on the typical change observed in the absorption and circular dichroism spectra, intercalation of fascaplysin is regarded as the major binding mode. The calorimetric titration curves showed an exothermic reaction which was exhausted at a 2:1 base pair/drug; ratio. This finding is in agreement with an intercalation model comprising nearest neighbor exclusion. In addition, significantly weaker non-intercalative DNA interactions can be observed at high drug concentration. By comparison of all these data with the binding behavior of known intercalating agents, it is concluded that fascaplysin intercalates into DNA.

Introduction

Fascaplysin (**1**) is a red pigment isolated in 1988 from the Fijian sponge *Fascaplysinopsis* Bergquist sp., and its structure was determined by spectral and X-ray analyses.[1] This natural product was reported to inhibit the growth of several microbes, including *Staphylococcus aureus*, *Escherichia coli, Candida albicans*, *Saccharomyces cerevisiae*, and to suppress proliferation of mouse leukemia cells L-1210 with $ED_{50} = 0.2\,\mu g/mL$.[1] Low cytotoxic effects on resting Ehrlich tumor cells, mouse lymphocytes, and erythrocytes were observed at high concentrations (50 μg/mL). However, after proliferative or mitogenic stimulation, incorporation of labeled thymidine, uridine, and leucine into DNA, RNA, and proteins, respectively, was strongly inhibited at concentrations of fascaplysin as low as 1 μg/mL.[2] Soni and co-workers[3] recently reported, that fascaplysin specifically inhibits cyclin dependent kinase 4 (Cdk4) causing a G1 arrest of tumor (U2-OS, HCT-116) and normal (MRC-5) cells. However, additional mechanisms of action have to be considered, e.g. fascaplysin may exert some of its biological activity through an interaction with DNA, similar to structurally related DNA intercalating cryptolepine (**2**)[4] and ellipticine.[5]

Herein, we report the results on titrations of calf thymus (CT) DNA with fascaplysin (**1**) monitored by isothermal titration calorimetry (ITC), which is a general method for measuring binding affinities and stoichiometries of binding phenomena,[6] by ultraviolet absorption spectroscopy (UV), and by circular dichroism (CD). While the thermal method directly addresses the thermodynamics of the binding process, the spectroscopic methods probe the change of the electronic properties of the drug molecule as it binds to DNA. Thus, the thermal and spectroscopic methods have different point of views complementing each other and revealing a complete picture of the binding process. A study on the DNA intercalating properties of (**1**) is presented and compared with those of cytotoxic agent cryptolepine (**2**, Chart 1), which has been demonstrated to efficiently intercalate into DNA and to act as a potent topoisomerase II inhibitor.[4]

Results

Usually, ITC experiments are performed by adding aliquots of the low-molecular weight partner (ligand or drug)

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to a reactor cell containing the high-molecular weight binding partner (macromolecule). In the present study, a solution of the macromolecule (DNA) had to be injected into a solution of the drug, due to limited solubility of fascaplysin and cryptolepine in buffered solutions.

The results of the ITC experiments are presented in Figure 1. The titration of a 0.2 mM solution of **1** with aliquots of a 4.3 mM CT-DNA produced exothermic signals which became continuously weaker as the titration progressed (Fig. 1a). At a molar ratio of 2 base pairs/drug, the signal essentially vanishes, that means saturation is achieved. The small remaining signal was due to dilution effects of CT-DNA as demonstrated with CT-DNA being added to pure buffer solution. Dilution of the CT-DNA (no drug present) gave only a small exothermic signal, virtually independent of the progress of the titration. For comparison, a similar titration experiment was performed with cryptolepine (Fig. 1b). Again, a continuously decreasing exothermic signal was observed reaching the baseline level at a molar ratio of 2 base pairs/drug molecule. Reaching saturation at 2 base pairs/drug molecule is consistent with intercalation and with previously published data obtained from DNA absorbance titration experiments of cryptolepine.[4]

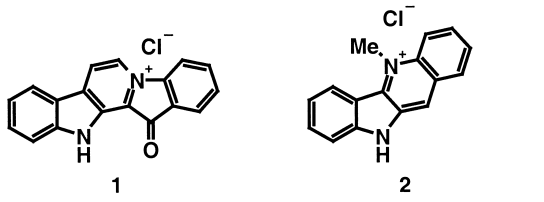

Chart 1. Chemical structures of fascaplysin (**1**) and cryptolepine (**2**).

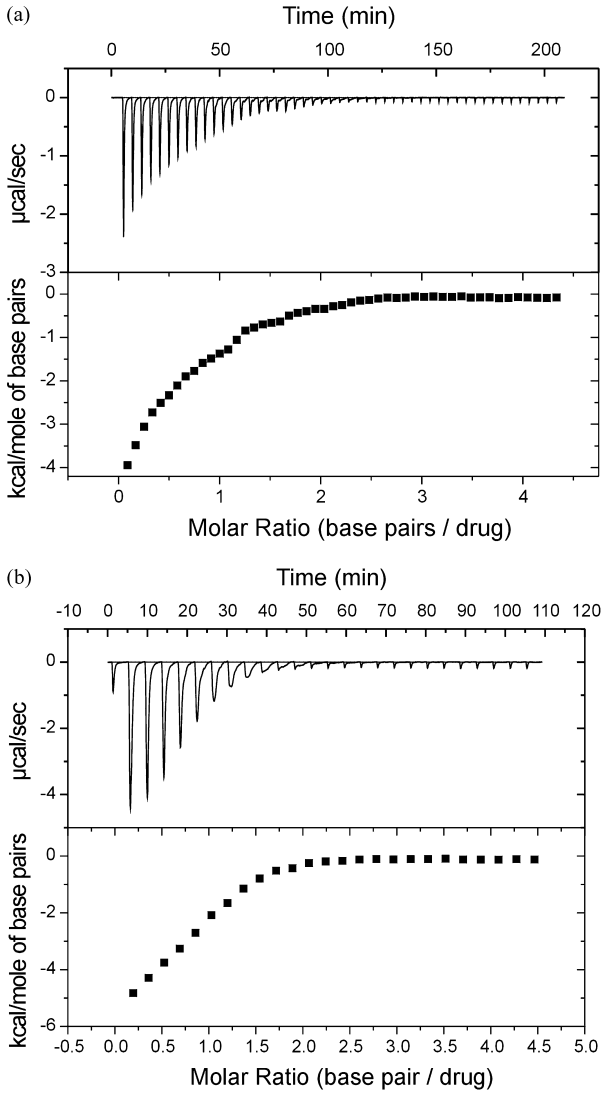

Figure 1. Isothermal titration calorimetry of (a) 0.2 mM fascaplysin (**1**) solution and (b) of 0.2 mM cryptolepine (**2**) solution with 4.3 mM (base pair) solution of calf thymus DNA. Upper panel: baseline corrected raw data (dissipated power as a function of time). Lower panel: integrated heat under each peak as a function of the molar base-pair/drug ratio.

Simulations of ITC experiments were performed assuming a binding model with nearest neighbor exclusion, as typically applied for intercalators such as ethidium bromide.[7a,b] The simulations were run with varying binding constants but with the same binding enthalpy ($\Delta H_m = -10$ kcal/mol) for all curves. As presented in Figure 2, none of the

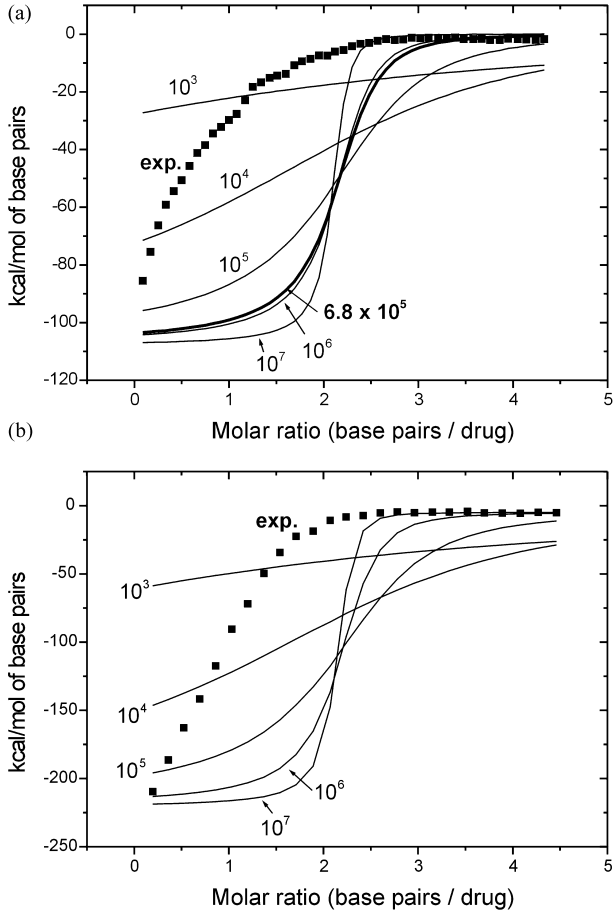

Figure 2. Comparison of experimental and simulated ITC experiments with (a) fascaplysin (**1**) and (b) with cryptolepine (**2**). Assumed binding enthalpy was calculated $\Delta H_m = -10$ kcal/mol. The binding constants used for the simulation are shown near the curves. The curve for **1** representing $K = 6.8 \times 10^5\ M^{-1}$ was obtained with the absorbance titration experiment assuming non-sequence specific binding with nearest neighbor exclusion.

simulated curves fit the experimental data. Other binding enthalpies and binding constants also failed to provide better correlations. Similar DNA binding behavior was demonstrated for both compounds.

An absorbance titration experiment was used to determine the binding constant of **1** to DNA. The experiment was carried out by adding aliquots of a 2.8 mM DNA solution to a 21 μM solution of the compound. The buffered solution of **1** alone shows an absorption band centered at 413 nm (Fig. 3). Bathochromic and hypochromic effects were observed upon addition of DNA. A red-shift of 15 nm and about 40% hypochromicity was determined at high DNA/drug ratios. The isosbestic points, present at 333 and 448 nm, are indicative for a single binding mode. Therefore, a binding model with non-cooperative binding and nearest neighbor exclusion, often referred to as the McGhee–von Hippel model,[7a,b] was used to fit a Scatchard plot representation of the absorbance data obtained at 413 nm (Fig. 4), resulting a binding constant $K = 6.8 \times 10^5\ M^{-1}$ (base pairs). However, an unsatisfactory fit was obtained indicating a more complex binding behavior. Thus, a two-site model comprising two independent non-cooperative types of binding[7c] was applied to fit to the experimental data. An excellent fit was obtained as shown in Figure 4, yielding two affinity constants ($K_1 = 2.5 \times 10^6\ M^{-1}$, $K_2 = 7.5 \times 10^4\ M^{-1}$) and the numbers of binding sites per base pair ($n_1 = 0.30$, $n_2 = 0.33$). These results are in good agreement with those obtained for known intercalating agents such as cryptolepine,[4] ellipticines,[5] and acridines.[8]

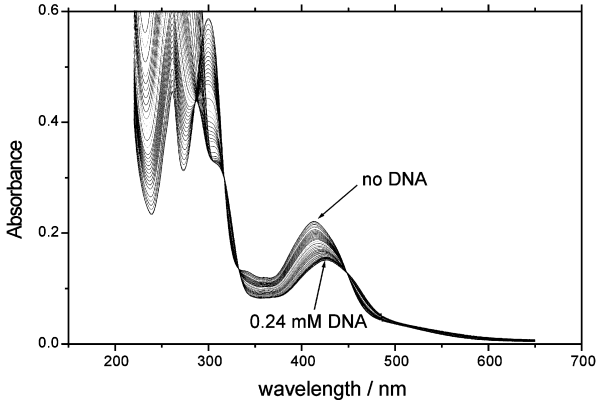

Figure 3. Titration of 21 μM fascaplysin (**1**) with 2.8 mM (base pairs) calf thymus DNA at pH 8.0. Spectra are uncorrected for contributions of DNA.

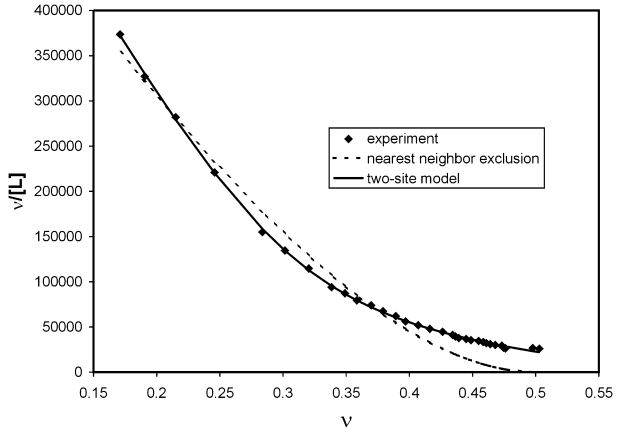

Figure 4. Scatchard plot of data from Fig. 3 evaluated at 413 nm. The data was fitted to a model with nearest neighbor exclusion for intercalative binding (dotted line) and to a two-site model (solid line); ν = number of bound fascaplysin molecules per nucleic acid base pair; [L] = molar concentration of free fascaplysin.

Circular dichroism (CD) experiments were performed to further study DNA binding properties of **1**, which does not exhibit any optical activity in the absence of DNA. CD spectra were measured at various ratios of fascaplysin/DNA concentrations. As displayed in Figure 5, a maximum (positive CD) at 325 and a minimum (negative CD) at 415 nm was induced as the DNA concentration was increased from 0 to about 1.5 base pairs/fascaplysin. Further increase of the DNA concentration, that means lowering the loading of DNA with fascaplysin, caused decreasing CD signals at 325 and 415 nm. The intensity of the induced CD signal strongly depends on the DNA/drug ratio. This variation of molar dichroism and the low value for $\Delta\epsilon^{max}$ ($\Delta\epsilon^{max} = 2.5\ M^{-1}cm^{-1}$) is consistent with an intercalative mode of binding to DNA[9] and has already been observed for other intercalating agents such as cryptolepine,[4] ellipticine derivatives,[10] ethidium bromide,[11] and acridine orange.[12]

Discussion

The absorption spectroscopic data display clear isosbestic points (Fig. 3) indicating a single binding mode. Saturation occuring at the typical base pair/drug ratio of 2 is in agreement with DNA intercalation. However, the Scatchard plot representation in Figure 4 reveals that the simplest model for intercalative binding (no sequence specificity, nearest-neighbor exclusion) fails to properly reflect these data. Therefore, we applied a two-site

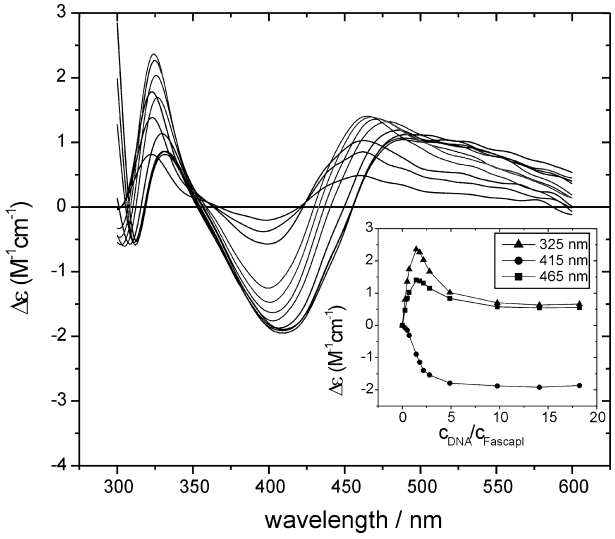

Figure 5. Titration of fascaplysin (**1**) with CT DNA monitored with CD spectroscopy (smoothed spectra, baseline subtracted). The concentration of **1** was kept constant at 62 μM and the DNA concentration was continuously decreased. Inset: CD signal at various wavelengths as a function of the fascalysin/DNA concentration ratio.

model assuming two independent non-cooperative binding modes as already described for cryptolepine.[4] With this model, an excellent correlation of the experimental data was obtained suggesting sequence specific intercalation into DNA. The electronic interactions between fascaplysin and the various intercalation sites of the DNA may cause similar spectral shifts, essentially independent of the exact sequence. This would justify the existence of isosbestic points in the UV data. In addition, the biphasic CD signals give further support for a sequence specific intercalation mode. Similar behavior of the CD signal has been observed for cryptolepine[4] and other typical intercalators such as ethidium bromide[11] and acridine orange.[12] However, in the case of ethidium bromide, the UV absorbance data perfectly fit the simple nearest-neighbor exclusion model comprising no sequence specificity.[7b]

The ITC data cannot be interpreted solely with an intercalative binding mode. On one hand, saturation of the signal is observed at a base pair/drug ratio of 2, providing evidence for an intercalative binding mode. On the other hand, as shown in Figure 2, the nearest neighbor exclusion model completely fails to reflect the experimental data. Assuming sequence specific interactions with varying individual binding enthalpies and affinities may help explain the continuous rise of the integrated heat signal. However, in such a model signal saturation should be observed at base pair/drug ratios much higher than 2, because drug molecules would preferably translocate from low-affinity to the limited number of high-affinity intercalation sites with increasing DNA concentration until all drug molecules are located in high-affinity sites. In order to account for the saturation at base pair/drug ratio of 2 and the constantly increasing signal at lower ratios, we thus postulate the existence of additional binding sites with much lower affinities than the intercalation sites. At low DNA concentrations, drug molecules occupy all possible intercalation sites and in addition bind to non-intercalative sites. As more DNA is added, those molecules occupying non-intercalative sites will change position and intercalate until the base pair/drug ratio becomes approximately 2 when all drug molecules can be accommodated in high-affinity intercalation sites.

Obviously, the nature of these postulated non-intercalative binding sites cannot be described in detail. The values of the affinity constants are estimated to be less than $10^5\,M^{-1}$. Possible binding modes include groove or surface binding. Since these additional binding modes remain "invisible" in the absorption spectroscopic analysis, as shown by the existence of isosbestic points, binding of the drug to the DNA surface is the most likely process. In this case, only minor effects on the UV spectrum are expected. The positive charge on both drugs, fascaplysin and cryptolepine, leads to favorable electrostatic interaction with the DNA phosphate groups, enhancing loose surface binding. Similar external binding has been observed and kinetically resolved for the aminoacridine molecules rivanol and proflavine which are also positively charged.[15] It was shown for these two molecules that the external binding is typically about one order of magnitude weaker than intercalation. Insertion kinetics was in the millisecond time domain. Even though ITC is not able to resolve such rapid kinetics, it can still monitor the external binding through the total time-integrated heat released in the external binding process. In summary, ITC data provide evidence for intercalative binding and for additional low-affinity binding modes, presumbaly external binding to the double-helix.

Conclusions

Herein, we could demonstrate DNA intercalating properties of fascaplysin by using isothermal titration calorimetry, absorption spectroscopy, and circular dichroism. Binding mode and affinity constants of this natural product are comparable to those of other typical DNA intercalators. Conclusively, some of its biological activity can be attributed to interference with the genetic material.

Experimental

Drug. Synthetic fascaplysin (**1**) was used throughout the experiments described in this report. The material was obtained in a five-step synthesis following the procedure of Radchenko and co-workers.[13] The main analytical characteristics (IR, UV, MS, ^1H, ^{13}C NMR) of synthesized **1** were identical to those published for the natural product.[1]

Biochemicals. Highly polymerized calf thymus (CT) DNA sodium salt was obtained from Sigma (Cat. No. D-3664). Its concentration in solution (expressed in base pairs) was determined by UV spectroscopy assuming $\epsilon = 13{,}200\,M^{-1}cm^{-1}$.[14] All experiments were carried out in buffer solution consisting of 10 mM TRIS with 1 mM EDTA adjusted to pH 8.0.

Isothermal titration calorimetry. ITC experiments were performed on a MCS ITC titration calorimeter from Microcal Inc. The system was equipped with a 250-μL injection syringe. The temperature was 25 °C in all experiments. Raw data were integrated using Origin Software Version 2.9 that came with the calorimeter. Simulations shown in Figure 2 were performed by calculating the number of bound molecules per base pair (ν) before and after each injection step.[7b] From these ν values and the assumed binding enthalpy, the heat release for each injection step could be evaluated.

Absorption spectroscopy. The UV spectroscopic titration was carried out directly in a 1 cm quartz cuvette using a diode array UV spectrometer (Hewlett Packard, model 8453). The cuvette initially held 2.5 mL of a 21 μM solution of fascaplysin and a 2.8 mM solution of DNA was added stepwise by means of a dispenser (Hamilton, Microlab 500) equipped with a 25 μL syringe and adequate teflon tubing.

Circular Dichroism. The CD titration was carried out on a Jasco model J-710 spectropolarimeter using a 1 cm path length cuvette. A buffered mixture of 62 μM drug and 1.1 mM base pairs DNA was gradually diluted with a 62 μM solution of fascaplysin solution ensuring a constant concentration of fascaplysin and a decreasing concentration of DNA. Spectra were smoothed using the spectrometer software's default parameters and the baseline (buffer solution) was subtracted from all spectra.

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