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Calorimetric and Spectroscopic Studies of Hoechst 33258: Self-association and Binding to Non-cognate DNA

Niklaas J. Buurma and Ihtshamul Haq*

Centre for Chemical Biology, The Department of Chemistry, The University of Sheffield, Dainton Building, Brook Hill, Sheffield, South Yorkshire, S3 7HF, UK

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Sequence and structure-specific molecular recognition of DNA by small molecules is an important goal in biophysical chemistry and drug discovery. Many candidate ligands possess flat aromatic surfaces and other molecular features that allow them to self-associate. In addition, non-specific binding to the target is a complicating feature of these interactions. Therefore, multiple equilibria are present and need to be accounted for in data analysis in order to obtain meaningful thermodynamic parameters. In order to address these issues we have systematically examined the bis-benzimidazole dye Hoechst 33258 (H33258) in terms of self-aggregation and binding to DNA oligonucleotides lacking any cognate minor groove A·T sites. This model system has been interrogated using isothermal titration calorimetry (ITC), circular dichroism (CD), fluorescence spectroscopy and pulsed gradient spin echo NMR. Three distinct binding events and ligand self-aggregation have been identified and, where possible, quantified. H33258 self-aggregation involves a step-wise aggregation mechanism, driven by stacking interactions. The DNA binding process includes two specific binding modes and non-specific DNA-templated H33258 stacking. We have written novel ITC data-fitting software (IC-ITC; freely available to the biophysics community), which simultaneously fits ligand aggregation and ligand–DNA binding. Here, this numerical analysis, which uses simulated annealing of complex calorimetric data representing multiple coupled equilibria, is described.

Keywords: drug-DNA interactions; isothermal titration calorimetry; non-specific binding; ligand self-association; coupled equilibria; data analysis

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Introduction

Nucleic acids are the biological target for many anticancer, antibiotic and antiviral drugs and, hence, DNA and RNA are important targets in drug development. Sequence and/or structure-specific targeting of DNA offers the tantalizing prospect of controlling numerous genetic diseases by selectively inhibiting gene expression. Global inhibition of DNA replication or targeting of DNA/RNA hybrids potentially opens routes to novel antibiotic and antiviral agents. One of the most successful approaches in sequence-selective targeting of DNA thus far is the polyamide approach pioneered by Dervan and co-workers,[1] which has already led to successful blocking of tumor growth in nude mice.[2] To exploit the full potential of DNA recognition in medicine and biotechnology there is a strong need for new drugs and biosensors that interact with DNA. Over 20 years of studying ligand–DNA complexes with X-ray crystallography[3] and NMR has led to a fairly detailed understanding of the structural factors involved in DNA binding. However, our understanding of the thermodynamic principles guiding DNA binding and sequence selectivity has lagged behind. This is mainly because ultra-high-sensitivity instruments for measuring thermodynamics, for example isothermal titration calorimetry (ITC), have become commercially widespread only relatively recently. An improved understanding of binding thermodynamics will certainly inspire further development of

*Corresponding author. E-mail address: I.Haq@Sheffield.ac.uk.

Abbreviation used: ITC, isothermal titration calorimetry.

DNA-binding drugs, thereby offering new prospects for the treatment of major diseases.

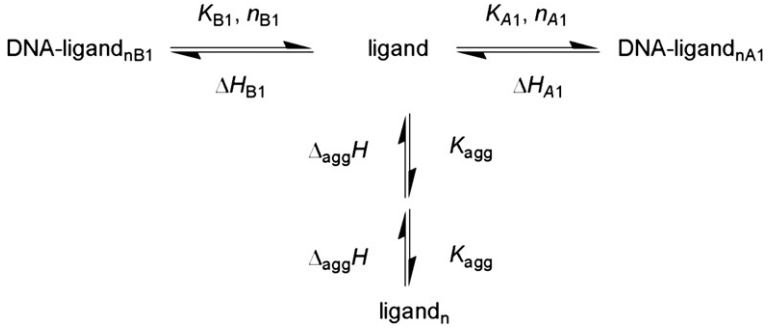
Most small molecules that bind to DNA are largely planar aromatic compounds of considerable hydrophobicity. Almost perversely, it is precisely this set of properties that makes compounds good intercalators and/or minor groove binders, which also favors self-aggregation in aqueous solution. This is especially the case for intercalators where π-stacking provides an important driving force for both intercalation into adjacent DNA base pairs and self-aggregation. Intercalators such as ethidium bromide, proflavine, and acridine orange,[4,5] and end-stacking ligands like the camptothecins[6] have all been shown to aggregate. Equally, minor groove binders berberine[7] and Hoechst 33258 (H33258),[8,9] combined intercalator/minor groove binders such as daunomycin,[10] related doxorubicin[11] and methylene blue[12] have been reported to aggregate in aqueous solution. Ligand self-association almost certainly has *in vivo* consequences but more immediately this phenomenon presents a clear difficulty when undertaking biophysical studies of ligand binding.

For most experimental techniques used in the study of DNA-binding, aggregation of DNA-binding compounds does not cause significant problems. As Chaires *et al*. noted, for tight binding to DNA free ligand concentrations are always very low and hence most ligand molecules exist as monomers; this is especially the case for UV/vis or fluorescence titrations. Under these conditions, ligand self-association will not interfere with the interpretation of the binding data and the binding isotherms will not need correction. So, if binding is tight, for all practical purposes self-association may be ignored for these conditions.[10] Unfortunately we often need to examine ligands that bind in the range 10^4–10^5 M^{-1}, and some techniques intrinsically require relatively high concentrations of ligand; for example, in the injection syringe during ITC experiments or for sensitivity reasons in the case of NMR. For the case of ITC, the presence of high concentrations of relatively strongly self-aggregating ligand in the injection syringe means that the non-constant (de)aggregation heat effects (during the course of the titration) become important. This is, of course, true whether the ligand is a small molecule or a self-associating protein. To circumvent these problems, one can use the model-free ITC protocol as described by Chaires during his study of daunorubicin binding to DNA.[13] In model-free ITC, there is no attempt to measure binding constants but rather concentrations are chosen so as to measure only a statistically meaningful value of ΔH. As Brad Chaires notes, in experiments where binding sites become saturated, ligand self-association presents a "daunting" difficulty.[14] Ironically, older experimental procedures naturally restricted the user to the model-free ITC protocol.[15,16]

H33258 is the archetypal DNA minor groove binder and is known to be A/T-selective. However, multiple binding modes were found by Loontiens and divided into sequence-mediated, charge-mediated, dye-mediated, and structure-mediated interactions.[8] Apart from binding in the minor groove of A+T-rich DNA, H33258 acts as a "non-classical intercalator" towards G+C-rich DNA.[17–22] A third binding mode involves DNA-templated stacking of H33258.[17] DNA-templated H33258 stacking is accompanied by charge neutralization and eventually leads to insoluble dye–DNA aggregates at high [H33258]/[DNA] ratios.[8,17,23–25] In addition, H33258 has been found to bind cooperatively with certain specific oligonucleotide sequences.[26,27] Finally, as a typical planar aromatic hydrophobic dye, H33258 has been found to self-aggregate.[8] To summarize, a range of interactions are possible, and presumably occur simultaneously, for H33258 in combination with DNA. These interactions include H33258 self-aggregation and DNA binding involving different binding sites. Interactions relevant for the work presented here are summarized in Scheme 1.

In view of the range of possible H33258–DNA interactions and the relative lack of thermodynamic data on these (in fact, contradicting reports have been published on the thermodynamics of H33258 binding to DNA.[8,28,29,30]), we have chosen H33258 for a full calorimetric and spectroscopic analysis of its binding modes and sequence selectivity.

AT-specific minor groove binding of H33258 known as Type 1 or sequence-mediated binding is perhaps one of the most studied drug–DNA interactions to date, both structurally and thermodynamically. In fact, we published a paper ten years ago that represented a convergence between thermodynamic and structural data.[28] However, non-specific, Type 2, background binding as well as its linkage to ligand de-aggregation is less well understood. In order to address this we have designed and synthesized a



Scheme 1.