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Crystal structure of a berenil - dodecanucleotide complex: the role of water in sequence-specific ligand binding

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The three-dimensional structure of a complex between the dodecanucleotide d(CGCGAATTCGCG) and the anti-trypanocidal drug berenil, has been determined to a resolution of 2.5 Å. The structure has been solved by molecular replacement and refined to an *R* factor of 0.177. A total of 49 water molecules have been located. The drug is bound at the 5'-AAT-3' region of the oligonucleotide. At one end of the drug the amidinium group is in hydrogen-bonded contact with N3 of the adenine base complementary to the thymine of the AAT. The other amidinium group does not make direct interactions with the DNA. Instead, a water molecule mediates between them. This is in hydrogen-bonded contact with an amidinium nitrogen atom, N3 of the 5' end adenine base and the ring oxygen atom of an adjacent deoxyribose. Molecular mechanics calculations have been performed on this complex, with the drug at various positions along the sequence. These show that the observed position is only 0.8 kcal/mol higher in energy than the best position. It is suggested that there is a broad energy well in the AATT region for this drug, and that water molecules as well as the neighbouring sequence, will determine precise positioning. More general aspects of minor groove binding are discussed.

Key words: berenil/crystal structure/dodecanucleotide/ligand binding/molecular modelling/water molecules

Introduction

The recognition of specific DNA sequences by small molecules is currently the focus of considerable interest (Dervan, 1986; Lown, 1988), not least because of their potential as artificial gene regulators and as selective chemotherapeutic agents. Ligands that interact non-covalently with DNA have been shown by footprinting (Van Dyke *et al.*, 1982; Lane *et al.*, 1983) and biophysical (Zimmer and Wähnert, 1986) methods to bind predominantly to AT sequences. Crystallographic studies of several such agents complexed to oligonucleotides have shown that these are located in the minor grooves of AT regions by virtue of a combination of specific hydrogen bonding and close ('isohelical') fit to the shape of the minor groove (Kopka *et al.*, 1985; Coll *et al.*, 1987, 1989; Pjura *et al.*, 1987; Teng *et al.*, 1988; Carrondo *et al.*, 1989). We report here the crystal structure of a complex between a minor groove ligand and a DNA sequence that for the first time shows direct involvement of water molecules in the recognition process.

The 1,3-*bis*(4-phenylamidinium)triazene compound berenil (Figure 1) has veterinary application as an anti-trypanosomal agent and has cytotoxic and anti-viral properties (De Clercq and Dann, 1980). It binds reversibly to double-helical DNA primarily at AT-rich regions (Newton, 1975; Braithwaite and Baguley, 1980; Baguley, 1982), as shown most recently by a footprinting study with DNA fragments (Portugal and Waring, 1987). Berenil interacts with the kinetoplast DNA of *Trypanosoma cruzi* cells (Bernard and Riou, 1980), which has been found to have bending at its phased A tracts (Marini *et al.*, 1982; Burkhoff and Tullius, 1987). The footprinting revealed that strong-binding sequences are at least three base pairs long, with a possible preference for alternating AT sites. The drug also binds to DNA on reconstituted nucleosome core particles, in common with netropsin and other minor groove binders. It changes the rotational orientation of the DNA by exposing otherwise hidden AT sequences (Portugal and Waring, 1986). Molecular modelling studies have suggested that berenil interacts via the minor groove of B-DNA (Gresh and Pullman, 1984; Pearl *et al.*, 1987; Gago *et al.*, 1988), with the drug appearing to span two base pairs and hydrogen-bonding preferentially to adjacent thymine bases via their O2 atoms. We have co-crystallized berenil with the well-studied self-complementary duplex sequence d(CGCGAATTCGCG) (Drew and Dickerson, 1981), and report here on its crystal structure, together with the results of molecular modelling studies using this sequence.

Results

Crystal structure

The observation of continuous electron density (Figure 2) in the minor groove of the d(CGCGAATTCGCG) double helix, together with the subsequent satisfactory refinement of a berenil molecule in this position, unequivocally places the drug at the AT region (Figure 3). It is asymmetrically disposed with respect to the diad axis of the duplex and is bound to the AAT sequence. The berenil molecule spans some three base pairs, interacting primarily with the two adenines A5 and A18 on opposite strands (Figure 4), in

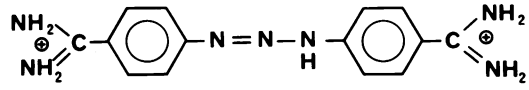

Fig. 1. Chemical structure of berenil.

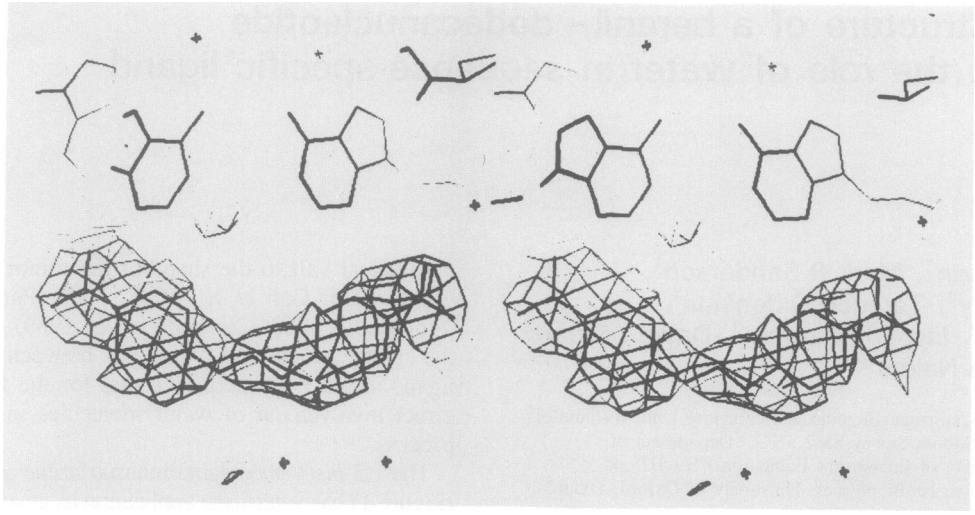

Fig. 2. Stereo view of the final omit difference Fourier electron density map with a berenil molecule superimposed on the density displayed by TOM. Phases were calculated using all atoms except those of berenil. The density displayed is at the 2σ level.

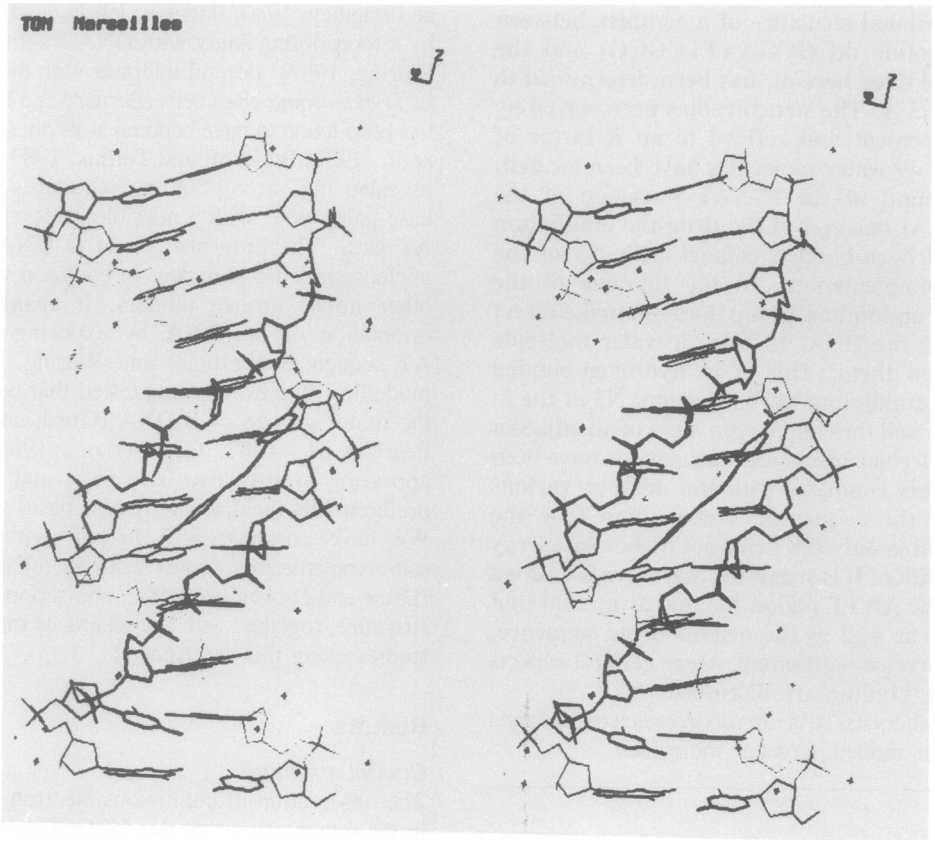

Fig. 3. Stereo view of the d(CGCGAATTCGCG)–berenil complex. Some of the water molecules are shown in this view.

agreement with the estimate of the site size from DNase I footprinting. The overall disposition of the drug is quite distinct from previous proposals based on molecular modelling. The footprinting data strongly disfavour a binding-site model with just two contiguous AT base pairs, and generally favour binding sequences with three or more A and T residues, suggestive of flanking sequence effects.

The phenyl rings of berenil lie parallel to the hydrophobic minor groove walls whilst the terminal amidinium groups are in close contact with the adenine bases A5 and A18. The N3 atom of A18 is linked by a hydrogen bond of length 2.98 Å to a nitrogen atom of one amidinium, which is located between the two TA base pairs (T7-A18 and T8-A17) and is therefore significantly out of the base pair plane at this point. At the other end of the ligand, the amidinium does not directly contact N3 and A5. Instead, a water molecule mediates between this charged group and N3 of A5, with hydrogen-bond distances of 2.80 and 3.10 Å respectively (Figure 5). This water molecule, which is in the plane of the A5-T20 base pair, thus effectively bridges the respective hydrogen bond donor/acceptor groups and clearly plays a crucial role in the interaction of berenil.

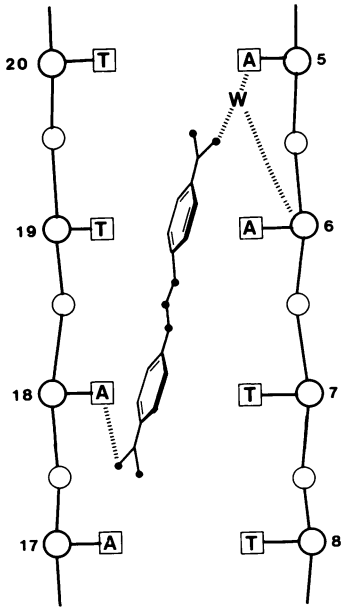

Fig. 4. Schematic representation of the berenil bound to the d(CGCGAATTCGCG) duplex. Dashed lines indicate hydrogen bonds, as detailed in the text.

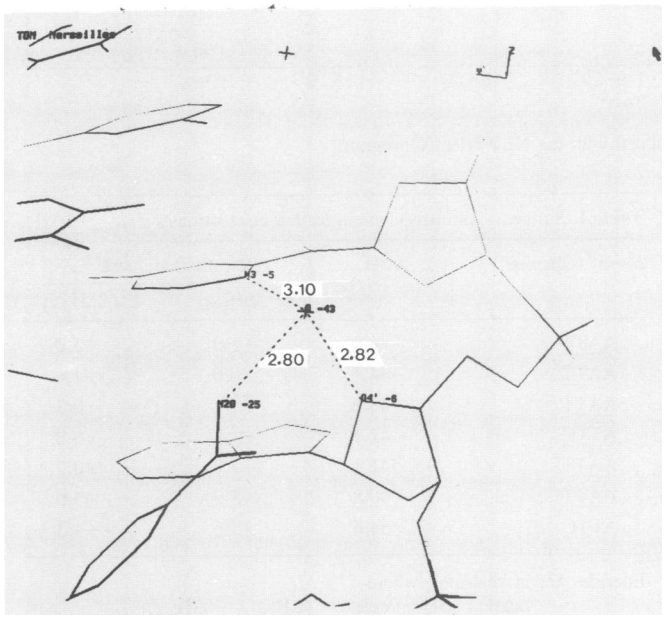

Fig. 5. Detail of the hydrogen-bonding network at one end of the bound berenil. The bridging water molecule is shown. Adenine 5 is at the top, with adenine 6 below.

The water molecule also forms a hydrogen bond with the O4′ ribose ring atom of A6, at a distance of 2.82 Å. This water molecule is in a different position from that found in this general region in the crystal structure of the native dodecamer, where it is a component of an extended minor groove water network (Drew and Dickerson, 1981). In that case the water is very weakly hydrogen-bonded to both the N3 atom of adenine 5 and O2 of cytosine 21. It is apparent then that this minor groove-binding drug effectively displaces any ordered water network in the minor groove, at least in the AT region. The readily located waters are now in the major groove and around the phosphate groups, many of them actually bridging phosphate oxygens, in striking contrast to the native structure where the waters are found mainly in the grooves. A specialized case of bridging involves the triazene group of berenil, which does not directly participate in base recognition, where the hydrogen atom present in the triazene bridge must point outward from the minor groove. We find that the triazene nitrogen at the 5′ end of the binding site is close to a water molecule at the mouth of the minor groove (3.17 Å separation). The major groove waters are mostly hydrogen-bonded to polar atoms of the bases in a monodentate manner, with O6 and N7 of guanines being preferred. There are several localized base–water–base interactions, especially in the GC regions.

Figure 6 shows that the berenil molecule has widened the minor groove compared to the native structure by ~1.5 Å at its maximum. This is similar to changes observed for netropsin and Hoescht 33258. The significant widening extends for ~2 bp.

The berenil molecule is positioned in the minor groove (Figure 3) so that the phenyl rings are between the more hydrophobic sugar and C5′ atoms of the two backbone strands. The more polar triazene group of the drug is between the two phosphodiester groups of the backbone.

Molecular modelling

Binding and perturbation energies (which measure conformational energy change), were obtained for each modelled complex. The latter reflect the distortions induced by the drug during binding. The binding energies and individual perturbation energies for each drug–DNA complex are given in Table I. These show that berenil binds preferentially at a d(. . AATT . .) site which involves spanning three base pairs and hence hydrogen bonds are formed with non-adjacent bases (adenines 1, 3). This conclusion is in accord with results from footprinting (Portugal and Waring, 1987) and our recent modelling studies of diverse oligonucleotide duplexes (Jenkins and Neidle, unpublished data). Previous analyses have suggested that the drug binds favourably to adjacent thymine in d(. . TA . .) sequences, involving only a 1,2-bp stretch (Gresh and Pullman, 1984; Pearl *et al.*, 1987; Gago *et al.*, 1988). It is noteworthy that the octamer sequence finds a d(. . AATT . .) binding site which differs by only 0.5 kcal/mol from the overall minimum energy complex. Indeed, an enthalpy difference of ~1 kcal/mol separates the three lowest energy (. . AATT . .) (. . AATT . .) and (. . AATT . .) binding sites (see Table I), suggesting that the interaction of berenil with the 8mer duplex involves a broad enthalpy well spanning the 4 bp d(. . AATT . .) sequence, rather than one global minimum.

Examination of each of the six structures resulting from energy minimization reveals little geometric distortion of either the berenil or the oligonucleotide. In the case of the energetically unfavourable d(CGAATTCG) model, however, significant distortion is suffered by the DNA in such a way as to reduce specific steric clashes involving the guanine residues with consequential loss of favourable hydrogen-bond contact with the berenil.

As observed in earlier work (Pearl *et al.*, 1987) the berenil ligand retains the general planarity and conformation suggested by the crystal structure with minimal induced twisting or bending. Phenyl-amidinium torsion angles are

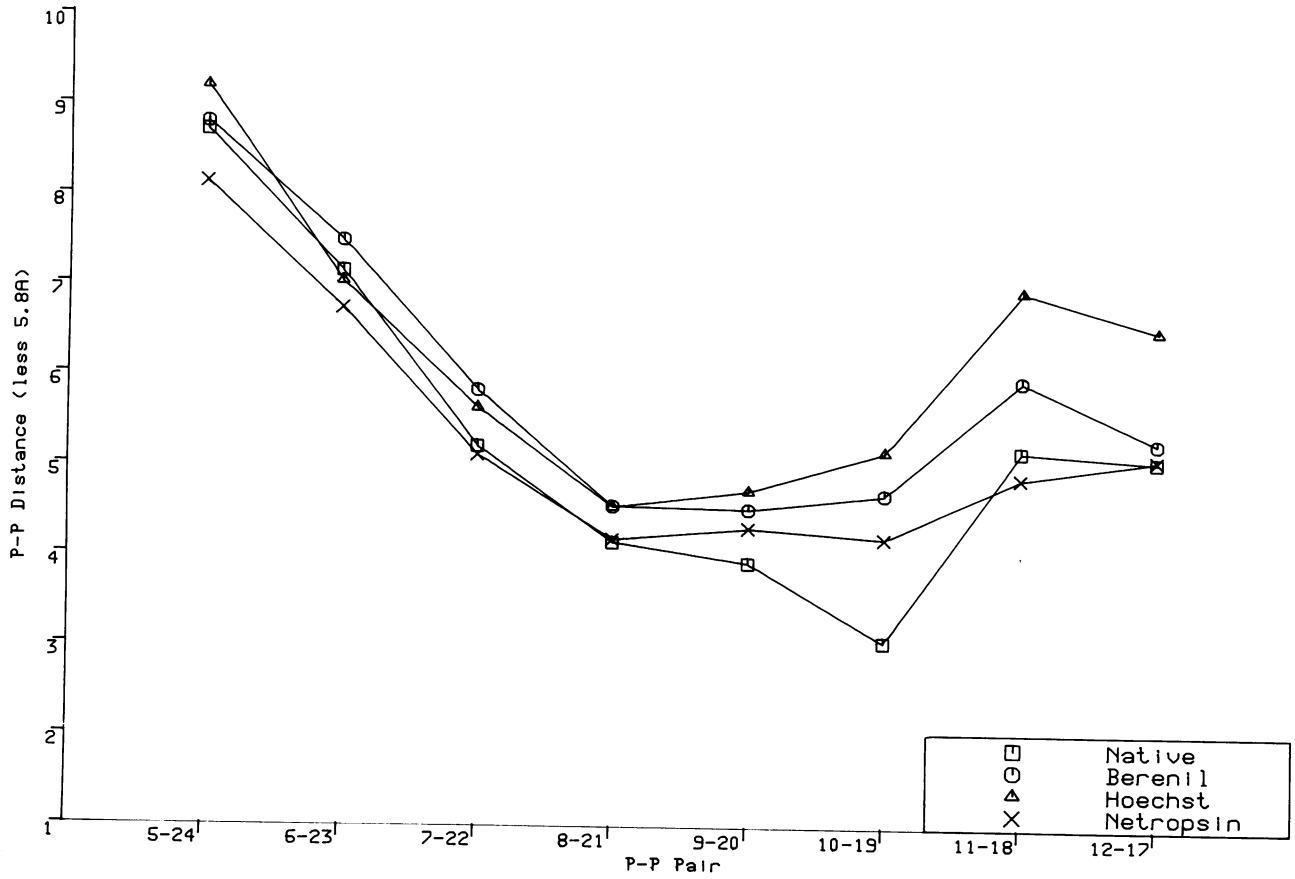

Fig. 6. Plot of minor groove widths in various drug–DNA complexes, calculated with the NEWHELIX program.

confined to $< \pm 21°$, or $\pm 6-12°$ in the favoured models, whilst all torsions involving the triazene bridge are restricted to only $\pm 5°$. There is no evidence of induced rotation of any amidine group such as to make hydrogen-bond contacts with oxygen atoms of the phosphate backbone. The sugar puckers in all oligomer models were qualitatively unchanged from the starting C2′-*endo* conformation.

Further, the initial hydrogen-bond contacts are generally retained, at least in part, with O2 (C or T) . . . H-N(amidine) and N3(A) . . . H-N(amidine) distances of 2.2–2.4 Å. Where hydrogen-bonded contact to the bases has been lost, this has been compensated by favourable ribose O4′ . . . H-N(amidine) contacts between the charged amidine group and the 5′–3′ or 3′–5′ phosphodiester backbones.

Secondary interactions with the ribose O4′ atoms, rather than the oxygen atoms of the phosphate, play a significant role in determining the overall binding energy, particularly in favoured models.

Table I. Summary of energy minimization calculations

5′–3′ sequence[a]	ΔΔH	Perturbation energy[b]	
		ΔH_{DNA}	ΔH_{drug}
. . AATT . .	−44.6	+1.0	+1.0
. . AATT . .	−44.1	0.0	+1.6
. . AATT . .	−43.8	+1.1	+0.7
. . ATTC . .	−42.3	+1.1	+0.9
. . AATT . .	−41.1	+2.0	+0.7
. . AATT . .	−40.8	+3.9	+0.6
. . ATTC . .	−34.6	+5.6	+4.3

Energies are in kcal/mol, where

$$\Delta\Delta H = \Delta H \text{ complex} - (\Delta H_{DNA} + \Delta H_{drug})$$

[a]Underscore indicates spanned binding site.
[b]The calculated energies for the free d(CGAATTCG) octamer duplex and berenil were −380.1 and −0.5 kcal/mol, respectively. Perturbation energies (ΔH_{DNA} and ΔH_{drug}) indicate the difference in energy for the fragments in the optimal complexes with respect to their free states.

Discussion

It is apparent that both the length of the berenil ligand and the nature of the interactions with the adenines do not permit symmetrical binding across the diad axis of this sequence. Why does berenil not bind between A6 and A17, since this appears to be an equivalent AT binding site? (There is no indication of electron density in this region.) The exocyclic N2 of guanine G16 presumably prevents such binding, due to steric hindrance in the minor groove region where interaction with N3 of A17 would occur. Flanking sequences thus appear to play an important role in discriminating between different binding sites. Interactions of this type have been examined in detail by us in a molecular mechanics study using a large number of sequences (Jenkins and Neidle, unpublished data). This study also indicates that a replacement of A5 by thymine would not remove the necessity for a bridging water molecule, since a thymine O2 would not come sufficiently close to the amidinium group for hydrogen bond formation. There is no opportunity in the present dodecamer sequence for such an interaction to occur. The two DNA

fragments used in the footprinting study (Portugal and Waring, 1987) contain few of the 16 distinct AT tetranucleotide sequences, hence the full sequence preference order for berenil is not known experimentally. The observed position of the berenil molecule has the hydrophobic phenyl rings contacting the hydrophobic regions of the backbone and the triazene group neighbouring the polar phosphodiester groups. This may be a further factor in the exact location of the drug within a general AT region. No thermodynamic data are available for berenil binding to polynucleotides; that for netropsin (Marky and Breslauer, 1987) shows that the process is enthalpy-driven. The present results suggest a similar situation for berenil although the incorporation of (redistributed) solvent at the drug binding sites for some sequences will enhance an entropic contribution.

The present structural result suggests that berenil should bind well to an AATT site in genomic DNA and is supported by the fact that the sole AATT site in the footprinted 102mer fragment examined is in the region of the sequence most strongly protected from DNase I. In general, we suggest that berenil does not easily tolerate either C or G bases at the 3′ side of the binding site, since we find that AAT(T) rather than AAT(C) is preferred for the drug. The nature of the flanking sequence at the 5′ end may be of secondary importance.

The widening of the minor groove of the AATT region compared to the native dodecamer reflects the interplay of two opposing factors: (i) the intrinsic narrowness of the AT sequence minor groove, and (ii) the widening essential for effective ligand binding. Recent hydroxyl radical footprinting studies (Fox, personal communication) have suggested that the site size is in accord with the structural results presented here. The overall bending of the dodecamer is not significantly reduced compared to the native structure, although extrapolations to DNA in solution must be made with caution in view of the influence of crystal packing forces. The AT-selective DNA groove binder distamycin strongly diminishes the bending of kinetoplast DNA (Wu and Crothers, 1984) and is found to decrease the bending of the sequence d(CGCAAATTTGCG) in the crystal structure of its complex by 4° (Coll *et al.*, 1987). Berenil may therefore be considerably less perturbing in its effect on kinetoplast DNA structure.

Site-specific recognition of DNA by proteins is, in general, probably via the major groove, primarily for steric reasons. Non site-specific proteins, such as histone H1 (Turnell *et al.*, 1988) or DNase I (Suck *et al.*, 1988), appear to interact with the minor groove. Crystal structures of protein–oligonucleotide complexes have shown a few instances where a water molecule mediates between an amino acid residue and a base. Especially relevant is the phage 434 operator–repressor complex (Aggarwal *et al.*, 1988) where Arg^{43} is bridged via three water molecules to base pairs in the minor groove. The coordination to N3 of adenine is strikingly similar to that in the present structure, implying that the role of water molecules in these circumstances is to fill in the gaps not occupied by respective 'side-chains'. In the case of berenil, the triazene group and the phenyl rings together impart selectivity towards AT, whereas the amidinium moieties select particular bases. The water molecule at the 5′ end thus fulfills an essential role enabling an adenine to be sensed by the drug. The possibility of such interactions will have to be taken into account in the future design of sequence-specific agents. Bridging water molecules have been found to be important in stabilizing the intercalative drug daunomycin when bound to the sequences d(CGTACG) and d(CGATCG) in the crystal (Wang *et al.*, 1987; Moore *et al.*, 1989).

Materials and methods

Crystal structure analysis

The dodecanucleotide sequence was synthesized using an Applied Biosystems synthesizer using phosphoramidite chemistry and purified by HPLC on a reverse phase C8 column. Berenil was synthesized at the Institute of Cancer Research. Crystals were obtained by vapour diffusion at 278K from a solution containing 3 mM DNA dodecamer, 10 mM sodium cacodylate (pH 7.0), 30 mM $MgCl_2$, 2 mM berenil and 20% v/v 2-methylpentan-2,4-diol (MPD) against a 50% v/v MPD reservoir. The crystals of the complex are yellow in colour and form highly elongated needles. These have unit cell dimensions of a = 24.51, b = 39.98, c = 66.23 Å and are in the same space group ($P2_12_12_1$) as the native dodecamer, which has dimensions of a = 24.87, b = 40.39, c = 66.20 Å; the two are therefore isomorphous. X-ray crystallographic data were collected on a Xentronics area detector mounted on a Rigaku RU200 rotating anode X-ray source. The data collected extend to a resolution of 2.5 Å. A total of 5762 reflections were collected, which merged to 2195 unique reflections with an overall merging agreement of 3.7%, of which 1759 reflections had intensity over the 2σ level.

The structure was solved by molecular replacement, using the native d(CGCGAATTCGCG) duplex coordinates as a starting model. This was initially refined as a rigid body with the least-squares program CORELS (Sussman *et al.*, 1977). Successive rounds of refinement using both CORELS and the restrained-refinement program (Westhof *et al.*, 1985) NUCLIN resulted in an R factor of 0.201, using data from 8 to 2.5 Å. At this point ($2F_O$-F_c) difference and 'omit' maps were calculated using the PROTEIN package (Steigemann, 1874) and viewed on a Silicon Graphics Iris 3130 using the graphics package TOM (Cambillau, 1988). These showed numerous putative water peaks and an extended continuous volume of density in the minor groove of the duplex. This was unequivocally fitted to a berenil molecule in terms of size and overall shape. The DNA, ligand and a total of 49 water molecules located to date have all been included in subsequent refinements. The temperature factures for the water molecules ranged from 10 to 71 $Å^2$, with a mean of 32 $Å^2$. The bridging water molecule has a B of 30 $Å^2$. Details of the water arrangement will be discussed in a subsequent paper. The current R factor is 0.177 for the 2σ data. Final refined coordinates, including those for the water molecules, have been deposited in the Brookhaven Protein Data Bank.

Molecular modelling

The structure for the double-stranded d(CGAATTCG) core 8mer sequence from the Dickerson dodecamer was generated in the B conformation (Arnott *et al.*, 1976). Initial Cartesian coordinates for berenil were derived from an analysis of the crystal structures of berenil itself (Pearl *et al.*, 1987) and structurally related 1,3-diaryltriazenes (Walton, Jenkins and Neidle, unpublished data). The molecule was not considered to be symmetric about the central nitrogen atom, reflecting the non-equivalence of the 1,3-triazene nitrogen atoms.

Atomic partial charges for berenil were calculated using the semi-empirical MNDO method within the AMPAC (QCPE) program; additional geometric and force-field parameters were obtained by interpolation. Those for the DNA were taken from Weiner *et al.* (1984, 1986).

Docking of the berenil within the minor groove of the 8mer duplex was accomplished using the interactive molecular graphics program GEMINI (1989), implemented on a Silicon Graphics IRIS 4D/20G workstation. Structures (Table I) were generated for the seven possible modes of interaction involving both adjacent (1,2 bp models) and non-adjacent (1,3 bp models) bases but where a guanine was explicitly excluded from the 5′ → 3′ strand. Previous work (Jenkins and Neidle, unpublished data) has shown that the amino group at the 2-position in a spanned guanine prevents positioning of the ligand. Indeed, preferred models for this interaction favour guanines only in flanking regions. During these procedures berenil was positioned in such a way that favourable hydrogen bonds were formed between the berenil amidine NH donors and thymine (or cytosine) O2 or adenine N3 acceptor atoms, whilst minimizing the overlap of the van der Waals surface with that of the oligonucleotide.

Each of the bound complexes, together with the isolated DNA and drug,

was subjected to molecular mechanics full geometry minimization using an all-atom force field (Weiner *et al.*, 1986) of the form:

$$\Delta H = E_{\text{non-bonded}} + E_{\text{electrostatic}} + E_{\text{torsion}} + E_{\text{angle}} + E_{\text{bond}}$$

A distance-dependent dielectric function of the form $\epsilon = 4r_{ij}$ was used (Whitlow *et al.*, 1986; Orozco, Laughton, Herzyk and Neidle, to be published) to damp the strong electrostatic interactions which result from explicit exclusion of solvent in these models. All calculations were performed on a VAX 11/750 and Alliant FX40 computers using the EMPMDS program (Haneef, 1986). Convergence was judged to have been achieved during the energy refinements after at least 2000 cycles, when the r.m.s. gradient was ~0.15 kcal/mol Å in all cases.

The binding energy ($\Delta\Delta H$) for interaction of berenil with the oligonucleotide was calculated as

$$\Delta\Delta H = \Delta H_{\text{complex}} - (\Delta H_{\text{DNA}} + \Delta H_{\text{drug}}),$$

where each of the enthalpy terms represents the energy after minimization, as described.

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