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Hetero-association of caffeine and aromatic drugs and their competitive binding with a DNA oligomer

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Abstract NMR spectroscopy has been used to elucidate the molecular basis of the action of caffeine (CAF) on the complexation with DNA of mutagens such as ethidium bromide, propidium iodide, proflavine and acridine orange, and anticancer drugs such as actinomycin D and daunomycin. The hetero-association of CAF and each of the aromatic ligands in 0.1 mol L^{-1} phosphate buffer (pD = 7.1) has been investigated as a function of concentration and temperature by 500 MHz ^{1}H NMR spectroscopy and analysed in terms of a statistical-thermodynamic model, in which molecules form indefinite aggregates for both self-association and hetero-association. The analysis leads to determination of the equilibrium constants of hetero-association and to the values of the limiting chemical shifts of the hetero-association of CAF with each of the aromatic molecules. The hetero-association constants between CAF and each of the aromatic drugs/dyes are found to be intermediate in magnitude between those for self-association of CAF and the corresponding drug/dye. The most probable structures of the 1:1 CAF + ligand hetero-association complexes have been determined from the calculated values of the induced limiting chemical shifts of the drug protons. Knowledge of the equilibrium constants for self-association of CAF and the aromatic ligands, for their hetero-association and their complexation with a DNA fragment, the deoxytetranucleotide 5′-d(TpGpCpA), enabled the relative content of each of the CAF-ligand and CAF-ligand-d(TGCA) complexes to be calculated as a function of CAF concentration in mixed solutions. It is concluded that, on addition of CAF to the solution, the decrease in binding of drug or mutagen with DNA is due both to competition for the binding sites by CAF and the aromatic molecules, and to formation of CAF-ligand hetero-association complexes in the mixed solution; the relative importance of each process depends on the drug or mutagen being considered.

Keywords Caffeine · Aromatic drugs · NMR · Association · DNA binding

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Introduction

Investigations of the hetero-association of different molecules with conjugated aromatic planar rings and their competitive binding to receptors deal with some important aspects of molecular interactions. They are of interest not only from the physicochemical point of view, leading to determination of the influence of the structures of the chromophores and their side chains on association affinity, but also, from the pharmacological point of view, hetero-association complexes and competitive binding may influence the activity of drugs. Two widespread examples of this approach are the interaction of drugs with aromatic molecules from food sources(e.g. polyphenols, methylxanthines) and when drugs are used in combination (Alberts et al. 1981; Adel et al. 1993). Hence, it is important to investigate quantitatively the hetero-association of drugs (and drugs with aromatic molecules from food) in order to quantify the effect on the competitive binding of different aromatic molecules to receptors such as DNA (Pal and Ghosh 1995) or proteins (Dalmark and Johansen 1982; Wang et al. 1998). In the present work the influence has been examined of one of the most widely occurring "food" aromatic molecules, caffeine, on the intercalation of drugs and mutagens with DNA.

Caffeine (1,3,7-trimethylxanthine, CAF) and its derivatives are regularly consumed in sources such as tea,

coffee, cola beverages and chocolate (Spiller 1984). It is generally accepted that some of the biological activity of CAF results from its interaction with biopolymers, such as enzymes and nucleic acids (Witte and Bohme 1972; Fritszche et al. 1980; Kan et al. 1980; Selby and Sancar 1990). In cell systems, it has been shown that CAF is capable of reducing the toxicity of a typical DNA intercalator, ethidium bromide (Kimura and Aoyama 1989) and the efficacy of a number of aromatic anti-cancer drugs, such as doxorubicin and itsanalogues, and of novatrone, ellipticine and others (Ganapathi et al. 1979; Ross et al. 1979; Iliakis et al. 1986; Traganos et al. 1991a, 1991b). The effect of CAF on drug efficacy in cellular systems has been carried out with the drugs in the concentration range 0.01–1 mM and CAF in the concentration range 1–20 mM. From such work it was concluded (Traganos et.al. 1991a, 1991b; Kapuscinsky and Kimmel 1993; Larsen et al. 1996) that CAF forms complexes with aromatic molecules, which effectively lowers the concentration of free ligand andthereby reduces the biological activity of the drugs, i.e. it was assumed (Larsen et al. 1996) that CAF acts as an ''interceptor'' of biologically active aromatic molecules, which bind to DNA by intercalation. This hypothesis needs to be tested.

The hetero-association of CAF with different aromatic molecules has been investigated using different mathematical models and analytical procedures to interpret the experimental results (Weller et al. 1984; Aradi and Foldesi 1985, 1989; Kapuscinsky and Kimmel 1993; Chen and Shiao 1994; Baxter et al. 1996; Larsen et al. 1996). However, most of the proposed models of molecular hetero-association only cover limited sets of conditions and are not applicable to the general case. A model used to analyse optical spectroscopy measurements of CAF-drug complexation (Larsen et al. 1996) only considered formation of 1:1 hetero-complexes without taking into account the self-association of aromatic molecules in solution. Another model used to interpret optical spectroscopy data on the hetero-association of aromatic molecules took into consideration the monomer-dimer equilibrium (Kapuscinsky and Kimmel 1993) and in a recent paper (Zdunek et al. 2000) the indefinite association model was used for self-association ofone component, although the second component has negligible self-association under the conditions of the experiment. In order to achieve a good signal-to-noise ratio in quantitative NMR spectroscopy, relatively high concentrations (millimolar range) of analytes are required and models for NMR analysis need to take into account the possibility of formation of stacks higher than dimers for self-association and hetero-association (Martin 1996). A model developed for NMR investigations of the equilibrium association of CAF and methyl gallate (Baxter et al. 1996) is not satisfactory as it uses rather approximate expressions for the monomeric concentrations of one of the components in the mixed solution. Utilization of dimer models for molecular self-association in the analysis of the hetero-association of aromatic compounds (Aradi and Foldesi 1985, 1989) is confined to relatively small concentrations of the interacting molecules, as is the graphical method for determination of hetero-association parameters (Chen and Shiao 1994). A general statistical-thermodynamic model of mixed-association of two substances forming indefinite aggregates for both self-association and hetero-association was used by Weller et al. (1984) for NMR studies of the hetero-association of CAF and 5′-AMP; however, the equations used in this model do not allow the concentration of each type of complex to be calculated, which is necessary for the analysis of competitive binding. A similar statistical-thermodynamic model of hetero-association, which also takes into account formation of indefinite aggregates for both self-association and hetero-association of molecules, has recently been developed in our laboratory to provide analytical expressions for interpretation of NMR parameters of the interactingmolecules in mixed solutions. The model and analysis enables both the structural and thermodynamic properties of hetero-association complexes to be determined from measurements of proton chemical shifts of the molecules as a function of concentration and temperature (Davies et al. 1999). The model was tested for typical DNA intercalators, the phenanthridine drugs ethidium bromide (EB) and propidium iodide (PI), in which it was shown that the same results were given in two sets of experiments, one where the concentration of EB was constant and PI varied, and vice versa. The same model and analysis have been used in this work to determine the distinctive structural features and thermodynamic parameters of the hetero-association of CAFwith mutagenic agents such as EB and PI, two acridine dyes (proflavine, PF; acridine orange, AO) and with anticancer drugs such as actinomycin D (AMD) and the anthracycline antibiotic daunomycin (DAU).

In order to investigate the molecular basis of the ''protector'' or the ''interceptor'' action of CAF with respect to biologically active aromatic molecules which intercalate into DNA, it is necessary to calculate the relative content of each of the ''ligand-DNA'' complexes in the presence of CAF. This requiresknowledge of the equilibrium constants of self-association of CAF and the different ligands, the equilibrium constants for their hetero-association and also the equilibrium constants for their complexation with DNA (Veselkov et al. 2000). As a structural and thermodynamic analysis of the complexation of a number of aromatic ligands (EB, PI, PF, DAU and CAF) has been carried out previously with the same deoxytetranucleotide, 5′-d(TpGpCpA), in 0.1 M phosphate buffer (pD 7.1, $T = 298$ K) (Davies et al. 1996b, 2000; Eaton et al. 1998), we report, in this work, the NMR analysis of the self-association of CAF and its hetero-association with different aromatic ligands (EB, PI, PF, AO, DAU and AMD) under the same experimental conditions. The results enable the distinctive features of the complexation of aromatic ligands with a DNA oligomer to be calculated in themixed solution at different CAF concentrations (up to the CAF:ligand ratio of 100:1 covered in the experiments

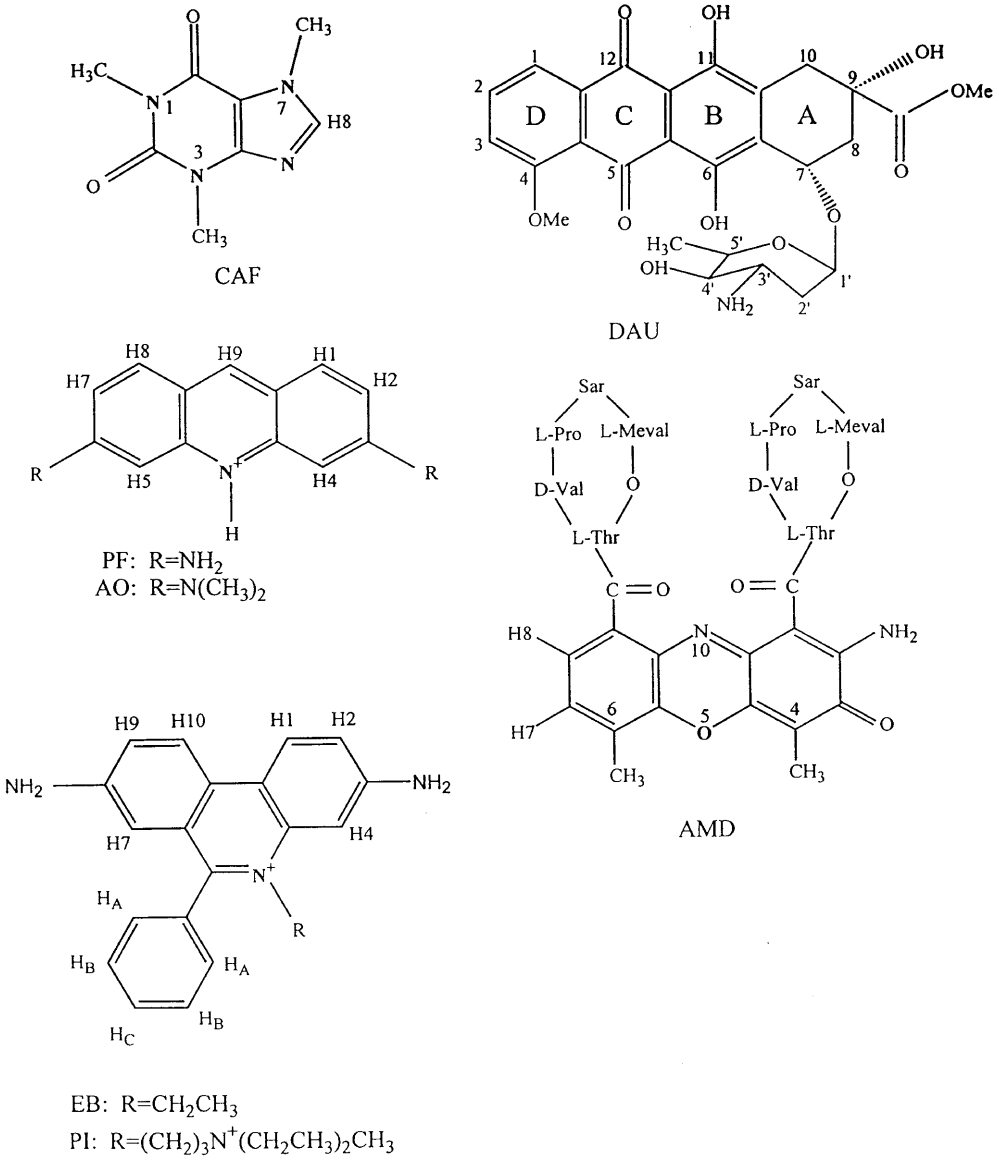

Fig. 1 Structures of caffeine (CAF) and aromatic ligands: proflavine (PF) and acridine orange (AO); ethidium bromide (EB) and propidium iodide (PI); daunomycin (DAU); actinomycin D (AMD)

with cells), thus giving insight into the molecular basis of the action of caffeine as a protector of DNA against intercalating drugs and mutagens in cell systems.

Materials and methods

Caffeine and the aromatic ligands (structures presented in Fig. 1) were purchased from Sigma (EB, PI, PF, AO and AMD) or Fluka (DAU) and were used without further purification. The samples were lyophilized from D_2O solutions and re-dissolved in 0.1 M phosphate buffer in 99.95% D_2O (pD 7.1) containing 10^{-4} M EDTA. The concentrations of the stock solutions of the aromatic molecules were measured spectrophotometrically on appropriate dilution using the following molar extinction coefficients: $\epsilon = 9740$ L mol^{-1} cm^{-1} ($\lambda = 273$ nm) for CAF (Lilley et al. 1992); $\epsilon = 5860$ L mol^{-1} cm^{-1} ($\lambda = 480$ nm) for EB (Bresloff and Crothers 1981); $\epsilon = 5900$ L mol^{-1} cm^{-1} ($\lambda = 493$ nm) for PI (Patel and Canuel 1977); $\epsilon = 4.1\times10^4$ L mol^{-1} cm^{-1} ($\lambda = 444$ nm) for PF (Albert 1966); $\epsilon = 5.6\times10^4$ L mol^{-1} cm^{-1} ($\lambda = 492$ nm) for AO (Stone and Bradley 1961); $\epsilon = 2.45\times10^4$ L mol^{-1} cm^{-1} ($\lambda = 440$ nm) for AMD (Chen et al. 1993); $\epsilon = 11500$ L mol^{-1} cm^{-1} ($\lambda = 477$ nm) for DAU (Huang and Phillips 1977; Chaires et al. 1982).

500 MHz ^{1}H NMR spectra were recorded on a Bruker DRX spectrometer with the residual water peak saturated during relaxation. Signal assignments of the non-exchangeable protons of the drugs were obtained previously using both two-dimensional homonuclear COSY (TOCSY) and NOESY (ROESY) experiments (Davies et al. 1996a, 2000). Chemical shift measurements of the non-exchangeable protons of the aromatic molecules were made as a function of concentration at two temperatures (298 and 308 K, but only 298 K for the CAF + AMD system) and measurements were made at one concentration as a function of temperature in the range 278–353 K; the three independent sets of NMR measurements were all made in the fast-exchange condition on the NMR timescale. It was shown previously (Altona et al. 1976) that the chemical shift of tetramethylammonium bromide (TMA) is essentially independent of pH, ionic strength and temperature and so chemical shifts were measured relative to TMAas an internal reference and recalculated with respect to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate), i.e. $\delta_{DSS} = \delta_{TMA} + 3.178$ (ppm). The sample temperature was regulated using the Bruker BVT-3000 unit.

For each system measured, each of three NMR data sets consisted of a minimum of 14 experimental observations

(i.e. concentration or temperature dependence) for different numbers of protons for each aromatic molecule: AMD (3), CAF (4), AO/PF (4), EB/PI (6). For CAF self-association there were data from 16 concentrations to calculate K, δ_m and δ_c (see below) independently for each of four protons at 298 and at 308 K and data from four CAF protons at 16 different temperatures to calculate ΔH and ΔS, i.e. a total of 192 data points. For calculations on the hetero-association of CAF with AMD there is a minimum of 196 (2×14×7) data points, for CAF with AO or PF a minimum of 360 (3×15×8) data points, and for CAF with EB/PI a minimum of 450 (3×15×10) data points.

The calculated fits of the models of the equilibrium reactions to the experimental chemical shift data were assessed by the magnitude of the discrepancy function, Δ:

$$\Delta = \sum_{i=1}^{n} (\delta_{ei} - \delta_i)^2 \quad (1)$$

where n is the number of experimental points; δ_{ei} and δ_i are the experimental drug proton chemical shifts for the i-th concentration or temperature and the calculated values using the theoretical models (Veselkov et al. 1985). Minimization of Δ was performed using initial approximations produced by the law of accidental numbers over a wide range of variations of the parameters. A large statistical set of data enabled the whole field of the possible values of minimization parameters to be investigated over a wide range of their variations, in order to determine the global and not a local minimum. The values of Δ obtained in the calculations are in the range 10^{-5}–10^{-6} for at least 14 experimental points in each data set, which corresponds to an average deviation between observed and modelled chemical shifts in the range 0.002–0.0003 ppm, assuming an error of 0.001 ppm in the measurements of the chemical shifts; these results indicate a very good fit to the experimental data. Magnitudes of the equilibrium constants were calculated for each proton independently and the average value and mean deviation determined.

The structures of the CAF dimer and 1:1 hetero-association complexes have been determined by analysis of the calculated values of induced proton chemical shifts in the dimer, $\delta\delta = (\delta_m - \delta_d)$, or in the hetero-complex, $\delta\delta = (\delta_m - \delta_c)$, where δ_m, δ_d and δ_c are the drug chemical shifts in the monomer, dimer and 1:1 hetero-complex forms, respectively. The mutual orientation of the molecules in the dimers or 1:1 complexes were determined by comparison of the induced proton chemical shifts and their theoretical values, as described in previous work (Davies et al. 1996a; Davies and Veselkov 1996). The theoretical values of the induced proton chemical shifts were derived from quantum-mechanical calculations of iso-shielding curves for aromatic molecules given by Giessner-Prettre and Pullman (1987). The discrepancy between experimental and theoretical values of induced proton chemical shifts of the molecules in the complexes did not exceed 5%. The spatial representation of structures was obtained with the help of Mathematica 2.2 software (Wolfram Research). Although the structures derived from limiting chemical shifts may be averages over several different geometries, calculations from the NMR data have been made assuming one structure, termed "the most probable" structure in this work.

Results

Self-association of CAF in aqueous solution

As a pre-requisite to complete analysis of the hetero-association of CAF with the different drugs, it is necessary to determine the thermodynamic characteristics of self-association of all the molecules in solution. Self-association of all the aromatic ligands, except CAF, has been studied previously under the same solution conditions and the published values of the self-association constants and thermodynamic parameters for EB, PI, PF, AO, AMD and DAU (Davies et al. 1996a, 1999, 2000) are summarized in Table 1. In order to check the reproducibility of the method and analysis for subsequent investigations of hetero-association, the NMR measurements of the self-association of AO and PF have been repeated in the present work and the results are the same,within error limits, as those previously published (Davies et al. 1996a). Determination of the structure and thermodynamics of the self-association of CAF under the same solution conditions as for the other ligands (0.1 M phosphate buffer, pD 7.1, $T = 298$ K) is reported in this work. The experimental concentration dependences of the chemical shifts of four CAF protons (not shown) at 16 different concentrations have been analysed, as in previous work (Baxter et al. 1996; Davies et al. 1996a; Veselkov et al. 1985), using the indefinite non-cooperative association model, in which the equilibrium constants K_j are assumed to be equal for the equilibria:

Table 1 Self-association parameters of aromatic ligands[a]

Ligand	K (L mol^{-1})[b]	K (L mol^{-1})[c]	σ[c]	$-\Delta H°$ (kJ mol^{-1})	$-\Delta S°$ (J mol^{-1} K^{-1})	Ref
EB	305 ± 14	347 ± 18	0.89 ± 0.06	23.4 ± 3.3	31 ± 5	Davies et al. (1996a)
PI	63 ± 6	67 ± 6	0.98 ± 0.05	26.2 ± 5.5	54 ± 16	Davies et al. (1999)
PF	698 ± 68	1050 ± 100	0.42 ± 0.06	46.0 ± 8.4	101 ± 17	Davies et al. (1996a)
AO	4600 ± 600	6900 ± 460	0.45 ± 0.05	38.1 ± 4.6	57 ± 8	Davies et al. (1996a)
AMD	1440 ± 160[d]	1400 ± 100	1.49 ± 0.1	31.8 ± 6.3	47 ± 11	Davies et al. (1996a)
DAU	720 ± 130	580 ± 120	1.34 ± 0.06	34.0 ± 6.0	60 ± 15	Davies et al. (2000)
CAF	11.8 ± 0.3	10.5 ± 0.6	1.08 ± 0.02	21.0 ± 0.4	50 ± 1	This work

[a]Determined from NMR measurements in 0.1 mol L^{-1} phosphate buffer solutions, pD 7.1, T = 298 K. The results are averages for three proton signals of AMD, four for AO, PF and CAF, six for EB and PI and eight for DAU. The self-association parameters for CAF, AO and PF were determined in this work. The results for AO and PF were the same, within error limits, as those published previously

[b]Calculated using the indefinite non-cooperative model in which the dependence of chemical shift δ on concentration x is given by: $\frac{\delta-\delta m}{\delta i-\delta m} = \left(\frac{2Kx+1-\sqrt{4Kx+1}}{2Kx}\right)$ where δ_i is the proton chemical shift for the CAF molecule in the aggregate and δ_m is the proton chemical shift of the monomer, i.e. at infinite dilution (Davies et al. 1996a)

[c]Calculated using the cooperative model in which the dependence of the observed proton chemical shift δ on concentration x_0 is given by: $\frac{\delta-\delta m}{\delta i-\delta m} = 1 - \frac{x_1}{x_0} - \frac{\sigma K x_1^2}{x_0(1-Kx_1)}$ where δ_m, δ_i are the proton chemical shifts in the monomer and in the molecules situated inside the aggregate, respectively; x_1 is the monomer concentration of the drug (Davies et al. 1996a)

[d]Calculated using the dimer model for self-association of AMD, since previous work using equilibrium centrifugation measurements (Crothers et al. 1968) has shown that AMD aggregates only to dimers in aqueous solution

$$X_j + X \overset{K_j}{\longleftrightarrow} X_{j+1} \quad (2)$$

The mean value of the self-association constant, $K = 11.8 \pm 0.3$ L mol^{-1}, for the four non-exchangeable protons of CAF at $T = 298$ K, presented in Table 1, is in good agreement with the K values determined previously for similar solution conditions using the indefinite association model (Fritszshe et al. 1980; Lilley et al. 1992; Kapuscinsky and Kimmel 1993; Baxter et al. 1996).

A previous study of CAF self-association by IR spectroscopy (Falk et al. 1990) suggested that the dimerization constant K_1 is substantially larger than the equilibrium constants (K_2, K_3, etc.) for formation of higher order aggregates. In order to test this conclusion and to estimate the probability of formation of complexes of higher order than dimers, the experimental results have also been analyzedusing the indefinite cooperative model of molecular self-association (Davies et al. 1996a), where the equilibrium constants of reactions (2) are assumed to be equal for all $j \geq 2$ ($K_2 = K_3 = ... = K_j = K$), $K_1 = \sigma K$, where σ is the cooperativity coefficient. [The case $\sigma = 1$ corresponds to the non-cooperative model considered above. For $\sigma < 1$ the system is cooperative, i.e. when dimer formation creates energetically favourable conditions for subsequent molecular association. When $\sigma > 1$ the self-association process is anti-cooperative.] The mean values of the calculated parameters K and σ for the four CAF protons are presented in Table 1. It can be seen that the cooperativity parameter, σ, is close to one for CAF, indicating that self-association of these molecules is non-cooperative, i.e. the dimerization constant K_1 is equal to the equilibrium constants for formation of higher order associates (K_2,K_3, ..., K_j). This resultis contrary to the conclusions made from the IR study (Falk et al. 1990), but it confirms the use of the indefinite non-cooperative model, which was previously assumed for self-association of CAF in aqueous solution (Fritszshe et al. 1980; Baxter et al. 1996).

The thermodynamic parameters for molecular self-association of CAF were determined, as previously (Davies et al. 1996a), from the experimental temperature dependence of the four proton chemical shifts of the CAF molecules (not shown), using the additive model for the observed proton chemical shift and van't Hoff's formalism. The calculated values of enthalpy (ΔH) and entropy (ΔS) of CAF self-association, presented in Table 1, are somewhat higher than values obtained in previous work (Horman and Dreux 1984; Lilley et al. 1992), which may be due to different solution conditions used in the experiments.

The mutual orientation of CAF molecules in the dimer was determined by quantitative comparison of $\Delta\delta$ and their theoretical values derived from quantum-mechanical calculations of iso-shielding curves for CAF (Kan et al. 1980). Within the limits of the model of molecular association, the assumption about the equal influence of neighbouring molecules is generally accepted, i.e. $(\delta_i - \delta_m) = 2(\delta_d - \delta_m)$ (Mitchell and Sigel 1978), where δ_i is the proton chemical shift in the aggregate. The calculated most probable structure of the CAF dimer has the planes of the molecules parallel to each other and situated 0.34 nm apart with a twist angle $\psi \approx 90°$, which is in good agreement with the structures obtained previously by NMR spectroscopy (Fritszshe et al. 1980; Kan et al. 1980), by Monte Carlo simulations (Danilov and Shestopalova 1989), and the weighted average ($\psi \approx 93°$) of the nine conformations of comparable energy determined by recent NMR and molecular modelling studies (Falk et al. 1998).

Hetero-association of CAF with aromatic drug molecules

The structural and thermodynamic parameters for the formation of complexes between CAF and the aromatic ligands (EB, PI, PF, AO, AMD, DAU) havebeen determined from measurements of the proton chemical shifts of both compounds in mixed solutions as a function of concentration and temperature. Measurements were made by keeping the CAF concentration constant and changing the content of the aromaticligand in solution; this experimental procedure was adopted because of the substantially higher magnitude of the self-association constant of all the ligands compared with CAF (Table 1), and therefore changes of ligand concentration affects the equilibrium distribution of the aggregates to a greater extent than keeping the ligand constant and varying the concentration of CAF in solution. The dependence of hetero-association chemical shifts on concentration and temperature are shownin Fig. 2a and b, respectively, using one of the systems studied, CAF + PF, as an example. All signals move to low frequency with increasing the ratio of PF:CAF (Fig. 2a) and with decreasing temperature (Fig. 2b), consistent with increasing aggregation due to stacking of aromatic molecules in solution. Similar curves (not shown) for the concentration dependence of chemical shifts were also observed at 308 K (analogous sets of curves are observed for hetero-association of all the drugs with CAF).

In the general model of molecular hetero-association (Davies et al. 1999) there is a dynamic equilibrium that includes indefinite self-association as well as indefinite hetero-association reactions of different types, as shown in the following scheme:

$$\begin{aligned}
&A_1 + A_i \overset{K_A}{\longleftrightarrow} A_{i+1} && \text{(a)}\\
&P_1 + P_j \overset{K_P}{\longleftrightarrow} P_{j+1} && \text{(b)}\\
&P_j + A_i \overset{K_{het}}{\longleftrightarrow} P_j A_i && \text{(c)}\\
&P_j A_i + P_l \overset{K_{het}}{\longleftrightarrow} P_j A_i P_l && \text{(d)}\\
&A_k + P_j A_i \overset{K_{het}}{\longleftrightarrow} A_k P_j A_i && \text{(e)}
\end{aligned} \quad (3)$$

where A_1 and P_1 correspond to the monomers of ligand and CAF, and A_i, A_k, P_j, P_l are the aggregates con-

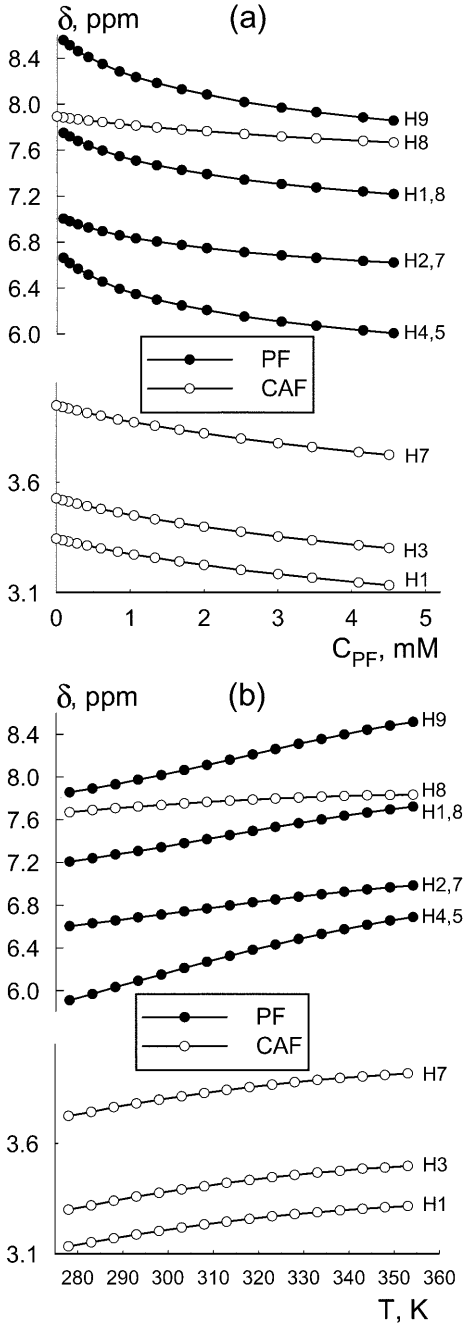

Fig. 2a, b Hetero-association of PF + CAF in 0.1 M phosphate buffer, pD 7.1. Changes of chemical shifts on: **a** concentration of PF ($CAF_0 = 2.0$ mM = const), $T = 298$ K; **b** temperature ($PF_0 = 2.5$ mM, $CAF_0 = 2.0$ mM). The error limits of the measurements and analysis (± 0.002 ppm) are much smaller than the symbols used to plot the experimental points. The fitted curves for concentration (and temperature) dependence of chemical shifts were calculated using Eqs. (4) and (5) and derived values of K_{het} and δ_c (and ΔH, ΔS) listed in Table 2

taining i, k monomers of the ligand and j, l monomers of CAF, respectively. The equilibrium constants for the self-association reactions of the ligands (K_A) and CAF (K_P) and for the hetero-association of drug molecules (K_{het}) are assumed to be independent of the number of molecules in the aggregates and complexes. As the self-association constant for CAF, K_P, is substantially smaller than K_A for the ligands (Table 1), estimates show that the hetero-complexes, $A_kP_jA_i$, where P_j aggregates of CAF are flanked by the ligand aggregates (A_k and A_i), are unlikely to form in solution, and consequently Eq. (3e) is neglected in the present case.

The dependence of observed proton chemical shifts of the ligand molecule for hetero-association between CAF and ligand can be written in the form (Davies et al. 1999):

$$\delta_A = \frac{[a_1]}{[a_0]}\left\{\delta_{mA}\left[2(1+K_A[a_1]) - \frac{1}{(1-K_A[a_1])^2}\right] + 2\delta_{dA}\left[\frac{1}{(1-K_A[a_1])^2} - 1 - K_A[a_1]\right] + \frac{\delta_{cA}K_{het}[p_1]}{(1-K_A[a_1])^2(1-K_P[p_1])}\left[1+\frac{K_{het}[p_1]}{(1-K_P[p_1])}\right]\right\} \quad (4)$$

and the corresponding expression for CAF is given by:

$$\delta_P = \frac{[p_1]}{[p_0]}\left\{\delta_{mP}\left[2(1+K_P[p_1]) - \frac{1}{(1-K_P[p_1])^2}\right] + 2\delta_{dP}\left[\frac{1}{(1-K_P[p_1])^2} - 1 - K_P[p_1]\right] + \frac{\delta_{cP}K_{het}[a_1]}{(1-K_P[p_1])^2(1-K_A[a_1])}\left[1+\frac{K_{het}[p_1]}{(1-K_P[p_1])^2}\right]\right\} \quad (5)$$

The values of δ_{mA}, δ_{dA}, δ_{mP}, δ_{dP} and the equilibrium constants K_A and K_P (Table 1) have been determined previously (Davies et al. 1996a, 1999, 2000). It follows that theobserved concentration dependence of the proton chemical shifts of the ligand and CAF in mixed solutions (e.g. Fig. 2a) is a function of two unknown quantities, δ_c and K_{het}, which have been determined using the computational procedure described previously (Davies et al. 1999). The magnitudes of the calculated parameters K_{het} and δ_c at 298 K summarized in Table 2 are averages of values calculated for each of the ligand and CAF protons listed, e.g. the average of eight independent determinations for CAF + PF. The values of the discrepancy function obtained in the calculations (e.g. $\Delta = 5.3\times10^{-5}$ for $T = 298$ K) indicate a very good fit to the experimental data. The reliability of the analysis can be seen, for example, for curves of the concentration dependences of chemical shifts in Fig. 2a, which were calculated using Eqs. (4) and (5) and the derived values of K_{het} and δ_c listed in Table 2. A similar good fit of experimental and calculated dependence of chemical shifts on concentration was found for complexation of CAF with all the aromatic ligands summarized in Table 2.

Table 2 Hetero-association parameters of CAF and aromatic ligands (in 0.1 mol L^{-1} phosphate buffer solutions, pD 7.1, $T = 298$ K)[a,b]

Proton of ligand	δ_c (ligand) (ppm)	Proton of CAF	δ_c (CAF) (ppm)	K_{het} ($L\ mol^{-1}$)	$-\Delta H°_{het}$ ($kJ\ mol^{-1}$)	$-\Delta S°_{het}$ ($J\ mol^{-1}\ K^{-1}$)
EB + CAF[c,d]						
H1	7.76	H8	7.09	–	–	–
H10	7.70	H7	2.79	–	–	–
H9	7.25	H3	2.44	62 ± 4	22.7 ± 3.0	42 ± 11
H4	7.09	H1	2.07	–	–	–
H2	7.08	–	–	–	–	–
H7	6.37	–	–	–	–	–
PI + CAF						
H1	7.70	H8	7.09	–	–	
H10	7.21	H7	2.75	–	–	
H9	6.96	H3	2.35	28 ± 5	21.1 ± 3.6	43 ± 13
H4	6.95	H1	1.82	–	–	–
H2	7.03	–	–	–	–	–
H7	6.15	–	–	–	–	–
PF + CAF						
H9	8.16	H8	7.03	–	–	–
H(1,8)	7.54	H7	3.08	–	–	–
H(2,7)	6.91	H3	2.66	160 ± 17	24.4 ± 0.5	40 ± 7
H(4,5)	6.19	H1	2.54	–	–	–
AO + CAF[c]						
H9	8.24	H8	6.83	–	–	–
H(1,8)	7.55	H7	2.90	–	–	–
H(2,7)	7.09	H3	2.50	264 ± 21	20.4 ± 1.0	22 ± 4
H(4,5)	6.15	H1	2.32	–	–	–
AMD + CAF						
H(7,8)	7.46	H8	7.40	–	–	–
4-CH_3	2.34	H7	3.39	–	–	–
6-CH_3	1.58	H3	3.12	246 ± 48	27.2 ± 4.3	49 ± 12
	–	H1	2.88	–	–	–
DAU + CAF						
	–	H8	6.55	–	–	–
	–	H7	2.59	72 ± 4	23.5 ± 1.2	43 ± 4
	–	H3	2.06	–	–	–
	–	H1	1.81	–	–	–

[a]The magnitudes of the hetero-association parameters for CAF with each of the ligands are averages of values for each listed proton of the ligand and CAF

[b] ^1H NMR measurements of the hetero-association between CAF and the aromatic ligands have also been measured at $T = 308$ (not presented). The hetero-association constants calculated using these data are: EB + CAF, 43 ± 4 L mol^{-1}; PI + CAF, 21 ± 3 L mol^{-1}; PF + CAF, 107 ± 14 L mol^{-1}; AO + CAF, 191 ± 10 L mol^{-1}; DAU + CAF, 53 ± 3 L mol^{-1}; the values are consistent with the $\Delta H°_{het}$ and $\Delta S°_{het}$ values determined from variable temperature experiments

[c]The values in previous work (Larsen et al. 1996) for the hetero-association constants for the EB + CAF and AO + CAF systems calculated from optical spectroscopy measurements at ambient temperature using a 1:1 hetero-association model are: $K_{CAF+EB} = 85 \pm 4$ and $K_{CAF+AO} = 258 \pm 5$ L mol^{-1}

[d]The value in previous work (Zdunek et al. 2000) for the hetero-association constant for EB + CAF is 64.6 ± 2 L mol^{-1} (0.1 M NaCl at $T = 298$ K) using optical spectroscopy measurements and the indefinite association model for EB self-association

For each of the drugs it can be seen from Table 2 that the magnitude of the hetero-association constant (K_{het}) is intermediate between the values of the self-association constant for CAF ($K_P \approx 12$) and for the respective drugs (Table 1). The magnitude of K_{het} for CAF + AO determined in this work (264 ± 21 L mol^{-1}) at 298 K is within experimental error of that observed previously (258 ± 5 L mol^{-1}) at ambient temperature by optical spectroscopy (Larsen et al. 1996). The value for CAF + EB in this work, 62 ± 4 L mol^{-1}, is in good agreement with recent results by optical spectroscopy measurements using the indefinite association model (Zdunek et al. 2000), but is somewhat smaller than that determined previously, 85 ± 4 L mol^{-1} (Larsen et al. 1996) using a 1:1 hetero-association model. It should be noted that equilibrium centrifugation measurements of the self-association of AMD have shown (Crothers et al. 1968) that there is no detectable formation of aggregates higher than dimers even at concentrations approaching saturation, probably as a result of steric interactions due to the large pentapeptide lactone side chains. The experimental results for the CAF-AMD system were analyzed, in this work, using the dimerization model for AMD self-association (i.e. in Eq. 3, $i = 1$). Calculations of the hetero-association of CAF and AMD were also made using higher order aggregates than dimers for AMD. In this case the calculated discrepancy function Δ was larger for the indefinite association model compared

to the dimer model for AMD (and the K value was 20% smaller), providing supporting evidence that the dimerization model for AMD is sufficient for hetero-association complexation. On the other hand, use of the indefinite association model for all aromatic ligands other than AMD gave significantly smaller values for the discrepancy function than when the dimer model was used for hetero-association with CAF.

The thermodynamic parameters $\Delta H°_{het}$ and $\Delta S°_{het}$ for hetero-association of CAF with different aromatic ligands were determined from measurements of the proton chemical shifts of the molecules in the mixed solution as a function of temperature using the additive model for the experimental proton chemical shifts, as in previous work (Davies et al. 1999). The error limits of the measurements are smaller than the symbols used to plot the experimental points in Fig. 2b. The corresponding van't Hoff's plots, $lnK = f(1/T)$, for the systems studied are presented in Fig. 3. The data plotted in Fig. 3 are the averages of the calculated parameters for different protons at each temperature. The data were fitted with a linear least-squares program and the derived values of enthalpy and entropy of the hetero-association of CAF with different aromatic ligands in solution are summarized in Table 2. It is found that the enthalpies of formation of the hetero-complexes are intermediate in magnitude between the values of $\Delta H°$ for self-association of CAF and the aromatic ligands in aqueous solution (Table 1).

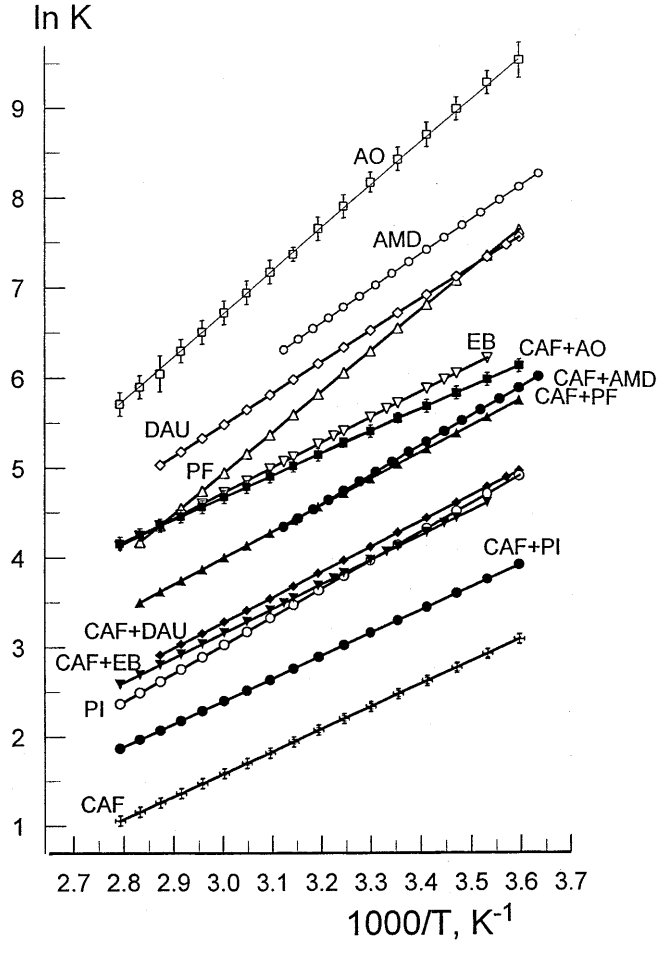

Fig. 3 Van't Hoff plot of ln$K$ vs. $1/T$ for formation of CAF dimer (*crosses*), ligand dimer (*open symbols*) and for formation of hetero complexes (*filled symbols*) in solution: CAF + EB, CAF + PI, CAF + PF, CAF + AO, CAF + DAU, CAF + AMD. Owing to the scale factor the magnitude of the error limits in K for self-association of AO is shown in the van't Hoff's plot as it is greater than that for the other ligands in Table 1. The error limits in self-association K for CAF are smaller than the symbols used to plot the experimental data. The resulting error limits in K_{het} for CAF + AO are represented by vertical lines on each data point

It should be noted that the fitted curves for the temperature dependence of the chemical shifts in Fig. 2b were calculated using Eqs. (4) and (5) and derived values of K_{het}, δ_c, ΔH and ΔS listed in Table 2. The calculated discrepancy function between experimental and calculated values was within the experimental error of the measurements (± 0.002 ppm). Error limits in K values as a function of temperature are shown, as an example, for self-association reactions of CAF and AO and the hetero-association of CAF and AO in Fig. 3. The error limits for CAF self-association are smaller than the symbols used to plot the results, whereas those for AO are for the largest self-association constant of all the aromatic ligands listed in Table 1. The resulting error limits in K_{het} for CAF + AO are only slightly larger than the symbol used to represent the data point at all temperatures, as shown in Fig. 3.

Structure of the CAF-drug hetero-association complexes

Attempts to investigate the structures of the CAF-ligand hetero-association complexes by 2D NOE (or ROE) experiments were not successful, because no intermolecular cross-peaks were observed in NOESY (or ROESY) spectra of mixed solutions of CAF and the aromatic drugs. This is likely to be a consequence of the relatively small hetero-association constants and the existence of a complex distribution of different self-association and hetero-association complexes in mixed solution which lead to relatively small intensities of the cross-peaks. Hence, the most probable structures of the 1:1 CAF-ligand hetero-association complexes were determined in the same way as for the CAF dimer using the limiting proton chemical shifts, δ_c, of both CAF and the ligands (Table 2). Utilization of iso-shielding curves for both CAF (Kan et al. 1980) and the ligand (Giessner-Prettre and Pullman 1987) enables the structure of the hetero complex to be determined with greater accuracy than the structure of the self-associated dimer in aqueous solution. The calculated most probable structures of the 1:1 hetero complexes of CAF with various aromatic ligands (PF, PI, DAU and AMD) in Fig. 4 show that the planes of the chromophores of the aromatic ligands and the CAF molecule are parallel to each other (situated 0.34 nm apart) and that there is extensive overlap

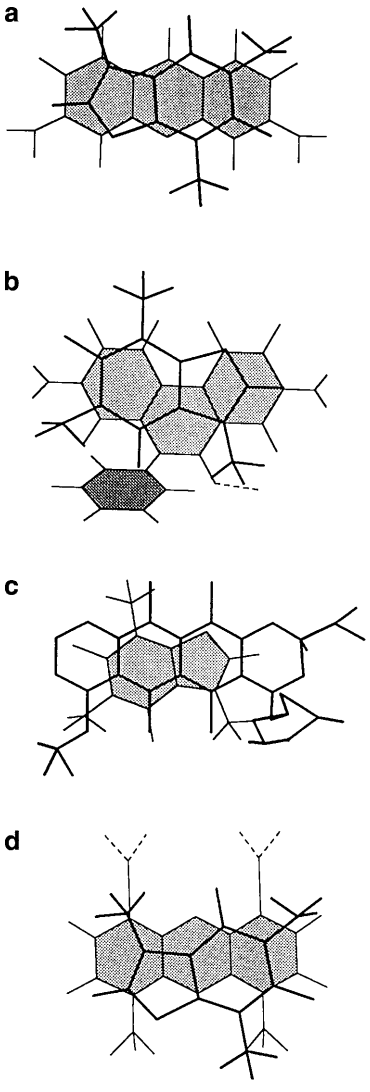

Fig. 4a–d Views of the calculated most probable NMR structures of 1:1 CAF-ligand hetero-association complexes looking perpendicular to the planes of the drug chromophore: **a** CAF + PF; **b** CAF + PI; **c** CAF + DAU; **d** CAF + AMD

of the aromatic rings, indicating a substantial role of dispersive interactions when the hetero complex is formed. The calculated structures of CAF-EB and CAF-AO complexes (not shown) are in good agreement with the structures of the hetero complexes for the same molecular systems determined by molecularmodelling calculations (Larsen et al. 1996).

The presence of CAF in the mixed solution with DAU has practically no effect on the chemical shift of the non-exchangeable protons in rings A and D of the chromophore of DAU, and therefore it is possible that the CAF molecules in the hetero complexes with DAU are stacked with the central aromatic rings (rings B and C), which do not contain any protons from which the structure may be determined. Hence, calculation of the hetero-association parameters for CAF with DAU was carried out, in the main, using the experimental concentration dependences of the CAF proton chemical shifts in the mixed solution. The structure determined for the CAF + DAU hetero complex (Fig. 4c) is in reasonable agreement with theoretical calculations (Larsen et al. 1996) of the structure of the hetero complex of CAF with doxorubicin, which is a near analogue of DAU.

Discussion

Hetero-association of CAF with different aromatic ligands in aqueous solution

It is seen from Table 2 that, on binding with CAF, the magnitudes of the hetero-association constant K_{het} differ substantially for the aromatic ligands with different chromophore structures and side groups, e.g. the hetero-association constants of CAF with the phenanthridine drugs EB and PI are smaller than for CAF with the acridine drugs PF and AO. As shown in Fig. 4, the centres of all the aromatic rings in the acridine chromophore are situated on a straight line, enabling more extensive overlap of CAF with the acridine rings in the hetero complex compared with the phenanthridine chromophore, where the centres of the aromatic rings are not collinear. Hence, it is likely that there are greater contributions of dispersive interactions of the molecules in 1:1 PF-CAF (AO-CAF) complexes than in the EB-CAF (PI-CAF) hetero complexes. The side chains of the chromophores may also influence the probability of hetero complex formation owing to additional intermolecular interactions and steric factors, i.e. by comparison of K_{het} of CAF + PI with CAF + EB and comparison of CAF + PF with CAF + AO (Table 2). The thermodynamic parameters determined for these molecular systems are consistent with this result (Table 2), i.e. comparison of $\Delta H°$ and $\Delta S°$ values for hetero-association of CAF with PF or AO indicates different contributions of dispersive interactions (more negative $\Delta H°$ value for PF-CAF compared to AO-CAF complex formation) and hydrophobic interactions (more positive $\Delta S°$ value for AO-CAF compared to PF-CAF complex formation), because AO contains hydrophobic methyl side groups compared to PF (Fig. 1).

Relative content of "ligand-DNA" complexes in the presence of CAF in aqueous solution

Calculation of the relative content of "ligand-DNA" complexes in the presence of CAF in the solution can be made from a knowledge of the equilibrium constants for self- and hetero-association of CAF and the aromatic ligands (Tables 1, 2) and their complexation with deoxyoligonucleotides obtained under the same experimental conditions (Table 3) (Veselkov et al. 2000). This, in turn, should lead to an understanding of the molec-

Table 3 Summary of the equilibrium constants for complex formation between different ligands and the deoxytetranucleotide 5′-d(TGCA)[a,b]

Ligand	K_1 (10^{-3} L mol^{-1})	K_2 (10^{-3} L mol^{-1})	K_3 (10^{-3} L mol^{-1})	K_4 (10^{-3} L mol^{-1})	K_5 (10^{-3} L mol^{-1})
EB[c]	27 ± 6	72 ± 9	10 ± 3	141 ± 25	0.6 ± 0.3
PI[d]	36 ± 6	189 ± 24	19 ± 4	101 ± 28	0.7 ± 0.2
PF[e]	1.2 ± 0.2	15 ± 2	7 ± 1	17 ± 3	1.5 ± 0.7
DAU[f]	53 ± 13	560 ± 13	7 ± 1	19 ± 1	–[g]
CAF[d]	0.034 ± 0.004	0.246 ± 0.018	0.060 ± 0.010	0.042 ± 0.015	0.016 ± 0.006

[a]Determined from NMR measurements in 0.1 mol L^{-1} phosphate buffer solutions, pD 7.1, T = 298 K

[b]In previous work (Davies et al. 1996b; Eaton et al. 1998; Davies et al. 2000; Veselkov et al. 2000) on drug-oligonucleotide complexation, a number of possible reaction schemes were considered prior to the general scheme in Eq. (7). If only K_1 and K_2 reactions are considered, the fit of the data is not good (the discrepancy function Δ is ca. 10^{-4}, corresponding to chemical shift differences of ca. 0.01 ppm). The discrepancy function Δ decreased progressively for successive addition of reactions governed by K_3, K_4, K_5, where it stabilized at ca. 10^{-6}, which corresponds to chemical shift differences of ca. 0.001 ppm, the limit of experimental error. Addition of further reactions did not provide a better fit

[c]Davies et al. (1996b)

[d]Veselkov et al. (2000)

[e]Eaton et al. (1998)

[f]Davies et al. (2000)

[g]The value of this association constant turned out to be negligible and was not included in the calculations

ular basis for the influence of CAF on the efficacy of intercalative binding into DNA of biologically active aromatic molecules in aqueous solution. All of the models of reactions used in subsequent calculations have been published previously (Davies et al. 1996a, 1996b, 1999).

Quantitative analysis of the complexation of each of the ligands (A)[1] with the DNA fragment, 5′-d(TpGpCpA), has been investigated in our laboratory (Davies et al. 1996b, 2000; Eaton et al. 1998) using the same general model summarized in Eq. (7). In addition to the self-association reactions of the ligand (Eq. 2) and oligonucleotide:

$$N + N \overset{K_N}{\longleftrightarrow} N_2 \tag{6}$$

complexation reactions were included for the ligand with both the single-stranded (N) and double-stranded (N_2) form of the oligonucleotide:

$$\begin{array}{ll} \text{(a) } A + N \overset{K_1}{\longleftrightarrow} AN & \text{(b) } A + N_2 \overset{K_2}{\longleftrightarrow} AN_2 \\ \text{(c) } A + AN \overset{K_3}{\longleftrightarrow} A_2N & \text{(d) } A + AN_2 \overset{K_4}{\longleftrightarrow} A_2N_2 \\ \text{(e) } AN + N \overset{K_5}{\longleftrightarrow} AN_2 & \end{array} \tag{7}$$

where A and N are the monomer forms of the ligand and tetranucleotide, respectively. In Eq. (7) the possibility of 1:2 complex formation of the drug molecule A (CAF or ligand) with the tetranucleotide duplex (AN_2) was considered in two different ways, i.e. direct binding of A with the duplex (reaction b) and formation of this complex by interaction of the tetranucleotide monomer with the 1:1 complex AN (reaction e), where the drug molecule acts as a ''nucleation centre'' (Davies et al. 1996b).

The equilibrium constants K_1–K_5 of complexation of each of the drugs (EB, PI, PF, DAU and CAF) with both the monomer and duplex form of the deoxytetranucleotide d(TpGpCpA) (Davies et al. 1996b, 2000; Eaton et al. 1998) are summarized in Table 3. Investigations of the AO-d(TGCA) and AMD-d(TGCA) systems were found to be in intermediate chemical exchange on the NMR timescale over the concentration and temperature range studied, and so the K values for complexation of AO and AMD with d(TGCA) could not be calculated from NMR data using the additive modelconsidered previously (Davies et al. 1996b), as it relies on systems being in fast exchange. The equilibrium constants for complexation of CAF with d(TGCA) are approximately 2–3 orders smaller than for binding of the other ligands with the same tetranucleotide. The observed differences in the K values for ligand binding with 5′-d(TGCA) are due both to the distinctive structural features of the chromophores of the ligands and to the nature of their side chains and groups (Davies et al. 1996b, 2000; Eaton et al. 1998).

In addition to self-association reactions (2), hetero-association reactions (3) and reactions (7) of drug complexation with d(TGCA), the following reactions of hetero complex formation between the drugs and the monomer and duplex form of the tetranucleotide (analogous to the complexation reactions summarized in Eq. 7c and d) needed to be taken into account:

$$\begin{array}{ll} AN + P \overset{K_3^P}{\longleftrightarrow} APN \text{ (a)} & AN_2 + P \overset{K_4^P}{\longleftrightarrow} APN_2 \text{ (b)} \\ PN + A \overset{K_3^A}{\longleftrightarrow} APN \text{ (c)} & PN_2 + A \overset{K_4^A}{\longleftrightarrow} APN_2 \text{ (d)} \end{array} \tag{8}$$

where A, P and N are the monomer forms of ligand, CAF and tetranucleotide, respectively; $K^{\mathrm{P}}{}_3$, $K^{\mathrm{P}}{}_4$ and $K^{\mathrm{A}}{}_3$, $K^{\mathrm{A}}{}_4$ are the equilibrium constants of CAF- and drug-nucleotide complexation, respectively. It is assumed that the equilibrium constants $K^{\mathrm{P}}{}_3$, $K^{\mathrm{P}}{}_4$ (CAF) or $K^{\mathrm{A}}{}_3$, $K^{\mathrm{A}}{}_4$ (drug) for ligand binding with the 1:1 (AN or PN) complex and with the 1:2 (AN_2 or PN_2) complex are equal to the corresponding K_3 and K_4 values in Eq. (7). Such an assumption is reasonable, because the binding of aromatic ligands to DNA corresponds to the

[1]For CAF-d(TGCA) interaction the same model of complexation has been applied, i.e. A = P in the case of CAF in Eq. (6)

"excluded neighbour" model (McGhee and von Hippel 1974), according to which intercalation of the ligand molecule between adjacent base pairs is impossible; hence it follows that the mutual influence of each of the ligands, not containing large side chainsor groups, is negligible when intercalated into the oligonucleotide sequence. The computational procedure described in previous work (Davies et al. 1996b; Davies and Veselkov 1996) has been used to calculate the equilibrium concentrations of P, A and N by solving a system of nonlinear equations, based on the mass law equations for reactions (2), (3), (7) and (8) and the mass conservation law, and, hence, determination of the relative content of different complexes in solution (Veselkov et al. 2000).

The relative decrease in the content of the aromatic ligand-deoxytetranucleotide duplex has been calculated as a function of tetramer concentration for different amounts of CAF in the mixed solution using the equilibrium constants summarized in Tables 1, 2 and 3. An example for one of the systems studied, CAF + DAU, is presented in Fig. 5 and analogous results are observed for all the other drugs. At $r_N(=N_0/A_0)>1$, i.e. when the tetranucleotide concentration exceeds the drug content (A_0 for the drug was taken as 1 mM), all the calculated dependences tend towards constant values. The degree of saturation of the curves depends substantially on the concentration of CAF in solution (where $r_P=P_0/A_0$, the ratio of CAF and drug concentrations in solution). The largest changes in the complexation of DAU with the tetranucleotide duplex are observed in the range of $r_P=2$–20. When the CAF content in solution is further increased ($r_P \geq 40$) this effect becomes less pronounced. In principle, such an analysis enables the optimum concentration of CAF to be determined for any defined reduction in ligand binding to DNA. For example, at a CAF to drug ratio of $r_P=40$ (Fig. 5), the relative decrease of drug + d(TGCA)$_2$ complexes amounts to 55–60%, whereas at $r_P=100$ it is ca. 80%, and the intercalative binding of DAU with DNA is only about 20%.

What is the molecular basis for the action of CAF on drug-DNA binding?

It was assumed previously (Traganos et al. 1991a, 1991b; Kapuscinsky and Kimmel 1993; Larsen et al. 1996) that the protective properties of CAF in cells against intercalating agents, which inhibit cell growth, is due to the formation of hetero complexes between CAF and aromatic ligands, thus leading to a lower effective concentration of intercalator in solution, and therefore to a decrease of its biological activity. The present work enables this assumption to be tested quantitatively. Using the equilibrium constants summarized in Tables 1, 2 and 3, the relative proportions of the CAF-tetranucleotide and the drug-CAF-nucleotide hetero complexes APN and APN$_2$ were calculated as a function of r_P, the ratio of CAF and drug concentrations in solution. The results for EB, PI, PF and DAU shown in Fig. 6 indicate that

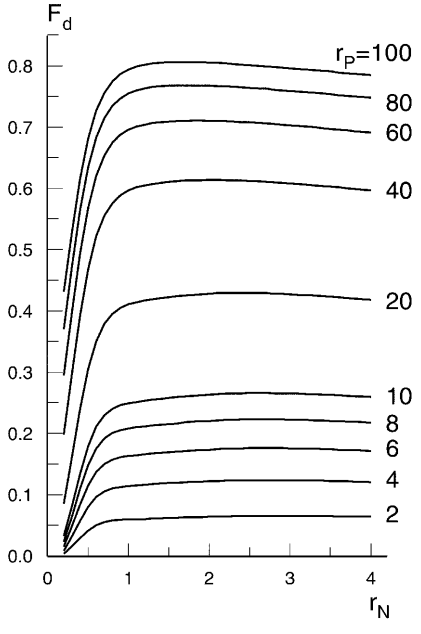

Fig. 5 Relative decrease in the content of the complexes of DAU with the 5′-d(TpGpCpA) duplex calculated (from results in Tables 1, 2, 3) as a function of $r_N=N_0/A_0$ at different ratios (r_P) of CAF to ligand concentration in solution; $T=298$ K; $F_d=(f_0-f_p)/f_0$, where f_p, f_0 are the fractions of the complexes between ligand and duplex of the deoxytetranucleotide in the presence of CAF and without CAF in solution, respectively

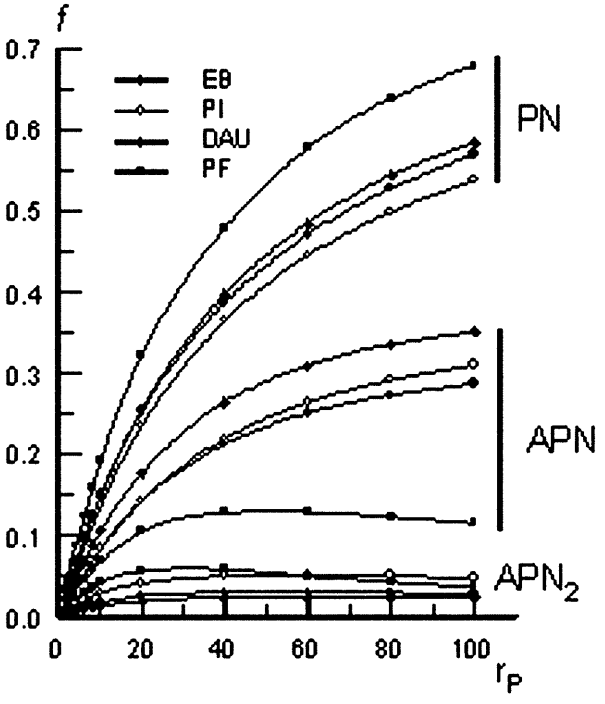

Fig. 6 Relative content, $(C_i/C_0)_{CAF}$, of the complexes of CAF (P) and aromatic ligands (A) with tetranucleotide (N) 5′-d(TpGpCpA) calculated (from results in Tables 1, 2, 3) as a function of r_P, the ratio of CAF to drug concentration in the mixed solution at $N_0=1$ mM. *PN*: complexes of CAF with nucleotide; *APN* and *APN$_2$* are hetero-association complexes

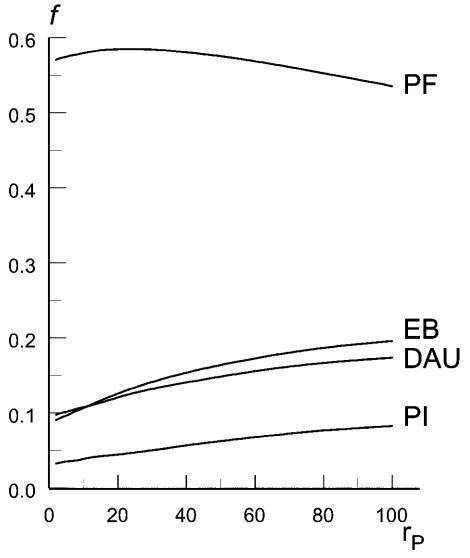

Fig. 7 Ratio (f) of the content of "CAF-ligand" hetero-association complexes with respect to the total amount of complexes of CAF with the deoxytetranucleotide, 5′-d(TpGpCpA), calculated (from results in Tables 1, 2, 3) as a function of the ratio (r_P) of CAF and ligand concentrations in the mixed solution of CAF + d(TGCA) with aromatic ligand ($N_0 = 1$ mM, $T = 298$ K)

the relative proportions of the CAF-tetranucleotide (PN) complexes increase with increasing CAF concentration, i.e. CAF "blocks" the binding sites of the ligand on the deoxytetranucleotide. The relative proportions of CAF-ligand-DNA hetero complexes (APN and APN_2) depend on the relation between the equilibrium constants for complexation of the drug and CAF with the deoxytetranucleotide, as well as for drug-CAF hetero-association in solution. As shown in Fig. 6, the contribution of the APN complex to the general equilibrium for the mixed solution of PF-CAF-d(TGCA) is substantially smaller than for the other ligand-CAF-d(TGCA) mixtures, which is a result of the more pronounced hetero-association of CAF with PF compared with the other ligands inaqueous solution (Table 2).

In order to determine which process prevails in the effect of CAF on the degree of intercalative binding of aromatic ligands with DNA (i.e. competition by drug and CAF for the binding sites of the oligonucleotide, or formation of "CAF-ligand" hetero complexes in solution), the proportion of the "CAF-ligand" hetero complex was calculated relative to the complexes of CAF with the deoxytetranucleotide at different CAF concentrations in solution, using the equilibrium constants summarized in Tables 1, 2 and 3. It is seen from the results of such calculations in Fig. 7 that, for systems characterized by relatively small hetero-association constants (EB + CAF, DAU + CAF and PI + CAF in Table 2), the contribution of the "CAF-ligand" hetero complex to the decrease in drug binding with the tetranucleotide is no more than 20%, even at the maximum ratio of CAF to drug concentration considered, $r_P = 100$. However, for the acridine drug PF the contribution of hetero complex formation with CAF is appreciably higher (more than 50%) compared with EB, DAU and PI (Fig. 7), owing to the relatively large hetero-association constant of complexation between CAF and PF in aqueous solution (Table 2). Our results have shown that the assumption in previous work (Traganos et al. 1991a, 1991b; Kapuscinsky and Kimmel 1993; Larsen et al. 1996) is not really correct, i.e. that the protective properties of CAF, acting as an "interceptor" of biologically active aromatic molecules intercalating into DNA, are due only to formation of CAF-ligand hetero complexes in solution at relatively high CAF concentrations. The competitive binding of CAF with DNA also has to be taken into account (Veselkov et al. 2000).

The model and analytical method outlined in this work has general application to understanding molecular complexation processes in multi-component equilibria, as it enables the relative importance of each complexation reaction to be determined quantitatively. For example, in order to elucidate the molecular basis for the action of CAF as a protector of DNA complexation with mutagens and with aromatic antibiotics, it has been shown, in this work, that it is necessary to take into account not only the hetero-association of the molecules but also the competition between CAF and the ligand for the oligonucleotide binding sites.

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