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THERMODYNAMICAL MODEL FOR INSERTION AND AGGREGATE BINDING OF CAFFEINE TO THE HOMOPOLYMER POLY(RIBOADENYLATE) AND MODEL CHOICE BY DATA ANALYSIS *

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This paper describes the model used to estimate the parameters of caffeine-poly(riboadenylate) (poly(A)) interactions from corresponding ^{1}H-NMR measurements. The model of insertion and aggregate binding describes the non-cooperative insertion of a molecule C into an interspace between two monomers of a homopolymer in competition with aggregate binding. It contains two binding constants, K_1 for insertion and K_2 for the interaction of monomeric A units of the polymer with C molecules in bound aggregates, and two cooperativity parameters, K_{CC} for the stacking of C molecules within aggregates and τ which is thought to be due to conformational adaptation of the polymer to those bound aggregates which cover more than one A unit. In contrast to other models, the size of a binding site (within the aggregates) is less than one monomeric unit, with n denoting the maximum number of C molecules per A unit in bound aggregates. The model is developed for general n by means of the method of sequence-generating functions. For $n = 2$ and $n = 3$, the correctness of the model treatment was checked by the matrix method. The model is applicable to the binding of aggregates to homopolymers, which are flexible enough to fit their structure to the aggregates.

1. Introduction

The binding of caffeine to poly(riboadenylate) was investigated by NMR spectroscopy. The aim of these studies was to determine the structure of the caffeine-poly(A) complex as a model for the caffeine-DNA complex [2]. Analogously to ref. 1, this paper deals with the choice of a proper model for the description of the NMR data and with the treatment of this model for the purpose of parameter estimation, especially the estimation of interaction shifts characterizing the structure of the complex.

In section 2 the inspection of the NMR data includes the application of model-free analysis to binding data. This method is very powerful but seldom used in the literature. Using this method it becomes apparent that there are two binding mechanisms which are assumed to be a non-cooperative insertion of single caffeine molecules between two adenine bases of poly(A) and, in competition, binding of caffeine aggregates to poly(A). Bound aggregates are both bound products of self-association and outside bound monomers (aggregates of unit length), besides aggregates in solution. As explained in section 2, the data suggest that the maximum number, n, of aggregate-bound caffeine molecules per A unit is greater than one.

The different states of interaction contributing to the chemical shift are identified in section 3. In section 3, the binding model is treated. The relative populations of the different states of interaction are calculated as functions of the total con-

* This paper and the preceding one [1] are theoretical counterparts to the paper of ref. 2.

centrations and the binding parameters. For general n, the method of sequence-generating functions is used. The particular cases $n = 2$ and $n = 3$ were checked by the matrix method. Section 4 explains the procedure of parameter estimation by means of our general data fitting programme for non-linear regression analysis.

For a fixed set of binding parameters, the model behaviour is discussed in section 5. It shows the binding of an associating ligand to a linear homopolymer flexible enough to fit its structure to the aggregate structure.

The model is treated for the purpose of parameter estimation by means of a computer programme. During this treatment, two implicit equations, the interaction equation and the balance equation, must be solved simultaneously. As this problem is characteristic for complicated binding models, its solution is demonstrated in the appendix.

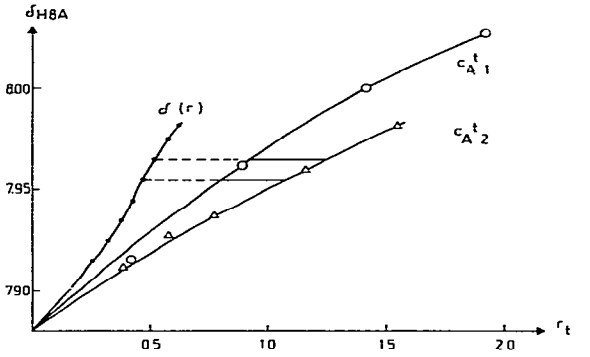

Fig. 1. δ_{H8A} as a binding state-specific signal plotted vs. $r_t = c_C^t/c_A^t$ for $c_{A1}^t = 0.051$ M and $c_{A2}^t = 0.095$ M. The choice of some constant values of δ_{H8A} defining certain binding states (each characterized by its binding ratio r) is indicated by horizontal lines. As equal binding states correspond to equal caffeine concentrations in solution c_{CS}, the values of r and c_{CS} may be estimated from their intersections with the curves by $c_{C,1}^t = c_{CS} + rc_{A1}^t$ and $c_{C,2}^t = c_{CS} + rc_{A}^t$. Additionally, as a result of this model-free analysis, δ_{H8A} as a function of r was introduced, taking the numerical values on the abscissa to be values of r instead of r_t.

2. Choice of a proper model

As in the investigation of caffeine-AMP mixed association [1], three A proton chemical shifts ($j = 1$–3) and four C proton chemical shifts ($j = 4$–7) were measured (see fig. 1 of ref. 1). The titration was done at fixed poly(A) concentration c_A^t, the caffeine concentration c_C^t being varied. The resulting courses of both A and C proton chemical shifts vs. c_C^t are displayed in fig. 2 of ref. 2.

The different courses of $\delta_{H8A}(c_C^t)$ and $\delta_{H2A}(c_C^t)$ cannot be explained by a single binding mechanism. Particularly, the non-monotonic course of δ_{H2A} suggests the existence of at least two binding mechanisms.

The data shown in fig. 2 of ref. 2 indicate enlargement of the distance between H8A positions (situated near the sugar phosphate backbone in poly(A)) over the whole c_C^t range, i.e., by both binding mechanisms. The distance between H2A positions is also enhanced by the second binding mechanism, but in the first binding mechanism these protons are clearly influenced by the approach of some interaction partner.

From this behaviour, together with self-association of C in solution, the two mechanisms may be specified to be (1) insertion of single C monomers between A units and (2) binding of C aggregates at poly(A), where n, the maximum number of aggregate-bound C molecules per A unit, has a value greater than one. For stereochemical reasons, $n = 2$ has been assumed (see section 6).

In order to confirm these assumptions, model-

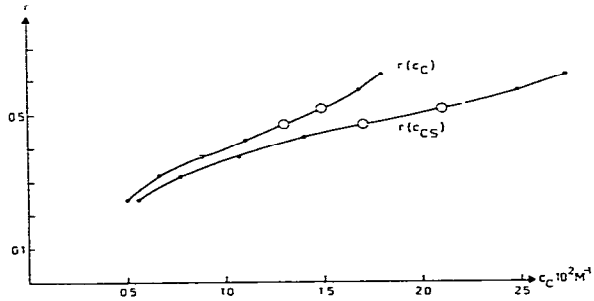

Fig. 2. The binding ratio as constructed by model-free analysis (see fig. 1) shown as a function of the caffeine concentration in solution ($r(c_{CS})$, lower curve) and of the concentration of free monomeric caffeine ($r(c_C)$, upper curve), taking into account the self-association of caffeine. The open circles correspond to the two binding states indicated by horizontal lines in fig. 1.

free analysis has been performed in a similar way to those described in refs. 3–7. Being intensive quantities [1] which reflect the extent of binding, r, the measured chemical shifts of A protons are directly applicable as polymer state specific signals. δ_{H8A} has been chosen because of its most pronounced and monotonic dependence on caffeine concentration. As usual, from two titrations at different polymer concentrations c_A^t, the binding ratio r (sum of concentrations of all caffeine bound by any binding mechanism/total concentration of A units) has been derived as a function of the caffeine concentration not bound to poly(A) (fig. 1). Taking into account the self-association of caffeine as resulting from ref. 2. we obtain a plot of r vs. the concentration of free monomeric caffeine c_C, which clearly indicates the onset of a second binding mechanism with enhanced amounts of caffeine bound per A unit at high caffeine concentrations (fig. 2).

Furthermore, as any constant value of δ_{H8A} specifies a value of r, a plot of δ_{H8A} vs. r may be drawn, which is additionally introduced in fig. 1. If there were only one type of binding with a single interaction shift, the chemical shift (δ_{H8A}) should exhibit a linear dependence on r. Therefore, its obvious deviation from a straight line also shows that more than one binding mechanism is indicated in the measured signal.

These results suggest a model that contains beside the insertion of single caffeine molecules between two A units an outside aggregate binding with more than one caffeine per A unit.

3. Statistical treatment of the binding model

Models that describe structural transitions or ligand binding on linear polymers regarding special binding mechanisms have been derived from statistical thermodynamics by either the matrix method or the method of sequence-generating functions [12–16] and other equivalent methods [17,18]. Ref. 10 must be considered in connection with refs. 14, 20, and 21. In our case, the method of sequence-generating functions has the advantage that it refers to any number of caffeine molecules bound per A unit in aggregated form. The case of general n is intentionally treated both to demonstrate the general applicability of the method of sequence-generating functions and to provide formulas for cases where n must be assumed to be greater than two.

3.1. Introduction of statistical weight

In this binding model, K_1 is the equilibrium constant of insertion and K_2 that of the first caffeine-adenine binding contact in bound aggregates. If, however, an aggregate covers more than one A, then its binding constant is K_2 at the first A, but the binding contact at each of the following As contributes a factor of $K_2\tau$. This means that $K_2\tau$ is a (dimensionless) measure of cooperativity (see section 5) concerning the interaction (via conformation changes of the backbone) of occupied A units. To minimize the number of parameters, τ is assumed equal at each subsequent binding contact. K_{CC} describes the stacking interaction of neighbouring caffeine molecules in bound aggregates as well as in aggregates in solution. The maximum number of Cs that bind per monomeric unit of poly(A) in aggregate binding is n.

In the method of sequence-generating functions, three states (fig. 3) of A units are considered, viz., free, inserted and aggregate-bound, designated by u, v and w, respectively. We define a chain element to comprise one A and its right-hand interspace.

The notation 'u' is used only if an A unit together with its full right-hand interspace is not occupied by any ligand. Unoccupied states which do not meet this condition are considered as gaps within sequences of bound aggregates, i.e., as w states.

The notation 'v' is used for an A unit with a caffeine molecule inserted in its right-hand interspace. The next A-unit, as influenced by the inserted caffeine as well, is assumed not to be a possible binding contact for aggregates, for steric reasons. Although not stringent at large n, this assumption is reasonable for $n = 2$ as used in the evaluation of caffeine-poly(A) data. Moreover, this assumption may easily be lifted in the derivation of sequence-generating functions.

In order to calculate the sequence-generating

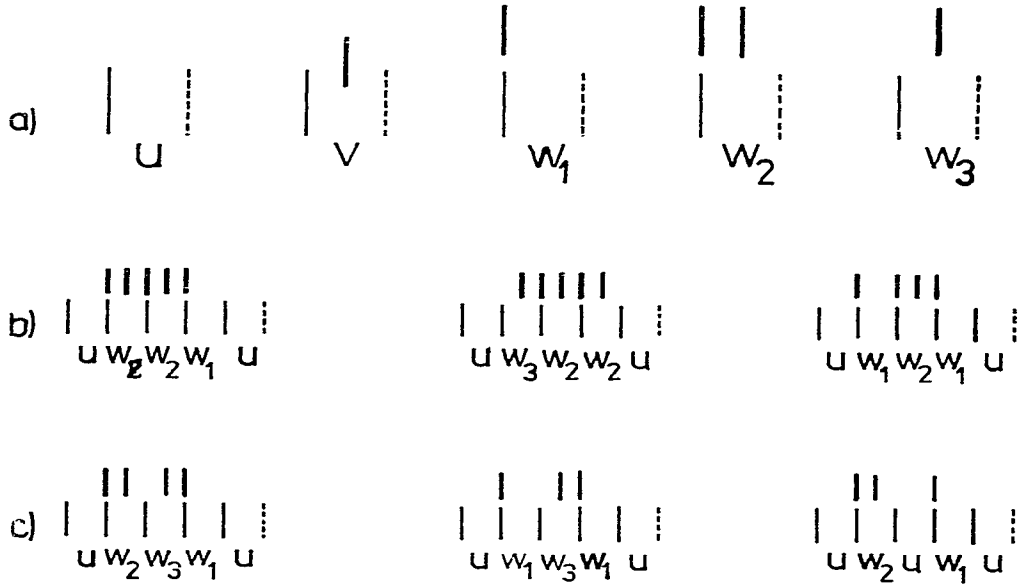

Fig. 3. States and sequences illustrating the formation of sequence-generating functions (for $n = 2$). (a) Possible states of a single chain element. Note that w_3 may only be followed by w_1 or w_2. These five states are exactly those that have been used for $n = 2$ to apply the matrix method to this system of interaction. Dashed lines always indicate adenine bases that no longer belong to the element under consideration but mark the right border of interspace. (b and c) Demonstration of aggregate binding (reduced in scale). (b) Examples of non-interrupted w sequences without and with a gap of type a. ($uw_2w_2w_1u$) Gapless aggregate which starts and stops at a binding contact; number of binding contacts covered $k = 3$, number of caffeine molecules comprised $m = (k-1)n + 1 = 5$. ($uw_3w_2w_2u$) Gapless aggregate with both continuation to the right and to the left. ($uw_1w_2w_1u$) w sequence containing a type a gap (unit length being maximum length for $n = 2$). (c) Distinction between gaps of type b and an interruption of a w sequence. ($uw_2w_3w_1u$) Type b gap of unit length. ($uw_1w_3w_1u$) Type b gap of maximum length of $2n - 2 = 2$. (uw_2uw_1u) Interrupted w sequence, consisting of two gapless aggregates of unit length, one of them with a continuation to the right.

functions, relative statistical weights are introduced containing contributions from binding states (symbolized by g) and interactions (denoted by γ). In particular, $\gamma_{ij} = 1$ denotes no interaction and $\gamma_{ij} = 0$ forbidden sequences. In detail, the model assumes:

$g_u = 1$

$g_v = K_1 c$ where c denotes the free monomeric ligand concentration,

$\gamma_{xy} = 1$ for $x = u, v$ and $y = u, v$

as no interaction is assumed in all these cases.

For aggregate binding the situation is more complicated. The statistical weight of a w state consisting of an aggregate that comprises m Cs and covers k binding contacts without any gap is given by (fig. 3b))

$$g_{mk} = c(K_{CC}c)^{m-1} K_2 (K_2 \tau)^{k-1} \quad (1)$$

The treatment of gaps within sequences of bound aggregates will be discussed below (fig. 3b and c).

3.2. Calculation of the sequence-generating functions

U_v is the sequence-generating function for a sequence of states u preceded by a state v.

$$U_v = \sum_{j=1}^{\infty} u_{vj} x^{-j} \quad (2)$$

where u_{vj} is the statistical weight of a sequence of j

states u preceded by a state v

$$u_{vj} = \gamma_{vu} g_u^j \gamma_{uu}^{j-1} = 1 \tag{3}$$

The result is

$$U_v = \frac{1}{x-1} \tag{4}$$

The calculation of U_w yields the same result

$$U_w = U_v = U \tag{5}$$

Similarly,

$$V_u = \sum_{j=1}^{\infty} v_{uj} x^{-j} \tag{6}$$

where

$$v_{uj} = \gamma_{uv} g_v^j \gamma_{vv}^{j-1} = (K_1 c)^j \tag{7}$$

The result is

$$V_u = \frac{K_1 c}{x - K_1 c} \tag{8}$$

and again

$$V_u = V_w = V \tag{9}$$

The determination of W_u and W_v is much more complicated. Aggregates that start from a binding contact must not follow state v, for steric reasons. They may, however, follow state u. Therefore, W_u and W_v, indeed, differ from one another. If, however, this assumption is lifted, they are identical to one other.

For the discussion intended, it is convenient to consider first of all, gapless aggregates that start from and stop at binding contacts (fig. 3b). Their continuations to the left and to the right (at the border to states u or v) and the gaps within sequences of aggregates will be dealt with separately (fig. 3b and c).

For such aggregates, eq. 1 applies with $m = (k-1)n + 1$.

$$g_{(k-1)n+1,k} = c(K_{CC}c)^{(k-1)n} K_2 (K_2\tau)^{k-1} \tag{10}$$

The power of x that must be multiplied with aggregate weights has to be considered carefully. x is the partition function per monomeric unit. A monomeric unit comprises one A and its right-hand interspace. Therefore, the aggregates under consideration affect k monomeric units and contribute the factor x^{-k}.

The contribution to W (whether W_u or W_v) of such gapless aggregates (GLA) of length of $k = 1$, or $k = 2$, or ..., is

$$g_{GLA} = \sum_{k=1}^{\infty} cK_2 x^{-1} \left[K_2 \tau (K_{CC}c)^n x^{-1} \right]^{(k-1)}$$

$$= \frac{cK_2}{x - K_2\tau(K_{CC}c)^n} \tag{11}$$

Continuations to the left (CL) (fig. 3b) of length of 0 or 1 or ... $n-1$ contribute to g_{GLA} the factor

$$g_{CL} = 1 + x^{-1} \sum_{i=1}^{n-1} (K_{CC}c)^i$$

$$= 1 + x^{-1} \frac{K_{CC}c - (K_{CC}c)^n}{1 - K_{CC}c} \tag{12}$$

because any continuation to the left, except of length 0, covers an additional monomeric unit (via its interspace).

Continuations to the right (CR) (fig. 3b and c) of length 0 or 1 or ... $n-1$ contribute to g_{GLA} the factor

$$g_{CR} = 1 + \sum_{i=1}^{n-1} (K_{CC}c)^i = \frac{1 - (K_{CC}c)^n}{1 - K_{CC}c} \tag{13}$$

because no continuation to the right covers an additional monomeric unit.

According to the assumption that an A-unit neighbouring an inserted caffeine cannot be a binding contact of an aggregate-bound caffeine, state v can only precede state w if there is a left-hand continuation (CL) of non-zero length representing caffeine molecules (partly) covering the preceding interspace. Therefore,

$$g_{CL,u} = g_{CL} \tag{14}$$

but

$$g_{CL,v} = g_{CL} - 1 \tag{15}$$

Exclusively this causes the difference between W_u and W_v.

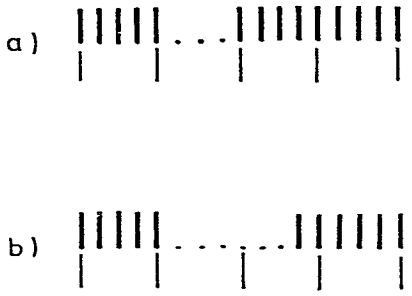

Fig. 4. Maximum size of gaps of type a or b for $n = 4$. (a) $n - 1 = 3$. (b) $2n - 2 = 6$.

Yet, gaps within sequences of aggregates must be considered. Gaps may be of two types: (a) those not covering binding contacts (GNB) (fig. 3b); (b) those covering one binding contact (GCB) (fig. 3c). Their contributions to W will now be discussed.

One should proceed carefully with these considerations because the introduction of too large a gap of type b might create a state u (fig. 3c). Gaps of type a are of length $n - 1$ at most (see fig 4).

From combinatorics we find $n - j$ ways to place such a gap of length j by removing j neighbouring C molecules from a sequence of $n-1$ C molecules between neighbouring gapless aggregates, thereby eliminating $j + 1$ CC contacts. Conversely, with i denoting the number of C molecules not removed by forming the gap, ($i \leqq n-2$), there are $i + 1$ ways of distributing them to the left and to the right of the gap. This way we find that ($i + j = n - 1$) type a gaps of length 1 or 2 or ... $n - 1$ have the weight (where the sum reads backwards)

$$g_{GNB} = \sum_{i=0}^{n-2} (i+1)(K_{CC}c)^i = -\frac{(K_{CC}c)^{n-1}}{1-K_{CC}c}\left[n - \frac{1-(K_{CC}c)^{-n}}{1-(K_{CC}c)^{-1}}\right] \quad (16)$$

where $i = 0$ describes an empty interspace between two occupied neighbouring binding contacts. Any additional factor x^{-1} does not appear, since no additional monomeric unit is covered by putting a gap of type a between gapless aggregates.

Gaps of type b are at most of length $2n - 2$ because at least one C must be attached to the right gapless aggregate (see fig. 4). Otherwise this would give rise to a state u contradicting our definition of a gap (fig. 3c).

Large gaps with $n \leq j \leq 2n - 2$ C molecules removed may be treated in a similar way to type a gaps with an additional factor of $K_{CC}c$ contributed by the unremovable right-hand CC contact. Smaller gaps with $1 \leq j < n$ are restricted by the condition that they must cover the binding contact, otherwise being type a gaps. There are j ways of creating small gaps of length j, giving rise to $2n - 1 - i$ possibilities for putting $n \leq i \leq 2n - 2$ C molecules to the left or to the right. Therefore, ($i + j = 2n - 1$) type b gaps of length of 1 or 2 or ... $2n - 2$ have the weight

$$g_{GCB} = \left[\sum_{i=0}^{n-1} i(K_{CC}c)^i + \sum_{i=n}^{2n-2} (2n-1-i)(K_{CC}c)^i\right]x^{-1} = \frac{x^{-1}}{(1-K_{CC}c)^2} \times \left[1-(K_{CC}c)^n\right]\left[K_{CC}c-(K_{CC}c)^n\right] \quad (17)$$

where $i = 0$ is a forbidden state with no contribution, because this would exceed the maximum size of type b gaps and give rise to a state u (figs. 3c and 4). The state with $i = 1$ can be realized only in one way within a non-interrupted w sequence (fig. 3c). The factor x^{-1} appears because one additional monomeric unit of poly(A) is involved in a type b gap.

Now, all expressions needed are available in order to formulate W_u and W_v. The weight of all possible sequences of aggregates, containing any number of single gapless aggregates, separated from each other by type a or b gaps, that are preceded by u, may be written

$$W_u = g_{CL,u}g_{GLA}\left\{1 + (g_{GNB} + g_{GCB})g_{GLA} + \left[(g_{GNB} + g_{GCB})g_{GLA}\right]^2 + \ldots\right\}g_{CR} \quad (18)$$

W_v is obtained from W_u by writing $g_{CL,v}$ instead of $g_{CL,u}$. Any immediate sequence of gaps is forbidden in order not to create states u within aggregate states.

In a more compact notation W_u and therefore W_v may be written as

$$W_u = \frac{g_{CLu} g_{GLA} g_{CR}}{1 - (g_{GNA} + g_{GCA}) g_{GLA}} \tag{19}$$

After introducing the expressions just derived the final result is

$$W_u = \frac{\left[1 + x^{-1} \frac{K_{CC}c - (K_{CC}c)^n}{1 - K_{CC}c}\right] \frac{K_2 c}{x - K_2 \tau (K_{CC}c)^n} \frac{1 - (K_{CC}c)^n}{1 - K_{CC}c}}{1 - \frac{1}{1 - K_{CC}c}\left\{-n(K_{CC}c)^{n-1} + \frac{1 - (K_{CC}c)^n}{1 - K_{CC}c}\left[1 + x^{-1} K_{CC}c\left(1 - (K_{CC}c)^{n-1}\right)\right]\right\} \frac{K_2 c}{x - K_2 \tau (K_{CC}c)^n}} \tag{20}$$

W_v is obtained from W_u by omitting the term '1' at the first position of the first factor in the numerator.

An independent combinatorial derivation of the sequence-generating functions W_u and W_v yielded equivalent results.

3.3. Derivation and solution of the interaction equation

Now, all the sequence-generating functions are ready to be introduced into the general interaction equation for x [12], which (by use of the notation in eqs. 5 and 9) may be written as

$$G(c, x) = 1 - UV(W_u + W_v) - UW_u - VW_v - UV = 0 \tag{21}$$

After many rearrangements the result is in a fairly compact notation, expressed in powers of x,

$$G(c, x) = x^2 - Ex - F = 0 \tag{22}$$

with

$$E = 1 - A + C^{n-1}BD - M/(1 - C)^2 \tag{23}$$

$$F = (1 + A)\left[M/(1 - C)^2 - C^{n-1}BD\right] + N/(1 - C)^2 \tag{24}$$

$$A = K_1 c, \quad B = K_2 c, \quad C = K_{CC} c, \quad D = \tau K_{CC} \tag{25}$$

$$M = B\left(nC^{n-1} - (n-1)C^n - 1\right) \tag{26}$$

$$N = B(1 - C^n)^2 \tag{27}$$

The largest root of eq. 22 is

$$x_1 = \frac{E}{2} + \sqrt{\frac{E^2}{4} + F} \tag{28}$$

Thus, for any value of n the system equation is quadratic in x and may be solved for x_1 analytically. This must be due to the assumption of nearest neighbour interaction.

These results were checked by using the matrix method for $n = 2$ and $n = 3$ with a different definition of states and their statistical weights (fig. 3a). In both cases, the coefficients E and F of the interaction equation coincide with those resulting from the method of sequence-generating functions (eqs. 23 and 24).

3.4. The balance equation of ligand concentration

The solution of the interaction equation (eq. 28) still contains c, the free monomeric ligand concentration, as an unknown. To determine fully this solution, besides the system equation, $G(c, x) = 0$ (eq. 22), we need the balance equation of ligand concentration, $H(c, x) = 0$, where

$$H(c, x) = c_C^t - \frac{c}{(1 - K_{CC}c)^2} - c_A^t r \tag{29}$$

(the index of x has been omitted) c_C^t is the total caffeine concentration, the second term the concentration of caffeine in solution, according to the isodesmic model of self-association (eq. 6 of ref. 1), and the third term the concentration of caffeine bound to poly(A), r being the quotient of the concentration of bound ligands divided by the poly(A) concentration in monomeric units, c_A^t.

The dependence of $H(c, x)$ on x is due to r because, in terms of partition function formalism,

r is given by

$$r = \frac{1}{N}\frac{\partial \ln Z}{\partial \ln c} = \frac{\partial \ln x}{\partial \ln c(x = x_1)}$$

$$= -\frac{\partial G/\partial \ln c}{\partial G/\partial \ln x(x = x_1)}$$

The algorithm of solving the equation system

$G = 0$

$H = 0$

is given in the appendix. Of course, any derivative of G has to be taken at the values of the solution (c, x).

3.5. The concentrations of system components

The connection between the chemical shifts measured and the parameters to be estimated is mediated by a similar set of equations to those in ref. 1. In the corresponding notation

$$(\delta_{j,})_A = \delta_{j,A} + 2\Delta\delta_{j,A,ins}c_{A,ins,i}/c^t_{A,i} + \Delta\delta_{j,A,att}c_{A,att,i}/c^t_{A,i} \quad (30)$$

for $j = 1$–3 and

$$(\delta_{j,})_C = \delta_{j,C} + \Delta\delta_{j,C,ins}c_{C,ins,i}/c^t_{C,i} + \Delta\delta_{j,C,att}c_{C,att,i}/c^t_{C,i} + \Delta\delta_{j,C,agg}c_{C,agg,i}/c^t_{C,i} \quad (31)$$

for $j = 4$–7.

The factor 2 in eq. 30 accounts for the fact that each of the two monomeric units of poly(A) involved in insertion contributes its interaction shift $\Delta\delta_{j,A,ins}$.

$c_{C,agg}$ denotes the concentration of all caffeine molecules involved in aggregates at the polymer as well as in solution.

The relative concentration $c_{A,ins}/c^t_A$ of A interspaces carrying inserted Cs in solution is the same as that of an individual poly(A) chain because interaction between polymers is neglected. Therefore,

$$\frac{c_{A,ins}}{c^t_A} = \frac{\partial \ln x}{\partial \ln K_1(x = x_1)} = -\frac{\partial G/\partial \ln K_1}{\partial G/\partial \ln x(x = x_1)} \quad (32)$$

For the same reason, the relative concentration of binding contact As where Cs are attached is

$$\frac{c_{A,att}}{c^t_A} = \frac{\partial \ln x}{\partial \ln K_2(x = x_1)} = -\frac{\partial G/\partial \ln K_2}{\partial G/\partial \ln x(x = x_1)} \quad (33)$$

and, corresponding to a 1 : 1 relation

$$\frac{c_{C,ins}}{c^t_C} = \frac{c^t_A}{c^t_C}\frac{c_{A,ins}}{c^t_A} \quad (34)$$

and

$$\frac{c_{C,att}}{c^t_C} = \frac{c^t_A}{c^t_C}\frac{c_{A,att}}{c^t_A} \quad (35)$$

$c_{C,agg}$ has to be composed from the contributions of inner and outer caffeine molecules of aggregates at the polymer and in solution (as derived for self-association in ref. 1, eqs. 3, 19 and 20). The quotient of C concentration bound in aggregates divided by c^t_A is given by

$$r_{agg} = \frac{c_{C,out,P} + c_{C,in,P}}{c^t_A} = r - \frac{c_{C,ins}}{c^t_A}$$

$$= \frac{\partial \ln x}{\partial \ln c} - \frac{\partial \ln x}{\partial \ln K_1} \quad (36)$$

(the notation in derivatives of G is now omitted)

The model allows one to separate the concentration of Cs involved in attachment to initial binding contacts $c_{C,att,init}$ from that of Cs attached to consecutive binding contacts $c_{C,att,cons}$ as follows

$$\frac{c_{C,att,cons}}{c^t_A} = \frac{\partial \ln x}{\partial \ln \tau} \quad (37)$$

$$\frac{c_{C,att,init}}{c^t_A} = \frac{\partial \ln x}{\partial \ln K_2} - \frac{\partial \ln x}{\partial \ln \tau} \quad (38)$$

Twice the concentration of initial binding contacts is the concentration of outer Cs in bound aggregates

$$c_{C,out,P} = 2c_{C,att,init} \quad (39)$$

From eqs. 36 and 39, $c_{C,out,P}$ and $c_{C,in,P}$ may be calculated separately, in order to be introduced into the last line of eq. 31 in a proportion of 1 : 2 (see eq. 3 in ref. 1).

4. Parameter estimation

The total model of caffeine-poly(A) binding contains five non-linear system parameters

$$K_1, K_2, K_{CC}, \tau, n \tag{40}$$

and 25 linear measurement parameters, viz. (see eqs. 30 and 31)

$$\delta_{j,A},\ \Delta\delta_{j,A,ins},\ \Delta\delta_{j,A,att} \text{ for } j = 1\text{–}3 \tag{41}$$

$$\delta_{j,C},\ \Delta\delta_{j,C,ins},\ \Delta\delta_{j,A,att},\ \Delta\delta_{j,C,agg} \text{ for } j = 4\text{–}7$$

Some of the parameters were known in advance or assumed and were kept fixed during data analysis of binding.

K_{CC}, $\delta_{j,C}$ and $\Delta\delta_{j,C,agg}$ were taken from ref. 2, $\Delta\delta_{j,C,agg}$ being identified with $\Delta\delta_{j,CC}$. $\delta_{j,A}$ was directly measured at $c_C^t = 0$. $n = 2$ was assumed for steric reasons.

Thus, a grand total of 17 parameters had to be estimated. As in ref. 1, this has been done by using the general curve-fitting programme ALAU.

In the experiments, NMR data on caffeine-poly(A) mixtures of constant poly(A) concentration (0.051 M) and variable caffeine concentration (from 0 to 0.13 M) had been prepared (see fig. 2 in ref. 2). Table 1 shows the parameter values estimated from those data, their errors being omitted (see table 3 in ref. 2). The data material was insufficient to allow complete parameter estimation. The parameter K_1 could only be scanned. Thereby, K_{AC}^2/K_{AA} served as an upper limit of its value [2]. Finally, $K_1 = 10\ M^{-1}$ was chosen in ref. 2 because for this value (see table 4 in ref. 2) the interaction shifts of insertion estimated agree very well with those obtained from the structural model. Fig. 2 in ref. 2 shows the curves of chemical shifts vs. c_C^t fitted with the parameter values estimated. A discussion of the results may be found in ref. 2.

5. Discussion of the model behaviour

Figs. 5–7 demonstrate the model behaviour for the case of 0.051 M poly(A) and varying concentration of monomeric caffeine in solution with the set of parameters $K_1 = 10\ M^{-1}$, $K_2 = 25.3\ M^{-1}$, $K_{CC} = 10.5\ M^{-1}$, $\tau = 0.53$ M, and $n = 2$. In fig. 5 one recognizes that the binding fraction of inserted caffeine (r_{ins}) is always appreciably smaller than that of aggregated caffeine (r_{agg}). At small caffeine concentrations, this is caused simply by the ratio of K_1 and K_2. At concentrations c_C larger than about 0.015 M, r_{ins} starts to decrease. In

Table 1

Best estimates of parameter values

Parameter values estimated of caffeine binding to poly(A). Complete parameter estimation was impossible due to insufficiency of data material. K_1 could only be scanned in a reasonable interval of values. The four values of K_2 and τ, respectively, correspond by data fitting to the four K_1 values scanned when n was fixed as equal to two.

$K_1\ (M^{-1})$	Interaction shifts of binding caffeine-poly(A) (ppm)							
	A,ins				A,att			
	10	20	30	60	10	20	30	60
H8A	−0.05	−0.05	−0.05	−0.07	0.25	0.29	0.31	0.37
H2A	−0.22	−0.13	−0.11	−0.08	−0.02	−0.01	0	0.01
H1'A	0.06	0.04	0.03	0.02	0.02	0.02	0.01	0.01
	C,ins				C,att			
H8C	−0.44	−0.23	−0.16	−0.06	−0.19	−0.22	−0.24	−0.29
H7C	−0.60	−0.31	−0.21	−0.08	−0.09	−0.13	−0.16	−0.24
H3C	−0.79	−0.40	−0.27	−0.09	−0.04	−0.10	−0.15	−0.27
H1C	−0.66	−0.32	−0.21	−0.06	−0.03	−0.09	−0.13	−0.24
$K_2\ (M^{-1})$					25.3	31.7	38.0	59.1
τ (M)					0.53	0.37	0.30	0.18

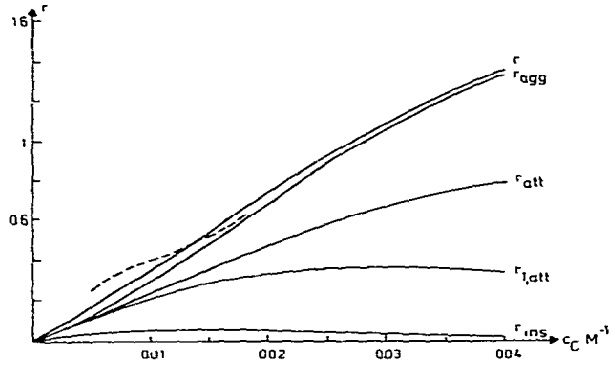

Fig. 5. Binding fractions r of bound caffeine molecules per monomeric unit of poly(A). Parameter set: $K_1 = 10\ M^{-1}$, $K_2 = 25\ M^{-1}$, $K_{CC} = 10\ M^{-1}$ and $\tau = 0.53$ M. r, of all bound caffeine; r_{agg}, of all aggregate-bound caffeine; r_{att}, of all aggregate-bound caffeine attached to an A; $r_{1,att}$, of bound caffeine attached at initial binding contacts, i.e., number of aggregates per monomeric unit of poly(A); r_{ins}, of all inserted caffeine. For comparison the result of model-free analysis $r(c_C)$ is given as a dashed curve.

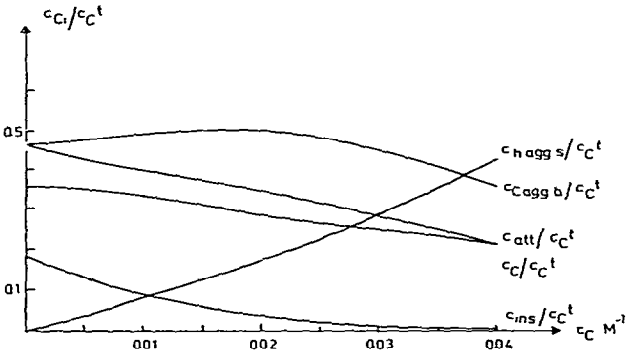

Fig. 7. Concentration fractions of caffeine molecules in different states. c_C^t, total concentration; $c_{h,agg,s}$, concentration of caffeine molecules in higher aggregates (dimers, trimer, etc.) in solution; $c_{C,agg,b}$, concentration of caffeine molecules in the aggregate bound state; c_{att}, concentration of caffeine molecules attached to a base A of poly(A); c_C, concentration of monomeric caffeine molecules in solution; c_{ins}, concentration of caffeine molecules inserted between two bases A of poly(A).

competition for the binding sites, the aggregate binding overcomes the insertion. Apart from the ratio of binding constants, the reason for this behaviour is given by the fact that only one caffeine molecule per monomeric unit may be inserted, but two (generally n) molecules per monomeric unit may be bound in an aggregate.

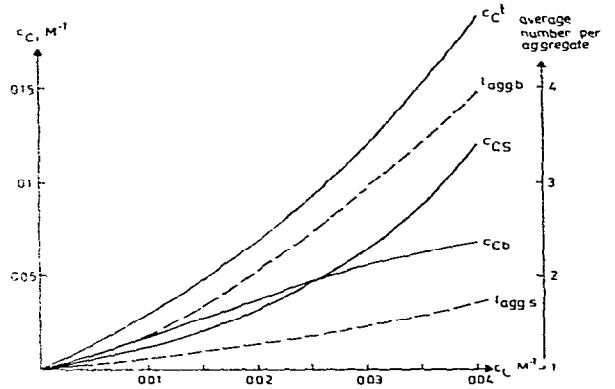

Fig. 6. Dependence of caffeine partial concentrations on the concentration of free monomeric caffeine ($c_A^t = 0.051$ M; parameters as in fig. 5). c_{Cb}, concentration of polymer-bound caffeine; c_{CS}, concentration of all non-polymer-bound caffeine; c_C^t, total caffeine concentration; $l_{agg,b}$, mean length bound; $l_{agg,s}$, mean length of aggregates in solution.

The general phenomenon that at sufficiently high ligand concentration a binding mechanism which needs less space becomes superior to another binding mechanism even with a higher binding constant will be briefly demonstrated using our model as an example. If the state 'aggregate bound' is lengthened by one A unit, the statistical weight of the polymer state is enhanced by a factor of

$$K_2 \tau K_{CC}^2 c_C^2 \qquad (42)$$

whereas the lengthening of the state 'inserted' gives the factor

$$K_1 c_C \qquad (43)$$

per monomeric unit. Thus, a 2-fold concentration increase gives rise to a factor of 4 in eq. 42, but a factor of 2 in eq. 43; a 10-fold concentration increase results in a factor of 100 or 10, respectively. This also explains why, for instance, in the case of ligand binding to DNA the 'weaker' outside binding replaces the 'stronger' intercalation at high ligand concentration [19].

Fig. 5 further shows that the number of bound aggregates ($r_{1,att}$) starts to decrease at concentrations $c_C > 0.03$ M, whereas the number of binding contacts (r_{att}) is further increasing (saturation at

$r_{att} = 1$). Thus, joining of aggregates by filling gaps then outweighs the initiation of new aggregates.

For the sake of comparison, in fig. 5 the function $r(c_C)$ as a result of model-free analysis shown in fig. 2 is represented by a dashed line.

Fig. 6 shows that up to concentrations of 0.025 M monomeric caffeine there is more caffeine bound to polymer (c_{Cb}) and less non-bound caffeine (c_{Cs}) in solution. At higher concentration the non-bound part predominates increasingly, because there is only a fixed concentration of poly(A) available for caffeine binding, whereas the self-association in solution is non-limited.

The numerical value of $\tau(< 1)$ might suggest consideration of aggregate binding as an anti-cooperative process, with energy consumption by destacking [2] overcompensating energy gain by binding. However, both the stronger increase of average aggregate length at polymer ($l_{agg,b}$) in comparison to that in solution ($l_{agg,s}$) (fig. 6) and the sigmoidal shape of r_{agg} in fig. 5 indicate the cooperative character of aggregate binding.

If we consider aggregates of length 3, they may be attached to the polymer in two ways, covering one or two A units as binding contacts. The corresponding binding constants are K_2 or $K_2^2\tau$, respectively. Thus, the involvement of a second A unit in aggregate binding is favoured by a factor of $K_2\tau = 13.3$. This is one contribution to the cooperativity of aggregate binding.

A second contribution originates from the interaction between Cs within the aggregate, which leads to a tendency to avoid gaps and to form longer aggregates, even for a constant number of binding contacts occupied and a constant amount of aggregate binding (r_{att} = constant, r_{agg} = constant). Consider two aggregates, each of length 2, attached to one A unit per aggregate, and leaving a gap of length 1 between them. The statistical weight of this sequence of states is given by the expression

$$K_2^2 K_{CC}^2 c_C^4$$

By rearrangement of one caffeine molecule, a state would be created which represents the binding of one aggregate of length 4 without any gap. In this case, the statistical weight is given by

$$K_2^2 \tau K_{CC}^3 c_C^4$$

The ratio of these two statistical weights is

$$K_{CC}\tau = 5.55 > 1$$

This is the second contribution to the cooperativity of aggregate binding.

Thus, aggregate binding is, indeed, a cooperative phenomenon. The energy loss caused by conformational constraints leading to poly(A) destacking is overcompensated by both AC and CC interaction.

Note that for each gapless aggregate one pure binding constant K_2 (without the factor τ) is introduced (eqs. 11 and 18). This means that a gap (as well as a state u or v) interrupts the conformational constraint on the polymer induced by aggregate binding.

In fig. 7, the contributions of the caffeine concentrations in the different states to the total caffeine concentration are demonstrated. Caused by the cooperative character of aggregate binding, the portion of the aggregate-bound caffeine increases at first, but starts to decrease at high concentration, whereas the portion of inserted caffeine decreases from the beginning. The only component which increases more and more is the portion of aggregates in solution.

6. Concluding remarks

The underlying data material did not allow determination of all parameters of the model. For n, a value of 2 was assumed. With regard to the phosphate–phosphate distance in poly(A) and the stacking distance in C-aggregates [2], no greater value of n is possible in this case. K_1 was scanned. From the results of C-AMP mixed association, a reasonable upper limit for K_1 was deduced. From the structural model the value $K_1 = 10\ M^{-1}$ was chosen.

The impossibility of determining all parameters in this case is not a property of the model. The parameters K_1, K_2, K_{CC}, τ and n are independent. They can be determined in common from a sufficient set of data. For further use of the model, some hints on how to obtain such a set of data will be given.

In the present case, all parameters (interaction

shifts as well as binding parameters) had to be obtained from the measurement at a single poly(A) concentration. In contrast, model-free analysis demonstrates the possibility of determining separately the binding behaviour and the dependence of the measured signal on the binding ratio from consistent measurements at two polymer concentrations at least. To obtain a good estimate also for n, measurements should be extended to saturation of aggregate binding, i.e., to high ligand concentration in the solution. For the determination of K_1 it seems essential to perform measurements also at low binding ratios. According to the principles of design of binding experiments (H. Schütz, unpublished results) the determination of all parameters requires, besides measurements at a medium polymer concentration, supplementary measurements at both high and low polymer concentration.

Appendix: An algorithm of solving the system of implicit equations in the interaction

The regula falsi method is applied to $H(c, x)$, considered as a function of c.

(1) c covers the interval $0 \leq c \leq c^t$. The regula falsi needs two marginal values c_- and c_+. A practical choice is $c_- = 0$ and $c_+ = \min(c^t, 0.999/K_{CC})$.

(2) Calculation of both $x_- = 1$ from $c_- = 0$ and x_+ from c_+ according to eq. 28.

(3) Calculation of both $\partial G/\partial \ln c$ and $\partial G/\partial \ln x$ for (c_-, x_-) and $c_+, x_+)$, respectively, from eq. 22.

(4) Calculation of both $H(c_+, x_+)$ and $H(c_-, x_-)$ from eq. 29.

(5) Application of the regula falsi method to $\{c_+, H(c_+, x_+)\}$ and $\{c_-, H(c_-, x_-)\}$, and determination of the zero c_z, $c_- < c_z < c_+$.

(6) Calculation of x_z from c_z according to eq. 28 and of $H(c_z, x_z)$ from eq. 29.

(7) Substitution of that $\{c, H(c, x)\}$ in item 5 by $\{c_z, H(c_z, x_z)\}$ for which the H terms have the same sign. Iteration of the regula falsi method up to $|H/c^t| \leq 10^{-7}$.

(8) Calculation of x from the iteratively determined value c, according to eq. 28.

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