BIS-ACTINOMYCIN D: A NEW SYNTHETIC SEQUENCE-SPECIFIC LIGAND

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1. Introduction

Actinomycin D (AMD) is an anti-tumour antibiotic extensively studied in the past 20 years ([1,2] and literature cited therein). AMD has been found to inhibit DNA-dependent RNA synthesis. It is generally agreed that biological activity of AMD is a result of complex formation between the antibiotic molecule and DNA. The binding reaction between AMD and DNA exhibits a pronounced specificity for GC pairs and requires normal double-helical conformation of DNA [1,3]. The physical nature of the binding site for AMD on DNA is not yet established. Although it is known that in the complex each AMD molecule covers 5 basepairs, at least one of which must be GC [3,4] the origin of binding specificity and the orientation of AMD molecule with regard to a GC pair are not yet known.

Several detailed molecular models for the AMD–DNA complex have been proposed. Jain and Sobell have suggested that the phenoxazone chromophore of AMD is intercalated into the DNA helix preferentially between GC and CG basepairs whereas the 2 pentapeptide rings are located in the minor DNA groove and participate in hydrogen-bond formation with guanine bases [5]. By contrast, Müller and Crothers [3] have considered that electronic interaction rather than hydrogen bonding between an intercalated phenoxazone chromophore of AMD and a guanine base is a primary factor responsible for GC-specificity of the binding reaction. In the alternative model [6], the phenoxazone chromophore and the two pentapeptide rings of AMD are located in the DNA minor groove. Stabilization of the complex is provided by a hydrogen bond between the quinone oxygen of the AMD chromophore and 2-amino group of guanine as well as by hydrogen bonds which connect 3 DNA phosphate groups with the NH_2 group of the chromophore and carboxamide groups linked to the chromophore ring in positions 1 and 9.

It is of interest to try to design derivatives of AMD that exhibit higher binding specificity than AMD. It is possible to introduce small and bulky substituents (such as H–Val–Glo–NH–, H–(Val–Glo)$_2$–NH, and H–(Val–Glo)$_3$–NH–, where Glo is a glycolic acid residue) in position 7 of the phenoxazone chromophore of AMD with retention of biological activity and DNA binding properties [3,7–9]. Here, we describe DNA binding properties of bis-AMD, a new type of synthetic sequence-specific ligand in which 2 actinomycin-like molecules are linked covalently by a flexible chain attached to position 7 of the antibiotic chromophore.

2. Materials and methods

The structure of the derivatives that have been studied here is shown in fig.1. These compounds have been synthesized from corresponding monomer analogs of AMD as in [10]. Poly(dG) · poly(dC) and poly(dG–dC) · poly(dG–dC) were obtained from P. L. Biochemicals (USA), poly(dA–dC) · poly-(dG–dT) was purchased from Boehringer Mannheim (FRG). Calf thymus DNA (42% GC, ϵ_{260} = 6650), *Micrococcus lysodeikticus* DNA (72% GC, ϵ_{260} = 6850), *Escherichia coli* DNA (50% GC, ϵ_{260} = 6650), *Clostridium perfringens* DNA (28% GC, ϵ_{260} = 6300) were from Sigma (USA). All polynucleotides were used as supplied, without further purification. All polynucleotide solutions were dialysed for 6 h against 0.05 M phosphate buffer (pH 6.8) containing 5 × 10^{-4} M EDTA. Absorption isotherms for the binding of AMD derivatives and bis-AMD to DNA were determined by spectrophotometric titration using a Cary 118 spectrophotometer. Small increments of concen-

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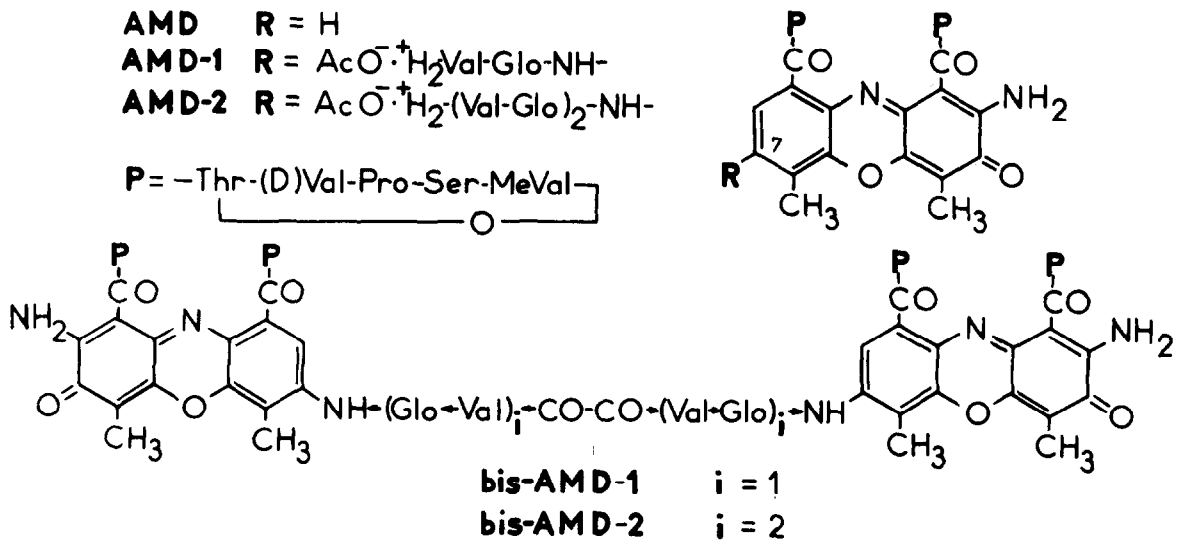

Fig.1. Chemical formulae of actinomycin D (AMD), AMD analogs (AMD-1 and AMD-2) and dimeric compounds (bis-AMD-1 and bis-AMD-2). Indicated are CO → NH directions in the two halves of the connecting chain of bis-AMD. Glo is the glycolic acid residue.

trated ligand solution (5×10^{-4} M) were added to DNA solution in 0.05 M phosphate buffer (pH 6.8) containing 5×10^{-4} M EDTA and 0.2 M NaCl. Absorbancy readings were taken at 425 nm after 60 min wait. In some cases longer periods were required for establishment of equilibrium. All binding experiments were carried out at 22°C.

3. Results and discussion

The absorption spectra of bis-AMD-1 and bis-AMD-2 as well as spectra of corresponding monomer analogs are shown in fig.2. The shapes of the absorption spectra for the monomeric and dimeric compounds are similar, but the molar extinction coefficients calculated for bis-AMD-1 and bis-AMD-2 are twice as large as those determined for AMD-1 and AMD-2, respectively. The observed additivity of the absorption spectra obtained for the monomeric and dimeric compounds clearly demonstrates that the 2 AMD-like fragments of bis-AMD do not interact with each other in solution.

When bis-AMD-1 is bound to DNA the observed spectral changes are very similar to that detected on binding of AMD-1 (fig.2). However, the amplitudes of the spectral changes accompanying the complex formation between bis-AMD-1 and DNA are 2-times

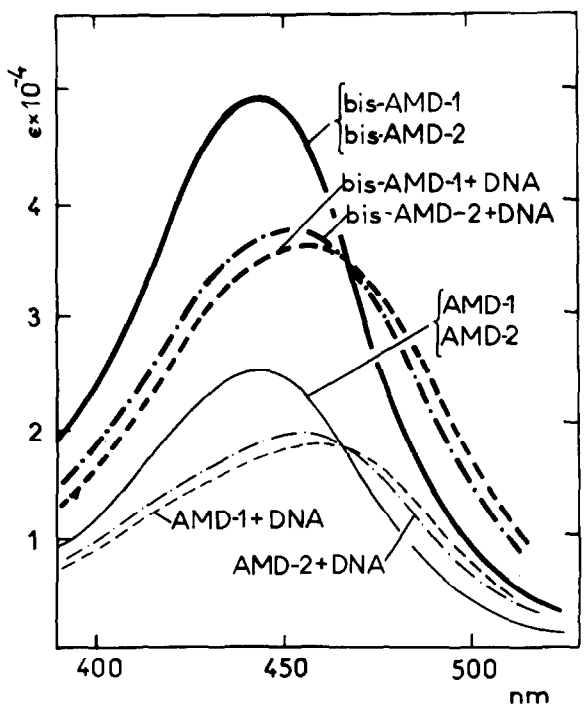

Fig.2. Absorption spectra of bis-AMD-1, bis-AMD-2 and AMD analogs (AMD-1, AMD-2) in the free state and in the complex with calf thymus DNA. ε is the molar extinction coefficient. Ligands were 4×10^{-6} M, DNA was 4×10^{-4} M (basepairs). The spectra were measured in 0.05 M phosphate buffer (pH 6.8) containing 5×10^{-4} M EDTA.

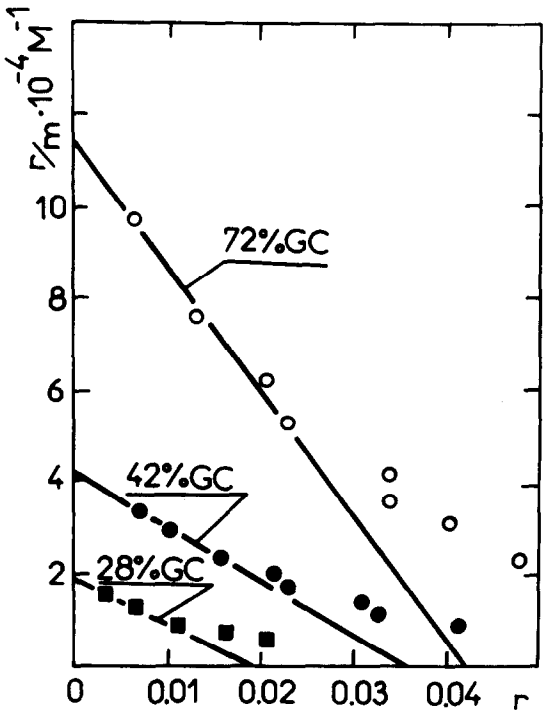

Fig.3. Scatchard plots for the binding of bis-AMD-1 to various DNAs. r is the ratio of mol bound bis-AMD-1/mol DNA base-pairs. m is the molar concentration of free bis-AMD-1 in solution. The solid lines show the initial slopes of binding isotherms. Data points are shown for titrations of *Micrococcus lysodeikticus* DNA (○), calf thymus DNA (●) and *Clostridium perfringens* DNA (■).

greater than the corresponding changes observed on binding of AMD-1. This strongly suggests that the two AMD-like fragments of bis-AMD-1 bind to DNA in virtually the same manner as does AMD-1.

Fig.3 shows Scatchard isotherms obtained for binding of bis-AMD-1 to various naturally occurring DNAs. Similar data were obtained for binding of bis-AMD-2. From fig.3, one can conclude that the initial slopes and the intercepts of the binding isotherms on the vertical axis depend strongly on GC content of DNA. It is well-known that intercept of a binding isotherm on the vertical axis, $\lim_{r\to 0} r/m$, is equal to an average intrinsic binding constant of a ligand to an isolated site on DNA [4,11–13]. If ligand carries n reaction centers which are capable of interacting with GC pairs only, the average binding constant K is given by [12,13]:

$$K = \lim_{r\to 0} r/m = K_0\, S^n\, X_{GC}^n \qquad (1)$$

Here, S is the stability constant which characterizes the interaction between a single ligand reaction center and GC pair. X_{GC} is the fraction of GC pairs in DNA. K_0 is the constant which characterizes unspecific interactions between ligand and DNA.

From eq. (1) one can conclude that the total number of ligand reaction centers, n, can be determined from the slopes of experimental plots of $\ln K$ *vs* $\ln X_{GC}$. Fig.4 shows such plots calculated for the binding of the monomeric and dimeric AMD analogs to various naturally occurring DNAs. From fig.4 one can conclude that the best fit between experimental and theoretical plots of $\ln K$ *vs* $\ln X_{GC}$ calculated for binding of AMD, AMD-1 and AMD-2 takes place when $n = 1$. This means that each monomer AMD molecule carries one GC-specific reaction center, irrespectively on the presence or absence of a bulky substituent in position 7 of the antibiotic chromophore ring. This result dis-

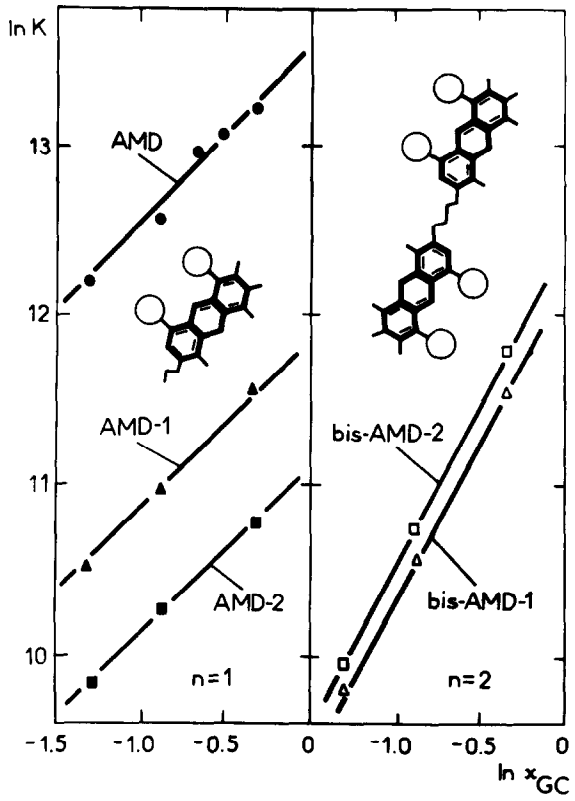

Fig.4. Plots of $\ln K$ against $\ln X_{GC}$ calculated from binding bis-AMD and AMD analogs to various DNAs. The experimental data are compared with the theoretical curves $\ln K = \ln K_0 + n\ln S + n\ln X_{GC}$ calculated for $n = 1$ and $n = 2$ (solid lines).

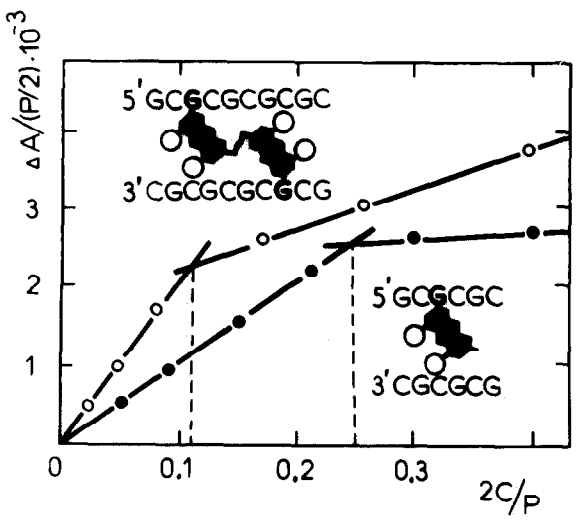

Fig.5. Titration of poly(dG–dC) · poly(dG–dC) with AMD-1 (•–•) and bis-AMD-1 (○–○). ΔA is the difference in the absorbance at 425 nm of ligand solutions in the absence and presence of poly(dG–dC) · poly(dG–dC). The concentration of poly(dG–dC) · poly(dG–dC), $P/2$, was 2×10^{-4} M (basepairs). C is the total molar concentration of the ligand in the titration assay.

agrees with Jain and Sobell's model for AMD–DNA complex predicting that the antibiotic molecule carries 2 GC-specific reaction centers associated with the carbonyl oxygens of threonyl residues in the 2 pentapeptide lactones of AMD [5]. Evidently, the AMD chromophore rather than peptide rings carries determinants of specificity [3,6].

In the case of binding of bis-AMD-1 and bis-AMD-2 the best fit between experimental and theoretical plots of $\ln K$ *vs* $\ln X_{GC}$ takes place when $n = 2$. This finding coupled with our other observations strongly suggest that the 2 monomer fragments of bis-AMD bind specifically and that each fragment carries one GC-specific reaction center.

Fig.5 shows typical titration curves obtained for binding of AMD-1 and bis-AMD-1. From measured saturation level of binding of AMD-1 we conclude that each bound AMD-1 molecule occupies 4 basepairs. By contrast, the titration curve for binding of bis-AMD-1 exhibits no well-defined saturation plateau, but shows a sharp break in which the slope of the titration curve changes. We interpret these observations as indicating that bis-AMD-1 can form strong and weak complexes with DNA and that the strong complex is saturated when one bis-AMD-1 molecule is bound/9 or 10 basepairs. Evidently, in a strong complex, both monomeric fragments of bis-AMD-1 are bound to DNA while in a weak complex 1 monomeric fragment is bound, the other dangling free. The site size for strong binding of bis-AMD-2 is found to be nearly equal to that determined for binding of bis-AMD-1.

It is of interest to compare DNA binding properties of bis-AMD with predictions of recent models proposed for AMD–DNA complex. According to Jain and Sobell's model [5] atomic groups linked to position 7 of the intercalated AMD chromophore are projected into the major DNA groove whereas the antibiotic pentapeptide rings are located in the minor groove. For intercalation of two chromophores of bis-AMD into DNA this complex geometry seems to

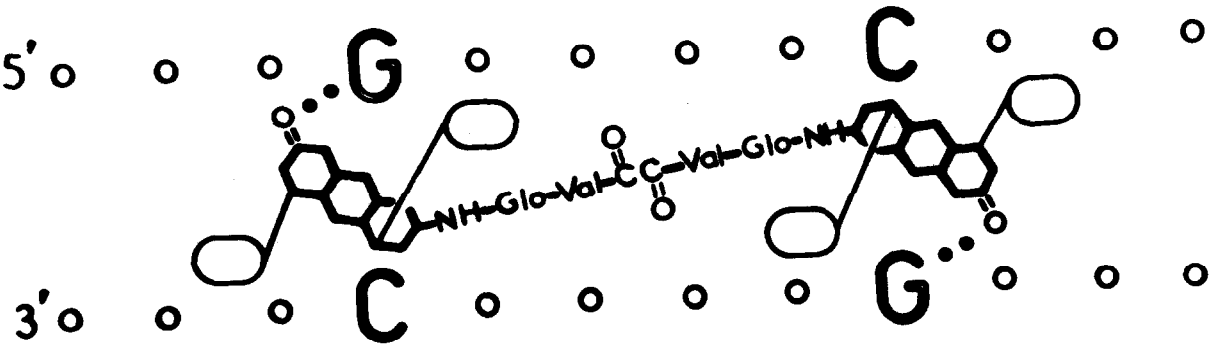

Fig.6. Schematic illustration of the proposed model for the strong complex between bis-AMD-1 and DNA. The 2 phenoxazone chromophores of bis-AMD-1 are located in the minor DNA groove and are hydrogen bonded to 2 guanine bases lying in the opposite polynucleotide chains and separated from each other by 4 basepairs. Open circles symbolize DNA bases which are not implicated in specific interaction with bis-AMD-1. The dotted lines represent hydrogen bonds.

be very unlikely since it requires insertion of the bulky connecting chain of bis-AMD between DNA basepairs.

Examination of outside-binding model for AMD–DNA complex [6] led us to a conclusion that bulky substituents in position 7 of the antibiotic chromophore are projected into the minor DNA groove. In addition, the connecting chain in the molecule of bis-AMD-1 seems to be long enough to allow for hydrogen bonding of the 2 chromophores to 2 guanine bases lying in the opposite polynucleotide chains and separated by 4 basepairs (fig.6). In the complex, the 2 AMD-like fragments of bis-AMD are probably related by 2-fold rotational symmetry. The NH bond of guanine N2 forms an angle of ~20° with the dyadic axis of DNA indicating that the NH bonds in GC and CG basepairs form an angle of ~40°. This provides a basis for discrimination of GC and CG basepairs by a potential hydrogen bond acceptor (presumably, the quinone oxygen of the phonoxazone chromophore). The proposed model agrees with our experiments showing that the binding constant of bis-AMD-1 to poly(dG–dC) · poly(dG–dC) is ~100-times larger than that determined from binding to poly(dG) · poly(dC) (the experimental values of $\lim_{r\to o} r/m$ are 4.3×10^5 M^{-1} and 4×10^3 M^{-1} for binding to poly(dG–dC) · poly(dG–dC) and poly-(dG) · poly(dC), respectively). In the latter polymer all guanine bases are present in 1 polynucleotide strand, thereby allowing for hydrogen bonding of 1 AMD-like fragment of bis-AMD only. Similarly, at low extents of binding bis-AMD-1 interacts strongly with calf thymus DNA ($\lim_{r\to o} r/m = 4 \times 10^4$ M^{-1}) and forms less-stable complexes with poly(dA–dC) poly-(dG–dT) ($\lim_{r\to o} r/m = 2 \times 10^3$ M^{-1}) under the same conditions. Other experiments show that bis-AMD-2 exhibits the same sequence specificity. These experiments as well as data showing that each bis-AMD molecule carries 2 GC-specific reaction centers clearly demonstrate that bis-AMD-1 and bis-AMD-2 exhibit greater selectivity of binding that does AMD.

Earlier, DNA binding properties of the AT-specific ligand bis-netropsin have been reported [14]. This synthetic ligand inhibits selectively initiation of transcription from certain procaryotic promoters [15]. Synthesis of a second GC-specific ligand, bis-AMD, is important for further progress in designing of sequence-specific ligands.

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