Kinetic evidence that echinomycin migrates between potential DNA binding sites

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ABSTRACT

The hypothesis that echinomycin locates its preferred nucleotide sequences in DNA by a process of "shuffling" between potential binding sites has been tested. Immediately after reacting with calf thymus DNA the antibiotic is relatively weakly bound inasmuch as the complex dissociates quite rapidly when detergent is added. If the complex is allowed to equilibrate for various periods of time after mixing, an increasing proportion of the bound antibiotic dissociates slowly on addition of detergent. The kinetics of appearance of the slowly-dissociating form, and its dependence upon ionic strength, are fully consistent with the shuffling model. In contrast the dissociation profiles from poly(dG-dC) and poly(dA-dT) are independent of mixing time.

INTRODUCTION

The biochemical target for several clinically important antitumour drugs seems to be DNA, which they may recognise with varying degrees of specificity [1,2]. A well known example is actinomycin D which binds selectively to DNA at the sequence GpC[3-5]. A different pattern of specificity is seen with the bifunctional intercalating quinoxaline antibiotic echinomycin (Figure 1) which also binds best to G+C rich DNAs [6,7] and has recently been shown to recognise the sequence CpG [8,9].

Crystallographic and model building studies have indicated the molecular interactions which probably serve as determinants of sequence-recognition by such ligands [2-4, 10-12]. But how do they locate their preferred binding sites in natural, heterogeneous DNA containing a plethora of potential intercalative sites? Only a kinetic approach can address this question experimentally. Studies with both actinomycin and echinomycin have revealed that their dissociation profiles can be described by the sum of three exponential terms [13-16]. This has been interpreted as evidence for the existence of three broad classes of intercalative binding sites having different affinities for the antibiotics. The most preferred (i.e. tightest) binding sites in each case are identified as those

Figure 1. Structure of echinomycin.

characterised by the slowest rate of dissociation. Moreover, there is evidence that the slow dissociation of echinomycin from those sites is primarily responsible for its high biological activity [17,18].

The association reaction between actinomycin and DNA can be resolved into five components which could reflect conformational adjustments in the interacting molecules [13,14] and/or a process of "shuffling" whereby the antibiotic interacts initially with a variety of nucleotide sequences, and subsequently migrates along the DNA lattice so as to attain optimum binding [15]. We favour the latter hypothesis. By contrast, the situation with echinomycin is less complicated: its association with natural DNA appears as a one-step process [19]. Presumably, if the antibiotic does shuffle between its potential binding sites this reaction is not accompanied by any detectable absorbance changes.

In this paper we report the results of a series of simple experiments to test the hypothesis that echinomycin first binds rather non-specifically to many nucleotide sequences in DNA and then, over a period of minutes, becomes redistributed on to slowly-dissociating sites by a process of "shuffling".

MATERIALS AND METHODS

All experiments were conducted at 20°C in a Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid] NaOH buffer, pH 7.0, $I = 0.01$ containing 2mM Hepes, 10μM EDTA and 9.4mM NaCl. Reagent grade water from a Millipore Milli Q2 system was used throughout. Calf thymus DNA (highly polymerized sodium salt, type I) was obtained from Sigma Chemical Co., St Louis, MO, USA. Poly(dG-dC) and poly(dA-dT) were purchased from the Boehringer Corp. Nucleic acid concentrations are expressed with

respect to nucleotides and are based on the following values for $\epsilon(P)_{260}$: calf thymus DNA, 6600; poly(dG-dC), 7100; poly(dA-dT), 6700. Echinomycin was obtained from Drs H. Bickel and K. Scheibli of CIBA-Geigy Ltd., Basel, Switzerland. Solutions of echinomycin were prepared by shaking the solid material with buffer for two hours. The excess antibiotic was removed by filtration and the concentration of the solution estimated from the absorbance at 245nm using an extinction coefficient of 48,200 [20].

All dissociation experiments were performed in 40mm optical path length semi-micro quartz cuvettes. Typically 3ml of a nearly saturated antibiotic solution (5 μ M) was mixed with 0.2ml of a concentrated DNA solution to yield a final DNA concentration of 500 μ M and a drug/nucleotide ratio (D/P) of 0.01. This complex was then dissociated by adding 0.8ml of a solution of sodium dodecyl sulphate (SDS) (10%, w/v), and the absorbance change at 320nm was monitored using a Unicam SP8-200 spectrophotometer as previously described [15-17]. Whenever possible readings were continued for at least four times the longest observed dissociation time constant. Because of the small absorbance change ($\Delta A=0.05$) the bandwidth was set to high energy (10nm) with full scale deflection on the chart recorder set to 0.05A.

The decay was analysed as previously described [15-17,19] using a computer program written by Johnson and Schuster [21] according to a non-linear least-squares Gauss-Newton method. The program decomposes the decay into the sum of three exponentials so as to satisfy the equation

$$A_t = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + A_3 e^{-t/\tau_3}$$

RESULTS

If echinomycin first binds rather non-specifically to many nucleotide sequences in DNA and subsequently redistributes itself onto preferred binding site(s), then shortly after mixing a large proportion of the ligand molecules should be bound to weaker sites and this should be reflected in the dissociation profile. Such is found to be the case. Representative profiles are shown in Figure 2, where the echinomycin and DNA were left in contact for different periods of time before being dissociated by the addition of detergent. When the reactants have fully equilibrated (after at least 30 mins) the decay is characterized by two exponentials with time constants of 670 $\pm$ 160s and 45 $\pm$ 15s, and 32% of the total decay is represented by the slower dissociating species. This behaviour is similar to that previously described [16] except that at this very low ratio of antibiotic

Figure 2. Detergent-induced dissociation of echinomycin from calf thymus DNA. The complexes were left for various times before dissociating by addition of SDS. In each plot the antibiotic concentration was 5 μ M; the DNA concentration 500 μ M; and the final SDS concentration was 2% (w/v). The ordinate represents the natural logarithm of the fractional absorbance change. (Δ) The complex was left for 30s before dissociating. The fitted line is a single exponential with a time constant of 37.7s. ($\circ$) The complex was left for 5 mins before dissociating. The fitted curve is described by the sum of two exponentials with parameters $\tau_1=37$ s (80%), $\tau_2=597$ s (20%). ($\bullet$) The complex was left to equilibrate for several hours before dissociating. The fitted curve represents the sum of two exponentials with parameters $\tau_1=31$ s (66%), $\tau_2=757$ s (34%).

to nucleotides the intermediate time constant τ_2 has vanished. In contrast, when the reactants have been mixed for shorter periods of time the magnitude of the slower component is decreased, although the kinetic constants remain effectively the same. After the shortest mixing interval (30s) the slowest component falls below the level of detection, and the whole decay is characterized by a single exponential with a time constant equal to that of the faster dissociating species. The magnitude of the slower component increases exponentially as a function of pre-equilibration time (Figure 3) and the time constant for its appearance is 273 ± 16 s, which is consistent with the hypothesis that the antibiotic shuffles between potential binding sites until optimal binding is attained.

Figure 3. Amplitude of the slowest component in the dissociation reaction as a function of time after mixing echinomycin and DNA at $I=0.01$. The amplitude is expressed in absolute absorbance units on the left ordinate and as a percentage of the total absorbance change on the right, plotted for various pre-equilibration times. The curve fitted to the points is an exponential described by a time constant of 274s.

The rate at which the antibiotic appears on the tighter binding sites will depend on the rate of dissociation from the weaker sites as well as the relative abundance of the two classes of sites. However, any molecule which dissociates from the DNA is more likely to reassociate with the most abundant (weaker) sites, so that the overall rate of appearance of the slow dissociating species will generally be less than the rate of dissociation from the weaker sites. Under the experimental conditions in Figure 2 the putative shuffling takes place at ionic strength 0.01, whereas the addition of 2% SDS to initiate dissociation raises the resultant ionic strength to 0.1. Increased salt concentration is known to speed up the dissociation of the antibiotic [16]. It is therefore not possible to compare directly the rate of appearance of the slow component with the dissociation of the weaker species. To investigate this problem further experiments were performed in which the antibiotic and DNA were left to equilibrate at $I=0.1$ before dissociation by addition of SDS. For all pre-equilibration times the dissociation profile was described by the sum of two exponentials with time

Figure 4. Amplitude of the slowest component in the dissociation reaction as a function of time after mixing echinomycin and DNA at $I=0.10$. The ordinate represents the amplitude expressed in absolute absorbance units. The curve fitted to the points is an exponential described by a time constant of 175s.

constants of 300 ± 60 s and 31 ± 6 s, faster than at the lower ionic strength, as expected. Once again the relative amplitudes of the two components were found to vary with the length of time for which the antibiotic and DNA had been in contact. The amplitude of the slower component increased exponentially as a function of pre-equilibration time (Figure 4) with a time constant of 175 ± 17 s.

In a similar series of experiments performed with poly(dG-dC) and poly(dA-dT) the dissociation profiles were found to be independent of the mixing time. After preequilibration for only 30s the dissociation profiles were completely described by single exponentials with time constants of 53 ± 6 s for poly(dG-dC) and 14.0 ± 3.2 s for poly(dA-dT) similar to the values of 55 ± 9 s and 17.2 ± 1.3 s seen with complexes which had been preequilibrated for several hours. The amplitude of the absorbance changes were also unaffected by the premixing time. It appears that for these synthetic polymers the antibiotic rapidly attains binding to the most preferred site (CpG in poly(dG-dC)) either by never binding to the other sequence or by migrating from it onto the tight sites very rapidly.

DISCUSSION

The results presented here establish that the profile of dissociation of echinomycin from natural DNA depends on the length of time for which the reactants have been in contact. Shortly after mixing the slower dissociating species represents only a very small proportion of the decay, increasing to 32% after longer mixing intervals. The rate of appearance of the slowly dissociating species is somewhat slower than the rate of dissociation from the weaker (more abundant) binding sites, as anticipated.

Without recourse to any detailed analysis of kinetic models two simple theories can be proposed to account for these observations. Either the ligand redistributes over its potential binding sites, or slow conformational changes occur in the antibiotic or the DNA which alter the dissociation rate. It is difficult (if not impossible) to eliminate the hypothesis of conformational changes purely on the basis of kinetic data, but the available evidence clearly favours the "shuffling" hypothesis for the following reasons.

Firstly, the dissociation of the antibiotic from poly(dG-dC) and poly(dA-dT) is independent of the premixing time and contrasts markedly with the reaction seen with natural DNA. If conformational changes in the antibiotic were responsible for the time dependence seen with natural DNA there should be an equally pronounced dependence with the synthetic DNAs which contain a much larger proportion of the putative tight binding sites. The fact that the two types of DNA behave so differently in this respect provides compelling support for the shuffling hypothesis.

Secondly, a mechanism involving redistribution of ligand molecules is consistent with the results of stopped-flow experiments [19] in which the association reaction with natural DNA was found to be completely described by a single exponential, whereas the reaction with poly(dG-dC) required the sum of two exponentials, explained on the basis of ligand initially binding to both types of sequences (CpG and GpC) and subsequently migrating on to the preferred sequences (CpG). Shuffling was not detectable with natural DNAs because the spectral properties of echinomycin are very similar when bound to different nucleotide sequences.

Thirdly, increasing the ionic strength speeds up the rate at which optimal binding is attained, and is also known to increase the dissociation rates [16]. Elevated ionic strength is likely to slow down any conformational changes as a result of increased rigidity of the DNA helix,

although structural changes in the DNA cannot be excluded.

Fourthly, the rate at which optimal binding develops is five-fold slower than the dissociation of the antibiotic from its weaker binding sites, and so falls close to the expected time scale. It would be fortuitous if conformational change(s) happened to occur at exactly the right rate to generate maximal confusion with ligand migration.

While each of these observations alone is not sufficient to prove unambiguously that ligand redistribution does occur, when taken together they provide coherent evidence for such a scheme, which would be comparable in all respects to the mechanism by which actinomycin binds to its preferred sites [15]. We are also aware of the possibility that ligand migration might help to explain why the calculated echinomycin-DNA equilibrium binding constant, derived from the quotient of measured on- and off-rates [16,19], is 3-6 times larger than that determined directly by solvent partition analysis.

One obvious difference between the present results and those reported for actinomycin D is that with echinomycin the slowest dissociating species only accounts for 32% of the total decay, whereas with actinomycin D at a similar binding ratio the slow component accounts for nearly 70% of the total. We note that the sequence to which echinomycin binds best (CpG) occurs in calf thymus DNA at only one third the frequency of GpC, the preferred sequence for actinomycin binding [22]. On the other hand, the difference might arise because echinomycin displays less pronounced sequence selectivity than actinomycin. It is also worth noting that in the experiments with actinomycin D the slowest component was still detectable immediately after mixing whereas with echinomycin it was below the level of detection when the reactants were left in contact for 30s or less. This may indicate that few echinomycin molecules bind directly to the optimal, tightest sequence, either because it occurs at very low frequency, or because the rate constant for association with this sequence is unusually low.

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