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DRUG–DNA DISSOCIATION KINETICS

IN VITRO TRANSCRIPTION AND SODIUM DODECYL SULPHATE SEQUESTRATION

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Abstract—The rate of dissociation of actinomycin D from DNA was measured by sodium dodecyl sulphate (SDS) sequestration (37°) from calf thymus DNA and a 24 base pair (bp) synthetic DNA containing one high affinity AGCT site for the drug. The time constants were 276 and 142 sec, respectively, and suggest a stabilising effect though positive cooperativity in heterogenous DNA, or from specific neighbouring sequences. The time constant for dissociation of actinomycin D from the AGCT site was 2900 sec as measured by an *in vitro* transcription assay at 37°, and suggests that, under conditions of active transcription of the DNA, the drug–DNA complex has additional stabilising contributions, possibly by a cage effect from RNA polymerase, or by additional drug–RNA polymerase contacts.

There has been considerable debate in recent years as to the mode of action of anti-cancer drugs which bind reversibly to DNA [1–4]. Much speculation has centred on the role of the rate of dissociation of these drugs from their DNA substrate [5–8], and there has been recent support for the notion that the longer a drug resides on DNA, the greater its cytotoxic effect [9]. As a consequence of this interest in drug–DNA dissociation rates, there has been a commensurate effort to find simple procedures to quantitate these dissociation rates. The earliest procedure utilised the tedious, but rigorous T-jump method [10], but this has largely been replaced by a much simpler and widely employed sodium dodecyl sulphate (SDS) induced sequestration method [11–14]. This procedure relies on an essentially unidirectional sequestration of drug by SDS micelles, with the drug kinetics being monitored by a change of absorbance accompanying the process from bound to free drug. Recently, a more physiological system was demonstrated in which the drug dissociation rate was measured under conditions of active transcription of the DNA by RNA polymerase [15]. Use of this procedure resulted in the unexpected phenomenon that actinomycin D dissociated some five times slower from the transcribed DNA than measured by SDS sequestration from naked calf thymus DNA. This difference was attributed to an averaging of dissociation rates from a range of differing affinity drug binding sites observed during SDS sequestration, as compared to discrete sites in the *in vitro* transcription assay. Alternatively, the difference may be due to the extremes of solvent conditions for the two methods, given that the SDS sequestration procedure requires the presence of 1–2% SDS.

We have addressed this problem of apparent inconsistency of drug–DNA kinetic data, as determined by different methods, by synthesizing the same drug binding site analysed by the *in vitro* transcription assay [16], and monitoring the rate of dissociation of drug from that site under SDS sequestration conditions. The transcription assay probed over 200 base pairs (bp) from the *lac* UV5 promoter, and detected six high affinity actinomycin D binding sites, five of which were GpC [16]. In this assay, the rate of elongation of RNA past the drug binding site is a direct measure of the rate of dissociation of the drug from that site. When multiple drug binding sites are present, "read-through" of RNA polymerase from one site to other downstream sites occurs, and must be taken into account when analysing such multi-site transcription data [16]. To avoid any possible errors arising from this source, we have limited our comparative analysis of SDS sequestered and transcription derived drug dissociation kinetics to the first drug site detected in the transcription assay [16].

MATERIALS AND METHODS

SDS buffer. The buffer used for SDS sequestration studies comprised 40 mM Tris (pH 8.0), 3 mM $MgCl_2$, 400 mM NaCl and 0.1 mM EDTA. These conditions were analogous to those employed in the *in vitro* transcription assays [16], except that, in order to improve the solubility of SDS, NaCl was used instead of KCl, and bovine serum albumin (BSA) was omitted.

Materials. Calf thymus DNA was obtained from Calbiochem (San Diego, CA) and was dissolved in the SDS buffer, filtered (0.2 μm, Millipore, Bedford, MA) and quantitated spectrophotometrically using a base-pair molar extinction coefficient of 13,200 M^{-1} cm^{-1} (260 nm). Actinomycin D (AMD) was also purchased from Calbiochem and was dissolved in SDS buffer and quantitated spectrophotometrically using $E = 24{,}450\ M^{-1}\ cm^{-1}$ (440 nm). SDS (mol-

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ecular biology grade) was purchased from Sigma (St. Louis, MO) and was dissolved in SDS buffer to yield a 10% (w/v) solution.

Sephadex G-50 and T4 polynucleotide kinase were purchased from Pharmacia (Uppsala, Sweden). [γ-^{32}P]ATP was obtained from Bresatec (Adelaide, S.A., Australia). All other chemicals were analytical reagent grade and prepared using Type I water from a Milli-Q 4-stage water purification system (Millipore).

Synthesis of 24'mer. Complementary synthetic oligodeoxynucleotides, corresponding to 28–47 of the transcribed 497 bp UV5 DNA [16], were synthesized on a model 381A Applied Biosystems DNA synthesizer. Purity and yields of the DNA were assessed by trityl cation assay (Applied Biosystems User Bulletin, No. 13, November, 1984), HPLC analysis of the trityl containing species, and by 5' end-labelling of the detritylated oligonucleotide using [γ-^{32}P]ATP and T4 polynucleotide kinase according to the methods of Maniatis *et al.* [17].

Following cleavage from the solid support, deprotection and then detritylation, the oligonucleotides were finally taken up in 1.0 ml of Milli-Q water. Partial purification was accomplished by gel filtration through a 6.5 cm by 1.5 cm Sephadex G-50 column, where only the first A_{260} absorbing fractions were pooled. Subsequent end-labelling analysis revealed insignificant oligodeoxynucleotides less than 12 bases long.

To anneal the complementary strands, equimolar amounts of the two oligonucleotides were combined in a buffer containing 100 mM NaCl, 10 mM Tris·HCl (pH 8.0) and 0.1 mM EDTA, heated to 90° for 10 min and allowed to cool slowly overnight to 26° (25° below the calculated melting temperature of the annealed 24'mer). Verification of the annealed product was obtained by electrophoresis through a 20% non-denaturing polyacrylamide gel against suitable molecular weight markers, followed by staining with ethidium bromide.

SDS sequestration. AMD (0.75 ml, 47 μM) was added to DNA (0.25 ml, 3.5 mM bp) and 1.0 ml SDS buffer to yield a drug to DNA (bp) ratio of 1:25 and allowed to equilibrate for 1 hr. SDS (0.5 ml, 10%) was then added rapidly, the solution mixed well, and the absorbance then monitored at 425 nm in a Cary 118 spectrophotometer.

RESULTS

SDS dissociation kinetics. To assess the validity of the kinetics determined by the transcription assay, SDS studies were undertaken with calf thymus DNA and a synthetic 24 bp fragment of DNA under conditions employed during the elongation phase of the transcription assay [16]. The sequence of the synthetic fragment corresponds to the 28–47 region of the transcribed 497 bp UV5 DNA, and has an AMD site in the centre of this region [16].

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5'- AATTCAGGAAACAGCTATGACCAT
    GTCCTTTGTCGATACTGGTATTAA - 5'
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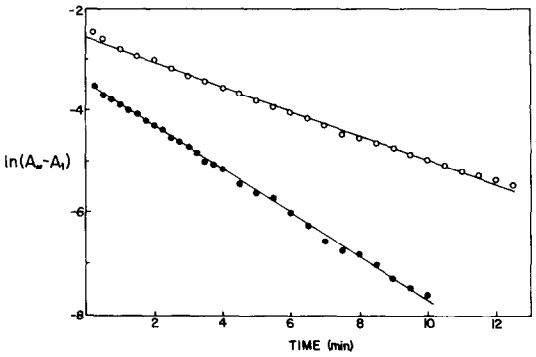

Fig. 1. SDS sequestration of actinomycin D from DNA. First-order kinetic plots are shown for the dissociation of AMD from the synthetic 24'mer (●) and from calf thymus DNA (○) at 37°, at a drug loading of 0.04 per bp.

Table 1. Time constants for the dissociation of actinomycin D (AMD) from DNA

DNA	Method	Drug/bp	τ (sec)
24'mer	SDS	0.04	142
	SDS	0.01	140
497 bp UV5	T (I = 0.4)	—	2900
fragment	T (I = 0.1)	—	2700
Calf thymus	SDS	0.04	276

The time constants are for *in vitro* transcription detected (T) and SDS sequestered dissociation of AMD from DNA at 37°. The same solvent conditions were used for both methods [16]. The drug/bp value in the transcription assay is not meaningful because some of the AMD is bound to protein in the transcription buffer.

The two 24'mers were synthesized so that, following annealing, Eco RI ends remained for potential genetic manipulations.

The SDS-induced dissociation profiles of AMD from calf thymus DNA and the synthetic 24'mer are shown in Fig. 1. The profiles were obtained at low drug loading (0.04 AMD/bp) to ensure that there was one AMD molecule for each synthetic DNA (Fig. 1). The linear nature of the first-order plot for the oligomer extended over four half-lives and confirms that the decay process is adequately described by a single dissociation event. For calf thymus DNA, the first-order plot exhibited a faster process (or processes) in the first 30 sec, but was adequately described by a single event for the next 12 min. These results are consistent with previous observations of a unique dissociation process of actinomycin D from a single class of binding site, as in poly dG·poly dC [18, 19], whereas multiple dissociation processes have been routinely observed for calf thymus DNA [19–21].

The time constants were 142 and 276 sec for the synthetic DNA and calf thymus DNA respectively. The kinetics of dissociation of AMD from the synthetic DNA were not altered by a decrease of drug loading to 0.01 per bp, or by diluting the drug–DNA solution 5-fold (Table 1). These results suggest strongly that the dissociation of AMD from the syn-

thetic DNA is a single-step independent process from one site, and this is assumed to be AGCT, based on the well documented GpC preference of AMD for this sequence [18, 22–24]. The dissociation from calf thymus DNA can be explained in terms of the antibiotic dissociating simultaneously from a range of binding sites which are resolvable as at least two kinetic events [19–21], the slower dissociating site under our high ionic strength transcription conditions being by far the more predominant.

DISCUSSION

The SDS-induced dissociation of AMD was some 17 times faster from the 24′mer than from the same sequence of DNA used in the *in vitro* transcription assay, and this confirms the original observation [15] that transcription-detected dissociation events are slower than the comparable sequestration process. This now raised questions as to why this difference occurs, whether one of the values is somewhat artifactual, and which procedure yields the more meaningful result as a screening process for possible DNA-directed drug activity.

There are two possible extremes to account for the observed kinetic differences, one being that the SDS procedure destabilises the drug–DNA complex (resulting in unrealistically small time constants) or, alternatively, that the transcription assay contributes additional stabilisation of the drug–DNA complex (resulting in enhanced time constants). Destabilisation of the drug–DNA complex could be attributed to the increase of ionic strength accompanying the addition of 2% SDS to the solution, but this is considered unlikely since this results in only a 15% increase of ionic strength and would not account for the observed effects—the dissociation of charged intercalators varies with log I [13], and AMD, being uncharged, would be expected to be even less sensitive to changes of ionic strength. Furthermore, no major changes of the drug dissociation kinetics were observed in the transcription assay for elongation conditions varying from 0.1 to 0.4 M ionic strength (Table 1).

Destabilisation could also be due to the effect of SDS in enhancing the hydrophobicity of the solvent, thus partially denaturing the DNA. Conventional melting curves of calf thymus DNA in the presence and absence of 2% SDS revealed no discernible differences of stability between the two conditions (within ±0.1°), and this serves to discount altered hydrophicity as a possible destabilising element. It therefore appears unlikely that the SDS sequestration procedure yields artifactual dissociation values, although it is acknowledged that the process is an approximation since it assumes a unidirectional dissociation event with negligible contributions from reverse processes. This conclusion is supported by the detection of virtually identical values for the dissociation of daunomycin from DNA, as measured by SDS sequestration and the thermodynamically rigorous T-jump procedure [11], and by the initial observations of Muller and Crothers [20] that the same time constants were obtained from SDS sequestration and dissociation by dilution for a range of actinomycin derivatives.

The alternative explanation assumes a stabilising effect inherent in the transcription assay, but not in the sequestration procedure. This could arise from a "cage effect" where the drug is constrained in a small volume by the physical size of RNA polymerase, known to cover up to 30 bp of DNA, and extending approximately 9 bp downstream of the active catalytic site [25], which is also the location of the drug binding site [15, 16]. Such a cage effect would result in a higher effective free concentration of drug following dissociation from the DNA, and hence a higher probability of re-binding, resulting in a longer DNA residence time. The physical bulk of RNA polymerase surrounding the drug site could also result in additional drug-polymerase contacts, and this too could enhance the drug residence time. Both of these factors would be expected to be exacerbated by large drugs such as AMD with its extensive pentapeptide appendages, but would be anticipated to be diminished for drugs with smaller pendant groups.

An additional, but smaller stabilising factor has also been noted from a comparison of the SDS sequestered time constants for calf thymus DNA and the 24′mer (Table 1). The 24′mer contains the high affinity AGCT site for AMD [16, 23], but the drug residence time was only half that observed with heterogenous calf thymus DNA (which yields an average time constant for a range of equal or lesser affinity drug sites). It therefore appears that multiple drug sites are stabilised either by conformational effects transmitted along the DNA from neighbouring sites, as suggested by previous observations of positive cooperativity of AMD binding to DNA [16, 18], or by specific sequences surrounding the AMD sites. The existence of such stabilizing effects is also implied by the residence time of 1500 sec obtained transcriptionally for the AGCT site in the relatively short 203 bp fragment used previously [15], compared to a value at 2900 sec obtained from a 497 bp fragment containing an additional 312 bp downstream sequence [16].

In summary, we conclude that the 17-fold difference between the rate constants for the dissociation of AMD from DNA, as measured by transcription and SDS sequestration, was due mainly to a stabilisation associated with the presence of RNA polymerase (either a cage effect or direct additional drug–polymerase contacts). Stabilisation also arose, to a lesser degree, from positive cooperative binding of AMD to DNA. Both techniques yield legitimate kinetic parameters, with the SDS sequestering procedure being much simpler, considerably faster, but yielding only approximate rate constants. In contrast, the *in vitro* transcription assay is more complex and slower, but yields a physiologically more meaningful estimate of drug life-time at individual sites, and these can be up to 20-fold longer than measured by SDS sequestration. The difference, however, is likely to be less for drugs which are physically smaller than AMD.

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