Enzymatic Activation of DNA Cleavage by Dynemicin A and Synthetic Analogs†

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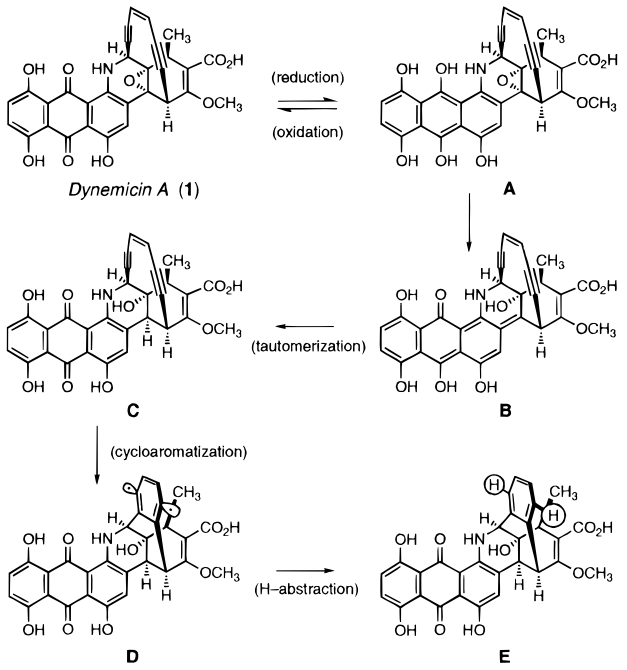
ABSTRACT: Dynemicin A (**1**), a member of the enediyne family of natural products, binds to double-stranded DNA ($K_B \sim 10^4$ M^{-1}) and in the presence of millimolar concentrations of a reducing cofactor such as NADPH or GSH reacts to cleave DNA. In this work, we show that the two flavin-based enzymes ferredoxin−$NADP^+$ reductase and xanthine oxidase catalyze the reductive activation of **1** by NADPH and NADH, respectively. The enzyme-catalyzed reductive activation of **1** leads to more rapid and efficient cleavage of DNA, even with 10−20-fold lower concentrations of the stoichiometric reductant. Significantly, the enzymatic systems are also found to activate the tight-binding ($K_B \geq 10^6$ M^{-1}) synthetic dynemicin analogs **3** and **5** toward DNA cleavage. These same analogs do not undergo reductive activation with NADPH or NADH alone, where evidence has been obtained to support the proposal that the DNA-bound drugs are protected from reductive activation. The new enzymatic activation processes described may have important implications for chemistry occurring with **1** and synthetic analogs in vivo, as well as for the future development of dynemicin-based anticancer agents.

Isolated from the fermentation broth of the microorganism *Micromonospora chersina* (Konishi et al., 1990, 1991), dynemicin A (**1**) is an exceedingly potent cytotoxin with LD_{50} values in the picogram to nanogram per milliliter range against a variety of tumor cell lines (Kamei et al., 1991). Dynemicin A (**1**) is structurally unique among the naturally occurring antitumor agents, possessing features characteristic of both the anthracycline (Priebe, 1995) and enediyne antibiotics (Nicolaou & Dai, 1991). Like other members of the enediyne antibiotic family, dynemicin A has been proposed to function in vivo as a DNA-damaging agent following reductive activation. The anthraquinone is postulated to serve as a DNA-binding element by intercalation into the DNA base stack, while the (Z)-enediyne bridge is proposed to lie within the minor groove of DNA (Semmelhack et al., 1990; Langley et al., 1991; Cardozo & Hopfinger, 1991; Wender et al., 1991; Elbaum et al., 1995). In vitro experiments have shown that **1** brings about single- and double-stranded cleavage of B-form DNA in the presence of a reducing cofactor such as NADPH[1] or a thiol, and this activity has been rationalized by the pathway shown in Scheme 1 (Sugiura et al., 1990, 1991; Kusakabe et al., 1995). The proposed cleavage mechanism involves reductive activation of the anthraquinone moiety, which triggers a reaction cascade leading to Bergman cycloaromatization and formation of a reactive 1,4-biradical intermediate that is postulated to abstract hydrogen atoms from the deoxyribose backbone of DNA (Semmelhack et al., 1990; Langley et al., 1991; Cardozo & Hopfinger, 1991; Wender et al., 1991; Elbaum et al., 1995). Sugiura and co-workers have provided evidence for the intermediacy of the biradical **D** in experiments conducted in vitro (Shiraki & Sugiura, 1990; Miyoshi et al., 1991; Shiraki et al., 1992; Kusakabe et al., 1993).

Scheme 1



In addition to extensive studies of the DNA-cleaving activity of dynemicin A, considerable effort has been directed toward the total synthesis of dynemicin A and dynemicin models (Nicolaou et al., 1993; Wender et al., 1993, 1995; Magnus et al., 1993; Toshima et al., 1993; Shair et al., 1994, 1995, 1996). We recently described the completion of a multistep, enantioselective synthetic route to dynemicin A (**1**) that, by virtue of its convergence, also provided access to a wide range of dynemicin analogs (Myers et al., 1994, 1995a,b). Preliminary DNA binding and cleaving experiments with **1** and synthetic analogs **2**−**6** provided insights into the dynamic process of DNA cleavage by **1** (Myers et al., 1995a). One of the striking findings from our preliminary

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[1] Abbreviations: bp, base pair(s); DMSO, dimethyl sulfoxide; GSH, glutathione; NADH, β-nicotinamide adenine dinucleotide disodium salt; NADPH, β-nicotinamide adenine dinucleotide phosphate tetrasodium salt; PAGE, polyacrylamide gel electrophoresis; UV, ultraviolet.

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work was that the tight-binding analogs **3** and **5** ($K_B \geq 10^6$ M^{-1}, versus $K_B \sim 10^4$ M^{-1} for **1**) failed to produce any detectable DNA cleavage in the presence of GSH (20 mM) or NADPH (20 mM), although both **3** and **5** underwent efficient reductive activation in the absence of DNA. Under forcing conditions (Cohen, 1995), those involving the use of a dilute solution of DNA (5 μM), an increased ratio of drug to bp of DNA (1:10), and an increased concentration of reductant (GSH, 50 mM), DNA cleavage by dynemicin methyl ester (**3**) was observed. These findings led us to propose that reductive activation of the tight-binding dynemicin analogs did not occur at any appreciable rate with the activating agents NADPH or GSH when the analogs were bound to DNA. This proposal was found to be generally true for compounds **1**−**6**, as supported by kinetics studies of the rates of reaction of **1**−**6** in the presence of varying concentrations of DNA, as well as by monitoring the rates of DNA cleavage at varying concentrations of drug and DNA ([DNA]/[drug] and [[^{32}P]DNA]/[carrier DNA] held constant). In further studies, described herein, we have found that flavin-based enzymatic reductants dramatically accelerate the rates of DNA cleavage by **1** and dynemicin analogs, to include the tight-binding analogs **3** and **5**. These findings may have important implications for chemistry occurring with **1** in vivo, as well as for the future development of dynemicin-based anticancer agents.

MATERIALS AND METHODS

Materials. Sonicated and deproteinized calf thymus DNA was purchased from Pharmacia and was stored frozen at −20 °C as a solution in sterile water. Plasmid pBR322 was obtained as an aqueous solution from Boehringer Mannheim and was precipitated with ethanol prior to use. Restriction endonucleases were obtained from New England Biolabs or Boehringer Mannheim and were used according to the supplier's recommended protocol in the activity buffer provided. Radioactive nucleotides were purchased from Amersham Life Science or Dupont-NEN. NADH, NADPH, and GSH were commercial reagents (Sigma). Ferredoxin−$NADP^+$ reductase (EC 1.18.1.2, specific activity of 5 units/mg) and xanthine oxidase (EC 1.1.3.22, specific activity of 0.11 unit/mg) were purchased as lyophilized powders (Sigma). Stock solutions of these enzymes in sterile water were stored frozen at −20 °C. All other chemicals were purchased from Aldrich, Sigma, or GIBCO and were of the highest purity available. NICK spin columns loaded with Sephadex G-50 gel were purchased from Pharmacia.

General Procedures. Compounds **1**−**6** were manipulated as solutions in DMSO. Concentrations of drug solutions were determined by UV spectroscopy; extinction coefficients were determined experimentally: **1**, ϵ(560 nm) = 10 670 M^{-1} cm^{-1}; **2**, ϵ(540 nm) = 6440 M^{-1} cm^{-1}; **3**, ϵ(560 nm) = 10 820 M^{-1} cm^{-1}; **4**, ϵ(540 nm) = 7090 M^{-1} cm^{-1}; **5**, ϵ(560 nm) = 11 330 M^{-1} cm^{-1}; and **6**, ϵ(540 nm) = 6800 M^{-1} cm^{-1}. The 3′-^{32}P-end-labeled 193 bp restriction fragment has been previously described (Myers et al., 1995a,b). Standard techniques were employed for DNA manipulations (Sambrook et al., 1989).

General Equilibrium Dialysis. Dialysis experiments were conducted at 23 °C in a standard dialysis buffer solution (Tris-HCl aqueous buffer solution, 30 mM, pH 7.5; NaCl, 50 mM) using a Spectra/Por 2 dialysis membrane (molecular weight cutoff of 12000−14000, flat tube diameter of 10 mm). Dialysis membranes were soaked in water for at least 1 h prior to use. In a typical experiment, one end of a dialysis membrane, 20 cm in length, was sealed with a loop knot. A solution (ca. 3 mL) of known concentrations of drug and double-stranded calf thymus DNA (ratio of 1:20 drug:bp DNA) in dialysis buffer solution was added carefully to the dialysis bag and the open end was sealed with a loop knot. The sealed dialysis bag was then suspended in a known volume of dialysis buffer solution and was vigorously shaken (∼100 rpm) with an Eppendorf mixer for ∼10 h to allow the drug to fully equilibrate across the membrane. A range of concentrations of drug and DNA (at a constant ratio of 1:20) was examined for each drug. The concentrations of drug inside and outside the membrane were determined by UV spectroscopy, and these values were used to calculated the DNA binding constant, according to the equation K_B = [DNA·drug]/[DNA][drug]$_{free}$, where the concentration of drug in the compartment external to the membrane was defined as [drug]$_{free}$ and the concentration of drug within the dialysis membrane represented the sum [DNA·drug] + [drug]$_{free}$.

*DNA Cleavage Experiments with **1**−**6***. Reactions were performed at 37 °C in 1.6 mL Eppendorf tubes in a total reaction volume of 100 μL (10% aqueous DMSO by volume). A 10 μL aliquot of a solution of a given synthetic anthraquinone **1**−**6** (0.5 mM) in DMSO was combined with an aqueous solution of double-stranded calf thymus DNA (10 mM bp, 10 μL), 3′-labeled restriction fragment (∼150 kcpm), NaCl (500 mM, 10 μL), Tris-HCl aqueous buffer solution (500 mM, 6 μL, pH 7.5), and sufficient water to achieve a final volume of 100 μL. Reaction mixtures were incubated at 37 °C for 15 min prior to the addition of a reducing agent. Reactions were initiated at 37 °C by the addition of an aqueous solution of GSH (200 mM, 10 μL), NADPH (200 mM, 10 μL), or NADH (200 mM, 10 μL), thus producing the following concentrations of solution components at the onset of the reaction: **1**−**6** (0.05 mM); double-stranded calf thymus DNA (1.0 mM bp); GSH, NADPH, or NADH (20 mM); NaCl (50 mM); and Tris-HCl buffer (30 mM). Reactions employing an enzyme/NAD(P)H system were carried out in an identical fashion with the following exception. An aliquot of an aqueous solution of ferredoxin−$NADP^+$ reductase (19 μM, 10 μL) or xanthine oxidase (12 μM, 10 μL) was added to the reaction mixture prior to incubation and activation with NADPH (10 mM, 10 μL) or NADH (10 mM, 10 μL), respectively. This produced the following concentrations of solution components at the onset of the reaction: ferredoxin−$NADP^+$ reductase (1.9 μM) and NADPH (1 mM) or xanthine oxidase (1.2 μM) and NADH (1 mM). Control reactions were carried out on a $^1/_2$ scale (50 μL total volume) as described above with the omission of the indicated reaction component(s) (drug, cofactor, or enzyme/cofactor). Each reaction solution was incubated at 37 °C for 10 h in the absence of light, and the cleavage products were precipitated by the addition of an aqueous solution of sodium acetate (3 M, 11 μL, pH 5.2) and ethanol (350 μL) and were analyzed subsequently by PAGE, as previously described (Myers et al., 1995a,b). The amount of background radiation in the control lane (typically 3−5% of the total radioactivity in the lane) was subtracted from each cleavage lane before calculation of the percent of DNA cleaved. The percent of DNA cleaved is defined as

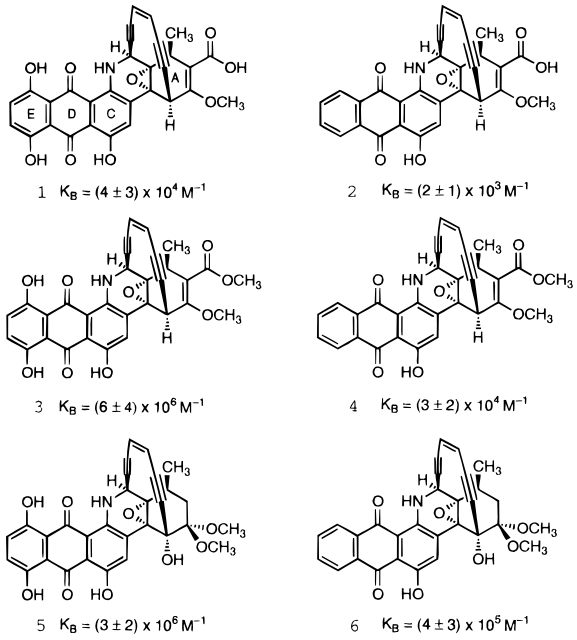

FIGURE 1: Dynemicin A (**1**) and synthetic analogs **2**−**6**. Binding constants were determined by equilibrium dialysis in aqueous Tris buffer solution (30 mM, pH 7.5; NaCl, 50 mM) at 23 °C.

the amount of radioactivity of all migrating fragments divided by the total radioactivity in the lane. "Relative cleavage" is defined as the percent of DNA cleavage relative to the lane of highest cleavage intensity (assigned a value of 100).

RESULTS AND DISCUSSION

Equilibrium constants for the binding of **1**−**6** to double-stranded calf thymus DNA were redetermined, using an equilibrium dialysis method previously described (Myers et al., 1995a), providing the values indicated below each structure (Figure 1). The measurements were complicated by the generally poor aqueous solubility of this class of compounds, and multiple determinations of the data were necessary. Our results were essentially as before, albeit with increased estimates of the errors involved, and a revision upward of the binding constant of compound **6** from $(4 \pm 1) \times 10^4$ to $(4 \pm 3) \times 10^5\ M^{-1}$. The measured binding constants can be considered to be accurate to within 1 order of magnitude and correlate well with results from DNA-cleaving assays using GSH and NADPH as activating agents. For example, incubation of **1**−**6** (50 μM) with a 3′-^{32}P-end-labeled 193 bp restriction fragment (*Eco*RI/*Ssp*I) from plasmid pBR322 and calf thymus DNA (1 mM bp, [DNA]/[drug] = 20) in the presence of GSH (20 mM) as the activating agent at 37 °C for 10 h and analysis of the DNA cleavage products by PAGE (Figure 2) using an 8% denaturing gel showed that the tight-binding analogs **3** and **5** failed to bring about any detectable DNA cleavage. Similar results were obtained in the presence of NADPH (20 mM, see Figure 3 below), with the exception that analog **6** [$K_B = (4 \pm 3) \times 10^5\ M^{-1}$] also failed to produce any detectable DNA cleavage with this reductant. Although initial experiments with **6** and NADPH did produce DNA cleavage (Myers et al., 1995a), we now believe that these earlier experiments inadvertently incorporated an unknown contaminant (perhaps a metal ion) that catalyzed the reduction reaction, for further experiments with **6** and NADPH failed

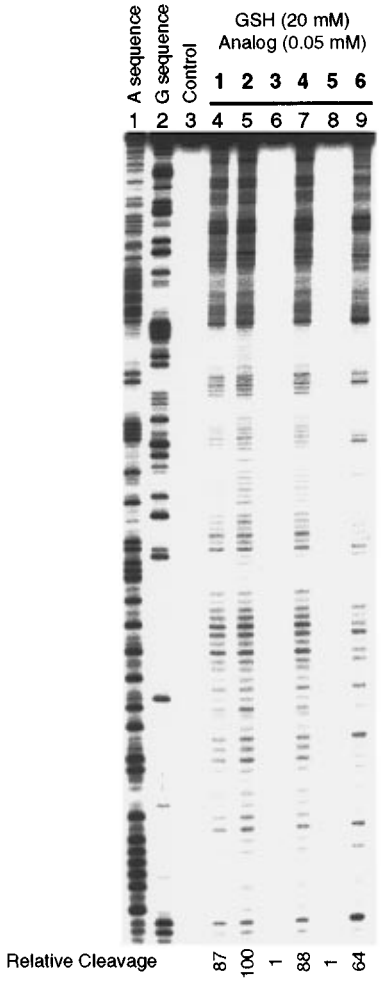

FIGURE 2: Cleavage of a 3′-labeled 193 bp restriction fragment of plasmid pBR322 (*Eco*RI/*Ssp*I) by **1**−**6** at 37 °C (pH 7.5, 10 h) using GSH (20 mM). Storage phosphor autoradiogram of an 8% denaturing polyacrylamide gel. All reaction mixtures contained calf thymus DNA (1 mM bp), 3′-end-labeled 193 bp restriction fragment (∼50 kcpm), and NaCl (50 mM) in Tris-HCl (30 mM, pH 7.5) with 10% DMSO by volume: lanes 1 and 2, Maxam−Gilbert A and G sequencing reactions, respectively; lane 3, GSH only and no added drug for 10 h; and lanes 4−9, synthetic anthraquinone **1**−**6** (0.05 mM) as indicated.

to produce any detectable cleavage in many repetitions of the original experiment. These data together with the kinetics measurements referred to above suggest that dynemicin analogs with binding constants greater than $\sim 10^5\ M^{-1}$ will not bring about the cleavage of DNA (10 h reaction period) using these activating agents, with activation by GSH somewhat more rapid than that by NADPH. The use of a thermodynamic parameter (K_B) to estimate what is in essence a kinetic phenomenon is of course tenuous and ultimately must defer to an analysis of the relevant kinetic parameters, to include the on and off rates for drug binding.

Drawing from the extensive literature concerning the reductive activation (Iyer & Szybalski, 1964; Lin et al., 1972; Moore, 1977) of the clinically important quinone-containing antitumor agents mitomycin C (Gutteridge & Toeg, 1982; Doroshow, 1983a; Kleyer & Koch, 1984; Fisher et al., 1985; Phillips et al., 1989; Lown, 1988), adriamycin, and daunomycin (Pan et al., 1984; Prisots & Sartorelli, 1986; McGuiness et al., 1991; Li & Kohn, 1991; Tomasz, 1995), we investigated the use of two flavin-based enzymatic systems, ferredoxin−NADP$^+$ reductase and xanthine oxidase, as

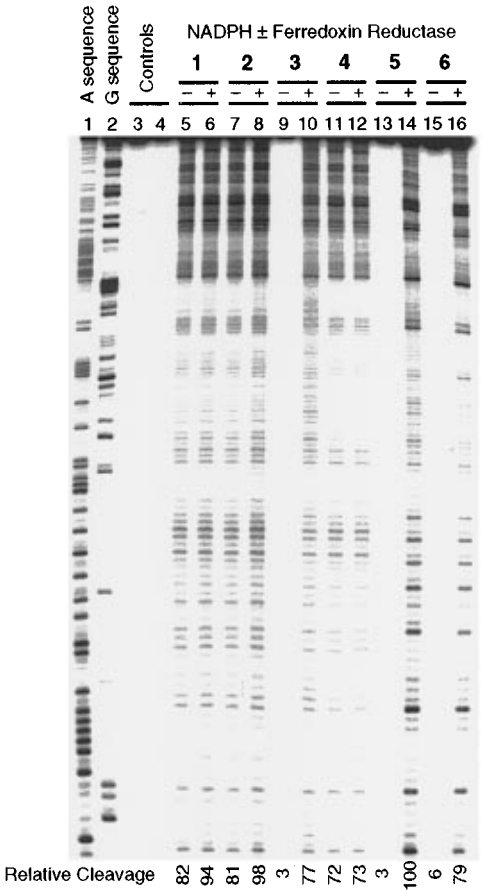

FIGURE 3: DNA cleavage by **1**−**6** using NADPH or ferredoxin−NADP$^+$ reductase/NADPH at 37 °C (pH 7.5). Storage phosphor autoradiogram of an 8% denaturing polyacrylamide gel. All reaction mixtures contained calf thymus DNA (1 mM bp), 3′-end-labeled 193 bp restriction fragment (∼50 kcpm), and NaCl (50 mM) in Tris-HCl (30 mM, pH 7.5) with 10% DMSO by volume: lanes 1 and 2, Maxam−Gilbert A and G sequencing reactions, respectively; lane 3, NADPH (20 mM) only and no added drug for 10 h; lane 4, ferredoxin−NADP$^+$ reductase (1.9 μM) and NADPH (1 mM) only and no added drug for 4 h; and lanes 5−16, **1**−**6** (0.05 mM) and NADPH (20 mM) in the absence (−) of ferredoxin−NADP$^+$ reductase for 10 h or **1**−**6** (0.05 mM) and NADPH (1 mM) in the presence (+) of ferredoxin−NADP$^+$ reductase (1.9 μM) for 4 h.

potential catalysts for the reduction of **1**−**6** with NADPH and NADH (Halliwell, 1978; Bachur et al., 1979; Doroshow, 1983b). Ferredoxin−NADP$^+$ reductase is a 41 kDa protein isolated from spinach leaves (Karplus et al., 1991), while xanthine oxidase is a 300 kDa mammalian enzyme that utilizes NADH as a cofactor (Hille & Nishino, 1995) and has been identified in the nuclei of mammalian cells (Hashimoto, 1974) and shown to catalyze the reduction of the anthracycline anticancer drugs adriamycin and daunomycin by NADH (Pan & Bachur, 1980, and references cited therein). Both are low-potential, flavin-based electron transferases and are commercially available in pure form. The relevant oxidation/reduction potentials for this study are as follows: GSH, −260 mV (Millis et al., 1993); NADPH, −320 mV (Loach, 1976); NADH, −310 mV (Loach, 1976); ferredoxin−NADP$^+$ reductase/NADPH, −440 mV (Batie & Kamin, 1981); and xanthine oxidase/NADH, −420 mV (Hunt et al., 1993). For comparison, the anthraquinone antibiotics adriamycin and daunomycin exhibit reduction potentials in the range of −300 to −450 mV (Svingen & Powis, 1981; Land et al., 1983; Kawakami & Hopfinger, 1990).

Initial experiments established that both enzymes catalyzed the rapid and efficient cleavage of DNA by all dynemicin analogs examined (**1**−**6**), in the presence of 10−20-fold lower concentrations of the stoichiometric reductant (NADPH in the case of ferredoxin−NADP$^+$ reductase and NADH in the case of xanthine oxidase) than were used in cleavage experiments lacking enzyme. For example, incubation of the 3′-^{32}P-end-labeled 193 bp restriction fragment described above and calf thymus DNA (1 mM bp) with analogs **1**−**6** (50 μM) in the presence of ferredoxin−NADP$^+$ reductase (1.9 μM, 0.04 equiv) and NADPH (1 mM) at 37 °C for 4 h followed by PAGE provided the data shown in Figure 3. For comparison, parallel reactions were conducted lacking enzyme, with a 20-fold higher concentration of NADPH (20 mM, 37 °C, odd lanes 5−15), and for a longer incubation period (10 h). For reference, the total concentration of NADPH and NADP$^+$ in most tissues is estimated to be 10−100 μM, and the NADPH:NADP$^+$ ratio (∼100−200) greatly favors the reduced form (Srivastava & Bernhard, 1987). By contrast, the concentration of NADH and NAD$^+$ is ∼1−10 μM in most tissues, and the NAD$^+$:NADH ratio (∼1000) favors the oxidized form (Lehninger et al., 1993). Similar results were obtained using xanthine oxidase (1.2 μM) as catalyst and NADH (2 mM) as the stoichiometric reductant (37 °C, 4 h; data not shown). In both cases, each of the six compounds **1**−**6** led to DNA cleavage in the enzyme-catalyzed reductions, whereas compounds **3**, **5**, and **6** produced no detectable cleavage products in the absence of enzyme and a 10−20-fold increase in the concentration of the stoichiometric reductant. Enzyme-catalyzed DNA cleavage reactions always proceeded with greater efficiencies than the corresponding noncatalyzed reactions.

The DNA cleavage patterns were found to be independent of the reductant employed and, as previously noted (Myers et al., 1995a,b), showed only modest variation within the series **1**−**6**. The former is illustrated for dideoxydynemicin (analog **2**) in Figure 4, showing a comparison of the cleavage patterns produced by each of the reductants GSH (20 mM), NADPH (20 mM), ferredoxin−NADP$^+$ reductase/NADPH (1.9 μM and 1 mM, respectively), NADH (20 mM), and xanthine oxidase/NADH (1.2 μM and 1 mM, respectively). Although none of the analogs can be said to produce highly sequence specific DNA cleavage, the observed specificity is maintained with each reducing agent employed. This supports the idea that all reductants produce a common activated intermediate from reductive activation of a given analog (Svingen & Powis, 1981; Land et al., 1983; Kawakami & Hopfinger, 1990), regardless of whether the reduction proceeds by one- or two-electron processes. The data do not allow us to distinguish between two-electron reduction mechanisms (as shown in Scheme 1, hydroquinone intermediates) and one-electron processes (semiquinone intermediates, not shown), for disproportionation pathways can be invoked that transform two-electron reduction products into one-electron reduction products (Mukherjee et al., 1989). This feature remains a mechanistic ambiguity in metabolic pathways of mitomycin C (Wilson et al., 1985; Peterson & Fisher, 1986; Danishefsky & Egbertson, 1986; Tomasz et al., 1988; Hoey et al., 1988; Siegel et al., 1992; Sartorelli et al., 1993; Cummings et al., 1995; Li et al., 1996) and the anthracyclines (Bachur et al., 1978; Pan et al., 1981; Karasch & Novak, 1983; Gianni et al., 1983; Keyes et al., 1984; Doroshow, 1986; Sulikowski et al., 1991; Riley & Workman,

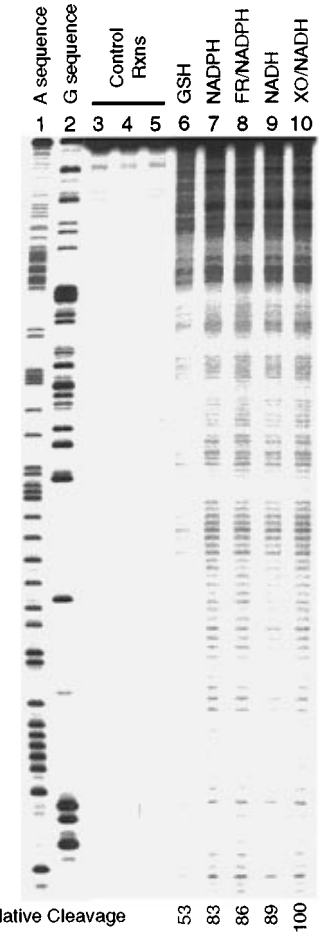

FIGURE 4: Comparison of DNA cleavage by **2** (37 °C, pH 7.5) using different cofactor systems for reductive activation. Storage phosphor autoradiogram of an 8% denaturing polyacrylamide gel. All reaction mixtures contained calf thymus DNA (1 mM bp), 3′-labeled 193 bp restriction fragment (∼50 kcpm), **2** (0.05 mM), and NaCl (50 mM) in Tris-HCl (30 mM, pH 7.5) with 10% DMSO by volume: lanes 1 and 2, Maxam−Gilbert A and G sequencing reactions, respectively; lane 3, drug only and no reducing cofactor for 10 h; lane 4, ferredoxin−$NADP^+$ reductase (1.9 μM) and NADPH (1 mM) only and no added drug for 1 h; lane 5, xanthine oxidase (1.2 μM) and NADH (1 mM) only and no added drug for 2 h; lane 6, **2** and GSH (20 mM) for 10 h; lane 7, **2** and NADPH (20 mM) for 10 h; lane 8, **2**, ferredoxin−$NADP^+$ reductase (1.9 μM), and NADPH (1 mM) for 1 h; lane 9, **2** and NADH (20 mM) for 10 h; and lane 10, **2**, xanthine oxidase (1.2 μM) and NADH (1 mM) for 2 h.

1992; Gaudiano et al., 1992; Lown, 1993; Cullinane et al., 1994; Beall et al., 1995) as well.

Given the potential importance of nuclear and mitochondrial enzyme-catalyzed processes for the reductive activation of the mitomycins and adriamycins (Backer & Weinstein, 1980; Bachur et al., 1982; Kennedy et al., 1982; Gigli et al., 1988), the demonstration herein of the efficacy of such reductants in the in vitro activation of **1** (and **2**−**6**) provides the first evidence that this is a feasible, if not likely, pathway for the in vivo activation of dynemicin A. Earlier experiments using GSH (20 mM) and NADPH (20 mM) as reductants suggested that the design of dynemicin analogs with increased DNA binding affinity might not be a prudent direction for improving antitumor properties (Myers et al., 1995a), assuming DNA cleavage as the basis for antitumor activity. Although it is conceivable that the more powerful enzymatic reductants may reduce the DNA-bound form of the drug, it is equally probable that they are simply more efficient in reducing the free drug in solution. The question of whether the enzyme-based reductants activate via DNA-bound or free forms of the drug remains an unresolved issue at this point. The kinetic assays previously used to evaluate GSH and NADPH reductions cannot be used for the enzymatic reductions because the latter involve catalytic quantities of enzymes and the kinetic assays require that the reduction proceed under pseudo-first-order conditions. In this regard, it will be interesting to synthesize a dynemicin analog with nanomolar or greater affinity for DNA to determine if there is a binding threshold beyond which even the enzymatic reductants fail to activate the drug. The new enzymatic activation processes reported herein suggest that in vivo reductive activation of tight-binding analogs is a feasible process, at least for agents with micromolar binding constants, and to that extent should prove useful for the future design of dynemicin analogs.

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