Redox-Active Daunomycin–Spin-Labeled Nucleic Acid Complexes

John C. Ireland, Gary T. Pauly, Elizabeth V. Bobst, and Albert M. Bobst*

Department of Chemistry, University of Cincinnati, Cincinnati, Ohio 45221

Received May 8, 1986; Revised Manuscript Received August 18, 1986

ABSTRACT: Interaction studies between daunomycin (DM) and enzymatically spin-labeled nucleic acid duplexes reveal two modes of binding by electron spin resonance (ESR) spectroscopy. At a low drug/nucleotide (D/N) ratio, the drug binds in the intercalative mode with only a slight reduction in base mobility. Saturation in the intercalative mode is achieved at a lower D/N ratio for B′ DNA than for B DNA. After full intercalation, further addition of DM seems to destabilize the helix and to allow the formation of redox-active DM stacks complexed to the nucleic acid lattice. These stacks will irreversibly oxidize all the nitroxides covalently bound to the 4- or 5-position of the pyrimidine base. Interactions between DM and spin-labeled single-stranded nucleic acids lead directly to the formation of redox-active complexes, while mixing of the drug with spin-labeled nucleic acid building blocks not incorporated in a nucleic acid lattice causes no ESR signal change. Complete disappearance of the ESR signal of spin-labeled nucleic acids extrapolates to a D/N value which is a constant for a particular lattice system and is independent of spin-labeling content.

Daunomycin (DM)[1] is an anthracycline quinone widely used in the treatment of certain solid tumors and leukemias in humans. Although the primary mode of action of DM most likely involves intercalation of the drug chromophore into duplex DNA, other findings indicate that different modes of action involving potentially cell-lethal mechanisms are also possible, most of which require the redox cycling properties of the drug's quinone moiety. A comprehensive review of the biochemical and biophysical properties of DM has been published (Gianni et al., 1982).

The subject of this paper concerns the ESR-observable interactions of DM with spin-labeled nucleic acid building blocks, spin-labeled single-stranded RNA and DNA strands, and the spin-labeled duplexes $(dA)_n(dT)_n$ and $(dA\text{-}dT)_n$. This laboratory has invested considerable effort in developing spin-labeled RNA and DNA systems to ellucidate their local base dynamics (Kao et al., 1983; Bobst et al., 1984a; Kao & Bobst, 1985) and to study the nucleic acid binding properties of various proteins (Bobst et al., 1982, 1984b, 1985; Kao et al., 1985). Unlike the high molecular weight DNA binding ligands previously investigated, the present study monitors the interaction of nitroxide labels with a small positively charged intercalating ligand which possesses a chemically reactive quinone functionality.

Evidence is presented for the differential destabilization of B′ and B DNA duplexes in the presence of high DM concentrations. Also, it is shown that DM will act as an oxidizing agent toward potential electron donors which are covalently attached to nucleic acid lattices.

Materials and Methods

Daunomycin hydrochloride was obtained from Sigma Chemical Co. and in some instances purified by HPLC (Bioanalytical Systems Inc.). Stock solutions of DM were prepared by wetting the drug crystals with ethanol, followed by addition of either cacodylate buffer (0.01 M sodium cacodylate, 0.01 M NaCl, and 0.001 M EDTA, pH 7.2) or HPLC buffer (70% MeOH/30% 50 mM ammonium phosphate, pH 7.0), and care was taken to avoid undue exposure of the drug solutions to light. Stock solutions were stored at 4 °C and used within 3 weeks. HPLC purification was achieved on a 30-cm μBondapak C-18 reverse-phase column at a flow rate of 1.5 mL/min at 2000 psi. Column effluent was monitored at 480 nm, and the DM's retention time was 17.5 min with the HPLC buffer listed above.

The enzymatically spin-labeled nucleic acids were prepared by approaches developed in this laboratory. The single-stranded RNAs $(1s^4U,C)_n$ with different $1s^4U/C$ ratios were obtained according to Bobst et al. (1981). $(DUAT,dT\text{-}dA)_n$ was prepared according to Toppin et al. (1986). The spin-labeled nucleic acids containing DUAP instead of DUAT such as $(DUAP,dT)_n$ and $(DUAP,dT\text{-}dA)_n$ were prepared in a manner analogous to $(DUAT,dT)_n$ and $(DUAT,dT\text{-}dA)_n$. The enzymatically spin-labeled nucleic acids were examined for their sizes by gel electrophoresis and consisted of 300–600 nucleotides. The annealing to form the B′ duplex $(DUAP,dT)_n(dA)_n$ was achieved as described earlier for other B′ duplexes (Bobst et al., 1984a). The chemical structures of the nitroxide spin probes utilized in this study are given in Figure 1.

All ESR spectra were recorded with a Varian E-104 Century Series spectrometer interfaced with an Apple II plus microcomputer (Ireland et al., 1983). The microwave power was 10–12 mW and the modulation amplitude 1 G. The spectra are the result of time averaging 4 times with 1-min scans. The initial ESR spectra are all normalized to 18 cm

* Correspondence should be addressed to this author.

[1] Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; $(A)_n$, poly(adenylic acid); $(dT)_n$, poly(thymidylic acid); $(dA\text{-}dT)_n$, alternating copolymer of deoxyadenosine and thymidine; $(1s^4U,C)_n$, copolymer of $1s^4U$ and cytidine; $(DUAP,dT)_n$, copolymer of DUAP and thymidine; $(DUAT,dT)_n$, copolymer of DUAT and thymidine; $(DUAP,dT\text{-}dA)_n$, alternating copolymer of DUAP, thymidine, and deoxyadenosine; $(DUAT,dT\text{-}dA)_n$, alternating copolymer of DUAT, thymidine, and deoxyadenosine; $\tau_\perp$, correlation time for rotation perpendicular to the principal axis of diffusion; ESR, electron spin resonance; D/N, ratio of drug DM vs. nucleotide; D/S, ratio of drug DM vs. spin-labeled nucleotide; DM, daunomycin; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid. Structures of DM and the abbreviated uridine analogues $1s^4U$, DUAP, and DUAT are given in Figure 1.

0006-2960/86/0425-6890$01.50/0

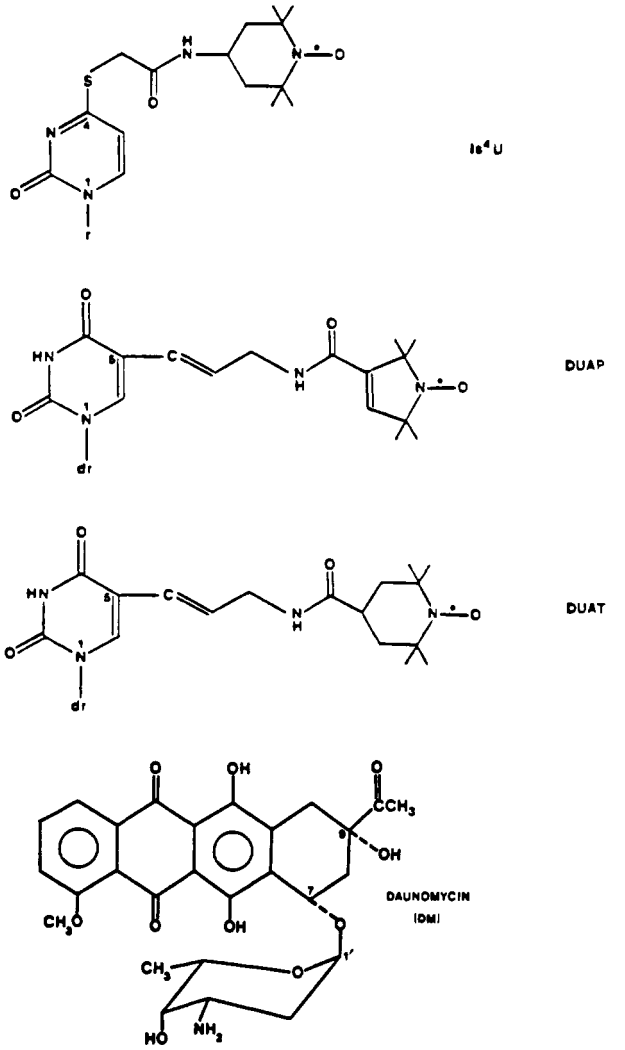


FIGURE 1: Chemical structure of the nitroxide spin-labeled nucleic acid building blocks 1s⁴U, DUAP, and DUAT and of DM.

which corresponds to the full vertical displacement of a digital plotter which serves as the primary data output device.

Titration experiments were performed in an E-258-3 Varian flat quartz cell in an E-238 cavity. The drug DM was introduced in incremental (2–5 μL) additions to a known molar quantity of spin-labeled polynucleotide solution. Mixing of sample components was achieved with a plastic tuberculin syringe temporarily attached to the neck of the ESR cell. Solutions were mixed gently for approximately 5 min and allowed to stand 10 min before measurement. To assure equilibrium, several time course experiments were performed which involved several different mixing and standing times. These tests as well as previously published results (Wang & Finch, 1980) indicated that the DM–nucleic acid interaction occurs rapidly.

The r value can be calculated from the known polymer concentration, C_N, or spin-label concentration, C_s, and the bound ligand concentration C_B^{DM}, where $a = C_s/C_N$ and C_{bp} corresponds to the base pair concentration with the following relationship:

$$r = C_B^{DM}/C_{bp} = 2C_B^{DM}/C_N = 2aC_B^{DM}/C_s$$

The r' value is defined here as the ratio of total ligand concentration, C_T^{DM}, to base pair concentration and can be expressed similarly:

$$r' = C_T^{DM}/C_{bp} = 2C_T^{DM}/C_N = 2aC_T^{DM}/C_s$$

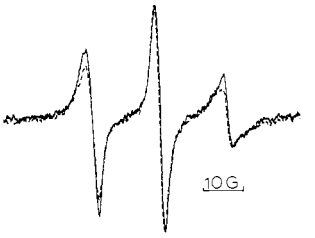


FIGURE 2: ESR spectra of 28 nmol of $(DUAT,dT\text{-}dA)_n$ ([DUAT]/([dT] + [dA]) = 0.03) in 0.01 M NaCl, 0.001 M EDTA, and 0.01 M sodium cacodylate, pH 7.2 (—), in the absence of DM and (---) with DM at r = 0.2.

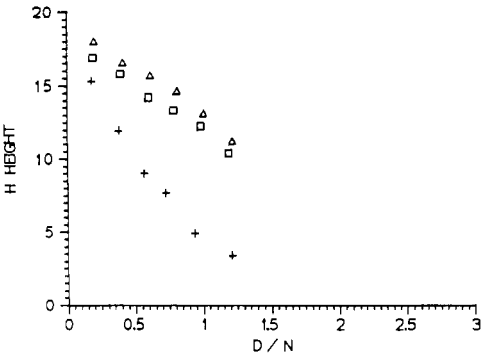


FIGURE 3: Normalized center-field H_0 peak heights vs. D/S for the titration with DM in 0.01 M NaCl, 0.001 M EDTA, and 0.01 M sodium cacodylate, pH 7.2, of (△) 23 nmol of $(DUAT,dT\text{-}dA)_n$ ([DUAT]/[dT,dA] = 0.02), (□) 26 nmol of $(DUAP,dT\text{-}dA)_n$ ([DUAP]/[dT,dA] = 0.02), and (+) 25 nmol of $(DUAP,dT)_n(dA)_n$ ([DUAP]/[dT,dA] = 0.019).

Results

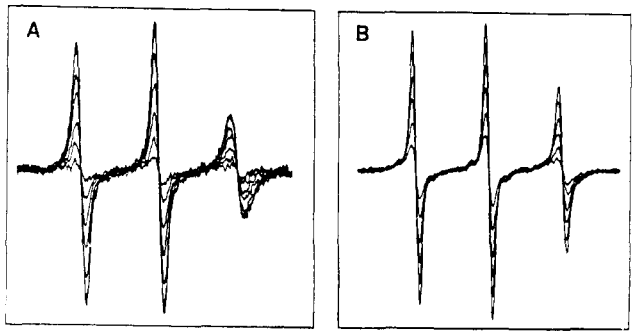
ESR Titrations of B and B′ DNA Duplexes Containing Enzymatically Incorporated Nitroxide Radicals. Visible absorption titrations of various unlabeled and spin-labeled duplex lattice systems with DM were performed as published earlier (Phillips et al., 1978; Chaires et al., 1982). The presence of the nitroxide label in the nucleic acid lattices did not seem to affect the apparent association constants reflecting the drug binding to the lattice in the intercalative mode which occurs at relatively low r values (Ireland, 1984). However, extending the usual range of titration by adding relatively large amounts of DM to the spin-labeled duplexes revealed the existence of two distinct ESR effects. At $r < 0.3$, the addition of DM causes only a small change in the ESR line shape of the B duplex $(DUAT,dT\text{-}dA)_n$ as shown in Figure 2. When a motional model for site specifically labeled RNA and DNA duplexes (Kao et al., 1985) is applied, the small reduction in the low- and high-field peak heights would indicate a slightly larger $\tau_\perp$, reflecting a very small reduction in base mobility. A similar reduction in base mobility is seen with $(DUAP,dT\text{-}dA)_n$, and an even smaller reduction in base mobility is observed with the B′ duplex $(DUAP,dT)_n(dA)_n$ (data not shown). A much more pronounced effect in terms of overall signal intensities is observed with $r > 0.3$ as shown with Figure 3. After the initial subtle line-shape change observed at low r values which cause only a minimal loss of the center field peak height (H_0), further addition of DM results in a linear decrease of H_0 without further ESR line-shape change. The linear decrease is considerably steeper for the B′ than for the B duplex. H_0 extrapolates to 0 at D/N = 1.3 for the B′ duplex and at D/N = 3.2 for the B duplex. Note that D/N reflects here the total drug over nucleotide ratio and corresponds to $r'/2$ (see Materials and Methods and Table I).

ESR Titrations of Spin-Labeled Nucleotide Monomers with DM. Before consideration of potential interactions of DM with

Table I: Titration of Spin-Labeled Nucleic Acids with DM[a]

lattice	C_s/C_N[b]	D/S[c]	D/N[d] (%)
$(ls^4U,C)_n$	0.015	153	2.2 ± 10
$(ls^4U,C)_n$	0.045	44	1.9 ± 10
$(ls^4U,C)_n$	0.07	30	2.1 ± 10
$(DUAP,dT)_n$	0.038	35	1.3 ± 10
$(DUAP,dT)_n(dA)_n$	0.019	70	1.3 ± 10
$(DUAP,dT\text{-}dA)_n$	0.02	160	3.2 ± 10
$(DUAT,dT\text{-}dA)_n$	0.02	160	3.2 ± 10

[a] 0.01 M sodium cacodylate, 0.01 M NaCl, and 0.001 M EDTA, pH 7.2. [b] $C_s/C_N = a$ (percent labeling). [c] $D/S = C_T^{DM}/C_s$. [d] $D/N = C_T^{DM}/C_N = r'/2$.



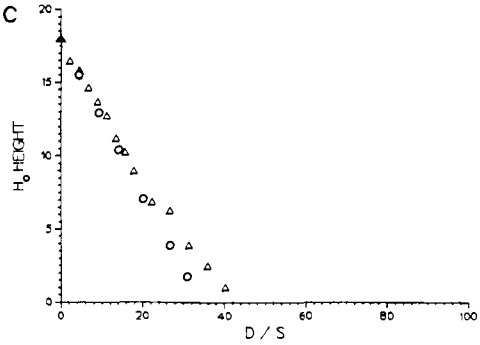


FIGURE 4: (A) Superimposed ESR spectra of 12.4 nmol of $(ls^4U,C)_n$ ([ls^4U]/[C] = 0.045) during titration with DM in 0.01 M NaCl, 0.001 EDTA, and 0.01 M sodium cacodylate, pH 7.2. (B) Superimposed ESR spectra of 9.9 nmol of $(DUAP,dT)_n$ ([DUAP]/[dT] = 0.038) during titration with DM. (C) Normalized center-field H_o peak heights vs. D/S for the titrations of $(ls^4U,C)_n$ (△) and $(DUAP,dT)_n$ (○) with DM.

spin-labeled lattice systems, the chemical reactivity of DM toward a spin-labeled nucleotide was investigated. For that purpose, a solution of pls^4U and pC was prepared at a concentration which would mimic the spin and nucleotide concentrations in a typical single-stranded spin-labeled polynucleotide. The ESR line shape remained unchanged with addition of DM to the unpolymerized nucleotides, and there was also no loss of signal amplitude with a D/S ratio of up to 50. The observed loss is therefore lattice dependent.

ESR Titrations of Single-Strand Spin-Labeled Polynucleotides with DM. Due to the single positive charge carried by the glycosidic amine function of DM at neutral pH, the drug is capable of participating in an electrostatic interaction with the polyanionic phosphate lattice backbone of either DNA or RNA. Therefore, ESR titrations of a spin-labeled single-strand RNA lattice and DNA lattice were performed to investigate the interaction of DM with these lattices where classical intercalation has not been reported. Figure 4A shows the ESR spectra of $(ls^4U,C)_n$ with 4.5% ls^4U in the presence of increasing amounts of DM, and in Figure 4B, $(DUAP,dT)_n$ containing 3.8% DUAP is titrated with DM. Figure 4A,B demonstrates that the addition of DM to either the RNA or

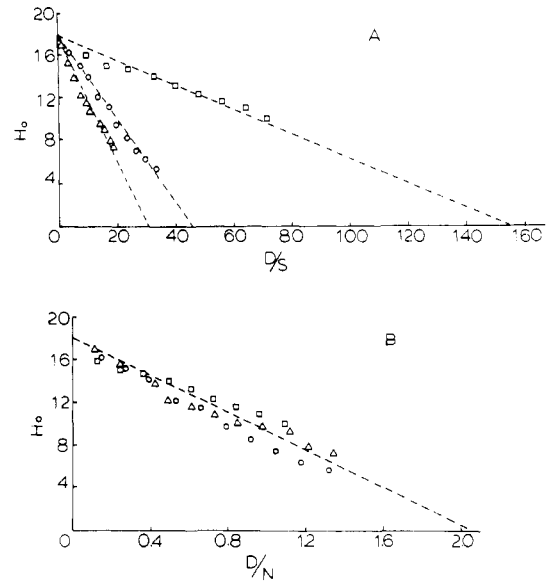


FIGURE 5: Normalized center-field H_o peak heights vs. D/S (A) and vs. D/N (B) for the titrations of $(ls^4U,C)_n$ ([ls^4U]/[C] = 0.015) (□), $(ls^4U,C)_n$ ([ls^4U]/[C] = 0.045) (○), and $(ls^4U,C)_n$ ([ls^4U]/[C] = 0.07) (△) with DM.

the DNA spin-labeled lattice does not cause any broadening of the observed ESR line shapes. However, one observes a substantial loss of signal which is linear with respect to the amount of drug added.

Irreversible Oxidation of the Spin-Label Covalently Bound to Lattice. Attempts to recover the lost ESR signal observed in a $(ls^4U,C)_n$DM complex using a competition approach with unlabeled nucleic acids as described in studies using gene 32 (Bobst et al., 1982) or gene 5 protein (Bobst et al., 1984b) as ligands were not successful with the spin-labeled nucleic acid–DM complex. This ruled out the possibility of a loss in H_o due to excessive broadening as a result of spin exchange or large correlation time of the nitroxide radicals. Recovery of the loss of ESR signal by bubbling oxygen through the $(ls^4U,C)_n$DM complex solution in an attempt to regenerate the nitroxide radicals, which could have been lost by the formation of hydroxylamines due to a simple reduction process, was not successful. In contrast to the reduction reaction of nitroxides to hydroxylamines which is known to be reversible, oxidation of the nitroxide radical is irreversible, leading first to the formation of an oxoammonium salt which then may decompose via several pathways (Keana, 1984). The possibility that the usually stable nitroxide radical in $(ls^4U,C)_n$ was destroyed in the presence of DM was tested with digestion experiments using a published procedure to determine the amount of nitroxide labeling in an RNA lattice (Bobst, 1972). The RNA lattice $(ls^4U,C)_n$ containing 4.5% ls^4U was first titrated with DM until DM/N = 0.7, and this resulted in a 27% loss of H_o. Then a digestion of the $(ls^4U,C)_n$DM complex with 0.1 N KOH for 22 h gave an H_o value which was about 29% smaller than the H_o value obtained from digesting uncomplexed $(ls^4U,C)_n$. These results present strong evidence that DM will indeed irreversibly oxidize nitroxide radicals covalently attached to a nucleic acid lattice.

Dependence of Nitroxide Signal Loss as a Function of Percent Labeling. In Figure 5A, the loss of H_o of $(ls^4U,C)_n$ with different amounts of incorporated ls^4U is plotted as a function of D/S which corresponds to the DM to ls^4U ratio. It is obvious that the lattice with the highest incorporation of nitroxides gives the steepest linear decay and the H_o extrapolates to 0 at D/S = 30 (see Table I). The lattice with the

lowest nitroxide incorporation shows a linear decay which extrapolates at D/S = 153. By use of the relationship given for r', the data of Figure 5A can be replotted by expressing H_0 as a function of the D/N ratio as shown with Figure 5B. It is apparent that a plot of H_0 vs. D/N gives the same linear decay for all lattices tested with a D/N ratio on the order of 2 to complete the oxidation of all spin-labels. This implies that the lattice may be acting as a nucleus on which the drugs are microscopically concentrated (stacked) and become redox active toward the nitroxide radicals.

Stability of the Nitroxide Signal at Two Different D/N Ratios. As stated under Materials and Methods, great precaution was taken to assure that the binding equilibrium had been reached in the drug–lattice interaction by using different mixing and standing times. Since the apparent irreversible oxidation suggests that we are no longer dealing with a simple equilibrium phenomenon, the ESR signal of $(1s^4U,C)_n$ with 7% labeling was monitored at D/N = 0, D/N = 0.38, and D/N = 0.7 over a period of 60 min. The signal intensity remained essentially constant during this time period for the three different D/N ratios. Thus, the initial addition of DM is causing the observed effect with a rapid chemical oxidation. Although a large excess of DM over spin-labeled nucleotides is present at D/N = 0.38 and D/N = 0.7, the nitroxide destruction if fixed at a given D/N ratio for at least an hour. This observation implies that the ESR signal loss cannot be the result of a simple weak and reversible association between DM and lattice.

Chemical Reactivity of HPLC-Purified DM. Given the observation that an irreversible oxidation of the nitroxide radical was occurring upon addition of DM, we attempted to prove that DM itself was responsible for the process. It is unlikely that a drug–Fe(III) complex is the oxidant, because no detectable absorbance was determined at 600 nm [the absorbance maximum for the drug–Fe(III) complex], and EDTA was present in sufficient excess to prevent a significant concentration of drug–Fe(III) complex (Eliot et al., 1984). In an approximation to HPLC conditions reported earlier (Gray & Phillips, 1981), the major peak fraction was collected and its concentration determined at 480 nm. The HPLC-isolated DM was stored at 4 °C in darkness, and the drug was used without further manipulations to prevent its potential degradation. A titration of $(1s^4U,C)_n$ with HPLC-purified DM dissolved in the HPLC solvent system is shown in Figure 6. One indeed observes a loss of signal as a function of D/S, although the loss is less pronounced and nonlinear relative to DM prepared in the titration buffer. To verify a dependency of signal loss on solvent conditions, nonpurified DM was dissolved in the methanol/ammonium phosphate HPLC solvent system with a solution concentration approximately equivalent to that of the HPLC-purified DM solution. This resulted in a titration curve which is superimposable with the one observed with HPLC-purified DM, thereby strongly supporting the hypothesis that the nitroxide destruction is not caused by a DM contaminant, but by DM itself.

Amount of DM Required To Irreversibly Destroy Nitroxide Radicals Covalently Bound to Different Nucleic Acid Systems. The amounts of DM required to fully destroy the nitroxide radicals attached to various nucleic acid lattices were extrapolated from the shown titration data and are tabulated in the form of D/S and D/N (see Table I). In the case of the single-stranded RNA $(1s^4U,C)_n$, the value is on the order of 2 and does not depend on the percent labeling, a. For the single-stranded DNA $(DUAP,dT)_n$ and the double-stranded B′ DNA $(DUAP,dT)_n(dA)_n$, the D/N is also constant and of

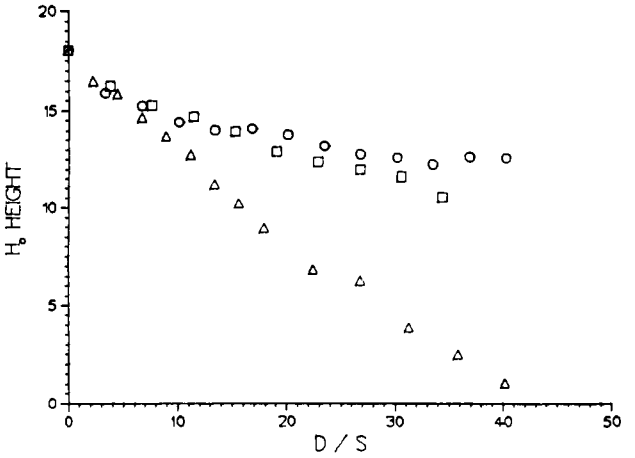


FIGURE 6: Comparison of normalized center-field H_0 peak height vs. D/S for the titration of 12 nmol of $(1s^4U,C)_n$ ($[1s^4U]/[C] = 0.045$) with non-HPLC- and HPLC-purified DM under different solvent conditions. (△) Titration in titration buffer (0.01 M NaCl, 0.001 M EDTA, and 0.01 M sodium cacodylate, pH 7.2) with 2.4×10^{-4} M DM prepared in titration buffer. (○) Titration in titration buffer with 2.3×10^{-4} M DM prepared in the HPLC solvent system (70% MeOH/30% 50 mM ammonium phosphate, pH 7). (□) Titration in titration buffer with 2.3×10^{-4} M HPLC-purified DM in the HPLC solvent system.

the order of 1.3. This suggests a similar mechanism for the destruction of the nitroxide which presumably occurs in both instances at the single-strand level. In the case of the double-stranded B DNA $(DUAP,dT\text{-}dA)_n$ or $(DUAT,dT\text{-}dA)_n$, the value is considerably larger and on the order of 3.

Discussion

The DNA binding interactions of DM have been extensively quantitated by a variety of techniques [for a review, see Gianni et al. (1982) and references cited therein]. Because of the irreversible nature of the ESR phenomenon detected in the present study, equilibrium binding measurements of DM–spin-labeled nucleic acid complexes by ESR were precluded. However, we established by visible absorption titrations that the nitroxide label had no effect on the apparent association constant determined at relatively small r values (Ireland, 1984). Comprehensive investigations of the DM/nucleic acid complexation process have also been established at relatively low r values in the literature (Graves & Krugh, 1983; Chaires et al., 1982, 1985; Chaires, 1985).

It was shown earlier in an interaction study between DM and $(dA)_n(dT)_n$ or $(dA\text{-}dT)_n$ that r is about a factor of 2 larger for the alternating duplex than for the homopolymer duplex (Phillips et al., 1978). A larger r value was also observed for the alternating duplex in a comparative thermodynamic analysis of DM binding to $(dA)_n(dT)_n$ and $(dA\text{-}dT)_n$ (K. J. Breslauer, private communication). Thus, it takes less DM to saturate $(dA)_n(dT)_n$ than $(dA\text{-}dT)_n$ in the intercalation mode. This property of the two different lattices could explain our observation shown in Figure 3, where we establish that the D/N ratio needed for the complete titration of the nitroxide radicals is considerably larger for the B DNA duplexes $(DUAP,dT\text{-}dA)_n$ or $(DUAT,dT\text{-}dA)_n$ than for the B′ DNA duplex $(DUAP,dT)_n(dA)_n$.

A model for the titration of B or B′ DNA with DM from small to large r values is proposed with Figure 7. At low r values, DM intercalates to give the complex symbolized with Figure 7B which displays an $r = 0.35$ as suggested for $(dA\text{-}dT)_n$. It has also been established that DM intercalates with a DNA duplex in such a way that the drug's amino sugar moiety is located in the minor groove (Quigley et al., 1980;

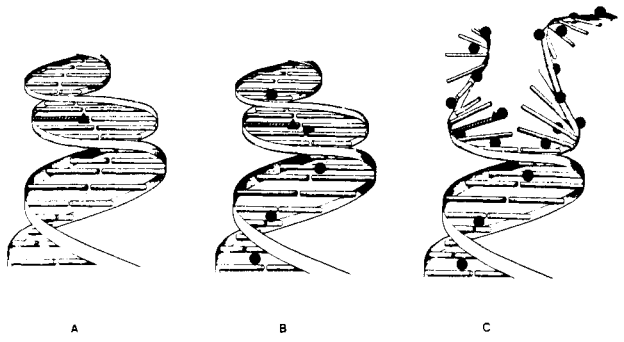


FIGURE 7: Model for sequential destabilization of a B or B′ DNA duplex with DM and subsequent formation of DM stacks. (A) DNA duplex with nitroxide-labeled base [indicated by (—▲)]. (B) DNA duplex partially saturated with DM at intercalation sites [drug molecules indicated by (●)]. (C) Formation of DM stacks along single-stranded regions with concomitant oxidation of nitroxide radical [indicated by (—▲●)].

Yen et al., 1983). The formation of such a complex should cause no more than a small ESR line-shape change when the reporter group is located in the major groove (Bobst et al., 1984a). The very subtle ESR line-shape change observed upon intercalation of $(DUAT,dT\text{-}dA)_n$ with DM is in full agreement with the intercalation model. The ESR line-shape observed at $r = 0.2$ most likely reflects a minor decrease in base dynamics due to DM intercalation.

After the lattices are saturated with DM in the intercalative binding mode, a second type of binding, involving electrostatic interactions between the negatively charged phosphate groups and the positively charged DM ligands, may become operational. This second mode of binding is possibly preceded by a DM-induced denaturation of the duplex upon its saturation with DM. The denaturation would lead to the formation of single-stranded regions which would be the target site for a buildup of DM stacks as shown in Figure 7C. It was recently reported that several intercalators including mitoxantrone can lead to destabilization of the secondary structure of the nucleic acid at relatively high r values (Kapuscinski & Darzynkiewicz, 1985). The intercalator-induced destabilization model would explain the same D/N value of 1.3 determined for the complete destruction of the nitroxide in $(DUAP,dT)_n$ or $(DUAP,dT)_n(dA)_n$ (Table I), since the same DM stacking process at the single-strand level could then be applied. Furthermore, if the titration data shown in Figure 4 for $(DUAP,dT)_n$ are replotted in Figure 3 as D/N instead of D/S, the linear height decay as a function of D/N is essentially superimposable for $(DUAP,dT)_n$ and $(DUAP,dT)_n(dA)_n$. This similarity in slope would be anticipated if the same molecular mechanism is involved in the destruction of the nitroxide radicals.

Many reports have shown the formation of superoxide anion radicals with anthracycline antibiotics (Kalyanaraman et al., 1980; Carmichael et al., 1983). Some evidence was also presented for the alkylation of nucleic acids and proteins by the one- and two-electron reduction products of these anthracyclines (Sinha & Gregory, 1981). Titration of the spin-labeled nucleic acid building block pls^4U with DM shows no ESR signal loss, thereby ruling out a simple redox process occurring between the nitroxide radical and the quinone moiety of the drug. In contrast, titration of the single-stranded spin-labeled lattice systems $(ls^4U,C)_n$ and $(DUAP,dT)_n$ with DM causes a significant loss which can be quantitated. The titration data on $(ls^4U,C)_n$ of different ls^4U content strongly support the model of DM stack formation among the single-stranded nucleic acid backbone. We propose that the formation of such stacks is a prerequisite for the irreversible oxidation of nitroxide radicals covalently bound to the nucleic acid bases.

Whereas the DM intercalative binding mode has only a small effect on the base dynamics, the subsequent type of binding, which presumably involves electrostatic interactions between the negatively charged phosphate groups and the positively charged DM ligands, causes the quinone moiety of the DM to act as a strong oxidant. Many reports have shown the potential of DM to display chemical reactivity. It is also believed that the detailed chemical reactions taking place after the quinone reduction depend on the presence or absence of molecular oxygen. With oxygen present, several reports have shown the formation of superoxide anion radicals with anthracycline antibiotics (Kalyanaraman et al., 1980; Carmichael et al., 1983). Without oxygen, intramolecular elimination of the C-7 glycoside occurs (Rao et al., 1978). Some evidence was also presented for the alkylation of nucleic acids and proteins by the one- and two-electron reduction products of these anthracyclines (Sinha & Gregory, 1981). Thus, the observed formation of chemically reactive DM under certain conditions is well established. Here, we present evidence for redox-active DM–nucleic acid complexes as monitored from the viewpoint of the oxidative destruction of nitroxide radicals covalently attached to various nucleic acids. As shown with Figure 7C, the formation of DM stacks along single-stranded nucleic acids gives rise to redox-active DM. On the other hand, in the absence of DM stacks, as in the case of the titration of the spin-labeled nucleic acid building block pls^4U, the unstacked quinone moiety of the drug will not oxidize the nitroxide radical.

The DM stack model shown in Figure 7 implies that the second mode of binding is preceded by a DM-induced destabilization of the duplex after its saturation with DM in the intercalative mode. The proposed destabilization leads to the formation of single-stranded regions which act as target sites for the DM stack formation as shown in Figure 7C. Double-strand destabilization of DNA by several intercalators including mitoxantrone at high r values was recently reported (Kapuscinski & Darzynkiewicz, 1985). The intercalator-induced destabilization model valid at large r values would explain why we determine the same D/N value of 1.3 for a complete destruction of the nitroxides in $(DUAP,dT)_n$ or $(DUAP,dT)_n(dA)_n$ (Table I). Also, if the titration data shown in Figure 4 for $(DUAP,dT)_n$ are replotted in Figure 3 as D/N instead of D/S, the linear ESR height decays as a function of D/N are superimposable for $(DUAP,dT)_n$ and $(DUAP,dT)_n(dA)_n$. This similarity in slope would be anticipated if the destruction of the nitroxide radicals covalently bound to the nucleic acids is caused by the same redox-active DM–nucleic acid complex. Further evidence for the formation of stable DM stacks along the single-strand nucleic acid backbone comes from titration data on $(ls^4U,C)_n$ of different ls^4U content (Table I). The D/N value is constant for the $(C)_n$ lattices and does not depend on the amount of ls^4U present in $(C)_n$.

In conclusion, the binding of DM to B and B′ DNA in the intercalative mode causes only a slight decrease in the base dynamics as determined from the ESR line-shape changes. Complete intercalation is achieved earlier for B′ DNA than for B DNA. After saturation of the duplexes in the intercalative mode, a further increase in the DM concentration causes a destabilization of the helix, and DM stacks are formed along the single-stranded nucleic acids. These DM stacks complexed to the nucleic acid chain are redox active and will irreversibly

destroy nitroxide radicals covalently attached to positions 4 or 5 of the pyrimidine bases.

Registry No. DM, 20830-81-3; $(1s^4U,C)_n$, 104532-53-8; (DUAT, dT-dA)$_n$, 104548-86-9; (DUAP, dT)$_n$, 104532-54-9.

REFERENCES

Bobst, A. M. (1972) *Biopolymers 11*, 1421–1433.

Bobst, A. M., Langemeier, P. W., Torrence, P. F., & DeClercq, E. (1981) *Biochemistry 20*, 4798–4803.

Bobst, A. M., Langemeier, P. W., Warwick-Koochaki, P., Bobst, E., & Ireland, J. (1982) *J. Biol. Chem. 257*, 6184–6193

Bobst, A. M., Kao, S.-C., Toppin, R. C., Ireland, J. C., & Thomas, I. E. (1984a) *J. Mol. Biol. 173*, 63–74.

Bobst, A. M., Ireland, J. C., & Bobst, E. V. (1984b) *J. Biol. Chem. 259*, 2130–2134.

Bobst, E. V., Bobst, a. M., Perrino, F. W., Meyer, R. R., & Rein, D. C. (1985) *FEBS Lett. 181*, 133–137.

Carmichael, A. J., Mossoba, M. M., & Riesz, P. (1983) *FEBS Lett. 164*, 401–405.

Chaires, J. B. (1985) *Biopolymers 24*, 403–419.

Chaires, J. B., Dattagupta, N., & Crothers, D. M. (1982) *Biochemistry 21*, 3927–3932.

Chaires, J. B., Dattagupta, N., & Crothers, D. M. (1985) *Biochemistry 24*, 260–267.

Eliot, H., Gianni, L., & Myers, C. (1984) *Biochemistry 23*, 928–936.

Gianni, L., Cordon, B. J., & Myers, C. E. (1982) *Rev. Biochem. Toxicol. 5*, 1–82.

Graves, D. E., & Krugh, T. R. (1983) *Biochemistry 22*, 3941–3947.

Gray, P. J., & Phillips, D. R. (1981) *Photochem. Photobiol. 33*, 297–303.

Ireland, J. C. (1984) Ph.D. Thesis, University of Cincinnati, Cincinnati, OH.

Ireland, J. C., Willett, J. A., & Bobst, A. M. (1983) *J. Biochem. Biophys. Methods 8*, 49–59.

Kalyanaraman, B., Perez-Reyes, E., & Mason, R. P. (1980) *Biochim. Biophys. Acta 630*, 119–130.

Kapuscinski, J., & Darzynkievicz, Z. (1985) *Biochem. Pharmacol. 34*, 4203–4213.

Kao, S.-C., & Bobst, A. (1985) *Biochemistry 24*, 5465–5469.

Kao, S.-C., Poplnaszek, C. F., Toppin, C. R., & Bobst, A. M. (1983) *Biochemistry 22*, 5563–5568.

Kao, S.-C., Bobst, E. V., Pauly, G. T., & Bobst, A. M. (1985) *J. Biomol. Struct. Dyn. 3*, 261–268.

Keana, J. F. W. (1984) in *Spin Labeling in Pharmacology* (Holtzman, J. L., Ed.) pp 1–85, Academic Press, New York.

Phillips, D. R., DiMarco, A., & Zunino, F. (1978) *Eur. J. Biochem. 85*, 487–492.

Quigley, G. J., Wang, A. H.-J., Ughetto, G., Van Der Marel, G., Van Boom, J. H., & Rich, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7204–7208.

Rao, G. M., Lown, J. W., & Plambeck, J. A. (1978) *J. Electrochem. Soc. 125*, 534–539.

Sikic, B. I., Ehsan, M. N., Harker, W. G., Friend, N. F., Brown, B. W., Newman, R. A., Hacker, M. P., & Acton, E. M. (1985) *Science (Washington, D.C.) 228*, 1544–1546.

Sinha, B. K., & Gregory, J. L. (1981) *Biochem. Pharmacol. 30*, 2626–2629.

Toppin, C. R., Pauly, G. T., Devanesan, P., Kryak, D., & Bobst, A. M. (1986) *Helv. Chim. Acta 69*, 345–349.

Wang, G. M., & Finch, M. (1980) *Drug Chem. Toxicol. 3*, 213–225.

Yen, S.-F., Germon, W., & Wilson, W. D. (1983) *J. Am. Chem. Soc. 105*, 3717–3719.

Disulfide Bond Formation between the Active-Site Thiol and One of the Several Free Thiol Groups of Chymopapain

Ibolya Kőrődi, Bence Asbóth, and László Polgár*

Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, Hungary H-1502

Received February 24, 1986; Revised Manuscript Received July 3, 1986

ABSTRACT: Chymopapain, a cysteine protease of papaya latex, has been purified with the use of fast protein liquid chromatography. Two homogeneous fractions were analyzed for thiol content and thiol reactivity. It was found that peak 1 and peak 2 contained two and three thiol groups, respectively, per mole of enzyme. This result is inconsistent with the general belief that chymopapain contains one essential and one nonessential thiol group and suggests that a significant portion of the thiol groups was oxidized in the previous preparations. Such an oxidation can account for some of the inconsistent results reported in the literature. An irreversibly oxidized nonessential thiol group may modify the catalytic function of chymopapain especially if it is close to the active site. That one thiol group resides indeed in the vicinity of the essential thiol group is clearly demonstrated by the biphasic reactions of chymopapain with disulfide compounds such as 2,2′-dipyridyl disulfide and 5,5′-dithiobis(2-nitrobenzoate). In the first step of these reactions a mixed disulfide is formed between the enzyme and the reactant, which is followed by a first-order, intramolecular reaction leading to the liberation of the second half of the disulfide compound. Furthermore, on addition of one Hg^{2+} ion, 2 mol of thiol group, one essential and one nonessential, disappears concomitantly. Formation of a disulfide bond between the catalytically competent thiol group and another free thiol group of chymopapain under physiological conditions may be of regulatory importance.

Chymopapain has been distinguished from the other cysteine proteases of papaya latex by its additional thiol group and classified as a dithiol cysteine protease (Brocklehurst & Salih, 1983). In the course of chromatography of papaya latex it is eluted from a cation-exchanger column as a broad peak between papain and papaya proteinase A. The broad chymopapain peak can be separated into several fractions, all containing essential and nonessential thiol groups, but less than

0006-2960/86/0425-6895$01.50/0