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Mechanism of Monofunctional and Bifunctional Alkylation of DNA by Mitomycin C†

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Received August 31, 1987; Revised Manuscript Received December 4, 1987

ABSTRACT: The relative amounts of monofunctional and bifunctional alkylation products of DNA with mitomycin C (MC) depend on whether one or both masked alkylating functions of MC are activated reductively; adduct **8** is the result of one function and adducts **7** and **9**, formed as a pair, are the result of both functions being activated [Tomasz, M., Lipman, R., Chowdary, C., Pawlak, J., Verdine, G. L., & Nakanishi, K. (1987) *Science (Washington, D.C.) 235*, 1204–1208]. To determine the mechanism governing this differential reactivity of MC with DNA, MC–*Micrococcus luteus* DNA complexes formed under varying conditions in vitro were digested to nucleosides and adducts. Adduct distribution, analyzed by high-performance liquid chromatography, served as the measure of monofunctional and bifunctional activation. H_2/PtO_2 and xanthine oxidase/reduced nicotinamide adenine dinucleotide (NADH) activated MC mostly monofunctionally, and $Na_2S_2O_4$ activated the drug bifunctionally under comparable conditions. Excess MC selectively suppressed, but excess PtO_2 selectively promoted, bifunctional activation by H_2/PtO_2; excess xanthine oxidase and/or NADH also had promoting effects. O_2 tested in the $Na_2S_2O_4$ system was inhibitory. 10-Decarbamoyl-MC acted strictly monofunctionally under all conditions. Monoadducts bound to DNA were converted to bis adducts upon rereduction. A mechanism with the following features was derived: (i) Activation of MC at C-1 and C-10 is sequential (C-1 first). (ii) A one-time reduction is sufficient for both. (iii) Activation of the second function may be selectively inhibited by kinetic factors or O_2. (iv) **7** and **9** are coproducts of bifunctional activation; their ratio depends on the DNA base sequence. (v) Activation of the second function involves an iminium intermediate. Direct applications to the action of MC in vivo are discussed.

Mitomycin C (MC,[1] **1**) is an antibiotic and antitumor agent used in clinical cancer chemotherapy. It is also a weak mutagen and carcinogen and inhibits bacterial cell division. One of its most interesting properties is the ability to bind covalently to DNA, both monofunctionally and bifunctionally, resulting in the latter case in the formation of covalent cross-links between the complementary strands (Szybalski & Iyer, 1967). These effects make MC unique among the known antibiotics. They also represent the ultimate molecular basis for its biological activity, as indicated by the parallels with a number of known DNA-targeted agents: selective inhibition of DNA replication, strong induction of the SOS response and sister chromatid exchange, and cross-resistance or cross-hypersensitivity of bacterial and mammalian cells to UV light and MC [see Tomasz et al. (1986) for specific references].

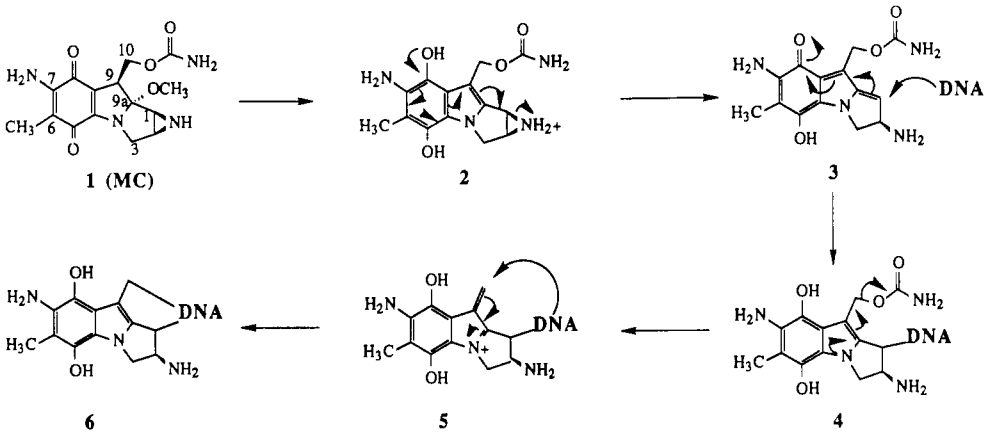
Under physiological conditions the covalent reactivity of MC with DNA requires enzymatic or chemical reduction (Iyer & Szybalski, 1964). This phenomenon led to designation of MC as a "bioreductive alkylating agent" (Lin et al., 1976). From their original observation, Iyer and Szybalski (1964) postulated a mechanism for the reductive activation of MC: the C-1 aziridine and C-10 carbamate groups are two masked alkylating functions, which become "allylic" (therefore activated) upon reduction of the quinone system and consequent spontaneous elimination of methanol from the 9/9a position (see **2**); they then may be displaced by two nucleophiles of DNA, resulting in a MC–DNA cross-link. Moore (1977) amended this hypothesis by speculating that both displacements are of S_N1 types, facilitated by resonance with the indolohydroquinone system of reduced MC, taking place sequentially as shown in Scheme I (**2** → **6**). Experimental investigations of the activation mechanism in model systems employing a large variety of enzymatic and chemical reducing systems as well as model low molecular weight nucleophiles verified most aspects of this scheme (Tomasz & Lipman, 1981; Kohn & Zein, 1983; Hornemann et al., 1983; Bean & Kohn, 1983; Pan et al., 1984; Peterson & Fisher, 1986; Danishefsky & Egbertson, 1986; Egbertson & Danishefsky, 1987). Evidence was

†This research was supported by NIH Grant CA 28681 and a PSC-CUNY Faculty Research Award (both to M.T.) and by NIH Grant RCMI RR03037 to the Center for Gene Structure and Function at Hunter College.

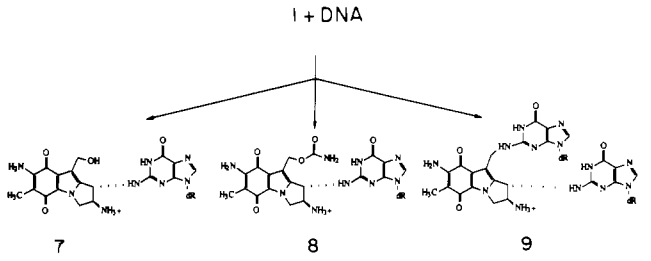
[1] Abbreviations: MC, mitomycin C; UV, ultraviolet; br, binding ratio; NADH, reduced nicotinamide adenine dinucleotide; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; CHO, Chinese hamster ovary.

0006-2960/88/0427-3182$01.50/0

Scheme I



Scheme II



also offered by several investigators that these model reactions for Scheme I take place in the semiquinone rather than the hydroquinone reduction state of MC (Danishefsky & Egbertson, 1986; Egbertson & Danishefsky, 1987; Andrews et al., 1986; Kohn et al., 1987). The products and mechanism of the reactions of MC with DNA itself have not been known, however. The general assay for "cross-linked" DNA (Szybalski & Iyer, 1967) and association of the UV chromophore of MC with DNA (Tomasz et al., 1974) were the only experimental indications of the alkylation process until lately. Recently, however, the reaction products of reductively activated MC with DNA itself were isolated and their structures elucidated (Tomasz et al., 1986, 1987), as shown in Scheme II. It is apparent from these structures that reductively activated MC reacts with the N^2 position of guanines, in the minor groove of DNA, either monofunctionally (**7**, **8**) or bifunctionally (**9**). Adduct **9** was shown to form between dG residues in opposite strands of duplex DNA, and therefore, it represents the long sought for MC–DNA cross-link previously inferred only from the altered renaturation kinetics of MC-treated DNA (Szybalski & Iyer, 1967). This adduct was also shown to be formed in vivo upon injection of rats with MC (Tomasz et al., 1987). Structures **7–9** verify directly that the DNA-reactive sites of the drug are its C-1 and C-10 positions as originally postulated by Iyer and Szybalski (1964).

The formation of three different major adducts between MC and the guanines of DNA indicated that the mechanism of the interaction of the drug with DNA was complex. Adding to product structural complexity, the distribution of the three adducts showed a striking dependence on the conditions of the reductive activation in vitro, reflecting monofunctional activation by H_2/PtO_2 or reductases but bifunctional activation when $Na_2S_2O_4$ was used (Tomasz et al., 1987). Since occurrence of these adducts in vivo is intrinsically related to the biological effects of MC, we wanted to determine the mechanism that governed the formation of the adducts, explained the observed wide variations of their distribution in experiments in vitro, and had fundamental validity for the mode of action of MC in vivo. An examination of the relationship between the mode of activation of MC and the mode of its reaction with DNA was conducted to achieve this goal, as follows.

Materials and Methods

Materials and their sources are as follows: *Micrococcus luteus* DNA, Worthington Biochemicals (sonicated before use); mitomycin C, Bristol Laboratories, Syracuse, NY; 10-decarbamoyl-MC, synthesized as described (Kinoshita et al., 1970); all other materials, as described previously (Tomasz et al., 1986).

Reactions of DNA with MC under Variations in the Reductive Activating Conditions and Isolation of the Resulting MC–DNA Complexes. (A) H_2/PtO_2 as Reducing Agent. (i) Standard reaction: *M. luteus* DNA (0.67 μmol/mL), MC (0.33 μmol/mL), and PtO_2 catalyst (100 μg/μmol of MC) were mixed in 0.015 M Tris-HCl (pH 7.4) and hydrogenated by a published procedure (Tomasz et al., 1983). Isolation and determination of the binding ratio (br, mole of MC per mole of mononucleotide) of the resulting MC–DNA complex were accomplished as described (Tomasz et al., 1974). (ii) Reaction with a 5-fold excess of MC: this is the same as the standard reaction, above, except for a 5-fold excess of MC (1.65 μmol/mL) used in this reaction. (iii) Reaction with a 10-fold excess of PtO_2: this is the same as the standard reaction, above, except for a 10-fold excess of PtO_2 (1000 μg/μmol of MC) used in this reaction.

(B) $Na_2S_2O_4$ as Reducing Agent. (i) Standard reaction: DNA (0.67 μmol/mL) and MC (0.33 μmol/mL) in 0.015 M Tris-HCl (pH 7.4) were treated with 0.06 M aqueous $Na_2S_2O_4$ (1.5 mol/mol of MC) under anaerobic conditions (bubbling He gas) for 25 min. Isolation and determination of the br of the resulting MC–DNA complex were accomplished as described (Tomasz et al., 1974). (ii) Reaction in the presence of air: This is the same as the standard reaction, except air instead of He gas was bubbled through the reaction mixture.

(C) Xanthine Oxidase/NADH as Reducing Agent. (i) Standard reaction: DNA (0.67 μmol/mL), MC (0.67 μmol/mL), and NADH (0.67 μmol/mL) were incubated in 0.015 M Tris-HCl, pH 7.4, with xanthine oxidase (0.5 unit/μmol of MC) for 20 min at 37 °C under a helium atmosphere. Isolation and determination of the br of the resulting MC–DNA complex were accomplished as described (Tomasz et al., 1986). (ii) Reaction with a 10-fold excess of NADH: This is the same as the standard reaction, above, except a 10-fold excess of NADH and a 2-fold excess of xanthine oxidase were used in this case.

Reactivation of DNA-Bound Monofunctional Mitomycin Residues. (A) $Na_2S_2O_4$ as Reactivator. The MC–DNA complex, br 0.065 (1 μmol/mL), prepared by the H_2/PtO_2

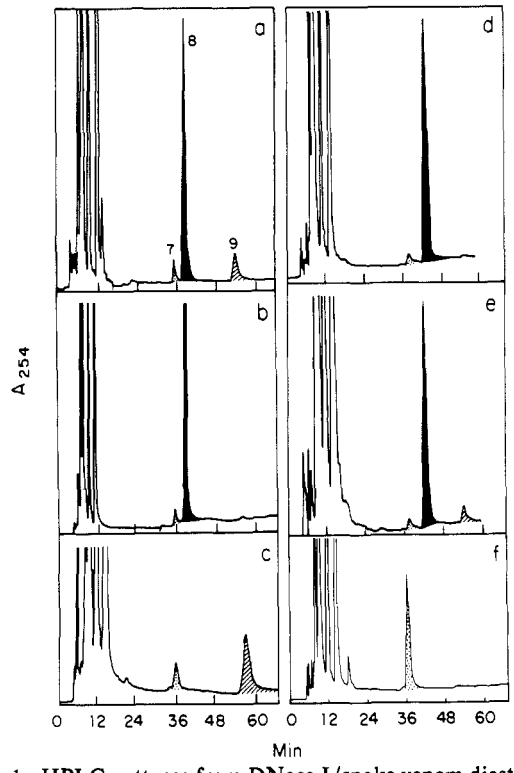


FIGURE 1: HPLC patterns from DNase I/snake venom diesterase/alkaline phosphatase digests of MC–DNA complexes formed under various activating conditions: (a) H_2/PtO_2 standard reaction; (b) H_2/PtO_2, 5-fold excess of MC; (c) H_2/PtO_2, 10-fold excess of PtO_2; (d) xanthine oxidase/NADH, standard reaction; (e) xanthine oxidase/NADH, 10-fold excess of NADH, 2-fold excess of xanthine oxidase; (f) H_2/PtO_2 standard reaction, analogue 10-decarbamoyl-MC used instead of MC for complex formation with DNA. The cross-identity of peaks in the various experiments is indicated by their identical shading.

activation method was treated with 1.5 μmol/mL $Na_2S_2O_4$ under anaerobic conditions in 0.015 M Tris-HCl buffer, pH 7.4, for 25 min at room temperature. The purple color of the complex disappeared. The mixture was reexposed to air, and the return of the color was observable. The complex was isolated by Sephadex G-100 chromatography as usual (Tomasz et al., 1974).

(*B*) *H_2/PtO_2 as Reactivator.* The same complex as above was treated with 100 μg/mL PtO_2 and H_2 gas for 25 min. No color change was observed. The complex was isolated as above.

Preparation of Complexes of 10-Decarbamoyl-MC with DNA. Using either $Na_2S_2O_4$ or H_2/PtO_2 as reducing agent, the procedures were analogous to those with MC above in the standard reactions, except that the concentration of 10-decarbamoyl-MC was 1.34 μmol/mL in these reaction mixtures.

Analysis of Adduct Distribution in MC–DNA Complexes. The complexes were digested to nucleosides and nucleoside–drug adducts by DNase I, snake venom diesterase, and alkaline phosphatase in combination. The resulting digests were separated by HPLC, yielding characteristic adduct distribution patterns (Tomasz et al., 1986). Quantitation of peaks was accomplished by measurement of peak areas by triangulation.

HPLC Separations. A reversed-phase column (Beckman Ultrasphere ODS; 1.0 × 25 cm) was used; flow rate was 2.0 mL/min; eluant was 8:92 CH_3CN/0.02 M potassium phosphate, pH 5.0.

RESULTS

Distribution of Adducts 7–9 in MC–DNA Complexes Formed under Various Reductive Conditions. (i) H_2/PtO_2 activation of MC: Under the standard (arbitrary) conditions (Figure 1a) mostly **8** and small amounts of **7** and **9** were obtained. On the other hand, the presence of a larger excess of MC completely suppressed formation of **7** and **9** (Figure 1b).[2] Excess PtO_2 suppressed **8** (Figure 1c). (ii) Xanthine oxidase/NADH (standard reaction) yielded hardly any **7** and **9** (Figure 1d). When the amounts of enzyme and NADH were increased compared with the standard reaction, the relative amounts of **7** and **9** increased appreciably (Figure 1e).

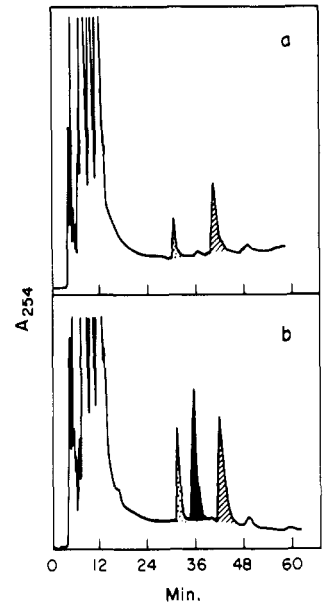


FIGURE 2: Effect of O_2 on adduct distribution: HPLC patterns from DNase/snake venom diesterase/alkaline phosphatase digests of MC–DNA complexes formed under (a) anaerobic $Na_2S_2O_4$ activation conditions and (b) $Na_2S_2O_4$ activation in the presence of air.

Effect of O_2 on Adduct Distribution. Using $Na_2S_2O_4$ as activating agent, the standard anaerobic reaction yielded the pair **7** and **9** (Figure 2a); when air was bubbled through the reaction mixture, however, **8** became the predominant adduct (approximately 50% of the total three; Figure 2b).

Adducts Formed with 10-Decarbamoyl-MC. 10-Decarbamoyl-MC readily formed a complex with DNA under H_2/PtO_2, xanthine oxidase/NADH, or $Na_2S_2O_4$ activating conditions. The single major adduct after enzymatic digestion was identified as **7** (Figure 1f); no trace of **9** could be detected. The only minor adduct, eluting later, is the 1″-β isomer of **7** (unpublished results).

Conversion of Monofunctionally Bound MC Residues in DNA to Bifunctional Ones by Reductive Reactivation. A MC–DNA complex, br 0.065, was prepared under H_2/PtO_2 activation (standard reaction; Materials and Methods), and its adduct distribution was determined by enzymatic digestion, followed by HPLC analysis of the digest (**7**:**8**:**9** = 5:80:15). An undigested portion of the complex was reduced with $Na_2S_2O_4$ for a brief period and then allowed to be reoxidized by air. Compared with the original, this complex showed no change in br but a large change in adduct distribution (**7**:**8**:**9** = 30:0:70). This indicated that the reduction of the MC residues, bound in the original, mostly monofunctionally substituted complex, converted all of the DNA-bound **8** type adducts into DNA-bound **7** and **9** (Figure 3). In an analogous experiment using H_2/PtO_2 for the reduction of the complex,

[2] A small amount of **7**, apparent in Figure 1b, originates from **8** during the enzymatic digestion of the MC–DNA complex (Tomasz et al., 1986). [See also footnote *a* to Scheme III.]

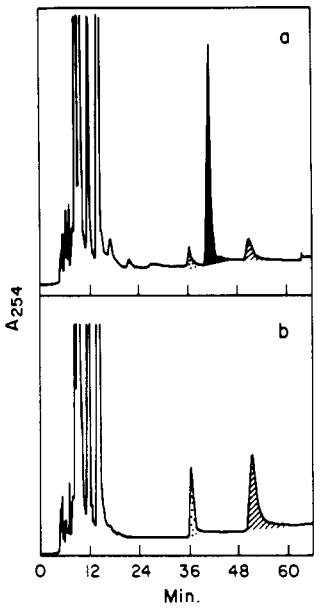


FIGURE 3: Conversion of monofunctionally bound MC residues in DNA to bifunctional ones upon reactivation: HPLC patterns of the nuclease digest of (a) MC–DNA complex and (b) same complex after exposure to $Na_2S_2O_4$ as reactivator.

Scheme III: Summary of MC Adduct Distribution under Various Reaction Conditions[a]

MC + DNA	H_2/PtO_2, std reaction	84% **8**, 16% **7** + **9**
	H_2/PtO_2, excess MC	96% **8**, 4% **7** + **9**
	H_2/PtO_2, excess PtO_2	0% **8**, 100% **7** + **9**
	XO, std reaction	95% **8**, 5% **7**, 0% **9**
	XO, excess NADH	88% **8**, 12% **7** + **9**
	$Na_2S_2O_4$, std reaction	0% **8**, 100% **7** + **9**
	$Na_2S_2O_4$, air	53% **8**, 47% **7** + **9**
10-decarbamoyl-MC + DNA	$Na_2S_2O_4$ or H_2/PtO_2	100% **7**

[a] The yields of **7** and **8** are corrected for the fact that 3% of **8** is converted to **7** during enzymatic hydrolysis of the MC–DNA complexes (Tomasz et al., 1986). XO = xanthine oxidase.

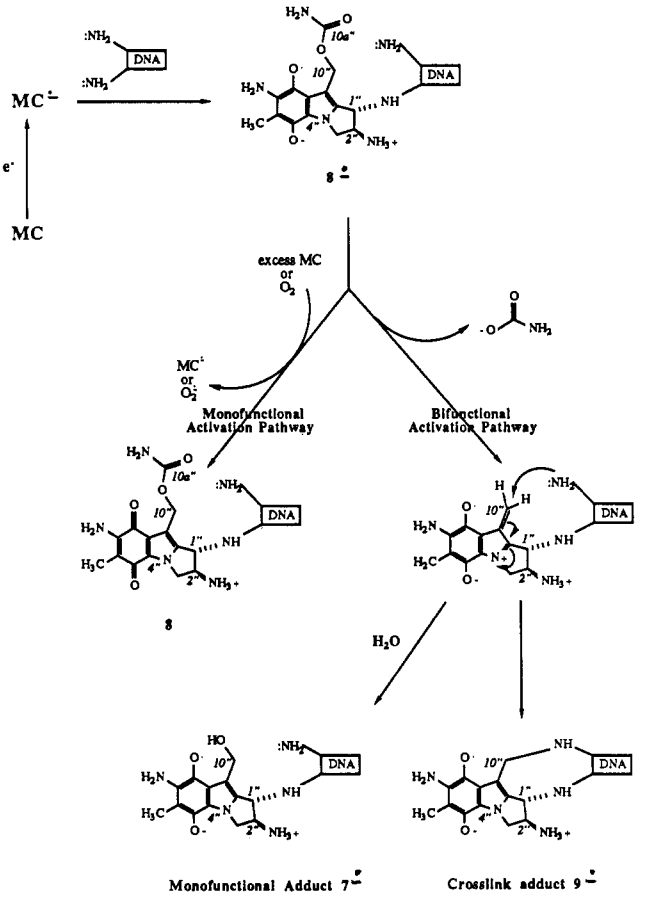
no change in the original adduct distribution was observable.

All of these results are summarized in Scheme III.

Discussion

In order to explain the earlier observed dependence of monofunctional versus bifunctional activation of MC on the reducing conditions (see the introduction), we proposed a mechanism featuring reduction kinetics rather than redox potentials as the critical factor (Tomasz et al., 1987). Crucial for the rationale of this proposal was the discovery by Peterson and Fisher (1986) of autocatalytic activation of MC in which a catalytic amount of electron equivalents induces stoichiometric activation of MC in a chain reaction to yield monofunctionally substituted mitosenes as final products. [A similar chain reaction in the case of mitomycin B was reported also by Egbertson and Danishefsky (1987)]. We postulated that after an initial reduction and alkylation step the monoadduct species $8^{\bullet-}$ may react further by either of two pathways: (i) electron transfer to unreacted MC (autocatalytic activating conditions analogous to the Peterson–Fisher mechanism), thereby losing its activated state and giving monoadduct **8** as end product, or (ii) retro-Michael elimination of the C-10″

Scheme IV



carbamate to give $9^{\bullet-}$, which is receptive to a second nucleophilic attack giving bifunctionally alkylated end products **7** and **9** (Scheme IV). The actual balance of the two paths should depend on the particular reaction conditions in a predictable, experimentally testable manner. The results presented here represent such experimental testing, as follows.

In our system DNA itself is the substrate reacting with MC; adduct **8** is the product of monofunctionally activated MC (along with <5% of its 1″-β isomer; Tomasz et al., 1986), while adducts **7** and **9** are diagnostic coproducts of bifunctional activation formed as an obligatory pair (Tomasz et al., 1987). The latter findings are explained by considering that in DNA only a fraction of the guanines are at cross-linking distance from another guanine, i.e., those in a GpC, CpG, or GpG sequence; only that fraction can give **9** upon reaction with MC. MC bound to other guanines will react with H_2O at its second activated position at C-10″; therefore, the product in that case will be **7** (Scheme IV; Tomasz et al., 1987). The present experiments consisted of varying the reaction conditions and observing their effects on the distribution of **7**–**9**. A simple summary is given in Scheme III.

The first conclusion drawn from the results is that the initial monofunctional step of DNA binding always occurs at C-1 of MC, yielding monoadduct **8**. This simply follows from the fact that an alternative, C-10-linked monoadduct has not been observed. That **8** is precursor in its reduced form to **7** or **9** was also shown directly, by conversion of DNA-bound **8** in a second, separate reduction step to **7** and **9** (Figure 3, Scheme III).

Reduction Kinetics Determine the Monofunctional/Bifunctional Activation Ratio. Hydrogen/PtO_2, observed in previous studies to act as a monofunctional activating agent of MC (Tomasz & Lipman, 1981; Hashimoto et al., 1980;

Tomasz et al., 1983, 1986; Kohn & Zein, 1983), was shown now to be fully capable of bifunctional activation, simply when a larger amount of PtO_2 was used (Figure 1a,c). Xanthine oxidase/NADH also switches from a purely monofunctional to a partly bifunctional activating catalyst when the enzyme + NADH/MC ratio is increased (Figure 1d,e). In contrast, a *larger excess of MC* dramatically suppresses bifunctional product formation (Figure 1a,b). In these experiments the *rate of reduction* of MC is what varies, and the results indicate clearly that this determines the product distribution. Note that H_2/PtO_2 gives the same, full bifunctional activation as $Na_2S_2O_4$, under appropriate conditions (Figures 1c and 2a). These results constitute proof for the concept that underlies this mechanism (Scheme IV), which predicts that if the reduction rate is slow, the monofunctional, autocatalytic activation pathway predominates because $\mathbf{8}^{\bullet-}$ is inactivated by excess MC faster than it is formed [as shown first by Peterson and Fisher (1986) for activation of MC in the absence of DNA; in their case the nucleophile was water]. Alternatively, if the reduction rate is fast, $\mathbf{8}^{\bullet-}$ will accumulate and have a long enough lifetime to undergo the second activation step ("bifunctional activation pathway").

S_N1 Displacement of the C-10″ Carbamate Group. The second activation step of MC is indicated in Scheme IV as S_N1 displacement of carbamate, aided by iminium ion formation. This is more likely than S_N2 displacement on theoretical grounds. The net negative charge of the anion radical should greatly stabilize such an intermediate. Model experimental studies (Zein & Kohn, 1986) provided support for the iminium mechanism, at least in methanolic solutions and in the absence of DNA. That the displacement of the C-10″ leaving group is indeed the critical step toward the bifunctional pathway (Scheme IV) is confirmed by the finding that 10-decarbamoyl-MC and DNA yield **7** but not **9**, even when the strictly bifunctional activating agent $Na_2S_2O_4$ is used (Figure 1f; Tomasz et al., 1988), indicating the lack of bifunctional potential of 10-decarbamoyl-MC. This result is consistent with previous reports that this analogue does not cause cross-links in DNA in vitro (Carrano et al., 1979) or in vivo (Otsuji & Murayama, 1972). Evidently, bifunctional activation requires a good leaving group at C-10″, such as carbamate rather than OH^-; i.e., elimination from C-10″ is rate determining in the second activation step.

Effect of O_2 on the Activation Pathway. It is remarkable that the overall binding of MC to DNA in the presence of O_2 was decreased by only 20% as compared with the anaerobic counterpart of this experiment. Thus, it seems that, despite the potential inhibitory effect of O_2 on the reduction step itself, much of the MC has become monofunctionally activated. However, the overall activation pattern has changed from a purely bifunctional (**7** and **9**; Figure 2a) to a mixed mono- and bifunctional one (**7**–**9**; Figure 2b). This result, as originally predicted from Scheme IV, indicates that O_2 indeed inactivated some of the initial monofunctional product $\mathbf{8}^{\bullet-}$ and thereby selectively decreased the extent of bifunctional activation.

These results, all taken together, confirm the proposed mechanism governing monofunctional versus bifunctional alkylation of DNA by MC. The main characteristic of this mechanism is the potential for either autocatalytic or stoichiometric reduction kinetics. The results also apply to reductive alkylation reactions of MC and its analogues in general. Several additional comments regarding Scheme IV may be made:

(i) The formulation of the reduction as one-electron type was adapted in light of several recent reports implicating the MC semiquinone radical anion ($MC^{\bullet-}$) rather than the previously assumed MC hydroquinone (Scheme I; Iyer & Szybalski, 1964) as the species giving rise to the subsequent activation steps (Danishefsky & Egbertson, 1986; Andrews et al., 1986; Kohn et al., 1987). The present findings have no bearing on this matter and adapt themselves to either formulation; i.e., if the MC hydroquinone proves to be the active species, then OH should be substituted for O^- in position 8″ in the appropriate formulas in Scheme IV.

(ii) A feature of the present mechanism is that, regardless of whether the semiquinone or hydroquinone is the active species, once it is formed it will suffice for activation of both alkylating functions of MC. Although it is demonstrated that $Na_2S_2O_4$ is capable of reactivating monofunctionally bound MC in a subsequent process to form cross-links (Figure 3), such second reduction is not necessary under conditions of the bifunctional pathway. It also seems a priori unlikely, on the basis of steric considerations, that in vivo the MC already bound to DNA monofunctionally could be a further substrate to a reductase, as proposed by Andrews et al. (1986).

DNA Alkylation Mechanisms and Cellular Toxicity of MC. Since MC–DNA adduct patterns are diagnostic of the partitioning of MC between the monofunctional and bifunctional activation pathways, analysis of the adducts formed in vivo should give direct information on the course of activation in the cell. The cross-link adduct **9** was shown to form in cellular DNA upon injection of rats with a high dose of MC (Tomasz et al., 1987); recent results in our laboratory (Chowdary & Tomasz, 1987a,b) indicate formation of **7** and **9** in CHO cells treated with MC, also under high-dose conditions. Thus, in these cells bifunctional activation of MC has occurred, indicating the absence of excess (unreduced) MC at the MC–DNA binding sites, because excess MC would have inactivated the second function (Scheme IV). O_2 apparently did not cause such inactivation either. In accord with the latter point, Fracasso and Sartorelli (1986) as well as Marshall and Rauth (1986) detected the formation of cross-linked (i.e., bifunctionally alkylated) DNA in MC-treated tumor cells under aerobic conditions using the alkaline elution technique.

It is known, however, that MC is less toxic to aerobic cells than to hypoxic ones and the antitumor activity of MC against hypoxic solid tumors has been attributed to this selectivity (Sartorelli, 1986). The differential toxicity can only be observed at very low drug doses in cell cultures. In view of the mechanism (Scheme IV) it is attractive to speculate that the effect is due to increased bifunctional activation of MC, and therefore more cross-linking of DNA, in the hypoxic cells than in the aerobic ones, since in the latter O_2 may inhibit selectively the bifunctional pathway. The present work demonstrates the existence of such inhibition in a chemical system (Figure 2), but the in vivo systems tested so far (rat, CHO cells) showed fully bifunctional activation under aerobic conditions (Tomasz et al., 1987; Chowdary & Tomasz, 1987a,b). The latter findings do not invalidate the above theory, however, since the experiments were conducted under such high MC dose conditions that the O_2 effect would have been "swamped out". More sensitive adduct detection techniques, when developed, will allow the use of lower, physiologically more relevant doses of MC in order to probe the proposed correlation among cellular O_2 tension, adduct distribution, and MC toxicity.

ACKNOWLEDGMENTS

We thank Dr. W. T. Bradner of Bristol Laboratories, Syracuse, NY, for a generous supply of mitomycin C.

Registry No. MC, 50-07-7.

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Renaturase and Ribonuclease H: A Novel Mechanism That Influences Transcript Displacement by RNA Polymerase II in Vitro†

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Received September 18, 1987; Revised Manuscript Received January 8, 1988

ABSTRACT: I have previously reported an activity in HeLa cells which facilitates transcript displacement by purified mammalian RNA polymerase II in vitro. I have shown that this activity copurifies with one of two separable ribonuclease (RNase) H activities in HeLa cells. The RNase H activity in question has characteristics similar to those reported for RNase H2b from calf thymus. RNase H proteins purified from several other sources including *Escherichia coli* also show renaturase activity. When the renaturase/RNase H protein is present during transcription by purified RNA polymerase II, transcripts are truncated close to the 5′ end, and the remainder of the transcript is displaced normally from its template by the polymerase. Since RNA polymerase II dependent transcripts in vivo normally require the presence of the 5′-triphosphate terminus for capping, the in vivo significance of RNase H as a renaturase factor is presently not understood. However, the in vitro action of renaturase/RNase H suggests that the mechanism of this reaction may involve R-loop displacement after formation of a short single-stranded region of DNA on the template strand following hydrolysis of a hybrid transcript oligonucleotide by RNase H.

To understand the molecular details of eukaryotic transcription, a system containing only purified components would be of considerable value. Recent studies have shown that there are a large number of genetic elements and protein factors needed in conjunction with purified RNA polymerase II for accurate and efficient transcription of different genes. Although the majority of the known factors are implicated in correct initiation of transcription, it is to be expected that additional factors will be important in the elongation and termination phases of transcription as well.

† Supported by NIH Grant RO1-GM34963 to Michael J. Chamberlin.

0006-2960/88/0427-3187$01.50/0