MtmMII-mediated C-Methylation during Biosynthesis of the Antitumor Drug Mithramycin Is Essential for Biological Activity and DNA-Drug Interaction*

Received for publication, November 11, 2003, and in revised form, December 3, 2003
Published, JBC Papers in Press, December 5, 2003, DOI 10.1074/jbc.M312351200

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The antitumor drug mithramycin consists of a polypeptide chromophore glycosylated with a trisaccharide and a disaccharide. Two post-polypeptide methylations take place during mithramycin biosynthesis. One of these methylations has been shown to be very relevant for biological activity, that is, the introduction of a methyl group at aromatic C-7. We have purified to 282-fold the MtmMII methyltransferase involved in this reaction. The protein is a monomer, and results from kinetic studies were consistent with a model for the enzyme acting via a compulsory order mechanism. The enzyme showed high substrate specificity and was unable to operate on structurally closely related molecules. Structural predictions suggest that the molecule is integrated by two domains, an essentially all-α-amino domain and an αβ-carboxyl domain displaying a variation of the Rossmann-fold containing the cofactor binding site. Although 7-demethyl-mithramycin did not show any biological activity, it was able to reach the nucleus of eukaryotic cells, with subsequent binding to DNA. Mithramycin and 7-demethylmithramycin were able to form similar complexes with Mg2+, although their respective DNA binding isomers were very different. The binucleotide binding model fit well the isomers recorded for both compounds, predicting that the C-7 methyl group was essential for high affinity binding to specific GC and CG sequences. Considering previous structural studies, we propose that this effect is performed by positioning the group in the floor of the minor groove, allowing the interaction with the third sugar moiety of the trisaccharide, d-mycaroose, which is involved in sequence selectivity.

Polyketides comprise a family of compounds synthesized via linear poly-β-ketones by repetitive head-to-tail additions of acyl units. These compounds may undergo a variety of further reactions, including cyclizations, methylations, hydroxylations, glycosylations, oxidations, and reductions. The result is an extremely diverse family of natural products, many of which display useful biological activities with clinical (antibiotics, antifungal, antitumor drugs, antiparasitic, and immunosuppressive agents) or agricultural (insecticides, herbicides) applications. The aurolic acid group of antitumor drugs comprise mithramycin (MTM),1 chromomycins, olivomycins, chromocyclomycin, durbamycin, and UCH9 (1) (Fig. 1). All of these compounds belong to the large and important family of aromatic polyketides. Their structures consist of a tricyclic chroomophore that is glycosylated at two different positions of the aglycon with saccharides of various chain lengths (1). The only exception is chromocyclomycin, which possesses a tetracyclic chroomophore. The polypeptide aglycon of these compounds is derived from the condensation of ten acetates in a series of reactions catalyzed by a type II polyketide synthase (2). These compounds are active against Gram-positive bacteria but not against Gram-negative because of a permeability barrier. While these compounds are too toxic to be used as antibiotics, they show good antitumor activity and some of them are in clinical use. They interact with GC-rich regions of DNA in a non-intercalative way in the presence of Mg2+ ions, which are essential for activity, forming 2:1 antibiotic-Mg2+ complexes and inhibiting replication and transcription (3).

MTM is produced by Streptomyces argillaceus as well as other streptomycetes. It has been clinically used for the treatment of testicular carcinoma, Paget’s bone disease, and other bone growth disorders and is also used for control of hypercalcemia in patients with malignant disease (4). The biosynthetic pathway for MTM biosynthesis has been extensively studied by biochemical and genetic means (2, 5–12). Although MTM is a tricyclic aromatic polypeptide with a side chain, its biosynthesis proceeds through tetracyclic tetracycline-like intermediates (6). The MTM polypeptide skeleton derives from one acetyl-CoA and nine malonyl-CoA units and, through condensation reactions, gives rise to a decaketide (5). This molecule is a substrate for several aromatizations and cyclizations to produce 4-demethyl-premithramycinone (the first biosynthetic intermediate so far isolated) and, after an O-methylation step, it renders premithramycinone (2, 6). This tetracyclic intermediate is then glycosylated through a series of biosynthetic intermediates with the addition of d-olivo, d-oliose, and d-mycaro sugar moieties finally making a trisaccharide chain, and generating 9-demethyl-premithramycin A3 (9-DMPA3). This intermediate is then C-methylated to premithramycin A3 (PMA3) (11) prior to the addition of a disaccharide of d-olivo-d-olivo to form premithramycin B (9). All of these biosynthetic intermediates have been isolated from mutants blocked at different steps in biosynthesis and share a common feature, which is the pres-

*This work was supported by a grant from the Plan Regional de Investigación del Principado de Asturias (GE-MEDO1-05). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: MTM, mithramycin; MtmMII, mithramycin methyltransferase; PMA3, premithramycin A3; 9-DMPA3, 9-demethyl-premithramycin A3; 7-DMTM, 7-demethyl-mithramycin; PMA1, premithramycin A1; PMC, premithramycinone; AdoMet, S-adenosylmethionine; SAH, S-adenosylhomocysteine; PBS, phosphate-buffered saline; TSB, Trypticase soy broth; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid.
ence of a tetracyclic aglycon. The final tricyclic structure of MTM is obtained after the oxidative cleavage of the fourth ring of premithramycin B, in a reaction catalyzed by a monooxygenase and followed by the ketoreduction of the 4'-position of the generated aliphatic chain (9, 12). This renders the fully biologically active compound. None of the other biosynthetic intermediates obtained by insertional inactivation shows any biological activity. One outstanding case is 7-DMTM, a product accumulated by a mutant lacking the mtmMII methyltransferase gene, whose structure resembles that of MTM, varying only in the absence of the C-7 methyl group (11). Interestingly, 7-DMTM displays no activity over several tumoral cell lines (13).

Biological methylations have been found to play a crucial role in drug metabolism and biosynthesis. In secondary metabolic pathways, S-adenosyl-methionine (AdoMet) is commonly utilized as a ubiquitous methyl donor, while folate-dependent methylations are mainly found in primary metabolism in different organisms, including mammals (14). Substrates of AdoMet-dependent methyltransferases include lipids, proteins, polynucleotides, polysaccharides, and small molecules as polyketides. The methylated functional groups include carboxyl, phenol, and hydroxyl groups (oxygen atoms), aliphatic and aromatic amines (nitrogen atoms), thiol and sulfides (sulfur atoms), olefins and ring carbons (carbon atoms), halides and metal ions (14). The structure of MTM contains nine methyl groups. Six of them are part of the sugar moieties, because each deoxysugar unit contains a C-6 methyl group derived from the dehydration step affecting C-4 and C-6 during its biosynthesis; another methyl side chain is attached at C-3 of the β-mycarose moiety, the third sugar of the trisaccharide chain. The remaining three methyl groups are part of the aglycon: C-5' is derived from the acetate starter unit, meanwhile methyl groups at C-1' and C-7 are transferred by methyltransferases coded by the mtmMI and mtmMII genes, respectively (11).

In this report, we have purified the MtmMII methyltransferase and characterized the methylation step occurring at the aromatic C-9 of the aglycon of 9-DMPA3. A conformational model for the protein based on structural predictions is also presented. Finally, we analyze the relevance of the methylation to the biological activity of the drug and propose a model to explain drug-DNA interaction and its structural basis.

EXPERIMENTAL PROCEDURES

Reagents—Phenyl Sepharose High Performance, Sephacryl S-200 HR, Superdex 200, and S-adenosyl-1-[methyl-3H]methionine (specific activity 2.92 TBq/mmol) were purchased from Amersham Biosciences. DTT and alkaline phosphatase were from Roche Diagnostics Gmh. Ethylene glycol, octanol, olivomycin, AdoMet, and SAH were purchased from Sigma-Aldrich. Acrylamide and bisacrylamide were from Bio-Rad, EDTA and ammonium sulfate from Merck, and Tris from USB. Calf thymus DNA was purchased from Fluka & Riedel; phosphate-buffered
After addition of enzyme, the reaction mixture was incubated at 35 °C for 20 min. A small concentration (113 mM) of S-adenosyl-L-3-methyl-
3H-homocysteine was added subsequently; samples were taken at different
times, processed as described (18) and used to calculate the rate of
isoetric exchange.

**Biological Assays**—The biological activities of MTM and 7-DTMT were tested by performing bioassays against Micrococcus lutes ATCC
10240 as previously described (19).

**Structural Predictions**—Secondary structure predictions and fold
recognition were performed using the PSIPRED (20–23) and 3D-PSSM
(24) servers.

**Fluorescence Microscopy**—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100
IU/ml α-penicillin, and 100 μg/ml streptomycin in a humidified at-
mosphere of 5% CO₂. For fluorescence experiments, cells were subcul-
tured onto microscope slides by incubation at 37 °C for 2 min with
0.0125% Trypan blue 0.002% EDTA, followed by the addition of complete
medium, washing, and resuspension in fresh medium. After growth,
slides were washed with PBS, and MTM or 7-DTMT were added at 1
mg/ml in the same buffer. After 30 min at 37 °C in 5% CO₂, cells were
washed with PBS and intrinsic fluorescence was observed in a Leica
DMR-XA microscope using a filter L5 Blue BP 1480/40 BP 527–530.

**Determination of Physico-chemical Parameters**—MTM and 7-DTMT hydrophobicities were determined measuring their partition coeffi-
cients (Ko) between water and octanol. 1 ml aliquots of both molecules
(40 μl dissolved in Tri-HCl buffer 50 mM pH 2.6 at 30 °C) were extracted by addition of 1 ml of octanol followed by vigorous shaking.
The final concentrations in both solvents were determined spectrophoto-
metrically at 280 nm.

For the determination of the pKₐ of the molecules, the compounds were dissolved at a final concentration of 30 μM in a wide range three-
fold different concentrations of MgCl₂ (1–1000 μM). After incubation with Mg²⁺, samples did not exceed 0.05. Scattering effects were eliminated by subtrac-
tion of the values measured for control solutions.

**Absorbance and Fluorescence Measurements**—Absorption and fluo-
rescence spectra were recorded with a Kontron 930 (Unikon Instru-
ments) spectrophotometer and a PerkinElmer LS50B luminescence
spectrometer, respectively. All fluorescence measurements for MTM and 7-DTMT and their complexes with Mg²⁺ were carried out at an
excitation wavelength of 470 nm to avoid time-dependent decrease of
the fluorescence emission produced by photodegradation of the mole-
cules (25), and an emission wavelength of 540 nm. No correction for
optical filtering effects was made, because the absorbance of the sam-
ple did not exceed 0.05. Scattering effects were eliminated by subtrac-
tion of the values measured for control solutions.

**Binding Experiments**—All binding experiments were carried out at
30 °C in 20 mM Tri-HCl buffer, pH 8.0. Formation of the complex antibiotic-Mg²⁺ was followed measuring fluorescence quenching in-
duced by addition of different concentrations of MgCl₂ to MTM or
7-DTMT. Samples were allowed to equilibrate over 1 h at 30 °C.

To carry out binding experiments involving antibiotic-Mg²⁺ com-
plexes and calf thymus DNA, the correspondent molecule (3 μM) was
preincubated with Mg²⁺ (10 μM) to ensure the complete formation of
the complex. Small aliquots of DNA were then added, and fluorescence
values were taken when no further changes in the measurements were
observed. During the titration, the total dilution was restricted to 5% of
the initial volume, and the appropriate correction was incorporated in
the calculation.

**Analysis of Binding Data**—Results from fluorimetric titrations were
evaluated by linear and nonlinear regressions using the program
Statistica for Windows. Interactions were analyzed utilizing the following
procedures.

1) The Scatchard plot shown by Equation 1,

\[ r(C_p) = K(n − r) \]

(Eq. 1)

where \( r = C / C_p \), \( C_p \), \( C_p \), and \( C_r \) are the concentrations of bound, free ligand, and DNA respectively. \( n \) is the binding stoichiometry, expressed as ligand bound per nucleotide, and \( K \) is the intrinsic binding constant

(25). Experimental estimations of these values were performed from
spectroscopic titration experiments as previously described (25).

2) Neighborder exclusion model shown by Equation 2 (28).

\[ r \frac{1}{C_p} = K(1 − nr) \left( \frac{1 − nr}{1 − (n − 1)n} \right)^{−1} \]

(Eq. 2)

3) Neighborder exclusion with cooperative ligand-ligand interactions

M. J. Fernández Lozano, unpublished results.
shown by Equations 3 and 4 (26),

\[ \frac{r}{C_i} = K(1 - nr) \left[ \frac{2 wr - 1}{2(w - 1)(1 - nr)} \right]^{1/2} \left[ 1 - \frac{(n + 1)r + R}{2(1 - nr)} \right]^{1/2} \]  
(Eq. 3)

\[ R = \left( 1 - (n + 1)r + 4wr(1 - nr) \right)^{1/2} \]  
(Eq. 4)

where \( \omega \) is the cooperativity parameter and \( n \) is the exclusion parameter, expressed as base pairs.

4) Dinucleotide binding model shown by Equation 5 (26),

\[ r_D = \sum_{M} \frac{f_{MN}K_{MN}C_i}{1 + K_{MN}C_i} \]  
(Eq. 5)

where \( r_D \) is the binding ratio now expressed as moles of ligand per mole of dinucleotide and \( f_{MN} \) is the dinucleotide frequency. Although there are 16 possible dinucleotides, only 10 of them are unique, so that Equation 5 has 10 terms. Dinucleotide frequencies have been experimentally determined (27) and may be fixed as constant for a given DNA (26).

RESULTS

Characterization of the MtmMII C-Methyltransferase—MtmMII was purified 282-fold with a 19% recovery in a four-step purification procedure with a purity of more than 90%. The first stage in the purification was the fractionation of the cell-free extract by ammonium sulfate precipitation, followed by three chromatographic steps using hydrophobic interaction (phenyl-Sepharose), gel filtration (Sephacryl S-200), and affinity (adenosine-agarose) (Fig. 2, A, B, and C). A summary of the procedure is shown in Table I and Fig. 2 D. The purified protein showed a molecular mass of ~36.5 kDa as deduced from SDS-PAGE gels (Fig. 2 D) and 36.1 kDa as calculated by gel filtration on a Superdex 75 column in the presence of 0.15 M NaCl at a flow rate of 0.1 ml/min (data not shown). Keeping in mind that the molecular weight deduced from the primary structure of the protein is 35529.5 (11), these experiments clearly suggest that MtmMII is a monomer. Enzyme activity was shown to be unaffected by the addition of different mono or divalent cations, and was stable for at least several months when kept at 70 °C in 20% glycerol. Analysis of the optimal pH of the reaction showed the typical bell-shaped curve with an optimum at slightly alkaline pH, between 8 and 8.5.

Steady State Initial Velocity Studies—When the initial velocity was measured varying the concentration of 9-DMPA3 at different fixed concentrations of AdoMet, a set of intersecting lines was obtained by regression analysis of the data at each AdoMet concentration (Fig. 3). To obtain estimates of the kinetic constants, the full data set was then fitted by performing nonlinear regression analysis to the equation of Alberty in Ref. 28, obtaining the following parameters:

\[ V = 19.5 \mu mol/min \cdot mg protein^{-1}; K_a = 23.2 \mu M; K_b = 2.95 \mu M; K_m = \]
shown in Fig. 4. A hyperbolic plot was obtained with depletion at high concentrations of the pair AdoMet/SAH. In contrast, varying the pair AdoMet/SAH, a hyperbolic plot was obtained (Fig. 4B).

When the effect of increased concentrations of the pair AdoMet/PMA3 on the rates of exchange between [3H]AdoMet and [3H]PMA3 was measured, a hyperbolic plot was obtained. In contrast, varying the pair AdoMet/SAH, a hyperbolic plot with depletion at high concentrations of the pair AdoMet/SAH was obtained (Fig. 4B).

In similar experiments, the rates of isotopic exchange at equilibrium were measured varying the concentration of the substrate, 9-DMPA3, and one of the different products, SAH and PMA3. In both cases, plots showing depletion at high concentrations of the pair varied were obtained (Fig. 4C).

Use of MtmMII for the Methylation of Alternative Substrates—Some alternative molecules were assayed as potential substrates for MtmMII. The study involved the use of several MTM biosynthetic intermediates as 4-DMPC, PMC, PMA1, 7-DMTM and some structural closely related molecules as olivomycin, daunorubicin, and tetracycline. No transference of radioactivity from [3H]AdoMet could be detected in any of these experiments, indicating that MtmMII possesses high specificity for its natural substrate, 9-DMPA3.

Predicted Structural Features of the MtmMII—It was not possible to calculate a three-dimensional model of MtmMII, since no suitable targets were found in the Protein Data Bank. On the other hand, secondary structure prediction of MtmMII indicates that this molecule is an αβ protein (Fig. 5). The existence of two different regions could be determined in the enzyme: an N-domain (amino acids 1–150), essentially constituted by α-helices, and a C-domain, integrated by the rest of the molecule, in which 7 α-helices and 7 β-strands are disposed alternatively with the exceptions of helices C’-C and D-E and strands 6–7, which are consecutive. This motif at the C-domain, is a variation of a Rossmann-fold, usually found in nucleotide- or dinucleotide-binding proteins (Fig. 6). Three motifs, previously described as well conserved in different polyketide methyltransferases (11), can be localized at the edges of predicted structural elements (Fig. 5), motif I between strand 1 and helix A, and motives IV and VI at the ends of strands 4 and 5, respectively. On the other hand, a comparison of the amino acid sequence of MtmMII with proteins in data bases using the Basic Local Alignment Search Tool (BLAST) program (31) showed similarities mainly with O-methyltransferases that catalyze the transference of methyl groups to cyclic compounds, such as caffeic acid, catechol, or hydroxyindole. Highest similarities were placed mainly at the C-domain, because the N-terminal domain was less conserved in the different proteins. On the other hand, results did not change when data bases were scanned using the sequence of each domain independently. Fold recognition using the mGenThreader method (20–23) gave rise to high confidence results for two proteins of known three-dimensional structure, chalcone O-methyltransferase and isoavone O-methyltransferase from Medicago sativa (32). Although the primary sequences of these proteins are not very closely related, their secondary structures correlate very well with the one predicted for MtmMII (Fig. 5) and, moreover, they are integrated by two domains, as proposed for MtmMII. Structural alignment allows us to propose that at least two of the previously described well conserved domains (I and II) are probably involved in binding of the methionine moiety from AdoMet. It is possible to propose a role for some other well conserved regions, as motifs II, III, and V, placed at the N terminus of strands 2 and 3 and the C terminus of helix C, respectively; all probably involved in the binding of the adenosine group of AdoMet. No conserved regions of known function could be assigned to the N-domain.
Influence of C-7 MTM Methylation on Biological Activity—The influence of the presence of the methyl group on the biological activity of the molecule was first tested by bioassay against *M. luteus*. Results showed that MTM was able to inhibit bacterial growth at concentrations of 5 μg·mL⁻¹, becoming the inhibition very clear at 25 μg·mL⁻¹. However, the molecule lacking the C-7 methyl group, 7-DMTM, showed no biological activity even at concentrations of 200 μg·mL⁻¹ (data not shown).

The ability of these compounds to diffuse through the eukaryotic cell membrane and to penetrate HeLa cells was examined taking advantage of the intrinsic fluorescence of both molecules. Although small amounts of MTM and 7-DMTM appeared to be associated to the cell membrane, most appeared located at the nucleus, leaving the rest of the cell mainly free of fluorescence (Fig. 7). These experiments indicate that both compounds are able to locate to and accumulate into the cell nucleus, which is in apparent contradiction with the previously described differences in activity over several eukaryotic tumor cell lines (13).

Influence of C-7 Methylation on MTM Physico-chemical Properties—Hydrophobicity of both molecules was compared by measuring their respective water/octanol partition coefficients. The values obtained were 0.196 and 0.201, so that ∆G° were 0.961 and 0.946 kcal·mol⁻¹ for MTM and 7-DMTM, respectively. To determine the influence that the presence of the
methyl group at C-7 could have on the ionization of the phenol group, apparent $pK_a$ values were determined for both compounds, obtaining values of 5.5 and 5.8, respectively. These results indicate that C-7 methylation does not cause significant changes in physico-chemical properties.

MTM-Mg$^{2+}$ and 7-DMTM-Mg$^{2+}$ Interactions—Mg$^{2+}$ addition produced changes in the 7-DMTM absorption and fluorescence spectra that were very similar to those previously described for MTM (25) (data not shown), allowing us to carry out spectrometric titrations of the drug-Mg$^{2+}$ interactions.

Fluorometric titrations of MTM and 7-DMTM with Mg$^{2+}$ gave rise to composite curves (Fig. 8). After addition of the ion, the curve trends to a first plateau at concentrations under 300 $\mu$M Mg$^{2+}$ (Fig. 8A); further additions produced an increase which reaches a final plateau at concentrations under 10 mM Mg$^{2+}$ (Fig. 8B), indicating in both cases that two equilibria are responsible for the titration profiles over the analyzed range of concentrations.

Interactions of Drug-Mg$^{2+}$ Complexes with DNA—Interactions of drug-Mg$^{2+}$ complexes with DNA were analyzed at physiological concentrations over 10 mM Mg$^{2+}$. The fluorescence properties of drug-Mg$^{2+}$ complexes were employed to study the binding to DNA, since this interaction causes decoupling of the fluorescence of these ligands (25). Fluorescence emission intensities were plotted as a function of the input concentrations of DNA (Fig. 9). The results were clearly different for both molecules, reaching MTM saturation at DNA values much smaller than 7-DMTM.

The above experimental data were fitted to different models to evaluate binding parameters for ligand-DNA interactions (see “Experimental Procedures”). Representation in the form of Scatchard plot (Fig. 10, A and B) gave rise to $K$ values of $4.4 \times 10^4$ M$^{-1}$ and $9.4 \times 10^4$ M$^{-1}$ for MTM and 7-DMTM, respectively (Table II), although the fitted isotherm did not adequately account for the curvature seen in the data, specially for 7-DMTM. Better results could be obtained by fitting to the neighbor exclusion model, which produced $K$ values of 1.2 $\times$ 10$^5$ M$^{-1}$ and 1.9 $\times$ 10$^5$ M$^{-1}$ showing again an important difference in the affinity of both molecules for DNA (Table II). Introduction of additional terms in this model, trying to account for the ligand-ligand interactions did not improve the fitting significantly, giving rise to $\omega$ values of <1 for both molecules (Table II).

To determine the kinetic mechanism of the reaction, we also performed isotopic exchange reactions at equilibrium. Results obtained when the pair AdoMet/PMA3 was varied were consistent only if the products are incorporated and released from the catalytic center of the enzyme in a specific order; first AdoMet and later 9-DMPA3. Any type of random bi-bi mechanism would produce hyperbolic plots, while random on-ordered-off or Thorell-Chance mechanisms would give a different combination of plots (30). Raising the concentration of the second substrate, 9-DMPA3, together with any of the products, SAH or PMA3, makes the enzyme-AdoMet and (in the case of the first product) enzyme-PMA3 species scarce, increasing other forms, and the exchange rates are lowered. Nevertheless, if the concentration of the first substrate, AdoMet, is raised, depletion would be observed only when associated with a simultaneous rise in the first product, SAH, because the enzyme-PMA3 becomes scarce; otherwise the plot might be hyperbolic. All kinetic information described for the reaction mechanism of MtmMII can be represented by Scheme 1. The substrates bind in a compulsory order to the enzyme to form a ternary complex in which the exchange reaction takes place; the products are also released in a compulsory order. Ordered Bi-Bi mechanisms have been described for some AdoMet-dependent methyltransferases (33, 34) even involving processive movement and rapid exchange of a macromolecular substrate, without dissociation (35); nevertheless, other enzymes operate by different mechanisms (36).

Some enzymes involved in the biosynthesis of polyketide compounds are able to catalyze the corresponding reaction by incorporating alternative substrates, which makes them very interesting for their potential utility in the emerging field of combinatorial biosynthesis (37). Unfortunately, MtmMII exhibited a high dependence on the substrate structure, because
DNA binding isotherms. Association; B curves for drug-Mg²⁺/H₉₂₆₂ MTM (12 a function of Mg²⁺ similar distances and geometries for the calculated hydrogen non-methylated) and guanine rings. These resulted in very simplified models that included phenolic rings (methylated and phenolic hydroxyl O-8 (3), we performed molecule could be determined. On the other hand, since structural changes could be related with the specific presence of the group because saturation at DNA values were much more reduced for MTM than for 7-DMTM. Scatchard plot analysis of the data pointed to a higher affinity of the methylated molecule for DNA. Nevertheless, this model did not account for the presence of curvature in the plots, mainly for 7-DMTM. These curves could be better explained by neighbor exclusion models, improving the fitting of the results, and indicating again that the presence of the methyl group at C-7 increases drug-DNA binding affinity by more than 60×. Nevertheless, fittings were not as good for 7-DMTM, which showed an important statistical deviation from the theory. In fact, although the neighbor exclusion principle has been used to explain binding of some antibiotics to DNA (42), theoretical and experimental studies have thoroughly questioned it (26). Results did not improve when cooperative effects were considered.

Another possibility is to assume heterogeneity of binding sites. The simplest case in this scenario is the dinucleotide binding site model, whose binding isotherm is described by 10 terms, due to the 10 unique dinucleotide combinations (26). Application of this model resulted in good statistical significance for both molecules, and pointed out to the existence of relevant differences. MTM showed enhanced affinity for the dinucleotide pairs GC and CG, increasing their respective K values about 100 and 150× with respect to the medium values determined for the rest of the dinucleotide combinations. On the contrary, the absence of the methyl group at C-7 suggests a great decrease of the affinity for DNA and, more remarkable, an important change in the specificity for dinucleotide combinations. It is important to reflect that these models do not consider the possibility of changes in the DNA lattice, although NMR studies have showed that binding of MTM resulted in perturbations in the helical parameters compared with standard B-DNA and A-DNA helical values (3). Anyway, our results indicate that the presence of C-7 methyl group conditions the increase in the affinity of the molecule for specific sequences of DNA.

The biosynthetic pathway of MTM has evolved to include a methyl group at C-7 in the molecule, reaction catalyzed by the

Fig. 8. Fluorometric titrations of MTM (12 μM) and 7-DMTM (12 μM) as a function of Mg²⁺ concentration and DNA binding isotherms. A, first part of the composite curves for drug-Mg²⁺ association; B, second part of the composite curves for drug-Mg²⁺ association.

Fig. 9. Binding isotherms for the association of drug-Mg²⁺ complexes with calf thymus DNA: ○, MTM and □, 7-DMTM.
MtmMII methyltransferase. According to all the preceding data, we can conclude that, although the sequence similarity within the protein family that includes this molecule is very distant, structural predictions indicate that the tertiary structure of this enzyme presents a common fold with other methyltransferases. Nevertheless, this enzyme has clearly evolved to catalyze this concrete reaction, since no other closely related molecules are accepted as substrates. Interestingly, binding studies suggest that the presence of this chemical group conditions the high affinity binding to specific sequences of DNA.

A potential explanation for these observations could be derived from the examination of the structure of the MTM dimer-DNA complex (3). The E-sugar ring of the trisaccharide is positioned in the floor of the minor groove and in close contact with the C-7 methyl group of the opposite MTM monomer, which could contribute to the positioning and binding of this sugar moiety (Fig. 11). Interestingly, differences in both the hydrogen bonding and the hydrophobic nature of the substituents on the E-sugar result in reduced sequence selectivity for MTM dimer-DNA complexes relative to chromomycin dimer-DNA complexes (3). On the other hand, within the aureolic acid group members, there is a direct close relationship between the size of the substituent chemical group present at C7 position.

**Table II**

| Model                                | MTR | 7-DMTM | MTR | 7-DMTM | MTR | 7-DMTM | MTR | 7-DMTM |
|--------------------------------------|-----|--------|-----|--------|-----|--------|-----|--------|
| Scatchard plot                       | $4.4 \times 10^6$ | $9.4 \times 10^4$ | 0.02 | 0.02   | 89.2 | 66.4   |
| Non-cooperative neighbor exclusion   | $1.2 \times 10^5$ | $1.9 \times 10^3$ | 26.5 | 20     | 93   | 72.8   |
| Cooperative neighbor exclusion       | $1.2 \times 10^5$ | $1.9 \times 10^3$ | 26   | 21.7   | 0.49 | 0.5    | 93   | 72.2   |

**Scheme 1**

**FIG. 10.** Fitting of the MTM and 7-DMTM DNA-binding data to different models. A, MTM; B, 7-DMTM. Straight lines, Scatchard plots; dashed lines, neighbor exclusion model. C, affinity profile for the dinucleotide model; straight line, MTM; dashed line, 7-DMTM.

**FIG. 11.** Detail of the MTM-Mg$^{2+}$ dimer-DNA interaction. A, DNA strands. B, MTM aglycon. C, D, and E, trisaccharide from the opposite MTM molecule. E-sugar is displayed as a ball model.
and the length of the oligosaccharide present in the molecule (Fig. 1). Our results suggest that the presence of the C-7 methyl group in the molecule would allow an optimal interaction of E sugar along the minor groove of the DNA, that is essential for high affinity binding and sequence selectivity.

Acknowledgments——We thank Dr. Francisco González for discussions and for his ab initio studies of simplified models, Dr. Santiago Cal for discussions and HeLa cell cultures, and Dr. Alvaro Ohaya and J. M. Argüelles Collada for fluorescence microscopy.

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