In vitro Binding of Nitracrine to DNA in Chromatin

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In the presence of sulfhydryl compounds nitracrine, an anticancer drug, binds covalently to DNA. The accessibility of DNA in chromatin both to nitracrine and to 8-methoxypsoralen, which was used as a reference compound in this study, when assayed in NaCl concentrations from 0 to 2 M show similar characteristics. The initial decrease reaches a minimum at 0.15 M NaCl above which dissociation of non-histone proteins and histones at higher ionic strengths is demonstrated by an increase in accessible sites. The relative accessibility of DNA in chromatin to nitracrine is, however, lower than that found for 8-methoxypsoralen. Partial dissociation of chromatin with 0.7 M NaCl increases the accessibility of DNA in chromatin when assayed in the absence of NaCl but has no apparent influence when estimated at ionic strength close to physiological conditions.

Introduction

The anticancer drug, nitracrine (Ledakrin, C-283), 1-nitro-9-(3,3-N,N-dimethylaminopropylamino)acridine undergoes metabolic activation in the cell and subsequent covalent binding to DNA [1–7]. In the presence of sulfhydryl compounds the drug and several of its biologically active analogs form irreversible complexes with DNA and RNA [3, 8–12]. Covalent binding of drugs to chromatin may provide information on the properties of DNA in nucleoprotein, particularly if coupling factors do not introduce uncontrolled perturbation in the studied structure. As the presence of thiols in the course of the reaction is unlikely to introduce substantial changes in chromatin these experiments aim to exploit this property of nitracrine to assay the accessibility of DNA within a nucleoprotein complex.

Materials and Methods

Chemicals

8-Methoxyl-[^{3}H]methoxypsoralen, 140 mCi/nmol (Amersham, England) was diluted with the unlabelled drug (Sigma, U.S.A.) to activity 1.2×10^4 cpm/nmol. 9-[^{14}C]Nitracrine (activity $3-6 \times 10^3$ cpm/nmol) was previously described [9].

Binding of nitracrine or 8-methoxypsoralen to native chromatin

Native calf thymus chromatin was isolated according to the method of Bonner *et al.* [13] as modified by Turoverova and Vorobev [14] and used following centrifugation in 1.7 M saccharose [13, 14] and dialysis against 10 mM Tris/HCl (pH 7.2). The nitracrine complexes were formed in the presence of the drug (0.1 mM, if not otherwise specified, 2 mM Tris/HCl (pH 7.2) and 5 mM dithiothreitol for 1 h [8–11] at chromatin DNA or free DNA concentration of 400 μg/ml and NaCl concentration as indicated. After 1 h unreacted drug was removed during the deproteinisation of chromatin with chloroform/isoamyl alcohol [15] and the amount of [^{14}C]nitracrine irreversibly bound to DNA was estimated as described [9]. The complexes of nitracrine with free DNA were similarly treated. Photoreaction of [^{3}H]8-methoxypsoralen (0.046 mM) with chromatin (or DNA) at DNA concentration of 400 μg/ml was carried out by irradiation as described [10] and the stoichiometry of the purified complexes was estimated by radioactivity measurements as for nitracrine. DNA and protein in chromatin were estimated according to Burton [16] and Lowry *et al.* [17] respectively. The protein/DNA ratio was found to be 1.8 (w/w).

Binding of nitracrine to partially dissociated chromatin

Native calf thymus chromatin diluted to a DNA concentration of 2 mg per ml was dissociated with 0.35 M NaCl [18] or 0.7 M NaCl [19]. The dissociated proteins were then separated by centrifugation in a glycerol gradient [19] containing 0.35 or 0.7 M NaCl,

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Dedicated to the late Professor Bronisław Filipowicz (1904–1988) formerly the Head of the Department of General and Physiological Chemistry School of Medicine in Łódź.

Reprint requests to Prof. M. Gniazdowski.

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respectively. Native chromatin (also passed through the glycerol gradient but no NaCl added) and the salt treated chromatin were subsequently dialyzed overnight against 10 mM Tris/HCl, pH 8 containing 0.1 mM EDTA and, when indicated, 0.15 M NaCl. The protein/DNA ratios in two experiments were 1.7 ± 0.04 (0.35 M NaCl) and 1.4 ± 0.1 (0.7 M NaCl). The dialyzed chromatin preparations were suspended in a Potter homogenizer, and then reacted with nitracrine for 1 h in the presence of 0.15 M NaCl (if not otherwise specified) and dithiothreitol. The stoichiometry of the complexes following purification was determined (see above).

Results and Discussion

Covalent binding of drugs to chromatin may provide information on the properties of DNA in nucleoprotein, particularly if coupling factors do not introduce uncontrolled perturbation to the studied structure. The following facts indicate that nitracrine in the presence of dithiothreitol, actually forms a covalent complex with DNA [20]: i) [^{14}C]nitracrine in the complex is precipitated with trichloroacetic acid with an efficiency similar to that of DNA; ii) the complex is not dissociated by urea, sodium dodecyl sulfate, high ionic strength or organic solvents; iii) neither thermal nor alkaline denaturation considerably affect the efficiency of trichloroacetic acid precipitation of [^{14}C]nitracrine-DNA.

The mode of the thiol-dependent binding of the drug to DNA has not been fully understood. A reduction of the nitro group was suggested to be a step leading to an active derivative [8] but susceptibility of the nitro group in the position 1, of aminoalkyl chain in the position 9 and hydrogen atoms in the position 2 and 4 of the acridine ring in nitracrine to nucleophilic substitutions by thiols and other reagents [21, 22] may indicate on the potentially multiple sites of attachement of the drug molecule to DNA. Actually a substitution of the tritium atoms in the positions 2, 3 or 4 was observed in the course of the thiol-dependent nitracrine binding to DNA [23] indicating the either positions to be involved in the adduct formation. Most of the drug molecules attached to DNA bear both the acridine ring and the aminoalkyl side chain [23]. The drug exhibits a preference to purines, particularly to guanine residues in DNA [9]. Pilot experiments on the chromatographic resolution of the hydrolyzed complexes indicate on the at least three types of adduct [24] as inferred from the earlier studies [23].

The presence of thiols in the course of the covalent binding of nitracrine to DNA is unlikely to introduce substantial changes to chromatin. An additional advantage in using this drug to probe protein-DNA interactions is the fact that its irreversible binding to histone or albumin under similar conditions is fivefold lower than to DNA [20]. As an indirect experimental approach has already indicated, the protein components in native chromatin reduce the accessibility of DNA to nitracrine [25, 26]. The level of drug binding to the DNA of native calf thymus chromatin in the absence of NaCl is 30–40% of that found with naked DNA (Fig. 1, 2). It does not change substantially when assayed at a constant drug to DNA ratio and the DNA concentration varied from 200 to 400 μg/ml (not shown). The accessibility of DNA in chromatin to nitracrine is considerably lower than that found by Reynolds and Hurley with anthramycin [27] but higher than was reported by Metzger and Daune for calf thymus chromatin using a carcinogenic aminofluorene derivative [28]. Comparative time-course binding experiments show that (Fig. 1) the proportion of DNA accessible to the

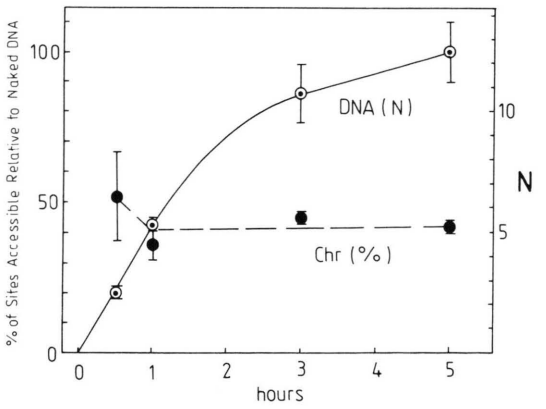

Fig. 1. Time course of nitracrine binding to naked DNA (⊙) and chromatin (●). Native calf thymus chromatin and naked DNA with nitracrine and dithiothreitol (concentration of NaCl = ○) were incubated for the time indicated. DNA was then purified. The number of the drug molecules bound per 10^3 DNA nucleotides (N, right scale) to naked DNA (controls) and the relative amount of the drug bound to chromatin, expressed as a percentage of the corresponding controls (left scale), were estimated. The range values are denoted by the vertical bars on the points which are mean values of 2 independent experiments. See Materials and Methods for further details.

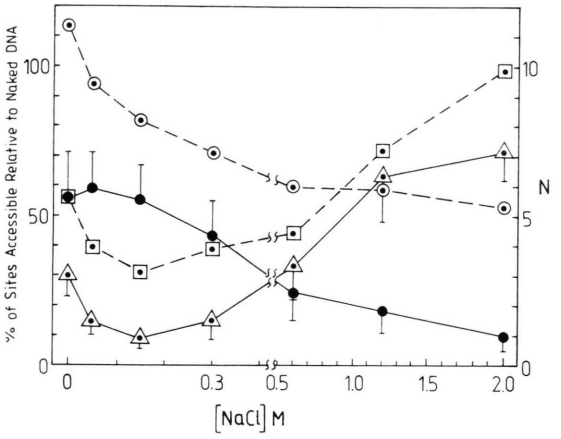

Fig. 2. Effects of salt on nitracrine and 8-methoxypsoralen binding to naked DNA and chromatin. Native calf thymus chromatin and naked DNA were incubated with nitracrine and dithiothreitol or were irradiated with 8-methoxypsoralen in the presence of increasing concentrations of NaCl. DNA was then purified. The number of nitracrine (●) and 8-methoxypsoralen (⊙) molecules bound to naked DNA (N) and the relative amounts of nitracrine (△) and 8-methoxypsoralen (⊡) bound to chromatin, expressed as a percentage of the corresponding controls were estimated. The data are mean values from three independent experiments with nitracrine and from one experiment with 8-methoxypsoralen. See Materials and Methods and Fig. 1 for further details.

drug following a small decrease within the first hour remains essentially constant up to 5 h.

Derivatives of furocoumarins have recently been used in numerous elegant studies as molecular probes for chromatin DNA structure (*e.g.* see ref. [29–33]). In the experiments presented in Fig. 2, binding of nitracrine to free DNA and DNA in chromatin at different ionic strengths is compared to 8-methoxypsoralen photobinding under identical conditions. The binding curve for 8-methoxypsoralen closely resembles that obtained for the effect of ionic strength on 4,5′,8-trimethylpsoralen binding to supercoiled Col E1 plasmid DNA [31]. Accessibilities of DNA in chromatin to nitracrine and 8-methoxypsoralen show qualitatively similar characteristics. The initial decrease reaches a minimum at 0.15 M NaCl which corresponds to aggregation of chromatin. Removal of non-histone proteins and histone H1 at 0.7 M NaCl, and further dissociation of histones, is demonstrated by an increase in accessible sites and is consistent with the results reported by Hyde and Hearst [31]. DNA, however shows, lower accessibility to nitracrine than to 8-methoxypsoralen. As recently reported [33] at 0.15 M NaCl 8-methoxypsoralen reacts with chromatin to a level of about 30% which is somewhat higher than 20% found with trimethylpsoralen [31] while only 10% of DNA is accessible to nitracrine (Fig. 2). As both drugs form covalent complexes with DNA in different reactions, show different base specificity [9, 34] and probably indulge in different steric interactions prior to their coupling to DNA [35], the observed lower accessibility of DNA to nitracrine may be due to several factors. The simplest explanation, namely that the affinity of the nitroacridine derivative (or its by-product transiently formed) for DNA is just lower than that of 8-methoxypsoralen, does not agree with the other data. As shown in our laboratory, photo-binding to DNA is more efficiently inhibited by an intercalating ligand, ethidium bromide, and non-intercalating peptide-type antibiotics, netropsin or distamycin A, than the thiol-dependent binding of nitracrine [35]. Perhaps the highly hydrophobic 8-methoxypsoralen molecules may more easily penetrate the aggregated chromatin than charged nitracrine molecules. The latter seems to be supported by the data indicating that the accessibility of chromatin DNA to trimethylpsoralen is higher than that to its ionic aminoethyl derivative [31] and recent experiments pointing on the importance of hydrophobic properties in furocoumarin-DNA interactions [36].

In order to assay how partial dissociation of chromatin influences accessibility of DNA at ionic strength close to physiological conditions native chromatin and chromatin partially depleted of proteins were examined in the nitracrine reaction system at 0.15 M NaCl. Native chromatin was subjected both to 0.35 M NaCl [18] or 0.7 M NaCl [19] to remove non-histone proteins and histone H1. Naked DNA and chromatin preparations were reacted with nitracrine in the presence of 0.15 M NaCl at the increasing drug to DNA ratio. The numbers of nitracrine molecules bound to naked DNA and to DNA in native chromatin (Fig. 3A) and to DNA in chromatin treated with 0.35 M NaCl (Fig. 3B) or 0.7 M NaCl (Fig. 3C) were plotted against the drug to DNA ratio during the complex formation. As shown for the reaction of the aminofluorene derivative with chromatin [28] a linear relationship is observed between the adduct formation and the drug concentration, both for DNA and DNA extracted from various chromatin preparations (Fig. 3). The calculated intercepts

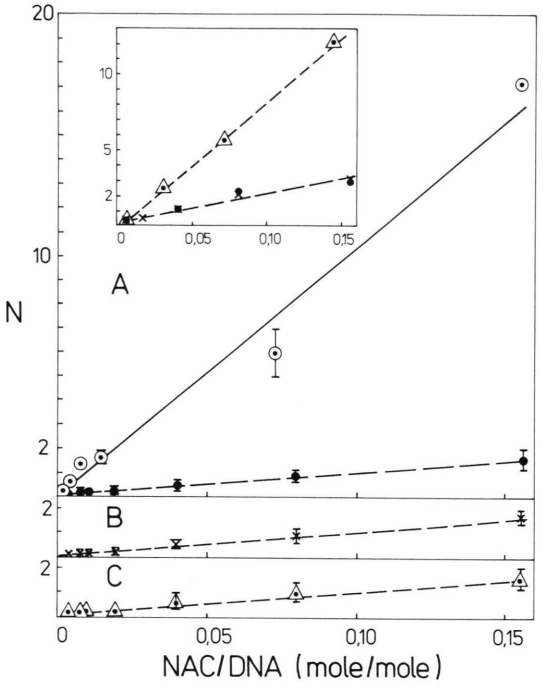

Fig. 3. Plot of the number of nitracrine molecules (N) bound A) to naked DNA (⊙) and native chromatin (●), B) to chromatin dissociated with 0.35 M NaCl, and C) to chromatin dissociated with 0.7 M NaCl. Native calf thymus chromatin was treated with 0.35 or 0.7 M NaCl and centrifuged in the glycerol gradient containing 0.35 or 0.7 M NaCl correspondingly. Both preparations and native chromatin were then dialyzed against the buffer containing 0.15 M NaCl. The data of two independent experiments are plotted against the drug/DNA ratio (mol of drug per mol of DNA nucleotide) during reaction of native or protein depleted chromatin with nitracrine in the presence of dithiothreitol (2 mM) and NaCl (0.15 M). The inset shows the binding plot of nitracrine to native (●), and dissociated with 0.35 M (×) and 0.7 M NaCl chromatin (△) as described above but no NaCl was present in the nitracrine reaction medium. No corresponding control DNA was included in this experiment. See Materials and Methods and Fig. 1 for further details.

(Table I) are negligible. Hence the ratios of the slopes are equal to the respective ratios of accessibilities [28]. The partially deproteinized chromatin preparations show an accessibility of about 10%, as low as that found for native chromatin (Fig. 3, Table I). The lack of increase of accessibility following the salt treatment is due to the ionic strength (0.15 M NaCl) during the drug binding. When the binding of the drug is assayed in the absence of NaCl (see the inset to Fig. 3A) following dissociation of chromatin with 0.35 M NaCl no changes in the amount of nitracrine bound observed. On the other hand, an increase of about fourfold in accessibility is shown for chromatin following dissociation with 0.7 M NaCl, when assayed at low ionic strength. These observations can be compared to those reported by Metzger and Daune [28]. A small increase in the accessibility to aminofluorene derivative follows dissociation of non-histone proteins from both calf thymus and chicken erythrocyte chromatin with 0.35 M NaCl. Similarly to our results erythrocyte chromatin depleted of histones H1 and H5 exhibits an over threefold increase of the carcinogen binding as compared to native chromatin. Negligible changes in accessibility seen at the moderate ionic strength (Table I) may be due to an aggregation of chromatin although there was no visible precipitation of chromatin previously treated with 0.7 M NaCl following dialysis to 0.15 M NaCl. It was suggested that the accessibility of native chromatin DNA may be re-

Table I. Accessibility of DNA in native and partially dissociated chromatin. The slope of N *versus* nitracrine/DNA in the absence of NaCl (see the inset to Fig. 3A) for native and 0.35 M NaCl treated chromatin is 19.7, that for chromatin dissociated with 0.7 M NaCl is 86.2 while the intercepts are 0.3 and 0.5, respectively.

	Slope of N *versus* drug/DNA	Intercept of N *versus* drug/DNA	% of accessible DNA
Naked DNA	105	−0.01	100
Native chromatin	10.1 ± 2.5	0.07	9.6 ± 2.4
Chromatin treated with 0.35 M NaCl	10.0 ± 2.0	0.08	9.5 ± 1.9
Chromatin treated with 0.7 M NaCl	10.4 ± 1.7	0.12	9.6 ± 1.6

duced by an increased internal stabilization of nucleosomes under the ionic conditions close to those of physiological conditions [31]. It can be concluded from this experiment, that despite partial removal of histone H1 and non-histone proteins a highly stabilized structure present at 0.15 M NaCl is still preserved. Our data are consistent with the results obtained by Osipova *et al.* [37] indicating that at this ionic strength the oligonucleosome chain without H1 folds up to a compact configuration typical for oligonucleosomes with H1 at the lower salt concentration.

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