Acyltransferase-mediated Binding of N-Hydroxyarylamides to Nucleic Acids

Frederick A. Beland, William T. Allaben, and Frederick E. Evans

Department of Health, Education and Welfare, Food and Drug Administration, National Center for Toxicological Research, Jefferson, Arkansas 72079

ABSTRACT

N-Hydroxyarylamides are metabolically activated to nucleic acid-binding species by the action of N,O-acyltransferase (AT). The substrate specificity of these enzymes in rat, guinea pig, monkey, baboon, pig, and human liver has been examined by measuring the AT-mediated nucleic acid binding of the N-formyl, N-acetyl, and N-propionyl derivatives of N-hydroxy-2-aminofluorene. Human and pig enzymes catalyzed binding in the order formyl >> acetyl > propionyl, while for the other species the order was acetyl > propionyl > formyl. Ammonium sulfate fractionation of the cytosols suggested that the baboon and rat have at least two different AT's: one with a higher specificity for the formyl derivative; the other with a marked preference for acetyl and propionyl compounds. Only one form, with a high formyl group specificity, was detected from human liver.

The identity of the in vitro AT-mediated DNA adducts from rat, baboon, and human liver was established. In each instance, one adduct accounted for >75% of the bound 2-aminofluorene (AF) residues. This product had a high-pressure liquid chromatography retention time and pH-dependent partition characteristics identical to those of an adduct synthesized by an acid-dependent (pH 4.6) reaction of N-hydroxy-2-aminofluorene with calf thymus DNA. This synthetic adduct has been identified as N-(deoxyguanosin-8-yl)-2-aminofluorene by nuclear magnetic resonance, mass, and ultraviolet light spectroscopy. Moreover, it was identical to the product obtained from the alkaline (pH 12) hydrolysis of N-(deoxyguanosin-8-yl)-2-acetylaminofluorene. Since an arylaminated (i.e., aminofluorene) residue(s) is the major product found in rat liver DNA following administration of N-hydroxy-N-acetyl-2-aminofluorene, these data suggest that AT may play a major role in the formation of this DNA-carcinogen adduct.

INTRODUCTION

Carcinogenic arylamines, such as AAF, are activated to DNA-binding species by sequential N-hydroxylation and O-esterification (27). In rat liver, this metabolic sequence results in the formation of both acylated (approximately 20%) and nonacylated (approximately 80%) arylamine-DNA adducts (11, 18, 19). Two AAF-DNA adducts have been identified (Adducts II and III in Chart I) (19, 29) and are thought to arise from a sulfotransferase-catalyzed formation of a reactive sulfuric acid ester (8, 15). High hepatic levels of this enzyme have been associated with a marked susceptibility to N-hydroxy-AAF-induced liver tumors (8). On the other hand, AF adducts have been suggested to arise by reaction of DNA with N-acetyl-arylamines which are formed by the action of AT on N-hydroxy-AAF (5, 13). This enzyme has been detected in a variety of tissues (13) and species (14) and has been extensively purified from rat liver (2). Although the importance of AT in the formation of N-hydroxyarylamide-induced liver tumors is not clear, this enzyme may play a crucial role in susceptible tissues that are devoid of sulfotransferase activity (14). Previous studies have identified the AT-mediated guanosine-AF adduct (5, 13); however, the AT-catalyzed DNA adducts have not been fully characterized. We have now examined the substrate specificity of this enzyme from rat, guinea pig, monkey, baboon, pig, and human liver by measuring the AT-catalyzed nucleic acid binding of N-formyl, N-acetyl, and N-propionyl derivatives of N-hydroxy-AF. The identity of the major in vitro AT-mediated DNA adducts from rat, baboon, and human liver was also investigated.

MATERIALS AND METHODS

\[\text{[ring-}^{3}H]\text{-N-Hydroxy-2-acylaminofluorenes (specific activity: formyl, 48.3 mCi/mmol; acetyl, 45.3 mCi/mmol; propionyl, 51.8 mCi/mmol); and [acetyl-}^{3}H]\text{-N-hydroxy-AAF (specific activity, 50 mCi/mmol).}\]

N-hydroxy-AAF was synthesized by the method of Lotlikar et al. (22). Immediately prior to use, the compound was suspended in argon-purged 95% ethanol, stirred for 5 min under an argon atmosphere, and then filtered to remove any 2,2'-azoxyfluorene that formed upon storage. The hydroxylamine prepared in this manner had a half-life of 2 hr [as measured by Fe^{2+}-reducing equivalents (12)] and did not contain any nitrosofluorene [as indicated by the absence of UV absorbance at 365 nm (22)]. The ethanol filtrate was evaporated under reduced pressure, and the N-hydroxy-AF residue was dissolved in methanol for the reaction with DNA.

Animals were obtained from the following sources: female rhesus monkeys and baboons from D. E. Hill, University of Arkansas for Medical Sciences, Little Rock, Ark.; castrated male pigs (Yorkshire/Hampshire/Duroc cross) from J. Hunter, Cabot, Ark.; female guinea pigs (strain 9; NIH) and Sprague-Dawley rats (250 to 300 g) from our breeding colony. Rat, guinea pig, rhesus monkey, and pig livers were obtained from animals killed immediately prior to the experiments. Baboon liver was quick-frozen in liquid nitrogen and stored at —70° until analysis. Human liver samples were obtained at autopsy.

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1 A preliminary account of this work has appeared (1).
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: AAF, 2-acetylaminofluorene; N-hydroxy-AAF, N-hydroxy-2-acetylaminofluorene; N-hydroxy-2-acetylaminofluorene; AF, 2-aminofluorene; AT, N,O-acyltransferase; N-hydroxy-AF, N-hydroxy-2-aminofluorene; NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; NOE, nuclear Overhauser effect.

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Acyltransferase-mediated Nucleic Acid Adduction

Chart 1. Metabolic activation of acylaminofluorenes. Adducts Iii and Iii (R, acetyl) have been previously shown to be formed in vivo in rats, presumably through a 3'-phosphoadenosine 5'-phosphosulfate-dependent sulfotransferase activation (8, 15). Intramolecular N-O-acyltransfer provides a mechanism for the formation of nonacylated adducts (i.e., Adduct I), the major DNA-bound products in rat liver (5, 13).

from males 48 to 85 years old. Immediately following death, the bodies were stored at 4°, and the samples were taken 4 to 18 hr postmortem.

The following AT isolation procedures were carried out at 4°. Livers were suspended in 4 volumes of argon-purged 50 mM sodium pyrophosphate: 1 mM dithiothreitol buffer, pH 7.4 (13), and homogenized with a Polytron (Brinkman, Westbury, N. Y.) for 30 sec at 75% maximum setting. The mixtures were then centrifuged at 105,000 x g for 60 min to obtain the cytosol fractions which were refrigerated in an argon atmosphere until AT analysis. Human, baboon, and rat enzymes were further purified by sequential ammonium sulfate precipitation. To accomplish this, a saturated solution of ammonium sulfate was added to the cytosol to 10% final concentration. The precipitate which formed was collected by centrifugation (9000 x g, 30 min), resuspended, and dialyzed against 50 mM sodium pyrophosphate: 1 mM dithiothreitol buffer (pH 7.4). The supernatant again treated with ammonium sulfate until 20% saturation was reached, and the centrifugation process was repeated. This sequence was continued until 90% saturation was obtained (13). Protein concentrations were determined by the technique of Lowry et al. (23). Acyltransferase activity was quantitated by the tRNA-binding assay described previously (13).

In experiments where DNA binding was investigated, 2 mg calf thymus DNA per ml (type I; Sigma Chemical Co., St. Louis, Mo.) was substituted for tRNA. After incubation, the mixture was successively extracted with equal volumes of phenol and diethyl ether. The DNA was then precipitated by the addition of 2 volumes of ethanol and collected by centrifugation. To effect hydrolysis, the DNA was dissolved in 10 ml 5 mM bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane-HCl (pH 7.1), 0.1 mM EDTA buffer and dialyzed overnight. Magnesium chloride (10 mM) was added, and the solution was incubated at 37° with DNase I (Sigma DN-Cl, 0.1 mg/mg DNA) for 6 hr. The pH of the hydrolysis solution was then adjusted to 8.0 by the addition of 700 μl 1 M Tris-HCl buffer (pH 8.0). Venom phosphodiesterase (Sigma type VII, 0.02 unit/mg DNA) and alkaline phosphatase (Sigma type III, 0.5 unit/mg DNA) were added, and the hydrolysis was resumed. After 6 hr, phosphodiesterase and alkaline phosphatase were again added, and the incubation was continued for an equal period of time.

Preliminary purification of the DNA hydrolysate was accomplished by passing the mixture through a Sephadex LH2O column (10 x 1.5 cm; Pharmacia, Piscataway, N. J.). Nonconjugated nucleosides, enzymes, and salts were eluted with water, and then the adducts were isolated by eluting with methanol at a flow rate of 2 ml/min. The adduct fraction was concentrated in a vacuum, filtered through a fine-finned glass filter, and chromatographed with a Waters HPLC Model 600A chromatograph, equipped with a U6K injector, a Model 440 UV detector, a Model 660 solvent programmer, and a 0.05 cm x 30 cm. The individual adducts were separated with a convex gradient (Program 2) from 20 to...
56% aqueous methanol in 30 min and subsequent linearly programming (Program 6) to 100% methanol in 2 min. A flow rate of 2 ml/min was maintained throughout the elution.

To investigate the acid-dependent covalent DNA binding of N-hydroxy-AF, 10 mg calf thymus DNA (Sigma, type I) was dissolved in 10 ml 10 mM sodium acetate, 12.5 mM sodium chloride, pH 4.6. After purging the system with argon, a methanolic solution of N-hydroxy-AF (15 mg/10 ml) was added, and the mixture was incubated overnight at 37°C. The resulting solution was extracted 2 times with an equal volume of chloroform:isooamy alcohol:phenol (24:1:25), 3 times with diethyl ether, and brought to 0.1 M in sodium chloride solution, and the AF-DNA was precipitated by the addition of the 2 volumes of ethanol. This DNA was then dissolved and hydrolyzed by the procedure outlined above.

NMR spectra were obtained on a Bruker Model WH 270 spectrophotometer in the Fourier transform-mode. UV spectra were obtained on a Beckman Model 25 spectrophotometer and field-desorption mass spectra were obtained on a Varian Model CH-5 spectrophotometer. Radioactivity was determined with a Searle Model 6893 spectrophotometer using Scintisol. pH-dependent partitioning was done by using the method of Moore and Koreeda (28). All experiments were conducted under low-UV fluorescent lights (General Electric, Gold, No. 4060).

RESULTS

AT-mediated Nucleic Acid Binding. The substrate specificity for AT-catalyzed binding of N-hydroxyfluorenylamides to nucleic acid was established by comparing the binding of the N-formyl, N-acetyl, and N-propionyl hydroxamates to exogenous tRNA using 105,000 x g cytosol fractions isolated from rat, guinea pig, monkey, baboon, pig, and human livers (see "Materials and Methods"). Two patterns of substrate activation were observed (Table 1), and these varied with different species. Rat, guinea pig, monkey, and baboon cytosols demonstrated more tRNA binding with the acetyl derivative than with propionyl and formyl variants. Human and pig liver cytosols, however, showed a much greater specificity for the formyl derivative and very little activity with acetyl and propionyl substrates. This marked contrast in activity between the latter 2 species and the others suggested that human and pig liver cytosols had a unique type of AT. Another possibility was that each of these species had at least 2 types of AT, but that the formyl-specific enzymes predominated in human liver, while the acetyl-specific enzymes were more prevalent in the other animals. In an attempt to distinguish between these possibilities, the cytosols from rat, baboon, and human liver were treated with saturated ammonium sulfate. This fractionation of the rat and baboon cytosols revealed at least 2 AT's: one precipitated with 50 to 60% saturated ammonium sulfate (Chart 1) with a high specificity for the acetyl and formyl derivatives; the other precipitated at 60 to 70% saturation with a high activity for the formyl derivative. In contrast, the human cytosol was not separated into fractions with different specificities by this procedure. Maximum activity with all 3 substrates was found in the 70% fraction.

AT-mediated DNA Adducts. The identity of the AT-catalyzed DNA-AF adduct(s) was established by using N-hydroxy-2-formylaminofluorene as the substrate for the human liver enzyme, the acetyl derivative for the baboon preparations, and the acetyl and propionyl analogs for rat liver incubations. These particular substrate-cytosol combinations were chosen because they had demonstrated maximum activity in the tRNA-binding assay. After ethanol precipitation, the DNA was enzymatically hydrolyzed to mononucleosides, and the adducts were separated as described in "Materials and Methods." Each cytosol preparation produced the same 2 major peaks of radioactivity; a typical profile is shown in Chart 3 which illustrates the result with baboon liver cytosol. With all 3 cytosols, the first peak contained 8% of the radioactivity and had a retention time identical to N-(deoxyguanosin-8-yl)-2-acetylaminofluorene. (Chart 1, Adduct II). This suggested that intermolecular N-O-acetyltransfer occurred during the incubation. Thus, if N-hydroxy-AF was the substrate, N-acetoxy-2-acetylaminofluorene would be the AT-generated reactive intermediate. [This latter compound has previously been shown to react with DNA and give Adducts II and III (6).] By analogy, N-hydroxy-2-formylaminofluorene was used in the formation of N-formyl-2-formylaminofluorene. To test this hypothesis, incubations were conducted using rat liver cytosol with [acetyl-3H]-N-hydroxy-AF as the substrate. If intermolecular acetyltransfer had occurred, the radioactive label would be retained in the product. However, when the adduct fraction was chromatographed by HPLC no radioactivity was associated with either of the adduct peaks. Furthermore, when the HPLC was monitored at 340 nm, both peaks showed appreciable UV absorbance (A268/A246 = 1.2). Since arylamidated adducts show very little UV absorbance beyond 320 nm (19, 21), we concluded that under these conditions very little, if any, intermolecular N-O-acetyltransfer was occurring, and that the minor adduct was not a N-(deoxyguanosin-8-yl)-2-acetylaminofluorene.

With all 3 cytosols, the great majority of the radioactivity migrated as a single band with a retention time of 25 min. In each of the incubations, this peak was isolated, and the pH-
Acytransferase-mediated Nucleic Acid Adduction formation under basic conditions. To obtain further evidence on the identity of the adduct, a large scale incubation was conducted with rat liver cytosol and N-hydroxy-AAF. UV spectra (pH 1, 7, 13) obtained on this AT-mediated product were the same as those reported for N-(guanosin-8-yl)-AAF (21). These UV spectral properties as well as the pH-dependent characteristics were identical to those observed from a product obtained from an acid-catalyzed reaction of N-hydroxy-AF with DNA. Since this latter compound could be obtained in greater quantities, it was characterized by conventional spectroscopic techniques.

Characterization of Acid-catalyzed N-hydroxy-AF DNA Product. N-hydroxy-AF was reacted with calf thymus DNA at pH 4.6 to yield a product with approximately 1 AF residue per 5 bases. This DNA was hydrolyzed enzymatically and subjected to LH2O chromatography as described in "Materials and Methods." HPLC analysis of the methanolic fraction from this

dependent partitioning characteristics of the adduct were established by the method of Moore and Koreeda (28). Identical results were obtained in all instances; a typical result is shown in Chart 4. The presence of both acidic and alkaline pKₐ's was consistent with a deoxyguanosine adduct (28). Moreover, increased aqueous solubility at alkaline pH ruled out N-1- or O²-products since substitution at these positions prevents anion

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column revealed only one AF product.

The identity of this product was deduced from analysis of its 270-MHz proton NMR spectrum (Chart 5). Resolution was sufficient to assign all the protons (Chart 5 and Table 2); the deoxyribose protons and the AF bridgehead protons were readily assigned by homonuclear decoupling, intensity measurements, and comparison with the parent compounds. The AF aromatic protons were assigned with the aid of homonuclear decoupling and are in complete agreement with previous NMR studies on fluorene and aminofluorene (3, 4, 24—26). However, several points deserve special mention. F1 appeared as a broad singlet (Chart 6), but decoupling unequivocally showed the presence of a small coupling to F3. This meta coupling was better revealed in F3 (Chart 6). The NOE was used to identify F8. It was anticipated that selective saturation of the bridgehead protons F9 should be accompanied by a NOE for F8 because of its relatively close position to the bridgehead protons. On the other hand, the relative distances of F7, F6, and F5 are such that they should not exhibit an appreciable NOE. Chart 6 shows the effect of saturating the bridgehead protons. The doublet experiencing the increase in intensity was assigned to F8. With this assignment, the remaining AF assignments readily followed from the homonuclear decoupling experiments. F1 also experienced an NOE. This was expected, and the result confirms its assignment. Since all the AF hydrocarbon protons were accounted for, covalent attachment must be through the AF nitrogen. The deoxyribose protons were also accounted for, which shows that the point of attachment on the DNA side of the bond must be through some part of the base. The remaining 3 resonances (4 protons) readily exchanged with D2O. Since all the naturally occurring bases have at least one hydrocarbon-type proton, the AF must be substituted at such a position. Attachment at C-8 of guanine was uniquely consistent with the data. The exchangeable protons were assigned to G(N)1 (δ = 10.59) and G(N)2a,b (δ = 6.47) protons of guanine and the arylamine proton F(N)2 (δ = 8.77) of AF. Large changes from the normal deoxyribose coupling constants were observed with this adduct. This reinforced the proposed structure, since the same effect has been observed previously in C-8 amino-substituted analogs (9, 10).

This assignment was fully supported by additional experiments. The field desorption-mass spectrum of the adduct after acetylation revealed peaks at 530 and 572, consistent with di- and triacetylated deoxyguanosine AF adducts. Finally, N-(deoxyguanosin-8-yl)-2-acetylaminofluorene can be deacetylated by treatment with weak base (0.1 N NaOH, 100°, 30 min). The product obtained from this reaction had identical HPLC, pH-dependent partition characteristics, and UV spectra as the acid-catalyzed AF adduct.
Acyltransferase-mediated Nucleic Acid Adduction

Arylaminated (i.e., AF) adducts are the major bound products found in rat liver DNA following administration of the carcinogen AAF (11, 18, 19). The structures of these adducts have not been determined, but they could conceivably arise from an AT-mediated process (see Chart 1). These experiments were designed to establish the feasibility of such a pathway by using liver cytosolic AT from a number of species and 3 different N-hydroxy-N-2-acylaminofluorenes as substrates.

A marked species variation was observed with human and pig cytosols demonstrating a distinct preference for the formyl derivative. The failure to detect appreciable activity for N-hydroxy-AAF in the human liver incubations may be a result of the manner in which the tissue was obtained. Acetyl-specific AT derived from rat liver is quite a labile enzyme, and special precautions must be taken to maintain its activity (2, 13). If such a species is present in human liver it could have readily degraded during the 4- to 18-hr postmortem period. To resolve this question, assays are currently being conducted using fresh human liver biopsy samples.

The proposed mechanism for AT involves an intramolecular acyl migration to form an acyloxyamine (5, 13). The acyl group could interact with the DNA and dictate the particular type of adduct formed. In this study, 3 different acyl groups were used as substrates, but in each instance the adducts formed were identical. Thus, at least in this limited series, the nature of the side chain does not appear to influence the kinds of adducts that will be formed.

The major DNA adduct obtained from the AT incubation was N-(deoxyguanosin-8-yl)-2-aminofluorene. This adduct was synthesized by 2 additional routes: by alkaline hydrolysis of N-(deoxyguanosin-8-yl)-2-acetylaminofluorene and by the reaction of N-hydroxy-AF with DNA under acidic (pH 4.6) conditions. The results obtained from this latter route confirm the original suggestions by Kriek (17) and by King and Phillips (16) on the acid-catalyzed formation of Adduct I (Chart 1) when reacting DNA with N-hydroxy-AF. We found this product to be quite stable, which conflicts with earlier statements on the instability of N-(guanin-8-yl)-arylamines (16, 20). The reason for this discrepancy is unknown. One reason may be that while our DNA samples were never subjected to a pH greater than 8, Kriek's (20) samples were at pH 9 during both hydrolysis and chromatography.

The formation of only one AF adduct from the acid-catalyzed reaction of N-hydroxy-AF with DNA was quite fortuitous and deserves special emphasis. Recent data obtained in this laboratory indicate that AF adducts are only slowly lost from mam liver DNA (7). Similar observations have recently been reported by Kriek and Hengeveld (20). Space-filling molecular models indicate that this AF adduct, Adduct I, could fit within the major groove of the DNA helix and, perhaps, escape detection by excision repair enzyme systems. This contrasts to the C8-AAF adduct, Adduct II, which because of the N-acetyl group causes significant perturbation of the double helix. The relative ease of synthesis of C8-AF adduct by the acid-catalyzed reaction should facilitate studies into its effect on the DNA's integrity.

In addition to N-(deoxyguanosin-8-yl)-2-aminofluorene, another adduct was found in the AT-catalyzed incubations. This product showed appreciable UV absorbance at 340 nm, which is consistent with an arylaminated adduct (21). It does not result from intermolecular acyltransfer, because when incubations were conducted with [acetyl-3H]-N-hydroxy-AAF as the substrate no radioactivity could be detected with the adduct. Experiments designed to establish the identity of this product and to determine if it occurs in vivo are currently in progress.

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