Isotope Effects on the Sequence-Specific Cleavage of DNA by Neocarzinostatin: Kinetic Partitioning between 4'- and 5'-Hydrogen Abstraction at Unique Thymidine Sites

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Abstract: In this article it is shown that in certain sequences cleavage of DNA at thymidine residues by activated neocarzinostatin occurs via a rate-limiting abstraction of either a 4'- or 5'-hydrogen from the deoxyribose moiety and that this partitioning can be modulated by deuteriation at either position. An analysis by gel electrophoresis of the *Hind*III–*Bam*HI restriction fragment (346 bp) of pBR322 revealed that in the first 45 nucleotides of the (+)-strand T_{14}, T_{17}, T_{27}, and T_{44} are strongly sensitive to deuterium substitution at C-4' ($k_H/k_D \sim 2.4$–5.5) and are less sensitive to deuteriation at C-5' ($k_H/k_D \sim 1.0$–2.6). All four of these T sites are located in GT steps. Other T residues are cleaved and exhibit variable sensitivity to 5'-deuteriation; however, they show no evidence of 4'-chemistry. The 5'-radical intermediate yields 3'-phosphate termini. The 4'-radical intermediate is demonstrated to partition between a modified abasic carbohydrate terminus and a 3'-phosphoglycolate terminus. The relative ratio of these two termini is, in turn, modulated by the structure of the thiol activator/reductant.

Introduction

The mechanistic analysis of DNA cleavers (such as, bleomycin) using specifically deuteriated ^{32}P end-labeled DNAs is proving to be a method of choice for probing the chemical events at individual sequence sites.[1] The approach combines the incorporation of nucleotides of high deuterium content into DNA with the quantitation of drug-induced damage at deuteriated sites by high-resolution gel electrophoresis to detect rate-limiting carbon–hydrogen bond cleavages in the deoxyribose backbone. An analysis of the cleavage of [4'-^{2}H]thymidine-labeled DNA by Fe^{II}·bleomycin provided the first proof of this concept.[1] The generality of the technique has been corroborated by our recent observation of isotope effects on the abstraction by neocarzinostatin (NCS).[2] This study demonstrated the power of the method for elucidating and quantitating the sequence-specific chemistry of minor damage pathways that are not easily detected by other approaches. Since NCS has been proposed to have a particularly rich and complex chemistry at certain thymidine residues which may involve both 4'- and 5'-hydrogen abstraction, we have applied our technique to probe the mechanistic subtleties of this chemistry.

NCS contains an unusual [7.3.0]dodecadiene–diyne epoxide, which upon binding to the minor groove of DNA and activation by thiol addition generates a bisradical species (NCS*).[3] The major damage by NCS* is strand scission caused by hydrogen abstraction from the 5'-position of thymidine and deoxyadenosine residues.[4] The newly generated 5'- and 3'-termini contain a nucleoside 5'-aldehyde and a 3'-phosphate, respectively (Figure 1). A recent report by Saito and co-workers has suggested that the NCS-mediated cleavage of the self-complementary hexanucleotide d(CGTACG) results in the formation of products that imply a partitioning between 4'- and 5'-hydrogen abstraction from the thymidine.[5] They failed to detect a significant amount of the phosphoglycolate terminus (<3%), a product diagnostic for 4'-chemistry in DNA cleavage by iron·bleomycin.[6] This suggested to us that our technique could provide direct evidence for 4',5' partitioning and permit a screening of a variety of DNA sequences for thymidine sites that are susceptible to this dual chemistry besides the sequence studied by Saito and co-workers.[5]

We demonstrate that in certain sequences thymidine is the site of cleavage by activated NCS via a rate-limiting abstraction of either a 4'- or a 5'-hydrogen and that this partitioning can be modulated by deuteriation at either position. Moreover, the 4'-radical intermediate also partitions between a modified abasic carbohydrate terminus and a 3'-phosphoglycolate terminus. The relative ratio of these two termini is, in turn, modulated by the structure of the thiol activator/reductant.

Experimental Section

General Procedures. Tetrahydrofuran (Baker) was distilled from lithium aluminum hydride (Aldrich) and stored over 4-Å molecular sieves (Aldrich). Formamide (U.S. Biochemicals) was distilled before use. All other commercially available chemicals and reagents were of the highest quality available and were used without further purification. [γ-^{32}P]ATP (>5000 Ci/mmol) was purchased from Amersham. B^2H_3–THF (98% ^{2}H) was obtained from Alfa Products. *Hind*III (50 units/μL) and *Bam*HI (10 units/μL) were obtained from Bethesda Research Laboratories. [4'-^{2}H]Thymidine (>95% ^{2}H) was prepared as previously described.[7] The NCS chromophore was extracted from the native proteinaceous drug according to published procedures.[8] Thin-layer chromatography was performed on 1000-μm silica gel GF plates from Analtech.

Synthesis of Thymidine 5'-Carboxylic Acid. Thymidine 5'-carboxylic acid was synthesized according to the method of Moss et al.[9] in 50% yield: mp 260–265 °C dec; (lit.[9] mp 263–265 °C dec); UV $\lambda_{max}(H_2O)$ 267 nm (lit.[9] λ_{max} 267 nm); ^{1}H NMR (D_2O/NaOD) δ 8.21 (1 H, s), 6.29 (1 H, dd, J = 8.8, 5.7 Hz), 4.49 (1 H, d, J = 5.0 Hz), 4.22 (1 H, s), 2.19 (1 H, dd, J = 5.7, 12.9 Hz), 1.96 (1 H, m, J = 12.9, 8.8, 5.0 Hz).

Synthesis of [5'-$^2H_2$]Thymidine. The reductive deuteriation of thymidine 5'-carboxylic acid was performed by a modification of the pro-

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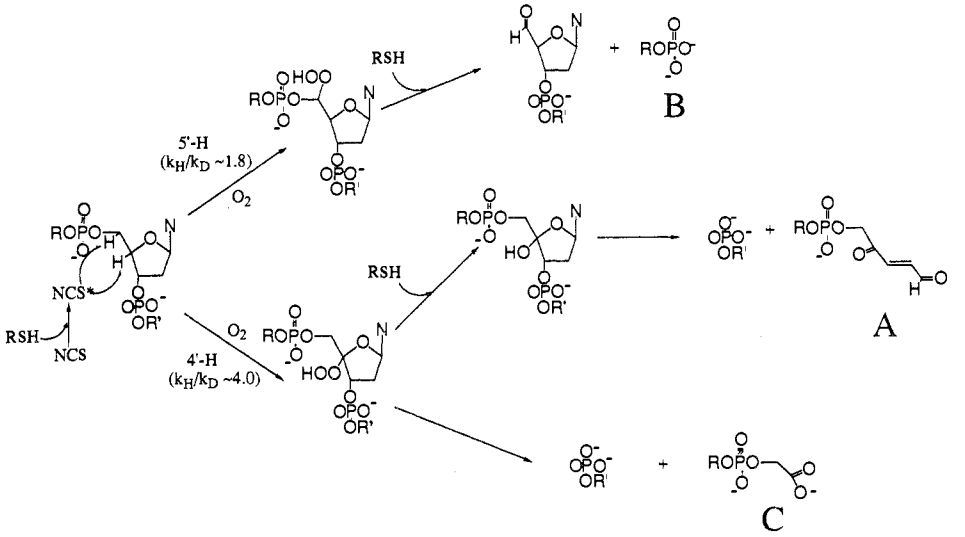

Figure 1. Proposed scheme for the cleavage at thymidine residues by NCS via partitioning between 4'- and 5'-hydrogen abstraction.

cedure of Brown and Subba Rao.[10] To a suspension of thoroughly dried (over P_2O_5) thymidine 5'-carboxylic acid (199 mg, 0.78 mmol) in anhydrous THF (20 mL) cooled to 0 °C under dry N_2 was added 0.85 M B^2H_3 in THF (4 mL, 3.4 mmol) by syringe under N_2. The reaction was allowed to slowly warm to room temperature. After 4 h the solution was again cooled to 0 °C and another aliquot of B^2H_3 was added (6 mL, 5.1 mmol). The reaction was then left 16 h at room temperature. The excess borane was quenched by the dropwise addition of H_2O and the solution was evaporated to a white powder. The powder was repeatedly dissolved in MeOH and evaporated to give a mostly clear colorless oil. Preparative TLC on 1000-μm silica gel GF eluting twice with 10% MeOH/$CHCl_3$ provided two major bands. The slower eluting band (major product) was extracted with MeOH, filtered, and evaporated to a clear oil, which could not be successfully crystallized (144 mg, 71% yield). UV data were consistent with that reported for thymidine,[11] and the ^1H NMR was identical with authentic thymidine except for a very small signal for the residual 5'-protons (~90% ^2H), and the expected simplification of the 4'-proton and the residual 5'-protons due to [5'-H,^{2}H]thymidine: UV $\lambda_{max}(H_2O)$ 267 nm; ^1H NMR (MeOH-d_4) δ 7.80 (1 H, d, J = 1.2 Hz), 6.27 (1 H, t, J = 6.8 Hz), 4.39 (1 H, m), 3.89 (1 H, d, J = 3.3 Hz), 3.78 (0.09 H, d, J = 3.3 Hz), 3.71 (0.09 H, d, J = 3.3 Hz), 2.21 (2 H, m), 1.87 (3 H, d, J = 1.2 Hz).

Synthesis of [5'-$^2H_2$]Thymidine 5'-Triphosphate. [5'-$^2H_2$]Thymidine (35 mg, 143 μmol) was converted to 3'-*O*-acetyl[5'-$^2H_2$]thymidine (10 mg) according to the method of Verheyden and Moffatt.[12] [5'-$^2H_2$]-Thymidine 5'-triphosphate was prepared from the acetylated thymidine according to the method of Ludwig and Eckstein.[13] The triphosphate was purified by chromatography on DEAE-Sephadex (1.3 × 16 cm) using a linear gradient of triethylammonium bicarbonate (0.05–1 M, 1.6 L total volume). The triphosphate was eluted at ~0.5 M salt, collected, and desalted by repeated evaporation from ethanol. The purified triphosphate was successfully incorporated in the subsequent DNA polymerization by using Sequenase (U.S. Biochemicals) to afford double-stranded DNA, as seen on a 0.8% agarose gel.

Preparation of Deuteriated DNA Fragments. The *Hind*III–*Bam*HI restriction fragment (346 bp) from pBR322 was cloned into M13mp19, and the single-stranded DNA (the (−)-strand) was isolated from infected *Escherichia coli*, JM101.[14] The deuteriated (+)-strand was prepared to form the double-stranded fragment in the following manner. The primer (−40 sequencing primer for M13, New England Biolabs; 0.2 μg, 38 pmol) in H_2O (6 μL) was phosphorylated at the 5'-end in a reaction containing 100 mM Tris (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, and 10 units of T_4 polynucleotide kinase. The reaction was incubated at 37 °C for 45 min. The single-stranded DNA (50 μg, 20 pmol) in 140 mM Tris (pH 8.0) and 140 mM NaCl was added to the kinase solution. The sample was heated at 65 °C for 10 min and then slowly cooled to room temperature over 1 h to permit annealing. The deuteriated (+)-strand was synthesized in a reaction containing 20 mM Tris (pH 7.5), 12 mM $MgCl_2$, 2 mM DTT, the annealed primer/template, 50 units of Sequenase (U.S. Biochemicals), 40 units of T_4 ligase, bovine serum albumin (BSA; 6 μg/mL), 1.2 mM ATP, 300 μM each of dATP, dGTP, dCTP, and TTP or [4'-^{2}H]TTP or [5'-$^2H_2$]TTP. The reaction was incubated at 37 °C for 2 h. An additional 160 units of ligase were added and the solution was maintained at 16 °C overnight. The double-stranded DNA (~100 μg) was extracted and purified according to published procedures[14] and diluted to 0.5 μg/μL with H_2O.

The deuteriated, double-stranded DNA (100 μg) was digested for 1 h at 37 °C with 50 units of *Hind*III in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 mM $MgCl_2$. The 5'-phosphate ends were hydrolyzed with 0.4 unit of calf intestine phosphatase (Boehringer) in 50 mM Tris (pH 9.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine at 37 °C for 1 h. The reaction was quenched by addition of 4 μL of 0.5 M EDTA and the sample was heated at 65 °C for 10 min. The linearized DNA was extracted and precipitated as before and resuspended to a concentration of 1.0 μg/μL.

5'-^{32}P end-labeling was accomplished with T_4 polynucleotide kinase. The reaction (45 μL) contained the phosphatase-treated DNA (7 μg) in 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, BSA (10 μg/μL), 20 units of T4 kinase, and 180 μCi of [γ-^{32}P]ATP. The reaction was incubated at 37 °C for 1 h and subsequently terminated by addition of 4.0 M ammonium acetate (200 μL) and cold absolute ethanol (750 μL). After 20 min in a dry ice/acetone bath the sample was precipitated, washed, and lyophilized and the pellet was resuspended in H_2O (22 μL).

The 346-bp *Hind*III–*Bam*HI fragment was released from the linear DNA by digestion with 10 units of *Bam*HI in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 100 mM NaCl at 37 °C for 1 h. The fragment was purified on a 5% disulfide cross-linked polyacrylamide gel.[15] Sequence verification was performed by the method of Maxam and Gilbert.[16]

Reaction of 5'-^{32}P End-Labeled DNA with NCS Chromophore. In a 1.5-mL Eppendorf tube, the 5'-^{32}P end-labeled restriction fragment (~180 000 cpm) containing T or a specifically deuteriated T was dissolved in a solution (43 μL) of 50 mM Tris-HCl (pH 7.5), 1 mM sodium EDTA, and sonicated salmon sperm DNA (16 μg). The solution was cooled on ice for 10 min followed by the addition of 250 μM NCS chromophore (5 μL; 19 bp of DNA/mol of NCS) and 50 mM glutathione (5 μL). The reaction mixture was maintained at 4 °C for 30 min and terminated by the addition of a "stop solution" (20 μL) containing 1 M sodium acetate and salmon sperm DNA (24 μg). The DNA was precipitated with absolute ethanol (175 μL) at −70 °C for 10 min and centrifuged at 14 000 rpm for 30 min at 4 °C. The supernatant was removed and the pellet was resuspended in 70% aqueous ethanol (1 mL), centrifuged at 14 000 rpm for 10 min, and dried under vacuum.

For alkali-treated samples, the dried pellet was dissolved in 1 M piperidine (100 μL) and heated at 90 °C for 30 min. After addition of 0.5

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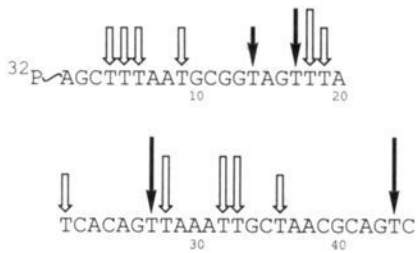

Figure 2. Histogram of T-site damage by NCS in the first 44 residues of the (+)-strand of the *Hind*III–*Bam*HI fragment of pBR322. Open arrows indicate T sites that exhibit essentially only 5′-hydrogen abstraction yielding 3′-phosphate termini (B; Figures 1 and 3). Filled arrows denote T sites that exhibit the distinctive three-band array (A–C; Figures 1 and 3) due to 4′- and 5′-hydrogen abstraction. Band B is slightly suppressed by 5′-deuteriation while bands A and C are strongly affected by 4′-deuteriation. Length of arrow approximates relative extent of damage at each site. Strand cleavage is also observed at several of the deoxyadenosine and deoxycytidine residues. Damage at these sites consists of 3′-phosphate termini (B).

M sodium acetate in 1 M acetic acid (200 μL), the DNA was precipitated with absolute ethanol (750 μL) at −70 °C for 10 min followed by centrifugation (14000 rpm) for 10 min. The pellet was resuspended, repelleted, and dried as described above.

Gel Electrophoresis of DNA Reaction Samples. The DNA pellet (untreated or alkali treated) was dissolved in 95% formamide (4 μL) containing 10 mM sodium EDTA and 0.1% bromophenol blue (pH 9.1). After being heated at 90 °C for 1 min, the sample was immediately cooled on ice and loaded onto a 20% polyacrylamide slab gel (30:1 acrylamide/*N*,*N*′-methylenebis(acrylamide)) containing 7 M urea. The gel was subjected to electrophoresis (35 mA, 2700 V) in a buffer (pH 8.3) containing 100 mM Tris, 100 mM boric acid, and 2 mM sodium EDTA at 50 °C until the bromophenol blue dye reached the bottom of the gel (~3.5 h). The gel was dried on Whatman 3 MM chromatography paper at 75 °C under vacuum for 45 min on a slab gel dryer. The dried gel was exposed to Kodak X-OMAT AR film for 40 h.

Quantitation of DNA Fragments. Peak volume quantitation was performed on a Molecular Dynamics 400A PhosphorImager. The dried gel was exposed to a 35 × 43 cm PhosphorImager screen for 16 h. The raw data were stored and quantitated by using the ImageQuant program and line graphs were prepared with Microsoft Excel. All cleavage data were normalized to the NCS-induced cleavage fragment at A_{15} of the (+)-strand of the *Hind*III–*Bam*HI fragment (346 bp) of pBR322. Cleavage at this adenosine residue is not subject to an isotope effect in these reactions. To quantitate a particular band, an image of the gel was displayed at 4× magnification. A rectangular box was drawn around a particular band and then the volume was quantitated by the ImageQuant program. The same rectangle was used to quantitate the corresponding band in each lane of the gel image. Background values were obtained as close to the analyzed band as possible in a blank area of the lane and were then subtracted from the appropriate band values to afford the corrected volumes. Isotope effects were determined by dividing the peak volume of a band in protio-DNA by the peak volume of the same band in the deuterio-DNA and correcting for differences in total counts per lane by normalizing to the A_{15} fragment.

Results and Discussion

Scope of 4′-Chemistry at T Residues. The findings of Saito and co-workers[5] prompted us to explore, in native DNA, the scope of partitioning between 4′- and 5′-chemistry that they observed for thymidine in the hexamer d(CGTACG). The *Hind*III–*Bam*HI fragment (346 bp) of pBR322 containing thymidine or [4′-^{2}H]-thymidine (95% ^{2}H) or [5′,5′-$^2H_2$]thymidine (90% ^{2}H) and 5′-^{32}P end-labeled on the (+)-strand was prepared.[1] An analysis of the cleavage of thymidine residues in a portion of this fragment with NCS is shown in Figure 2. Significant cleavage was observed for all of the 15 T sites in the 45-nucleotide region comprising the 5′-end. Four of the T sites (T_{14}, T_{17}, T_{27}, and T_{44}) exhibited pronounced product suppression when [4′-^{2}H]thymidine was present in the strand. A substantially smaller effect was observed with [5′-$^2H_2$]thymidine (vide infra). On the basis of our previous studies with bleomycin[1] and NCS,[2] this suggested the occurrence of a rate-limiting 4′-hydrogen abstraction, which is observed under the "one-hit" conditions employed. Significantly, these four T sites are found in GT steps and the 4′-deuterium isotope effect appeared to be independent of the residue located at the 3′-side of these residues. Analysis of several other DNA sequences has established that this phenomenon occurs predominantly at GT sequences, with AT steps affording substantially less 4′-chemistry (data not shown). While the lack of an observable 4′-deuterium isotope effect does not rule out 4′-chemistry at other T sites, the profile of products generated at GT sites proved to be a unique feature that distinguished the chemistry at these residues from that at other T sites.

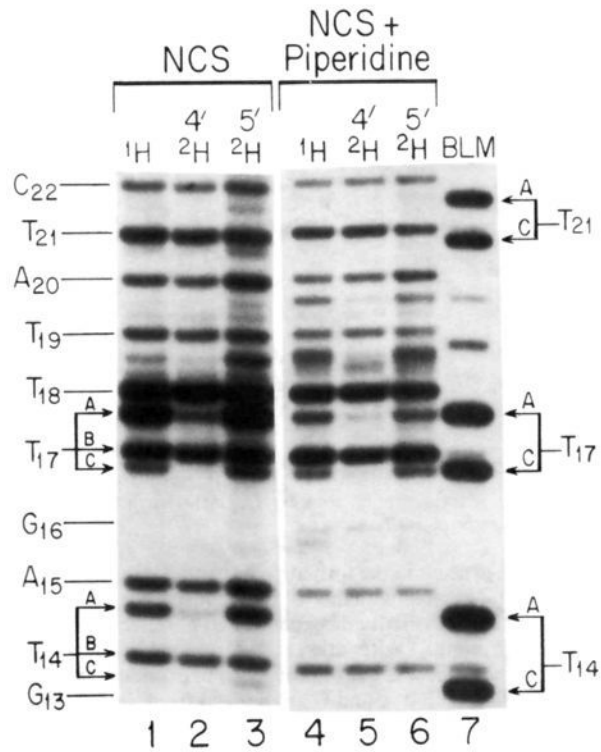

Figure 3. Deuterium isotope effects on the cleavage of the *Hind*III–*Bam*HI fragment of pBr322 by NCS at T residues. 5′-^{32}P end-labeled fragment (~180000 cpm) containing unlabeled T (lanes 1 and 4), [4′-^{2}H]T (lanes 2 and 5), or [5′,5′-$^2H_2$]T (lanes 3 and 6) was treated with 12.8 μM NCS chromophore, which was activated with glutathione as described in the Experimental Section. Lanes 1–3 were maintained under neutral conditions during the workup procedure; lanes 4–6 were treated with 1 M piperidine at 90 °C for 30 min to effect hydrolysis of alkali-labile intermediates. Lane 7 is a control reaction with iron·bleomycin and the 5′-^{32}P protio fragment that shows, as standards, both the 3′-phosphoglycolate terminus (band C) and the terminus (band A) derived by β-elimination from the 4′-hydroxy species.

Product Identification at GT Steps. Autoradiography of a high-resolution sequencing gel revealed a complex and isotopically sensitive array of bands associated with GT sites (Figure 3). For example, in the region between G_{13} and C_{22} three bands (A–C) were found to be associated with cleavage events at T_{14} and T_{17} under neutral conditions (Figure 3, lanes 1–3). Bands A and C were strongly suppressed to approximately the same extent by deuteriation at C–4′ while band B appeared less sensitive to isotopic substitution at either C-4′ or C-5′. This three-band pattern was a common feature of all GT steps analyzed and was not observed at other T sites (see T_{18}, T_{19}, and T_{21}).

The structural identification of each band was based on isotopic sensitivity, electrophoretic mobility, and literature precedent. Band B was easily assigned as corresponding to 3′-phosphate termini (Figure 1, B). This band is observed at virtually all NCS damage sites and represents the major cleavage pathway initiated by 5′-hydrogen abstraction.[3] Trapping of the 5′-radical by O_2 with subsequent thiol reduction of the 5′-hydroperoxide has been proposed to afford 5′-hydroxy intermediate. This intermediate decomposes to yield a 5′-nucleoside aldehyde-containing fragment and the observed 3′-phosphate-containing fragment B. That most

Table I. Deuterium Isotope Effects on T-Site Damage by NCS

residue	band	isotope effect[a] [4-^{2}H]T	[5′-^{2}H]T
T_{14}	A	3.5	0.9
	B	1.1	2.6
	C	2.4	0.9
T_{17}	A	4.0	0.8
	B	1.0	0.9
	C	2.7	0.8
T_{18}	B	1.1	1.0
T_{19}	B	0.9	2.1
T_{21}	B	1.0	1.6
T_{27}	A	4.1	1.1
	B	0.9	1.5
	C	5.5	1.1
T_{28}	B	1.2	2.0
T_{32}	B	1.1	1.3
T_{33}	B	1.1	1.2

[a] Determined by normalization to the A_{15} fragment (see Experimental Section). Normalization to other fragments such as A_{19} or A_{23} suggests an error of ±0.3.

cleavage sites result in the formation of band B only is consistent with the known predominance of 5′-chemistry for NCS.[3]

The isotopic sensitivity of bands A and C reflects a common mechanistic origin. Deuteriation at C-4′ resulted in a suppression of both bands that was quantitatively similar (vide infra). This clearly indicates that A and C are derived from a rate-limiting 4′-hydrogen abstraction and must partition from a common 4′-intermediate. Band C has been assigned as the 3′-phosphoglycolate terminus (Figure 1, C). Its identity is based on its electrophoretic mobility relative to the 3′-phosphoglycolate generated at the same d(GT) sites by iron·bleomycin (Figure 3, lane 7) and by an HPLC and TLC analysis of the NCS-induced cleavage of the 13-mer [5′-^{32}P]TTCTCATG*T*TTGA and of the 7-mer [5′-^{32}P]-TG*T*TTGA.[17] The amount of 3′-phosphoglycolate observed was substantially greater than that reported by Saito et al.[5] This appears to be a function of the thiol used as activator/reductant (vide infra).

The anomalously slow moving band A appears to be formed by the partial chemical decomposition of an alkali-labile lesion. Band A was converted to the corresponding 3′-phosphate terminus (band B) upon treatment with 1 M piperidine at 90 °C (lanes 4–6). In addition, band A was also observed in the iron-bleomycin reaction (lane 7), where the partitioning between pathways leading to 3′-phosphoglycolate or to an alkali-labile lesion has been well documented.[1,6] The chemical and electrophoretic properties of band A are consistent with the structure A (Figure 1), which is generated by a β-elimination of the 3′-phosphate from a ring-opened 4′-keto abasic site. The subsequent elimination of the 5′-phosphate requires more vigorous conditions. We suggest the trans geometry for A on the basis of recent studies on the chemical β-elimination of the 3′-phosphate from aldehyde abasic sites induced by sodium hydroxide and a primary amine.[18] This structural assignment is consistent with the findings of Saito et al.[5] They were able to trap the 4′-keto abasic site with hydrazine prior to β-elimination.

Band A is unusual in that it has not previously been identified as an intermediate by gel electrophoresis for either NCS or iron·bleomycin. In the case of iron·bleomycin, only 3′-phosphoglycolate termini are usually observed prior to piperidine treatment. Our preliminary investigations suggest that the formation of A is very sensitive to electrophoresis conditions, especially the pH of the loading buffer. At pH 9.1 significant amounts of A are generated while at pH 8.1 little is detected. This appears to be quite consistent with an initial, facile β-elimination under mildly alkaline conditions to give A. Studies are currently underway to elaborate this point.

Quantitation of Isotope Effects. The quantitation of isotope effects for several T sites in the fragment is shown in Table I. Bands A and C at T sites in GT steps were associated with large 4′-deuterium isotope effects (2.4–5.5). The generation of a common intermediate by 4′-hydrogen abstraction leading to both bands implies an identical isotope effect on both products. Variability in the chemical degradation of the 4′-keto abasic site leading to the formation of band A, as discussed above, is a likely explanation for the variability in the magnitude of the isotope effects between bands A and C. This variability is eliminated by quantitative conversion of the 4′-keto abasic site to its pyridazine derivative by treatment with hydrazine.[17] Thus, the results are consistent with the formation of a common 4′-radical intermediate.

In general, the isotope effects associated with 5′-hydrogen abstraction were substantially smaller, ranging from 1.0 to 2.6. Previous studies using [5′-^{3}H]T revealed isotope effects on tritium incorporation into NCS in the range of 3.2–3.5.[8,19]

Mechanistic Proposal. Figure 1 provides a reasonable explanation for the observed chemistry. Overall, the evidence suggests that the damage by NCS at GT steps is the result of a kinetic partitioning between 4′- and 5′-hydrogen abstraction. It is not clear if this partitioning occurs through a common DNA·NCS* complex. A common complex implies that total damage (4′- plus 5′-chemistry) at a GT site should remain constant upon deuteriation at either position. In analyses of DNA and oligomers, total damage does not appear to be constant, suggesting that damage at either position may occur through distinct binding modes. This point is under further investigation.

After abstraction of the 4′- and 5′-hydrogen by rate-limiting processes, the carbon-based radical is trapped by molecular oxygen to ultimately afford the corresponding peroxides.[20] The 5′-peroxide is reduced by excess thiol/reductant to the 5′-hydroxyl species, which spontaneously cleaves to yield the 5′-aldehyde terminus and the 3′-phosphate terminus (B). The formation of A and C is explainable by a partitioning of the 4′-peroxide. This species may undergo thiol reduction to the 4′-hydroxyl species and, ultimately, to the release of nucleic acid base, β-elimination, and A. In the case of bleomycin, the 4′-hydroxyl species is not formed from the 4′-peroxide but by other processes.[21] The 4′-peroxide may also undergo a competing fragmentation like that observed for iron·bleomycin leading to the formation of the 3′-phosphoglycolate terminus (C).[22]

The ability to detect phosphoglycolate, then, is necessarily linked to the efficiency of thiol reduction of the 4′-peroxide. Since the thiol is also required for NCS activation, dissecting both phenomena by changing the thiol structure may be possible. Our recent studies (data not shown) demonstrate that small, neutral thiols, such as β-mercaptoethanol and 4-hydroxythiophenol used by Saito and co-workers,[5] yield little phosphoglycolate, suggesting efficient reduction of the 4′-peroxide. Glutathione (used in this study) and 3-mercaptopropionate give, by comparison, significant amounts (>4-fold enhancement) of phosphoglycolate. This points to an accessibility of the peroxide at the DNA lesion to thiol that is sensitive to steric and electronic considerations.

Conclusions. The results demonstrate that scanning a DNA fragment for specific deuterium isotope effects on drug-induced cleavage is a useful method for dissecting out mechanistic contributions at individual sequence sites. The observation of both 4′- and 5′-hydrogen abstraction from T sites in GT steps confirms

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and extends the findings of Saito et al.[5] and suggests that this is a general property of all GT steps. Recent observations have demonstrated that the cleavage resulting from 4'-hydrogen abstraction at GT steps occurs most frequently as part of a staggered double-strand break.[23] Additional studies using oligomers to explore the effects of thiols on the partitioning between 4'- and 5'-hydrogen abstraction and on the reduction of peroxide intermediates are in progress.

Acknowledgment. This research was supported by the National Institutes of Health GM 34454 (J.W.K. and J.S.) and National Institutes of Health CA 44257 and GM 12573 (I.H.G.).

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Topographic Design of Peptide Neurotransmitters and Hormones on Stable Backbone Templates: Relation of Conformation and Dynamics to Bioactivity[1]

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Abstract: We have proposed that development of methods for controlling the side-chain topography of amino acid residues in peptides and proteins provides a new approach to the topographical design of biologically active peptides. An example of this approach is the use of the 1,2,3,4-tetrahydroisoquinolinecarboxylic acid (Tic) residue, which favors a gauche (-) side-chain conformation when in the N-terminal position, whereas in its acylated form (internal position), the most stable side-chain conformation is gauche (+). This approach has been tested by incorporating D-Tic or Tic at different positions of μ opioid receptor specific octapeptides such as D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (CTP, **1**), examination of the biological consequences of these modifications, and detailed ^1H NMR based conformational analysis. The compounds prepared and their biological activities were as follows: D-Tic-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (**2**; gauche (-), δ/μ = 7800, IC_{50} μ = 1.2 nM); Gly-D-Tic-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (**3**; gauche (+), δ/μ = 19, IC_{50} μ = 278.7 nM); and D-Phe-Cys-Tic-D-Trp-Orn-Thr-Pen-Thr-NH_2 (**4**; gauche (+), δ/μ = ~7, IC_{50} μ = 1439.0 nM). In the absence of a geminal pair of protons suitable for distance calibration, a new technique (Davis, D. G. *J. Am. Chem. Soc.* **1987**, *109*, 3471-3472) of transverse and longitudinal cross-relaxation rate measurements has been utilized in conjunction with other 2D NMR methods in order to determine the three-dimensional solution conformations for the peptides **1-4**, with subsequent application of restrained molecular dynamics (GROMOS). The average backbone conformations in peptides **1-4** were very similar, but the side-chain conformational preferences in the analogues differed, suggesting that the different affinities and selectivities for μ opioid receptors were primarily due to differences in the side-chain conformations of Tic (D-Tic), and thus due to differences in the topographies of these peptides, and not the backbone conformations. A detailed analysis of these relationships is presented.

Introduction

A dogma in molecular biology is that the biological function ("function" code) of biologically active peptides is determined by the conformations coded in their primary structure ("structure" code). For peptide neurotransmitters and hormones, transfer of their biological messages to the target cells via specific receptors requires at least two consecutive events: (1) binding of the hormone or neurotransmitter to its receptor; (2) transduction of the information from the hormone–receptor complex into the cell, leading to a biological response.[3] Since the structural, conformational, and dynamic properties of the peptide hormone and its receptor play a key role in both steps, their recognition and control are essential prerequisites to understanding the molecular basis of information transfer in these systems. In principle, the most direct approach would be the use of transferred nuclear Overhauser effect (TRNOE) studies of the neurotransmitter bound to its receptor. This method has provided insights into the conformations of small ligands bound to proteins.[4-6] Currently there are no isolated opioid receptors available. However, in principle this goal also may be pursued by examination of the conformational and dynamic properties of constrained synthetic hormone analogues carefully selected for their complementary biological properties.

Opioid peptides, owing to the multiplicity of opioid receptors, display a variety of biological actions. At least four major classes of opioid receptors have been postulated so far; μ, δ, κ, and ϵ. Recognition of the conformational and dynamic features of ligands

(1) Preliminary communication of this work was presented in part during the 10th American Peptide Symposium, May 23-28, 1987, St. Louis, MO, and the 13th International Conference on Magnetic Resonance in Biological Systems, Madison, WI, August 14-19, 1988. Symbols and abbreviations are in accord with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977). All optically active amino acids are of the L variety unless otherwise stated. Other abbreviations include the following: Tic, 1, 2, 3, 4-tetrahydroisoquinolinecarboxylic acid; Pen, penicillamine or β,β-dimethylcysteine; Orn, ornithine; TRNOE, transferred nuclear Overhauser effect; NOE, nuclear Overhauser effect; DIEA, diisopropylethylamine; *p*-MBHA, *p*-methylbenhydrylamine; TPPI, time-proportional phase increments; CTP, D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2-N^{α}-*tert*-butyloxycarbonyl; Cbz, benzyloxycarbonyl; GPI, guinea pig ileum; MVD, mouse vas deference.

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0002-7863/91/1513-2275$02.50/0