DNA Damage by Histone Radicals in Nucleosome Core Particles

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S *Supporting Information*

ABSTRACT: Although DNA binding proteins shield the genetic material from diffusible reactive oxygen species by reacting with them, the resulting protein (peroxyl) radicals can oxidize the bound DNA. To explore this possible DNA damage by protein radicals, histone H4 proteins containing an azoalkane radical precursor at defined sites were prepared. Photolysis of a nucleosome core particle containing the modified protein produces DNA damage that is consistent with selective C4′-oxidation. The nucleotide(s) damaged is highly dependent on proximity to the protein radical. These experiments provide insight into the effects of oxidative stress on protein-bound DNA, revealing an additional layer of complexity concerning nucleic acid damage.

γ-Radiolysis kills cells by damaging DNA via direct ionization (the "direct effect") of the biopolymer and through the reaction with hydroxyl radicals (HO·) formed by the ionization of water (the "indirect effect"). A great deal has been learned regarding the chemistry of DNA damage directly by studying the effects of γ-radiolysis and by independently generating the reactive intermediates formed in DNA.[1−3] Most chemical studies have been carried out on free DNA, but nuclear DNA is assembled in chromatin that is composed of monomeric nucleosome components in which ~145 bp of DNA completes ~1.6 turns around an octameric core of histone proteins.[4] Although DNA binding proteins protect the nucleic acid by absorbing the radiation energy and by scavenging HO·, the modified proteins produced can induce nucleic acid damage.[5−8] Much remains to be learned about the transfer of damage from protein radicals to DNA. We wish to report on DNA damage by protein radicals in which the reactive species are independently generated from chemically modified proteins.

Proteins and peptides that are oxidized by reactive oxygen species (e.g. HO·, $^1O_2$) can transfer this damage to bound DNA directly and by their transformation into reactive radicals.[9,10] For instance, one electron reduction of protein hydroperoxides formed by O_2 trapping of protein radicals produces reactive species such as alkoxyl radicals that react with nucleosides and DNA.[9,11] Protein radicals may also migrate from one residue to another via hydrogen atom abstraction, raising the possibility that oxidation of an amino acid that does not contact DNA may ultimately give rise to one that can.[12] Addressing these and other fundamental reactivity questions would be facilitated by a method for independently generating protein radicals at defined sites. Chemical modification of proteins with DNA damaging agents that generate reactive oxygen species or other types of reactive intermediates that are not formed naturally in proteins as a result of oxidative stress is a powerful approach for probing the biopolymers' interactions.[13−15] To our knowledge, this approach has not been used to produce the type of alkyl radical in an amino acid side chain that would be produced by reaction between HO· and a protein. Hydrogen atom abstraction from amino acid side chains by HO· is kinetically preferred over formation of the thermodynamically more stable backbone radicals resulting from α-hydrogen removal.[16,17]

To probe the reactivity of a histone-like radical with DNA in a nucleosome core particle, we synthesized modified histone H4 proteins (Scheme 1), which upon photolysis under aerobic

Scheme 1

a. 1,5-Dibromopentane, n-BuLi   b. 2, Gua·HCl, Tris (pH 8.5)
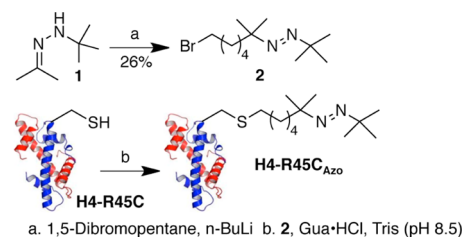
conditions form peroxyl radicals of the type expected when cells are exposed to oxidative stress. We took advantage of the absence of the amino acid cysteine in the wild type *Xenopus laevis* H4 protein to express histone H4 variants containing a single cysteine (Cys, C, e.g. the arginine 45 cysteine mutant, H4-R45C) in *E. coli*. The purified proteins were conjugated to a photolabile radical progenitor (2) containing an azoalkane ("Azo"). Facile tautomerization of azoalkanes to hydrazones under protic conditions led us to prepare an azoalkane that generates a tertiary alkyl radical.[18] Hydrogen atom abstraction from leucine, valine, and isoleucine can yield tertiary alkyl radicals. Alkylation of cysteine by 2, which was synthesized from the *tert*-butyl hydrazone of acetone (1), yields the photolabile H4 variant (e.g., H4-R45C_Azo, Scheme 1).[19] The modified protein was purified by reversed phase HPLC and characterized by mass spectrometry.[20] The alkyl chain bonded to the cysteine's sulfur needed to be longer than two carbons to prevent β-elimination of the radical. The shortest chain accessible by this synthetic method contained six carbons. The radicals generated from proteins containing the azoalkane are not exactly those produced by hydrogen atom abstraction from the native proteins. While their proximity to DNA will differ, their general reactivity (e.g. thermodynamics and bimolecular kinetics) should model the radicals generated from native histone H4.

Cysteine was introduced separately in the lysine rich amino terminus (H4−K5C), in the entry region of the octameric protein core (H4-L22C), and at a site (H4-R45C) within the core that X-ray crystallography places the modified amino acid proximal to the DNA in the minor groove approximately one-half of a helical turn in either direction from the dyad axis (superhelical locations (SHL) 0.5 and −0.5) (Figure 1).[21,22]
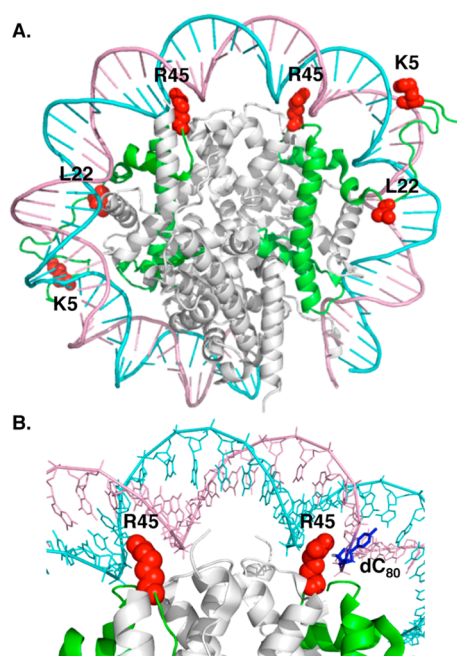

Figure 1. NCP showing positions of histone H4 modifications. (A) Complete NCP structure from PDB: 1KX5. (B) H4 Arg45 region and proximal nucleotide, dC_{80} (blue) from PDB: 3LZ0. Positions (red) of histone H4 (green) modified with photolabile radical precursor.

The HPLC purified modified histones (H4−K5C$_{Azo}$, H4-L22C$_{Azo}$, and H4-R45C$_{Azo}$) were characterized by ESI MS analysis of intact proteins and by LC/ESI-MS analysis of protease digested material.[20]

Folded octamers containing the H4 variants and wild type H2A, H2B, and H3 proteins were purified by FPLC. The viability of the azoalkane proteins as radical precursors was established by LC/ESI-MS analysis of photolyzed (350 nm) octamer containing H4-R45C$_{Azo}$ (Scheme 2, Figure 2). H4-R45C$_{Azo}$ was completely consumed upon photolysis (350 nm, 1 h) (Figure 2A). MS analysis of the intact protein could not resolve **3** and **4**. However, **3** and **4**, which are believed to arise via disproportionation of the photochemically generated radical pair (Scheme 2) were detected following thermolysin digestion of the photolyzed protein (Figure 2B).
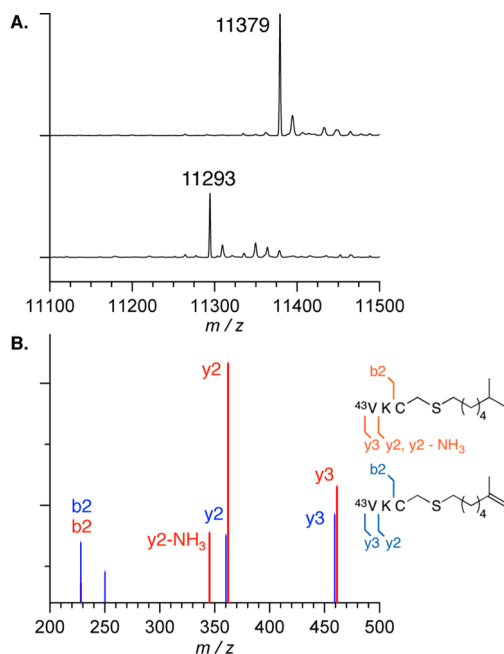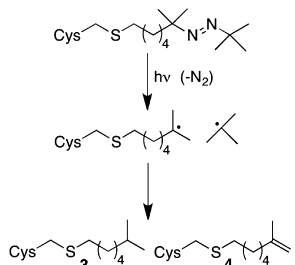
Scheme 2

Figure 2. MS analysis of photolyzed histone octamer containing H4-R45C$_{Azo}$. (A) H4-R45C$_{Azo}$ before (top) and after (bottom) photolysis. (B) Peptide fragment containing the modified cysteine from thermolysin digest of photolyzed H4-R45C$_{Azo}$.

Nucleosome core particles (NCPs) containing the histone variants were reconstituted with the 145 bp strong positioning 601 DNA sequence (**5a**; see Supporting Information for entire sequence) identified by Widom (individual strands 5′-^{32}P-labeled in separate experiments), and their homogeneity was established by HO· footprinting.[20,23] Direct strand scission, alkaline labile lesions (50 mM NaOH, 1 M piperidine, or Ir^{4+}/piperidine[24]), and DNA−protein cross-links were not detected following aerobic photolysis of NCPs containing H4-K5C$_{Azo}$ or H4-L22C$_{Azo}$, suggesting that alkyl radicals (and their respective peroxyl radicals) produced at these positions do not damage DNA.

In contrast, direct strand scission ($4.4 \pm 0.5\%$) was observed in the top strand at SHL 0.5 following aerobic photolysis of NCP containing H4-R45C$_{Azo}$. NaOH treatment increased the total cleavage to $6.8 \pm 1.3\%$ (Figure 3). However, treating the crude photolysate with $NaBH_4$ (100 mM) reduced the cleavage to $1.0 \pm 0.2\%$, suggesting that most of the observed direct strand scission was due to cleavage of abasic lesions catalyzed by the NCP during sample processing (see below and Figure 4 for additional information).[25−28] The half-lives of oxidized abasic sites in NCPs are as short as 15 min. Little if any increase in cleavage compared to NaOH treated material was detected upon piperidine or Ir^{4+}/piperidine treatment, which cleave DNA at modified nucleobases in addition to damaged sugars.[24] The effects of alkaline treatments on strand scission suggest that the protein (peroxyl) radical produces sugar damage, such as abasic lesions, but does not react with the nucleobase(s).[28] The strand damage is significantly quenched when β-mercaptoethanol (5 mM) is present during photolysis and suggests that similar reductions may occur in cells. In addition, β-mercaptoethanol (5 mM) completely eliminates strand damage when solutions are sparged with Ar prior to photolysis, suggesting that the thiol competes with O_2 for the protein and/or DNA radicals.[20] Furthermore, in contrast to previous studies in which protein
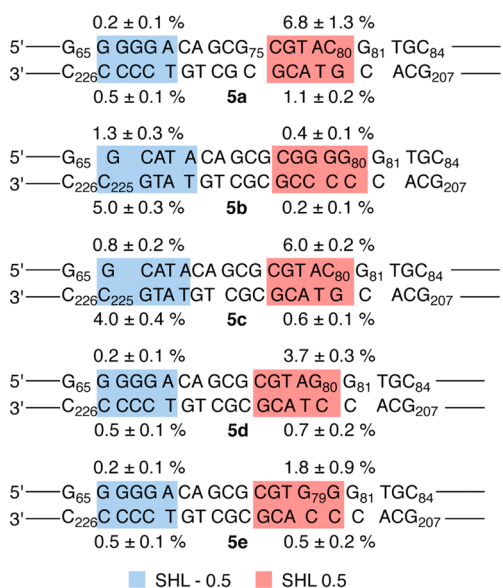
Figure 3. Sequence dependence of DNA strand scission upon photolysis of NCPs containing H4-R45C$_{Azo}$. Total strand scission following NaOH treatment in the region of SHL −0.5 and 0.5 on each strand is noted. Cleavage yields are the average ± std. dev. of at least four reactions.
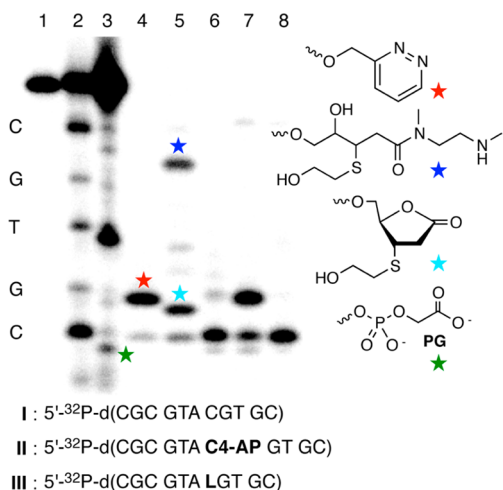

I : 5'-^{32}P-d(CGC GTA CGT GC)

II : 5'-^{32}P-d(CGC GTA **C4-AP** GT GC)

III : 5'-^{32}P-d(CGC GTA **L**GT GC)

Figure 4. Selective C4'-oxidation in photolyzed NCP containing H4-R45C$_{Azo}$ and **5a** (5'-^{32}P-G$_{75}$). 3'-Terminal damage was visualized in the NCP following digestion with Hinp1l. Lane: 1, **I**; 2, **I** + HO·; 3, **I** + bleomycin; 4, **II** + hydrazine; 5, **III** + DMEDA, BME; 6, NCP (*hν*); 7, NCP (*hν*) + hydrazine; 8, NCP (*hν*) + DMEDA, BME.

hydroperoxides were reduced, DNA−protein cross-links were not detected.[7] Strand damage was selective for the top strand at SHL 0.5. In addition, less than 1% damage was detected in either strand at SHL −0.5 where the other copy of H4-R45C$_{Azo}$ in the NCP should interact with the DNA (more below).
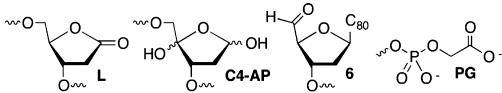

The damage at SHL 0.5 was examined in more detail by introducing the ^{32}P-label at the 5'-phosphate of dG$_{75}$ in **5a** (Figure 4). Digesting the photolysate with HinP1I provided DNA fragments (8 bp) that were readily resolved using 20% denaturing PAGE.[20] The higher resolution gel revealed that

~90% of the damage occurred at dC$_{80}$ and the remaining 10% at the adjacent dG$_{81}$. Cleavage dependence on NaOH was suggestive of abasic lesion formation.[28] A combination of reactions that selectively identify 2-deoxyribonolactone (L) or the C4'-oxidized abasic site (C4-AP) and independently synthesized oligonucleotides containing these lesions were used to determine the products in the damaged DNA.[28−30] For instance, L undergoes selective reactions with *N,N'*-dimethylethylene diamine (DMEDA) and *β*-mercaptoethanol (BME) (Figure 4, lane 5).[29] However, these diagnostic products were not observed following subjection of photolyzed H4-R45C$_{Azo}$ containing **5a** to these reactants (Figure 4, lane 8). In contrast, reaction of the photolyzed NCP with hydrazine (Figure 4, lane 7), a reagent known to react with C4-AP and yield cleaved DNA containing a 3'-terminal pyridazine (Figure 4, lane 4), indicated that this oxidized abasic lesion is the major product.[30] In addition, comparison of bleomycin treated DNA (Figure 4, lane 3) and photolyzed NCP (Figure 4, lane 6) indicated that a minor amount of 3'-phosphoglycolate (PG) was formed. PG is also indicative of C4'-hydrogen atom abstraction. DNA fragments containing 3'-terminal phosphates were the major product when photolyzed NCP was not subjected to any additional treatment. This is consistent with the facile cleavage induced at C4-AP by histone proteins.[27]

The selective formation of C4'-oxidation products at mostly a single nucleotide is consistent with DNA oxidation by a nondiffusible reactive species positioned near the minor groove. The C5'-position is also accessible from the minor groove. However, ^{32}P-labeling of T$_{86}$ in **5a** and digesting the photolyzed NCP DNA with MseI provided no evidence for the diagnostic 5'-aldehyde (**6**).[20,31,32] A fragment containing a 5'-phosphate at dG$_{81}$ was the sole 5'-terminal end product and, presumably, resulted from *β*-elimination at C4'-oxidized dC$_{80}$, which could be catalyzed by the histone proteins in the NCP.[27] Overall, these experiments suggest that the protein (peroxyl) radical (e.g., **7**, Scheme 3) selectively abstracts the C4'-hydrogen atom(s) of the

Scheme 3
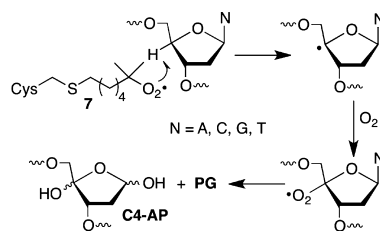
proximal nucleotide. The DNA radical reacts in the presence of O$_2$ to yield C4-AP as the major product with a minor amount of 3'-phosphoglycolate (PG).[33]

The sequence dependence on strand damage was examined by first exchanging the sequences at SHL −0.5 and 0.5 (**5b**, Figure 3), which reduced damage at SHL 0.5 in **5b** to <1%. In contrast, the damage at SHL −0.5 in **5b** (dC$_{225}$C$_{226}$ = 1:1) was comparable to the corresponding sequence at SHL 0.5 in **5a**, suggesting that the DNA sequence, and not the environments within the NCP, is the source of the difference in reactivity. Similar levels of strand damage were detected at SHL −0.5 and 0.5 in **5c**, providing additional support that the nucleic acid sequence/structure is determining strand damage. Introducing a 5'-dG$_{80}$G$_{81}$ (dG$_{80}$G$_{81}$ = 68:32) sequence at SHL 0.5 (**5d**) slightly reduced the total damage detected compared to that in **5a**. However, introducing a

third consecutive dG at SHL 0.5 ($dG_{79}GG_{81}$ in **5e**) reduced the observable damage to <2%. Treatment with Ir^{4+}/piperidine[24] revealed that nucleobase damage does not replace sugar oxidation in sequences containing three or more consecutive dG's. Finally, hydroxyl radical cleavage (using Fe·EDTA) of either free 5′-^{32}P-**5a** or NCP containing this DNA does not reveal any inherent loss in reactivity at the poly·dG sequence.[20] These data suggest that the radical (e.g. **7**) is less able to abstract hydrogen atoms from dG stretches. dG rich stretches of DNA are known to favor narrow and wide major and minor grooves, respectively.[34] However, the X-ray crystal structure of the NCP containing **5a** indicates that the minor groove of $G_{65}-G_{69}$ is only ~1 Å wider than that in the region of $A_{79}-G_{81}$.[20] Why contiguous dG's at either SHL −0.5 or 0.5 (e.g., **5a** and **5b**) reduce the protein (peroxyl) radical's ability to damage DNA warrants further investigation.

We believe that the proximity between the protein (peroxyl) radicals and DNA also explains why strand damage is observed when R45 is modified but not when either K5 or L22 is. The radical produced upon photolysis of H4−K5C$_{Azo}$ is not constrained close to the DNA due to its presence in the conformationally unrestricted tail region. The lack of DNA damage by H4−K5C$_{Azo}$ also indicates that protein radicals do not migrate from one amino acid to another under these conditions.[12] Examination of the NCP X-ray crystal structure containing **5a** indicates that L22 is further from the DNA than is R45 and is situated closer to the major groove.[20]

Overall, these experiments demonstrate that a protein-like radical in an NCP, whose formation initially protects DNA from oxidative stress, ultimately damages the nucleic acid in an O_2 dependent manner. Proximity plays a large role in the ability of a protein radical to damage DNA. This type of chemistry is not limited to nucleosomes and can be expected to occur with a variety of DNA binding proteins. Independent generation of alkyl radicals in proteins will greatly facilitate studies in other DNA−protein systems.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Procedures for all experiments. Complete sequences of all DNAs used to prepare nucleosome core particles. Representative autoradiograms of HO· cleavage, strand damage in NCPs, and 5′-end group analysis. Mass spectra of modified histone H4 proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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