Oxidatively Induced DNA−Protein Cross-Linking between Single-Stranded Binding Protein and Oligodeoxynucleotides Containing 8-Oxo-7,8-dihydro-2′-deoxyguanosine†

Mark E. Johansen, James G. Muller, Xiaoyun Xu, and Cynthia J. Burrows*

Department of Chemistry, University of Utah, 315 S. 1400 East, Salt Lake City, Utah 84112-0850

Received November 17, 2004; Revised Manuscript Received February 4, 2005

ABSTRACT: The formation of covalent cross-links between amino acid side chains and DNA bases in DNA−protein complexes is a significant pathway in oxidative damage to the genome, yet much remains to be learned about their chemical structures and mechanisms of formation. In the present study, DNA−protein cross-links (DPCs) were formed between synthetic oligodeoxynucleotides containing an 8-oxo-7,8-dihydro-2′deoxyguanosine (OG) or an 8-oxo-7,8-dihydro-2′-deoxyadenosine (OA) nucleotide and *Escherichia coli* singled-stranded binding protein (SSB) under oxidative conditions. Studies with various sequences indicated that DNA homopolymers and those lacking 8-oxopurines were less reactive toward DPC formation. DPCs were formed in the presence of HOCl, peroxynitrite, and the one-electron oxidants Na_2IrCl_6, Na_2IrBr_6, and $Na_3Fe(CN)_6$. Protein−protein cross-linking was also observed, particularly for oxidants of high reduction potential such as Na_2IrCl_6. The adducted oligodeoxynucleotides were sensitive to hot piperidine treatment leading to strand scission at the site of cross-linking. In addition, the covalent cross-links were somewhat heat and acid labile, which may be related to the difficulties encountered in obtaining complete characterization of trypsin digests of the DPCs. However, model reactions involving the single amino acids lysine, arginine, and tyrosine, residues known to be involved in base contacts in the DNA:SSB complex, could be studied, and the adduct formed between N^{α}-acetyllysine methyl ester and an 18-mer containing OG was tentatively characterized by electrospray ionization mass spectrometry as analogues of spiroiminodihydantoin and guanidinohydantoin. A mechanism involving nucleophilic attack of an amino acid side chain (e.g. the ϵ-amino group of lysine) at C5 of a 2-electron oxidized form of OG is proposed.

Reactive oxygen species responsible for oxidative DNA damage are produced as a result of tissue inflammation (*1*), exposure to environmental oxidants (*2*), ionizing radiation (*3*, *4*), and normal metabolic processes. Oxidation of DNA results in direct strand breaks, modified bases, and DNA−protein cross-links (*5*), all of which have been implicated in cancer, aging, immune system decline, cardiovascular disease, and neurological disorders (*6*−*9*). Structures and mechanisms pertinent to DNA strand breaks and base oxidation have been investigated in considerable detail in recent years (*3*, *8*); however, much less is known about the chemistry and biochemistry of DNA−protein cross-links (DPCs).[1] DNA is surrounded by and tightly associated with a variety of proteins that range in function from structural organization of the genome to the control of cellular processes. Oxidation of either DNA or its associated proteins results in the formation of electron-deficient species that may be subject to attack by nucleophilic residues on a bound partner leading to covalent cross-links. Background levels of DPCs in human white blood cells range from 0.5 to 4.5 per 10^7 bases (*10*), and DPCs were observed to accumulate on an age-related basis in mouse organs (*11*) and to be induced by exposure to metal ions such as iron and copper (*12*, *13*). Thus, DPC formation is an important and relatively abundant form of DNA damage. Despite the biological significance of DPCs, much remains to be learned about the structure and mechanism of formation of the cross-links.

In the studies reported so far, DPC formation is observed in a wide range of reaction conditions (e.g. aerobic vs anaerobic) using a variety of oxidants, and so a diversity of structures and mechanisms is anticipated. Several groups have studied the formation of hydroxyl radical-induced cross-links between tyrosine or lysine with thymine or cytosine (*14*−*19*). Many of these studies and others have concluded that protein oxidation occurs first in DPC formation resulting from radical formation at an amino acid residue with subsequent bond formation to the nucleobase (*20*−*25*). In contrast, an example of an oxidation of a nucleobase that resulted in a DPC was shown by Stemp and co-workers when guanine bases in DNA were specifically photooxidized with a DNA bound intercalator Ru(1,10-phenanthroline)$_2$dipyridophenazine^{+3} in the presence of histones or other proteins (*26*, *27*). Morin and Cadet observed formation of a covalent

† This work was supported by a grant from the National Science Foundation, 0137716, to C.J.B.

* To whom correspondence should be addressed. Tel: (801) 585-7290. Fax: (801) 585-0024. E-mail: burrows@chem.utah.edu.

[1] Abbreviations: AcArgOMe, N^{α}-acetylarginine methyl ester; AcLysOMe, N^{α}-acetyllysine methyl ester; AcTyrOMe, N^{α}-acetyltyrosine methyl ester; DPC, DNA−protein cross-link; ESI-MS, electrospray ionization mass spectrometry; Gh, 5-guanidinohydantoin; NaP_i, $NaH_2PO_4 + Na_2HPO_4$; OA, 8-oxo-7,8-dihydro-2′deoxyadenosine; OG, 8-oxo-7,8-dihydro-2′deoxyguanosine; SSB, single-stranded binding protein; Sp, spiroiminodihydantoin (or 2-imino-5,5′-spirodihydantoin).

10.1021/bi047580n CCC: $30.25
Published on Web 03/25/2005

adduct between guanine's C8 and the α-amino group of lysine that could be attributed to initial generation of a guanine radical followed by nucleophilic attack of lysine (*28*−*30*). However, structural identification of DNA−protein cross-links may be complicated by the fact that the redox potentials for amino acid side chain oxidation vs nucleobase oxidation are similar, generating competing pathways of adduction to amino acid vs base radicals. Furthermore, peptide-to-DNA electron transfer has been demonstrated for intercalated tyrosines in which a tyrosine radical was responsible for adduct formation although the initial radical formation was on a guanine base (*31*). In another example, DNA strand scission has resulted from initial formation of peptide hydroperoxides (*32*). Thus, the question of which partner, protein radical or DNA radical, is responsible for covalent bond formation can be a complex issue.

Our laboratory has recently focused on DNA−protein cross-links formed via further oxidation of the oxidized base lesion 8-oxo-7,8-dihydro-2′-deoxyguanosine (OG) (*33*). Although guanine is more readily oxidized than other bases native to DNA, its common oxidation product OG is orders of magnitude more sensitive to oxidation (*34*, *35*). When oxidation of an OG-containing DNA oligomer was triggered by Ir(IV) as a one-electron oxidant, an electron-deficient center on DNA was the clear precursor to adduct formation with a bound protein, the repair enzyme *Escherichia coli* MutY which preferentially binds duplexes containing the OG:A mispair. OG was shown to be the site of cross-linking on DNA, and lysine 142, an active site residue involved in binding to the OG:A mispair of the DNA substrate, was implicated as the nucleophile attacking the oxidized form of OG (*33*). Repeated attempts to characterize the MutY-OG DPC by mass spectrometry were unsuccessful likely due to iron−sulfur cluster oxidation and subsequent multiple oxidative events occurring in the protein (*36*). In the present work, we turned to *E. coli* single-stranded binding protein (SSB) because of its versatility in binding any sequence of single-stranded DNA and the lack of redox-active cofactors.

E. coli SSB is a 19 kD protein containing 177 amino acids that binds DNA nonselectively (*37*, *38*). The protein forms a homo-tetramer in the presence and absence of DNA that is stable over a wide range of conditions (*37*, *39*−*42*). SSB is thought to play an essential role as an accessory protein in DNA replication, recombination, and repair (*43*). Most organisms, including humans, have been found to possess SSBs, and they share extensive sequence similarities with *E. coli* SSB and appear to bind in a manner similar to ssDNA.

Several reports of cross-linking of SSB to DNA have appeared, some of which have also identified the site of the cross-link. Steen et al. (*44*) characterized a UV-induced (312 nm) cross-link of a DNA oligomer containing a 5-iodouracil to a SSB protein. The method used included enzymatic digestion of the protein and oligodeoxynucleotide and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry to identify the tryptophans 54 and 88 as the sites of the cross-linked residues. Merrill et al. (*45*) UV-cross-linked SSB to polythymidine oligodeoxynucleotides and isolated a tryptic peptide (amino acids 57−62) that contained the DPC. It was observed through peptide sequencing that phenylalanine 60 was the site of the cross-link. Sastry et al. (*46*) utilized a photoactive bis-alkylating molecule to create the DPC between SSB and DNA, but this appeared to cross-link at multiple sites on the protein and none were characterized. Most relevant to the present work is a study by Kulcharyk and Heinecke who cross-linked SSB to poly (dA), poly(dT), and poly(dC) using hypochlorous acid (*47*). Poly-(dG) could not be studied because it is not single stranded and forms higher order structures. In the presence of HOCl, it is likely that electrophilic sites on the protein were formed, and indeed recent studies by Davies and co-workers provided evidence for protein oxidation with HOCl as the precursor to DPC formation in nucleosomes (*23*).

	Sequence #
5′TCATGGGTC(**OG**)TCGGTATA3′	1
5′TCATGGGTCGTCGGTATA3′	2
5′TCATGGGTC(**OG**)TCGGTATATCAGTGCTATCACATTAGTGTA3′	1′
5′TCATGGGTCGTCGGTATATCAGTGCTATCACATTAGTGTA3′	2′
5′AAAAAAAAAGAAAAAAAA3′	3
5′AAAAAAAAA(**OG**)AAAAAAAA3′	4
5′AAAAAAAAA(**OA**)AAAAAAAA3′	5
5′AAAAAAAAAAAAAAAAA3′	6
5′CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC3′	7
5′CCCC(**OG**)CCCCCCCCCCCCCCCCCCCCCCCCCCCCC3′	8
5′CCCCCCCCCCC(**OG**)CCCCCCCCCCCCCCCCCCCCC3′	9
5′CCCCCCCCCCCCCCCCCC(**OG**)CCCCCCCCCCCCCC3′	10
5′CCCCCCCCCCCCCCCCCCCCCCCCCCCC(**OG**)CCCCC3′	11

FIGURE 1: Single-stranded DNA sequences used in oxidative cross-linking to SSB. Oligomers **1**−**6** are 18-mers, **1′** and **2′** are 40-mers containing the sequences of **1** and **2**, and **7**−**11** are variants of $(dC)_{35}$.

In the present study, *E. coli* SSB was cross-linked to an oligodeoxynucleotide containing an OG or an OA residue, as well as to native dG-containing oligodeoxynucleotides, using oxidants that will primarily oxidize the oxopurine base allowing for a subsequent nucleophilic attack by a site on the protein to form a covalent cross-link. The DPC was isolated and analyzed via several methods in order to characterize the cross-link and explore a mechanism of formation.

EXPERIMENTAL PROCEDURES

Materials. Na_2IrCl_6, Na_2IrBr_6, and $Na_3Fe(CN)_6$ were purchased from Alfa Aesar (Ward Hill, MA), and phosphoramidites were purchased from Glen Research (Sterling, VA). [γ-^{32}P]-ATP was obtained from Pharmacia-Amersham (Arlington Heights, IL), *E. coli* SSB from Promega (Madison, WI), and T4 polynucleotide kinase from New England Biolabs (Beverly, MA). Peroxynitrite was prepared as follows: a 25-mL volume of 1 M HCl was added to an ice-cold 50-mL solution of 50 mM sodium nitrite and 50 mM H_2O_2 while being stirred rapidly in a 250-mL beaker followed immediately by 25 mL of 1.5 M NaOH to quench the reaction. The concentration was determined by UV spectroscopy at 302 nM ($\epsilon = 1670\ M^{-1}\ cm^{-1}$), and the solution was used immediately after preparation.

Synthesis of Oligodeoxynucleotides. Oligodeoxynucleotides (Figure 1) were synthesized with an Applied Biosystems DNA/RNA synthesizer (ABI 392 B). Oligodeoxynucleotides containing OG or OA were deprotected manually by placing approximately 3 mL of a mixture of fresh NH_4OH and 1.75% β-mercaptoethanol into a 10 mL syringe and attaching it to the column containing the oligodeoxynucleotide on the solid support. It was then connected to a second 10-mL syringe at the other end of the column, and the solution was passed

through the column several times to get rid of air bubbles in the column. This was allowed to stand overnight at room temperature. The column was then incubated for 17 h at 55 °C. The oligodeoxynucleotides were lyophilized to dryness and stored in 6 M urea. Purification of DNA was performed by gel electrophoresis at 45 W using a 1.6 mm, 20%, 19:1 cross-linked polyacrylamide gel on a Gibco SA gel apparatus. Oligodeoxynucleotides were visualized by UV-shadowing and excised. These were then extracted from the gel by the crush and soak method. Gels were crushed and placed in a 10 mL syringe with ~3 mL of H_2O or a Costar Spin-X centrifuge tube filter. This was shaken at 4 °C overnight. The supernatant containing the oligodeoxynucleotides was then filtered from the syringe or centrifuged to remove solid gel in the case of the centrifuge tube filter. Supernatant was then placed into a microcentrifuge tube and dried in a Savant Speed-Vac Plus (SC110A). Samples were then desalted using a NAP-25 column containing Sephadex G-25 medium (Pharmacia Biotech). UV absorbance (Beckman spectrophotometer (DU 650)) was used to quantify DNA, and extinction coefficients were calculated using software provided with the Beckman spectrophotometer. Labeling was performed using 30 μCi of [γ-^{32}P]-ATP and 30 units of T4 polynucleotide kinase in the presence of 10 pmol of oligodeoxynucleotide for 30 min at 37 °C. Unreacted [γ-^{32}P]-ATP was separated from 5′-end labeled oligodeoxynucleotides using a MicroSpin G-25 column (Pharmacia-Amersham).

DNA−Protein Cross-Linking Reactions. E. coli single-stranded binding protein (SSB) was cross-linked to single-stranded oligodeoxynucleotides according to the following procedure. Two microliters of 200 nM 5′ ^{32}P-labeled oligodeoxynucleotide (40 nM final concentration) were added to 1 μL of 10x buffer (100 mM NaP_i (pH 7.0), 1.0 M NaCl), 1 μL of H_2O, and 5 μL of 1.2 μM SSB (diluted with 20 mM Tris-HCl (pH 7.5), 20% glycerol, at a final concentration of 600 nM) and allowed to incubate at room temperature for 15 min. Next, 1 μL of 1.0 mM Na_2IrCl_6, Na_2IrBr_6, or $Na_3Fe(CN)_6$ was added to initiate the cross-linking, and the solution was allowed to incubate for 15 min at room temperature. The reaction was quenched with 2 μL of 0.1 M EDTA (pH 8.0). Thirteen microliters of 2x SDS loading buffer (100 mM Tris (pH 6.8), 200 mM DTT (added at time of use), 4% SDS, 0.2% BB dye, and 20% glycerol) was then added to the solution. These samples were heated for 3 min at 90 °C, followed by cooling for 5 min at 20 °C. From each sample, 13 μL was placed in a well of a 15% 29:1 cross-link SDS polyacrylamide gel and electrophoresed at 250 V for approximately 30 min. The gels were exposed to a storage phosphor autoradiography screen overnight and visualized using a Molecular Dynamics Storm 840 PhosphorImager and later quantified using ImageQuant software.

Piperidine Treatment of SSB−DNA Cross-Link. SSB was cross-linked to DNA as described above and run on an SDS−PAGE gel. Cross-linked bands were excised from the gel. DPCs were then purified and desalted using the same method as described above. Sixty microliters of fresh 0.2 M piperidine was added to 10 μL of DPC (~60 μM) and heated to 90 °C for 30 min. An equal amount of H_2O was added to another 10-μL DPC sample and treated identically as a control. After 30 min the sample volume was reduced by vacuum and loaded onto a 15% 19:1 polyacrylamide gel with 7 M urea and analyzed by storage phosphor autoradiography.

Protease Digestion. A cross-link between SSB and DNA was formed and isolated as described above using [^{32}P]-5′-end-labeled DNA as a gel standard to locate unlabeled cross-link. Purified DPC was then taken up into 25 mM NH_4HCO_3 (pH 8), and an equal amount of trypsin (by weight, Promega) was added and incubated at 37 °C while shaken (~350 rpm) for 2−18 h. The digested DPC was then loaded onto a 15% polyacrylamide gel and run at 45 W for 1.5 h. Digested DPC bands were isolated and desalted as described above. Extra desalting steps included dialysis using 1 or 2 kDa molecular weight cutoff tubing into water for >18 h.

Model Studies with Amino Acids. A 2-μL aliquot of 200 nM 5′ ^{32}P labeled oligodeoxynucleotide (**1**) was added to 1 μL of 10x buffer (100 mM NaP_i (pH 7.2), 1.0 M NaCl) and 5 μL of 4 μM, 40 μM, 400 μM, 4 mM, 40 mM, or 200 mM aqueous α-amino and carboxy-terminal protected amino acids (AcLysOMe, AcArgOMe, or AcTyrOMe), and the mixture was allowed to incubate at room temperature for 15 min. Next, 2 μL of 0.5 mM Na_2IrCl_6 was added, and the existing solution was allowed to incubate for an additional 15 min at room temperature. The reaction was then quenched with 2 μL of 100 mM EDTA (pH 8) and dried under vacuum for 15 min. The resulting solid was dissolved in 12 μL of loading buffer (6 M urea). For each sample, a 4-μL aliquot was placed in a well of a 15% 19:1 polyacrylamide gel and electrophoresed at 45 W for 3.5 h. After electrophoresis gels were visualized by storage phosphor autoradiography with use of an Amersham Typhoon 9400 phosphorimager.

Preparation of Adducts for Electrospray Mass Spectrometry. One milliliter of 5 μM oligodeoxynucleotide (*1*) in 10 mM NaP_i (pH 7.5) and 100 mM NaCl was incubated with 28 mg AcLysOMe at room temperature for 30 min. To the existing solution were added 2 μL of 25 mM Na_2IrCl_6, and the subsequent mixture was incubated at room temperature for an additional 30 min. The reaction was then quenched with 2 μL of 100 mM EDTA (pH 8) and dried under vacuum for 8 h. Samples were then desalted using 2 kDa MW cutoff tubing into water for 8 h. DNA for MS analysis was prepared by adding 5 M ammonium acetate to give a final DNA concentration of 50 μM. The sample was incubated for 2 h at room temperature. Ethanol/isopropanol (50:50, v/v) was added (3 × volume), and the sample placed in a −80 °C freezer overnight. This was then centrifuged, and the supernatant was removed, allowing the pellet to dry. The DNA pellet was dissolved into 1 mM ammonium acetate/2-propanol (50:50, v/v) and submitted for mass spectrometry. DNA samples were analyzed by negative electrospray ionization (ESI) on Micromass Quattro II tandem mass spectrometer equipped with Zspray API source. Samples were introduced via infusion at a flow rate of 5 μL/min. The source and desolvation temperatures were 80 °C and 120 °C, respectively. The capillary voltage was set to 3.1 kV, sampling cone voltage to 58 V, and the extractor cone to 3V. The instrument was operated and data accumulated with Micromass Masslynx software (version 3.2).

RESULTS

Formation of DNA−Protein Cross-Links

Sequences of oligodeoxynucleotides used in cross-link formation with SSB are shown in Figure 1. Oligomer **1** is an 18-mer containing one OG residue in proximity to GG

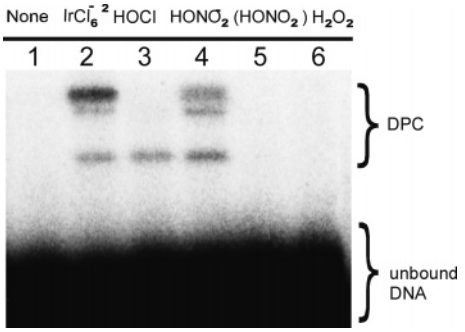


FIGURE 2: DPC formation with oxidants peroxynitrite and hypochlorous acid. All lanes were 30 nM oligomer **1** (5′-end labeled) and 461 nM SSB. Lane 1: no oxidant. Lane 2: $IrCl_6^{-2}$ (1.4 μM). Lane 3: HOCl (100 μM). Lane 4: peroxynitrite, freshly prepared (100 μM). Lane 5: peroxynitrite (100 μM) degraded by incubation at 37 °C for 30 min. Lane 6: H_2O_2 (100 μM). All reactions were conducted at pH 7.0, 10 mM NaPi, 100 mM NaCl, and were quenched with 2 μL of EDTA (0.1 M, pH 8).

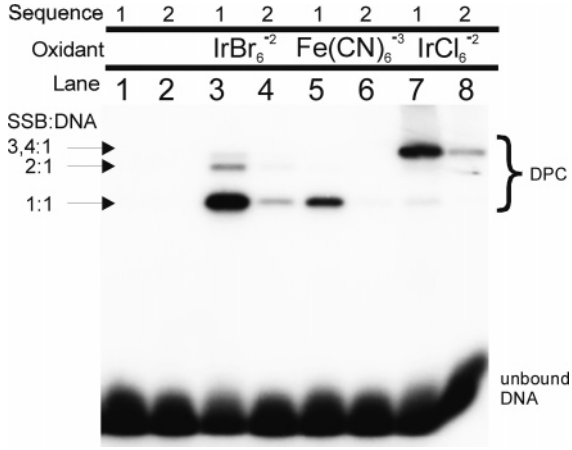
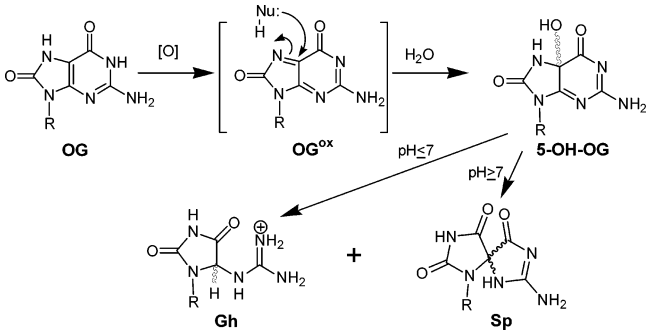


FIGURE 3: DPC formation with oxidants $IrCl_6^{-2}$, $IrBr_6^{-2}$, and $Fe(CN)_6^{-3}$. All ssDNA was 5′ end labeled. Lanes 1, 3, 5, and 7 contained oligomer **1**. Lanes 2, 4, 6, and 8 contained oligomer **2**. Lanes 1−2 did not have oxidant. Lanes 3−4 contained $IrBr_6^{-2}$. Lanes 5−6 contained $Fe(CN)_6^{-3}$. Lanes 7−8 contained $IrCl_6^{-2}$. All reactions were 36 nM ssDNA, 545 nM SSB, and 90 μM oxidant. All reactions were conducted at pH 7.0, 10 mM NaPi, 100 mM NaCl, and were quenched with 2 μL of EDTA (0.1 M, pH 8).

Scheme 1



and GGG sequences in the same strand that might act as competing sites for oxidation (*48*). Oligomer **2**, containing only native nucleotides with a dG in place of the dOG nucleotide, was used for comparison. Oligomers of 35−40 nucleotides in length are known to form stable complexes with SSB with one DNA strand binding to two subunits of the SSB tetramer under the conditions used (*49*−*51*), and so sequences **1′** and **2′** were designed as 40-mer analogues of **1** and **2** to test the role of sequence length on the ability to form cross-links. Because poly(dA) was thought to be the least reactive sequence, a series was also designed in which a single dG, dOG, or dOA residue was placed in the center of an 18-mer poly(dA) sequence providing oligomers **3**−**6**. Additionally, poly(dC) oligomers were investigated in order to provide a correlation to the crystal structure of SSB bound to $(dC)_{35}$ (*52*). An OG scanning experiment was performed in which OG was placed at 4 different sites along the oligomer, at positions 5, 12, 20, and 30 (oligomers **7**−**11**).

Cross-Linking Oxidants. DNA and protein were incubated for 15 min at room temperature in 10 mM sodium phosphate buffer (pH 7) containing 100 mM NaCl in order to form the DNA−protein complex. Oxidant was then added to initiate formation of the cross-link. The initial oxidants screened included HOCl and H_2O_2 for comparison to published work (*47*), as well as Na_2IrCl_6 and peroxynitrite, oxidants that are known to be highly reactive with OG. The formation of DPCs was readily apparent with some of these oxidants as shown in Figure 2. Na_2IrCl_6 is an outer-sphere one-electron oxidant whose redox potential at 0.9 V vs NHE is well matched to oxidation of OG and OA bases (0.7 and 0.9 V vs NHE (*34*)), and the products arising from OG oxidation using 2 equiv of this oxidant have been identified as the hydantoins Sp and Gh (Scheme 1) (*53*, *54*). Figure 2, lane 2 shows formation of high molecular weight adducts to the radiolabeled oligomer **1** using this Ir(IV) oxidant. Multiple bands are observed indicating cross-linking both between DNA and protein as well as between DNA and protein−protein cross-links (vide infra). Na_2IrCl_6 at 1.4 μM was considerably more effective than 100 μM HOCl (Figure 2, lane 3) at generating DPCs. Peroxynitrite, freshly generated from H_2O_2 plus $NaNO_2$, was also effective at forming DPCs (Figure 2, lane 4) consistent with its ability to oxidize OG in preference to other bases (*55*−*57*). However, if peroxynitrite was allowed to degrade at 37 °C for 30 min, it lost the ability to generate cross-links (Figure 2, lane 5). Hydrogen peroxide was ineffective at a concentration of 100 μM.

In order to further investigate the potential of one-electron oxidants to generate DPCs, a comparison was made of $IrCl_6^{-2}$, $IrBr_6^{-2}$, and $Fe(CN)_6^{-3}$ (Figure 3 and Supporting Information). All three oxidants caused cross-linking to occur with efficiency roughly corresponding to the redox potential of each oxidant: $IrCl_6^{-2}$ (0.90 V) ≈ $IrBr_6^{-2}$ (0.82 V) > $Fe(CN)_6^{-3}$ (0.42 V vs NHE) (*58*, *59*). A quantitative comparison of the extent of cross-linking appears in Table 1 in which all high molecular weight bands are added together. Note that the success of $Fe(CN)_6^{-3}$ in forming DPCs with the higher potential OG-containing sequences is likely driven by the large excess of oxidant being used. However, the distribution of high-molecular-weight adducts was different for the various oxidants and depended directly on redox potential, as described in the next section.

Protein−Protein Cross-Linking. Multiple high-molecular-weight bands appear by gel electrophoretic analysis of radiolabeled ssDNA cross-linked to SSB due to oxidative protein−protein covalent cross-linking in the SSB tetramer in addition to DNA−protein cross-linking (Figure 3). The higher order adducts likely involve only one strand of DNA adducted to a protein−protein dimer, trimer, or tetramer. A 1:1 SSB:DNA cross-link could be clearly distinguished from

Table 1: Comparison of Cross-Linking to SSB of Sequences Containing OG, OA, and G

	% DNA cross-linked to SSB[a]		
sequence[b]	$Fe(CN)_6^{-3}$	$IrBr_6^{-2}$	$IrCl_6^{-2}$
1 (OG)	12	42	23
2 (G)	0.61	5.1	5.9
1′ (OG)	12	46	41
2′ (G)	3.8	16	32
3 (G)			0.021
4 (OG)			7.3
5 (OA)			8.3
6 (A)			0.10
7 (C)			0.058
8 (5 OG)			0.30
9 (12 OG)			0.22
10 (20 OG)			0.28
11 (30 OG)			0.46

[a] Cross-linked yields refer to the sum of all DPC bands compared to unreacted oligomer. [b] Bases in parentheses are proposed to be the site of highest reactivity.

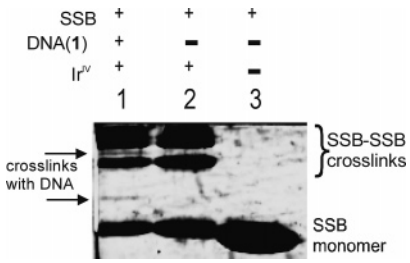


FIGURE 4: DPC formation vs protein−protein cross-link formation. SYPRO orange protein stained gel. Lane 1 contained 1.2 μM oligonucleotide 1, 18 μM SSB, and 72 μM $IrCl_6^{-2}$. Lane 2 contained 18 μM SSB and 72 μM $IrCl_6^{-2}$. Lane 3 contained 18 mM SSB only. All reactions were conducted at pH 7.0, 10 mM NaPi, 100 mM NaCl, and were quenched with 2 μL of EDTA (0.1 M, pH 8) and were electrophoresed for 30 min at 250 V on 15%, 29:1 cross-linked SDS−PAGE gel. Arrows indicate bands unique to DPC vs protein−protein cross-linking.

the 2:1, 3:1, and 4:1 adducts on the gel, the latter two being unresolvable under the conditions used. (Similarly, involvement of 2 DNA strands in the cross-linking to two or more proteins would be unresolvable from the topmost band.) Protein−protein cross-linking was also examined in the absence of DNA using $IrCl_6^{-2}$, and the dimer, trimer, and tetrameric cross-links could be observed by comparison to molecular-weight ladders and confirmed by ESI-MS analysis (see Supporting Information). The extent of protein−protein cross-linking was a function of both the amount of oxidant added and the oxidant's redox potential. For example, use of $IrCl_6^{-2}$, the strongest oxidant in the series, led to 94% protein−protein cross-linking of the total DPCs, $IrBr_6^{-2}$ to 14%, and $Fe(CN)_6^{-3}$ to zero protein−protein cross-linking that was also accompanied by a lower yield of the 1:1 DPC.

In order to investigate the extent of protein−protein cross-linking that does not result in formation of an adduct to DNA, gels were stained with Sypro Orange for visualization of all protein-containing bands. In addition to monomeric SSB, four high-molecular-weight bands could be distinguished (Figure 4). When the same gel was examined by storage phosphor autoradiography to visualize bands containing [^{32}P]-labeled ssDNA, only two minor bands could be identified separately from protein−protein cross-links (Figure 4, arrows). These were assigned as the 1:1 and 2:1 SSB:DNA cross-links; higher order DNA−protein−protein cross-links comigrated

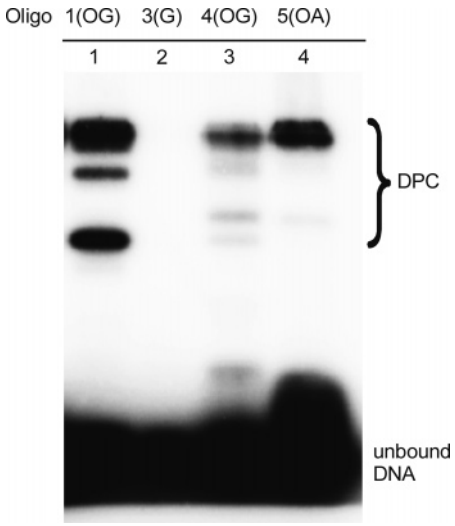


FIGURE 5: Cross-linking reaction with oligonucleotides 1, 3, 4, and 5. Lanes 1−4 contained 5′-end-labeled oligonucleotides **1**, **3**, **4**, and **5**, sequentially. All lanes were 36 nM ssDNA, 545 nM SSB, and 1 μM $IrCl_6^{-2}$ at pH 7.0, 10 mM NaPi, 100 mM NaCl, and were quenched with 2 μL of EDTA (0.1 M, pH 8).

with protein−protein cross-links. The intensity of the SSB:SSB cross-links compared to the resolvable DPCs suggests that a large fraction of the proteins are cross-linked to each other without forming a DNA−protein cross-link when Na_2IrCl_6 was used as oxidant.

The 40-mer sequence **1′** is long enough to span the binding sites of two SSB proteins and yet still forms cross-links that are very similar in pattern and yield to that of the shorter sequence **1** (Table 1 and Supporting Information). This would argue against formation of any protein−DNA−protein cross-links in which DNA forms two covalent bonds to two different SSB monomers.

The formation of protein−protein−DNA cross-links is also dependent on SSB tetramer stability. It is reported that the SSB tetramer is stable from conversion to the monomer form to as low a concentration as 30 nM (25 °C) for a wide range of conditions (*39*). While maintaining DNA and oxidant concentration steady, we varied the SSB concentration from 12 to 80 nM. A sudden increase of protein−protein cross-linking was observed in the DPCs at [SSB] ≥ 50 nM (see Supporting Information) suggesting that below this concentration the tetramer is not stable enough to form such protein−protein cross-links and only the monomer cross-links to DNA.

Cross-Link Dependence on Nucleobase and Sequence. While OG-containing oligomers (**1** and **1′**) were readily able to form cross-links to SSB, the parent oligomers containing dG nucleotides in place of dOG formed about 6-fold fewer DPCs (**2** and **2′**, see Table 1). Several other single-stranded 18-mer sequences (**3**−**6**) were placed under the same oxidizing conditions in the presence of SSB, for comparison to **1** and **2** (Figure 5 and Table 1). Poly(dA) is relatively resistant to oxidation, and so single-nucleotide substitutions were made at position 10 of an 18-mer in order to compare the reactivity of a single G (**3**), OG (**4**), or OA (**5**) to the homopolymer (**6**). The homopolymer of dA was unreactive toward DPC formation. Oligomer **4** formed about 1.5-fold fewer DPCs than **1** but much more than **3** which showed no cross-linking under the conditions tested. Interestingly,

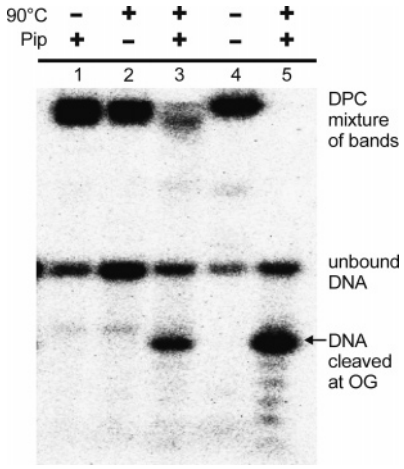


FIGURE 6: Piperidine and heat treatment of DPC. The DPCs of **1**:SSB were prepared and isolated as described in Experimental Procedures; subsequent treatments were ~60 μM in DPC. Lane 1: DPC treated with 0.2 M piperidine at room temperature. Lane 2: DPC heated to 90 °C for 30 min. Lane 3: DPC treated with 0.2 M piperidine and heated to 90 °C for 30 min. Lane 4: DPC treated with water at room temperature. Lane 5 was a control experiment with oligomer **1** oxidized with 3 μM $CoCl_2$, 60 μM $KHSO_5$ (7 mM NaPi, 70 mM NaCl, pH 7) at 25 °C for 30 min, then treated with 0.2 M piperidine at 90 °C for 30 min so as to cleave at the OG site of the oligonucleotide.

sequence **5** containing a 7,8-dihydro-8-oxoadenosine nucleotide (OA) showed strong reactivity toward cross-linking, comparable to the polyadenine sequence containing OG (**4**). Oxidative cross-linking of SSB to polydeoxycytidine (**7**) was barely detectable, although introduction of a single OG nucleotide at various positions of $(dC)_{35}$ enhanced cross-linking substantially (Table 1, oligomers **8**−**11**).

Characterization of DNA−Protein Cross-Links

Piperidine Lability of DPCs. Piperidine treatment of an oxidized OG adduct often results in base release and strand scission (*60*), but OG itself is not a piperidine-labile site. Although 6% cleavage of an OG-containing strand is observed after subjection to1 M piperidine at 90 °C for 2 h, the addition of β-mercaptoethanol reduces piperidine lability to nearly zero(*61*). Hot piperidine lability is therefore an indication that OG has undergone further oxidative modification. In the present set of experiments, it was important to first isolate the DPCs from un-cross-linked DNA since the latter material could include oxidized but un-cross-linked OG sites that would also generate the same cleavage band on a gel after piperidine treatment.

Bands representing the mixture of DPCs were collectively isolated from the gels and subjected to hot piperidine treatment (0.2 M piperidine, 90 °C, 30 min) and analyzed by gel electrophoresis (Figure 6, lane 3) alongside a control lane in which the oligomer alone was oxidized and treated with hot piperidine (Figure 6, lane 5). This experiment showed that most of the DPC underwent strand scission consistent with modification having occurred at the site OG. Had oxidatively generated cross-linking occurred at one of the G bases of **1**, one would anticipate observing bands above or below that of OG. For comparison, a sample of the isolated DPC bands was also simply diluted with H_2O and carried through the same manipulations and gel analysis without any base or heat treatment (Figure 6, lane 4). From this experiment, it was clear that no strand scission occurs at room temperature, pH 7; however, some loss of the protein adduct could be observed, even under these mild conditions, indicating that the covalent bond between DNA and protein could be reversible. Therefore, we examined heat and piperidine lability independently.

DPC Sensitivity to Heat and pH. The hot piperidine treatment revealed that DPCs might be reversibly formed. Thus, a comparison was made in which the isolated DPCs were treated at room temperature with 0.2 M piperidine for 30 min (Figure 6, lane 1) and at 90 °C, pH 7 for 30 min without piperidine. (Figure 6, lane 2). Although both conditions led to some reversal of the cross-link to yield a band of the same electrophoretic mobility as the starting oligodeoxynucleotide, heat appeared to have a greater effect than piperidine on this reaction. Heat alone led to 45% release of the protein under these conditions. Such lability is suggestive of Schiff base formation, and so the acid lability of the DPCs was also studied. Incubation of the DPCs at pH 2.5 or lower for a period of 2 h led to >50% cleavage of the DNA−protein linkage, although no DNA strand scission was observed (see Supporting Information). To further explore the possibility of Schiff base formation, $NaBH_4$ and $NaBH_3CN$ reductions were attempted, but these efforts failed to generate a more stable cross-link.

Site of Protein Cross-Linking. A polycytosine of 35 nucleotides in length (**7**) was synthesized for comparison to four other polycytosines with OG substituted at positions 5, 12, 20, and 30 (**8**, **9**, **10**, and **11,** respectively). The oligomer $(dC)_{35}$ was chosen for study because of a X-ray crystallographic analysis of this sequence with *E. coli* SSB that revealed several residues of the protein making base contacts to DNA (*52*). When the sequences were subjected to the cross-linking conditions described above, all OG-containing oligomers were able to form DPCs while the parent poly(dC) was unreactive (Table 1). This result supports the proposal that OG is the site of cross-link formation of the DNA. However, little information could be gained about the site of protein modification since all four OG-substituted sequences resulted in similar cross-linking efficiency within a factor of ~2. This may be a result of sliding of the DNA strand within the binding site so as to encounter the appropriate amino acid that acts as the nucleophile to then form the cross-link. Alternatively, there could be several amino acids available to form cross-links at different sites within the binding site. Curiously, the poly(dC) series **8**-**11** showed substantially reduced overall reactivity compared to the mixed sequences **1** and **2** (18-mers) and **1′** and **2′** (40-mers), and the poly(dA) series, **4** and **5** (18-mers), were intermediate in reactivity (Table 1).

Protein Digestion. Protein digestion of the DPCs was performed in an attempt to isolate and characterize a peptide segment responsible for cross-linking. High-molecular-weight bands were isolated from a SDS−PAGE gel as three samples; the lower band corresponding to a 1:1 DNA:SSB adduct, all three upper bands together representing DNA−protein−protein adducts, and residual material found in the wells of the gel. These were then digested with trypsin for cleavage at the carboxyl side of lysine and arginine residues. The result was that all three samples gave the same trypsin digestion bands suggesting that a single SSB:**1** cross-link as well as higher molecular weight bands all cross-link with

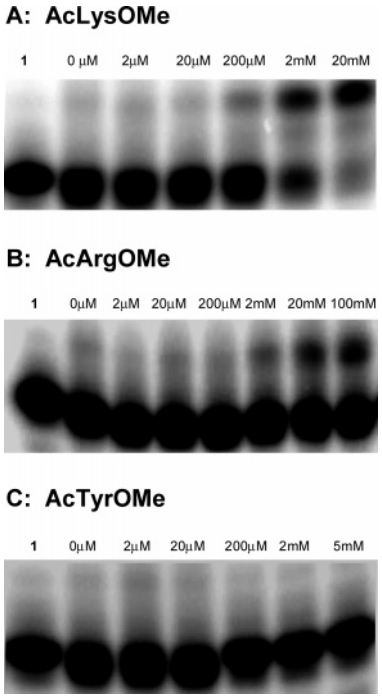


FIGURE 7: Oxidative cross-linking of amino acids to DNA 1. All experiments were conducted with [**1**] = 40 nM, [Na_2IrCl_6] = 100 μM, and protected amino acids in the concentrations shown. Lane 1 in each panel is DNA alone with no oxidant. AcTyrOMe is only water soluble to 5 mM; addition of 40% CH_3CN permitted studies up to 20 mM (data not shown) with no change in the DPC formation of either AcTyrOMe or AcLysOMe.

the same residues (see Supporting Information). When trypsin is added in excess, the DPC can be digested to produce one band on the PAGE gel that runs slightly slower than unreacted DNA.

A glutamic acid specific protease (V-8 protease) was also used on a mixture of DPC bands isolated from an SDS−PAGE gel (see Supporting Information). There was primarily one band seen suggesting one major site of cross-link formation for all DPCs. Numerous attempts were made to isolate the digested fragments and characterize them by electrospray (including in-gel digests) or MALDI mass spectrometry; however, these attempts failed to indicate nucleic acid content, and only protein−protein cross-linked samples could be analyzed. One concern is that the acidic nature of the MALDI matrices used may have led to reversal of the DNA−protein linkage. Peptide sequencing of the digests also failed to produce a discernible match to the protein sequence.

Amino Acid Model Studies

In order to gain insight to the identity of the protein component of the DPC, model studies were performed using oligodeoxynucleotide **1** and single amino acids with α-amino and carboxyl protection. Studies centered on 3 potential nucleophilic side chains, lysine's ϵ-amino group (AcLysOMe), arginine's guanidine group (AcArgOMe), and the phenolic oxygen of tyrosine (AcTyrOMe), since these 3 residues are present in the binding site of SSB and make contacts to DNA bases (see Discussion section). The results of concentration-dependent studies of these amino acids forming adducts to oligomer **1** in the presence of 100 μM Na_2IrCl_6 are shown in Figure 7, panels A−C. At a concentration of 2 mM, lysine was present in sufficient quantity to compete favorably with H_2O to form an adduct of substantially lower mobility. Arginine showed the same trend but was less reactive. Tyrosine, a poorer nucleophile, did not form a cross-link with oligomer **1** under these conditions, although we have previously seen evidence for tyrosine adducts to OG monomers. For tyrosine, it is likely that Ir(IV) reacts primarily with tyrosine rather than OG since AcTyrOMe is present in much higher concentration than DNA, and its redox potential is similar. The sharp bands observed in Figure 7, panel C for starting material in lanes containing high concentrations of AcTyrOMe are likely indicative that no oxidant is reaching DNA, since oxidation of OG to hydantoin products in oligomers leads to bands that have slightly different gel mobility. Thus, the overall tendency to form oxidized adducts was in the order Lys > Arg ≫ Tyr.

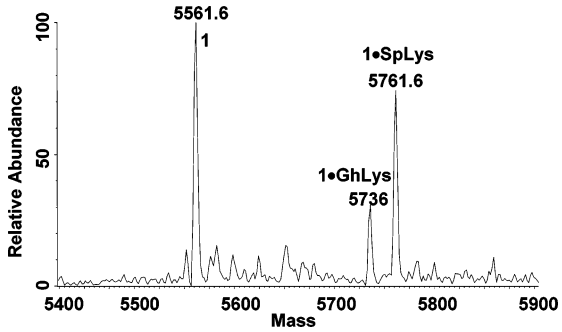


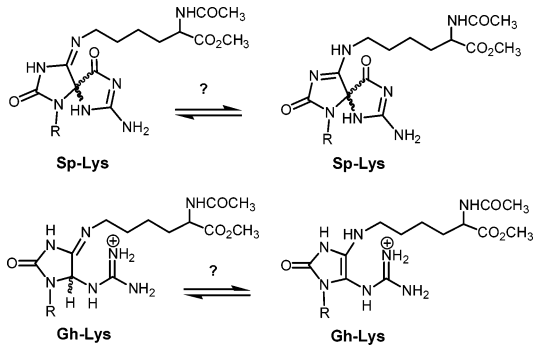
FIGURE 8: Negative ion ESI-MS analysis of oxidized adduct of **1** + Lys. DNA oligomer **1** was oxidized with Na_2IrCl_6 in the presence of AcLysOMe as described in Experimental Procedures. See Scheme 2 for proposed structures of the Gh and Sp-type adducts.

Since the lysine adduct was obtained in high yield, it was further analyzed by electrospray mass spectrometry (ESI-MS). Oligomer **1** (5 μM) was incubated at pH 7.5 (20 mM NaP_i, 100 mM NaCl) with AcLysOMe (140 mM) and Na_2IrCl_6 (50 μM). The crude reaction mixture showed 3 major peaks in the mass spectrum (Figure 8). The largest at 5561.6 amu corresponds to the unreacted starting oligodeoxynucleotide **1**. The major product peak at 5761.6 amu corresponds to an adduct of AcLysOMe (202 amu) with a loss of 2 amu as expected for an oxidized cross-link. A minor peak is also seen at 5736.0 representing a further loss of 26 amu. This product can be explained as a guanidinohydantoin-like product that could be formed from hydration (+18 amu) and decarboxylation (−44 amu) of an initial adduct. This chemistry represents the amine analogy (Scheme 2) to that of nucleophilic attack of H_2O at C5 of an oxidized OG residue (Scheme 1). We propose the structures Sp-Lys and Gh-Lys to represent the lysine adducts to oxidized OG sites in DNA.

DISCUSSION

Guanine, the most electron rich of the four DNA bases, is highly susceptible to oxidizing agents, and a major product of its oxidation is 8-oxo-7,8-dihydroguanine (OG). The levels of OG in human tissues are on the order of one in 10^6 (*62*), and its occurrence is more frequent under oxidative stress. OG has a lower redox potential than guanine (~0.7 V vs NHE (*63*−*65*) for OG; 1.3 V for G (*66*)), which makes OG

Scheme 2



highly reactive toward further oxidation (*34*, *35*, *55*, *56*, *58*, *67*, *68*). Guanine and 8-oxoguanine are therefore likely sites for cross-linking to protein nucleophiles under conditions that initiate DNA oxidation. We have identified the commercially available iridium(IV) salts Na_2IrCl_6 and Na_2IrBr_6 (0.9 and 0.82 V vs NHE, respectively) as oxidants that, because of their anionic nature, do not bind DNA and act as simple outer-sphere, one-electron oxidants modeling oxidation processes that might occur in the cell. Their reactivities are highest with the oxidized bases OG and OA, but they are also slightly reactive with G in the absence of 8-oxopurines (*58*). Peroxynitrite, formed in vivo from the reaction of superoxide with nitric oxide, is an intercellular messenger used by endothelium cells and neurons and is known to oxidize DNA bases (*69*, *70*). The OG base was found to be more highly susceptible to oxidation by peroxynitrite than G, generating some of the same products as Ir(IV) (*57*, *71*, *72*). Hypochlorous acid (HOCl) is formed in vivo by the enzyme myeloperoxidase in neutrophils and monocytes as part of a host defense system that can also cause damage to the host itself. Hypochlorous acid has been shown to cross-link SSB to DNA (*47*), and experiments with HOCl and H_2O_2 were repeated here for comparison.

Single-stranded binding protein was chosen for study because of its major role in the cell in protecting single-stranded DNA from digestion by nucleases, its ability to bind DNA independent of sequence, and the abundance of amino acid−nucleobase contacts that would favor formation of DNA−protein cross-links. Conditions were chosen that led to formation of the SSB tetramer that is capable of binding 4 oligodeoxynucleotides of 18-mer length or 2 oligomers of 35−40 nucleotides in length. All of the oxidants studied, with the exception of H_2O_2, led to formation of DPCs with observed reactivity in the order Ir(IV) > $HOONO^-$ > HOCl ∼ $Na_3Fe(CN)_6$. Protein−protein cross-linking was also observed and was a function of the strength of the oxidant; the stronger oxidant Na_2IrCl_6 led to extensive protein−protein cross-linking, but the weaker oxidants Na_2IrBr_6 and $Na_3Fe(CN)_6$ were more selective for DNA−protein cross-linking when the DNA contained a readily oxidized site, OG or OA.

No difference in reactivity was observed between an 18-mer and a 40-mer of similar sequence (e.g. **1** vs **1′**) as the DNA partner in formation of DPCs (Table 1). However, the base composition appeared to be a major factor in reactivity. As expected based on redox potentials, oligomers containing OG and OA were highly reactive toward oxidation and DPC formation compared to those only containing native bases (e.g. **1** vs **2,** and **4** and **5** vs **3** and **6**) with the general trend being OG ∼ OA > G > C, A. Although OG should be considerably more reactive than OA toward oxidation, the electron-deficient species resulting from OA oxidation could be less stable and more reactive leading to the approximately equal levels of DPC formation observed. Similar arguments have been put forth by Lewis and co-workers to explain the smaller than expected differences in G, GG and GGG sequences toward oxidation (*73*). Inexplicably, overall lower levels of DPC formation were observed when homopolymers formed the basis of the sequence. Even when they contained OA or OG, poly(dA) and poly(dC) sequences provided lower yields of DPCs (Table 1, **3**−**6** and **7**−**11**). This may point to a contribution of sequences containing dG as contributing factors in initiating oxidation and cross-link formation, even when one readily oxidizable OG exists. Further studies may help clarify the intricacies of the role of base content and sequence in DPC formation. A scanning experiment in which OG was placed at positions 5, 12, 20, or 30 of $(dC)_{35}$ was conducted to test the relative reactivity in various regions of the DNA:SSB complex that has been crystallographically characterized (*52*). Although slightly higher tendencies to form DPCs were observed when OG was located near the termini (positions 5 or 30), the differences are small and might be attributable to better accessibility to oxidant or more flexibility in the binding site rather than being located next to a particularly reactive protein side chain.

Some aspects of the chemical nature of the DPCs formed could be gleaned from their sensitivity to heat, acidity and piperidine treatment. Hot piperidine treatment is a convenient method for detecting certain DNA base modifications (*60*), and although OG and OA are not sensitive to this treatment, most of their further oxidation products lead to DNA strand scission at the nucleotide of modification under conditions of 0.2 M piperidine, 90 °C for 30 min. Most of the DPC material isolated from higher bands on the gel showed hot piperidine sensitivity leading to DNA strand scission at the OG or OA nucleotide. Therefore, oxopurines are the principle site in DNA responsible for the DPCs studied here. In those sequences lacking oxopurines, guanine is the likely site of oxidation and cross-link formation, but yields of cross-links were too low to verify this. It should be noted that recent studies by Stemp and co-workers in which G oxidation was responsible for DPC formation indicated that the adducts underwent DNA strand scission upon piperidine treatment and displayed heat sensitivity as well (*27*). This is a preliminary indication that the OG and G-derived cross-links may be structurally similar.

The DPCs were also found to be somewhat labile to heat and acid, but in these cases, the result was to cleave the DNA−protein linkage rather than the DNA strand. This type of reversion is characteristic of Schiff base formation since imines are sensitive to acid-catalyzed hydrolysis and more sensitive to hydrolysis even at pH 7 if the temperature is elevated. Attempts were made to stabilize the cross-link via reduction of the Schiff base with $NaBH_4$ and $NaBH_3CN$ without success. This failure could be due to the inaccessibility of the reagents to the cross-link or to the unusual context of the imine bond (see Scheme 2) lowering its reactivity toward these reducing agents.

The mechanism of OG oxidation involves trapping of an electron-deficient species by a nucleophile (*54*, *74*), typically water which is a poor but abundant nucleophile. Recent work

has implicated a 2-e$^-$ oxidized form of OG, OG^{ox} (Scheme 1), as a common intermediate generated from singlet oxygen oxidation of G and from Ir(IV), peroxynitrite and other oxidants acting on OG (*54*, *72*, *75*). This quininoid structure should be highly susceptible to nucleophilic attack at C5 leading to the formation of 5-OH-OG. The subsequent chemistry of 5-OH-OG depends on the reaction conditions and the context—the thermodynamically preferred product involves rearrangement to spiroiminodihydantoin, a lesion that has now been well characterized in several laboratories (*53*, *57*, *75*−*78*). In duplex DNA, or in single-stranded DNA at room temperature or below, the rearrangement is sterically encumbered by base stacking and hydrolysis at C6 followed by decarboxylation to the allantoin-like product guanidinohydantoin is preferred (*74*). The 5-OH-OG to Gh pathway can also be enforced in nucleosides using an acidic medium with pH <6. Under the reaction conditions used for oxidative cross-linking to proteins, both the Gh and Sp pathways would be expected for OG oxidation in single-stranded DNA. By comparison, we expected that our previous studies of MutY cross-linking to an OG-containing duplex probably led to predominantly a Gh-like adduct to Lys142 (*33*).

In the present work forming DPCs, we propose that an amino acid side chain serves as the nucleophile to form structures analogous to Gh and Sp by trapping OG^{ox} (or an OA^{ox} analogue). Support for this comes from model studies using protected amino acids such as AcLysOMe in which only the ϵ-amino group is available for reaction. ESI-MS evidence (Figure 8) points to formation of both a Gh-Lys and an Sp-Lys adduct as the major products when oligomer **1** was oxidized with Ir(IV) in the presence of excess AcLysOMe. If these structures represent some or all of the actual DPCs, they could explain both the hot piperidine sensitivity toward DNA cleavage as well as the heat and acid lability of the cross-link. The imine forms of these structures (Scheme 2, left side) would help explain their tendency to hydrolyze. However, the tautomers with endocyclic double bonds might predominate (Scheme 2, right side) and could explain the low reactivity observed toward borohydride reduction.

We do not know which amino acids of SSB are responsible for cross-linking; despite extensive efforts not detailed here (*79*), we were unable to obtain MS data on trypsin digests that would lead to a positive identification of the amino acid(s) involved. Future studies will attempt to stabilize the DPCs toward thermal and hydrolytic decomposition, which may facilitate isolation and characterization of the peptide−nucleotide cross-link. However, inspection of the X-ray crystal structure (*52*) of a fragment of the *E. coli* SSB tetramer bound to two $(dC)_{35}$ oligomers provides several ideas for amino acid−nucleobase interactions that could lead to oxidative cross-links. Figure 9 highlights a portion of the structure.

Early analysis of DNA binding by chemical modification studies uncovered lysine and tryptophan residues that are important in DNA binding (*80*, *81*), and photochemical cross-linking showed Trp54 and Trp88 to be closely interacting with DNA (*44*). Phe60 has also been implicated in DNA binding because of a cross-link formed to DNA when SSB was exposed to UV irradiation (*45*). The crystal structure confirmed the chemical studies that Trp54 and Phe60 make extensive interactions with the ssDNA. In addition, Lys43, Lys62, Lys73, Lys87, and the terminal amine are all within contact distance of the ssDNA backbone, and Tyr97 is close enough to cytosine 23 for nucleophilic attack. Arg84 contacts the phosphate backbone while Arg86 hydrogen bonds to a deoxyribose oxygen and is very close to the base as well. Arg21 is also very close to a base and may make some contacts (see Supporting Information). Thus, several arginine and lysine moieties as well as Tyr97 are potential nucleophiles in position for cross-linking. For this reason, lysine, arginine, and tyrosine were selected for amino acid cross-linking to DNA oligomer **1**. In addition, DNA binding of SSB is not sequence specific, and as such it is possible that the DNA can shift within the binding pocket making it possible for it to come into closer contact with some of these amino acids. Thus, the observed intrinsic reactivity of amino acid side chains was Lys > Arg ≫ Tyr, but positioning in the protein−DNA complex or flexibility and slippage could alter this order of reactivity.

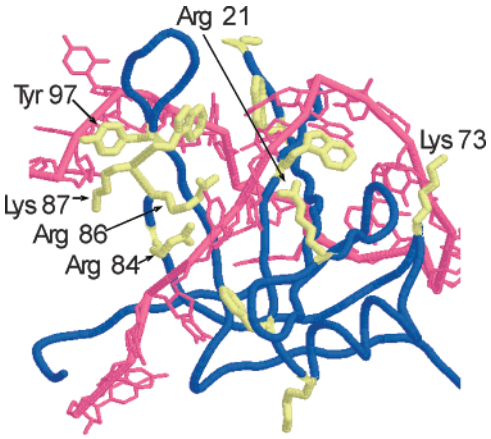


FIGURE 9: SSB contacts with ssDNA. SSB (blue) has several amino acid residues (yellow) that make contact with ssDNA (magenta) according to a crystallographic study of $(dC)_{35}$ bound E. coli SSB (PDB ID 1EYG, Raghunathan et al. *Nat. Struct. Biol.* **2000**, *7*, 6652−6657.) Trp40, Trp54, Trp80, Phe60, and Tyr97 all make stacking interactions with bases of the ssDNA. Lys73, Lys87, Arg21, Arg84, and Arg86 make phosphate backbone contacts with both Arg21 and Arg84 making base contacts as well.

CONCLUSIONS

E. coli single-stranded binding protein was cross-linked under oxidative conditions to ssDNA containing OG or OA at relatively high efficiency, with up to 50% of the DNA forming DPCs. Gel electrophoresis analysis points to OG (or OA) as the site of cross-linking, and revealed piperidine-induced DNA strand scission as well as heat or acid-induced cleavage of the DNA−peptide bond. Both in the presence and absence of DNA, protein−protein cross-linking was observed particularly with higher redox potential oxidants such as Na_2IrCl_6. Although trypsin digests of the DPC were not conducive to structural analysis by mass spectrometry, ESI-MS data confirmed that an amino acid model, AcLysOMe underwent oxidative cross-linking to OG in an oligodeoxynucleotide to yield hydantoin products, Gh-Lys and Sp-Lys, whose mechanism of formation appears to parallel that of OG oxidation followed by nucleophilic trapping by a solvent water molecule. Arginine was less reactive, but adducts could be detected by gel electrophoresis. SSB displays a number of arginine, lysine, and tyrosine side chains

contacting DNA bases in a crystal structure of the protein−DNA complex, and any of these nucleophiles remain as possible candidates for cross-linking to oxidized purines in DNA. The chemistry of lysine adduction to an oxidized oxopurine also parallels that observed for polyamines such as spermine which undergo facile cross-linking to oxidized guanines in DNA (*82*).

Because DNA is largely surrounded by lysine and arginine-rich proteins in the cell, it can be anticipated that oxidative damage to DNA will lead to the formation of electron-deficient species subject to nucleophilic attack by these amino acid residues. DNA−protein cross-links are indeed a significant outcome of oxidative stress in the cell, but progress in studying the biochemistry of these lesions is hampered by an inability to synthesize site specifically DNA−protein cross-links without resorting to unnatural bases. The placement of OG or OA at specific sites in synthetic oligodeoxynucleotides followed by oxidative cross-linking now allows control of at the least the DNA partner in the cross-link. This method has provided insight into the mechanism of formation of cross-links from oxidation initiated on the DNA strand and complements the existing work in which oxidation of proteins may alternatively elicit nucleophilic attack by a nucleic acid moiety. Additionally, the now well characterized phenomenon of long-range hole transport in DNA suggests that oxidation of DNA at distant site might also lead to DPC formation when the hole relocates to a particularly reactive site in a DNA−protein complex (*83*).

SUPPORTING INFORMATION AVAILABLE

Gel electrophoretic data for oligomers **1′** and **2′**, SSB tetramer concentration dependence, acid lability of DPC, and protein digests; ESI-MS data for protein−protein cross-linking; amino acid−nucleobase contacts for selected residues from PDB. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI047580N