CHEMBIOCHEM

DOI: 10.1002/cbic.200700686

Investigation of DNA–Protein Cross-Link Formation between Lysozyme and Oxanine by Mass Spectrometry

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Reactive nitrogen species are implicated in inflammatory diseases and cancers. Oxanine (Oxa) is a DNA lesion product originating from the guanine base through exposure to nitric oxide, nitrous acid, or N*-nitrosoindoles. Oxanine was found to mediate formation of DNA–protein cross-links (DPCs) in the cell extract. We have previously characterized two DNA–protein cross-links from the reaction between Oxa and glutathione: namely, the thioester and the amide. In this study, lysozyme was used to study site-specific modification on protein by Oxa moieties in DNA. With the aid of nanoLC coupled with nanospray ionization tandem mass spectrometry, addition of Oxa was found at Lys13, Lys97, Lys116, Ser85, and Ser86 of lysozyme when it was treated with 2′-deoxyoxanosine (dOxo). Furthermore, incubation of lysozyme with Oxa-containing calf thymus DNA, produced by treating DNA with nitrous acid, led to lysozyme modification at Lys116, Ser85, and Ser86. Interestingly, none of the cysteine residues was modified by dOxo, in contrast with our previous findings that dOxo reacted with oxidized glutathione disulfide, forming the thioester. This might be due to the half-life of the dOxo-derived thioester being 2.2 days at the pH of incubation. Furthermore, the sites of modifications on lysozyme are in good agreement with the solvent accessibility of the residues. Since repair of Oxa-derived DPCs has not been extensively investigated, these results suggest that these stable DPCs might represent important forms of cellular damage caused by reactive nitrogen species involved in inflammation-related diseases.*

Introduction

Excessive production of nitric oxide (NO) during infection and inflammation is known to damage DNA, and it plays an important role in cancers related to chronic infections and inflammation.[1,2] Direct treatment of DNA with NO leads to oxidation and deamination of bases, DNA strand breakage, formation of abasic sites, and DNA crosslinks.[3,4] Nitric oxide also reacts rapidly with superoxide anion to form peroxynitrite, which is considered to be a powerful in vivo nitrating agent for biomolecules, including proteins, nucleic acids, and lipids.[5–8] Furthermore, NO is known to be metabolized in vivo to nitrite, which can be oxidized by hydrogen peroxide (H_2O_2) in the presence or absence of metalloproteins to form reactive nitrogen oxide (RNOx) species, contributing to protein tyrosine nitration.[5] Moreover, nitrite is protonated to form nitrous acid (HNO_2) in the acidic environments of lysosomes and phagocytes. The formation and reactivity of HNO_2 may account for an important part of the bactericidal activity of phagocytes.[9,10] Oxanine (5-amino-3*H*-imidazo[4,5-*d*][1,3]oxazin-7-one, Oxa) is a DNA lesion product originating from reactions between the base guanine and NO, HNO_2, or the mutagen *N*-nitrosoindoles.[11–14] The mutagenic Oxa results in misincorporation during DNA replication and instability of the DNA helix.[15,16]

The *N*-glycosidic bond of 2′-deoxyoxanosine (dOxo) has been shown to be as stable as that of dGuo,[15] indicating that Oxa might constitute a persistent DNA lesion product. In addition, the *O*-acylisourea ring structure of Oxa is susceptible to nucleophilic attack by nucleophiles on proteins, forming DNA–protein crosslinks (DPCs).[17–19] DNA-binding proteins such as histone, high mobility group protein, and DNA glycosylases have been shown by gel electrophoresis to react with Oxa-containing oligonucleotides.[18] It has been reported that base and nucleotide excision repair enzymes can partially repair Oxa on oligonucleotides.[20–23] Although repair of DPCs by nucleotide excision repair enzymes has been demonstrated by Schiff base formation at an abasic site (2-deoxyribonolactone) or on aldehyde-derived 1,N^2-deoxyguanosine adducts with polypeptides,[24–26] it is not clear whether DPCs derived from Oxa can be repaired.

We have previously used the tripeptide GSH as a model to study the reactivities of protein side chains with Oxa-containing DNA and have characterized two forms of DPCs—namely, the thioester and the amide—produced through reactions of the *O*-acylisourea ring structure of Oxa with thiol and amino groups, respectively.[19] Although the possible sites of protein reacting with Oxa have been investigated,[18,19] factors other than nucleophilicity can influence the selectivity of a reaction site in a protein.[27] In this study, a highly sensitive and specific analysis based on nanoscale liquid chromatography coupled with nanospray ionization tandem mass spectrometry (nanoLC/NSI/MS/MS) was used to investigate the reactivity of the amino acid side chains on lysozyme when it reacts with dOxo and with Oxa-containing DNA.

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Results and Discussion

Reaction of dOxo with lysozyme

After mixtures containing lysozyme and 100 times molar excesses of dOxo had been incubated at 37 °C for 24 or 48 h, modifications of lysozyme at three out of its six lysine and two out of its ten serine residues were observed, according to nanoLC/NSI/MS/MS analysis of the tryptic digests. These sites of modification were Lys13, Lys97, Lys116, Ser85, and Ser86. The cysteine residues of the unmodified peptides were converted into carboxyamidomethylcysteine (CAM) residues by reduction of the protein with the reducing agent DTT, followed by alkylation with iodoacetamide prior to digestion with trypsin. Carboxyamidomethylation (+57) on cysteine, as well as addition of an Oxa (+152) or dOxo (+268) moiety, were set as variable modifications. The peptide fragments identified by BioWorks were assigned according to their *m/z* values and their fragment ion type: that is, "y" or "b" ions.

The collision-induced dissociation (CID) spectra of the three lysine-modified peptides were obtained by data-dependent MS acquisition. Interestingly, none of their cysteine residues had been modified by dOxo, which is in contrast to our previous report that dOxo reacted with the oxidized glutathione disulfide, forming the thioester.[19] This might be due to the short half-life of the dOxo-derived thioester, as well as the poor solvent accessibility of the cysteine residues (discussed later). The lower panel of Figure 1 A shows the CID spectrum of the parent peptide in which the b and y fragment ions are characterized by the SEQUEST algorithm, confirming the presence of the CAM moiety at the first residue of the C^{CAM}ELAAAM^{13}KR peptide. In the upper panel of Figure 1 A, a mass difference of 280 was observed between b_8' (*m/z* 1027.4 Da) and b_7 (*m/z* 747.2 Da) fragment ions in peptide C^{CAM}ELAAAM$^{13}K^{Oxa}$R, corresponding to a modification of oxanine (+152) at the eighth residue (Lys13). A mass difference of 280 between y_2' (*m/z* 455.2 Da) and y_1 (*m/z* 175.2 Da) fragment ions supports the above structural assignment. This result also indicated that lysine residue modified with oxanine was not cleaved by trypsin.

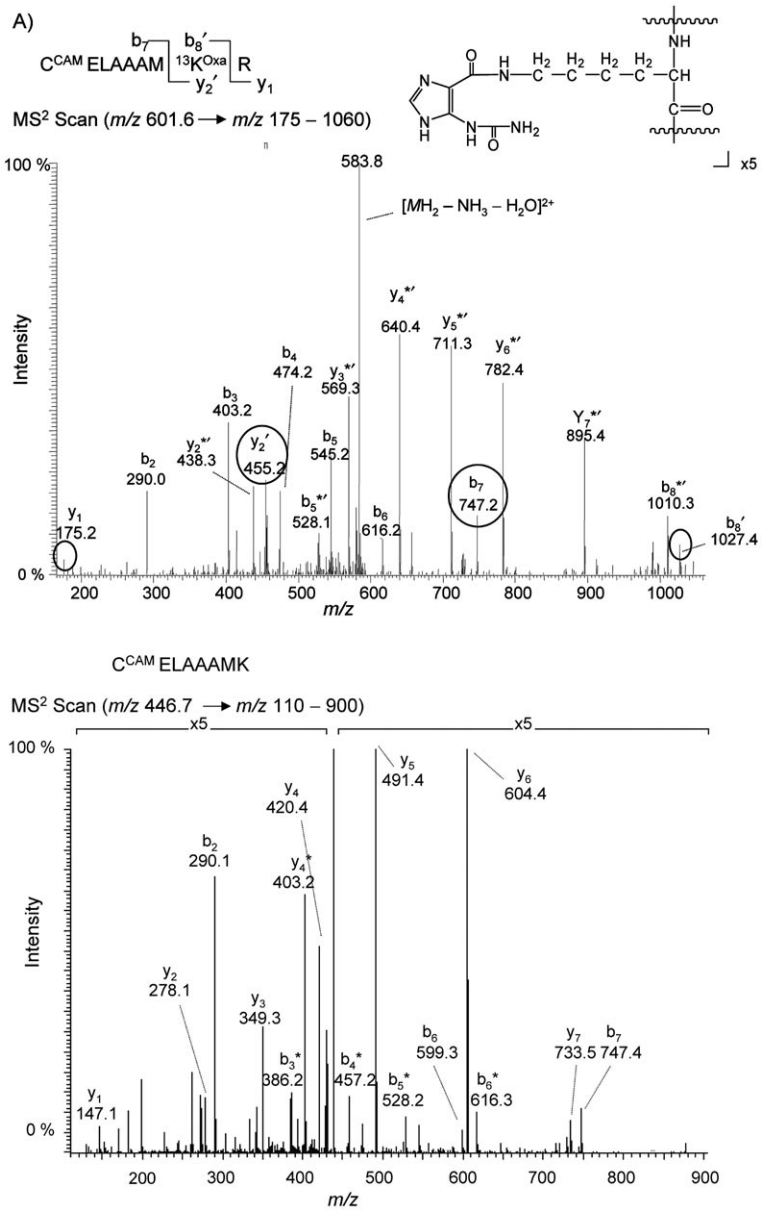


Figure 1. Modification of the lysine residues of lysozyme by dOxo. Collision-induced dissociation was carried out on the $[M+H]^{2+}$ ion A) at *m/z* 601.4 of C^{CAM}ELAAAM$^{13}K^{Oxa}$R (upper panel) and at *m/z* 446.7 of C^{CAM}ELAAAM^{13}K (lower panel), B) at *m/z* 978.6 of $^{97}K^{Oxa}$IVSDGNGMNAWVAWR (upper panel) and at *m/z* 902.2 of KIVSDGNGMNAWVAWR, and C) at *m/z* 743.6 of $C^{CAM116}K^{Oxa}$GTDVQAWIR (upper panel) and at *m/z* 667.4 of C^{CAM116}KGTDVQAWIR (lower panel). The " ′ " and "^{CAM}" superscripts represent Oxa- and carboxyamidomethyl modification, respectively. The "*" represents loss of a NH_3 molecule.

In Figure 1 B, a mass difference of 152 was observed between b_3' (*m/z* 493.3 Da) in the CID spectrum of the modified peptide (upper panel) and b_3 (*m/z* 341.5 Da) in that of the unmodified peptide ^{97}KIVSDGNGMNAWVAIR (lower panel), corresponding to a modification of oxanine. The unmodified y ions, including the y_{15} ion at *m/z* 1675.9 Da, in the CID spectrum of the modified peptide (upper panel) indicated that the peptide was not modified from the second to the last residue, which also supported the assignment of modification on the first residue, Lys97.

Similarly, a mass difference of 152 was observed between the b_2' (*m/z* 441.2 Da) fragment ion in the CID spectrum of the modified peptide (Figure 1 C, upper panel) and the b_2 (*m/z* 289.1 Da) fragment ion in that of the unmodified peptide C^{CAM}KGTDVQAWIR, corresponded to a modification of oxanine at the second residue (Figure 1 C, lower panel). Again, the lysine residue modified with oxanine was not cleaved by trypsin.

Modification of a single Oxa moiety was observed in the peptide NLCNIPCSALLSSDITASVNCAKK. This peptide contains three cysteine, four serine, and two lysine residues. All the

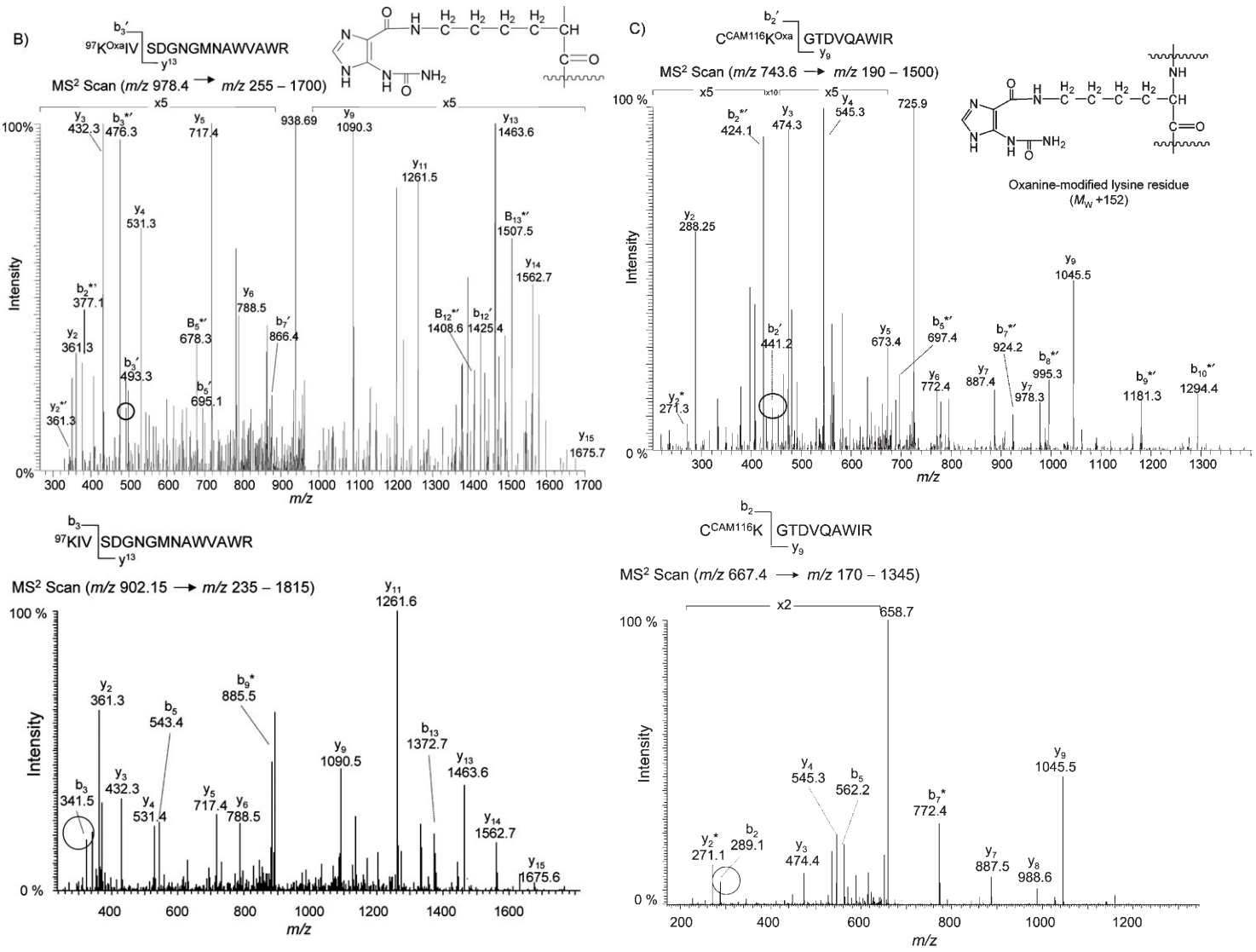


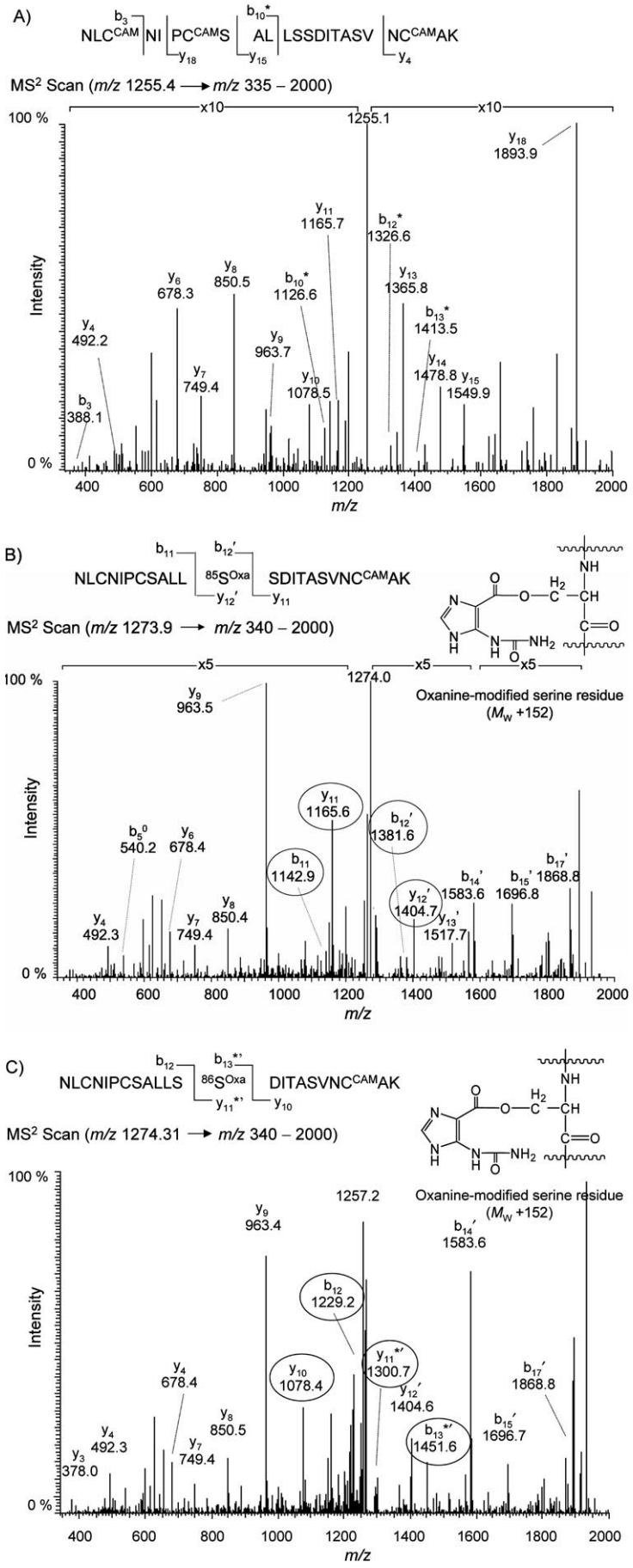
Figure 1 (continued).

three cysteine residues in the parent peptide were modified as CAM derivatives at positions 3, 7, and 21 (Figure 2A). Interestingly, only Ser85 and Ser86, but not Ser81 or Ser91, were modified by an Oxa, and not at the same time, as no doubly modified peptide was detected. Figure 2B shows the CID spectrum indicating modification of Ser85, the twelfth residue of the peptide, based on a mass difference of 239 between the b_{12}' (*m/z* 1381.6 Da) and b_{11} (*m/z* 1142.9 Da) ions and between the y_{12}' (*m/z* 1404.7 Da) and y_{11} (*m/z* 1165.6 Da) ions. On the other hand, a mass difference of 222 was observed between the b_{13}*′ ($b_{13}'-NH_3$, *m/z* 1451.6 Da) and b_{12} (*m/z* 1229.3 Da) fragment ions and between y_{11}*′ ($y_{11}'-NH_3$, *m/z* 1300.6 Da) and y_{10} (*m/z* 1078.5 Da) fragment ions, suggesting Oxa modification on Ser86 (Figure 2c). Furthermore, other fragment ions, including b_{14}' (*m/z* 1583.6 Da), b_{15}' (*m/z* 1696.8 Da), and b_{17}' (*m/z* 1868.8 Da) in Figure 2B and C provided supporting evidence for modification of one Oxa moiety, but not two, on the peptide.

Although addition of an Oxa (+152) and a dOxo (+268) moiety had been set as variable modifications for the peptide mapping in the reaction between dOxo and lysozyme, no modification of dOxo was observed. The 2-deoxyribose of dOxo was probably lost because of the formic acid solution added for centrifugation and during analysis by nanoLC/ESI/MS.

Dose-dependent formation of dOxo-modified peptides in lysozyme

The extent of dOxo modification on lysozyme sites was determined from the peak area ratios of the modified peptides versus those of the unmodified reference peptides in the selected reaction monitoring (SRM) mode. In the SRM experiment, the ion trap selects the precursor ion and acquires full-scan product ion spectra. The formation of a specific fragment ion from each precursor ion is used to construct a chromatogram with the specific reaction listed in Table 1. A typical set of nanoLC/NSI/MS/MS chromatograms of the unmodified and modified peptides in the tryptic digest obtained after the dOxo reaction with lysozyme are shown in Figure 3. Because



modification on the Lys residues led to miscleavage of the site by trypsin, the Lys-modified peptides are longer than the unmodified parent (or reference) peptides. Nonetheless, the retention times of the modified peptides are very close to those of their reference peptides, with a difference of less than 0.5 min.

The dose-response curves constructed in the SRM mode revealed a dose-dependent relationship between the concentrations of dOxo and the extents of modification at specific sites in a semiquantitative manner. At a specific modification site, the percentage of modification—defined as the ratio of peak area for the modified peptide versus the sum of peak areas of the modified and unmodified reference peptides—increases with increasing dOxo concentrations (Figure 4). The results suggest that these modifications are indeed due to the presence of dOxo. This quantification is in a relative sense, with the assumption that the ionization efficiency of the modified peptide is similar to that of the reference peptide. However, both Oxa and CAM modification on Cys might change the ionization efficiency of the peptide. Furthermore, the peptides with lysine modification were not cleaved by trypsin, so they were longer than their reference peptides.

Treatment of oxa-containing calf thymus DNA with lysozyme

It is of interest to know whether the sites of modification on lysozyme reacting with the nucleoside dOxo are the same when Oxa is present in DNA. Calf thymus DNA was incubated with HNO_2 to form Oxa, the yield of which (6.4%) was determined by HPLC with photodiode array detection on the mild acid hydrolysate of DNA.[19] Data-dependent MS analysis of the tryptic digest of the mixture of Oxa-containing DNA with lysozyme after incubation for 4 to 6 days revealed modification on Lys116, Ser85, and Ser86 of the lysozyme (Figure S1 in the Supporting Information). The CID spectra contained the characteristic fragment ions for structural assignments as described for Figure 3, and the sequence coverage of these analyses ranged from 91.5 to 95.3%.

Figure 2. Modification of the serine residues of lysozyme by dOxo. Collision-induced dissociation was carried out on the $[M+H]^{2+}$ ion A) at *m/z* 1255.4 of NLC^{CAM}NIPC^{CAM}SALLSSDITASVNC^{CAM}AK, B) at *m/z* 1274.5 of the modified peptide NLCNIPCSALL^{85}S^{Oxa86}SDITASVNC^{CAM}AK at Ser85, and C) NLCNIPCSALL^{85}S^{86}S^{Oxa}DITASVNC^{CAM}AK at Ser86. The observed b^+ and y^+ fragment ions are shown above the spectrum. The "′" and "^{CAM}" superscripts represent addition of Oxa and carboxyamidomethyl modification, respectively. The "*" and "°" represent loss of an NH_3 and of a H_2O molecule, respectively.

Table 1. Selected reaction monitoring (SRM) conditions with peptides containing lysine, serine, and their modifications in lysozyme by nanoLC/NSI/MS/MS.

Position	Parent and modified peptides	SRM conditions (*m/z*) (parent ion→daughter ion)
$^{13}K^{oxa}$	C^{CAM}ELAAAMK^{oxa}R	601.4 (2+)→290.2 (+1) (b_2)
^{13}K	C^{CAM}ELAAAMK	446.7 (2+)→290.2 (+1) (b_2)
$^{97}K^{oxa}$	K^{oxa}IVSDGNGMNAWVAWR	978.6 (2+)→432.3 (+1) (y_3)
^{97}K	IVSDGNGMNAWVAWR	839.2 (2+)→432.3 (+1) (y_3)
$^{116}K^{oxa}$	C^{CAM}K^{oxa}GTDVQAWIR	743.6 (2+)→673.4 (+1) (y_5)
^{116}K	GTDVQAWIR	523.6 (2+)→673.4 (+1) (y_5)
$^{85}S^{oxa}$	NLCNIPCSALLS^{oxa}SDITASVN-C^{CAM}AK	1274.5 (2+)→1381.6 (+1) (b_{12}')
$^{86}S^{oxa}$	NLCNIPCSALLSS^{oxa}DITASVN-C^{CAM}AK	1274.5 (2+)→1229.2 (+1) (b_{12})
^{85}S, ^{86}S	NLC^{CAM}NIPC^{CAM}SALLSSDITA-SVNC^{CAM}AK	1255.4 (2+)→963.5 (+1) (y_9)

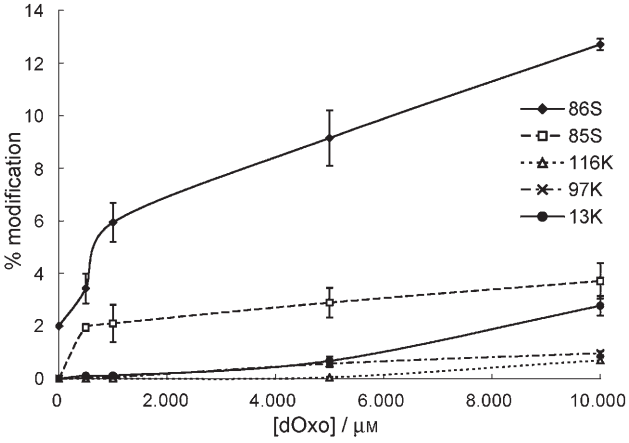


Figure 4. Dose-dependent formation of Oxa-modified peptides with increasing dOxo concentration. The percentage of modification at each reaction site was defined as the ratio of peak area for the modified peptide versus the sum of peak areas of the modified and unmodified reference peptides.

Stabilities of dOxo cross-links with GSH, NAc-Tyr, and NAc-Lys

We wondered whether the stability of dOxo crosslinks might have been the reason for not detecting modifications on cysteine and tyrosine, residues with a stronger nucleophilic side chain than Lys. We found that the half-life of dOxo-*S*-GSH, the thioester product of dOxo with glutathione,[19] at 37 °C was 11.2 days at pH 7.4 and 2.2 days at pH 8.0. On the other hand, the product of dOxo with *N*-acetyltyrosine has a half-life of 5.5 and 1.4 days at pH 7.4 and 8.0, respectively. The half-life of dOxo amide cross-linked product with glycine has been reported to be 1280 h (53.3 days) at pH 7.4.[17] We also examined the stability of dOxo cross-linked product with N^{α}-acetyllysine and determined its half-life as 91 and 101 days at pH 7.0 and 8.0,

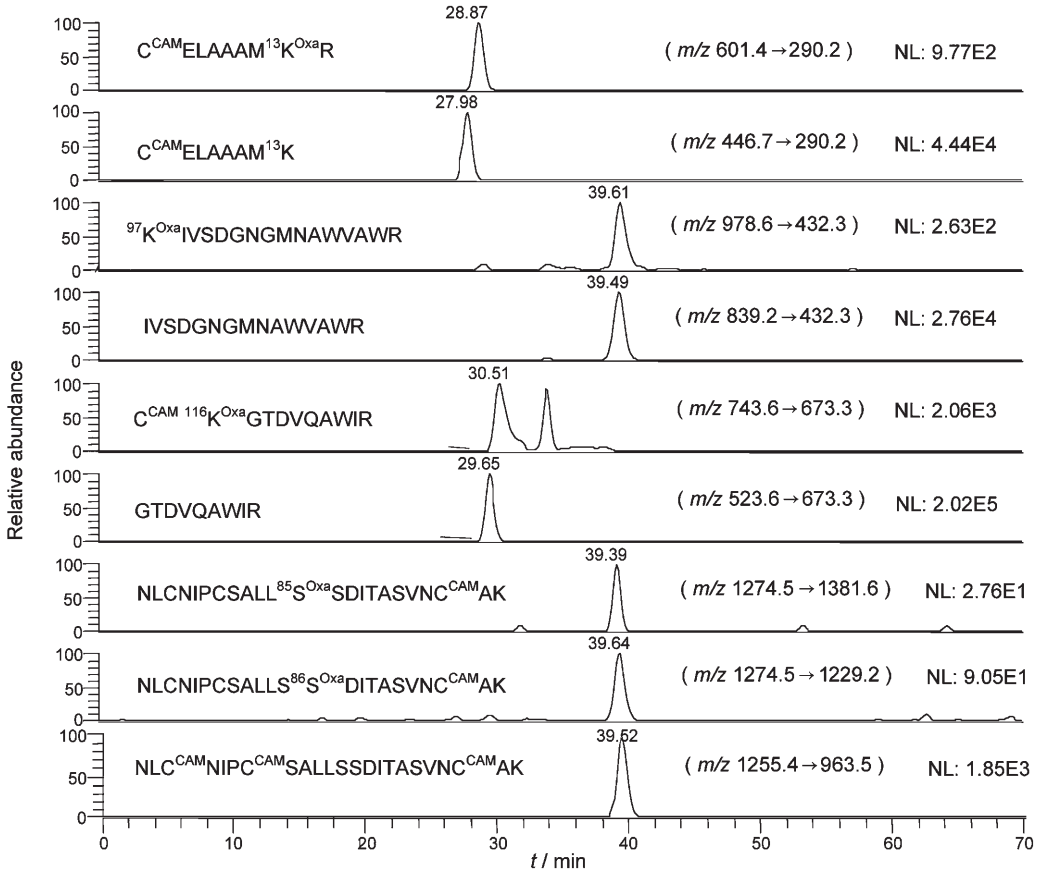


Figure 3. NanoLC/NSI/MS/MS chromatograms of the parent and modified peptides in the tryptic digests obtained after dOxo treatment of lysozyme analyzed under their selected reaction monitoring conditions listed in Table 1.

respectively. Unfortunately, we did not obtain sufficient amounts for half-life measurement of the cross-linked product between dOxo with *N*-acetylserine, due to the low reactivity of *N*-acetylserine with dOxo at or near neutral pH. When dOxo was incubated with *N*-acetylserine under strongly alkaline conditions, dOxo decomposed to a great extent before the product could be isolated. However, the detection of dOxo-serine cross-links in reactions of lysozyme with dOxo and with Oxa-containing DNA indicated that the environment of serine residues in the protein might increase its nucleophilicity.

The solvent-accessible surface area of residues with nucleophilic side-chains

There are eight cysteine residues in a lysozyme, forming four pairs of disulfide bonds. The six lysine, three tyrosine, ten serine, and eleven arginine residues are also possible sites of modification by dOxo. What are the factors determining the selectivity of lysozyme modification by dOxo? We show here that the modification sites of lysozyme correlate with the solvent-accessible surface areas of residues with nucleophilic side-chains, the secondary structures of the reactive residues, and the stabilities of the cross-links. With the aid of a computer program—GETAREA 1.1 (http://pauli.utmb.edu/cgi-bin/get_a_form.tcl)—the solvent-accessible surface areas (SASAs) of residues in lysozyme with nucleophilic side-chains were calculated and are listed in column 2 of Table 2. The results show that none of the cysteine residues of lysozyme is solvent-exposed, which explains in part why the most nucleophilic residues were not modified. Furthermore, three of the six lysine residues usually considered to lie on the outside of the protein—Lys13, Lys97, and Lys116—are found to be modified with dOxo. In the incubation mixture of Oxa-containing DNA and lysozyme, no modification on Lys13 and Lys97 was observed, which might be due to steric hindrance, as these two residues are part of the α-helix structure according to RasMol Molecular Graphics (Version 2.6),[28] for which the major source of data files is the Protein Databank at Brookhaven National Labs. On the other hand, Lys116 is part of a β-turn structure and was modified by the Oxa-containing DNA. It has been shown that the reactivities of the lysine ε-amino groups on lysozyme correlate with their surface accessibilities.[29,30]

None of the three tyrosine residues lies on the outside of the protein. Together with the instability of the cross-linked product, if formed, the absence of any detection of tyrosine cross-links can thus be explained. Interestingly, two of the three serine residues usually considered solvent-exposed—Ser85 and Ser86—are modified. Although Ser81 is considered highly solvent-accessible, the computer-generated structure revealed that Ser81 is part of a α-helix, while Ser85 and Ser86 are part of the coil. Although most of the arginine residues are solvent-exposed, they are not modified, possibly because of the high pK_a and low nucleophilicity of the side chain at the pH of the incubation.

Although Oxa lesions on DNA can be repaired by base and nuclear excision repair enzymes,[20–23] the observation that an Oxa moiety on an oligonucleotide reacts much more rapidly than other proteins with DNA glycosylases[18] indicates that Oxa inactivates base excision repair enzymes. It is of interest to determine factors governing the preference of protein targets of Oxa. Since DPCs might be a form of cellular lesion that is difficult to repair,[31–33] the full biological significance and consequences of Oxa-derived DPC formation remains to be investigated. To the best of our knowledge, this is the first study with convincing mass spectrometric evidence of Oxa-derived

Table 2. The solvent-accessible surface areas of residues with nucleophilic side chains.

Residue	Side chain [Å^2]	Ratio [%]	In/out
Cys (random coil: 102.3)			
6	37.46	36.6	m
30	0.09	0.1	i
64	0	0	i
76	0.077	0.8	i
80	8.48	8.3	i
94	0	0	i
115	1.14	1.1	i
127	5.63	5.5	i
Lys (random coil: 164.5)			
1	101.35	61.6	o
13	89.18	54.2	o
33	44.24	26.9	m
96	61.94	37.7	m
97	126.25	76.7	o
116	107.34	65.3	o
Tyr (random coil: 193.1)			
20	68.51	35.5	m
23	95.32	49.4	m
53	49.42	25.6	m
Ser (random coil: 77.4)			
24	29.98	38.7	m
36	1.2	1.5	i
50	19.72	25.5	m
60	0.16	0.2	i
72	7.33	9.5	i
81	81.11	100	o
85	48.84	63.1	o
86	51.65	66.7	o
91	0	0	i
100	28.56	36.9	m
Arg (random coil: 195.5)			
5	155.93	79.8	o
14	159.32	81.5	o
21	163.5	83.6	o
45	132.51	67.8	o
61	82.8	42.4	m
68	182.79	93.5	o
73	116.84	59.8	o
112	101.34	51.8	o
114	128.22	65.6	o
125	168.92	86.4	o
128	188.68	96.5	o

[a] Solvent-accessible surface areas were calculated with GETAREA 1.1 (http://pauli.utmb.edu/cgi-bin/get_a_form.tcl). The contributions from side chain atoms are listed in the second column. The next column lists the ratio of side chain surface area to "random coil" value per residue. The "random coil" value of a residue X is the average solvent-accessible surface area of X in the tripeptide Gly-X-Gly in an ensemble of 30 random conformations. Residues are considered to be solvent-exposed if the ratio value exceeds 50% (marked as "o") and to be buried if the ratio is less than 20% (marked as "i") in the last column. Residues with ratios between 20–50% are marked as "m".

DPC formation from DNA and a protein. This study should shed light on the mechanism of DPC formation by reactive nitrogen oxide species and their detection, repair, and role in inflammation-related diseases.

Experimental Section

Materials: Double-stranded calf thymus DNA and lysozyme from chicken egg white were purchased from Sigma. 2′-Deoxyoxanosine (dOxo) and oxanine (Oxa) were synthesized from 2′-deoxyguanosine and purified by previously published procedures.[19] All reagents are of reagent grade or above.

Dose-dependent reaction of dOxo with lysozyme: A solution (total volume of 100 μL) containing lysozyme (0.1 mM) and various concentrations of dOxo (0, 0.5, 1.0, 5 or 10 mM) in potassium phosphate buffer (200 mM, pH 8.4) was incubated at 37 °C for 24 h. SDS and DTT were added to a portion of the incubation mixture (14.2 μL, 20 μg lysozyme) to give final concentrations of 1% and 10 mM, respectively, and the system was then incubated at 95 °C for 5 min, followed by alkylation with iodoacetamide (final concentration 50 mM) in ammonium bicarbonate (10 mM) at room temperature for 30 min in the dark. The reagents were removed by addition of cold acetone (10 × volume) to the reaction mixture, which was kept at −20 °C for 15 min and then centrifuged for 10 min at 23000 *g* at 0 °C, followed by washing with 70% cold acetone (600 μL). The supernatant was removed, and the precipitate was dried under vacuum. The precipitate was dissolved in ammonium bicarbonate buffer (100 mM, pH 8.0, 40 μL), trypsin (10:1, *w/w*, 2.0 μg) was added, and the system was incubated at 37 °C for 18 h. Formic acid (0.1%, 160 μL) was added to the mixture, which was loaded onto a Nanosep 3K centrifugal device (Pall Life Sciences, Ann Arbor, MI), and centrifuged at 15000 *g* for 20 min. The filtrate was diluted with formic acid (0.1%), and a portion (2 μL) equivalent to 2 pmol of the starting lysozyme was filtered through a 0.22 μm Nylon syringe filter prior to nanoLC-nanospray ionization tandem mass spectrometry (nanoLC/NSI/MS/MS) analysis.

Synthesis of dOxo-containing DNA: The dOxo-containing DNA was obtained by incubating a solution containing calf thymus DNA (final concentration of 1.5 mg mL^{-1}) and sodium nitrite (0.5 M) in sodium acetate buffer (0.2 M, pH 3.7) in a final volume of 0.5 mL at 37 °C for 24 h. The reaction mixture was precipitated with cold ethanol (9.5 mL) and centrifuged at 22000 *g* at 0 °C for 20 min. The supernatant was removed, and the precipitate was washed twice with cold ethanol (70%, 3 mL), followed by centrifugation at 23000 *g* at 0 °C for 20 min. A portion of the precipitate was evaporated and hydrolyzed with HCl (0.1 N, 0.5 mL) at 60 °C for 30 min. The pH of the hydrolysate was adjusted to 5.5 prior to analysis by the following HPLC/UV system for oxanine content.[19]

HPLC chromatography was performed with a Hitachi L-7000 pump system with a D-7000 interface (Hitachi Ltd., Tokyo, Japan), a Rheodyne injector (Rheodyne, Inc., Cotati, CA), and an L-7450 A photodiode array detector (Hitachi Ltd., Tokyo, Japan) with a Prodigy ODS (3) column (4.6×250 mm, 5 μm column (Phenomenex, Torrance, CA)). Elution was with a linear gradient from ammonium formate buffer (50 mM, pH 5.5, 100%) to methanol in ammonium formate (50 mM, pH 5.5, 25%) from 0 to 25 min at a flow rate of 1.0 mL min^{-1}.

Reaction of dOxo-containing calf thymus DNA with lysozyme: A solution of dOxo-containing calf thymus DNA (final concentration 2.3 mg mL^{-1}) containing Oxa (0.1 mM) and lysozyme (0.1 mM) in potassium phosphate buffer (0.2 M, pH 8.4) in a final volume of 0.1 mL was incubated at 37 °C for 4 days. HCl was added to the reaction mixture to pH 1.0, and the mixture was heated at 70 °C for 30 min. The acid hydrolysate was neutralized to pH 7.4 with ammonium bicarbonate, and the small molecules were filtered through a Nanosep 3K centrifugal device with centrifugation at 15000 *g* for 20 min. The cross-linked lysozyme solution above the filter was denatured, alkylated, and digested with trypsin as described above, prior to nanoLC/NSI/MS/MS analysis.

NanoLC/NSI/MS/MS and data analysis: All MS/MS experiments for peptide identification were performed with an LTQ linear ion trap mass spectrometer (Thermo Electron Corp., San Jose, CA) fitted with a nanospray ionization source. The source was coupled online to an X'TremeSimple nanoflow LC system (Microtech, Orange, CA). Each sample (2 μL) was manually injected onto a Bio-Basic C18 column (150 mm × 75 μm, 5 μm, 100 Å, Thermo Electron Corp., Bremen, Germany). The mobile phases A and B were composed of 5% and 80% acetonitrile in formic acid (0.01%, pH 3.2), respectively. A linear gradient was employed from 5% B to 50% B from 0 to 60 min and from 50% B to 100% B over the next 15 min, and it then was held at 100% B for another 10 min at a flow rate of 300 nL min^{-1}. The column was equilibrated with 5% B for 20 min before the next run. The MS and MS/MS spectra were obtained at a heated capillary temperature of 200 °C with a spray voltage of 1.3 kV, a tube lens voltage of 75 V, a normalized collision energy setting of 45%, and an ion gauge pressure of 6.5×10^{-6} torr. Ions were isolated with a mass isolation width (*m/z*) of 1.0. Activation (*Q* value) for CID was set at 0.25 with an activation time of 30 ms. Mass spectrometers were operated in the data-dependent scan mode, automatically switched between full MS scan (*m/z* 250–2000) and MS/MS scan for the three most abundant peaks in a given MS spectrum. Peptide fingerprinting from the MS/MS data was performed by use of the Turbo SEQUEST algorithm incorporated in BioWorks version 3.2 (Thermo Electron Corp., San Jose, CA) to correlate the data against the NCBI equine protein database (National Center for Biotechnology Information, Bethesda, MD). Monoisotopic mass was used for the search. The mass tolerances for peptide and fragment ions were at 2.0 and 1.0 amu, respectively. The default charge was +2, and two trypsin miss cleavage were allowed. Carboxyamidomethylation (+57) on cysteine as well as addition of an Oxa (+152) or dOxo (+268) moiety were set as variable modifications.

The selected reaction monitoring (SRM) experiments were performed by selecting the precursor ion and acquiring full-scan product ion spectra. The formation of a specific fragment ion from each precursor was used to construct the chromatogram. The collision gas was argon, and the collision energy was 45%. The specific SRM conditions for peptides containing lysine, serine, and their modifications are listed in Table 1.

Stabilities of dOxo cross-links with GSH, NAc-Tyr, and NAc-Lys: The cross-linked products of dOxo with glutathione (GSH), *N*-acetyltyrosine (NAc-Tyr), and N^{α}-acetyllysine (NAc-Lys) were synthesized and purified from incubation mixtures of dOxo with GSH or NAc-Tyr or NAc-Lys by reported procedures.[19] The purified dOxo-GSH, dOxo-NAc-Tyr, or dOxo-NAc-Lys (0.1 mM) was incubated in potassium phosphate buffer (100 mM, pH 7.4 or 8.4) at 37 °C, and the reaction was monitored for the recovery of the starting material with time by the reversed-phase HPLC method reported previously.[19]

Abbreviations: CID, collision-induced dissociation; DDT, dithiothreitol; DPCs, DNA–protein cross-links; GSH, reduced glutathione; NAc, *N*-acetyl, dOxo, 2′-deoxyoxanosine (5-amino-3-β-(D-2′-deoxy-

ribofuranosyl)-3*H*-imidazo[4,5-*d*][1,3]oxazine-7-one); NSI/MS/MS, nanospray ionization tandem mass spectrometry; Oxa, oxanine (5-amino-3*H*-imidazo[4,5-*d*][1,3]oxazin-7-one); RNOx, reactive nitrogen oxide species; SASA, solvent-accessible surface area; SRM, selected reaction monitoring.

Acknowledgements

This work was supported by grants NSC 94-2113M-194-014 and NSC 95-2119M-194-004 from the National Science Council of Taiwan and from National Chung Cheng University (to H.-J.C.C.).

Keywords: DNA–protein cross-links · lysozyme · mass spectrometry · nitrogen oxides · oxanine

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Received: November 12, 2007
Published online on March 20, 2008

