Specific Disulfide Formation in the Oxidation of HIV-1 Zinc Finger Protein Nucleocapsid p7

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In vitro oxidation of the HIV-1 nucleocapsid protein p7 by the C-nitroso compound 3-nitrosobenzamide (NOBA) has been investigated. When reconstituted p7 was incubated with NOBA, three disulfide bonds were formed per molecule of p7, Cys 15–Cys 18, Cys 28–Cys 36, and Cys 39–Cys 49. These were identified using the proteolytic enzyme endoproteinase Lys-C and mass spectrometry. When the denatured protein (Apo-p7) was incubated with NOBA, a more random pattern of multiple S–S linkages was found. Oxidation of reconstituted p7 also occurred on treatment with cupric ions (Cu^{2+}), and the same three major disulfide bonds were formed as in the reaction with NOBA. These results suggest the interpretation that the oxidation reaction occurs at the zinc-binding centers while zinc cations are still bound and that the two zinc fingers are not identical in their chemical properties. This latter point is consistent with the independent biological roles reported previously for the two fingers in the viral infection cycle.

Introduction

The retroviral nucleocapsid protein of human immunodeficiency virus HIV-1 contains two CCHC-type zinc fingers (*1–3*), each of which binds zinc stoichiometrically with three cysteine thiols and a histidine imidazole group (*4–6*). These zinc complexes and the protein folding that they stabilize are essential for viral genome recognition during budding, genomic RNA packaging, and early events in viral infection (*7–9*).

Nuclear magnetic resonance experiments (*10*) have demonstrated that an 18-residue peptide with HIV-1 nucleocapsid N-terminal zinc finger sequence ejected zinc when it was treated with two C-nitroso compounds. The same peptide had been shown previously to have sequence-dependent, high-affinity binding to oligonucleotide d(ACGCC) (*11*). Addition of 3-nitrosobenzamide (NOBA)[1] and 6-nitroso-1,2-benzopyrone resulted in the dissociation of the complex, indicating the loss of the "finger" domain.

This susceptibility of retroviral zinc fingers is currently being exploited in the design of new agents with potential antiviral activity. Mutation of either of the two zinc fingers in the native HIV-1 nucleocapsid protein p7 yields noninfectious virus (*7, 9*), which suggests that the zinc fingers are ideal targets for the development of new antiviral drugs. The antiviral activity of NOBA has been demonstrated against HIV (*12*) and against SIV (*13*) in cell culture.

A detailed characterization of the reaction between the complete viral zinc finger protein p7 and C-nitroso compounds is of interest to the search for more effective antiviral drugs. In this report, we identify the products found in vitro in the reaction between the p7 protein and NOBA as a representative C-nitroso oxidizing agent. Only three disulfide bonds were identified by proteolytic cleavage and mass spectrometric analyses. This same specificity was also observed in the oxidation of p7 by Cu^{2+}. The patterns of disulfide formation are different in the two zinc complexes, consistent with an earlier observation (*9*) that the two zinc fingers in p7 are not functionally equivalent. A more diverse set of disulfide bonds was formed in control reactions using demetallated apo-p7.

Experimental Section

Chemicals. 3-Nitrosobenzamide (NOBA), mp 135 °C dec, was prepared by the oxidation of 3-aminobenzamide (Aldrich, Milwaukee, WI) by 2 equiv of 3-chloroperoxybenzoic acid in dimethylformamide solution at 5 °C as described previously (*14*). High-resolution EIMS: calcd for $C_7H_6N_2O_2$ 150.0429; found M^+ (m/z): 150.0429. 3,3′-Dicarbamoylazoxybenzene (the dimeric azoxy derivative of NOBA), mp 280–284 °C dec, was isolated as a side product (precipitate) from the NOBA synthesis reaction. High-resolution EIMS: calcd for $C_{14}H_{12}N_4O_3$ 284.0909; found M^+ (m/z): 284.0903.

Protein Preparation. The coding sequence for the nucleocapsid protein of HIV-1 (p7) was cloned[2] and expressed in *Escherichia coli* using an inducible expression vector p-Mal (New England Biolabs, MA). The protein was released from a fusion protein by proteolysis with factor Xa and purified as apo-p7 by reversed phase high performance liquid chromatography (HPLC). The protein was characterized by both microchemical and mass spectrometric amino acid sequencing (*2, 15*).

The p7 protein was reconstituted at 15 μM concentration in 50 mM Tris-HCl buffer at pH 7.5 with 2 equiv of $ZnCl_2$ to

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[1] Abbreviations: FAB, fast atom bombardment; NOBA, 3-nitrosobenzamide; ESMS, electrospray mass spectrometry; HPLC, high pressure liquid chromatography.
[2] Powell and Henderson, unpublished.

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generate native protein and its folded structure. The reconstituted Zn_2–p7 protein complex was also characterized as a three partner noncovalent complex by electrospray ionization mass spectrometry (*15*, *16*).

Oxidation of Reconstituted p7. The reconstituted p7 protein was treated with 20 molar equiv of NOBA or 10-fold excess of cupric chloride in 50 mM Tris-HCl buffer at pH 7.5. The reaction mixtures were incubated for times varying between 5 and 120 min at 37 °C. The reaction products of p7 were purified from reversed phase HPLC, using a C8 column (Aquapore RP-300, 4.6 × 250 mm, Applied Biosystems, San Jose, CA) and a solvent gradient: 0.08% trifluoroacetic acid in acetonitrile 10% to 40% in 20 min, in 0.1% trifluoroacetic acid in water.

Oxidation of Denatured Apo-p7. HIV-1 nucleocapsid protein p7 was denatured and separated from zinc cations on reversed phase HPLC at pH 2. The apoprotein was collected and freeze-dried. Reactions between 1 mL of a 15 μM solution of the apoprotein and 20 molar equiv of NOBA were carried out at pH 7.5 (50 mM Tris-HCl) at 37 °C, for times varying between 5 and 120 min, and were monitored by HPLC.

Mass Spectrometric Analysis. Electrospray ionization mass spectrometry (ESMS) was performed on a Vestec (Houston, TX) electrospray source fitted to a Hewlett-Packard (Palo Alto, CA) 5988A quadrupole mass spectrometer. All ions were detected with a Phrasor (Duarte, CA) high energy dynode ion detector, and data were collected with a Technivent (St. Louis, MO) Vector II software system. Samples were injected through a Rheodyne (Cotati, CA) 7125 loop injector fitted to a Harvard syringe pump (South Natick, MA) at 10 μL/min flow rate. The stainless steel needle was held at around 2.2 kV, and the source temperature was maintained at 240 °C. All samples were first dissolved in 6% acetic acid and then mixed with an equal volume of methanol. The instrument was calibrated using melittin and myoglobin.

Matrix assisted laser desorption mass spectrometry was carried out on a Kratos/Shimadzu Kompact III instrument, using the reflectron mode with a 337 nm UV laser. The matrix was α-cyano-4-hydroxycinnapinic acid used in a ratio to the sample of about 10 000:1. Prepro nerve growth factor and the protonated matrix dimer were used as external mass calibrants.

Fast atom bombardment (FAB) mass spectrometry was performed on the first two sectors of a JEOL HX110/HX110 tandem mass spectrometer (Tokyo, Japan). A JEOL FAB gun was operated at 6 kV with xenon as FAB gas. The accelerating voltage was 10 kV. Thioglycerol was used as the FAB matrix. The resolution was set at 500, and all data were collected with a JEOL DA 7000 data system. All molecular weights reported here are averages (*17*).

Proteolysis of Oxidized p7 and Apo-p7. Oxidized proteins (80 μg) were hydrolyzed by endoproteinase Lys-C (Boehringer Mannheim, Mannheim, Germany) without dithiothreitol, in 100 mM potassium phosphate at pH 7.9. The proteolysis was carried out at 37 °C for 10–15 min with a protease/protein ratio (w/w) of 1:50.

Peptide mixtures were purified on reversed phase HPLC (Aquapore RP 300 column, 4.6 × 250 mm, Applied Biosystems). The solvent gradient was 0.08% trifluoroacetic acid in acetonitrile (5% to 45% in 40 min) in 0.1% trifluoroacetic acid in water. Resolved peptides from reconstituted p7 were analyzed by electrospray and fast atom bombardment mass spectrometry. Peptides from apo-p7 were analyzed by laser desorption mass spectrometry.

Results

Reaction of NOBA and Cu^{2+} with Zn-Reconstituted p7. A solution of p7 reconstituted with zinc was incubated with NOBA as described in the Experimental Section. The reaction was followed by reversed phase HPLC. The peak comprising apo-p7 decreased in size, and an asymmetric peak appeared at shorter retention time (Figure 1). (Under the chromatographic conditions at pH 2, zinc is lost and the protein elutes as apo-p7.)

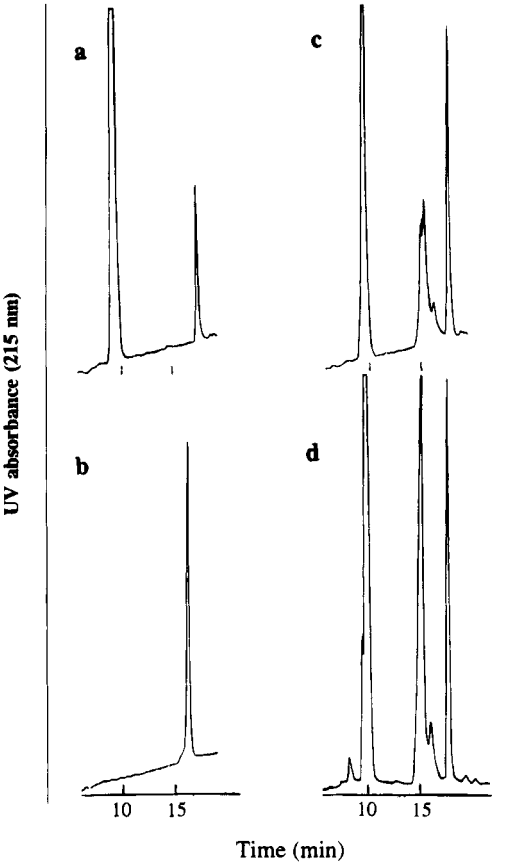

Figure 1. Analysis of p7 reactions with NOBA by reversed phase high performance liquid chromatography. (a) NOBA reagent before reaction with p7 (the sharp peak near the end of elution corresponds to the dimeric azoxy derivative of NOBA); (b) p7 protein before reaction; (c) p7 and NOBA reaction mixture after 2 h of incubation; (d) apo-p7 and NOBA reaction after 2 h of incubation.

The product was collected as one fraction from HPLC and characterized by ESMS (Figure 2). The major component was found to have a molecular mass of 6445.3 daltons. Apo-p7 has a calculated molecular mass of 6451.6 daltons, determined by ESMS as 6451.3 daltons. Thus the major product from the reaction of NOBA weighs 6 daltons less than apo-p7. The new protein was reduced by 2-mercaptoethanol to a protein that coeluted with apo-p7, consistent with disulfide formation.

Cupric chloride was also incubated with Zn-reconstituted p7. The chromatogram was similar to that from the NOBA reaction, and the peak area of apo-p7 was reduced by about half after 6 min. ESMS showed that the modified protein mixture has a molecular mass of 6445.7 daltons, again 6 daltons less than that of apo-p7. Two minor peaks eluting after the apo-p7 peak were found to have molecular masses of 12 896.6 daltons. These were not examined further in this study.

Reaction of NOBA with Apo-p7. The products formed in this control reaction eluted in a new peak with a shorter retention time under the same HPLC conditions used for the previous two analyses. This material was also found to have the molecular mass of fully oxidized p7 (6445.2 daltons).

Identification of the Positions of Disulfide Bonds. Oxidized proteins purified from the NOBA–p7, Cu^{2+}–p7, and NOBA–apo-p7 reactions were subjected to endoproteinase Lys-C hydrolysis. The molecular masses of the resulting peptides were determined by both ESMS and FABMS. Disulfide bridges were identified by com-

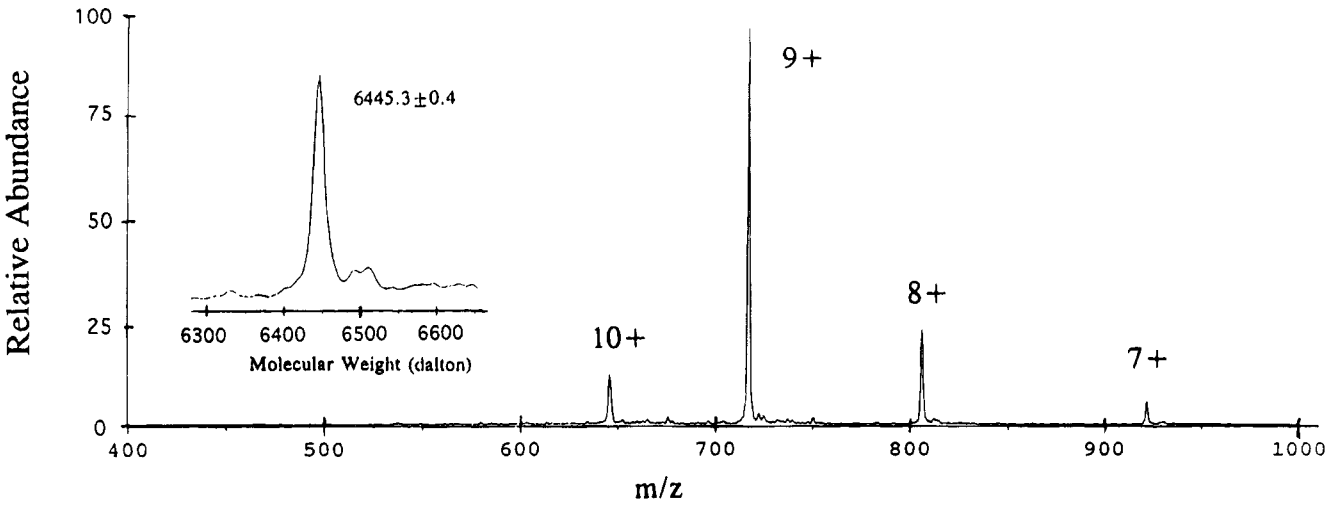

Figure 2. Mass spectrum of the peak eluting at 15 min produced by the reaction of Noba with p7 (Figure 1c).

Table 1. Molecular Masses of Peptides Produced by Endoproteinase Lys-C from NOBA- and Cu^{2+}-Oxidized p7[a]

peptide	calcd MW[b] (apo-p7)	obsd MW (apo-p7)	obsd MW (NOBA)	obsd MW (Cu^{2+})
[1–11]	1434.6	1435.1	1434.0	1434.0
[12–14]	372.5	372.5	372.8	372.7
[15–20]	670.8	670.6	668.8	669.2
[21–26]	653.7	653.6	653.7	653.0
[27–38]	1474.8	1474.2	1472.8	1472.6
[39–55]	1935.2	1935.2	1933.0	1932.9

[a] Sequence of p7 protein: MQRGNFRNQRKIIKC[15]FNC[18]GK-EGHIAKNC[28]RAPRKRGC[36]WKC[39]GKEGHQMKDC[49]TERQAN. [b] Molecular weights are calculated for unmodified peptides from reduced apo-p7 as isotopic averages (*17*).

paring the observed masses of the fragments to the theoretical masses of peptides resulting from all possible disulfide pairings. Peptides were also mapped from reduced apo-p7 as a control.

For the NOBA–p7 and Cu^{2+}–p7 reactions, fragments without Cys were easily identified on the basis of their measured and calculated molecular masses (Table 1). Three major peaks with molecular masses of 668.8, 1472.8, and 1933.0 daltons were also detected, which uniquely matched and were assigned as peptides [15–20], (Cys 15–Cys 18); [27–38], (Cys 28–Cys 36); and [39–55], (Cys 38–Cys 49). As further confirmation, the peptides were converted to their methyl esters and appropriate molecular masses were determined (data not shown). As shown in Table 1, the complete sequence of 55 residues was accounted for.

The same proteolytic cleavages and disulfide linkages were identified in p7 oxidized by Cu^{2+} ions (Table 1). Three disulfide-containing peptides were identified with molecular masses of 669.2, 1472.6, and 1932.9 daltons. These are consistent with pairing between the two cysteines within each peptide, [15–20], [27–38], and [39–55], respectively.

To elucidate the determinants of this specific pattern of S–S pairing, apo-p7 was incubated with NOBA. Digestion of the reaction product with endoproteinase Lys-C resulted in a larger mixture of peptides (Table 2). All possibilities of disulfide pairs were detected, except the linkage between Cys 36 and Cys 49. This wide variety stands in contrast to the trio formed in the folded protein. Table 3 summarizes the different patterns.

Table 2. Molecular Masses of Peptides Produced by Endoproteinase Lys-C from NOBA-Oxidized Apo-p7

obsd MW	peptide	calcd MW[a]
669.1	[15–20]	668.8
935.3	[34–41]	935.2
1148.6	[27–33] + [39–41]	1148.4
1241.1	[39–41] + [48–55]	1240.3
1491.8	[27–33] + [34–38]	1490.8
1778.3	[27–33] + [48–55]	1778.0
1318.0	[15–20] + [34–38]	1317.6
1513.5	[15–20] + [27–33]	1512.8
1622.4	[15–20] + [34–38] + [39–41]	1622.0
2159.3	[15–20] + [27–33] + [34–38]	2159.6
2251.3	[15–20] + [34–38] + [48–55]	2251.5

[a] Molecular weights are calculated for oxidized peptides from apo-p7 as isotopic averages (*17*).

Table 3. Disulfide Bonds Detected after NOBA Treatment

disulfide bonds	reconstituted p7	apo-p7
Cys 15–Cys 18	+	+
Cys 15–Cys 28, Cys 18–Cys 36[a]	–	+
Cys 15–Cys 28, Cys 18–Cys 39[a]	–	–
Cys 15–Cys 28, Cys 18–Cys 49[a]	–	–
Cys 15–Cys 36, Cys 18–Cys 39[a]	–	+
Cys 15–Cys 36, Cys 18–Cys 49[a]	–	+
Cys 15–Cys 39, Cys 18–Cys 49[a]	–	–
Cys 28–Cys 36	+	+
Cys 28–Cys 39	–	+
Cys 28–Cys 49	–	+
Cys 36–Cys 39	–	+
Cys 36–Cys 49	–	–
Cys 39–Cys 49	+	+

[a] Or alternative isomeric linkages.

Discussion

It was demonstrated previously that zinc was ejected and the three-dimensional folding was lost when a synthetic HIV-1 zinc finger peptide was treated with NOBA and NOBP (*10*). However, the reaction products were not determined. An oxidative mechanism of action of NOBP on poly(ADP-ribose) polymerase, which also contains two retroviral CCHC-type zinc fingers, has been proposed (*18*, *19*). Furthermore, the two zinc fingers in the HIV-1 nucleocapsid protein p7 were found to be not functionally equivalent, suggesting an independent role of each finger in viral RNA selection and packaging (*9*). The susceptibility of the two zinc fingers in the native p7 protein toward oxidizing reagents and their importance in the viral infectious cycle justify the determination of chemical mechanisms.

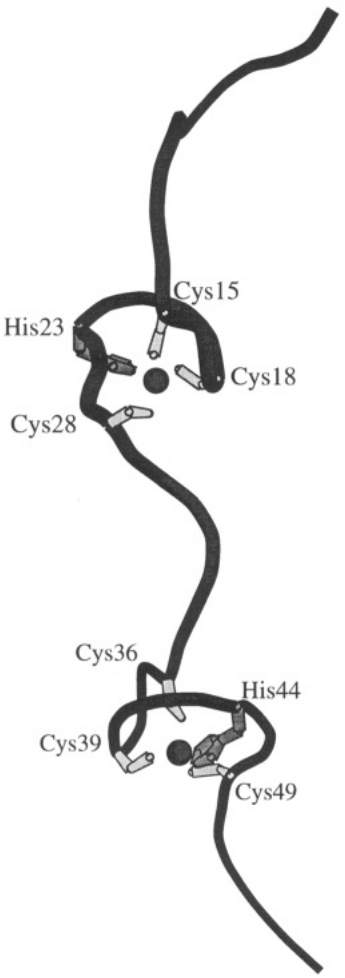

Figure 3. Backbone representation of HIV-1 NC protein, adapted from Summers et al. (1992) (*21*). The structure presented was transformed from ribbon model to tube model using the Molscript program. Balls represent the two zinc atoms; the amino acid residues chelating the zinc atom are pointed out from the main chain.

Since there are three cysteines in each zinc finger (Figure 3) and a total of six cysteines in HIV-1 nucleocapsid protein p7, a molecular mass decrease of six daltons in p7 after the NOBA treatment suggests the formation of three disulfide bonds. Such an oxidation reaction is predictable because C-nitroso compounds are known to be oxidizing reagents toward thiols (*18*, *19*). In other experiments we have determined that NOBA is reduced to 3-(hydroxyamino)benzamide by way of p7-bound adducts. Molecular masses of p7 adducts were observed by ESMS at 6602.4 and 6751.8 daltons, consistent with covalent bond formation between cysteine sulfur and the nitroso nitrogen atoms of 1 and 2 NOBA molecules. The hydroxylamine species at neutral pH readily dismutates and condenses with unreacted nitroso molecules to form the stable dimeric azoxy derivative 3,3′-dicarbamoylazoxybenzene, a process that commonly occurs during the mild reduction of aromatic nitroso compounds (*20*).

The more promiscuous pattern of disulfide pairing found to occur in the demetallated and denatured protein suggests that oxidation of the native or reconstituted protein by NOBA occurs at the Zn-binding centers while the metal cations are still bound.

Closer examination of the oxidation of reconstituted p7 reveals differences in the disulfide linkages formed within each of the two zinc fingers, Cys 15–Cys 18 in finger 1 and Cys 39–Cys 49 in finger 2. In one case the disulfide bond is connected with two intervening amino acid residues and in the other case with nine. It seems likely that the geometry and distribution of electron densities within the metal chelate coordinates disulfide pairing in a specific manner.

Asymmetry in the CCHC-type metal complexes may promote uneven electron distributions among the three thiolate centers in each finger (Figure 3). In addition, three different types of NH–S hydrogen bonds are present within each zinc finger, and there is unique hydrogen bonding between a glutamine side chain and cysteine sulfur in the N-terminal finger (*21*, *22*). Combined with the specific sequences within the each of the zinc binding motifs, these properties may result in different reactivities of the various cysteine residues toward NOBA. Differences in the chemical reactivities of the two zinc fingers in the HIV-1 nucleocapsid protein are consistent with earlier observations (*9*) of functional differences and the independent role of each finger in viral RNA selection and packaging and viral infection.

It is interesting that Cu^{2+} produced the same intramolecular disulfide pattern in the oxidation of p7. Since the oxidizing reagents are quite different, this further suggests that the intramolecular oxidation reaction is largely dependent on the chemical properties of metal-binding cysteines.

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