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Electrospray ionization mass spectrometric characterization of photocrosslinked DNA–*Eco*RI DNA methyltransferase complexes

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ABSTRACT

We describe a novel strategy combining photocrosslinking and HPLC-based electrospray ionization mass spectrometry to identify UV crosslinked DNA–protein complexes. *Eco*RI DNA methyltransferase modifies the second adenine within the recognition sequence GAATTC. Substitution of 5-iodouracil for the thymine adjacent to the target base (GAATTC) does not detectably alter the DNA–protein complex. Irradiation of the 5-iodouracil-substituted DNA–protein complex at various wavelengths was optimized, with a crosslinking yield >60% at 313 nm after 1 min. No protein degradation was observed under these conditions. The crosslinked DNA–protein complex was further analyzed by electrospray ionization mass spectrometry. The total mass is consistent with irradiation-dependent covalent bond formation between one strand of DNA and the protein. These preliminary results support the possibility of identifying picomole quantities of crosslinked peptides by similar strategies.

INTRODUCTION

Sequence-specific DNA–protein complexes reveal protein interactions with bases as well as the phosphodiester backbone. These interactions contribute to the overall binding energy and provide the basis for the enormous sequence discrimination shown by these proteins. Protein–DNA interactions also contribute to the catalytic mechanisms of enzymes such as endonucleases, methyltransferases and repair enzymes. Ultraviolet (UV) light-induced photochemical crosslinking of proteins to nucleic acids has been demonstrated for a variety of nucleic acid binding proteins. The amino acids proximal to the DNA can be identified through structural characterization of DNA–protein adducts resulting from photocrosslinking (1–3). However, low crosslinking efficiencies and application of standard peptide sequencing methods have necessitated the use of milligram quantities of both nucleic acid and protein. Furthermore, a finer structural analysis of the resultant adducts would be useful in defining the DNA–protein interface and possibly in the design of improved photoactivatable analogs.

Previous applications of photoactivatable analogs have relied on incorporating 5-bromodeoxyuracil (5-BrU) into DNA or 4-thiouridine into RNA; typical crosslinking yields are often <10% (3–5). An improvement on 5-BrU was made with 5-iododeoxyuracil (5-IdU) (6), because it is efficiently excited at 313 nm (7) with minimal overlap with DNA or protein absorbances. Thus 5-IdU is an excellent photoactivatable replacement for thymine that results in significantly lower levels of photoinduced protein degradation. Also, the van der Waals radius of iodine (2.15 Å) is only 8% larger than a methyl group. Replacement of thymine with 5-IdU leaves several DNA–protein complexes unaffected (6).

The well-studied *Eco*RI restriction/modification system provides an excellent opportunity to develop improved crosslinking strategies. The dimeric *Eco*RI endonuclease (R.*Eco*RI, mol. wt 31 059 Da) cleaves the recognition site G/AATTC and the monomeric methyltransferase (M.*Eco*RI, mol. wt 37 913 Da) transfers a methyl group from *S*-adenosylmethionine to the second adenine to form N^6-methyladenine (8). The X-ray crystal structures of a DNA dodecamer containing an *Eco*RI site and the DNA–R.*Eco*RI complex have been well studied (9–11). Our *in vitro* study revealed that M.*Eco*RI is an extremely efficient type II enzyme with a specificity constant (k_{cat}/K_m^{DNA}) for plasmid DNA of over 10^8 $s^{-1}M^{-1}$ (12). M.*Eco*RI is a bilobal enzyme (13) that has a catalytic domain containing the active site and AdoMet-binding regions, and a DNA-recognition region. Unlike the *Hha*I cytosine C^5 DNA methyltransferase that causes a 2° bend upon binding its recognition site, M.*Eco*RI is capable of bending DNA to ~52° (14). We also demonstrated that the target adenine base is stabilized in an extrahelical conformation prior to modification (15). Similarly, base flipping has been observed in M.*Hha*I (16) and suggested for other DNA modification enzymes (17,18). Thus, profound conformational changes can occur within the DNA–protein complex during sequence-specific DNA recognition and modification. One of our long term goals is to identify key residues which mediate these conformational changes.

Electrospray ionization mass spectrometry (ESI/MS) has proven to be an extremely powerful tool for the analysis of many large biomolecules. Previous studies were restricted primarily to homogeneous macromolecules that can form either positive ions (i.e. protein or peptide) or negative ions (i.e. DNA) in solution.

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Under the appropriate mass detection mode these ion species exhibit relatively high sensitivity. However, heteroconjugated species such as peptide–oligonucleotide hybrids pose special difficulties, since the two components generally possess conflicting requirements for ionization. Recently ESI mass analysis of a synthetic peptide–oligothymidylic acid heteroconjugate was described (19). Here we extend these studies, using ESI/MS-based methods to identify a photocrosslinked DNA–protein complex.

MATERIALS AND METHODS

Materials

All phosphoramidites and DNA synthesis reagents were obtained from Milligen/Biosearch except 5-IdU-CE and N^6-MedA-CE which were purchased from Glen Research. [γ-^{32}P]ATP (6000 Ci/mmol) was from Amersham. Sinefungin and 2-mercaptoethanol were purchased from Sigma.

M.*Eco*RI and R.*Eco*RI were purified from *Escherichia coli* strain MM294 harboring plasmid pPG440 (20). The concentration of M.*Eco*RI was determined spectrophotometrically ($E^{1\%}$ at 280 nm = 10.8) (8). Four DNA substrates 14 nt in length were prepared on a Biosearch 3810 DNA synthesizer using β-cyanoethyl phosphoramidites and purified on a Dynamax C18 reversed phase PureDNA column (Rainin Instrument Co.).

Top strand: d(GGCG**GAATTC**GCGG) (CT)
d(GGCG**GAAITC**GCGG) (CTI)

Bottom strand: d(CCGC**GAATTC**CGCC) (CB)
d(CCGC**GAMTTC**CGCC) (CBM)

(The M.*Eco*RI recognition site is in bold and underlined; I, 5-iodouridine; M, N^6-methylated adenine.) Oligonucleotides were diluted in 10 mM Tris, pH 8.0, 1 mM EDTA and the concentrations determined by absorbance at 260 nm. Complementary single strands were annealed and the double-stranded form was confirmed by autoradiography of ^{32}P-labeled DNA with non-denaturing polyacrylamide gel electrophoresis.

Determination of dissociation constants

The double-stranded DNA substrate was radiolabeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. Excess ATP was removed using a Bio-gel P6 column (BioRad) and the reaction cocktail diluted with buffer containing 10 mM Tris, pH 8.0, 1.0 mM EDTA and 100 mM NaCl. The apparent thermodynamic dissociation constants (K_d) for the various DNA substrates were determined using a standard gel mobility shift assay as previously described (21). The binding assay (30 µl) contained 0.1 nM DNA, 100 mM Tris, pH 8.0, 10 mM EDTA, 200 µg/ml BSA, 10 mM DTT, 20 µM sinefungin and various M.*Eco*RI concentrations (0–5.0 nM). Dissociation constants were determined by fitting data obtained by densitometry to a standard hyperbolic binding isotherm.

Photochemical crosslinking reaction

Small scale reactions (30 µl) contained 4 nM ^{32}P-labeled DNA and 20 nM M.*Eco*RI in the binding assay buffer as described above except that BSA was eliminated. Larger scale reactions (up to 0.5 ml) contained 10 µM unlabeled DNA and 10 µM M.*Eco*RI in the identical buffer. All reactions were conducted in 1.5 ml micro test tubes and incubated on ice during UV irradiation. The energy output of a tunable YAG 8010 Pump ND 6000 frequency-doubled dye (DCM in methanol) laser (Continuum Co.) at various wavelengths was kept at 20 mJ/pulse with a pulse width of 6 ns and the laser beam diameter was ~5 mm. Covalently crosslinked complexes were separated by SDS–PAGE and the photocrosslinking yields were quantified by densitometry as described above.

Purification of crosslinked samples

To determine an accurate mass of the crosslinked complex by ESI/MS the crosslinked samples were dialyzed against 10 mM NH_4OAc, pH 7.0, at 4°C for 12 h to remove salts and glycerol in the reaction buffer (MWCO 14 000–16 000; Spectrum Co.).

Electrospray ionization mass spectrometry

All ESI/MS analyses were performed on a VG Platform II mass spectrometer (Fisons Instrument). The N_2 nebulizing gas was maintained at 100 p.s.i., with a 5.0 l/min flow as drying gas. The DNA was brought up in a 90:9:1 methanol:H_2O:NH_4OH solution at a concentration of 5 pmol/µl. DNA samples were infused utilizing a Harvard Instruments Syringe Infusion Pump 22 at a flow rate of 10 µl/min. Experiments were performed in the negative ionization mode and the ESI source temperature was kept at 120°C. In most cases 15 continuum scans were averaged in the 'MCA' mode over the mass to charge range (*m/z*) 300–1700. The post-dialyzed crosslinked samples were loaded onto a protein desalting cartridge (Michrom BioResources Inc.) and washed with 10% acetonitrile in H_2O. M.*Eco*RI and the crosslinked DNA–M.*Eco*RI samples were eluted with acetonitrile:H_2O (80:20) at a flow rate of 50 µl/min and detected in positive ionization mode. The ESI source temperature was 70°C and the data were acquired in the continuum mode at 4 s/scan across the flow injection peak.

Table 1. Electrospray ionization (ESI) mass spectrometry data

Sample	Calculated mass (Da)	Measured mass (Da)
CT, d(GGCG**GAATTC**GCGG)	4345.1	4344.7 ± 0.43
CTI, d(GGCG**GAAITC**GCGG)	4456.1	4455.3 ± 0.08
CB, d(CCGC**GAATTC**CGCC)	4184.1	4183.6 ± 0.54
CBM, d(CCGC**GAMTTC**CGCC)	4198.1	4197.4 ± 0.29
M.*Eco*RI (325 amino acids)	37 913.4	37 916.9 ± 30.52
DNA–M.*Eco*RI complex	42 241.5	42 242.9 ± 21.28
CTI-I, d(GGCG**GAAUTC**GCGG)	4329.1	4328.2 ± 0.53

RESULTS AND DISCUSSION

Design and characterization of DNA substrates

Previous studies indicated that the thymine methyl moiety is critical for sequence-specific binding by M.*Eco*RI (22,23). Thus the thymine is a reasonable target for insertion of a photocrosslinking probe. Four oligodeoxynucleotides were synthesized using normal automated phosphoramidite synthesis technology (as described in Materials and Methods). Incorporation of modified bases was confirmed by ESI/MS analysis. Following oligonucleotide purification negative ionization electrospray mass spectrometry was used to obtain the mass spectra shown in Figure 1. A series of seven ions were detected in the mass to charge range 400–1200 with

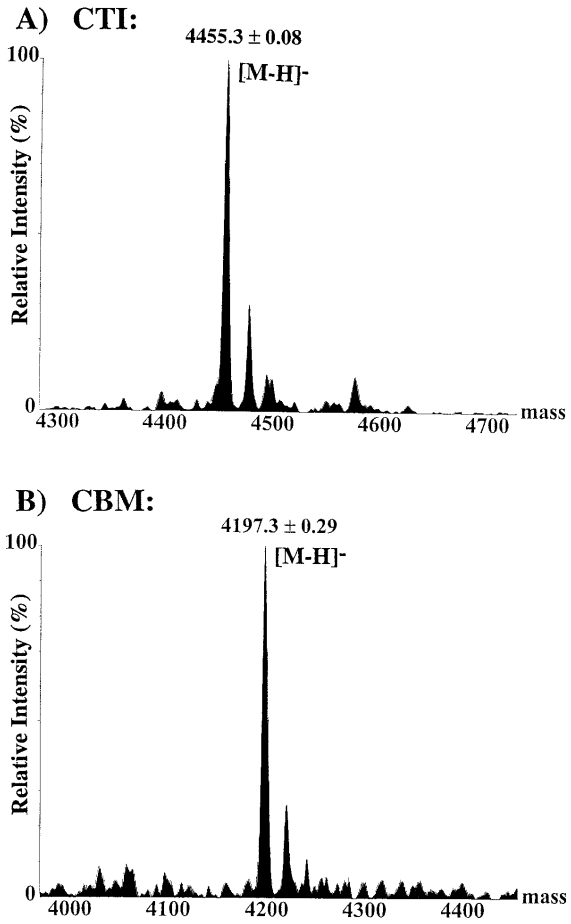

Figure 1. Negative electrospray ionization mass spectrometry of oligonucleotides. Oligonucleotides were dissolved in methanol:H_2O:NH_4OH (90:9:1) to a concentration of 5 µM and a 10 µl sample infused at a flow rate of 10 µl/min into the electrospray interface. Each spectrum is the average of 15 scans. (**A**) CTI, d(GGCGGAAITCGCGG). (**B**) CBM, d(CCGCGAMTTCCGCC). The ion signals adjacent to the major peak are the sodium adduction signals.

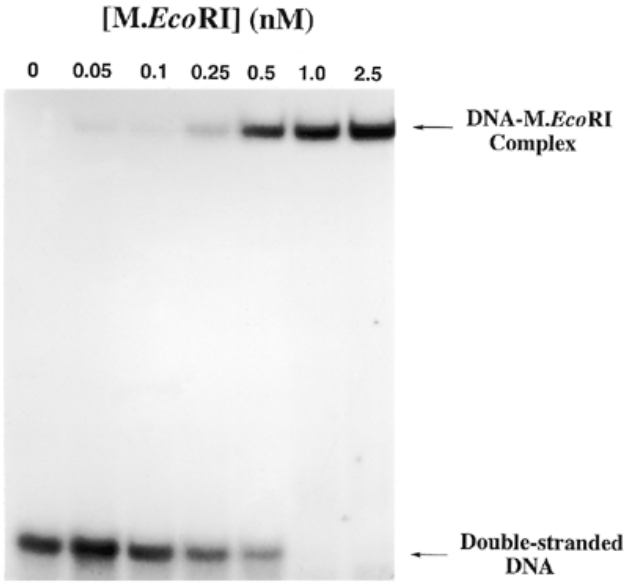

Figure 2. Gel mobility shift assay for K_d determination. Autoradiogram of gel mobility shift assay for determining the dissociation constant of M.*Eco*RI from 14 bp DNA. The binding reactions contained 0.1 nM ^{32}P-labeled DNA, 100 mM Tris, pH 8.0, 10 mM EDTA, 200 µg/ml BSA, 10 mM DTT, 20 µM sinefungin and various M.*Eco*RI concentrations (0–5.0 nM). After incubation at 37°C for 20 min the samples were loaded onto a 12% polyacrylamide gel and run at 200 V at 4°C for 2 h. Densitometric data was obtained from the upper bands of the gel shift autoradiogram and the dissociation constant was determined by fitting this data to a standard hyperbolic binding isotherm.

charged states ranging from –4 to –10. The data reveal that the 5-IdU-substituted strand (CTI) is 111 a.m.u. greater than the unmodified top strand (CT), consistent with incorporation of an iodine group into the oligonucleotide. Similar analysis for CBM and CB by mass spectrometry confirmed that a methylated group was incorporated into the oligonucleotide (Table 1). Our ESI mass spectra exhibit excellent mass resolution and accuracy under these optimal mass spectrometric conditions.

M.*Eco*RI affinity for 5-IdU-containing DNA (K_d)

Since the van der Waals radius of iodine is only 8% larger than the methyl group of thymine, replacement of thymine with 5-IdU was predicted to leave the DNA–protein complex unaltered. M.*Eco*RI binds to a double-stranded substrate in which one strand is substituted with 5-IdU in place of the first thymine (CTI) with similar affinity (K_d = 0.55 ± 0.02 nM) to the thymine-substituted duplex (0.43 ± 0.08 nM) (21; Fig. 2). The similarities in K_d indicate that the DNA–protein complex is not perturbed by incorporation of 5-IdU into the canonical site. However, our previous results indicated that the methyl group of the first thymine was in contact with M.*Eco*RI but showed a negative contribution to specificity (23). Thus the slight increase in K_d when the methyl group was replaced with a larger iodide group may be understandable. The similar binding affinity observed for 5-IdU-substituted DNA validated the use of this DNA for further photocrosslinking experiments.

DNA–protein photocrosslinking

5-IdU-substituted DNA was previously used for photocrosslinking to *Oxytricha nova* telomere protein subunits with irradiation at 308 and 325 nm (6). The crosslinked nucleoprotein complex was readily generated and detected by SDS–PAGE. However, the utilization of 308 nm light for crosslinking usually results in considerable protein degradation and decreases the crosslinking yield, in spite of a higher extinction coefficient at 308 nm than 325 nm (6). Protein damage may be caused by excitation of tryptophan residues.

Our crosslinking experiments have been optimized to minimize photodegradation and maximize crosslinking yield. We investigated the effect of short wavelength light on our 5-IdU-substituted DNA–protein complex. A time course of crosslinking at 308 nm is shown in Figure 3A. Following illumination of the radioactively labeled duplex DNA and an excess of M.*Eco*RI the reaction mixture was subjected to SDS–PAGE. The highest crosslinking yield was achieved within 1 min irradiation, with longer exposures resulting in degradation of the crosslinked complex. Two predominant secondary products were observed that may implicate susceptible sites in the polypeptide chain. Further mass spectrometric experiments are ongoing to reveal the exact cleavage positions in the protein. However, no DNA degradation products were observed.

A large scale crosslinking experiment was performed at 308 nm, followed by Coomassie staining of the samples on

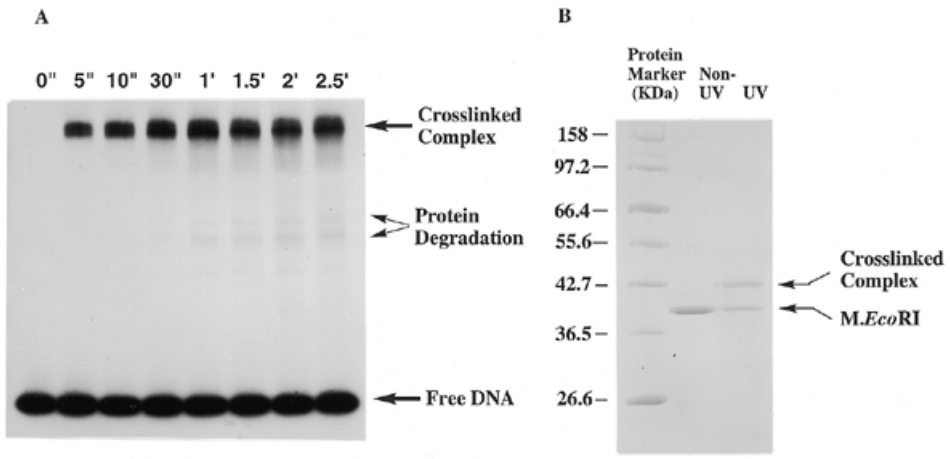

Figure 3. Photocrosslinking of DNA to M.*Eco*RI at 308 nm as a function of irradiation time. (**A**) 12% SDS–PAGE of the crosslinked complexes from reactions containing 4 nM ^{32}P-labeled DNA and 20 nM M.*Eco*RI in 100 mM Tris, pH 8.0, 10 mM EDTA, 10 mM DTT, 20 µM sinefungin and irradiated for the indicated times. Arrows indicate degradation products. (**B**) Coomassie stained SDS–PAGE of the large scale (10 µM M.*Eco*RI and DNA) crosslinking samples. Densitometry indicate that ~60% of the protein crosslinked to the DNA.

SDS–PAGE (Fig. 3B). Unlike the small scale reaction described earlier, equal amounts of DNA and protein were used, since the DNA–protein complex is the predominant species at these high concentrations. Approximately 60% of the protein is crosslinked to the DNA after 60 s irradiation (Fig. 3B, UV lane). The lower band in this lane corresponds to unreacted M.*Eco*RI (predicted mass 37 913 Da), while the upper band is presumed to contain the crosslinked complex consisting of a single enzyme molecule and a single strand of the oligonucleotide (CTI) lacking the iodide group (calculated mass 42 242 Da). M.*Eco*RI, like most DNA methyltransferases, functions as a monomer (12) and shows no covalent nucleoprotein complex formation in the absence of UV irradiation (Fig. 3B, non-UV lane). Our results indicate that all of the duplex DNA bound to the enzyme can be crosslinked with excess M.*Eco*RI (data not shown). Therefore, small scale crosslinking reactions with ^{32}P-labeled DNA result in relatively much higher crosslinking yields.

We applied a similar photocrosslinking strategy using R.*Eco*RI and the substrate described above. The R.*Eco*RI–DNA co-crystal structure shows that the nearest amino acids to the thymine C^5 methyl group are Gln115, Gly140 and Ala142, which are 3.66, 3.97 and 3.58 Å away respectively (11). This detailed information provides an opportunity to characterize the effectiveness of the 5-IdU-based photocrosslinking strategy. We could not detect formation of covalent complexes, in spite of demonstrating that the enzyme binds the DNA with an affinity comparable with the unmodified substrate (data not shown). The lack of any proximal aromatic residues may therefore explain the inability to crosslink R.*Eco*RI, since many characterized protein–DNA adducts involve such amino acids (4,24). However, our ability to crosslink a bending-deficient H235N M.*Eco*RI mutant shows that the method is not limited to wild-type M.*Eco*RI (unpublished results). Nevertheless, the requirements for successful DNA–protein photocrosslinking, including amino acid–DNA distances, and the types of amino acid residue which lead to good crosslinking are still not completely understood.

Irradiation at a longer wavelength (325 nm) was done to minimize protein degradation (6). 5-IdU-substituted DNA has relatively lower crosslinking yields at 325 nm, due presumably to the lower optical density at 325 nm (7), which necessitated longer irradiation times.

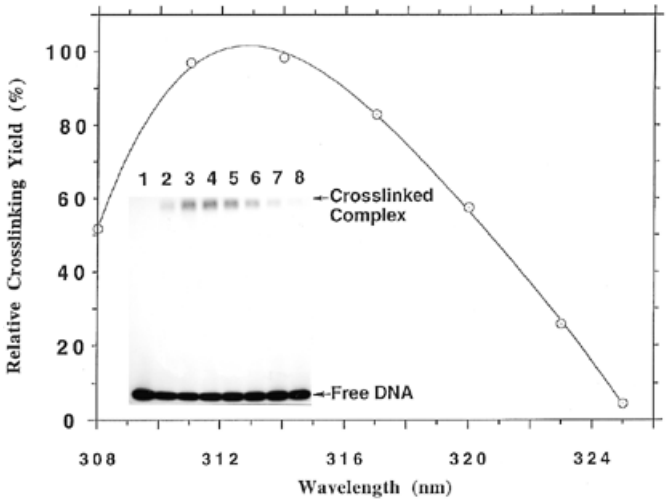

Figure 4. M.*Eco*RI crosslinking as a function of irradiation wavelength. Graph of relative crosslinking yields versus wavelength using the densitometric data obtained from the upper bands in the inset. Data was best fitted by a polynomial curve. (Inset) 4 nM ^{32}P-labeled DNA and 20 nM M.*Eco*RI in 100 mM Tris, pH 8.0, 10 mM EDTA, 10 mM DTT, 20 µM sinefungin irradiated at different wavelengths for 2 min on ice (lanes 1–8, non-UV and 308, 311, 314, 317, 320, 323 and 325 nm respectively). The crosslinked samples were processed by SDS–PAGE and autoradiography as described in Materials and Methods. Crosslinking yields were quantified by densitometry.

The photocrosslinking of DNA to M.*Eco*RI as a function of irradiation wavelength is shown in Figure 4. All samples were irradiated for 2 min to avoid protein degradation. A maximum crosslinking yield was achieved at 313 nm, consistent with previous data using this analog (7). Irradiation at 313 nm results in crosslinking yields that are at least five times higher than irradiation at 325 nm and no protein degradation was observed.

ESI/MS of M.*Eco*RI and DNA–M.*Eco*RI crosslinked complex

ESI mass analyses of covalent protein–nucleic acid complexes can be problematical, since proteins and nucleic acids are normally studied using positive or negative ionization methods

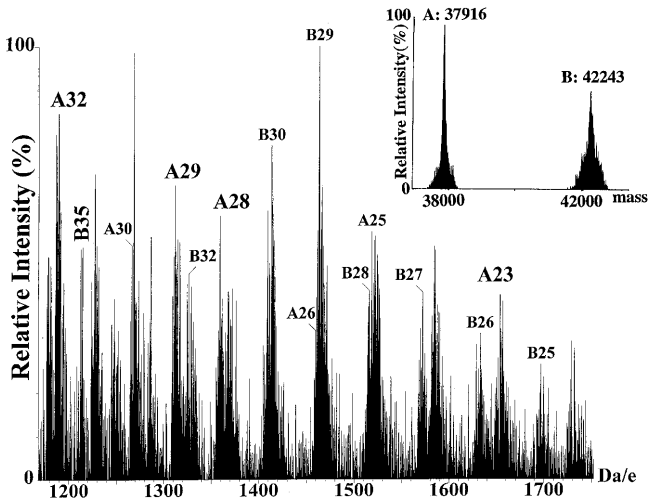

Figure 5. Electrospray ionization mass spectrum of M.*Eco*RI and DNA–M.*Eco*RI complex. The post-dialyzed crosslinked sample was loaded onto a protein desalting cartridge (Michrom BioResources Inc.) and washed with 10% acetonitrile in H_2O. M.*Eco*RI and crosslinked DNA–M.*Eco*RI complex were eluted with acetonitrile:H_2O (80:20) at a flow rate of 50 µl/min and detected in the positive ionization mode. (Inset) The whole mass spectrum was deconvoluted to yield a mass plot. Two species were resolved with masses consistent with the M.*Eco*RI and the crosslinked DNA–M.*Eco*RI complex.

respectively. For example, a recent analysis of a synthetic aminolinker–oligothymidylic acid by both positive and negative mode ESI could only identify ion signals corresponding to heteroconjugates in their HPLC-purified forms (19). We have recently developed an ESI/MS method to perform on-line LC-MS identification of crosslinked peptide adducts (unpublished results). Our results of ESI/MS on M.*Eco*RI and the crosslinked DNA–M.*Eco*RI complex are shown in Figure 5 and Table 1. M.*Eco*RI generated an envelope of ions (highlighted with an A) between *m/z* 1150 and 1700, corresponding to the +32 to +23 charge states. The M.*Eco*RI–DNA complex generated an envelope of ions (highlighted with a B) between *m/z* 1200 and 1800, corresponding to the +35 to +25 charge states. Deconvolution of the mass spectra for M.*Eco*RI and DNA–M.*Eco*RI complex is shown in the inset and yielded masses of 37 916 and 42 243 Da respectively. The mass of the DNA–M.*Eco*RI complex was consistent with the presence of a single strand of DNA (CTI with the loss of an iodo atom, mol. wt 4329.1). As expected for homolytic cleavage of the C–I bond, the mass was consistent with loss of the iodo moiety (25). The resulting uracilyl radical will likely react with nearby amino acids to form a covalent crosslinked complex. We were able to detect a small amount of the deiodinated CTI in this crosslinked sample using negative electrospray ionization mass spectrometry (data not shown). This deiodination of CTI was also directly observed by illuminating the single-stranded DNA in the absence of protein (see Table 1).

CONCLUSION

Our results clearly show that an *Eco*RI DNA methyltransferase–DNA complex was efficiently crosslinked and the intact complex can be analyzed by electrospray ionization mass spectrometry. ESI/MS analysis of the crosslinked DNA–protein complex also revealed that only a single strand of DNA formed a covalent bond with M.*Eco*RI following UV irradiation. The crosslinking yields were determined to be >60% for most of the reactions. The quantities of DNA–protein complexes and the resultant adducted peptides should be amenable to characterization by various mass spectrometric methods. The approach outlined in this paper may be readily extended to ESI/MS-based identification of adducted peptides similar to HPLC-ESI/MS analysis of post-translationally modified peptides (26–28). Our goal is to perform direct on-line identification of adducted peptides starting with microgram quantities of protein.

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