Proteinase K Activity Inhibited Near Amino Acids Carrying Large Substituents: Three PAH Diolepoxides Covalently Modify His-146 Human Serum Albumin Residue

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The chemical interaction between metabolites of polycyclic aromatic hydrocarbons (PAH) and proteins has received considerable attention in the past few years.[1-5] Recently, (±)-*r*-7,*t*-8-dihydroxy-*t*-9,*t*-10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*anti*-B*a*PDE) was shown to covalently modify human serum albumin (HSA) at an undetermined carboxylic acid group and at the N^{τ} of a histidine, most likely His-146.[4] In that work, HP and HPY adducted with *anti*-B*a*P(9,10)DE could be detected in a pronase digest by collision-induced dissociation (CID) tandem mass spectrometry (MS/MS), but only after peracetylation. The combination HP occurs three times in HSA (146-147, 338-339, 440-441). Only the detection of HPY pointed to the region 146-148, but that spectrum did not allow assignment of the modified amino acid. Reaction of the other two positions (His-338 and -440) could not be ruled out just because none of the corresponding tripeptides could be detected. This communication provides unambiguous confirmation of the modification of His-146 using a longer peptide sequence and shows that the same site is also covalently modified by two other PAH diolepoxides: (±)-*r*-8,*t*-9-dihydroxy-*t*-10,*t*-11-epoxy-8,9,10,11-tetrahydrobenzo[*a*]anthracene [*anti*-B*a*A(10,11)DE] and (±)-*t*-3,*r*-4-dihydroxy-*t*-1,*t*-2-epoxy-1,2,3,4-tetrahydrobenzo[*a*]anthracene [*anti*-B*a*A(1,2)DE].

The isolation and identification of longer peptides (11 and 12 amino acids long) were made possible by the finding that the action of another enzyme, proteinase K, which normally degrades proteins to free amino acids or di- or tripeptides (like pronase), is apparently stopped when the enzyme approaches an amino acid bearing a large substituent. An additional benefit is the resulting enrichment of the peptides derived from the protein molecules that have reacted with the diol epoxide. The former observation may be useful in other studies of proteins modified by large substituents on certain amino acids, such as the identification of active sites by chemical or photoaffinity labeling, digests of covalently linked protein–DNA complexes, etc.

Figure 1 shows the high-energy CID tandem mass spectrum[6,7,8] of a peptide isolated from the proteinase K digest of B*a*P(9,10)DE adducted HSA.[9,10] The abundant ion at m/z 257, indicative of *anti*-B*a*PDE adduction, is produced upon further fragmentation of the *anti*-B*a*PDE cation formed by cleavage between its C-10 carbon and N^{τ} of the histidine in the peptide. As shown in Scheme 1, this fragmentation initially produces an ion at m/z 303 (not observed in this spectrum), which then loses a molecule of H_2O followed by one of CO to produce the ion observed at m/z 257.[11] The ion at m/z 1406.0 corresponds to the protonated peptide molecule ion that has lost the adducting moiety as a neutral fragment (302 u). This fragmentation was

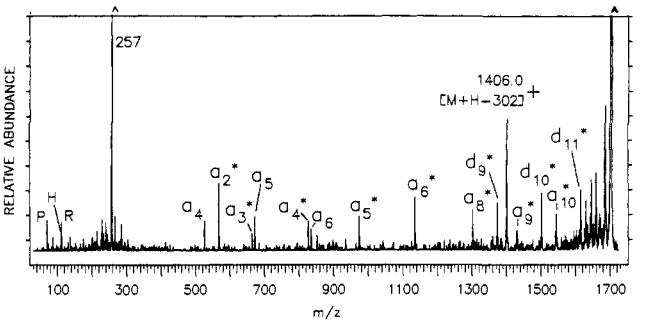
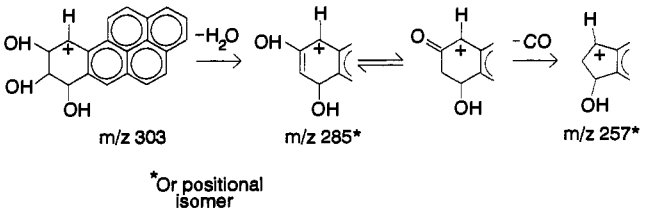


Figure 1. CID MS/MS spectrum of HSA 145–155 (RHPYFYAPELL) adducted at His-146 with *anti*-B*a*P(9,10)DE, $[M + H]^+ = m/z$ 1708.6. Product ions modified with *anti*-B*a*P(9,10)DE are indicated with an asterisk (i.e., a_2^*). The ion at m/z 257 is produced by the pathway shown in Scheme 1, and that at m/z 1406.0 corresponds to the unmodified peptide protonated molecule ion that is produced from loss of the modifying group as a neutral molecule from the precursor ion.

Scheme 1. Pathway Leading to the Abundant Product Ion Observed at m/z 257 in the CID MS/MS Spectra of *anti*-B*a*P(9,10)DE-Modified Peptides[a]



[a] The details of the pathway were determined using *anti*-B*a*P(9,10)DE-modified histidine,[4] the CID spectrum of which contains all the product ions shown. The longer the peptide, the less abundant these product ions become, although the m/z 257 ion is always the most abundant product ion in the spectrum. An analogous pathway produces the ion at m/z 233, which is observed in the CID spectra of *anti*-B*a*A(10,11)DE- and anti-B*a*A(1,2)DE-modified peptides.

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(1) Skipper, P. L.; Naylor, S.; Gan, L.-S.; Day, B. W.; Pastorelli, R.; Tannenbaum, S. R. *Chem. Res. Toxicol.* **1989**, *2*, 280–281.
(2) Day, B. W.; Skipper, P. L.; Rich, R. H.; Naylor, S.; Tannenbaum, S. R. *Chem. Res. Toxicol.* **1991**, *4*, 359–363.
(3) Naylor, S.; Gan, L.-S.; Day, B. W.; Pastorelli, R.; Skipper, P. L.; Tannenbaum, S. R. *Chem. Res. Toxicol.* **1990**, *3*, 111–117.
(4) Day, B. W.; Skipper, P. L.; Zaia, J.; Tannenbaum, S. R. *J. Am. Chem. Soc.* **1991**, *113*, 8505–8509.
(5) Hinson, J. A.; Roberts, D. W. *Annu. Rev. Pharmacol. Toxicol.* **1992**, *32*, 471–510.
(6) A JEOL HX110/HX110 tandem mass spectrometer was used, a description of which and its use in the fast atom bombardment (FAB) mode can be found in the literature.[7] A cesium ion gun was used with a mixture of glycerol and thioglycerol as the FAB matrix. Conditions for the high-energy CID spectra have been described.[8]
(7) Martin, S. A.; Biemann, K. *Int. J. Mass Spectrom. Ion Processes* **1987**, *78*, 213–228.
(8) Sato, K.; Asada, R.; Ishihara, M.; Kunihiro, F.; Kammei, Y.; Kubota, E.; Costello, C. E.; Martin, S. A.; Scoble, H.; Biemann, K. *Anal. Chem.* **1987**, *59*, 1652–1659.
(9) HSA (fatty acid free) was obtained from Sigma Chemical Co.; B*a*P-(9,10)DE, B*a*A(10,11)DE, and B*a*A(1,2)DE were obtained from the Midwest Research Institute (Kansas City, MO); 4-vinylpyridine, triethylphosphine, and dry tetrahydrofuran were obtained from Aldrich Chemical Co. (Milwaukee, WI); proteinase K was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). The method for adduction of serum albumin and isolation of adducted peptides differs from that in the earlier study:[4] 100 mg of HSA (25 mg/mL in phosphate-buffered saline, pH 7.2) was reacted with 1 mg of the diol epoxide (dissolved in 100 μL of dry tetrahydrofuran) for 2 h at 37 °C. The mixture was extracted (ethyl acetate followed by 1-butanol), and the protein was reduced and alkylated with triethylphosphine and 4-vinylpyridine[10] (8 M urea, pH 8.3, 2 h at 37 °C). The excess reagents were extracted (ethyl acetate), and the protein was digested with proteinase K (2% by weight, 4 M urea, pH 8.3, 6 h at 37 °C). The digested protein was acidified (acetic acid) and fractionated using a linear gradient of acetonitrile in the presence of trifluoroacetic acid on a reversed-phase HPLC column (Vydac Associates, protein C4, 10 mm i.d. 30 cm long). The fractions were dried, and those found to absorb light at 345 nm were analyzed by FAB mass spectrometry. Constant neutral loss scans (302 u for B*a*P(9,10)DE, 278 u for B*a*A(10,11)DE and B*a*A(1,2)DE) were used to identify the precursor ions likely to correspond to adducted HSA peptides. The sequences of the potentially adducted peptides were determined from the high-energy CID mass spectra.
(10) Andrews, P. C.; Dixon, J. E. *Anal. Biochem.* **1987**, *161*, 524–528.
(11) The fragmentation pattern was determined from the CID mass spectra of *anti*-B*a*PDE adducted model peptides, see ref 4 and the following: Zaia, J.; Biemann, K. Proceedings of the 40th ASMS Conference on Mass Spectrometry and Allied Topics, Washington, DC 1992; pp 1577–1578.

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used to facilitate the detection of such unmodified peptides using constant neutral loss scans.[12]

The remaining ions are due to fragmentation of the peptide chain and retention of the positive charge. Those labeled with an asterisk have the adducting moiety attached. The series of fragment ions labeled a_n^* and d_n^* (expected for an N-terminal arginine) serve to unambiguously identify the sequence of the peptide portion of the molecule.[13] The m/z of the a_2^* ion (produced by cleavage of the precursor ion between the α-carbon of His-146 and the adjacent carbonyl carbon with loss of a hydrogen[14]) identifies the adducted residue. From this spectrum, the peptide sequence was determined to be HSA 145–155 (RHPYFYAPELL, the modified amino acid is shown in bold), in which *anti*-B*a*PDE has reacted with His-146. Two other *anti*-B*a*PDE adducted peptides encompassing the same site were detected: HSA 144–154 (RRHPYFYAPEL), $[M + H]^+ = m/z$ 1751.2; and 144–156 (RRHPYFYAPELLF), $[M + H]^+ = m/z$ 2011.4. Thus, the CID MS/MS spectra of these large adducted peptides make it possible to identify the site of modification directly in contrast to the di- and tripeptides, where the PAH–triol moiety becomes the dominating factor and thus requires peracetylation to obtain a useful spectrum.[4]

The adduction sites for *anti*-B*a*A(10,11)DE and *anti*-B*a*A-(1,2)DE were also determined using high-energy CID mass spectra. Peptides reacted with these diol epoxides produce a CID profile qualitatively similar to that shown in Figure 1, although the diol epoxide fragment ion is now found at m/z 233 rather than at m/z 257 and is somewhat lower in relative abundance. The peptides identified were *anti*-B*a*A(10,11)DE-adducted RRHPYFYAPELL ($[M + H]^+ = m/z$ 1839.4)[15] and *anti*-B*a*A(1,2)DE-adducted RRHPYFYAPELL ($[M + H]^+ = m/z$ 1839.2),[16,17] corresponding to HSA 144–155 for either diol epoxide adduct. In both spectra, the m/z of an abundant a_3^* ion identifies His-146 as the adducted residue, and a series of a_n^* ions determines the sequence of the peptide portion of the molecules.

It is interesting to note that the two benzo[*a*]anthracene diol epoxides tested also modify the same HSA amino acid residue. This finding leads to the speculation that His-146 is the active residue associated with a PAH diol epoxide-binding pocket in the tertiary structure of HSA. From the present data, it cannot be concluded that His-146 is the exclusive site of stable adduct formation for the diol epoxides tested because binding to other sites may occur at a level below the detection limit of the present experiment. However, the larger, nested peptides (rather than the di- and tripeptides[4]) carrying the substituent are easier to detect and provide much more sequence information. This increases significantly the confidence in the assignment and greatly reduces the possibility of missing minor components resulting from reaction at other sites.

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(12) The molecular ion of a PAH diol epoxide-modified peptide produces an abundant product ion from the loss of the diol epoxide as a neutral molecule during CID. Thus, by acquiring a scan for the constant neutral loss of the PAH diol epoxide mass, modified peptides can be identified in the presence of unmodified peptides in the digest mixture.

(13) The peptide fragment ion nomenclature (Biemann, K. *Biomed. Mass Spectrom.* **1988**, *16*, 99–111) is adapted from the following: Roepstorff, R.; Fohlmann, J. *Biomed. Mass Spectrom.* **1984**, *11*, 601.

(14) Johnson, R. S.; Martin, S. A.; Biemann, K. *Int. J. Mass Spectrom. Ion Processes* **1988**, *86*, 137–154.

(15) Product ions observed in the CID spectrum of B*a*A(10,11)DE-adducted RRHPYFYAPELL (ion type is listed in boldface, followed by m/z value with the abundance relative to the m/z 233 ion in parentheses): $\mathbf{d_2^*}$ 200.2 (0.30), 233.2 (1.00); $\mathbf{a_3}$ 422.3 (0.18); $\mathbf{a_3^*}$ 700.4 (0.83); $\mathbf{a_4^*}$ 797.4 (0.36); $\mathbf{a_5^*}$ 960.8 (0.26); $\mathbf{d_6^*}$ 1031.4 (0.15); $\mathbf{a_6^*}$ 1107.5 (0.33); $\mathbf{d_7^*}$ 1178.2 (0.11); $\mathbf{a_7^*}$ 1270.3 (0.49); $\mathbf{a_8^*}$ 1341.1 (0.18); $\mathbf{a_9^*}$ 1438.6 (0.32); $\mathbf{d_{10}^*}$ 1509.6 (0.27); $\mathbf{[M + H - 278]^+}$ 1561.2 (0.40); $\mathbf{a_{10}^*}$ 1567.4 (0.21); $\mathbf{d_{11}^*}$ 1638.4 (0.29); $\mathbf{a_{11}^*}$ 1680.5 (0.37); $\mathbf{d_{12}^*}$ 1751.6 (0.50).

(16) Product ions observed in the CID spectrum of B*a*A(1,2)DE-adducted RRHPYFYAPELL (ion type is listed in boldface, followed by m/z value with the abundance relative to the m/z 233 ion in parentheses): $\mathbf{d_2^*}$ 200.1 (0.16), 233.0 (1.00); $\mathbf{a_3}$ 422.1 (0.065); $\mathbf{a_3^*}$ 700.2 (1.01); $\mathbf{a_4^*}$ 797.3 (0.30); $\mathbf{c_4^*}$ 842.0 (0.12); $\mathbf{a_5^*}$ 960.3 (0.48); $\mathbf{a_6^*}$ 1107.3 (0.53); $\mathbf{a_7^*}$ 1269.9 (0.76); $\mathbf{a_8^*}$ 1341.4 (0.14); $\mathbf{b_8^*}$ 1368.9 (0.097); $\mathbf{a_9^*}$ 1438.1 (0.38); $\mathbf{d_{10}^*}$ 1508.8 (0.20); $\mathbf{[M + H - 278]^+}$ 1560.7 (1.24); $\mathbf{d_{11}^*}$ 1637.7 (0.27); $\mathbf{a_{11}^*}$ 1680.9 (0.50).

(17) Two other *anti*-B*a*A(1,2)DE-adducted peptides containing the same sequence were detected and analyzed by CID MS/MS: HSA 144–148 (RRHPY), $[M + H]^+ = m/z$ 1005.9; and 144–153 (RRHPYFYAPE) $[M + H]^+ = m/z$ 1613.5.