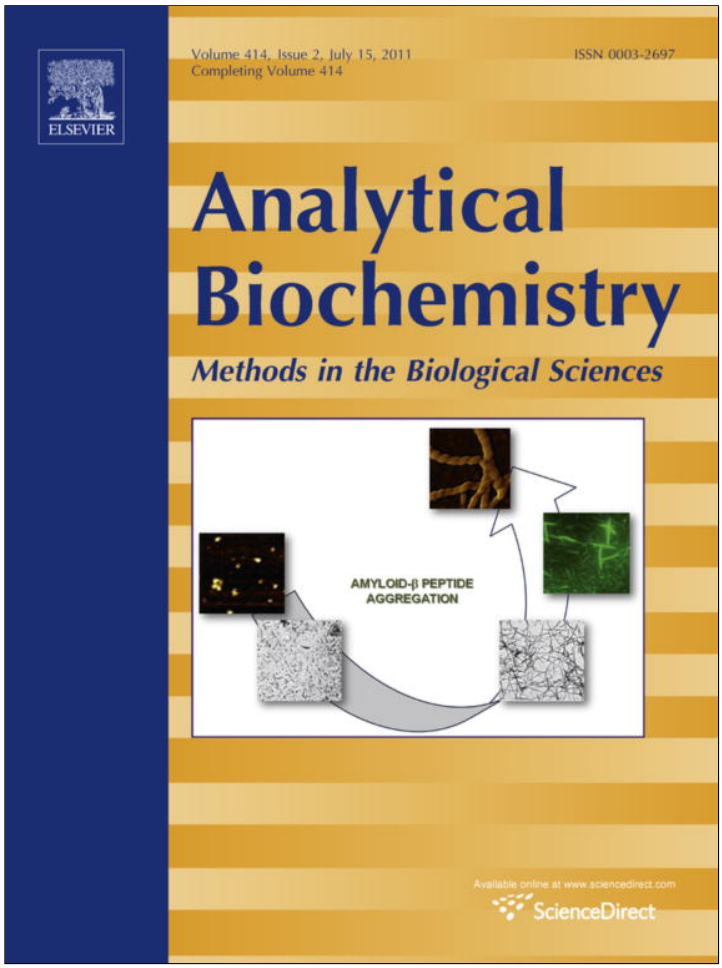

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Determination of the toluene diisocyanate binding sites on human serum albumin by tandem mass spectrometry

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ABSTRACT

Diisocyanates are highly reactive chemical compounds widely used in the manufacture of polyurethanes. Although diisocyanates have been identified as causative agents of allergic respiratory diseases, the specific mechanism by which these diseases occur is largely unknown. To better understand the chemical species produced when diisocyanates react with protein, tandem mass spectrometry was employed to unambiguously identify the binding sites of the industrially important isomers, 2,4- and 2,6-toluene diisocyanate, on human serum albumin at varying diisocyanate/protein ratios. The 2,4-isomer results in approximately 2-fold higher conjugation product ion abundances than does the 2,6-isomer, suggesting that the 2,4-isomer has a higher reactivity toward albumin. Both isomers preferentially react with the N-terminal amine of the protein and the ε-NH_2 of lysine. At a low (1:2) diisocyanate/protein ratio, five binding sites are identified, whereas at a high (40:1) ratio, near-stoichiometric conjugation is observed with a maximum of 37 binding sites identified. Binding sites observed at the lowest conjugation ratios are conserved at higher binding ratios, suggesting a subset of 5–10 preferential binding sites on albumin. Diisocyanate–protein conjugation results in a variety of reaction products, including intra- and intermolecular crosslinking, diisocyanate self-polymerization, and diisocyanate hydrolysis.

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Diisocyanates are highly reactive, low-molecular-weight chemicals widely used as crosslinking reagents in the manufacture of polyurethanes for diverse industries such as flexible furniture foams and rigid automotive foams. The reactive isocyanate (N=C=O) moieties are capable of nucleophilic addition with a variety of active hydrogen species, including amines, alcohols, phenols, and thiols [1]. Global production of diisocyanates, including methylene diphenyl diisocyanate (MDI)[1] and toluene diisocyanate (TDI), exceeds 4 million tons and increases yearly. Together, MDI and TDI account for more than 90% of the global diisocyanate market. TDI is available as either 2,4- or 2,6-isomers or, commonly, as 80:20 or 65:35 mixtures of 2,4-/2,6-isomers. TDI is the predominant isocyanate crosslinker used in the furniture/mattress industry [2].

Approximately a quarter of a million workers are involved in the global polyurethane manufacturing industry [2]. Because of the highly reactive nature of diisocyanates, occupational exposures are associated with adverse health effects. Diisocyanate exposure results in sensitization and asthma, contact dermatitis, and hypersensitivity pneumonitis [3]. Diisocyanate-induced asthma in occupationally exposed worker populations is estimated to range from 5% to 30% [3–6]. Furthermore, some occupational tasks, such as paint mixing/spraying and production of composite wood products, result in significant diisocyanate dermal exposures [7,8], which may also lead to immunological sensitization and subsequent development of asthma-like symptoms.

It is hypothesized that the isocyanate acts as a hapten that reacts with protein carriers (e.g., albumin) via nucleophilic attack; however, the ultimate form of these protein–isocyanate conjugates is poorly understood. Several protein targets of diisocyanates in vivo have been identified, most notably serum albumin [9–14], hemoglobin [15,16], tubulin, and actin [17]. However, the forms of the diisocyanate-conjugated products that function as allergens are as yet unknown. The diverse functional groups present in proteins, including amines, amides, thiols, alcohols, and carboxylic acids, present a large number of potential reaction sites for the isocyanate. However, previous studies have suggested that under physiological conditions, these are limited to N-terminal α-amines, the sulfhydryl group of cysteine, the hydroxyl groups of serine and tyrosine, the ε-amine of lysine, and the secondary amine of the imidizole ring of histidine [15].

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[1] *Abbreviations used:* MDI, methylene diphenyl diisocyanate; TDI, toluene diisocyanate; OAA, organic acid anhydride; HHPA, hexahydrophthalic acid; MHHPA, methylhexahydrophthalic anhydride; MS/MS, tandem mass spectrometry; qTOF, quadrupole time-of-flight; HSA, human serum albumin; HPLC, high-performance liquid chromatography; UPLC, ultra-performance liquid chromatography; CID, collision-induced dissociation; UHP, ultra-high-purity; HDI, hexamethylene diisocyanante.

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Similarly, organic acid anhydrides (OAAs), such as hexahydrophthalic acid anhydride (HHPA) and methylhexahydrophthalic anhydride (MHHPA), are capable of inducing airway disease at levels below 100 μg/m^3 [18]. These highly reactive sensitizing chemicals are widely used in the production of resins, paints, plastics, and adhesives. As is the case with diisocyanates, albumin has been identified as the major serum protein forming adducts with HHPA in vivo [19]. Several studies examining the binding sites of OAAs on serum albumin, both in vitro and in vivo, have been conducted [20,21] and identified a subset of residues that are preferentially bound. Furthermore, studies suggesting that conjugated serum albumin may be antigenic for both OAAs [22] and diisocyanates have been conducted [13,23].

Understanding the reaction of diisocyanates such as MDI and TDI with biological molecules is critical to understanding the mechanisms by which these chemicals affect living systems. Tandem mass spectrometry (MS/MS) performed on a quadrupole time-of-flight (qTOF) mass spectrometer [24] is particularly well suited for the analysis of modified proteins. Peptides derived from enzymatic digest may be fragmented and mass analyzed with high mass accuracy over a wide dynamic range [25]. To that end, our laboratory has begun examining the reaction products formed between diisocyanates and biological macromolecules by MS/MS [26]. Analysis of peptide–isocyanate conjugates by MS/MS revealed that isocyanates bind preferentially to the N-terminal amine of peptides. Furthermore, when a peptide with an N-terminal residue containing a side chain amine is reacted with a diisocyanate, intramolecular crosslinking with the second isocyanate becomes competitive with hydrolysis; however, the reactivity decreases as the residue is displaced further from the N terminus. The results of that study, when taken in aggregate with those of other laboratories, indicate that the N terminus of proteins is a likely target for adduction in isocyanate-exposed individuals. Kristiansson et al. [20] determined that at a 10-fold molar excess, HHPA bound to 37 sites on human serum albumin (HSA): the N-terminal aspartic acid and 36 lysine residues. More recently, Wisnewski et al. [27] examined the reaction products between MDI and HSA by high-performance liquid chromatography (HPLC)–MS/MS. Their data indicated 14 binding sites on albumin: 12 lysine and 2 asparagine residues. In addition, these authors suggested that the four "dilysine" (KK) motifs in HSA are important binding sites and that MDI shows reactive specificity for the second lysine.

The aims of this study were to comprehensively map the potential binding sites for TDI on HSA in vitro, to examine the concentration dependence of the binding sites that are observed to determine whether certain binding sites are kinetically favored, and to determine whether differences in binding chemistry are observed between the structural isomers 2,4- and 2,6-TDI.

Materials and methods

Chemicals and reagents

2,4- and 2,6-TDI, 3,4-dimethylphenyl isocyanate, and formic acid (98%, for MS) were acquired from Aldrich (St. Louis, MO, USA). HSA (>99%, globulin free, lyophilized), iodoacetamide, tributylphosphine, and porcine trypsin were acquired from Sigma Chemical (St. Louis, MO, USA). Acetone (HPLC grade), acetonitrile (HPLC grade), and 3 Å molecular sieves were acquired from Fisher Scientific (Pittsburgh, PA, USA). [Glu1]-Fibrinopeptide B (EGVNDNEEGFFSAR) was acquired from Protea Biosystems (Morgantown, WV, USA). Distilled deionized water (DDI, 18 MΩ) was produced in the laboratory by a Millipore Synthesis A-10 system (Billerica, MA, USA).

Preparation of TDI–albumin conjugates

Stock solutions of HSA (0.5 mg/ml) were prepared in 25 mM NH_4HCO_3 (pH 7.9). Dry acetone was prepared by incubation of HPLC-grade acetone on 3 Å molecular sieves. Stock solutions (7.0×10^{-3} M) of 2,4- and 2,6-TDI were prepared in dry acetone. Isocyanate was added to 1 ml of HSA stock solution (final isocyanate concentrations of 0, 7.0×10^{-6}, 3.5×10^{-5}, 7.0×10^{-5}, and 2.8×10^{-4} M), vigorously mixed for 5 s, and incubated at 37 °C with shaking (1500 rpm) for 1 h. Aliquots (100 μl) of each conjugate and control were taken for analysis. Disulfide bonds were reduced in both conjugates and control by reaction with tributylphosphine for 30 min at room temperature, followed by alkylation with iodoacetamide for 1 h at room temperature. Alkylation was quenched by further addition of tributylphosphine for 15 min at room temperature. Samples were twice dialyzed against 3 L of 25 mM NH_4HCO_3 using 3500 MWCO (molecular weight cutoff) mini dialysis units (Slide-A-Lyzer, Thermo Scientific, Waltham, MA, USA). Porcine trypsin was suspended in 25 mM NH_4HCO_3 and added to each aliquot at a 40:1 (protein/trypsin) ratio. Samples were incubated overnight at 37 °C with shaking (400 rpm). Samples were centrifuged at 14,000 rpm in a microcentrifuge (MiniSpin, Eppendorf, Hamburg, Germany) to pellet any insoluble material.

UPLC

Enzymatic peptides were separated on a Waters nanoACQUITY ultra-performance liquid chromatography (UPLC) system (Milford, MA, USA). Aliquots (1 μl) of the digest mixture were injected and trapped/desalted on a 5-μm symmetry C_{18} trapping column (180 μm × 20 mm) with 99.5/0.5 A/B (A: 0.1% formic acid; B: 0.1% formic acid in acetonitrile) at a flow rate of 15 μl/min for 1 min. Separation was performed on a 1.7-μm BEH130 C_{18} analytical column (100 μm × 100 mm) using gradient elution at a flow rate of 300 nl/min and a gradient of 99:1 to 60:40 A/B over 60 min.

MS/MS

The eluent from the UPLC system was directed to the nanoelectrospray source of a Waters SYNAPT MS qTOF mass spectrometer. Positive ion nanoelectrospray was performed using 10-μm PicoTip emitters (Waters) held at a potential of +3.5 kV. The cone voltage was held constant at +40 V for all experiments. Dry N_2 desolvation gas was supplied to the instrument via a nitrogen generator (NitroFlowLab, Parker Hannifin, Haverhill, MA, USA). [Glu1]-Fibrinopeptide B (100 fmol/μl in 75:25 A/B) was supplied to an orthogonal reference probe, and the $[M + 2H]^{2+}$ ion (m/z 785.84265 u) was measured as an external calibrant at 30-s intervals. Collision-induced dissociation (CID) was performed using ultra-high-purity (UHP) argon as collision gas. Spectra were acquired in an MS^e fashion [25]. Briefly, alternating 1-s mass spectra are acquired. The first spectrum acquired at low (6 eV) collision energy allows high mass accuracy precursor ion mass measurement. The second spectrum acquired at high (15–30 eV ramp) collision energy allows high mass accuracy fragment ion mass measurement. The fragment ion spectra may be temporally correlated with precursor spectra postrun. This method of data acquisition allows all precursor ions to be fragmented and analyzed rather than so-called data-dependent acquisition methods that require real-time decisions to be made on which ions to select for fragmentation and which may miss low-abundance precursor ions.

Data analysis

Data were analyzed with BioPharmaLynx version 1.2 (Waters), a software program for analysis of peptide mass maps and identification of sites of modification on known protein sequences. Default peptide mass map analysis criteria of 30 ppm mass error in both low and high collision energy mode were specified. Trypsin was specified as the digestion enzyme, and two missed cleavages were allowed. The submitted protein sequence was taken from P02768 (serum albumin precursor, *Homo sapiens*, http://www.uniprot.org/uniprot/P02768), and the signal and propeptides (residues 1–24) were removed. Custom modifiers were created for two bound forms of TDI. The first (TDI, $C_9H_6N_2O_2$, m/z 174.0429 u) represents TDI with both isocyanate moieties bound to a peptide via urea bonds. The second (TDI*, $C_8H_8N_2O$, m/z 148.0637 u) represents one isocyanate moiety bound to a peptide via a urea bond, whereas the second isocyanate moiety is hydrolyzed to the primary amine. Identification of a potential TDI binding site proceeded via a rigorous procedure that involved the following steps. First, a potential peptide–TDI conjugation product is observed with less than 30 ppm $m/\Delta m$ mass error in the analyte peptide mass map. Second, comparison of the analyte and control peptide mass map from unmodified HSA shows that observed m/z and chromatographic retention time are unique to analyte. Third, MS/MS data contain b_n- and y_n-type ions consistent with the assigned sequence and modifier.

Results and discussion

Mapping the binding sites of TDI on HSA

HSA was reacted in vitro (25 mM NH_4HCO_3, pH 7.9) to both 2,4- and 2,6-TDI at TDI/HSA ratios of 1:1 to 40:1. Thus, the protein–isocyanate conjugates produced were digested with trypsin, and the resultant proteolytic peptides were analyzed via UPLC–MS/MS to unambiguously determine TDI binding sites on the basis of their MS/MS fragmentation patterns. The data from this analysis are summarized in the TDI–HSA binding map presented in Fig. 1. A total of 37 binding sites were identified: the N-terminal amine on aspartic acid at position 1, 2 glutamine residues, and 34 lysine residues. The data summarized in Fig. 1 represent all binding sites identified for both structural isomers over all concentration ranges (1:1 to 40:1 TDI/HSA). Binding sites conserved at the lowest concentration (1:1) are highlighted in bold. Differences in binding chemistry observed as a function of TDI concentration are discussed in more detail in the next section.

Recently, Wisnewski et al. [27] published a report describing the binding sites observed on HSA on reaction with MDI. Similar to the data presented here, these authors prepared their MDI–HSA conjugates in vitro at a ratio of approximately 50:1 MDI/HSA and analyzed the conjugates by a combination of HPLC and MS/MS. The results of these experiments yielded 14 confirmed binding sites: 12 lysine and 2 asparagine residues. In general, the binding sites identified for MDI are in good agreement with those identified here for TDI. Our results confirm the binding of TDI to 11 of the 12 lysine residues identified for MDI. Our results do not confirm the binding of TDI to Lys162, nor did we observe binding to asparagine residues (although we did observe two different glutamine residues at 40:1 ratios). There is, however, a significant difference in the total reported number of binding sites for TDI–HSA presented here and the previously reported MDI–HSA study. We observed 37 binding sites (at 40:1 TDI/HSA) compared with 14 sites (at 50:1 MDI/HSA). TDI and MDI both share two reactive isocyanate moieties, although MDI is significantly larger than TDI. The larger size of MDI may prevent binding to a subset of the binding sites observed for TDI on the basis of steric hindrance. Alternatively, the difference in observed binding sites may be due to differences in the LC–MS/MS methods employed. Wisnewski et al. prescreened peptide digest HPLC eluent and collected putative diisocyanate-containing aliquots on the basis of 245-nm optical absorbance prior to analysis by nano-UPLC–MS/MS, whereas in the current study the entire peptide digest was analyzed by UPLC–MS/MS in an MS^e fashion [25], where all ions are fragmented in the mass spectrometer as they elute the LC and the fragment ions are realigned temporally with parent ions in silico postrun. Conventional data-directed methods of analysis require the instrumentation to make intelligent decisions on which ions to fragment on the basis of user-specified criteria; however, multiplexed methods of analysis such as MS^e are gaining popularity because every ion is fragmented and no data are "lost" because of a failure to meet selection criteria such as low abundance.

It is interesting to note that there is significant agreement between the results of the current study on TDI–HSA binding and the previously published report by Kristiansson et al. on HHPA–HSA binding [20]. Organic acid anhydrides are similar to diisocyanates insomuch as they are highly reactive sensitizing electrophiles that react preferentially with amines under physiological conditions. The 36 binding sites identified on HSA for HHPA compares favorably with the 37 binding sites identified here. Although

```
1    DAHKSEVAHR FKDLGEENFK ALVLIAFAQY LQQCPFEDHV KLVNEVTEFA
51   KTCVADESAE NCDKSLHTLF GDKLCTVATL RETYGEMADC CAKQEPERNE
101  CFLQHKDDNP NLPRLVRPEV DVMCTAFHDN EETFLKKYLY EIARRHPYFY
151  APELLFFAKR YKAAFTECCQ AADKAACLLP KLDELRDEGK ASSAKQRLKC
201  ASLQKFGERA FKAWAVARLS QRFPKAEFAE VSKLVTDLTK VHTECCHGDL
251  LECADDRADL AKYICENQDS ISSKLKECCE KPLLEKSHCI AEVENDEMPA
301  DLPSLAADFV ESKDVCKNYA EAKDVFLGMF LYEYARRHPD YSVVLLLRLA
351  KTYETTLEKC CAAADPHECY AKVFDEFKPL VEEPQNLIKQ NCELFEQLGE
401  YKFQNALLVR YTKKVPQVST PTLVEVSRNL GKVGSKCCKH PEAKRMPCAE
451  DYLSVVLNQL CVLHEKTPVS DRVTKCCTES LVNRRPCFSA LEVDETYVPK
501  EFNAETFTFH ADICTLSEKE RQIKKQTALV ELVKHKPKAT KEQLKAVMDD
551  FAAFVEKCCK ADDKETCFAE EGKKLVAASQ AALGL
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Fig.1. Map of the binding sites of 2,4- and 2,6-TDI on HSA. All observed binding sites are underlined. Favored binding sites observed at a 1:1 TDI/HSA molar ratio are in bold.

Table 1
Prominent tryptic TDI–albumin peptides observed from 10:1 TDI/HSA conjugate.

Peptide	Sequence	M_{Calc}	M_{Obs}	$m/\Delta m$ (ppm)	Modifier
1–10	DAHKSEVAHR	1322.6116	1322.5759	27.0	TDI
1–12	DAHKSEVAHRFK	1771.8177	1771.8350	9.8	2 TDI
1–12	DAHKSEVAHRFK	1745.8386	1745.8127	14.8	TDI*, TDI
137–144	KYLYEIAR	1202.6448	1202.6495	3.9	TDI*
137–145	KYLYEIARR	1322.6506	1322.6227	21.1	TDI
182–195	LDELRDEGKASSAK	1665.8323	1665.8422	5.9	TDI*
198–205	LKCASLQK	1120.5699	1120.5699	0.0	TDI
198–205	LKCASLQK	1094.5907	1094.5907	0.1	TDI*
198–209	LKCASLQKFGER	1609.8035	1609.8088	3.3	TDI
210–218	AFKAWAVAR	1166.6349	1166.6371	1.9	TDI*
275–286	LKECCEKPLLEK	1719.8324	1719.8350	1.5	TDI
349–359	LAKTYETTLEK	1443.7610	1443.7627	1.2	TDI*
373–389	VFDEFKPLVEEPQNLIK	2218.1309	2218.1440	5.9	TDI
411–418	YTKKVPQV	1117.5920	1117.5894	2.3	TDI
411–428	YTKKVPQVSTPTLVEVSR	2205.1792	2205.1968	8.0	TDI
414–428	KVPQVSTPTLVEVSR	1786.9941	1787.0011	3.9	TDI*
429–439	NLGKVGSKCCK	1423.6700	1423.6816	8.1	TDI
437–445	CCKHPEAKR	1332.6180	1332.6418	17.9	TDI*
437–445	CCKHPEAKR	1480.6818	1480.7252	29.3	2 TDI*
522–534	QIKKQTALVELVK	1652.9655	1652.9613	2.5	TDI
525–534	KQTALVELVK	1275.7570	1275.7570	1.5	TDI*
526–538	QTALVELVKHKPK	1812.0046	1812.0035	0.6	TDI*, TDI
539–557	ATKEQLKAVMDDFAAFVEK	2462.1941	2462.2319	15.4	TDI*, TDI
546–560	AVMDDFAAFVEKCCK	1963.8267	1963.8706	22.4	TDI
546–564	AVMDDFAAFVEKCCKADDK	2541.0764	2541.0632	5.2	TDI*, TDI
565–574	ETCFAEEGKK	1493.6610	1493.6792	12.2	2 TDI*

there is not site-for-site agreement between HHPA and TDI, significant overlap exists between the sites identified in the two reports, in particular, the N-terminal amine on Asp1 and the side chain ε-amine of Lys4, Lys199, Lys413, Lys444, Lys524, and Lys525.

When TDI reacts with serum albumin in solution, a variety of reaction products are observed. For example, Table 1 lists the TDI–albumin conjugated peptides observed for 2,4-TDI at a 10:1 diisocyanate/HSA ratio. In total, 26 TDI-conjugated peptides are observed with an average mass error ($m/\Delta m$) of 8.6 ppm. Peptides are observed conjugated to partially hydrolyzed TDI (TDI*, $C_8H_8N_2O$, m/z 148.0637 u) as well as nonhydrolyzed TDI (TDI, $C_9H_6N_2O_2$, m/z 174.0429 u). Because the isocyanate moiety is rapidly hydrolyzed in aqueous solutions, peptides of the general form $[M + TDI + nH]^{n+}$ are presumed to be intramolecularly crosslinked. A previous study in our laboratory to determine the binding sites of TDI on bioactive peptides by MS/MS determined that TDI was capable of forming intramolecular crosslinked products [26]. Similarly, in their recent study, Wisnewski et al. [27] observed several albumin peptides conjugated to MDI where the second isocyanate moiety was not hydrolyzed. These researchers also attributed this observation to the formation of intramolecular crosslinked products. The MS/MS data support this observation. For example, Fig. 2 presents the MS/MS fragment ion spectrum of the $[M + TDI + H]^+$ ion of serum albumin tryptic peptide 411–428 (YTKKVPQVSTPTLVEVSR). Observation of the unmodified y_n-ion series for y_1 to y_{13} indicates that the TDI is not bound to any of the 13 C-terminal residues. In contrast, the b_n-ion series b_4^* to b_{14}^* is observed at m/z 174.0429 u higher in mass than expected for the unmodified amino acid sequence, indicating that the TDI is bound at or before the lysine at position 4. The lack of a b_3 or b_3^* ion in this fragment ion spectrum suggests that the two residues' side chains are covalently linked via TDI. Formation of either the b_3 or b_3^* ion would have required significantly more energy to break both the amide bond of the peptide chain and the covalent side chain linkage formed by TDI.

Observation of TDI* species where one isocyanate moiety is hydrolyzed can be useful for resolving ambiguities between binding sites. For example, Fig. 3 presents the MS/MS fragment ion spectrum of the $[M + TDI^* + H]^+$ ion of serum albumin tryptic peptide 1–10 (DAHKSEVAHR). Observation of a prominent a_2^* ion indicates that the isocyanate is bound to the N-terminal amine of the aspartic acid residue at position 1 rather than the lysine at residue 4, as was suggested for MDI [27]. Observation of the $[M + TDI + H]^+$ ion (see Table 1) indicates that intramolecular crosslinking between the N-terminal amine and the ε-amine of lysine occurs; however, the N-terminal amine is the more favored binding site

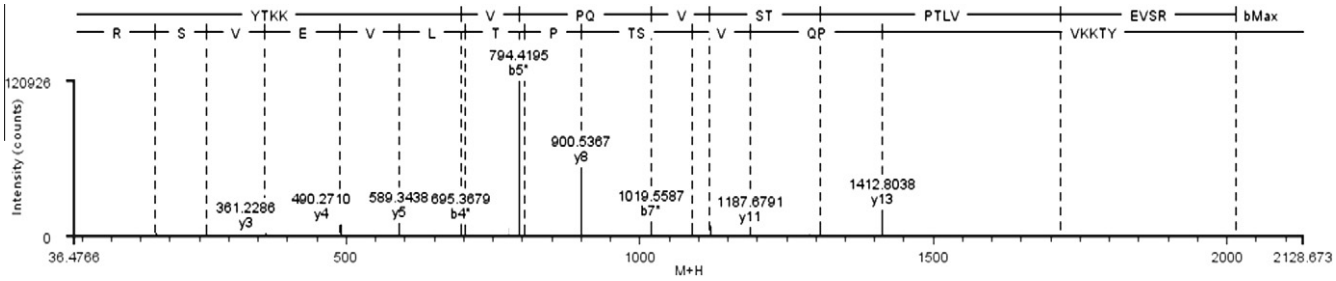

Fig.2. MS/MS fragment ion spectrum of the $[M + TDI + H]^+$ ion observed for HSA tryptic peptide 411–428 (YTKKVPQVSTPTLVEVSR). The two isocyanate groups of TDI react with the ε-NH_2 of the lysine residues at positions 413 and 414, forming an intramolecular crosslinked product.

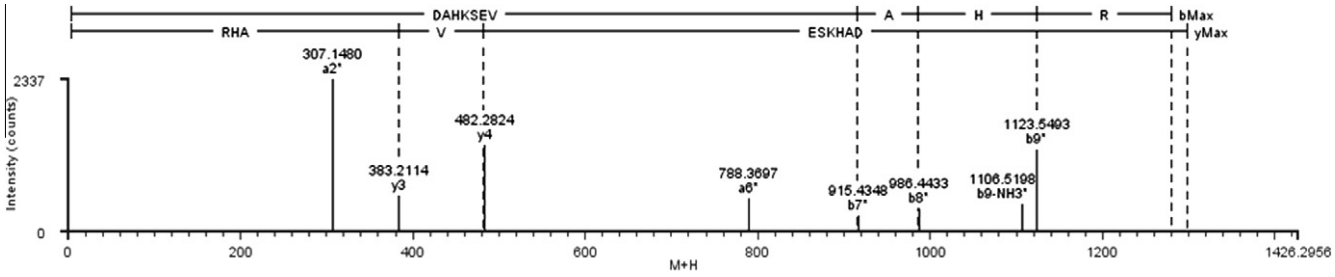

Fig.3. MS/MS fragment ion spectrum of the $[M + TDI^{*} + H]^{+}$ ion observed for HSA tryptic peptide 1–10 (DAHKSEVAHR). TDI is bound to the N-terminal NH_2 of the aspartic acid at position 1. The second isocyanate group of TDI is hydrolyzed to the amine.

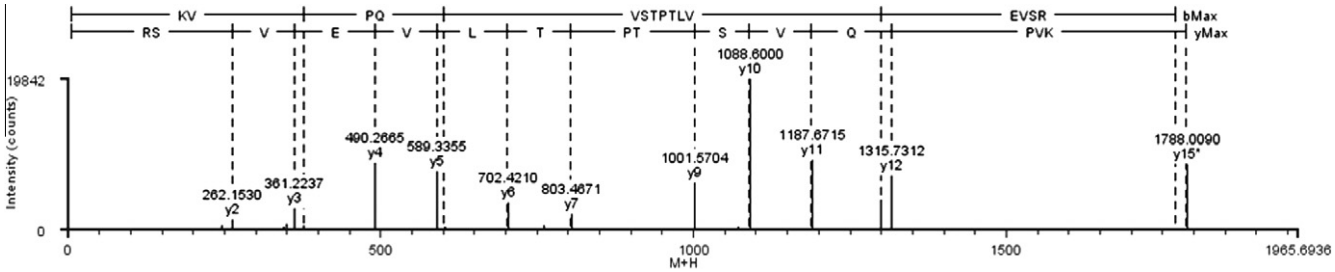

Fig.4. MS/MS fragment ion spectrum of the $[M + TDI^{*} + H]^{+}$ ion observed for HSA tryptic peptide 414–428 (KVPQVSTPTLVEVSR). TDI is bound to the ε-NH_2 of the lysine residue at position 414. The second isocyanate group of TDI is hydrolyzed to the amine.

on the basis of observed ion intensity. Ion intensities attributable to N-terminal amine binding exceed those of Lys4 binding by 2- to 10-fold depending on concentration of the isocyanate.

In their recent study on MDI–HSA conjugation, Wisnewski et al. highlighted the importance of dilysine motifs (KK) as particularly susceptible targets for MDI conjugation [27]. HSA contains four such motifs at positions 136–137, 413–414, 524–525, and 573–574. Of these, all but 573–574 were observed to conjugate MDI, in each case on the second of the two lysines. Our results with TDI–HSA conjugation differ in some important respects from those observed for MDI. First, in each case we observed TDI conjugation to both lysines in the dilysine motif, often by observation of the peptide $[M + TDI + H]^{+}$ ion, indicating intramolecular crosslinking between the two lysine ε-amines (see Table 1, HSA 411–418 $[M + TDI + H]^{+}$ ion). This difference may be attributable to the relative size of the two molecules. The elongated rigid MDI molecule is likely prohibited by sterics from binding to two adjacent lysine residues, whereas the two isocyanate moieties on TDI are in very close proximity. However, in some instances we observed independent binding to each lysine via two TDI^{*} molecules (see Table 1, HSA 565–574 $[M + 2TDI^{*} + H]^{+}$ ion). Second, we observed TDI binding to the 573–574 dilysine motif even at low isocyanate concentrations. It should be noted that the observed ion abundance for conjugation at these sites is low (~0.1–1% of the total isocyanate ion current). Our results support the hypothesis [27] that the second of the two lysines is more reactive insofar as we generally observed higher ion currents for ions that can be attributed to binding at the second of the two lysine residues. Fig. 4 presents the tandem mass spectrum of the $[M + TDI^{*} + H]^{+}$ ion of serum albumin tryptic peptide 414–428 (KVPQVSTPTLVEVSR), demonstrating that TDI is bound at the lysine at position 414, the second residue of the 413–414 dilysine motif. Observation of this ion also implies that on the protein molecules from which this ion was produced, isocyanate was not bound at position 413 because binding of TDI to a lysine residue prevents trypsin cleavage at that site. Lys414 has previously been reported to be a binding site for both MDI [27] and hexamethylene diisocyanante (HDI) [13]. This site is not conserved in other mammalian serum albumins (e.g., bovine, murine) and is hypothesized to be important to the human humoral immune response. Binding of the reactive electrophile HHPA was observed at three of the four HSA dilysine motifs (136–137, 413, and 524–525) [20], albeit not on the second of the two lysine residues in the case of Lys413.

Examining the concentration dependence of TDI binding on HSA

To determine what effect TDI concentration has on the binding sites observed, a number of experiments were performed at conjugation ratios varying from 1:2 to 40:1 TDI/HSA, and the results are summarized in Table 2. At a 1:1 molar ratio, the N terminus, Lys4, Lys199, and three of the four dilysine motifs (413–414, 524–525, and 573–574) were observed to be favored binding sites. In the extreme limiting case of a 1:2 ratio of 2,6-TDI/HSA, these sites were confined to just the N terminus, Lys4, Lys199, and the 413–414 dilysine. Increasing TDI concentration populated more binding sites up to a maximum of 37 at the highest concentration. Of the 12 lysine sites identified for MDI [27], 11 were observed, although several (notably Lys137, Lys212, Lys262, and Lys351) were observed only at ratios greater than 10:1 TDI/HSA. Fig. 5 plots the total observed ion current for four of the highly conserved binding sites as a function of isocyanate concentration. The N-terminal amine carries more than 40% of the total isocyanate ion current at low concentrations, but the total ion current does not increase at higher concentrations, indicating that this reaction product is essentially saturated at the lowest concentrations. Similarly, the 573–574 dilysine reaction product is saturated at very low concentrations, carrying 0.1–1% of the total ion current. In contrast, Lys199 (5–10% of the ion current) and the Lys413–414 KK motif (3–25% of the ion current) dramatically increase in intensity at higher concentrations. Although our results

Table 2
Comparison of TDI–albumin binding sites observed at varying conjugation ratios (TDI/HSA).

	2,4-TDI				2,6-TDI				
Residue	1:1	5:1	10:1	40:1	1:2	1:1	5:1	10:1	40:1
Asp1	X	X	X	X	X	X	X	X	X
Lys4*	X	X	X	X	X	X	X	X	X
Lys12			X	X				X	X
Lys73				X					X
Gln104				X					X
Lys106				X					X
Lys136				X					X
Lys137*			X	X				X	X
Lys159				X					X
Lys190			X	X				X	X
Gln196			X	X				X	X
Lys199*	X	X	X	X	X	X	X	X	X
Lys205			X	X				X	X
Lys212*			X	X				X	X
Lys262*				X					X
Lys274		X	X	X			X	X	X
Lys276			X	X				X	X
Lys281			X	X					X
Lys351*			X	X				X	X
Lys378		X	X	X				X	X
Lys402				X					X
Lys413	X	X	X	X	X	X	X	X	X
Lys414*	X	X	X	X	X	X	X	X	X
Lys432*		X	X	X				X	X
Lys436*			X	X				X	X
Lys439			X	X				X	X
Lys444	X	X	X	X		X	X	X	X
Lys524	X	X	X	X		X	X	X	X
Lys525*	X	X	X	X		X	X	X	X
Lys534		X	X	X		X	X	X	X
Lys536		X	X	X		X	X	X	X
Lys541*		X	X	X			X	X	X
Lys545		X	X	X			X	X	X
Lys557			X	X				X	X
Lys560			X	X				X	X
Lys573	X	X	X	X		X	X	X	X
Lys574	X	X	X	X		X	X	X	X

Note: Asterisks (*) indicated sites reported to be reactive to 4,4′-MDI by Wisnewski et al. [27].

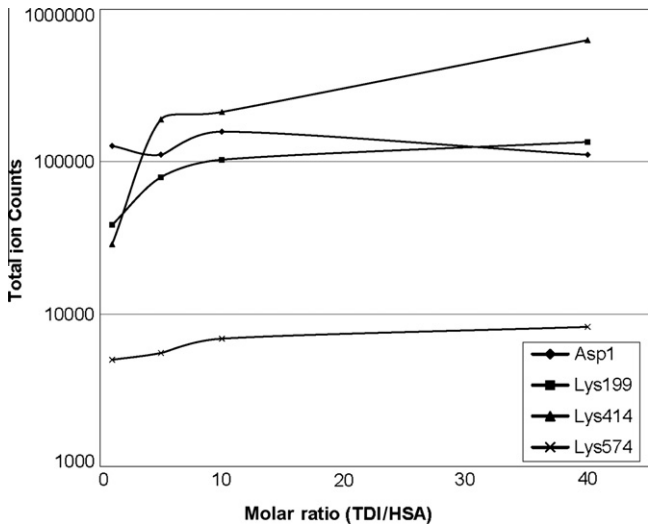

Fig.5. Comparison of the total [M + diisocyanate] ion current carried by four predominant binding sites of TDI as the TDI/HSA ratio is increased from 1:1 to 40:1.

confirm that dilysine motifs are reactive with TDI, under the conditions employed in this study, they contribute at most approximately 30% of the total isocyanate binding, whereas the N-terminal amine Lys4 and Lys199 are responsible for more than 75% of the total isocyanate binding at a 1:1 molar ratio. In addition, at high TDI concentrations (>10:1), a variety of more complex adducts are observed, including isocyanate polymerization (e.g., $[M + nTDI^{*} + H]^{+}$) and intermolecular crosslinked products (e.g., $[M + M' + TDI + H]^{+}$). We are currently pursuing immunological purification of isocyanate-bound proteins in an attempt to determine which of these sites are important for host recognition of isocyanate-haptenated proteins. Although we did not attempt exhaustive characterization of the polymerized TDI–HSA products for this study, we do not discount that such species could be immunologically active.

Differences observed in reactivity of 2,4- and 2,6-TDI toward HSA

Conjugation of serum albumin separately with 2,4- and 2,6-TDI allowed examination of the dataset with respect to differences in binding behavior between isomers. In each case, 2,6-TDI appeared to be less reactive, resulting in isocyanate–peptide ion intensities approximately half that of 2,4-TDI. Steric effects are well known to influence the reaction effects of isocyanates. Isocyanate in the 4-tolyl position is known to be more reactive than isocyanate in the 2-tolyl position due largely to the steric influence of the methyl group at position 1 [1]. Only slight differences in the "onset" of binding with a few residues were observed, in each case with the 2,4-TDI conjugated species being observed at lower isocyanate concentration than the 2,6-TDI species. 2,6-TDI did show a slight bias toward N-terminal binding at all concentrations, whereas 2,4-TDI showed a slight bias toward Lys199 and the dilysine motifs compared with 2,6-TDI.

Conclusions

Emerging bioanalytical techniques such as MS/MS are poised to make important contributions to the understanding of allergic disease. In this article, we have demonstrated the power of a multiplexed MS^{e} UPLC–MS/MS approach to the analysis of isocyanate-induced modifications on serum albumin, a putative carrier of isocyanate in vivo. This approach was employed to unambiguously identify the binding sites for the industrially important isomers, 2,4- and 2,6-TDI on HSA at varying diisocyanate/protein ratios. Both isomers preferentially react with the N-terminal amine of the protein and the ε-NH_2 of lysine. Binding sites observed at the lowest conjugation ratios are conserved at higher binding ratios, suggesting a subset of 5–10 preferential binding sites on albumin. Diisocyanate–protein conjugation results in a variety of reaction products, including intra- and intermolecular crosslinking, diisocyanate self-polymerization, and diisocyanate hydrolysis. The results presented here are in good agreement with recent studies on the binding of another occupationally relevant diisocyanate, MDI. In the aggregate, these studies indicate that there is a subset of important binding sites on serum albumin that are likely to play an important role in diisocyanate antigenicity.

Acknowledgments

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