Structural Elucidation of Isocyanate-Peptide Adducts Using Tandem Mass Spectrometry

Justin M. Hettick, Tinashe B. Ruwona, and Paul D. Siegel

Health Effects Laboratory Division, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA

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 isocyanates are reacted with model peptides, tandem mass spectrometry was employed to unambiguously identify the binding site of four commercially-relevant isocyanates on model peptides. In each case, the isocyanates react preferentially with the N-terminus of the peptide. No evidence of side-chain/isocyanate adduct formation exclusive of the N-terminus was observed. However, significant intra-molecular diisocyanate crosslinking was observed between the N-terminal amine and a side-chain amine of arginine, when Arg was located within two residues of the N-terminus. Addition of multiple isocyanates to the peptide occurs via polymerization of the isocyanate at the N-terminus, rather than via addition of multiple isocyanate molecules to varied residues within the peptide. The direct observation of isocyanate binding to the N-terminus of peptides under these experimental conditions is in good agreement with previous studies on the relative reaction rate of isocyanate with amino acid functional groups. (*J Am Soc Mass Spectrom* 2009, 20, 1567–1575) © 2009 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Diisocyanates are highly reactive chemicals widely utilized in the manufacture of polyurethane products, such as fibers, foams, insulation, and automotive products, including paints and protective coatings for truck beds. The most widely used isocyanates are the diisocyanates methylenebis(phenyl isocyanate) (MDI), toluene diisocyanate (TDI) and hexamethylene diisocyanate (HDI). TDI is often utilized in industrial applications in 80/20 and 65/35 mixtures of the 2,4- and 2,6-TDI isomers. Globally, TDI accounts for 34% of the isocyanate market [1]. Diisocyanate exposure results in sensitization and asthma, contact dermatitis, and hypersensitivity pneumonitis [2]. Diisocyanate-induced asthma in occupationally exposed worker populations is estimated to range from 5% to 30% [2–5]. The isocyanate ($\text{N}=\text{C}=\text{O}$) functional group may react with water to form amines, with alcohols to form urethanes, with amines to form ureas, with carboxylic acids to form amides, and following hydrolysis, with other isocyanates to form dimers and trimers. Previous studies [6, 7] present strong evidence that diisocyanates bind to such proteins as albumin and keratins in the airway. Furthermore, some occupational tasks, such as paint mixing/spraying and production of composite wood products, result in significant diisocyanate der-

mal exposures [8, 9] which may also lead to the development of asthma-like symptoms.

Although several protein targets of diisocyanates *in vivo* have been identified, the underlying antigenic forms of the diisocyanate are, as yet, unknown [10–12]. 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 physiologic 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 imidazole ring of histidine [13].

Tandem mass spectrometry, in particular collision induced dissociation (CID) of $[\text{M} + 2\text{H}]^{2+}$ ions produced via electrospray ionization (ESI) [14–17] is a powerful technique for the determination of not only primary sequence in peptides [18], but also post-translational modification [15] or chemical adduction. In this study, we have applied CID on a high-resolution quadrupole time-of-flight (qTOF) mass spectrometer [19] to characterize the adducts produced by the reaction of four commercially available bioactive peptides with four industrially relevant mono- and diisocyanates in an effort to (1) determine the number, chemical identity, and relative abundance of reaction products observed, and (b) identify the specific binding site of the isocyanate within the peptide. In particular, we have studied the adducts formed by 2,4- and 2,6-TDI because of the widespread

Address reprint requests to Dr. J. M. Hettick, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Health Effects Laboratory Division, MS L-2040, 1095 Willowdale Road, Morgantown, WV 26505, USA. E-mail: jhettick@cdc.gov

Figure 1. Structures of the isomers of toluene isocyanate, toluene diisocyanate and a hydrolysis product.

use of these chemicals in manufacturing, and their well-documented health effects. Due to the possibility of complex reaction products including inter- and intramolecular crosslinking with the diisocyanates, we have also evaluated the binding chemistry of the monoisocyanate analogues of 2,4- and 2,6-TDI, para-toluene isocyanate (PTI) and ortho-toluene isocyanate (OTI).

Experimental

Reagents

Unless otherwise specified, all reagents were acquired from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Formic acid (99.5%) was of mass spectrometry grade and acetonitrile and acetone were HPLC-grade. Ortho-toluene isocyanate (OTI), para-toluene isocyanate (PTI), 2,4-toluene diisocyanate (2,4-TDI, and 2,6-toluene diisocyanate (2,6-TDI) were dissolved in HPLC-grade acetone. The peptides leucine-enkephalin (Leu-enk, YGGFL), angiotensin I (DRVYIHPFHL), Substance P-amide (RPKPQQFFGLM-NH₂), and fibronectin-

adhesion promoting peptide (FAPP, WQPPRARI) were dissolved in 18 M Ω distilled deionized water (DDI) produced by a Millipore Synthesis A-10 (Billerica, MA, USA).

Preparation of Isocyanate-Peptide Adducts

Stock peptide solutions were prepared in DDI at a concentration of 0.5 mg/mL. Solutions of each of the four isocyanates were freshly prepared in 1 mL HPLC-grade acetone and infused at a 1:1 mol ratio into each peptide solution at a rate of 1.2 mL/h using a syringe pump (model 100; KD Scientific Inc., Holliston, MA, USA) with constant stirring. Reactions were carried out at room temperature in a chemical fume hood, and allowed to stir for 15 min following completion of the infusion. Peptide-isocyanate adduct solutions were filtered using 0.45 μ m filters (Millipore, Billerica, MA, USA) and stored at -20°C until analysis.

Mass Spectrometry

Aliquots of each peptide adduct solution were diluted 50:1 in 50/50 0.1% formic acid/HPLC-grade acetonitrile, yielding a final solution concentration of all peptide-NCO species of ~ 100 pmol/ μ L. Samples were infused at a rate of 5 μ L/min to a Micromass QTOF2 (Waters Corp., Milford, MA, USA) quadrupole time-of-flight mass spectrometer operated in positive electrospray (+ESI) mode. Sample analysis was performed at a capillary voltage of 2.5 kV with dry N₂ desolvation gas (NitroFlowLab; Parker Hannifin Corp., Haverhill, MA, USA) at a flow rate of 400 L/h and a temperature of 150°C . Ultra High Purity (UHP) Argon was used as a collision gas at collision energies of 5 eV (MS analysis) and 25–30 eV (MS/MS analysis). For peptide sequencing, the doubly charged $[M + 2H]^{2+}$ ion was selected by MS¹, with the exception of Leu-enkephalin (the $[M + H]^{+}$ ion was selected). The collision energy was set independently for each peptide to yield optimal relative abundance of sequence-specific fragment ions. Post-acquisition mass spectral charge deconvolution was performed with the “MaxEnt3” algorithm in the ProteinLynx 4.1 software suite (Waters Corp., Milford, MA, USA). The mass spectrometer was externally calibrated over the range 70–1570 u using the CID fragment ion mass spectrum of [Glu]¹-fibrinopeptide B (EGVNDNEEGFFSAR) $[M + 2H]^{2+}$ ions acquired at a collision energy of 35 eV.

Table 1. Relative ion intensities of ortho- and para-toluene isocyanate/peptide reaction products

Peptide	Sequence	M	M + OTI	M + PTI
Leu-enk	YGGFL	100	4.7	1.3
Angiotensin I	DRVYIHPFHL	13	100	18
FAPP	WQPPRARI	26	31	100
Substance P	RPKPQQFFGLM-NH ₂	0.35	100	32

Table 2. Relative ion intensities of 2,4-toluene diisocyanate/peptide reaction products

Peptide	Sequence	M	M + TDI*	M + TDI	M + 2TDI*	2M + TDI
Leu-enk	YGGFL	100	3.4	—	0.89	—
Angiotensin I	DRVYIHPFHL	83	100	29	47	23
FAPP	WQPPRARI	100	13	—	5.5	9.4
Substance P	RPKPQQFFGLM-NH ₂	33	17	100	9.6	50

*Hydrolysis product (amine group at either position 2 or 4).

Results and Discussion

MS Analysis of Isocyanate/Peptide Reaction Products

Four bioactive peptides were chosen for analysis (Leu-enk, angiotensin I, FAPP, and Substance P-amide) and reacted with two monoisocyanates (OTI and PTI) and two diisocyanates (2,4-TDI and 2,6-TDI). Structures and mass-to-charge data for each of these isocyanates is presented in Figure 1. The four peptides were chosen because they contain putative reactive amino acid residues in varied locations (e.g., N-terminus, internal, C-terminus). The resulting reaction products were analyzed by high-resolution qTOF-MS. The relative abundance and accurate mass (± 20 ppm) of the major reaction products was measured in qTOF-MS mode and the identity and relative intensity of the observed reaction products are listed in Tables 1, 2, and 3. The only reaction products observed for the monoisocyanates OTI and PTI (Table 1) were unreacted peptide $[M + 2H]^{2+}$ and the product formed by addition of one isocyanate to the peptide $[M + NCO + 2H]^{2+}$ (+133.0528 u). For Leu-enk, angiotensin I, and Substance P, the OTI adduct was two to five times more abundant than the PTI adduct.

The products of the reaction of peptides with the diisocyanate species 2,4- and 2,6-TDI were significantly more varied. A number of reaction products were observed (see Tables 2 and 3) including the anticipated mono-substituted $[M + dNCO^* + 2H]^{2+}$ (+148.0634 u) product. (The asterisk (*) indicates the diisocyanate product that has undergone hydrolysis at one isocyanate, see Figure 1). TDI hydrolyzes rapidly to a diamine in aqueous solution, with a half life of 5 to 30 s [20, 21]. Consequently, the +174.0429 u $[M + dNCO + 2H]^{2+}$ adduct is not observed unless the second isocyanate reacts at another site within the peptide to form an intramolecularly crosslinked species. In the case of Substance P, this intramolecular crosslinked species was the dominant reaction product, whereas it was only

a minor reaction product (ca. 30% relative abundance) for angiotensin I. Intramolecular crosslinking is not observed for leu-enk or FAPP, neither of which has residues with side-chain amines near the N-terminus. Addition of two hydrolyzed TDI molecules, $[M + 2dNCO^* + 2H]^{2+}$, was observed in low relative abundance, as was intermolecular crosslinking of two peptides via TDI, $[2M + dNCO + 2H]^{2+}$. Sabbioni and coworkers [13] suggested in 2001 that both isocyanate moieties of a diisocyanate would not react at the same time with one or more proteins (e.g., formation of $[M + dNCO + 2H]^{2+}$ and $[2M + dNCO + 2H]^{2+}$ should not be observed). However, under the conditions employed here, these products are formed, and in the case of Substance P, such crosslinking represents the dominant reaction product(s).

Addition of a second TDI molecule to the peptide is the result of polymerization of two diisocyanates to form a dimer bound to the peptide, rather than binding of two dNCOs to two different amino acid residues (e.g., side-chain adduction). This was determined on the basis of CID data (described below), as well as the observation of two TDI molecules bound to Leu-enkephalin, which has only one putative binding site, the N-terminal primary amine.

In general, we observe that isocyanates at the 2-position (OTI and 2,6-TDI) are far more abundant adducts than those at the 4-position (PTI and 2,4-TDI) under the aqueous conditions employed for this study. Although the isocyanate in the 4-position is roughly four times more reactive than that in the 2-position toward alcohols (during polyurethane production) the 2-position is more reactive toward amines, in good agreement with our observations [1, 22]. A comparison of the relative reactivity of the peptides with isocyanates is difficult to make as the pH of non-buffered aqueous solutions will vary with the chemical nature of the peptide dissolved. Although current investigations into the effect of pH on isocyanate-peptide binding are underway in our laboratory, previous studies have dem-

Table 3. Relative ion intensities of 2,6-toluene diisocyanate/peptide reaction products

Peptide	Sequence	M	M + TDI*	M + TDI	M + 2TDI*	2M + TDI
Leu-enk	YGGFL	100	3.7	—	0.11	—
Angiotensin I	DRVYIHPFHL	40	100	5.2	30	18
FAPP	WQPPRARI	100	14	—	4	4.7
Substance P	RPKPQQFFGLM-NH ₂	6.2	29	100	11	18

*Hydrolysis product (amine group at either position 2 or 4).

onstrated that buffers, such as phosphate and carbonate, act as competing nucleophiles in this system [23].

Tandem MS Analysis of Isocyanate-Peptide Adducts

To determine the isocyanate binding site, each peptide and its isocyanate adduct(s) was selected for sequencing by tandem mass spectrometry utilizing Ar collision-induced dissociation in the qTOF mass spectrometer. The MS/MS spectrum of each peptide and its corresponding isocyanate adducts are presented in Figures 2, 3, 4, 5, and 6. For each peptide, the tandem mass spectra of the monoisocyanate adducts (OTI and PTI) and the diisocyanate adducts (2,4- and 2,6-TDI) were very similar in terms of m/z and relative abundance of the

observed fragment ions. For simplicity, only the fragment ion spectra of the PTI and 2,4-TDI adducts are presented here. The fragment ion mass spectra of the OTI and 2,6-TDI adducts may be found in the Supporting Information, which can be found in the electronic version of this article.

Figure 2 presents the CID fragment ion mass spectra for Leucine-enkephalin (YGGFL) and the adducts produced by reaction with PTI and 2,4-TDI. The tandem mass spectrum of unmodified Leu-enk (Figure 2a) is characterized by abundant a- and b-type fragment ions indicative of N-terminal charge retention. A few low abundance y-type ions, indicative of C-terminal charge retention, are observed. Similarly, the fragment ion mass spectra of the peptide-isocyanate adducts (Figure 2b and c) are characterized by abundant N-terminal

Figure 2. Selected tandem mass spectra for Leucine-enkephalin (YGGFL). (a) Unmodified peptide ($[M + H]^+$ m/z = 556.2766); (b) PTI adduct ($[M + H]^+$ m/z = 689.3294); (c) 2,4-TDI adduct ($[M + H]^+$ m/z = 704.3400).

Figure 3. Selected tandem mass spectra for angiotensin I (DRVYIHPFHL). (a) Unmodified peptide ($[M + 2H]^{2+}$ $m/z = 648.8463$); (b) PTI adduct ($[M + 2H]^{2+}$ $m/z = 715.3728$); (c) 2,4-TDI adduct ($[M + 2H]^{2+}$ $m/z = 722.8781$).

charge retention ions. In each case, the base peak in the fragment ion spectrum is of the type $[b_1 + \text{NCO}]^+$ (denoted $b1^*$), which provides direct evidence that the isocyanate is bound to the N-terminal tyrosine of the peptide. Furthermore, tyrosine is the only immonium ion observed bound to the isocyanate. These immonium-isocyanate ions are useful because they provide direct evidence of isocyanate bound to a specific residue. A complete set of low abundance y_n^+ ions are observed; however, no ions of the type $[y_n + \text{NCO}]^+$ are observed, providing strong evidence that NCO is exclusively bound to the N-terminal tyrosine. Loss of PTI and 2,4-TDI are observed from a -, b -, and immonium ions, indicating that the urea bond formed when the isocyanate binds to the N-terminus is labile under CID conditions. Because of this, we cannot rule out low

abundance binding of NCO to other sites than the N-terminus, however, the spectra shown herein present no evidence of such binding. A complementary nonergodic fragmentation methodology, such as electron capture dissociation (ECD) [24], could help clarify this issue by reducing small molecule losses from peptide ions, but is not available on qTOF MS instrumentation.

The CID tandem mass spectra of angiotensin I (DRVYIHPFHL) and its PTI and 2,4-TDI adducts are presented in Figure 3. The tandem mass spectrum of the angiotensin I $[M + 2H]^{2+}$ ion (Figure 3a) shows abundant fragmentation with both N- and C-terminal charge retention, particularly for fragment ions containing histidine (e.g., y_2 and y_4). The tandem mass spectra of the isocyanate adducts (Figure 3b and c) yield complete

Figure 4. Selected tandem mass spectra for fibronectin adhesion promoting peptide (WQPPRARI). (a) Unmodified peptide ($[M + 2H]^{2+}$ $m/z = 512.2960$); (b) PTI adduct ($[M + 2H]^{2+}$ $m/z = 578.8227$); (c) 2,4-TDI adduct ($[M + 2H]^{2+}$ $m/z = 586.3280$).

sequence information, demonstrating that the isocyanate has bound to the N-terminal aspartic acid residue. Isocyanate is not observed bound to any C-terminal (y -type) fragment ions, nor is it observed bound to the histidine immonium ion. Observation of the prominent y_2 ion and lack of the $[y_2 + NCO]^+$ fragment ion suggests that neither the secondary amine on the side chain of histidine nor the C-terminus is reactive with isocyanate under these conditions. We conclude that isocyanate is bound to the N-terminal amine rather than the side-chain carboxylic acid of the N-terminal aspartic acid because no C-terminal binding of the isocyanate is observed in this or any other tandem mass spectra. The pK_a of the aspartic acid side chain is similar to C-terminal carboxylic acids, both of which should be deprotonated at non-acid pH, and therefore would be expected to have similar reactivity toward isocyanates.

For all four peptides examined in this study, we observe the apparent addition of two TDI molecules (+296.1468 u). To determine the binding site of the second TDI molecule, CID was performed on the $[M + 2TDI^* + 2H]^{2+}$ ion of angiotensin I (supporting data). The tandem mass spectrum (in particular, observation of the y_9 ion) unambiguously identifies the binding site of the second TDI molecule as the N-terminus. This suggests that one isocyanate group of TDI binds to the N-terminus and the second isocyanate group is hydrolyzed to the amine, which then reacts with a second TDI molecule. Thus, the two TDI molecules are polymerized at the N-terminus, rather than bound to two separate locations within the peptide.

Figure 4 presents the CID tandem mass spectra for fibronectin adhesion promoting peptide (WQPPRARI) and its PTI and 2,4-TDI adducts. The tandem mass spectrum of the FAPP $[M + 2H]^{2+}$ ion (Figure 4a)

Figure 5. Selected tandem mass spectra for Substance P-amide (RPKPQQFFGLM-NH₂). (a) Unmodified peptide ([M + 2H]²⁺ m/z = 674.3713); (b) PTI adduct ([M + 2H]²⁺ m/z = 740.8981); (c) 2,4-TDI adduct ([M + 2H]²⁺ m/z = 748.4034).

shows predominantly y-type ions, indicative of C-terminal charge retention. This is not an unexpected result, given the strongly basic residue arginine at positions 5 and 7 within the peptide. Abundant loss of NH₃ from fragment ions is observed, consistent with the tandem mass spectra of other peptides containing Arg, Lys, Gln, and Asn. In addition, a strong [b₇ + H₂O]⁺ ion is observed, as has been well documented for peptides with non-C-terminal protonated side-chain residues, such as Arg, Lys, and His [25]. From the tandem mass spectrometry data (Figure 4b and c), the isocyanate adduct was unambiguously assigned to the N-terminal tryptophan residue. No ions of the type [y_n + NCO]⁺ are observed, in particular, the lack of [y₂ + NCO]⁺ and [y₄ + NCO]⁺ ions suggests that the isocyanate does not react with the side-chain amine of the arginine residues. Further evidence of binding to the N-terminus is obtained through the observation of the

[Y + NCO]⁺ immonium ion and an unusual b₁ ion. Formation of acylium b₁ ions is generally considered to result in the loss of CO to form the stable a₁ immonium ion, however, a number of reports have described the formation of stable cyclic b₁ ions [26, 27].

The tandem mass spectrometry data for Substance P amide (RPKPQQFFGLM-NH₂) and its PTI and 2,4-TDI adducts are presented in Figure 5. Fragmentation of the [M + 2H]²⁺ ion (Figure 5a) is characterized by abundant a- and b-type ions, internal fragment ions containing lysine, and loss of NH₃. C-terminal y-type ions are observed in low relative abundance. The isocyanate adducts are localized to the N-terminal arginine residue (Figure 5b and c), as evidenced by [a₁ + NCO]⁺ and [b₁ + NCO]⁺ fragment ions. No [y_n + NCO]⁺ ions are observed. Of particular note, [y₉ + NCO]⁺ (Lys-containing) and [y₁ + NCO]⁺ (C-terminal Met amide-containing) ions are absent, suggesting no isocyanate adduction at either the

Figure 6. Tandem mass spectrum for Substance P-amide (RPKPQQFFGLM-NH₂) + 174 u adduct. ([M + 2H]²⁺ *m/z* = 761.3931).

C-terminal amide or the lysine side-chain at position 3. The most abundant product of the reaction of diisocyanates (2,4-TDI and 2,6-TDI) with Substance P is the [M + dNCO + 2H]²⁺ (+ 174.0429 u) adduct resulting from intramolecular crosslinking. The CID fragment ion spectrum of this ion is presented in Figure 6. The dNCO adduct from the diisocyanate is located on the N-terminal arginine, presumably bound to the N-terminus and an amine on the side chain of arginine. Similarly, a 174 u adduct is observed for the reaction of angiotensin I (which has an arginine at position 2) with diisocyanates (see Tables 2 and 3), although this adduct is observed in low relative abundance. Intramolecular crosslinking via diisocyanates with arginine residues further removed from the N-terminus (FAPP has arginine at positions 5 and 7) is not observed. We interpret this data in aggregate to indicate the diisocyanate rapidly binds to the N-terminal amine and may subsequently react with a side-chain amine of a residue in close proximity. We interpret the relative abundance of these products to indicate that the kinetics of the subsequent side-chain reaction are fast with respect to hydrolysis if the arginine is at position 1, but slow relative to hydrolysis if the arginine is removed to position 2.

Taken as a whole, the present experiments support the findings of Stark [28], who found that the rate of reaction of isocyanates with amino groups is related linearly to pK_a. That work suggested that at pH 7 and below, the N-terminus of peptides and proteins would react ~100 times faster than the ε-NH₂ group of lysine. Furthermore, Stark suggested the reaction proceeds via the uncharged -NH₂ species, rather than the -NH₃⁺ ion. The tandem mass spectrometry data presented here supports this hypothesis, as the isocyanate is observed bound preferentially to the N-terminal amine in each peptide. Similarly, Mason and Liebler [29] used phenyl isocyanate at pH 8.0 to label the N-termini of peptides generated by proteolytic digests. Other reports [13, 30] have suggested that the N-terminus of proteins (particularly albumin and hemoglobin) is the potential site of

diisocyanate adduction, and the results presented here demonstrate conclusively, on the basis of tandem mass spectrometry data, that the N-terminal α-amine of these four model peptides is the site of adduction.

Conclusions

Analysis of peptide-isocyanate adducts by tandem mass spectrometry reveals that isocyanates bind preferentially to the N-terminus of the four peptides examined under the conditions employed herein. 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. When the isocyanate-peptide reaction is carried out under 1:1 stoichiometric conditions, the extent of reaction depends on both the choice of isocyanate and peptide. In general, the isocyanate in the ortho position seems to be more reactive than that in the para position. The results of this study, when taken in aggregate with those of previous studies, indicate the N-terminus of proteins is a likely target for adduction in isocyanate-exposed individuals.

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Appendix A Supplementary Material

Supplementary material associated with this article may be found in the online version at [doi:10.1016/j.jasms.2009.04.016](https://doi.org/10.1016/j.jasms.2009.04.016).

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