

# Controlled Formation of Peptide Bonds in the Gas Phase

Sunyoung Lee, Stephen J. Valentine, James P. Reilly, and David E. Clemmer\*

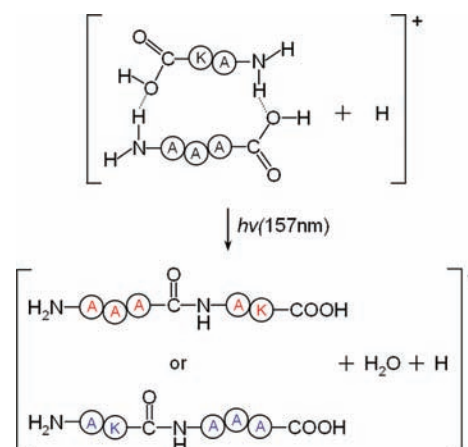
Department of Chemistry, Indiana University, Bloomington, Indiana 47405, United States

**ABSTRACT:** Photoexcitation (using 157 nm vacuum ultraviolet radiation) of proton-bound peptide complexes leads to water elimination and the formation of longer amino acid chains. Thus, it appears that proton-bound dimers are long-lived intermediates along the pathway to peptide formation. Product specificity can be controlled by selection of specific complexes and the incorporation of blocking groups at the N- or C-termini. The product peptide sequences are confirmed using collision-induced dissociation.

While investigating the photodissociation of ions, we found that it is possible to deliberately form peptide bonds in the gas-phase environment of a mass spectrometer. In this approach we create noncovalent, proton-bound dimer ions by electrospray ionization (ESI).<sup>1</sup> Specific complexes are mass-selected and irradiated with 157 nm (7.9 eV) vacuum ultraviolet (VUV) photons, and among the observed products are ions with mass-to-charge ( $m/z$ ) ratios that are consistent with elimination of H<sub>2</sub>O. Subsequent collision-induced dissociation (CID) of these ions reveals the formation of peptide bonds corresponding to the synthesis of longer chains of amino acids. That proton-bound dimers are long-lived intermediates along the pathway that leads to peptide formation is intriguing. Modification of the termini of individual reactants provides a means of controlling which sequences form. The formation of peptide bonds appears to be general over a wide size range and makes it possible to directly synthesize peptide-based hybrid molecules. Controlled bond formation in gas-phase ions is currently attracting significant attention<sup>2–5</sup> and should complement condensed-phase synthetic techniques,<sup>6</sup> providing rapid access to many new species for study. Additionally, studies of photoinduced bond formation in these types of species may provide insight into the origins of biologically relevant molecules.<sup>7–10</sup>

The first reported synthesis of a peptide was Fisher's generation of the diglycine in 1901.<sup>11</sup> His approach was to hydrolyze diketopiperazine. Over the next 50 years, syntheses were restricted to short sequences because of limitations imposed by solubility, undesirable side reactions, and the cumbersome nature of existing analytical techniques required for purification. In 1963, Merrifield revolutionized peptide synthesis with the introduction of solid-phase synthetic techniques to produce the tetrapeptide Leu-Ala-Gly-Val.<sup>12</sup> These methods were adopted widely to synthesize a range of peptide sizes and sequences<sup>13–16</sup> and were suitable for combinatorial approaches.<sup>17–19</sup> Despite these extraordinary advances, a number of bottlenecks remain, including the time required to carry out reactions; incomplete reaction steps that reduce yield and lead to unexpected sequences and other impurities; and related issues associated with solubility,

**Scheme 1.** Peptide Formation of AAAAK and AKAAA Pentapeptides from the [AK+AAA+H]<sup>+</sup> Complex upon 157 nm Irradiation

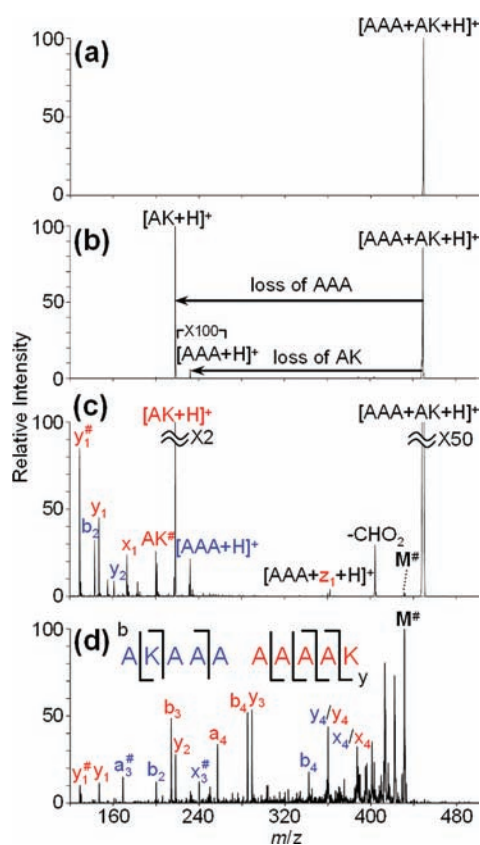


purification, and characterization.<sup>20,21</sup> The strategy described here leads to only small amounts of peptides and in the unusual vacuum environment. However, the synthesis and characterization steps are nearly instantaneous, and problems related to solubility of longer sequences in solution are alleviated in the gas phase by choosing appropriate shorter precursors.

Scheme 1 illustrates the approach. It is established that proton-bound dimers of two different peptides can be formed by ESI.<sup>22,23</sup> One low-energy configuration that is anticipated for such a complex is a head-to-tail arrangement between peptides that maximizes electrostatic interactions of the amino-terminus of each peptide with the carboxylic acid end of the other. Upon irradiation, elimination of a single water molecule from the complex results in the formation of a peptide bond. This leads to longer amino acid chains. In Scheme 1, we show the combination of a simple dipeptide (AK) with the tripeptide (AAA) to produce two pentapeptide sequences (AKAAA or AAAAK). Experimental data supporting these ideas are shown in Figure 1. Noncovalent, proton-bound [AAA+AK+H]<sup>+</sup> dimer ions ( $m/z = 449.2$ ) are produced by electrospraying a 49.5:49.5:1.0 (v:v:v) water:methanol:acetic acid solution containing  $\sim 10^{-5}$  M peptide precursors. Ions are transferred at 300 K through a drift tube and accumulated in a linear ion trap (LTQ Velos, Thermo-Electron, San Jose, CA) that has been modified to allow ion activation with light from a F<sub>2</sub> laser, as previously described.<sup>24</sup> Figure 1a shows the selected [AAA+AK+H]<sup>+</sup> precursor signal at  $m/z = 449.2$ . Upon collisional activation, these ions heat up and

Received: June 20, 2011

Published: September 12, 2011



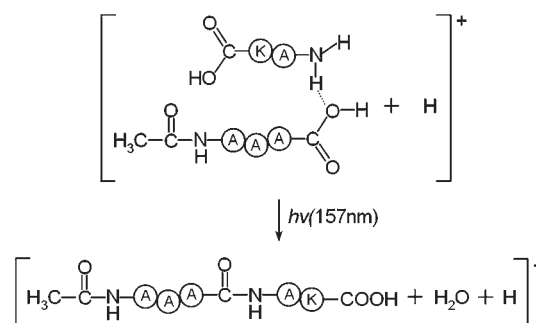
**Figure 1.** (a) Isolation spectrum of the  $[AAA+AK+H]^+$  complex ions, and MS/MS spectra of  $[AAA+AK+H]^+$  obtained by (b) CID and (c) 157 nm irradiation. Product ions from the  $[AK+H]^+$  and  $[AAA+H]^+$  are highlighted in red and blue, respectively. # indicates the loss of water. (d) CID spectrum of the water-loss product ( $M^\#$ ) in spectrum c. Peaks are assigned according to the two possible peptide sequences, AAAAK (red) and AKAAA (blue).

dissociate, primarily by the loss of the AAA neutral, producing the large  $[AK+H]^+$  peak ( $m/z = 218.2$ ). The AK neutral loss channel, resulting in  $[AAA+H]^+$  ( $m/z = 232.2$ ), is also observed, but the ion signal is much smaller. This analysis is consistent with our assignment of  $[AAA+AK+H]^+$  as a noncovalent complex.

When the accumulated  $[AAA+AK+H]^+$  complexes are irradiated with the  $F_2$  laser, we obtain the fragment ion spectrum shown in Figure 1c. In addition to peaks observed from dissociation of the noncovalent complex, many new types of ions that arise from breaking covalent bonds can be observed, similar to those previously reported in 157 nm photofragmentation studies of peptide monomer ions.<sup>25,26</sup> One difference that stands out is the observation of the  $[M-18+H]^+$  peak ( $M^\#$ ) at  $m/z = 431.2$ . This ion must arise from the loss of neutral water. While elimination of water or ammonia upon thermal activation of peptide monomer ions is well known,<sup>27</sup> there are no reports of this phenomenon for noncovalent peptide complexes. These types of species normally dissociate to form monomer units.

To understand the origin of water elimination, we have collisionally activated the  $[M-18+H]^+$  ion ( $M^\#$ ) that appears from photoexcitation (Figure 1c). The resulting CID spectrum is shown in Figure 1d. Interpretation of this spectrum is somewhat complicated. Assuming that both  $[AKAAA+H]^+$  and  $[AAAAK+H]^+$  are formed, peaks at  $m/z = 200.0$ ,  $342.2$ , and  $360.1$  correspond to the  $b_2$ ,  $b_4$ , and  $y_4$  ions from the AKAAA sequence. The

### Scheme 2. Peptide Formation of ac-AAAAK Pentapeptides from the $[ac-AAA+AK+H]^+$ Complex upon 157 nm Irradiation

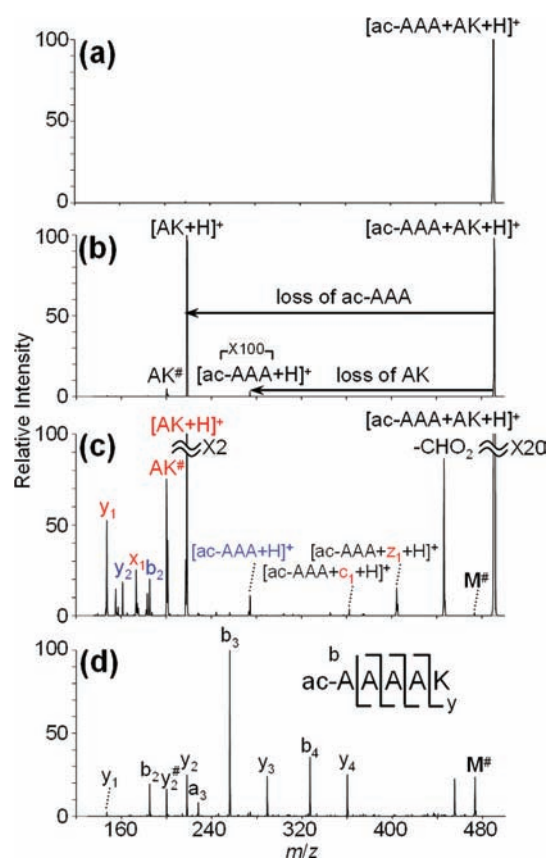


$m/z = 147.0$ ,  $214.0$ ,  $218.1$ ,  $257.2$ ,  $285.2$ ,  $289.2$ , and  $360.1$  fragments can be assigned to the  $y_1$ ,  $b_3$ ,  $y_2$ ,  $a_4$ ,  $b_4$ ,  $y_3$ , and  $y_4$  ions of AAAAK.

This interpretation requires that it is possible to eliminate water from either end of the complex (Scheme 1) and form peptide bonds that link the smaller units together to produce a longer amino acid chain. In this sense, the proton-bound complex presents itself as a well-defined, long-lived intermediate along the pathway to larger peptide formation. With this in mind, it should be possible to control which sequence is formed by simply blocking the end of one of the peptides. This prevents water elimination from one head-to-tail region of the complex, as shown in Scheme 2, where the amino terminus of the precursor AAA peptide is acetylated, to produce ac-AAA.

Figure 2 shows the results of experiments that are analogous to those described above; however, we first have acetylated the amino terminus of the tripeptide AAA. By blocking this group, photoexcitation of the noncovalent complex should produce only the ac-AAAAK sequence. As discussed above, we first select the proton-bound dimer precursor (Figure 2a) as the proposed intermediate for  $[ac-AAAAK+H]^+$  formation. A CID analysis confirms that this complex corresponds to the noncovalent  $[ac-AAA+AK+H]^+$  complex (Figure 2b). Upon 157 nm irradiation, a small peak corresponding to the  $[M-18+H]^+$  water-loss product at  $m/z = 473.3$  is detected (Figure 2c). Finally, Figure 2d shows the CID spectrum of the  $m/z = 473.3$  ion. This spectrum is remarkably simple, and a nearly complete set of y and b ions corresponding to only the ac-AAAAK sequence is observed. Additionally, this spectrum is indistinguishable from that obtained upon fragmentation of  $[ac-AAAAK+H]^+$  ions, where the precursor ac-AAAAK peptide (here, used as a standard) was produced by solid-phase synthesis. The AKAAA sequence has clearly been suppressed by acetylating the sequence AAA, providing exquisite reaction control. It is noteworthy that no evidence for cross-linking involving the lysine residue butylamine group is observed.

This phenomenon is observed for a range of amino acid chain lengths and sequences, as shown in Table 1. Thus, we can direct the formation of a range of well-known or exotic species for study. For example, combination of the pentapeptide ac-RPPGF and tetrapeptide SPFR can be used to produce the nonapeptide ac-RPPGFSPFR (acetylated bradykinin). Alternatively, inclusion of unnatural amino acids into the noncovalent intermediate complex allows formation of species such the tripeptide ac-AAA that incorporates a dodecylamine group at the C-terminal end (Table 1). Finally, it is well known that, by incorporating more



**Figure 2.** (a) Isolation spectrum of the  $[\text{ac-AAA+AK+H}]^+$  complex ions and MS/MS spectra of  $[\text{ac-AAA+AK+H}]^+$  obtained by (b) CID and (c) 157 nm irradiation. Product ions from the  $[\text{AK+H}]^+$  and  $[\text{ac-AAA+H}]^+$  are highlighted in red and blue, respectively. # indicates the loss of water. (d) CID spectrum of the water-loss product ( $M^\#$ ) in spectrum c. Peaks are assigned according to the peptide sequence, ac-AAAAK. Within experimental uncertainty, this spectrum is indistinguishable from CID spectra obtained for ESI-generated  $[\text{ac-AAA+AK+H}]^+$ , where the precursor ac-AAA AK peptide (here used as a standard) was produced by solid-phase synthesis.

**Table 1. Example Precursors and Peptide Sequences That Have Been Synthesized**

precursor peptides	synthesized peptides
GP	GPGP
LN	LNLN
AK, KL	AKKL, KLAk
GPGG	GPGGGPGG
GPGG, AK	GPGGAK, AKGPGG
Met-enkephalin (YGGFM)	YGGFMYGGFM
angiotensin II (DRVYIHPF), HL	DRVYIHPFHL (angiotensin I), HLDRVYIHPF
RPPGF, SPFR	RPPGFSPFR (bradykinin), SPFRPPGF
ac-RPPGF, SPFR	ac-RPPGFSPFR
AAA, AK	AAAAK, AKAAA
ac-AAA, AK	ac-AAAAK
ac-AAA, Met-enkephalin	ac-AAAYGGFM
ac-AAA, dodecylamine	ac-AAA-dodecylamine
bradykinin fragment 1–8 (RPPGFSPF)	RPPGFSPFRPPGFSPF

than two peptides into the ESI process, a complex combination of complexes can be obtained; thus, the approach extends naturally to make a range of related species. Because this is done inside the mass spectrometer, the method is rapid, selective, and sensitive, making it possible to produce and analyze species for study even with limited reagents. That gas-phase proton-bound dimers exist as long-lived intermediates along the pathway to peptide formation raises interesting possibilities about the mechanisms that direct bond formation. One possible mechanism that is consistent with the experimental findings is the Norrish type I reaction.<sup>28</sup> In this case, a hydrogen bond is formed between the oxygen on the C-terminus and the hydrogen on the N-terminus (Scheme 1). Photoexcitation leads to homolytic cleavage of the bond between the carbonyl carbon and the hydroxyl oxygen on the C-terminus of one peptide ion. This is followed by hydrogen abstraction from the N-terminus of the other peptide ion by the newly formed hydroxyl radical. The final step is radical recombination to form the peptide bond. It is of interest to elucidate the structures of such complexes, as well as the role and location of the excess proton.

## AUTHOR INFORMATION

Corresponding Author  
clemmer@indiana.edu

## ACKNOWLEDGMENT

The authors acknowledge David Smiley and Randy Arnold for synthesizing and characterizing peptide standards. We also thank a reviewer for drawing our attention to the Norrish mechanism and its consistency with experimental findings. This work is supported in part by a grant for the development of new instrumentation from the National Institutes of Health (1RC1GM090797-02).

## REFERENCES

- (1) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64–71.
- (2) Han, H.; McLuckey, S. A. *J. Am. Chem. Soc.* **2009**, *131*, 12884–12885.
- (3) Mentinova, M.; McLuckey, S. A. *J. Am. Chem. Soc.* **2010**, *132*, 18248–18257.
- (4) Mentinova, M.; McLuckey, S. A. *J. Am. Soc. Mass Spectrom.* **2011**, *22*, 912–921.
- (5) Ly, T.; Julian, R. R. *J. Am. Chem. Soc.* **2010**, *132*, 8602–8609.
- (6) Fields, G. B. *Solid-Phase Peptide Synthesis*; Academic Press: San Diego, CA, 1997.
- (7) Mason, S. F. *Nature* **1984**, *311*, 19–23.
- (8) Podlech, J. *Cell. Mol. Life Sci.* **2001**, *58*, 44–60.
- (9) Yang, P.; Xu, R.; Nantia, S. C.; Cooks, R. G. *J. Am. Chem. Soc.* **2006**, *128*, 17074–17086.
- (10) Nantia, S. C.; Cooks, R. G. *Angew. Chem., Int. Ed.* **2006**, *45*, 554–569.
- (11) Fischer, E.; Fourneau, E. *Ber. Dtsch. Chem. Ges.* **1901**, *34*, 2868–2877.
- (12) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154.
- (13) Houghten, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 5131–5135.
- (14) Cho, C. Y.; Moran, E. J.; Cherry, S. R.; Stephans, J. C.; Fodor, S. P. A.; Adams, C. L.; Sundaram, A.; Jacobs, J. W.; Schultz, P. G. *Science* **1993**, *261*, 1303–1305.
- (15) Scott, W. L.; Martynow, J. G.; Huffman, J. C.; O'Donnell, M. J. *J. Am. Chem. Soc.* **2007**, *129*, 7077–7088.

- (16) Nandy, J. P.; Prakesch, M.; Khadem, S.; Reddy, P. T.; Sharma, U.; Arya, P. *Chem. Rev.* **2009**, *109*, 1999–2060.
- (17) Geysen, H. M.; Meloen, R. H.; Barteling, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3998–4002.
- (18) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82–84.
- (19) Jayawickreme, C. K.; Graminski, G. E.; Quillan, J. M.; Lerner, M. R. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 1614–1618.
- (20) Gisin, B. F.; Merrifield, R. B. *J. Am. Chem. Soc.* **1972**, *94*, 3102–3106.
- (21) Morihara, K.; Oka, T. *Biochem. J.* **1977**, *163*, 531–542.
- (22) Smith, R. D.; Light-Wahl, K. J.; Winger, B. E.; Loo, J. A. *Org. Mass Spectrom.* **1992**, *27*, 811–821.
- (23) Counterman, A. E.; Hilderbrand, A. E.; Srebalus Barnes, C. A.; Clemmer, D. E. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 1020–1035.
- (24) Zucker, S. M.; Lee, S.; Valentine, S. J.; Webber, N.; Reilly, J. P.; Clemmer, D. E. *J. Am. Soc. Mass Spectrom.* **2011**, *22*, 1477–1485.
- (25) Kim, T.-Y.; Schwartz, J. C.; Reilly, J. P. *Anal. Chem.* **2009**, *81*, 8809–8817.
- (26) Reilly, J. P. *Mass Spectrom. Rev.* **2009**, *28*, 425–447.
- (27) Paizs, B.; Suhai, S. *Mass Spectrom. Rev.* **2005**, *24*, 508–548.
- (28) Norrish, R. G. W.; Kirkbride, F. W. *J. Chem. Soc.* **1932**, 1518–1530.