Determination of the Limited Trypsinolysis Pathways of Tumor Necrosis Factor-α and Its Mutant by Electrospray Ionization Mass Spectrometry

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Electrospray ionization mass spectrometry (ESI-MS) is employed to directly analyze the limited trypsinolysis products of wild-type tumor necrosis factor-α (wtTNF-α) and its mutant, M3S. To determine the charge numbers of peaks of relatively small peptides in the ESI mass spectrum of a digest, a series of sodium-adduct ion peaks of each peptide are generated by adding a small quantity of NaCl to the digest before taking the spectrum. From the monitoring of the composition of proteolytic mixture as the incubation time is lengthened, it has been learned that the proteolysis of wtTNF-α by trypsin occurs sequentially: Arg2, Arg6, Arg32, Arg31, and Arg44, and that M3S is strongly resistant to the proteolysis. Since the cleavage sequence of wtTNF-α and the mutation-induced resistance of M3S are consistent with the structural features of the proteins, we can suggest a mutant more resistant to proteolysis than M3S, which has an additional point mutation, Ala35Leu or Ala35Ile.

Limited proteolysis has been used to probe the structures and dynamics of proteins in solution, and provides useful and reliable information complementary to those from NMR and X-ray crystallography (1, 2). Conventional method for analyzing a digest of limited proteolysis employs SDS–PAGE separation along with N-terminal sequencing (3). Recently, however, the separation of a proteolytic digest and the identification of the cleavage sites have been greatly facilitated by HPLC and mass spectrometry (MS) (4–7).

Usually the HPLC fractions of peptides separated from a proteolytic mixture are collected before the molecular mass determination of the peptide in each fraction using a mass spectrometric technique, such as matrix-assisted laser-desorption ionization or electrospray ionization (ESI)2 mass spectrometry (8–10). However, a more convenient and efficient on-line methodology, HPLC–MS, has become popular recently, where the ESI interface is the way of choice to directly analyze the eluate from an HPLC column with a mass spectrometer (7, 11–14).

In general ESI mass spectra of proteins or large peptides consist of a distribution of molecular ion peaks with various charge numbers. The molecular weight can immediately be determined from any two adjacent peaks within the distribution, assuming that they differ by only one charge, and that charging is due to the protonation of the basic sites in the analyte molecules (15, 16). But from relatively small peptides, we often obtain ESI mass spectra with only one peak, and thus the charge state of the peak cannot be calculated. In that case, we must guess the charge state of a fragment peptide with the nature and number of basic amino acids in the peptide (17).
and its adjacent sodium-adduct ion peak depends on the charge state of each peptide ion. In the present work, the charge states of all small peptide fragments were determined simultaneously from the ESI mass spectrum of a NaCl-added proteolytic mixture.

Recently, we have reported the design and X-ray structure of a human tumor necrosis factor-α (TNF-α) mutant (M3S) with low systemic toxicity in vivo (18, 19). The biochemical properties of TNF-α have been studied extensively because of its potential use as a therapeutic agent for cancer patients (20, 21). To reduce its serious systemic toxicity and enhance its in vivo stability, a great number of mutants have been constructed and evaluated for the clinical applicability (22–24). It has been found that M3S is much more resistant to the proteolysis by trypsin than wild-type TNF-α (wtTNF-α) (19, 25). Two cleavage sites of wtTNF-α, Arg6 and Arg44 (Fig. 1 shows the amino acid sequence of wtTNF-α.), in the limited proteolysis by trypsin were reported by Narhi et al. (3). According to the X-ray structure of wtTNF-α (25), Arg44, however, is on a rigid loop and the rigidity of the loop is gained by the interactions with an adjacent, flexible loop of Leu29–Leu36, which contains two possible cleavage sites, Arg31 and Arg32. Thus, it is quite surprising that the rigid loop containing Arg44 was reported to be vulnerable to trypsin, whereas Arg31 and Arg32 on the highly disordered segment were not. We have also learned that one of the mutations of M3S, Leu29Ser, provides the 29–36 loop with the mutation-induced increase in rigidity (19).

In this work, we have carefully studied the tryptic proteolysis of wtTNF-α and M3S using the direct ESI-MS analysis methodology on a time-course basis by sampling an incubation mixture at different time intervals. The trypsinolysis pathway of wtTNF-α inferred from the results of the study is well understood from its structure and explains the mutation-induced resistance of M3S to proteolysis. Moreover, our understanding of the structure-related pathway has enabled us to suggest a mutant more resistant to proteolysis than M3S.

**MATERIALS AND METHODS**

**Materials.** HPLC grade acetonitrile was purchased from Burdick & Jackson, and HPLC grade formic acid was from Janssen Chimica. TPCK-treated trypsin, ammonium bicarbonate, and cellulose dialysis tubing were from Sigma. Sodium chloride was from Aldrich. Plasmid pT7-TNF, having a DNA insert coding for human TNF-α, was cloned by using PCR from the U937 human monocyte cDNA library.

M3S construction. The Escherichia coli host strain in this study was BL21(DE3)(ompT rB-mB-) whose chromosome carries the T7 RNA polymerase gene under the control of lacUV5 promoter. The gene encoding M3S was inserted downstream of T7 promoter to induce high-level expression of the target protein. A double plasmid system including a plasmid for a bacterial chaperone was used to obtain M3S in a soluble form. Construction of these expression vectors and purification schemes was described previously (18).

Limited tryptic proteolysis. wtTNF-α powder mixed with sodium sulfate was dialyzed with cellulose tubing (cutoff value 1200) for 8 h at 4°C with 0.1 M ammonium bicarbonate buffer solution (pH 8.5). This is diluted to 50 pmol/μl of wtTNF-α with the same buffer solution. M3S was also dialyzed and diluted to the same concentration.

![FIG. 1. The amino acid sequence of wtTNF-α. The arrows indicate the cleavage sites of the protein in limited tryptic proteolysis of the present work.](image-url)
Tryptic digestion was started by addition of a small quantity of trypsin (2% by concentration of the substrate) into the wtTNF-α or M3S solution at 23°C. In the case of wtTNF-α, 5 μl of sample was taken from the incubation solution and then diluted with 45 μl of acidified acetonitrile/water (50/50) solution (0.5% formic acid) every minute to quench the reaction until 5 min. Then incubation was quenched every 5 min until 30 min, and quenched at 60, 120, and 180 min. In the case of M3S, incubation was quenched every 5 min until 30 min, and quenched at 60, 120, and 180 min by the same method.

Addition of sodium ions. Ten microliters of trypsin (10 pmol/μl) was added to 100 μl of wtTNF-α (50 pmol/μl) at 23°C. After 30 min incubation, NaCl was added to the concentration of 0.5 mM, and then incubation was quenched by 10-fold dilution with the acidified acetonitrile/water (50/50) solution (0.5% formic acid).

ESI–MS analysis. Mass spectrometric analysis was performed using a triple quadrupole mass spectrometer (Quattro, Micromass, Manchester, UK; 0.1% resolution, 0.01% accuracy) equipped with an ESI source. Each sample quenched with the acidified acetonitrile/water (50/50) solution was injected into the ESI probe by 10 μl. Samples were delivered by a syringe infusion pump with the flow rate of 10 μl/min via a fused silica capillary (o.d. 375 μm, i.d. 75 μm). This probe is coupled directly to the quadrupole analyzer. The stainless-steel capillary tube at the end of the probe was heated to 75°C, and the high voltage applied was 2.9 kV. Scan range was m/z 300–1500 for wtTNF-α, and m/z 400–1600 for M3S. Scan rate was 300 Da/s for both cases.

RESULTS AND DISCUSSION

Determination of the limited trypsinolysis pathway of wtTNF-α. The identity and purity of wtTNF-α prepared were checked by ESI–MS before digestion. Figure 2a shows an ESI mass spectrum of wtTNF-α, where a group of multiply protonated molecular ion peaks marked with their charge numbers forms a distinctive bell-shaped distribution. Using a deconvolution algorithm this m/z envelope is transformed to molecular mass of 17,351 (+2) Da (Fig. 2b) that agrees exactly with the value calculated from the amino acid sequence of wtTNF-α (Fig. 1). The peak at 17,351 Da is the almost exclusive one in the transformed mass spectrum, saving us further purification of wtTNF-α.

To monitor the pathway of limited trypsic proteolysis of wtTNF-α, proteolysis experiments were conducted by varying the incubation time. Figure 3 shows mass spectra of the aliquots withdrawn from the incubation mixture at different times. The concentration of intact wtTNF-α drastically decreases upon the starting of the digestion and its peaks become undetectable after 2 min, while the peaks of some large fragments such as 3–157 and 7–157 peptides become predominant during this period. After 4 min the peaks of small peptide fragments begin to appear, and as the incubation period is lengthened, their intensities gradually increase at the expense of those of large fragments.

To determine the charge numbers of peaks of small fragments in the ESI mass spectrum of a digest (from A through F in Fig. 3), we added a small quantity of NaCl to the digest. ESI then produced adduct ions resulting from the attachment of sodium ions to the peptides (Fig. 4). The adduct ion peaks of a peptide generally form a series of peaks with a constant mass interval and with decreasing intensities. The charge number of each peptide ion can be determined from the mass interval between the peptide peak and its adjacent sodium-adduct ion peak. In the case of a singly protonated peptide, the peak with one sodium ion must be higher than the peptide peak by 22 m/z (A in Fig. 4). If a peptide is doubly or triply protonated, the adjacent adduct ion peak will be higher by 11 or 7 m/z, respectively (B, C, E, and F in Fig. 4). The results of charge assignments and consequent mass determinations of peptides in the digests are summarized in Table 1.

Since the mass of each peak was determined very accurately, we could correctly assign, from the amino acid sequence of wtTNF-α, each corresponding fragment to a specific segment within the protein. In Table 1 the segment of each peak is shown with its charge...
We have found that major cleavages occur at the C-terminal sites of Arg$^{2}$, Arg$^{6}$, Arg$^{31}$, Arg$^{32}$, and Arg$^{44}$. It was, however, reported previously that trypsin limitedly cleaved wtTNF-$\alpha$ at Arg$^{6}$ and Arg$^{44}$ (3). The apparent discrepancy between the two results seems to be due to the loss of some small fragments, which possibly occurred, during the dialysis of tryptic digests. But in the present study, since the tryptic digest is only diluted with an acidified acetonitrilewater solution, and then directly analyzed by ESI-MS, we can avoid such loss of fragment peptides.

The peak intensities of several peptides in Fig. 3 were plotted in Fig. 5 with respect to incubation time from 0 to 180 min. Since the relative abundances of peptide ions detected in an ESI mass spectrometer are known to strongly depend on the amino acid compositions of the peptides and their m/z values (17), peptides with little difference are grouped and compared in Fig. 5 so that an intensity of a peptide reflects its relative amount among the peptides in the same group at that incubation time. Figure 5a is the plot for wtTNF-$\alpha$ and two peptides, 3--157 (17,095 Da) and 7--157 (16,678 Da). wtTNF-$\alpha$ disappears drastically at the initial stage of incubation because the cleavage at Arg$^{2}$ converts the protein into 3--157 peptide with a fast rate. After a rapid increase, the intensity of 3--157 peptide gradually decreases due to the cleavage at Arg$^{6}$, with a concomitant increase of 7--157 intensity. Proteolysis at
Arg becomes almost negligible after 60 min. Considering that the N-terminus of wtTNF-α is very flexible (25), it is quite reasonable that Arg and Arg are cleaved first among the major cleavage sites.

The intensities of 7–32 and 7–31 peptides are compared in Fig. 5b. The former is slightly higher than the latter during their increases until 30 min. The 7–32 peptide is generated only from the cleavage at Arg in the 7–157 peptide, while the 7–31 peptide can be produced by the cleavage either at Arg in the 7–157 peptide or at Arg in the 7–32 peptide. Since the 7–32 peptide probably has a highly loose structure, compared with the 7–157 peptide, and Arg is located near one end of the peptide, it is quite reasonable to consider that the cleavage of 7–31 from 7–32 is faster than that from 7–157. Until 30 min the intensity of 7–32 is somewhat higher than that of 7–31, although the latter corresponds to the sum of cleavage products from 7–32 and 7–157. If the cleavage rate constant of Arg in 7–157 is $k_1$, that of Arg in 7–32 is $k_2$, and that of Arg in 7–157 is $k_3$, we can conclude $k_1 > k_2 > k_3$. If $k_1 < k_3$, then the intensity of 7–32 in Fig. 5b cannot be higher than that of 7–31. It should be also noted that in the 7–157 peptide Arg is cleaved faster than Arg.

After 30 min, the trend of intensities of the 7–32 peptide is reversed, while the intensity of the 7–31 peptide increases continuously until 120 min and then decreases. As the incubation time is lengthened, the total concentration of fragments rapidly increases, which would saturate all the active sites of trypsin molecules present in a limited amount. Therefore, in this situation the intensity of the 7–157 peptide whose cleavage rate is relatively slow would

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**FIG. 4.** Charge-state assignments of small fragment peptides (A–F) generated from the tryptic proteolysis of wtTNF-α. This ESI mass spectrum was obtained by adding a small quantity of NaCl to the incubation mixture at 30 min. For each peptide, there is a series of sodium-adduct ions. The mass interval between two adjacent peaks in the series is responsible for the charge state of the peptide. The result of the charge state assignments is summarized in Table 1.
remain almost constant as shown in Fig. 5b. Instead, the cleavage at Arg\textsuperscript{31} in the more vulnerable 7–32 peptide will be going on so that the intensity of the peptide decreases, and thus that of 7–31 increases. The intensity of 7–31 eventually decreases when it starts to be digested at Lys\textsuperscript{11}.

It should be noted from Table 1 that although we have found the 7–31, 7–32, 32–44, and 33–44 peptides, the peptide corresponding to the 7–44 segment was not detected. These results strongly suggest that the cleavages at Arg\textsuperscript{31} and Arg\textsuperscript{32} precede the cleavage at Arg\textsuperscript{44}. If it is the case, we might have detected fragments 32–157 and 33–157. The reason why we could not detect the fragments would be threefold: the steady-state concentration of the intermediate peptides, 32–157 and 33–157, is very low; their solubility in the quenched solution is probably smaller than the solubility of short peptides like 7–31, 7–32, and 7–44; and since the mass spectrum of such long peptides has multiple peaks, the intensity of each peak can be much smaller than is expected for a single-peak spectrum.

Recently, the X-ray structure of wtTNF-\(\alpha\) has revealed that the segment containing Leu\textsuperscript{43}–Arg\textsuperscript{44}–Asp\textsuperscript{45}–Asn\textsuperscript{46} is rigid but Arg\textsuperscript{31}–Arg\textsuperscript{32}–Ala\textsuperscript{33} is on a highly flexible loop, and that the Arg\textsuperscript{31}–Arg\textsuperscript{32}–Ala\textsuperscript{33} sequence is close to the hydrogen bonds and the hydrophobic interactions involving the loop containing Arg\textsuperscript{44} (19, 25). It is highly probable that the flexible Arg\textsuperscript{31}–Arg\textsuperscript{32}–Ala\textsuperscript{33} sequence could be cleaved first by trypsin. The cleavage could result in disruption of the interactions involving the loop containing Arg\textsuperscript{44}, and the loop could then become flexible, and vulnerable to trypsin. This cleavage sequence suggested from the structural feature of wtTNF-\(\alpha\) is consistent with the sequence inferred from the results of time-course analysis.

We have investigated the pathway of limited tryptic proteolysis of wtTNF-\(\alpha\) with the results presented in Figs. 3 and 5. It has been found that wtTNF-\(\alpha\) undergoes major cleavages at Arg\textsuperscript{2}, Arg\textsuperscript{6}, Arg\textsuperscript{31}, Arg\textsuperscript{32}, and

### TABLE 1

<table>
<thead>
<tr>
<th>Label</th>
<th>Measured m/z(^a)</th>
<th>Segment</th>
<th>Measured mass</th>
<th>Expected mass</th>
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<tbody>
<tr>
<td>A</td>
<td>436.46 (±0.09)</td>
<td>[3–6]+H(^+)</td>
<td>435.46 (±0.09)</td>
<td>435.44</td>
</tr>
<tr>
<td>B</td>
<td>621.2 (±0.2)</td>
<td>[33–44]+2H(^+)</td>
<td>1,240.3 (±0.5)</td>
<td>1,240.4</td>
</tr>
<tr>
<td>C</td>
<td>699.2 (±0.2)</td>
<td>[32–44]+2H(^+)</td>
<td>1,396.4 (±0.3)</td>
<td>1,396.6</td>
</tr>
<tr>
<td>D</td>
<td>729.1 (±0.1)</td>
<td>[7–32]+4H(^+)</td>
<td>2,912.4 (±0.6)</td>
<td>2,912.2</td>
</tr>
<tr>
<td>E</td>
<td>919.7 (±0.2)</td>
<td>[7–31]+3H(^+)</td>
<td>2,756.0 (±0.5)</td>
<td>2,756.1</td>
</tr>
<tr>
<td>F</td>
<td>971.7 (±0.2)</td>
<td>1–157</td>
<td>17,349 (±2)</td>
<td>17,351</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3–157</td>
<td>17,095 (±1)</td>
<td>17,095</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7–157</td>
<td>16,678 (±1)</td>
<td>16,678</td>
</tr>
</tbody>
</table>

\(\text{a}\) Each m/z value and its uncertainty are the average and the standard deviation of the corresponding m/z values in Figs. 3d–3h, respectively.

![FIG. 5. Time variations of the peak intensities of limited tryptic proteolysis fragments of wtTNF-\(\alpha\): (a) wtTNF-\(\alpha\) (○), 3–157 peptide (●), and 7–157 peptide (△); (b) 7–31 peptide (○) and 7–32 peptide (●). In the case of a, intensities represent the peak intensities of transformed mass spectra of the peptides.](image-url)
Arg\textsuperscript{44}, and that there seems to be a sequence among the cleavages: Arg\textsuperscript{29}, Arg\textsuperscript{30}, Arg\textsuperscript{32}, Arg\textsuperscript{31}, and Arg\textsuperscript{44}. This proteolysis sequence partially reflects the structure-function relationship of wtTNF-\(\alpha\), and therefore it would be a very valuable information particularly in designing the proteolysis-resistant mutants of wtTNF-\(\alpha\).

Resistance of M3S to limited proteolysis by trypsin. M3S (16561 Da) is a TNF-\(\alpha\) mutant containing three substitutions of Leu\textsuperscript{29}Ser, Ser\textsuperscript{52}Ile, and Tyr\textsuperscript{56}Phe, and a deletion at the N-terminus for seven amino acids. The deletion of the N-terminal segment that is disordered in the structure of wtTNF-\(\alpha\) excludes Arg\textsuperscript{2} and Arg\textsuperscript{3} from the possible cleavage sites of M3S. Comparative analysis of the X-ray structures of wtTNF-\(\alpha\) and M3S showed that due to the mutation-induced formation of a hydrogen bond between the Ser\textsuperscript{28} OH and the amide nitrogen of Arg\textsuperscript{31}, the 29–36 region of M3S became more rigid than that of wtTNF-\(\alpha\) (19). It is thus expected that an increased resistance of M3S to proteolysis could be attained with the mutation-induced change in rigidity.

To verify the expected resistance to proteolysis of M3S, tryptic digestion was carried out in the same way as wtTNF-\(\alpha\). ESI mass spectra obtained after 5- and 30-min incubations are shown in Fig. 6. No fragments can be found from 5-min incubation, and after 10 min, peaks of fragments begin to appear (data not shown). But, even at 30 min, as shown in Fig. 6b, the intensities of peaks for the fragments are still very small, which demonstrates that M3S is more resistant to the trypsic digestion than wtTNF-\(\alpha\).

Now it has been learned that an increased rigidity of the 29–36 loop improves the resistance of TNF-\(\alpha\) to proteolysis; a mutant more resistant than M3S can be designed by introducing a greater rigidity to the loop. For example, when Ala\textsuperscript{35} of M3S, the side chain of which faces toward the interior of the protein, is replaced by Leu or Ile, it has been found by using a crystallographic modeling program, CHAIN (Version 5.4, Baylor College of Medicine), that the bigger side chain of Leu or Ile makes better van der Waals contacts with Val\textsuperscript{17} and the C\textsuperscript{\#} and C\textsuperscript{\#'} of Arg\textsuperscript{32} without any disturbance of the loop conformation. Therefore, the additional mutation, Ala35Leu or Ala35Ile, would enhance the rigidity of the loop by the stronger hydrophobic interactions, making the mutant more resistant to proteolysis than M3S.

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**REFERENCES**


![FIG. 6. Time-course ESI-MS analysis of M3S digested with trypsin. (a) and (b) show ESI mass spectra of the aliquots withdrawn from the incubation mixture at 5 and 30 min, respectively. Peaks of intact M3S are marked with asterisks. From the transformed spectrum of (a), the molecular mass of M3S was measured to be 16,563 (23 Da that agreed well with the value calculated from the amino acid sequence of M3S. A and B peaks correspond to the 33–44 and 32–44 peptides, respectively.](image-url)