High sequence-coverage detection of proteolytic peptides using a bis(terpyridine)ruthenium(II) complex†

Akihiro Ito,[a] Taka-aki Okamura,*[a] Ken Masui,[a] Maki Kaneko,[a] Ryoji Masui,[b] Kojiro Ake,[b] Seiki Kuramitsu,[b] Minoru Yamaguchi,[c] Hiroki Kuyama,[c] Eiji Ando,[c] Shigemi Norioka,[d] Takashi Nakazawa,[e] Susumu Tsunasawa,[c] Hitoshi Yamamoto[a] and Norikazu Ueyama*[a]

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The use of a bis(terpyridine)ruthenium(II) complex for peptide labeling (〈Ru〉–CO labeling) supplied high intensity peaks in mass spectrometry (MS) analysis that overcame the contribution of protonation or sodiated adduction to peptides. 〈Ru〉–CO-labeled insulin A- and B-chains were detected simultaneously in comparable peak abundance by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The mass spectra of chymotryptic peptide fragments of 〈Ru〉–CO-labeled insulin also simultaneously indicated both N-terminal fragment ions, and amino acid sequences were determined easily by matrix-assisted laser desorption/ionization post-source-decay (MALDI-PSD). The sensitivity of detecting 〈Ru〉–CO-labeled peptide fragment ions was not dependent on the length or the sequences of the peptides. The 〈Ru〉–CO labeling method was applied to tryptic myoglobin fragments. The method indicated that each fragment ion is detected nearly equal in abundance and enabled the desired fragment ions to be distinguished from matrix clusters or their in-source fragments in lower mass regions. The desired fragment ions can be found in the mass region higher than 670.70 (= 〈Ru〉–CO). This method provided a high sequence coverage (96%) by peptide mass fingerprinting (PMF). Application of this method to a protein mixture (myoglobin, lysozyme and ubiquitin) successfully achieved high sequence-coverage characterization (>90%) of these proteins simultaneously.

Introduction

Tandem time-of-flight mass spectrometry (TOF-MS) is a powerful technique for the amino acid sequencing of proteolytic peptides. Peptide mass fingerprinting (PMF) and peptide sequence tagging (PST) are effective methods used in protein sequencing.[1–6] PMF was developed by some workers in 1993.[7] In short, an unknown protein of interest is cleaved into peptides by a protease such as trypsin. The proteolytic peptides are measured by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) or electrospray ionization mass spectrometry (ESI-MS). These masses are compared with the masses of the peptides which code for the genome. PST was developed in the mid 1990s.[2,8] PST employs MS/MS (*e.g.* collision-induced dissociation; CID) data produced by the tandem MS method and consists of short strings of amino acid differences deduced from the fragment spectrum. Matrix-assisted laser desorption/ionization post-source-decay (MALDI-PSD) also indicates fragment ion types similar to those observed under high energy CID conditions.[9–11] Using a combination of these MS analyses and genome database searching, protein sequencing is automated. However, some target peptide ions could not be detected because of low ionization efficiency caused by the acidic side chain in a positive mode.[12] Therefore, the above methods sometimes lead to ambiguous protein identification.[13–15] To reduce ambiguity, additional information reflecting the primary sequence of a target protein is required, namely improvement of the PMF sequence coverage.

Charged derivatization of peptides has been reported to improve the sensitivity of MS analysis for the desired fragment ions.[16–20] The N-terminal sulfonation of peptides reduces the intensity of N-terminal fragment ions and enhances the relative intensity of y-series ions (C-terminal fragment ions) in MS/MS analysis.[18–20] However, some of these methods give a more complicated spectrum by MS analysis than that without such a modification. This problem is caused by in-source fragmentation of the derivatized peptides, as the derived moiety is cleaved easily in MS analysis.[14]

We have demonstrated the amino acid sequencing of bovine ubiquitin through the use of a chemically inert bis(terpyridine)ruthenium(II) complex (〈Ru〉–COONSu, Fig. 1) as an N-terminal-labeling reagent in MALDI-PSD.[21] The 〈Ru〉–CO moiety is thermodynamically, chemically, and

[a]*Department of Macromolecular Science, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan. E-mail: tokamura@chem.sci.osaka-u.ac.jp (T.O.); ueyama@chem.sci.osaka-u.ac.jp (N.U.)*
[b]*Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan*
[c]*Life Science Laboratory, Shimadzu Corporation, Kyoto 604-8511, Japan*
[d]*Graduate School of Frontier Biosciences, Osaka University, Suita, Osaka, 565-0871, Japan*
[e]*Department of Chemistry, Nara Women's University, Nara 630-8506, Japan*
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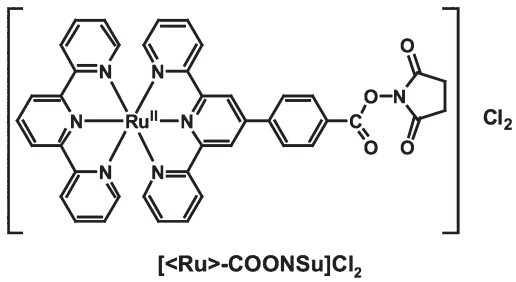

Fig. 1 Active ester of bis(terpyridine)ruthenium(II) complex.

photochemically stable. The cationic character causes a high ionization efficiency in MS analysis. The ⟨Ru⟩ moiety has an intense absorption ($\varepsilon \approx 5 \times 10^4$ M^{-1} cm^{-1}) near to the N_2 laser frequency (337 nm) in MALDI-TOF-MS. The laser beam probably excites directly the ligand and/or the metal center and brings about effective ionization even without the assistance of any matrix. This excitation occurs partially in MALDI-TOF-MS measurements and causes photochemical reduction, giving formal ruthenium(I) species. In MALDI-TOF-MS spectra, this divalent cationic reagent gives mainly singly-charged ions including both deprotonated ⟨Ru^{II}⟩–CO-labeled and ⟨Ru^{I}⟩–CO-labeled fragments. The ⟨Ru^{II}⟩/⟨Ru^{I}⟩ ratio is usually found to be approximately 1 : 1 in various measurements, as mentioned in a previous paper.[21] This phenomenon is characteristic of MALDI and such a reduction has not been found in ESI-MS. In both MALDI- and ESI-MS analyses, the ⟨Ru⟩–CO-labeled peptides were detected with high sensitivity.[22] The PSD or collision-induced decay analysis of ⟨Ru⟩–CO-labeled fragments enabled easy amino acid sequencing. The detection limit was approximately 10 fmol, which is sufficient for the analysis of each visible spot in two-dimensional electrophoresis separation, whereas detection by gel staining requires at least 100 fmol.[23] Application of this method to PMF should improve the sequence coverage.

In this study, we describe the simultaneous detection of proteolytic peptides as a technique for improving the sequence coverage of the PMF method. Initially, bovine insulin was reacted with the ⟨Ru⟩–CO labeling reagent. Bovine insulin is composed of two peptide chains (A- and B-chains), and each peptide shows an individual isoelectric point (pI). The ⟨Ru⟩–CO-labeled insulin was measured by MALDI-TOF-MS to examine the effect of the peptide chains' pI on the ionization process during MS analysis. The chymotryptic peptides of ⟨Ru⟩–CO-labeled insulin were also measured by MALDI-TOF-MS to investigate the dependence on the length of the peptides. The technique of PMF analysis using ⟨Ru⟩–CO labeling was applied to tryptic myoglobin fragments and tryptic fragments of a protein mixture (myoglobin, lysozyme and ubiquitin).

Experimental

Materials

[(tpy)Ru(tpyC_6H_4COONSu)]Cl_2 ([⟨Ru⟩–COONSu]Cl_2) was synthesized by a procedure similar to that reported in a previous study.[21] Bovine insulin, myoglobin (from horse heart), ubiquitin (from bovine red blood cells), lysozyme (from chicken egg white) and α-chymotrypsin were purchased from Sigma (St Louis, USA). Trypsin Gold was purchased from Promega (Madison, USA). All other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

Reduction and alkylation

Insulin (1.1 mg, 200 nmol) was dissolved in 600 μL of 0.5 mol dm^{-3} Tris buffer (0.5 mol dm^{-3} Tris HCl, 7 mol dm^{-3} guanidine hydrochloride, and 10 mmol dm^{-3} EDTA). Dithiothreitol (3.70 mg, 24 μmol) was added to the solution. The solution was incubated at 56 °C for 50 min. Iodoacetamide (8.8 mg, 48 μmol) was then added, and the mixture was shaken for 1 h at room temperature in the dark. The excess reagents were removed using the Float-A-Lyzer dialysis tube [molecular weight cut off (MWCO) 1000]. The reaction products were characterized by MALDI-TOF-MS.

Guanidination

The guanidination of ε-amino groups was performed following Beardsley and Reilly's method[24] with some modifications. The solution of *O*-methylisourea (21 mg, 81 μmol) in 7 mol dm^{-3} NH_3 aq. (0.1 mL) was added to the carbamidomethylated insulin solution and incubated for 10 min at pH 10.6 and 65 °C. After incubation, 300 μL of 10% trifluoroacetic acid (TFA) was added to the solution in an ice bath. The excess reagents were removed using the Float-A-Lyzer dialysis tube (MWCO 1000). The solution was concentrated to 60 μL in a vacuum centrifuge and desalted on a $ZipTip_{C18}$ pipette (Millipore, Bedford, USA) for the MALDI-TOF-MS measurement.

⟨Ru⟩–CO labeling

[⟨Ru⟩–COONSu]Cl_2 (0.13 mg, 150 nmol) and triethylamine (1.5 μL) were added to the guanidinated insulin solution (600 μL, *ca.* 40 nmol). The mixture was incubated at 40 °C for 24 h. A 20 μL portion of the solution was desalted on a $ZipTip_{C18}$ pipette for the MALDI-TOF-MS analysis.

Chymotryptic digestion

The solution was concentrated to 50 μL under reduced pressure. After cooling to room temperature, 300 μL of 50 mmol dm^{-3} $NaHCO_3$ buffer and 10 μL of chymotrypsin aqueous solution containing 11 μg of chymotrypsin were added. The mixture was incubated at 37 °C overnight. A 20 μL portion of the solution was purified using a $ZipTip_{C18}$ pipette for MALDI-TOF-MS analysis.

In-gel digestion of myoglobin

Myoglobin was resolved by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Each observed spot was cut into small pieces and collected in a microtube. The samples were reduced and alkylated in a similar way as described above for the in-solution method. The reactant was digested by trypsin, as described for the chymotryptic digestion. The tryptic peptides were guanidinated and ⟨Ru⟩–CO-labeled by a method similar to that mentioned in the previous section. A 20 μL portion of the

solution was purified using a ZipTip$_{C18}$ pipette for MALDI-TOF-MS analysis.

Preparation of 〈Ru〉–CO-labeled peptides for a protein mixture

One mL of each of myoglobin, lysozyme and ubiquitin aqueous solution (1 μmol dm^{-3}) were mixed. The mixture was reduced, alkylated, digested by trypsin, guanidinated, and 〈Ru〉–CO-labeled, successively, by a similar as mentioned above. A 20 μL portion of the solution was purified using a ZipTip$_{C18}$ pipette for MALDI-TOF-MS analysis.

Physical measurements

Sample preparation. α-Cyano-4-hydroxycinnamic acid (CHCA) or 2,5-dihydroxybenzoic acid (DHB) was saturated in a 50% acetonitrile aqueous solution containing 0.1% TFA. The saturated solution was diluted 20 times to produce a matrix solution. First, 1 μL of the matrix solution was applied to the sample plate. This was followed by the addition of 1 μL of sample solution and was allowed to dry under ambient conditions.

MALDI-TOF/TOF-MS. MALDI-TOF/TOF-MS spectra were recorded in a positive mode on a Bruker Ultraflex TOF/TOF mass spectrometer controlled by the Flexcontrol 2.2 software package. External calibration of MALDI mass spectra was carried out using singly-charged monoisotopic peaks of a mixture of human angiotensin II (*m*/*z* 1046.54), angiotensin I (*m*/*z* 1296.68), bombesin (*m*/*z* 1619.82), and adrenocorticotropic hormone (*m*/*z* 2093.08).

MALDI-TOF-MS. MALDI-TOF-MS and PSD spectra were recorded in a positive-ion mode on Shimadzu/Kratos AXIMA-CFR in a reflectron mode controlled by the KOMPACT 2.3.5 software package. External calibration of MALDI mass spectra was carried out using the monoisotopic mass of human angiotensin II (*m*/*z* 1046.54) or bovine insulin (*m*/*z* 5734.56). Metastable ions that were generated by laser-induced decomposition of the selected precursor ions were analyzed. Precursor ions were accelerated to 20 kV and selected in a timed ion gate.

Results and discussions

Simultaneous detection of 〈Ru〉–CO-labeled insulin

Initially, 〈Ru〉–CO labeling of bovine insulin was carried out according to the procedure shown in Fig. 2. The insulin A-chain has two acidic and no basic amino acid residues. The insulin B-chain has two acidic and four basic amino acid residues, including one Lys residue. The pI of the A-chain is lower than that of the B-chain. In addition, a positive charge was introduced to the B-chain by guanidination. Therefore, the insulin B-chain is detected easily in a positive-ion mode.

Guanidinated insulin and 〈Ru〉–CO-labeled insulin were measured by MALDI-TOF/TOF-MS to estimate the effect of the 〈Ru〉–CO moiety (Fig. 3). In the case of guanidinated insulin, the fragment ions of the A-chain (+ H^+: *m*/*z* 2566.3, calcd. 2566.2; + Na^+: *m*/*z* 2588.2, calcd. 2588.0) were observed

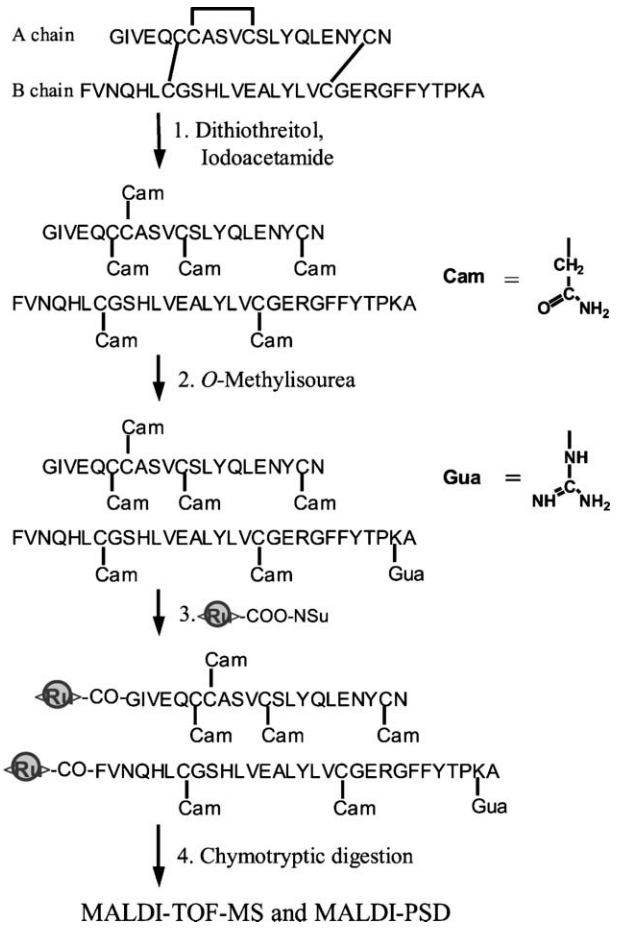

Fig. 2 Procedure for N-terminal 〈Ru〉–CO labeling of insulin: (1) all cystine residues are reduced by dithiothreitol and *S*-alkylated using iodoacetamide; (2) all ε-amino groups of lysine residues are guanidinated with *O*-methylisourea; (3) N-terminal amino acid residues are selectively labeled with the 〈Ru〉–CO labeling reagent; (4) derived proteins are digested by α-chymotrypsin.

with a lower intensity than that of the B-chain (+ H^+: *m*/*z* 3511.9, calcd. 3511.7) – a result of the restricted protonation of the acidic residues [Fig. 3(a)].

On the other hand, the analysis of 〈Ru〉–CO-labeled insulin indicated both fragment ions of the 〈Ru〉–CO-labeled A- (*m*/*z* 3237.2, calcd. 3236.2) and B-chains (*m*/*z* 4240.1, calcd. 4240.8) in comparable peak abundances, as shown in Fig. 3(b). The similar intensity is probably caused by similar physicochemical properties after derivatization. The large 〈Ru〉–CO moiety results in there being less of a dependency of the intensity on the bonded peptides in MS analysis. Moreover, this labeling enhances the relative peak intensity. The 〈Ru〉–CO-labeled insulin gives an intense peak using only low pmol quantities of sample in Fig. 3(b) where the amount of labeled sample is less than one-hundredth of the unlabeled one (labeled sample: 1.5 pmol, unlabeled sample: 200 pmol). To compare directly the increase of absolute intensity after derivatization, an equimolar mixture of labeled and unlabeled angiotensin II was analyzed by MALDI-TOF-MS (Fig. 4). Generally, a broad isotope distribution (multi-isotope) reduces the peak intensity in MS analysis. However, in this case, the intensity of the 〈Ru〉–CO-labeled fragment was observed to be about three

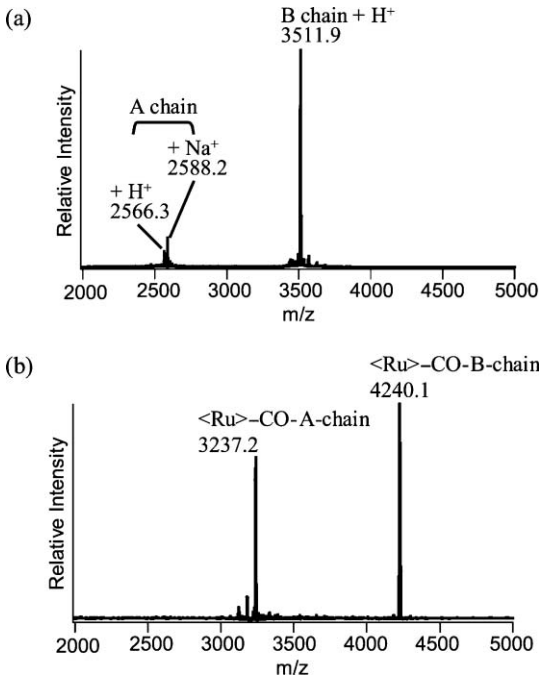

Fig. 3 MALDI-TOF/TOF-MS spectra of (a) 200 pmol of guanidinated insulin, and (b) 1.5 pmol ⟨Ru⟩–CO-labeled insulin.

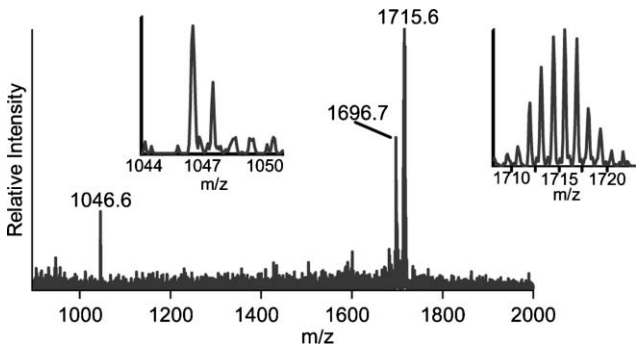

Fig. 4 MALDI-TOF-MS spectrum of ⟨Ru⟩–CO-labeled and unlabeled angiotensin II.

times that of the unlabeled one, though the isotope distribution lowers the peak height by about 30% (Fig. 4). These results suggest that the ionization efficiency of the ⟨Ru⟩ moiety surpasses the contribution of the protonation or sodiated adduction to peptides. The intensity of the individual isotopic form is reduced by spreading the abundance across several ions, but this also increases confidence in the assignment. In Fig. 3(b), the sobtained fragment peaks show characteristic isotope distribution patterns that are in agreement with the calculated one (Fig. 5).‡ The ⟨Ru⟩–CO labeling method allowed simultaneous detection of the fragment ions of the insulin A- and B-chains despite the contribution of protonation or sodiated adduction. In addition, this method enabled easy distinction of the desired peaks by their characteristic isotope distribution pattern.

‡ In this calculation, half of the ruthenium ions were assumed to be reduced to Ru^{I}.[21]

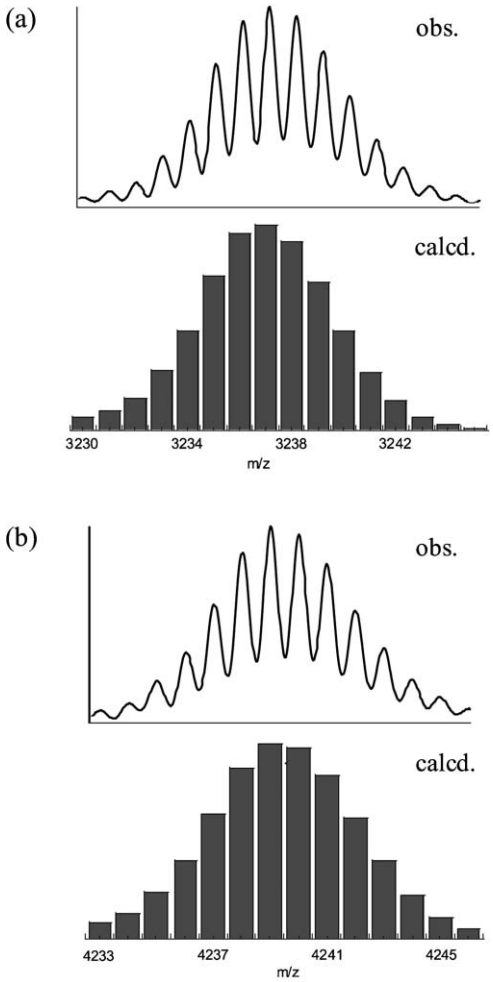

Fig. 5 Expanded spectrum (upper) and its calculated spectrum (lower) of the peak at (a) *m*/*z* 3237.2, and (b) *m*/*z* 4240.1 in Fig. 3(b).

The chymotryptic peptide fragments of ⟨Ru⟩–CO-labeled insulin were also examined by MALDI-TOF-MS (Fig. 6). Two ⟨Ru⟩–CO-labeled fragment peaks appeared at *m*/*z* 958.07 (f_A) and *m*/*z* 933.40 (f_B) with approximately the same peak abundance as observed for the original ⟨Ru⟩–CO-labeled chains. Another peak was caused by the [⟨Ru⟩–COOH + 2DHB − H_2O]$^+$ ion. These results suggest that the ⟨Ru⟩–CO

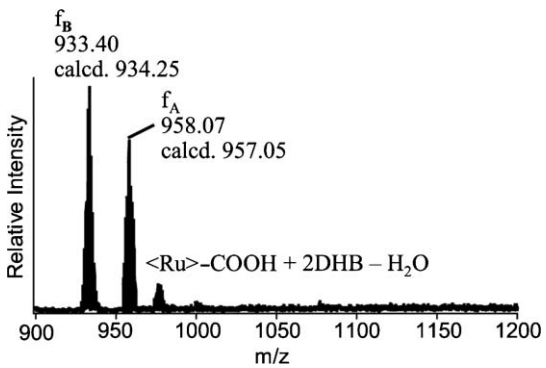

Fig. 6 MALDI-TOF-MS spectrum of ⟨Ru⟩–CO-labeled insulin digested by α-chymotrypsin.

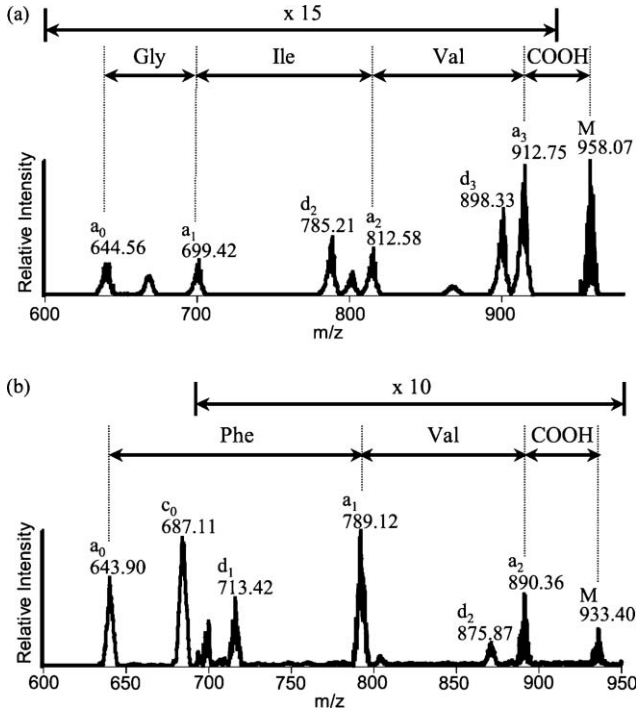

Fig. 7 MALDI-PSD spectra of the fragment ions (a) f_A at *m/z* 958.07, and (b) f_B at *m/z* 933.40.

labeling gives uniform intensity for various peptides with individual sequence and length. The observed ⟨Ru⟩–CO-labeled fragment peaks were analyzed by MALDI-PSD to determine the amino acid sequences (Fig. 7). Each spectrum indicates predominantly a_n and d_n fragment ions because the ⟨Ru⟩–CO-labeled peptides are cleaved easily at the C_α-carbonyl and C_β–C_γ bonds.[21,22] These fragment ions allow accurate determination of the amino acid sequences of the desired fragment ions by reading successively from the N-terminal amino acid residue to the C-terminus. The ⟨Ru⟩–CO labeling method enabled us to detect simultaneously all ⟨Ru⟩–CO-labeled fragment ions and to determine their sequences.

PMF analysis of tryptic peptides of myoglobin using a ⟨Ru⟩–CO labeling reagent

The procedure of PMF analysis using ⟨Ru⟩–CO labeling is shown in Fig. 8. Myoglobin has 20 acidic and 31 basic amino acid residues (Chart 1). Myoglobin produces 20 fragments by tryptic digestion, and these fragments were guanidinated (see Table S1,† homoarginine residues are represented by K*). After step 3 in Fig. 8, guanidinated peptides were analyzed by MALDI-TOF-MS [Fig. 9(a)]. Fig. 9(b) shows the MALDI-TOF-MS spectrum of the self-digested peptides of trypsin. In Fig. 9, these spectra are clearly different from one another. The self-digested fragments of trypsin were not observed in Fig. 9(a). Therefore, the influence of self-digested peptides of trypsin in Fig. 9(a) can be disregarded. In Fig. 9(a), most fragments arise from the protonation of tryptic fragments, and others were observed to be sodiated adduct ions (Na^+ or K^+). In the lower mass range (<*m/z* 600), the spectrum was complicated because of ions from the matrix (DHB) cluster

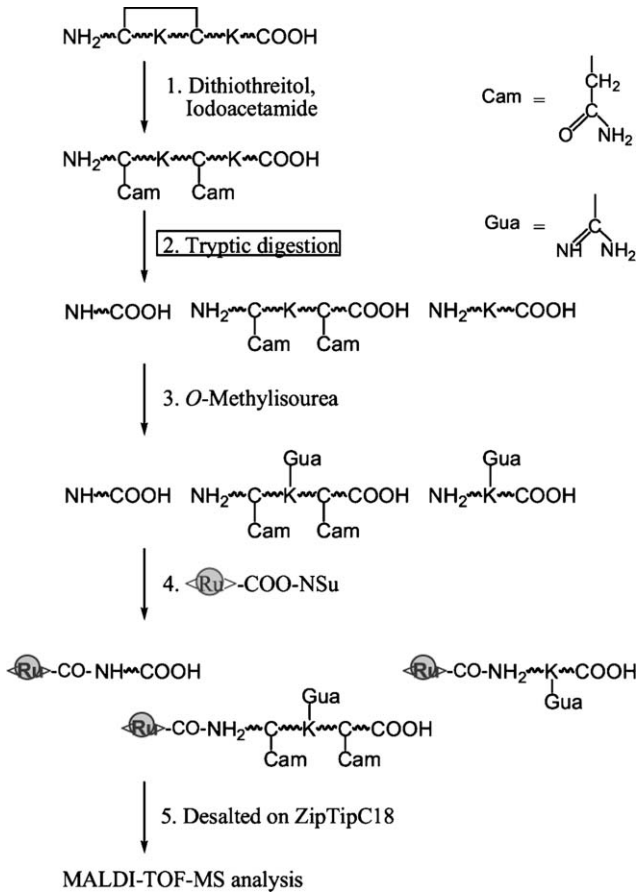

Fig. 8 ⟨Ru⟩–CO labeling procedure of enzymatic peptides: (1) all cystine residues are reduced by dithiothreitol and *S*-alkylated using iodoacetamide; (2) derived proteins are digested by trypsin; (3) all ε-amino groups of lysine residues are guanidinated with *O*-methylisourea; (4) peptides are labeled with ⟨Ru⟩–CO labeling reagent; (5) derived peptides are desalted on $ZipTip_{C18}$.

Myoglobin from horse heart

GLSDGEWQQV LNVWGKVEAD IAGHGQEVLI RLFTGHPETL EKFDKFKHLK 50
TEAEMKASED LKKHGTVVLT ALGGILKKKG HHEAELKPLA QSHATKHKIP 100
IKYLEFISDA IIHVLHSKHP GNFGADAQGA MTKALELFRN DIAAKYKELG 150
FQG 153 M.W. 16,949

Chart 1 Amino acid sequence of myoglobin. The shading indicates indicate basic amino acid residues. The underlining indicates acidic amino acid residues.

and its derivatives. In this range, the peaks (f_{16}, f_{18}, and f_{19}), which can be distinguished clearly from the DHB cluster ions, were assigned. The observed fragment ions (monoisotopic mass) with the calculated mass numbers are listed in Table S2.† Fragments f_9, f_{12}, and f_{13} were not detected. These fragments have comparatively high acidity, for which the pI values are 4.53, 5.84 and 4.00, respectively (Table S1†). Therefore, the peak intensity of these fragment ions is relatively low in a positive-ion mode. Other acidic fragments (*e.g.* f_2: *m/z* = 1859.0, pI = 4.37; f_3: *m/z* = 1607.8 pI = 4.65; f_6: *m/z* = 1314.5,

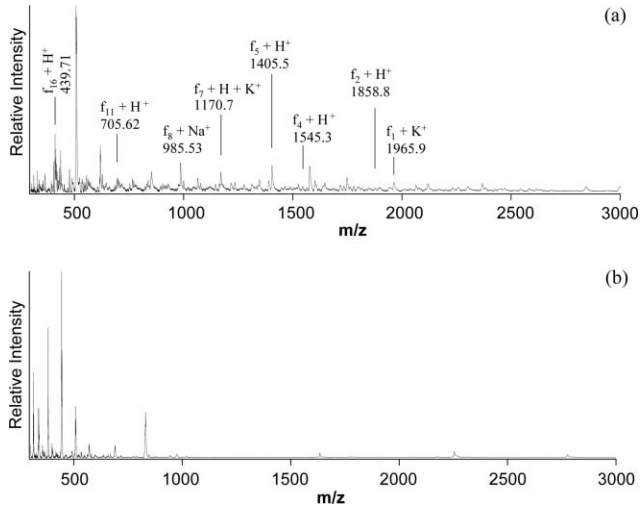

Fig. 9 MALDI-TOF-MS spectra of (a) tryptic peptides of myoglobin, and (b) self-digested peptides of trypsin.

pI = 5.40) were also obtained as very minor peaks. On the other hand, basic fragments f_5 (m/z = 1405.5, pI = 8.76) and f_7 (m/z = 1170.7, pI = 9.18) were observed as relatively intense peaks in Fig. 9(a). In this case, the sequence coverage was 75%.

After ⟨Ru⟩–CO labeling (Fig. 8, step 4), the labeled peptides were desalted using a $ZipTip_{C18}$ pipette. These peptides were analyzed by MALDI-TOF-MS (Fig. 10). All fragment ions are singly-charged as found in the previous measurements.[21,22] The observed fragment ions with their calculated mass numbers are listed in Table S3.† Some fragment ions appeared in the form with an elimination of the C-terminal arginine or homoarginine residue. By use of the ⟨Ru⟩–CO labeling method (Fig. 10 and Table S3†), all fragment ions were observed as more being intense peaks than the unlabeled peptide fragments [Fig. 9(b)]. In addition, acidic fragments f_9, f_{12}, f_{13} and lower mass fragments f_{15}, f_{17}, f_{20} were also detected, which were not observed without the labeling (Fig. 9). High sequence coverage (96%) was achieved by the ⟨Ru⟩–CO labeling method. This method shifted the desired fragment ions to a higher mass region (MW > 700), in which background is low in MALDI-TOF-MS and hence the method gave better spectra. Therefore, the method enables desired fragment ions to be distinguished from undesired ones, such as matrix clusters or self-digested fragments of protease, and improves the sequence coverage of the proteolytic peptides.

Application of PMF analysis using the ⟨Ru⟩–CO labeling reagent on a protein mixture

The analysis method was applied to a protein mixture (myoglobin, lysozyme and ubiquitin). The ⟨Ru⟩–CO-labeled fragments were desalted using a $ZipTip_{C18}$ pipette and analyzed by MALDI-TOF-MS (Fig. 11). The predicted fragments with their calculated mass numbers are summarized in Table S1 and S4.† The observed fragments are listed in Table S5† with their calculated mass numbers. In this case, some fragment ions also appeared in the form with an elimination of the C-terminal arginine or homoarginine residue. In addition, some fragments were caused by the cleavage of the guanidyl group or by dehydration. The ⟨Ru⟩–CO-labeling method provided the high sequence-coverage characterization of the three proteins simultaneously (myoglobin: 95%; lysozyme: 97%; ubiquitin: 91%).

Conclusions

The introduction of ⟨Ru⟩–CO moieties to peptides achieved high sequence coverage in PMF analysis. The ⟨Ru⟩–CO-labeled insulin A- and B-chains were observed in comparable peak abundance in the MS spectra, despite the fact that the A-chain exhibits weak intensity without the labeling. Application of this labeling method to PMF improved the sequence coverage. This method also provided high sequence-coverage characterization simultaneously for the individual proteins within a protein mixture. Furthermore, this method brought easy amino acid sequencing into MS/MS analysis. Therefore, this labeling method is applicable for PST and is easily adaptable to Mascot searching, as all fragments shift only by the same molecular weight. The use of our analytical method for protein identification should allow accurate and rapid determination.

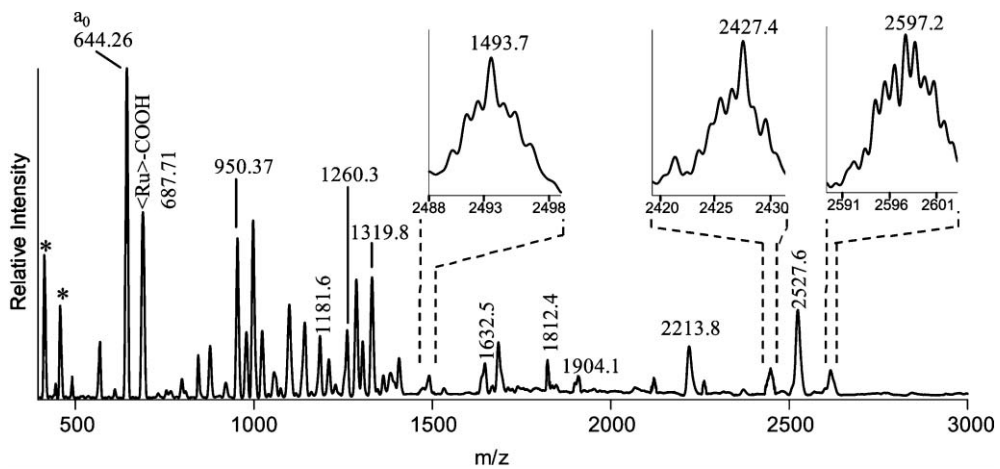

Fig. 10 MALDI-TOF-MS spectrum of ⟨Ru⟩–CO-labeled peptides of tryptic myoglobin using a DHB matrix. Peaks marked by asterisks correspond to DHB clusters and their derivatives. Insets are enlarged spectra around m/z 1493.7, 2427.4 and 2597.2.

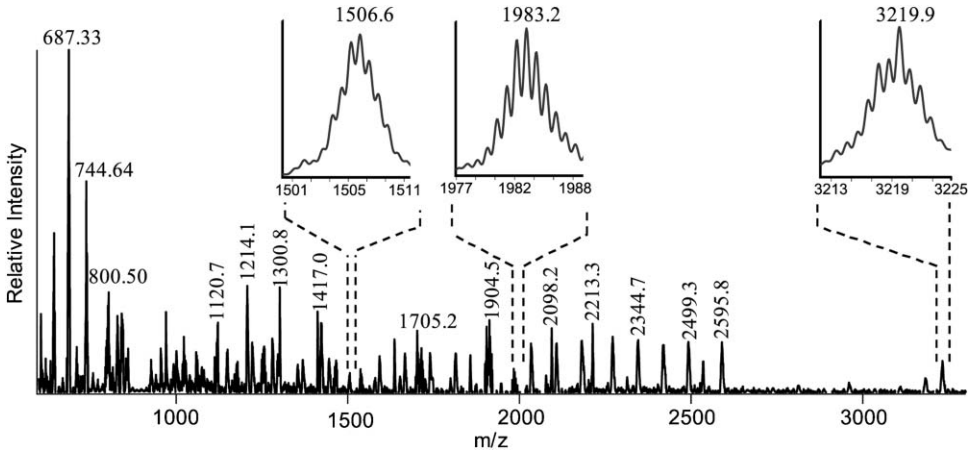

Fig. 11 MALDI-TOF-MS spectrum of ⟨Ru⟩–CO-labeled tryptic fragments of protein mixture. Insets are enlarged spectra around *m/z* 1506.6, 1983.2 and 3219.9.

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References

1 J. R. Yates, III, *J. Mass Spectrom.*, 1998, **33**, 1–19.
2 M. Mann and M. Wilm, *Anal. Chem.*, 1994, **66**, 4390–4399.
3 O. N. Jensen, A. V. Podtelejnikov and M. Mann, *Anal. Chem.*, 1997, **69**, 4741–4750.
4 P. A. Demirev, J. Ramirez and C. Fenselau, *Anal. Chem.*, 2001, **73**, 5725–5731.
5 B. Warsheid and C. Fenselau, *Anal. Chem.*, 2003, **75**, 5618–5627.
6 Y. An, Z. Fu and C. Fenselau, *J. Mass Spectrom. Soc. Jpn.*, 2005, **53**, 1–6.
7 W. J. Henzel, T. M. Billeci, T. M. Stults, S. C. Wong, C. Grimley and C. Watanabe, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 5011–5015; P. James, M. Quadroni, E. Carafoli and G. Gonnet, *Biochem. Biophys. Res. Commun.*, 1993, **195**, 58–64; M. Mann, P. Hojrup and P. Roepstorff, *Biol. Mass Spectrom.*, 1993, **22**, 338–345; D. J. C. Pappin, P. Hojrup and A. J. Bleasby, *Curr. Biol.*, 1993, **3**, 327–332; J. R. Yates, III, S. Speicher, P. R. Griffin and T. Hunkapiller, *Anal. Biochem.*, 1993, **214**, 397–408.
8 E. Mortz, P. B. O'Connor, P. Roepstroff, N. L. Kelleher, T. D. Wood, F. W. McLafferty and M. Mann, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 8264–8267; A. Shevchenko, O. N. Jensen, A. V. Podtelejnikov, F. Sagliocco, M. Wilm, O. Vorm, P. Mortensen, A. Shevchenko, H. Boucherie and M. Mann, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 14 440–14 445.
9 R. Kaufmann, B. Spengler and F. Lutzenkirchen, *Rapid Commun. Mass Spectrom.*, 1993, **7**, 902–910.
10 K. Biemann, *Annu. Rev. Biochem.*, 1992, **61**, 977–1010.
11 D. Fabris, M. M. Vestling, M. M. Cordero, V. M. Doroshenko, R. J. Cotter and C. Fenselau, *Rapid Commun. Mass Spectrom.*, 1995, **9**, 1051–1055.
12 N. P. Ewing and C. J. Cassady, *J. Am. Soc. Mass Spectrom.*, 2001, **12**, 105–116; A. Beck, M. Deeg, K. Moeschel, E. K. Schmidt, E. D. Schleicher, W. Voelter, H. U. Häring and R. Lehmann, *Rapid Commun. Mass Spectrom.*, 2001, **15**, 2324–2333.
13 T. Keough, M. P. Lacey and R. S. Youngquist, *Rapid Commun. Mass Spectrom.*, 2002, **16**, 1003–1015.
14 U. Hellman and R. Bhikhabhai, *Rapid Commun. Mass Spectrom.*, 2002, **16**, 1851–1859.
15 P. Chen, S. Nie, W. Mi, X.-C. Wang and S.-P. Liang, *Rapid Commun. Mass Spectrom.*, 2004, **18**, 191–198.
16 J. Zaia and K. Biemann, *J. Am. Soc. Mass Spectrom.*, 1995, **6**, 428–436.
17 K. D. W. Roth, Z.-H. Huang, N. Sadagopan and J. T. Watson, *Mass Spectrom. Rev.*, 1998, **17**, 255–274.
18 T. Keough, R. S. Youngquist and M. P. Lacey, *Anal. Chem.*, 2003, **75**, 156A–165A.
19 T. Keough, R. S. Youngquist and M. P. Lacey, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 7131–7136.
20 D. Wang, S. R. Kalb and R. J. Cotter, *Rapid Commun. Mass Spectrom.*, 2004, **18**, 96–102.
21 T. Okamura, T. Iwamura, A. Ito, M. Kaneko, M. Yamaguchi, H. Yamamoto, N. Ueyama, H. Kuyama, E. Ando, S. Norioka, T. Nakazawa, R. Masui and S. Kuramitsu, *Chem. Lett.*, 2005, **34**, 332–333.
22 A. Ito, T. Okamura, H. Yamamoto, N. Ueyama, K. Ake, R. Masui, S. Kuramitsu and S. Tsunasawa, *Anal. Chem.*, 2005, **77**, 6618–6624.
23 H. Katayama, K. Satoh, M. Takeuchi, M. Deguchi-Tawarada, Y. Oda and T. Nagasu, *Rapid Commun. Mass Spectrom.*, 2003, **17**, 1071–1078.
24 R. L. Beardsley and J. P. Reilly, *Anal. Chem.*, 2002, **74**, 1884–1890.