Determination of Unique Amino Acid Substitutions in Protein Variants by Peptide Mass Mapping with FT-ICR MS

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Peptide mass mapping plays a central role in the structural characterization of protein variants with single amino acid substitutions. Among the 20 standard amino acids found in living organisms, 18, all but Leu and Ile, differ from each other in molecular mass. The mass differences between amino acids range from 0.0364 to 129.0578 Da. The mass of the mutated peptide or the difference between normal and mutated peptides uniquely determines the type of substitution in some cases, and even pinpoints the position of the mutation when the involved residue is found only once in the peptide. Among 75 pairs of amino acid residues that are exchangeable via a single nucleotide replacement, 53 show specific change in exact mass, while only 25 in nominal mass. On the other hand, precise measurement, at least to the third decimal place, greatly enhances the capacity of the peptide mass mapping strategy for structural characterization. This notion was verified by an analysis of three Hb variants using MALDI-FTICR MS. In addition, the baseline resolution of two 1 kDa peptides with a single amino acid difference, Lys or Gln, which have the smallest (0.0364 Da) difference among residues, was achieved by measurement at a mass resolving power of 342,000. The results indicated that the smallest difference, 0.0040 Da between [$\Delta 29.9742$ for Glu-Val] and [$\Delta 29.9782$ for Trp-Arg], among all types of amino acid substitutions derived from a single nucleotide replacement can be discriminated at the present performance level. Therefore, FTICR MS is capable of identifying all 53 types of substitutions, each of which is associated with a unique mass difference, except for the Leu and Ile isomers. (J Am Soc Mass Spectrom 2006, 17, 508–513) © 2006 American Society for Mass Spectrometry

Many diseases are caused by a single nucleotide substitution in the coding region of the responsible gene. This type of mutation produces mutated proteins with single amino acid substitutions, unless the resulting codon terminates translation or the mutated mRNA or proteins are quite unstable. The mass spectrometric strategy of characterizing the mutated proteins was established a decade ago, mostly utilizing hemoglobin (Hb) variants as a model protein [1–6]. The approach involves two steps: molecular mass measurements of intact proteins [7], and peptide mass mapping of enzymatic digests [8, 9]. Detection of the molecular mass change caused by an amino acid substitution is an essential part of either analysis.

Among the 190 pairs formed by the 20 standard amino acids, only an exchange of Leu and Ile results in the same elemental composition, and therefore cannot be characterized on the basis of molecular mass. Other types of exchange accompanying a mass shift from 0.0364 Da for Glu-Lys to 129.04578 Da for Trp-Gly are theoretically amenable to the mass-based strategy. More importantly, according to the point mutation rule, the size of a mass shift uniquely defines the type and position of the substitution in many cases, as discussed in earlier studies [1, 10]. In addition, another rule of genetics in nature, where the vast majority of amino acid substitutions are caused by single nucleotide substitutions, facilitates the mass-based elucidation, because only 40% of the 190 different types of amino acid substitutions are derived from a single nucleotide substitution. Although some types of amino acid mutations are indistinguishable from each other because they involve the exact same change in elemental composition, the capability of this strategy depends on the resolution of the measurement. For example, two amino

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Table 1. Mass differences between peptides of wild-type and mutant globins

	Amino acid	<i>m/z</i>		Error ppm
		Observed	Theoretical	
Wild	97His	1126.5659	1126.5645	1.25
Moriguchi	97Tyr	1152.5703	1152.5689	1.21
Mass-shift	His-Tyr	−26.0044	−26.0044	0.38
Wild	92Arg	1087.6270	1087.6264	0.64
J Capetown	92Gln	1059.5846	1059.5839	0.51
Mass-shift	Arg-Gln	28.0424	28.0425	4.28
Wild	22Glu	1314.6670	1314.6654	1.19
G Coughatta	22Ala	1256.6616	1256.6599	1.36
Mass-shift	Glu-Ala	58.0054	58.0055	2.14

acid substitution types, Phe-Asp and Tyr-Asp, give the same nominal mass shift of 48 Da, but differ from each other by 0.03639 Da being $\Delta 48.00000$ Da versus $\Delta 48.03639$ Da, respectively.

Human Hb is a tetrameric protein in erythrocytes and is comprised of two globin molecules, α and β subunits, each of which bears a prosthetic heme. The subunits of adult Hb (Hb A) are composed of 141 amino acid residues ($M_r = 15,126.4$) for α and 146 amino acid residues ($M_r = 15,867.2$) for β , respectively. Most of the Hb variants are caused by an amino acid substitution in one of these subunits and are derived from a single nucleotide change in the gene. In most cases, the exception being highly prevalent mutations such as that causing sickle cell disease, Hb mutations are heterozygotic and thus both the normal peptide and its mutated counterpart are present in the patient.

Herein, precise measurement of the difference in the masses of normal and mutated peptides was carried out by Fourier transform ion cyclotron resonance (FTICR) MS [11]. The results demonstrate precise measurements to greatly enhance the capability of peptide mass mapping for structural characterization of amino acid substitutions in protein variants.

Materials and Methods

Materials

Three hemoglobin variants, Hb Moriguchi ($\beta 97\text{His} \rightarrow \text{Tyr}$), Hb G Coughatta ($\beta 22\text{Glu} \rightarrow \text{Ala}$), and Hb J Cape Town ($\alpha 92\text{Arg} \rightarrow \text{Gln}$), with known structures [2, 4, 5] were employed in this study. An enzymatic digest of globins was prepared according to methods described previously [2, 10]. The mutated peptide of Hb G Coughatta was purified by reversed-phase chromatography for MS/MS.

Synthetic peptides, LQQCPFEDH (1115.4706 Da) and LKQCPFEDH (1115.5070 Da), were purchased from Sigma (St. Louis, MO).

MALDI-FTICR MS

MS was performed in positive ion mode using a MALDI-FTICR mass spectrometer (IonSpec, Lake For-

est, CA) equipped with an actively shielded 7-tesla superconducting magnet (Cryomagnetics, Oak Ridge, TN). An air-cooled Nd:YAG laser system (New Wave, Fremont, CA) was used for ionization and desorption. The sample was mixed with 50 mg/mL recrystallized 2,5-dihydroxybenzoic acid on a stainless steel target. All mass spectra were acquired in single non-repetitive shot mode followed by a single scan, because multiple shot mode and accumulation of scans impair mass accuracy. Each measurement was repeated 20 times. The protonated molecules for the peptides, except those involved in the mutation, were used for the internal mass calibration.

Fragmentation of the peptides was accomplished by sustained off resonance irradiation and collision-induced dissociation (SORI-CID).

Statistical Analysis

Confidence intervals (CI) were calculated with Student's *t*-distribution.

Results

In the peptide mass mapping for detection of mutations, tryptic digestion is most often used and the resulting peptides have a mass of 1000 Da on average. The mutated peptides are βT11 (peptide no.11 of tryptic digest of β -globin chain) for Hb Moriguchi and βT3 for Hb G Coughatta, and the molecular masses for the corresponding wild peptides are 1125.5567 and 1313.6575, respectively. For Hb J Cape Town, digestion with lysylendopeptidase was applied, as the substitution of Gln for Arg turns off the tryptic cleavage at the mutated site, and the peptide αL9 (peptide no. 9 of lysylendopeptidase digest of α -globin chain) with an exact molecular mass of 1086.6186 for the wild type is the target in this case. All these mutations are heterozygotic and the variants are present at $\sim 50\%$, as are their normal counterparts. The FTICR MS of these peptides represented the mass accuracy within a few ppm (Table 1).

Figure 1. MALDI-FT-ICR mass spectra of tryptic digest of carbamidomethylated α and β globins of Hb Moriguchi. (a) Full spectrum. (b) Depicted spectrum for protonated β T11 region. The ions for normal and mutated (β T11') peptides are observed at m/z 1126.5638 and 1152.5689, respectively, since the mutation was heterozygotic. Normal β T11 sequence is LHVDPENFR.

Hb Moriguchi (β 97His→Tyr)

The normal sequence of β T11 is LHVDPENFR. The MALDI-FTICR mass spectrum of a tryptic digest is presented in Figure 1, and the signals at m/z 1126.5638

and 1152.5689 are the ions for wild and mutated β T11 peptides, respectively. The difference in nominal mass is 26 Da, suggesting five different types of mutations: His→Tyr (26.0044), Ala→Pro (26.0157), cmCys (carbamidomethylcysteine)→Trp (26.0487), Ser→Ile (26.0520), and Ser→Leu (26.0520) (exact mass in parentheses and unmodified cysteine excluded). In light of the point mutation rule, only His→Tyr is ascribed to the molecular mass shift of this peptide, because none of the other residues, Ala, Cys, or Ser, are present in the wild β T11 peptide. This assumption can be confirmed statistically by precise measurement of the mass shift as follows.

The mean of 20 mass difference measurements was 26.00439, and the standard deviation was 0.00046. Therefore, the 99% confidence interval (CI) is [26.004094, 26.004686], and only Tyr→His among the five types of substitutions described above lies within the 99% CI (Table 2). The position of the mutation was unequivocally demonstrated to be at the single Tyr, the second residue of β T11.

Hb J Cape Town (α 92Arg→Gln)

The normal sequence of α L9 peptide is LRVDPVNFK. In the MALDI-FTICR mass spectrum, the ions at m/z 1087.6267 and 1059.5841 are derived from wild and mutated peptides, respectively (Figure 2). The mean of 20 mass difference measurements was 28.04238 Da, and the 99% CI was [28.041950, 28.042803]. Only the Arg→Gln mutation giving the calculated shift of 28.0425 Da lies within the 99% CI (Table 2), while the nominal mass difference of 28 Da suggested five different types of mutations: Asp→Ser (27.9949), Glu→Thr (27.9949), Arg→Lys (28.0061), Val→Ala (28.0313), and Arg→Gln. Since one Arg residue is present in the sequence, the substitution is uniquely located at that position.

Hb G Couthatta (β 22Glu→Ala)

The normal sequence of β T3 peptide is VNVDEVG-GEALGR. The signals at m/z 1256.6614 and 1314.6670 are protonated β T3 from mutated and wild globins, respectively (Figure 3a and b). The difference in nominal mass suggests four different types of mutations: Trp→Lys (57.9844), Asp→Gly (58.0055), Glu→Ala

Table 2. The 99% Confidence Intervals (CI) for each measured mass-shift and the nearest (*) and the second nearest mass-shifts

	CI	Theoretical mass shift	Mutation
Hb Moriguchi	[26.004094,26.004686]	26.0044 26.0157	(Tyr-His)* (Pro-Ala)
Hb J Cape Town	[28.041776,28.042984]	28.0313 28.0422	(Val-Ala) (Arg-Gln)*
Hb G Couthatta	[58.004628,58.006082]	58.0053 58.0207	(Glu-Ala)* (Trp-Gln)

Figure 2. MALDI-FT-ICR mass spectra of the Lys-C digest of carbamidomethylated α and β globins of Hb J Cape Town. (a) Full spectrum. (b) Depicted spectrum for protonated α L9 region. The ions for normal and mutated (α L9') peptides are observed at m/z 1087.6267 and 1059.5841, respectively. Normal α L9 sequence is LRVPVNFK.

(58.0055). and Trp→Gln (58.0207). The measured difference was 58.0054 ± 0.0014 Da, and the 99% CI was [58.004628, 58.006082]. These results ruled out two types of substitutions, Trp→Lys and Trp→Gln, leaving two possible mutations, Asp→Gly and Glu→Ala. Since in total three Asp or Glu candidate residues are present in the sequence of β T3, the type and position of the mutation cannot be uniquely determined solely by the mass shift. SORI-CID was thus used to determine the structure of the mutated peptide in this case. In the MS/MS spectrum shown in Figure 3c, the y_9 ion at m/z 812.4564 contained the Glu→Ala mutation at either the fifth or the ninth residue from the N-terminal. Finally, the mutated site was determined to be the former by the MS/MS/MS spectrum, in which the normal mass for the y_7 ion was generated from the y_9 ion observed in the MS/MS spectrum (Figure 3d).

Minimal Mass Shift of Gln-Lys in a 1 kDa Peptide

The smallest mass shift produced by an amino acid substitution is 0.0364 Da. This shift is derived from an exchange of Gln for Lys or vice versa. Theoretically, separation of two 1 kDa peptides bearing a mass difference of this size requires a resolution of 30,000, which is readily achievable with a broadband scan. To demonstrate the separation capacity of FTICR MS, two homologous peptides, LQQCPFEDH (1115.4706 Da) and LKQCPFEDH (1115.5070 Da), were synthesized and the mixture was analyzed. The baseline resolution of these peptides was achieved by the MALDI-FTICR mass spectrum at a mass resolving power of 342,000 (Figure 4).

Discussion

Although structural characterization of protein variants is currently performed by DNA sequence analysis in most cases, MS still plays a central role in characterizing some systemically abundant proteins that can be easily obtained [12, 13]. A typical example is Hb, a number of mutations of which have been characterized by MS [2, 8, 14, 15]. Analysis of the mass of intact proteins is useful for detecting mutations in small proteins such as globins, but the determination of the type and position of such a mutation awaits peptide mass mapping of an enzymatic digest. In analysis of either proteins or peptides, precise measurement of a mass shift derived from the point mutation is essential [16, 17], while the Leu and Ile isomers, which can be exchanged with a single nucleotide replacement, are not amenable to this strategy. In the present study, the amino acids with the next smallest difference, Lys and Gln, could be discriminated in a 1 kDa peptide, the average size of tryptic peptides (Figure 4).

In some cases, the type of mutation is uniquely determined by the size of the molecular mass change in peptides, and the site may also be identified when the residue appears only once in the sequence [2]. In this respect, the present study has demonstrated that the precise measurement greatly enhances the capability of peptide mass mapping compared with conventional analysis on a nominal-mass basis. This notion is supported by the following theoretical considerations.

Among 190 different pairs of standard amino acids, 23 have unique differences in nominal mass. For example, His and Glu are the only pair that differs from each other by 8 Da, but this type of amino acid substitution requires a double nucleotide replacement in the codon. On the other hand, there are 75 pairs of residues that can be exchanged with single nucleotide substitutions, and only six pairs among them, His-Asp (22 Da), Arg-Cys (53 Da), Arg-Pro (59 Da), Arg-Ser (69 Da), Trp-Cys (83 Da), and Trp-Gly (129 Da), have unique mass differences even on a nominal-mass basis. These six can be readily characterized by straightforward conventional measurements. However, this highlights

Figure 3. MALDI-FT-ICR mass spectra of the tryptic digest of α and β globins of Hb G Coushatta. (a) Full spectrum. (b) Depicted spectrum for protonated $\beta T3$ region. The ions for normal and mutated ($\beta T3'$) peptides are observed at m/z 1314.6670 and 1256.6614, respectively. (c) MS² product ion spectrum of the purified $\beta T3'$. The mutated peptide $\beta T3'$ was purified by reversed-phase chromatography and subjected to SORI-CID. Three y -series ions, y_4 , y_9 , and y_{11} are observed. (d) MS³ product ion spectrum of the y_9 observed in the MS² spectrum. The normal $\beta T3$ sequence is VNVDEVG-GEALGR.

that nominal mass measurements do not specify the type of mutation in many cases. For example, there are ten different pairs, Asp-Val, Glu-Leu, Glu-Ile, Cys-Ser, Ser-Ala, Tyr-Phe, Met-Asp, Phe-Met, Leu-Pro, and Ile-Pro, having a difference of 16 Da, of which five (Asp-Val, Cys-Ser, Ser-Ala, Tyr-Phe, and Leu-Pro) can be derived from a single nucleotide substitution. On the other hand, in terms of exact mass, only two, Ser-Ala and Tyr-Phe, in the latter subgroup have the same 15.9949 Da difference. In this way, among a total of 190 pairs, 121 have unique changes in terms of exact mass and, therefore, can be discriminated from each other by precise measurement. When limited to single nucleotide substitutions, 54 (72%) of 75 types of exchange have specific differences in size (including 0 for Leu-Ile), i.e.,

in exact mass, while only 25 types (33%) differ in nominal mass (Figure 5). Among the remaining 21 pairs that cannot be specified on the basis of exact mass, those with a difference of 14.0157 Da for CH_2 (Ala-Gly, Thr-Ser, Leu-Val, Ile-Val, and Glu-Asp) constitute the largest group.

Obviously, 54 different sizes of mass shift associated with amino acid substitutions represent the difference in elemental composition of residues. For example, the shift of +26.0044 by the mutation His→Tyr of Hb Moriguchi is calculated from the difference in the compositions of His and Tyr, $[C_6H_7N_3O]$ versus $[C_9H_9NO_2]$, namely $[C_3H_2O-N_2]$. The smallest difference between amino acids is 0.0364 Da, resulting from an exchange of Lys and Gln, and the difference in the

Figure 4. MALDI-FT-ICR mass spectra of a mixture of synthetic peptides with a mass difference of 0.0364 Da. The protonated peptides with sequences of LQQCPFEDH and LKQCPFEDH are observed at m/z 1116.4784 and 1116.5148, respectively, displaying baseline separation.

elemental composition is $[C_1H_4O_1]$. On the other hand, the smallest difference between substitutions (from a single nucleotide replacement) is 0.0040 Da, the difference between $[\Delta 29.9742$ for Glu-Val] and $[\Delta 29.9782$ for Trp-Arg], and the method described herein can discriminate this small difference as suggested by the accuracy

Figure 5. Amino acid replacement pairs with respect to the causative nucleotide substitution and resulting mass shift. There are 190 pairs (Groups A + B) of standard amino acids, and 75 (Group B) can be derived from single nucleotide replacements in the genetic code. Exchange in the Group A pair is extremely rare. In Group B, 54 pairs (Groups C + G) have a specific difference in exact mass between the amino acids involved, and can thus be discriminated from each other, while the others (F) do not. Among the 54 pairs (Groups C + G), 25 pairs (Group D) are unique in nominal mass. Therefore, 53 pairs (Group G), the exception being the isomer pair (Group C) of Leu and Ile, are determined by precise measurements. The number of pairs is indicated in parentheses.

shown in Table 1. In conclusion, peptide mass mapping by FTICR MS is capable of identifying all 53 types (106 types when A→B and B→A are counted separately) of substitutions which are caused by single nucleotide substitutions and have unique mass shifts.

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