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The peak height ratio of *S*-sulfonated transthyretin and other oxidized isoforms as a marker for molybdenum cofactor deficiency, measured by electrospray ionization mass spectrometry

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Abstract

Molybdenum cofactor deficiency is a fatal neurological disorder, which follows an autosomal-recessive trait and is characterized by combined deficiency of the enzyme, sulfite oxidase, xanthine dehydrogenase and aldehyde oxidase. Early detection of molybdenum cofactor-deficient patients is essential for their proper care and genetic counseling of families at risk. We demonstrate the use of *S*-sulfonated transthyretin (TTR) as a marker for molybdenum cofactor deficiency. Plasma or sera obtained from 4 patients with molybdenum cofactor deficiency and 57 controls were studied by electrospray ionization mass spectrometry (ESIMS) following selective enrichment of TTR by immunoprecipitation using protein G/A agarose. The data obtained from molybdenum cofactor deficiency samples indicated a strong increase in the peak height of *S*-sulfonated TTR. A more significant difference was revealed if the peak height ratio of *S*-sulfonated TTR and the sum of the other oxidized TTR were determined. By accurate determination of the ratio, the samples of molybdenum cofactor deficiency patients could clearly be distinguished from controls without molybdenum cofactor deficiency.

Keywords: *S*-sulfonated; Immunoprecipitation; Protein G/A agarose; Mass spectrometry; Posttranslational modification; Molybdenum cofactor deficiency

1. Introduction

We reported previously a unique isoform of serum transthyretin (TTR), which was *S*-sulfonated by excessive sulfite *in vivo* in a patient with molybdenum cofactor deficiency (MCD) [1]. Molybdenum cofactor is essential for the function of the enzymes sulfite oxidase, xanthine dehydrogenase and aldehyde oxidase [2]. MCD and isolated sulfite oxidase deficiency result in a severe clinical phenotype, which is, in most cases, characterized by nontreatable convulsions presenting early after birth. Besides neurological symptoms and anatomic brain anomalies, ectopic lenses, dysmorphic signs, hypertonicity and hypotonia are frequent features. Due to the lack of sulfite oxidase activity, sulfite accumulates and sulfate production decreases. The presence of elevated levels of sulfite leads to the accumulation of *S*-sulfocysteine formed by a direct reaction of sulfite with cysteine [2]. The formation of protein-sulfonated compounds ($RS-SO_3^-$) in the albumin and fibronectin of several animal species exposed to sulfur dioxide or to sulfite/hydrogen sulfite has been reported [3]. TTR contains a single free cysteine at position 10. We predicted the elevation of *S*-sulfonated TTR in the sera of sulfite oxidase-deficient patients, and demonstrated it in a single case of MCD by electrospray ionization mass spectrometry (ESIMS) [1]. To reveal the modified structure of TTR, ESIMS with immunoprecipitated TTR was successfully applied [4].

The *S*-sulfonated TTR signals were also clearly high in additional MCD samples, in contrast to most control samples that presented low *S*-sulfonated TTR peaks. However, elevated *S*-sulfonated TTR was also observed in some of the

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control samples, although those patients did not have MCD. Commercially obtained TTR also showed a rather high peak of *S*-sulfonated TTR (~ 15%). High peaks of *S*-sulfonated TTR were often detected (ca. 20%) in TTR prepared from rabbit sera. Our original ESIMS method with immunoprecipitated TTR has been applied in several institutions to detect TTR variants in sera from patients with familial amyloidotic polyneuropathy [5–7]. In some of the samples, which were studied in this regard and were not from MCD patients, rather high signals corresponding to *S*-sulfonated TTR, which is 80 Da larger than free TTR, were also reported [6]. To distinguish MCD from these ambiguous cases, we here describe an improved method for sample preparation and calculation of results.

2. Materials and methods

2.1. Specimens

Plasma or sera of 4 patients with MCD as diagnosed on the basis of clinical features, metabolite studies, enzyme assays in cultured skin fibroblasts and mutation analysis, and 57 samples obtained from children with a variety of diagnoses, but neither MCD nor isolated sulfite oxidase deficiency, were analyzed. One case of MCD was already reported previously [1]. The ages of the MCD patients at the time of sampling were 3 days, 3 months, 2 and 3 years. The ages of the controls were distributed from 1 month to 13 years.

2.2. Sample preparation

We previously reported a convenient method for preparing protein samples for application to soft ionization MS [8–10]. The test serum was mixed with antiserum against the target protein, and the generated immunoprecipitates were subsequently washed with 0.15 M sodium chloride and water. The mixture was applied directly to on-line high performance liquid chromatography (HPLC)/ESIMS. Most samples were successfully analyzed by this method. However, the preparation method was not good enough to determine an accurate ratio for the *S*-sulfonated TTR and TTR isoforms. The remaining albumin in immunoprecipitates coeluted with *S*-sulfonated TTR in the HPLC system, and disturbed the measurement of this fraction. To wash out serum protein extensively, we used protein G/A agarose to form large immune complex particles. Protein G plus/Protein A Agarose (50 μl of suspension) (Oncogene Research Products, cat# IP05, lot# D14269, Boston, MA) was washed three times with 0.5 ml of 0.15 M sodium chloride. Antihuman TTR (prealbumin) antiserum (25 μl) (SIGMA, lot# 19H4822, St. Louis, MO) was added to the agarose, and the mixture was

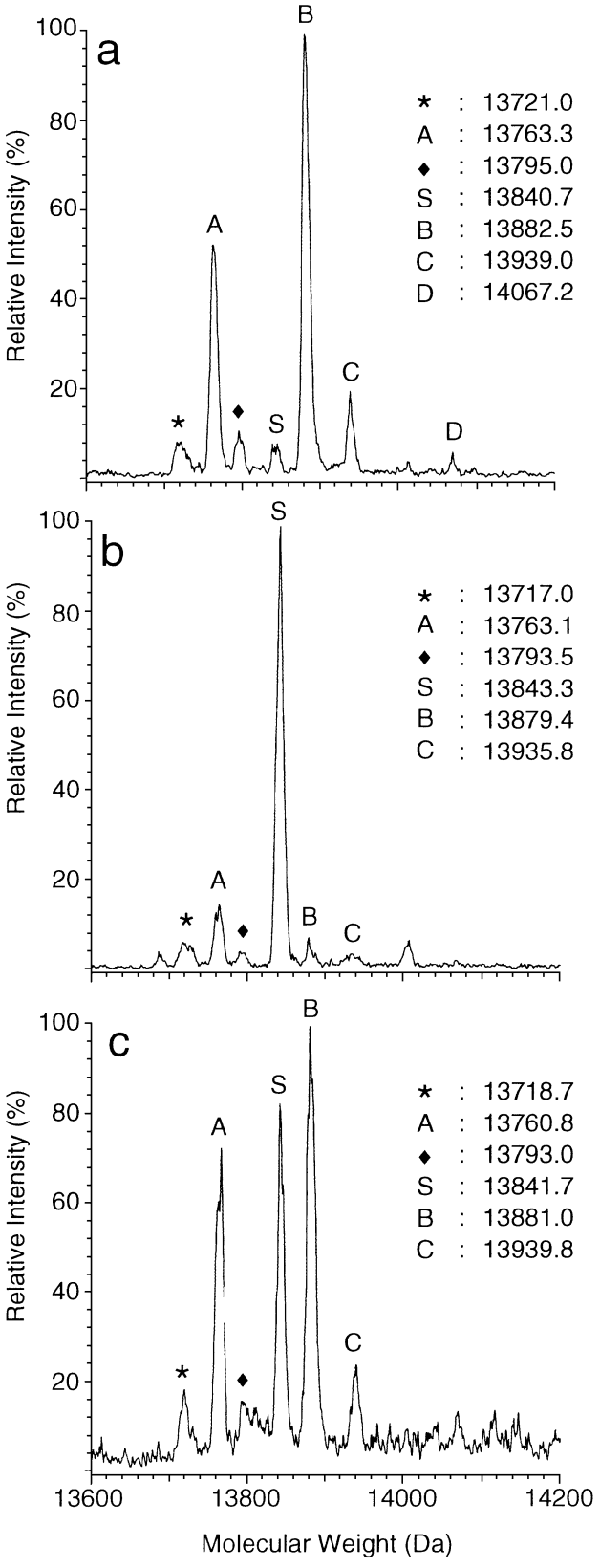

Fig. 1. Deconvoluted ESIMS spectra of TTR from a control infant (a), a patient with MCD (b), and a second infant without MCD (c), but with a rather high peak of *S*-sulfonated TTR. A, free TTR; B, TTR conjugated with cysteine; C, TTR conjugated with cysteinylglycine; D, TTR conjugated with glutathione; S, *S*-sulfonated TTR; *, modified TTR in which a side chain of cysteine residue is cleaved, and changed to glycine. The assignment of each peak was discussed in a previous paper [10]. ♦, the observed molecular mass corresponds to TTR in which cysteine is oxidized.

shaken for 30 min at room temperature. The resulting agarose–antibody complex was washed with 0.5 ml 0.15 M sodium chloride four times. Subsequently, a 10 μl sample serum was mixed with the washed complex and shaken for 30 min. The generated precipitates were washed with 0.5 ml of 0.15 M sodium chloride seven times. Each washing step consisted of turning the tube over several times, centrifuging for 5 s with a centrifuge and discarding the supernatant. Finally, the precipitate was suspended in 20 μl of 2% acetic acid and the agarose was removed by filtration, using an ultrafree-MC centrifugal 0.45 μm filter unit (Millipore, Bedford, MA). The filtration was repeated twice and the pooled filtrate was applied to LC/ESIMS.

2.3. Reversed-phase HPLC and mass spectrometry

On-line HPLC/ESIMS was used, with a PLRP-S reversed-phase column (1 × 50 mm, 1000 Å, 8 μm; Michrom Bioresources, Pleasanton, CA), as reported previously [4]. An ESI mass spectrometer, TSQ7000 (Thermo Quest, San Jose, CA) was utilized [4]. The scanning range was *m/z* 500–2000 in 3 s. Calibration was performed using the peptide Met–Arg–Phe–Ala and multiple charged ions of horse apo myoglobin, according to the manufacturer's instructions.

2.4. Calculation of the TTR ion peaks on ESIMS

The ratios of TTR isoforms' ion peak heights were determined on deconvoluted spectra, which were a summation of scans covering the whole TTR elution. The ratio of the peak heights of *S*-sulfonated TTR and the sum of the peak heights of the TTR conjugates with cysteine, cysteinylglycine and glutathione were calculated. Note the marks used in Fig. 1: (S)/(B + C + D).

3. Results

Deconvoluted ESIMS spectra of TTR prepared by immunoprecipitation using protein G/A agarose are shown in Fig. 1. The spectrum of TTR from a control infant (Fig. 1a) shows several peaks, which were similar to those previously reported by the original preparation method [4,10]. The observed mass of each peak corresponded to the theoretical molecular weight as assigned in the figure. The assignment of each peak was as reported previously [10]; the peak 80 Da larger than unmodified TTR was assigned to *S*-sulfonated TTR (S). The two major peaks in the control sample corresponded to free TTR (A) and TTR conjugated with cysteine (B). Samples of most of the remaining controls presented with patterns similar to that in Fig. 1a. The spectrum from a patient with MCD (Fig. 1b) showed a prominent *S*-sulfonated TTR peak, while the peaks of other oxidized forms of TTR and free TTR were relatively low. A similar pattern was reported for this patient using the earlier pretreatment method [1]. Spectra obtained from the other three MCD patients using the new method were nearly identical to that shown in Fig. 1b.

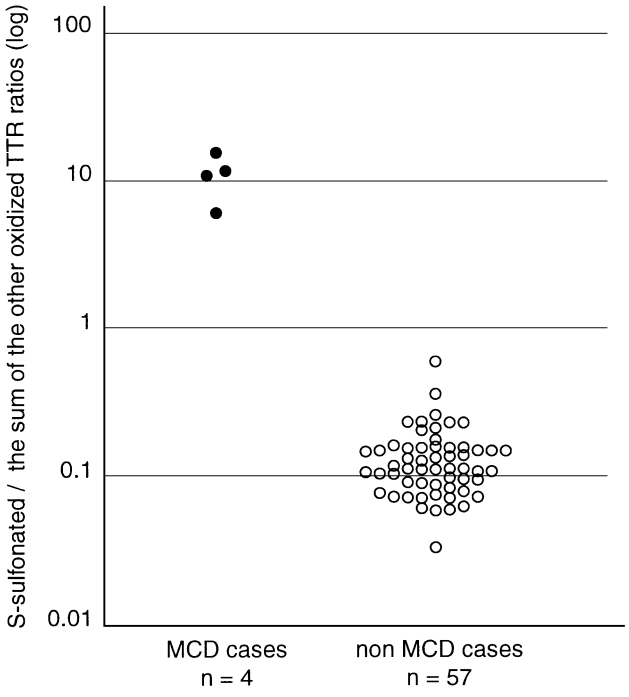

Fig. 2. Comparison of the ratio of peak height between patients with MCD and controls, calculated by the equation: (peak height of *S*-sulfonated TTR)/(the sum of the peak heights TTR conjugated with cysteine, cysteinylglycine and glutathione). Note the marks used in Fig. 1: (S)/(B + C + D).

Analysis of samples from most of the remaining controls showed some with patterns similar to that in Fig. 1a, but others with significantly higher signals of *S*-sulfonated TTR, as shown in Fig. 1c. However, a critical difference was noted between the spectra obtained from these controls and those of patients with MCD. Controls with high signals of *S*-sulfonated TTR also showed increased signals of TTR conjugated with cysteine, cysteinylglycine, and (sometimes) glutathione, while patients with MCD showed a specific elevation of *S*-sulfonated TTR. Thus, the significance of the elevated *S*-sulfonated TTR in any one individual is more accurately documented by calculating the peak height ratio of *S*-sulfonated TTR to that of the other oxidized derivatives (S)/(B + C + D). The mean and S.D. of the ratio for MCD cases ($n = 4$) and controls ($n = 57$) were 11.0 ± 3.8 and 0.14 ± 0.09, respectively (see Fig. 2).

No significant difference was found among the peak height ratios (*S*-sulfonated TTR)/(all oxidized isoforms in TTR) obtained from controls of different ages.

4. Discussion

Using an immunoprecipitation method previously reported by us [4], serum protein was not removed completely because of the washing limit for small amounts of precipitates (often invisible). On reversed-phase HPLC, *S*-

sulfonated TTR was eluted slightly later than the other isoforms of TTR, and co-migrated with the remaining serum protein, mainly albumin. Using the immunoprecipitation method with the help of protein G/A agarose, we obtained clearly visible precipitates, making the washing steps easy to perform. Sufficient washing removed the remaining albumin, and high-quality spectra were obtained for a wide range of TTR peaks on HPLC chromatography, which gave an accurate peak height ratio of the signal of *S*-sulfonated TTR to the sum of the signals of other TTR isoforms. With this improved procedure, we found a clear difference in the ratio (peak height of *S*-sulfonated TTR)/(sum of peak heights of total oxidized TTR) between MCD patients and controls.

S-sulfocysteine has been used for the diagnosis of sulfite oxidase deficiency. However, it is a strongly acidic and relatively unstable amino acid, which tends to be lost when urine is desalted with the H^+-form of a sulfonated ion-exchange resin [11]. In common routine amino acid analysis by ion-exchange chromatography, *S*-sulfocysteine elutes on a position corresponding to a void volume of chromatography, and is not separated from phosphoserine. *S*-sulfocysteine in urine can accurately be monitored by mass spectral analysis [2], and quantitation of this metabolite is now available as a service through the Duke University Mass Spectrometry Laboratory [12]. The assessment of peak height ratios proposed in this paper may represent a valuable additional diagnostic marker. By our experiences, TTR in plasma or sera can stay at ambient temperature for at least 1 week. *S*-sulfonated TTR in plasma or sera may be more stable than *S*-sulfocysteine in urine.

In some control samples, the peak height of *S*-sulfonated TTR was elevated in addition to that of other oxidized TTR isoforms. The mechanism of *S*-sulfonated TTR formation in control samples is unknown. We previously characterized a unique isoform of TTR, which was 46 Da smaller than free TTR [10]. The peak is marked by an asterisk in Fig. 1. To characterize this isoform, the component TTR-46 prepared by reversed phase HPLC was cleaved with trypsin. The peptides were analyzed by LC/ESIMS, and using reconstructed ion chromatograms, the ions corresponding to all normal expected peptides, and the ions 46 Da smaller than all normal peptides were plotted. A clear ion, 46 Da smaller than mass of normal T1 + 2(M + 2H: 729.9), was detected, but an ion corresponding to normal T1 + 2 was not detected. The collision-induced dissociation (CID) spectra of the abnormal ion suggested that Cys-10 is substituted by Gly [10]. The amino-terminal sequence of TTR-46 was also studied with a protein sequencer, and the first 15 residues were the same as normal sequence except position 10, which showed Gly instead of Cys [10]. It is possible that *S*-sulfonated TTR and TTR-Gly-10 are formed by an unknown common pathway, although additional experiments are needed to clarify this issue.

A European survey group has suggested that MCD is more common than generally thought, and that patients may well be overlooked because of a lack of awareness [13]. Although work with additional patient samples is needed to determine the general applicability of the assay described here, the study of TTR by ESIMS may represent a promising method for MCD screening. It may prove to be of special value in the diagnosis of patients with isolated sulfite oxidase deficiency, because that disorder is not associated with disturbed purine metabolism, as in patients with MCD deficiency, where low uric acid values can help establish the diagnosis [2].

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References

[1] M. Kishikawa, T. Nakanishi, A. Shimizu, M. Yoshino, Pediatr. Res. 47 (2000) 492–494.
[2] J.L. Johnson, M. Duran, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, New York, 2001, pp. 3163–3177.
[3] D.B. Menzel, D.A. Keller, K.H. Leung, Adv. Exp. Med. Biol. 197 (1986) 477–492.
[4] M. Kishikawa, T. Nakanishi, A. Miyazaki, A. Shimizu, M. Nakazato, K. Kangawa, H. Matsuo, J. Mass Spectrom. 31 (1996) 112–114.
[5] Y. Ando, P.I. Ohlsson, O.B. Suhr, N. Nyhlin, T. Yamashita, G. Holmgren, A. Danielsson, O. Sandgren, M. Uchino, M. Ando, Biochem. Biophys. Res. Commun. 228 (1996) 480–483.
[6] R. Theberge, L. Connors, M. Skinner, J. Skare, C.E. Costello, Anal. Chem. 71 (1999) 452–459.
[7] S. Ikeda, T. Tokuda, A. Nakamura, I. Ueno, T. Taketomi, N. Yanagisawa, Y.F. Li, Amyloid 4 (1997) 104–107.
[8] T. Nakanishi, N. Okamoto, K. Tanaka, A. Shimizu, Biol. Mass Spectrom. 23 (1994) 230–233.
[9] T. Nakanishi, M. Kishikawa, A. Miyazaki, A. Shimizu, Y. Ogawa, S. Sakoda, T. Ohi, H. Shoji, J. Neurosci. Methods 81 (1998) 41–44.
[10] M. Kishikawa, T. Nakanishi, A. Miyazaki, A. Shimizu, Amyloid 6 (1999) 48–53.
[11] H.J. Bremer, M. Duran, J.P. Kamerling, H. Przyrembel, S.K. Wadman, Disturbances of Amino Acid Metabolism: Clinical Chemistry and Diagnosis, Urban and Schwarzenberg, Baltimore, 1981.
[12] URL http://www.duke.edu/~mdfeezor/MSHome/MCDSulfo.html.
[13] H.A. Simmonds, G.F. Hoffmann, J.L. Perignon, V. Micheli, A.H. van Gennip, Lancet 353 (1999) 675–676.