Detection of a genetic variant, lysine→glutamic acid at position 372 of human serum albumin, by capillary electrophoresis and structural identification

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Abstract

A genetic variant of human serum albumin (alloalbumin) is detected by capillary electrophoresis (CE). Two albumin peaks, which were in the ratio of approximately one, were clearly separated. One of the peaks had the same migration time as normal albumin (Alb A) and the other (Alb X) had a longer migration time. SDS-polyacrylamide gel electrophoresis of CNBr fragments (CB) of Alb X indicated that the amino acid substitution was localized in the CB5 fragment (residue 330–446) of the molecule, because of anomalous migration of CB5 in the gel. The CE mapping of the tryptic peptides from the variant CB5 revealed clearly the existence of a new peptide, and the lack of two normal peptides. The sequence analysis of the variant peptide collected by CE micropreparation showed that the N-terminus of the variant peptide corresponded to that of T49 in Alb A. The substitution site, lysine→glutamic acid at the position 372, was revealed by sequence determination of the variant peptide purified by reversed-phase HPLC. ©1997 Elsevier Science B.V.

Keywords: Lysine; Glutamic acid; Human serum albumin

1. Introduction

Capillary electrophoresis (CE) is a useful technique for analysis of biologically important molecules [1–16]. It has also been used to detect small variations in the primary and/or secondary structure of peptides and proteins [6,7]. We have previously reported the separation of hemoglobin variants [14], and have also detected a genetic variant of human serum albumin [15]. Investigation of human albumin variants has been stimulated by recent improvements of methods for structural study of proteins and by interest in protein genetics and evolution, and some different amino acid substitutions and nucleotide mutations have been identified [17–22].

In this paper, we describe the detection of an alloalbumin and the strategy for identifying a variant peptide in alloalbumin using CE. The strategy consists of cyanogen bromide (CNBr) cleavage of the purified alkylated albumin, followed by SDS-polyacrylamide gel electrophoresis to identify the variant CNBr fragment, tryptic peptide mapping of the variant CNBr fragment, and micropreparation of the variant peptide using CE.

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2. Experimental

2.1. Chemicals and materials

Cyanogen bromide (CNBr) was purchased from Nacalai Tesque (Kyoto, Japan) and trifluoroacetic acid (TFA) was from Wako (Osaka, Japan). L-(Tosylamido-2-phenyl)ethylchloromethyl ketone (TPCK)-treated trypsin was from Sigma (St. Louis, MO, USA) and sodium 1-hexanesulfonate was from Tokyo Kasei (Tokyo, Japan). TSK 3000 SWXL column (30 cm×0.78 cm I.D.) for gel permeation HPLC was from Tosoh (Tokyo, Japan).

Serum samples were obtained from a hyperalbuminemia adult Japanese and a normal adult Japanese.

2.2. CNBr cleavage and screening variant CNBr fragment

The albumin variant purified by ion-exchange HPLC (data not shown) was reduced and carboxymethylated, and then cleaved by CNBr [17,20–22]. Fig. 1 shows a linear model of the primary structure of human serum albumin based on genomic sequence analysis [24]. The seven fragments in Fig. 1 were obtained by CNBr cleavage because of six methionine residues in the sequence. Analytical SDS-polyacrylamide gel electrophoresis showed that the CB5 fragment of the albumin variant exhibited anomalous migration in the gel (CB5* in lane 2 of Fig. 3A); it migrated more slowly, as if the variant CB5 had a higher molecular mass than normal CB5 (a polypeptide containing 117 amino acid residues). The same results of anomalous migration in CB5 fragments of albumin Naskapi and Mersin have been reported [18]. Possibly fragments of incomplete CNBr digestion and/or aggregates of fragments were also observed in the gel. The variant CB5 was separated by gel permeation HPLC under isocratic conditions using a mobile phase composed of 40% acetonitrile in 0.1% TFA at a flow-rate 1.0 ml/min (shaded area in Fig. 3B).

2.3. Tryptic digestion of the variant CB5

The separated variant CB5 was digested with TPCK–trypsin [17,20–22]. Tryptic peptides of human albumin are designated by the prefix T and are numbered consecutively in the sequence [17,20–22]. The normal CB5 has a potential yield of 18 tryptic peptides including single amino acid residues whose numbers were from T44 to T61.

2.4. Capillary electrophoresis (CE)

CE was performed on the CE-800 capillary electrophoretic system from JASCO (Hachioji, Japan). Uncoated fused-silica capillaries (50 cm total length, 30 cm effective length×50 μm I.D. for serum and 72 cm total length, and 50 cm effective length×50 μm I.D. for tryptic digest) were used. The capillary was cleaned with 0.1 M sodium phosphate (pH 2.5) for 5 min and equilibrated with the electrophoresis buffer for 5 min between each run [15]. For the separation of serum proteins, serum samples were diluted 20-fold in 20 mM sodium phosphate buffer (pH 7.0) containing 75 mM NaCl, and 100 mM sodium borate buffer (pH 10.0) was used as the electrolyte. Injection of the diluted serum into the capillary was made by the gravity method at 5 cm height for 10 s (3 nl). The tryptic digest (2 mg/ml) was introduced by electrokinetic injection at 15 kV for 20 s and was separated using a 30 mM sodium citrate buffer (pH 3.0) containing 100 mM sodium 1-hexanesulfonate. The separations were carried out at 15 kV constant voltage, and the temperature was controlled with blowers to dissipate the Joule heat [15]. Detection was at 200 nm.
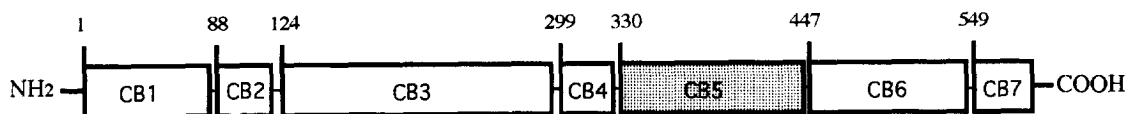

Fig. 1. Linear model of the primary structure of human albumin. The polypeptide has seven divisions of the CB fragments, numbered 1 to 7, and contains 585 amino acid residues [18,19,24]. The first residues of each CB fragment are numbered. The location of the variant fragment is indicated by a shaded box.

2.5. Micropreparation of the variant tryptic peptide by CE

A micropreparation of the variant peptide was performed according to the reported method [13]. The tryptic digest was concentrated three-fold (6 mg/ml) by lyophilization before preparation. Electrokinetic injection (90 s at 20 kV) was used, followed by a separation at 15 kV. As the variant peak was detected, the beginning- and ending-times of the peak were manually counted using a stopwatch. The time for the peptide to reach the end of the capillary at its peak-beginning was determined by the migration rate and the capillary length. The applied voltage was switched off when the peak had been migrated to the end of the capillary. A minivial, in which 10 μl of distilled water had been placed, was transferred to the outlet of the capillary, and the peptide was eluted into the minivial by applying voltage for a time span specific to each peptide peak.

2.6. Purification of the variant peptide by HPLC

The tryptic peptides of the variant CB5 were separated and purified by reversed-phase HPLC with a linear gradient of 0–60% acetonitrile in 0.1% TFA for 60 min.

2.7. Sequence analysis

Amino acid sequence determination was performed with an Applied Biosystems Model 471A protein sequencer. In order to confirm the sequence, conventional methods described previously [14,23] were also used.

3. Results and discussion

3.1. Separation of human serum proteins

The capillary electropherograms of human serum proteins are shown in Fig. 2. The components of normal adult serum proteins were clearly distinguished (Fig. 2A); γ-globulin fraction eluted first, followed by β, α2, α1, and finally albumin (γ, β, α2, α1, and Alb A in figure). Hyperalbuminemia serum showed a separation pattern almost identical to that
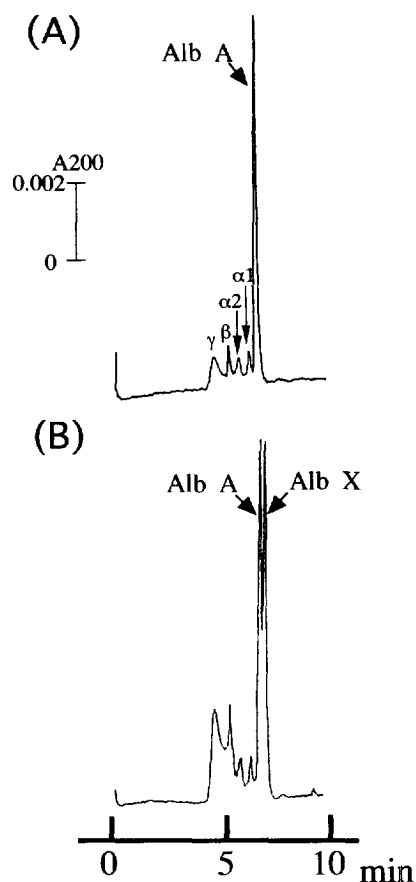
Fig. 2. Capillary electropherograms of serum from a normal volunteer (A) and a patient with hyperalbuminemia (B). The electrophoretic conditions are described in Section 2. Alb X in (B) showed an albumin variant, and Alb A in (A) and (B) was a normal albumin.

of the normal sample except for albumin (Fig. 2B). Two albumin peaks, which were in the ratio of approximately one, were detected (Alb A and Alb X in Fig. 2). The variant albumin (Alb X) migrated slower than the normal albumin (Alb A). This indicates that a net charge of the variant was more negative than that of Alb A.

3.2. Tryptic peptide mapping of the variant CB5 fragment

The tryptic peptide map of the separated variant CB5 (Alb X–CB5) is shown in Fig. 4A. More than 30 peaks were detected, although the CB5 fragment

of human serum albumin has a potential yield of 18 tryptic peptides including single amino acid residues. The number of peaks exceeds the 18 theoretical peptides, because of an impurity of the CB5 fraction which contained low amounts of CB3 and CB6 fragments (see Fig. 3B). Furthermore, it was difficult to designate and collect the variant peptide without contamination of adjacent peptides. We employed the electrophoretic buffer containing an ionic surfactant (sodium 1-hexanesulfonate) according to a previously reported method [16]. As shown in Fig. 4B, resolution was clearly improved. Three significant differences were evident on the electropherogram. A new peak appeared at the migration time of 38.5 min on the map (arrow Pv in Fig. 4B), which was absent
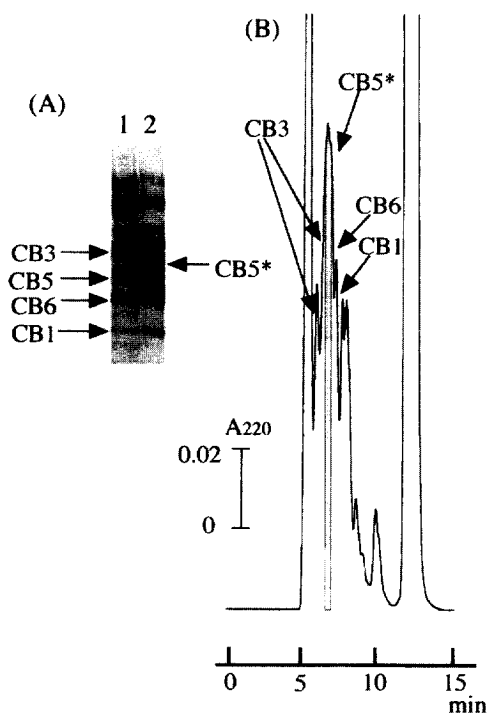
Fig. 3. SDS-polyacrylamide gel electrophoresis and separation of CNBr fragments of Alb X. The SDS-polyacrylamide gel electrophoresis patterns of CNBr fragments of Alb A (lane 1) and of Alb X (lane 2) show in (A) of the figure. The gel permeation HPLC of CNBr fragments of Alb X is shown in (B) of the figure. The variant CB5 was contained in the third peak with CB3 and CB6, and a shaded area was pooled and subjected to the following studies without further purification. An asterisk denotes the variant CB5 in (A) and (B) of the figure.
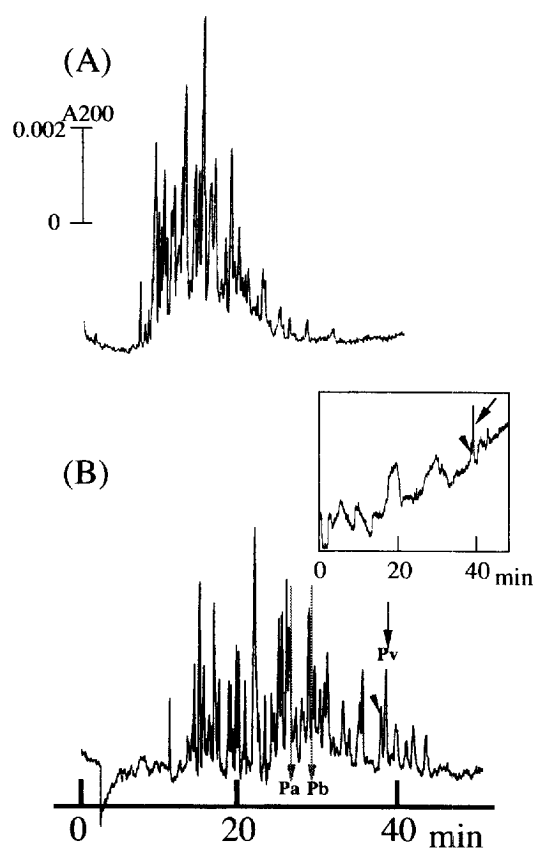
Fig. 4. CE Peptide mapping of variant CB5 fragment with and without sodium 1-hexanesulfonate. The tryptic digest of variant CB5 fragment was analyzed by CE and migrated as described in Section 2. (A) Map without sodium 1-hexanesulfonate. (B) Map from the variant CB5 with sodium 1-hexanesulfonate in electrolyte. An arrow Pv in (B) shows a variant peptide peak. Two shaded arrows, Pa and Pb, show the positions of the peptides which were absent from the map. An inset in figure shows the analysis of the variant peptide collected by CE micropreparation. The CE conditions were the same as in (B). An arrow in the inset shows a contaminated peak which has also been indicated in (B).

on the map of Alb A (not shown). In contrast, two peaks disappeared from the map of Alb X–CB5 (shaded arrows Pa and Pb in Fig. 4B). The disappearance of these peaks was closely related to the appearance of a new peak in the variant peptide map.

The resolution was effectively enhanced by sodium 1-hexanesulfonate, due to its hydrophobic interaction mechanism, as opposed to an exclusively ion-pairing mechanism [16].

3.3. Micropreparation and identification of the variant tryptic peptide

Micropreparation of the variant peptide was performed by using CE. The variant peptide was fractionated by the method described in Section 2. This procedure was repeated to obtain a sufficient amount. The collected fraction was again analyzed by CE before sequence studies. As shown in the inset of Fig. 4, the peptide peak (arrow), whose migration time was identical to that of Pv in Fig. 4B, was the major component (about 80% by area) of the fraction. The minor peak corresponded to the peak which migrated just before the new peak on the map (arrow heads in Fig. 4B and inset).

Successful micropreparation of peptides is largely dependent on high resolution and reproducibility of the CE separation. In fact, we were unsuccessful in collecting the two peaks in the tryptic Alb A-CB5, which were absent in the tryptic Alb X–CB5 (indicated in Fig. 4B), due to complex peaks. In addition, the gravity injection method might be better for quantification, although we employed the electrokinetic injection method because of its simplicity.

The collected variant peptide was subjected to sequence studies without further purification. It was difficult to estimate the first and the second residues because of many unexpected peaks. However, from the third to the fifth, PTH-alanine was detected at low yield. In spite of some ambiguous results and low recoveries, the result of the sequence analysis suggested that N-terminus of the variant peptide presumably corresponded to a Cys–Cys–Ala–Ala–Ala– of N-terminus of the tryptic peptide T49 in Alb

A, and the amino acid substitution probably occurred in T49 of Alb X (Fig. 5a).

3.4. Determination of the substitution site

The above finding was verified by sequence determination of the variant peptide purified from the tryptic peptides of Alb X–CB5 by a reversed-phase HPLC. Lys-372 of T49 was replaced by Glu-372 in the peptide (Fig. 5b). The same substitution was reported in albumins of the Naskapi type and Mersin which correspond to a single base mutation in the codon AAA to GAA [17–19].

4. Conclusion

In this report, we have identified a genetic variant of alloalbumin by CE and protein sequencing. We were unable to clarify the substitution site through direct sequencing of the peptide collected by CE. However, we believe that the proposed method is important and promising for the detection of genetic variants and internal sequence analysis of a number of proteins.

Acknowledgments

362   372   389
-Cys-Cys-Ala-Ala-Ala-Asp-Pro-His-Glu-Cys-Tyr-Ala-Lys Val-Phe-Asp-Glu-Phe-Lys-Pro-Leu-Val-Glu-Glu-Pro-Gln-Asn-Leu-Ile-Lys-
├────────────── Normal T49 ──────────────┤ ├────────────── Normal T50 ──────────────┤

                                                                    *
-Cys-Cys-Ala-Ala-Ala-Asp-Pro-His-Glu-Cys-Tyr-Ala-**Glu**-Val-Phe-Asp-Glu-Phe-Lys-Pro-Leu-Val-Glu-Glu-Pro-Gln-Asn-Leu-Ile-Lys-
                                                    Variant T49–50*

(a) ─➤─ ─➤─ ─➤─ ─➤─ ─➤─

(b) ─➤─

Fig. 5. Amino acid sequence analysis of the variant peptide. The variant peptide was identified by automated Edman degradation. The asterisk and boldface letters mark the substitution 372 Lys→Glu. The variant T49–50* identifies the new peptide generated by this substitution. The normal T49 and T50 correspond to Pa and Pb that are lacking in the tryptic peptide map of Alb X–CB5 (Fig. 4B). The arrows in (a) show sequence analysis of the peptide (Pv in Fig. 4B) obtained by CE micropreparation, and the arrows in (b) indicate sequence analysis of the variant peptide purified by the reversed-phase HPLC. Amino acid residues that were identified are indicated by small solid arrows and those that are probable are shown by shaded arrows.

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