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Short communication

Screening, purification, and identification of a copper-dependent FITC-binding protein in human plasma: Albumin

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Abstract

In this study, a protein purified by fluorescein isothiocyanate (FITC)-affinity chromatography from human plasma was identified as albumin by MALDI-TOF-MS. Albumin was found to conjugate with FITC-labeled molecules through a copper-dependent reaction. The formation of this complex was confirmed by methods including a newly developed "charcoal-based fluorescence assay" (CFA), gel-filtration, affinity chromatography, and ultrafiltration. The binding was identified as disulfide bridge formation. This is the first to demonstrate that copper induces a covalent binding of FITC-labeled molecules with albumin. In addition, the developed CFA method facilitates the screening of small fluorescent dyes binding to macromolecules.

Keywords: Albumin; FITC; Copper; Affinity; Purification

1. Introduction

Albumin is a major carrier protein with multiple functions in circulation. Binding of endogenous and exogenous ligands by albumin renders potential toxins such as metabolites and drugs harmless and transports them to disposal sites. Most of the metabolites and drugs are bound to Sudlow's site-I or site-II of albumin, with some exceptions [1,2]. Covalent interactions with albumin including glycosylation [3], acylation [4], thiolation [1,5] and S-nitrosation [6,7] are also found. Human serum albumin (HSA) is a 67 KD single chain protein with 17 S–S bonded cystines and one free thiol group at Cys-34 residue. The free thiol at Cys-34 is known to participate in numerous redox reactions. Low molecular weight thiol-containing molecules including cysteine, homocysteine, and glutathione were known to interact with albumin at Cys-34 via disulfide bridge formation [1,2]. Metal ions, such as copper, nickel, cobalt, and zinc, etc., are also known to be carried by albumin [1,2].

In our previous study, a method termed "charcoal-based fluorescence assay" (CFA) was used to analyze transglutaminase enzyme activity [8]. The enzyme is calcium-dependent and incorporates lysyl substrates, such as polyamines, into glutamyl substrates, such as casein [9]. Fluorescence-labeled polyamines such as fluorescein-5-isothiocyanate cadaverine (FITC-cad) and dansyl-cadaverine (dansyl-cad) are lysyl substrates of the enzyme [10]. CFA could rapidly analyze the incorporation of small fluorescent dyes into large protein molecules. Factor XIIIa, one type of the transglutaminases, is presented in blood plasma and activated by thrombin cleavage [11]. It was a surprise to find that in the presence of copper instead of calcium the "unactivated" plasma sample produced an intensive fluorescence signal when FITC-cad, but not dansyl-cad, was used as the lysyl substrate in the reaction. The intensive fluorescence signal was speculated to come from the binding of FITC-cad molecules

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with some unidentified plasma proteins. In this study, this plasma protein was purified and identified as HSA. The importance and application of this finding is discussed.

2. Experimental

2.1. Reagents and equipments

Human plasma was provided by Taiwan Blood Services Foundation (3F., No. 3, Nanhai Road, Jhongjheng District, Taipei 100, Taiwan). Human serum albumin (HSA; item number A3782, essentially fatty acid free, about 99% in purity), fluorescein isothiocyanate (FITC), and dansylcadaverine (dansyl-cad), were from Sigma–Aldrich (St. Louis, MO, USA). Fluorescein-5-isothiocyanate cadaverine (FITC-cad) was from AnaSpec, Inc. (San Jose, CA, USA). BioMag magnetic dextran-coated charcoal (MD-charcoal) concentrate (40 mg/ml) was purchased from Bangs Laboratories, Inc. (Fishers, IN, USA). MD-charcoal concentrate was diluted with water (1:4) and stored at 4 °C for subsequent application. Other chemicals used were analytical grade from either Sigma–Aldrich, or Merck (Darmstadt, Germany), unless otherwise stated.

HiPrep 16/60 Sephacryl S-200 high resolution column, gel-filtration calibration kit, and matrix of EAH Sepharose 4B were from Amersham Biosciences AB (Uppsala, Sweden). Microcon centrifugal filter unit (Microcon YM-30) was from Millipore (Bedford, MA, USA). Centrifuge 5415D was from Eppendorf AG (Hamburger, Germany). V96 microwell plate (#249945) was from Nalge Nunc International (Rochester, NY, USA). Water used in this study was prepared by Milli-Q system (Millipore, Bedford, MA, USA).

2.2. The binding of FITC-cad to albumin

A high-throughput charcoal-based fluorescence assay (CFA) modified from our previous publication was used to analyze the binding capacity of albumin for FITC-cad [8]. Typically, 30 μl per well of protein samples dissolved in TES buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 0.15 M NaCl) were added in microplate at 4 °C. After 30 μl reagent-A (100 mM Tris–HCl, pH 7.5, 10 mM $CuCl_2$, and 6 μM FITC-cad) was added into each well, the reaction was initiated by incubating the microplate in a 37 °C humidified incubator for 10 min, and terminated by adding 60 μl reagent-B (0.5 M Tris–HCl, pH 9.0, and 20 mM EDTA, 4 °C). To remove all of the free FITC-cad, 200 μl per well of suspended MD-charcoal was added, stand for 5 min at room temperature (25 °C), and finally the MD-charcoal was precipitated by magnet for 1 min. The fluorescent intensity was measured by microplate reader (Plate Chameleon, Hidex Oy, Finland) within an hour, with excitation wavelength setting at 485 nm and emission wavelength at 535 nm (Ex485/Em535), gain 35. Transfer of supernatant to another plate for fluorescence measurement is unnecessary because FITC-cad captured by the precipitated MD-charcoal does not exhibit fluorescent properties. Background levels of reactions were measured by replacing samples with TES buffer. The binding capacity of albumin for FITC-cad was expressed as net fluorescence per reaction in this study.

2.3. Preparation of FITC-affinity beads

FITC-affinity beads were prepared by coupling FITC molecules with EAH Sepharose 4B matrix. In detail, 40 μl of FITC stock solution (5 mg/ml in DMSO) supplemented with 1.96 ml of coupling buffer (0.1 M $NaHCO_3$, pH 9.0) was mixed with 1 ml of the drained matrix and incubated at 25 °C in dark for 2 h with constant shaking. The resulting matrix was washed with 1 M NaCl and TES buffer, and finally stored at 4 °C for later use.

2.4. Purification of copper-dependent FITC-cad binding protein from human plasma

Human plasma (79 mg/ml) was passed through 0.45 μm filters and stored at 4 °C before use. After a 10-fold dilution with TES buffer, 1 ml of the diluted plasma was applied to a Sephacryl S-200 column which was equilibrated with TES buffer before use. The fractionation process was performed at 4 °C with a flow rate of 0.5 ml/min, and the protein fractions were monitored by UV absorbance at 280 nm. The eluate of each fraction was analyzed by CFA for FITC-cad binding as described above.

Affinity purification was performed by mixing 0.25 ml of the protein sample (0.36 mg/ml) from gel-filtration chromatography (fractions 49–51) with 0.1 ml of drained matrix, supplemented with 5 mM $CuCl_2$. After incubating at 25 °C for an hour with shaking, the matrix was washed with 100 ml TES buffer at 4 °C, the beads were then treated with 0.2 ml SDS sample buffer (58.3 mM Tris, pH 6.8, 6% glycerol, 0.002% bromophenol blue, 2% SDS, and 1% 2-mercaptoethanol) at 95 °C for 15 min. The supernatant was collected for electrophoresis, and proteins were visualized by using GelCode E-zinc reversible stain kit (Pierce Biotechnology, Rockford, IL, USA).

2.5. The identification of FITC-affinity-purified protein

2.5.1. In-gel tryptic digestion

Designated protein band was excised from gel, and subjected to in-gel tryptic digestion procedures as described below. The dried gel piece in the vial was rehydrated by 30 μl of 100 mM dithiothreitol (DTT) (in 25 mM ammonium bicarbonate) for reduction and 55 mM iodoacetamide (in 25 mM ammonium bicarbonate) for alkylation sequentially. Then, 200 μl of 100% acetonitrile was then added to tube and incubated for 5 min at ambient temperature. The liquid was discarded; gel slice was washed twice and shrunk by the addition of acetonitrile. The dried gel pieces were incubated with trypsin (20 ng/ml in 25 mM ammonium bicarbonate; Promega, Madison, WI, USA) and digested overnight at 37 °C. Peptides were further desalted and concentrated with C18 spin column (Pierce).

2.5.2. Protein identification by matrix-assisted laser desorption/ionization time of flight mass spectrometer (MALDI-TOF-MS)

Peptides were mixed with α-cyano-4-hydroxycinnamic acid (10 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid) and spotted on to a MALDI target plate. MALDI-TOF and MALDI-TOF/TOF MS analysis were performed on 4700 Proteomics Analyzer (Applied Biosystems, Foster, CA, USA), and data were acquired in positive ion reflector mode. For MS analysis, mass spectra were obtained from 1000 accumulated shots in 800–4000 Da mass range. Ten best S/N peaks were selected for MS/MS analysis, air was used for collision gas and collision energy of 1 kV was applied. All the MS and MS/MS spectra were interpreted by GPS Explorer software (version 3.5, Applied Biosystems), which incorporated with a MASCOT search engine (Matrix Science, version 2.0.04) that was used for protein identification. Peptide mass fingerprinting method was used and data were search against NCBI (8600 Rockville Pike, Bethesda, MD) nonredundant database. Trypsin was chosen as the cleavage agent, one missing cleavage was allowed, cysteine carbamidomethylation and methionine oxidation were selected as variable modifications. Precursor ion and fragment ion error tolerances were set to 150 ppm and 0.2 Da, respectively.

2.6. Gel-filtration analysis for HSA–(FITC-cad) complex formation

An aliquote of 250 μl of HSA (4 mg/ml in TES buffer) was reacted with 250 μl of reagent-A described above, with or without copper; the reaction was terminated by adding 500 μl of reagent-B. The mixtures (1 ml) were then applied to a Sephacryl S-200 column with a flow rate of 0.5 ml/min at 4 °C. The formation of HSA–(FITC-cad) complex was monitored by measuring the fluorescence intensity of a 0.2 ml aliquote of each eluate fraction corresponding to the molecular weight of HSA determined by gel-filtration chromatography.

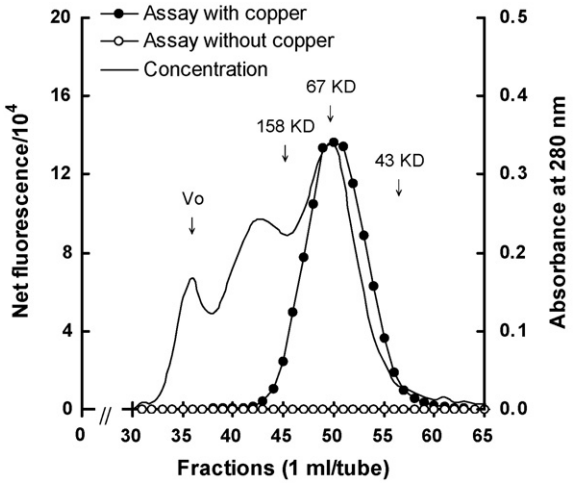

Fig. 1. Screening of the FITC-cad binding protein from gel-filtration chromatography. Human plasma proteins fractionated on a Sephacryl S-200 column were analyzed for FITC-cad binding ability in the presence or absence of copper, by using CFA method as described in Section 2. Protein fractions were monitored by UV absorbance at 280 nm, and molecular weights of standards were also indicated.

2.7. The stability of the interaction between HSA and FITC-cad

The HSA–(FITC-cad) complex was produced by reacting 30 μg of HSA with FITC-cad as described in "The binding of FITC-cad to albumin." After MD-charcoal precipitation, aliquots of 200 μl supernatant per well were collected and treated with 0.5 M NaCl, 0.1% sodium dodecyl sulfate (SDS), 1.2 M guanidine hydrochloride (Gu-HCl), or 10 mM DTT, at 25 °C for 30 min. The resulting mixtures were applied to Microcon YM-30 tubes and centrifuged at 12k × *g* for 10 min (Eppendorf 5415 D) to remove the released fluorescent dye. The resulting protein fractions were finally dissolved in 200 μl TES buffer, and adjusted to pH 9.0 with 1 M Tris buffer for fluorescence quantification.

3. Results and discussion

3.1. The purification and identification of a copper-dependent FITC-cad binding protein from human plasma

Human plasma was fractionated by Sephacryl S-200 column and analyzed by CFA as shown in Fig. 1. The intensive fluorescence signals were presented in the protein fractions with molecular weight of approximately 67 KD when copper was included in the reaction. By comparing dansyl-cad and FITC-cad in the interaction with gel-filtration purified protein samples

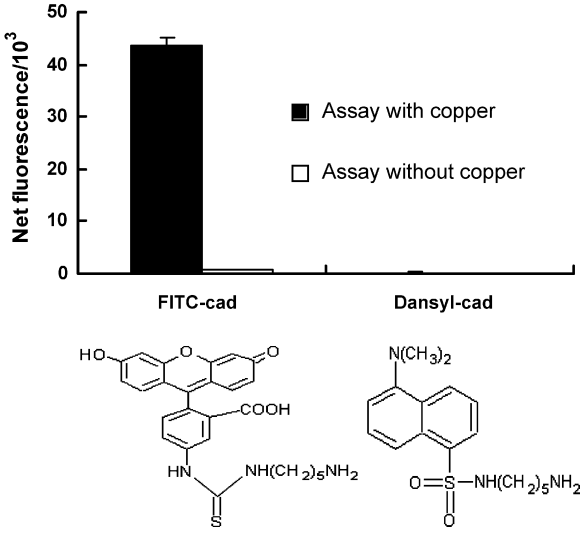

Fig. 2. Identification of essential interacting group of FITC-cad for complex formation. Protein fractions 49–51 from sephacryl S-200 gel-filtration (Fig. 1) were pooled for binding assay. Reaction mixtures containing protein samples and 3 μM FITC-cad (or dansyl-cad), 50 mM Tris, pH 7.5, with/without 5 mM $CuCl_2$ were incubate at 37 °C for 10 min and analyzed by CFA as described in Section 2. The fluorescence intensities were measured at Ex485/Em535, gain 35, for FITC-cad, and Ex340/Em535, gain 35, for dansyl-cad. Data presented are means ± S.D. ($n = 3$). The chemical structures of ligands were also illustrated.

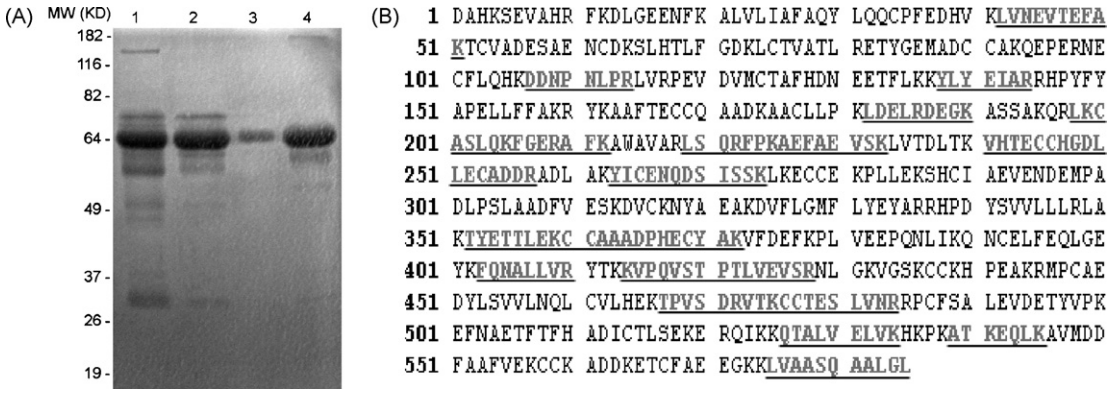

Fig. 3. Purification and identification of the FITC-binding protein. (A) Protein sample (pooled fractions 49–51) from gel-filtration chromatography (Fig. 1) was applied to FITC-affinity chromatography, and the purified protein was then analysis by gel-electrophoresis on a 12% SDS poly-acrylamide gel. Lane 1, human plasma, 5.2 μg; lane 2, gel-filtration column-purified proteins (fractions 49–51), 5.2 μg; lane 3, FITC-affinity purified protein; lane 4, commercial available HSA, 5.2 μg and (B) FITC-affinity purified protein was further identified. The single protein band from lane 3 of SDS-PAGE gel was excised for protein identification by MALDI-TOF-MS analysis. The peptides of the purified protein matched with 31% of total sequence coverage, to those of HSA are shown in bold gray and underlined.

using CFA method in the presence or absence of copper, the interaction site of FITC-cad was identified (Fig. 2). It was located at FITC itself while cadaverine was not essential for the binding. This protein was finally purified by FITC-affinity chromatography and demonstrated as a single band on electrophoresis gel (Fig. 3A). This single band of protein was then excised from gel and identified as albumin by peptide mass fingerprinting method as described in Section 2.5.2 (Fig. 3B).

3.2. The confirmation of the copper-induced HSA–(FITC-cad) complex

Commercial available HSA reacted with FITC-cad, with or without copper, was applied to gel-filtration to confirm the formation of HSA–(FITC-cad) complex. Only in the copper-supplemented reaction, the resulting HSA was eluted with an intensive fluorescence signal (Fig. 4). The result confirmed the existence of HSA–(FITC-cad) complex analyzed by CFA as shown in Figs. 1 and 2.

3.3. HSA binding with FITC-cad through a disulfide bridge

As shown in Fig. 5, the fluorescence signal was completely abolished under DTT treatment, indicating the linkage between HSA and FITC-cad is disulfide bond. The reduced fluorescence intensity by Gu-HCl treatment might also result from the disruption of the disulfide bond. There is only one free thiol at Cys-34 residue of HSA [1,2], thus, we speculated that it is the thiourea group of FITC-cad interacted with Cys-34 to form the complex through disulfide bridge.

3.4. Perspectives

Copper is essential for the binding of FITC-cad to HSA. Besides binding to the N-terminal region of albumin, copper also interacts with Cys-34 of HSA [7,12]. It is difficult to clearly identify the role of copper in the reaction at the time being. On the other hand, thiourea was known to interact with copper to form thiourea–copper complex [13], implying that the thiourea group in FITC-cad molecule may also interact with copper. Further studies are necessary to substantiate this speculation. Meanwhile, there are lots of drugs containing thiourea functional groups: e.g., thiourea-based gemfibrozil analogues as HDL-elevating agents [14]; pyridyl thiourea derivatives as non-nucleoside inhibitors of HIV reverse transcriptase [15]. There is a potential possibility that copper may induce the binding of albumin with thiourea-based drugs as observed in this study. The reaction mechanism remains to be further investigated.

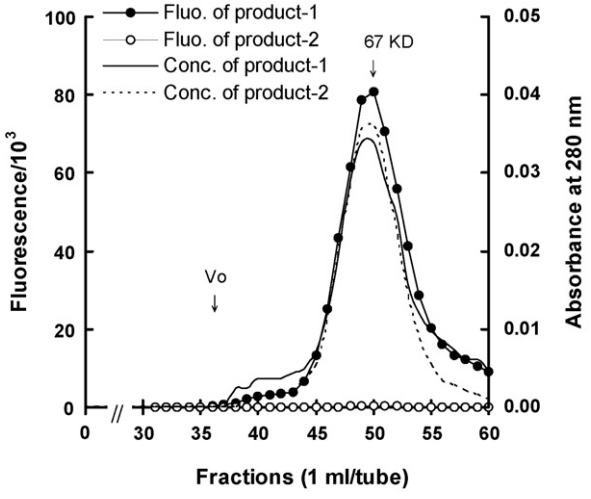

Fig. 4. Confirmation of the copper-induced complex formation. Commercial available HSA was used to confirm the HSA–(FITC-cad) complex formation by gel-filtration analysis. The resulting products from the reaction of HSA and FITC-cad, performed in the presence (product-1) or absence (product-2) of copper, were applied to a Sephacryl S-200 column, and the fluorescence (Fluo.) intensity of each fraction was measured at Ex485/Em535, gain 35. Protein concentrations (Conc.) were monitored by UV absorbance at 280 nm, and the molecular weight of albumin was also indicated.

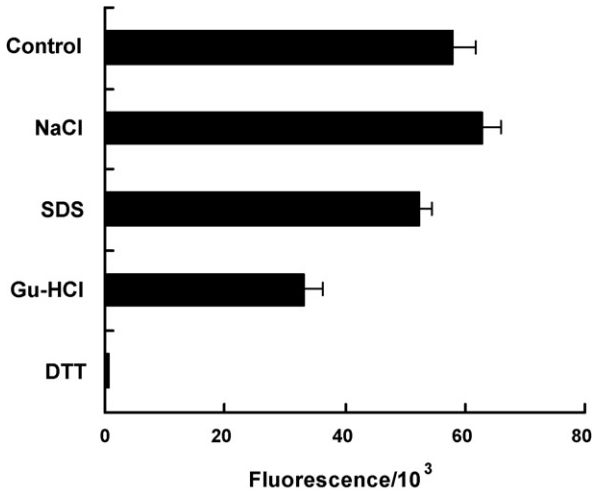

Fig. 5. Stability of HSA–(FITC-cad) complexes. HSA–(FITC-cad) complexes were treated with various reagents (0.5 M NaCl, 0.1% SDS, 1.2 M Gu-HCl, or 10 mM DTT) and then ultrafiltrated to remove the released fluorescent dyes. The remaining fluorescence intensities of the protein fractions were measured at Ex485/Em535, gain 35. Data represent means ± S.D. ($n = 3$).

4. Conclusion

This is the first to demonstrate that copper induces a rapid conjugation of FITC-labeled molecules with albumin via disulfide bridge formation. The assess of this study concludes: (i) FITC-affinity purification of albumin from human plasma is applicable; (ii) FITC-cad could be used as a fluorescent marker for detecting albumin in human plasma; (iii) FITC-labeled molecules could be reversibly anchored on albumin; and finally (iv) the "CFA" method could rapidly analyze the binding of small fluorescent dyes to large protein molecules as previously described, thus accelerating the binding assay in biochemical and pharmacokinetic analysis.

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