New Method for Identifying Genetic Variants of Human Proalbumin

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We describe a simple, rapid procedure for identifying the proalbumin nature of certain genetic variants of human albumin. Because both albumin and its precursor, proalbumin, exhibit multiple binding sites for Cu(I), we investigated the conditions for selective linkage of metal ions to the high-affinity N-terminal binding site of albumin. Guided by these data, we then developed a sensitive color test for detection of Cu(I)-albumin complexes after cellulose acetate electrophoresis of serum samples previously incubated with Cu(II). Use of dithiooxamide in ethanol–acetic acid solution allows protein fixation and copper staining in a single step. The simplicity of the procedure, together with the use of non-radioactive metal ions, makes it very useful for rapidly identifying proalbumin variants.

Although current methodologies allow one to examine the variability of proteins by studies at the genomic level, data concerning the polymorphism of human serum albumin have mostly been provided by studies carried out at the protein level. Indeed, analysis of restriction fragment length has evidenced mutations arising outside of coding regions (1, 2), and nucleotide sequencing of human albumin mRNA has been performed on only three different individuals (3–5). In contrast, more than 25 variants of human serum albumin have been detected by virtue of their abnormal electrophoretic mobilities (6, 7). Mutations responsible for these allotypes have been identified in only a few cases, each corresponding to substitution or deletion of a single nucleotide in the region coding for the C-terminal part of the molecule (8–12). Moreover, it is now established that mutations can affect not only the albumin molecule, but also the additional N-terminal hexapeptide, Arg-Gly-Val-Phe-Arg-Arg, possessed by, and which characterizes, its precursor, proalbumin (13). Two genetic variants, proalbumin Christchurch (14) and proalbumin Lille (15), have been reported, each resulting from a substitution at one of the two arginy1 residues at the dibasic site at which the normal propeptide is cleaved. Both of these mutations prevent excision of this basic propeptide and thus each of these proalbumin variants has a slower electrophoretic mobility than that of normal albumin.

Our data on the high frequency of proalbumin within albumin genetic variants (observed in the French population) show the need for precise identification of proalbumin variants (16). Up to now, their detection rested on three distinctive features of proalbumin molecules: their susceptibility to limited tryptic digestion, their inability to bind (radiolabeled) nickel, and their N-terminal amino acid sequence. The first is difficult to adjust, the second requires use of radioactive isotopes, and the third necessitates isolation of the proalbumin variant in high purity before its N-terminal amino acid sequence is determined.

Here we present a new method for the simple identification of proalbumin variants. It is based on a color test for detection of copper–albumin complexes, which is performed after cellulose acetate electrophoresis. Several regions of the albumin molecule participate in the transport of cupric ions. A first-class binding site, specific for Cu(I) and Ni(II) with high affinities, involves the first three amino acid residues. Several second-class binding sites, with lower affinity and specificity, are located on histidyl residues. When the first is blocked in the proalbumin molecule, the second ones still are available in both albumin and proalbumin. Owing to the existence of these second-class binding sites for Cu(I) or Ni(II), which are located beyond the N-terminal sequence, we had to strictly define the ratio Cu(I)/total albumin that allows saturation of albumin without labeling of proalbumin.
**Materials and Methods**

**Apparatus.** For serum protein electrophoresis, we used 140 × 57 mm gelatinized cellulose acetate membranes (Cellogel; Chemetron, Milano, Italy). To concentrate samples we used a Minicon B15 concentrator (Amicon Corp., Danvers, MA).

**Reagents.** Copper chloride and dithiooxamide were from Prolabo, Rhône-Poulenc, Paris, France. Proteins were stained with Amido Black 10B (Bender & Hobein, Munich, F.R.G.).

**Serum samples.** Human sera with heterozygous alloalbuminemia were provided by systematic screening of blood donors. We incubated 100 μL of each serum sample with, respectively, 6, 12, 18, and 24 μL of a 5 mmol/L CuCl₂ solution, providing a molar ratio for Cu(II)/albumin of 0.5, 1, 1.5, and 2. These mixtures were then concentrated to half their volume.

**Electrophoresis on cellulose acetate.** For this we used a pH 8.6 buffer containing, per liter, 7.5 mmol of barbital and 4 mmol of sodium barbital. Because our reaction is based on the reactivity of Cu(II) with albumin, which exhibits various binding capacities for various divalent cations, the buffer used should be devoid of such ions and, obviously, of chelators such as EDTA. For the semi-micro method we used, quadruplicate samples were electrophoresed for 60 min at 220 V.

**Copper staining.** Immediately after electrophoresis, the cellulose acetate membranes were immersed for 5 min in a 2 g/L solution of dithiooxamide in ethanol–acetic acid (95/5 by vol) for detection of albumin-bound Cu(II).

**Results**

**Conditions for selective staining of albumin-bound Cu(II).** As shown in Table 1, when the molar ratio Cu(II)/total albumin was 0.5, the first-class N-terminal site of albumin bound Cu(II), whereas proalbumin, owing to the blockage of this site, was devoid of metal ions. In contrast, when the ratio was higher, proalbumin linked Cu(II) by its second-class binding site, which is located in the albumin sequence of the molecule, and thus both albumin and proalbumin were detected by copper staining. For this reason, we selected a Cu(II)/total albumin molar ratio of 0.5 for standardizing the procedure.

**Sensitivity of staining of albumin-bound copper.** Because the proportion of Cu(II) to serum was so low, we assessed the sensitivity of the detection by staining albumin–copper complexes with the dithiooxamide reagent. Increasing amounts from 3 to 20 μL of the 5 mmol/L CuCl₂ solution were added to a constant volume (50 μL) of serum samples and 1-μL volumes of the mixtures were deposited onto a cellulose acetate membrane with an automatic applicator. After the samples had penetrated the membrane, it was immersed for 5 min in the dithiooxamide solution. The lowest amount of copper–albumin complex so detected corresponded to about 1 nmol of Cu(II).

The automatic applicator deposited 1 μL of sample, and human serum contains 0.6 nmol of albumin per microliter, so a convenient detection after electrophoresis on cellulose acetate was obtained with doubly concentrated sera, of which 4 μL was deposited by four times repeated application of samples.

**Identification of human proalbumin variants.** We thus analyzed several sera from blood donors who evidenced heterozygous alloalbuminemia or proalbuminemia. Figure 1 shows protein staining and copper staining of albumin variants Gent and B, and of proalbumin variants Christchurch and Lille. Both albumin and proalbumin were stained with Amido Black (Figure 1A). Albumin fractions that have bound Cu(II) are revealed by coloration with dithiooxamide (Figure 1B), whereas proalbumin fractions devoid of Cu(II) are unstained.

**Discussion**

Evidently, circulating proalbumins resulting from mutation affecting the propeptide sequence can be identified by a simple color test, performed after electrophoresis of sera on cellulose acetate. Our method is based on the reactivity of nonradioactive copper with albumin and proalbumin, respectively. One of the criteria by which proalbumin is distinguished is the nature of the N-terminal end of the albumin molecule. It has been established that the transport site for Cu(II) and Ni(II) on human serum albumin consists of a penta-coordinate structure involving the first three amino acid residues, Asp-Ala-His (17). The presence in proalbumins of an additional N-terminal hexapeptide results in the blockage of their high-affinity binding site. Furthermore, albumin exhibits other sites, with lower affinities for Cu or Ni, that are located on some histidyl residues of the albumin sequence (18), and these are available on proalbumin molecules for binding metal ions. Knowledge of these second-class binding sites prompted us to perform experiments based on the affinity of Cu(II) for proalbumin, using a Cu(II)/total albumin ratio <0.5.

Our previous works (19, 20) on hemocyanin, a protein that contains two copper atoms per monomeric subunit of

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**Table 1. Binding of Cu(II) on Albumin and Proalbumin**

<table>
<thead>
<tr>
<th>Molar ratio, Cu(II)/total albumin</th>
<th>Binding of Cu(II) Fraction</th>
<th>1st-class site</th>
<th>2nd-class site</th>
</tr>
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<tbody>
<tr>
<td>0.5</td>
<td>Albumin</td>
<td>+</td>
<td>-</td>
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<tr>
<td></td>
<td>Proalbumin</td>
<td>/</td>
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</tr>
<tr>
<td>1</td>
<td>Albumin</td>
<td>+</td>
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<td></td>
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<tr>
<td>1.5</td>
<td>Albumin</td>
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<tr>
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<td>Albumin</td>
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<tr>
<td></td>
<td>Proalbumin</td>
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*Slash indicates the unavailability of first-class binding site on proalbumin.

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**Fig. 1. Cellulose acetate electrophoresis of serum samples containing heterozygous alloalbumin or proalbumin**

A: Protein stained with Amido Black. B: Copper stained with dithiooxamide. 1. albumin Gent (fast-moving variant); 2. albumin A (slow-moving variant); 3. proalbumin Lille; 4. proalbumin Christchurch

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5 000 Da, made us familiar with reagents for detection of upper-protein complexes. But the necessity for relatively small amounts of copper chloride to be incubated with proalbumin also necessitated use of a highly sensitive reagent for detection of Cu(II)-protein complexes. A screening of copper agents performed for detection of hemocyanin evidence that dithiothreitol was very sensitive, specific for cupric ions, and usable in ethanol-acetic acid solution. This last feature allowed the fixation of protein and the detection of protein-bound Cu(II) in a single step, immediately after the electrophoresis.

Previously described methods for detection of proalbumins are not easy to perform. Limited tryptic digestion (21), which converts proalbumin into albumin, must be carried out with various amounts of enzyme, because the antiprose activity varies among serum samples. Labeling of albumin with 52Ni (22) requires handling of radioactive isotopes. And amino acid sequence determination is only possible in a few laboratories.

The simple procedure described here allows a rapid, reliable identification of proalbumin variants. It was used to study the 30 cases of genetic variants of human albumin that had been detected in the laboratory during the past 20 years, and it confirmed the proalbumin nature of nine alotypes suspected by the determination of their relative electrophoretic mobilities (23). Since two genetic variants, previously described as albumin Gainesville and albumin 'ollibauer, were shown to be respectively identical with proalbumin Christchurch (24) and proalbumin Lille (25), any variant with slow electrophoretic mobility must be examined by the present technique if its amino acid substitution has not been determined.

As was done for hemoglobin, a clear classification of enetic variants of human albumin must be established. The CSM family (Italian Committee for Standardization in Hematology and Clinical Biology) Albumin Study Group, in which we participate, is working in this direction with regard to the variants of European origin.

References


