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Structural changes and metal binding by proalbumins and other amino-terminal genetic variants of human serum albumin

(alloalbuminemia/bisalbuminemia/genetic polymorphism/copper-binding/Wilson disease)

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ABSTRACT **Proalbumins are rare genetic variants of human serum albumin containing a basic propeptide that is not removed during post-transcriptional processing because of a mutation in the site of excision, an Arg-Arg sequence. We have identified the amino acid substitutions in three different types of proalbumins designated Gainesville, Taipei, and Takefu. The first two proalbumins are identical to previously described proalbumins of the Christchurch and Lille types, respectively, and exhibit the characteristic properties of susceptibility to tryptic cleavage and of lower metal-binding affinity. Takefu is a third type of proalbumin and resists tryptic cleavage because of the substitution $Arg^{-1} \rightarrow$ Pro. Each of the first two types of proalbumins has been identified in geographically separate, ethnically diverse populations and therefore must have arisen by independent mutations. There is some tendency for mutations in albumin to cluster in the propeptide sequence. Although the substitution $His^{3} \rightarrow$ Gln in the genetic variant albumin Nagasaki-3 decreases metal-binding affinity, mutations further down the polypeptide chain have no such effect, nor is there any reduction of copper-binding affinity in albumin from patients with Wilson disease.**

Proalbumin, a serum albumin molecule that retains a basic amino-terminal hexapeptide because of incomplete post-transcriptional processing, does not occur normally in detectable amounts; however, a proalbumin may represent half of the circulating albumin in rare heterozygous individuals who have an inherited mutation in the propeptide sequence (1–4) and all of the albumin in extremely rare homozygotes (5). During cellular processing a cathepsin B-like enzyme removes the normal propeptide Arg-Gly-Val-Phe-Arg-Arg by cleaving the peptide bond to the amino-terminal aspartic acid of mature human albumin (6, 7). The genetic variant designated proalbumin Christchurch begins with the sequence Arg-Gly-Val-Phe-Arg-*Gln*-Asp-, in which the substitution $Arg^{-1} \rightarrow$ Gln prevents proteolytic removal of the mutated propeptide (1). A second genetic variant designated proalbumin Lille (2) has the amino-terminal sequence Arg-Gly-Val-Phe-*His*-Arg-Asp-. Proalbumins Pollibauer (4) and Tokushima (5) are identical to proalbumin Lille; the former is found in a few unrelated individuals of French and German origin, and proalbumin Tokushima is present in both heterozygous and homozygous conditions in a Japanese family (5). All of these proalbumins are inherited and have about a 50:50 distribution in heterozygous individuals. *In vivo* the propeptide is subject to limited cleavage by trypsin or cathepsin B (1–8). Proalbuminemia is asymptomatic. The only known functional difference is a decreased affinity for binding copper, as measured by radioautography of the binding of $^{63}Ni^{2+}$ (3–5, 9).

In a continuing structural study of genetic variants of albumin we have established that albumin Gainesville, discovered in a family of Irish descent in Gainesville, FL, by Lau *et al.* (10) is identical in structure and in related properties to proalbumin Christchurch from New Zealand (1). We have identified a variant (proalbumin Taipei) that is identical to the French proalbumin Lille and is present in heterozygous condition in an individual of Chinese ancestry living in Taiwan. We also report a type of proalbumin variant from Japan described and designated albumin Takefu by Nagata *et al.* (11). This has the propeptide Arg-Gly-Val-Phe-Arg-*Pro*- and differs from proalbumins Christchurch and Lille with respect to metal-binding affinity and susceptibility to limited proteolytic cleavage. Proalbumins Takefu and Christchurch represent a rare situation in that the same position is substituted differently for these two alloalbumins. The apparent clustering of mutations in the propeptide sequence in unrelated and genetically diverse individuals suggests that this segment may represent a hypermutable site or genetic "hot spot."

MATERIALS AND METHODS

Sera from individuals with an albumin genetic variant were obtained as follows: proalbumin Gainesville (10) from T. Lau (Gainesville, FL); proalbumins Y (serum 3433), Lille, and Pollibauer (3) from J. M. Fine (Paris); proalbumins Lille and Pollibauer (4) from M. Galliano (Pavia, Italy); proalbumin Takefu (11) from S. Migita (Kanazawa, Japan); proalbumin Taipei from C. Y. Chuang (Taipei, Taiwan); albumin Nagasaki-3 from C. Satoh (Hiroshima); albumin Naskapi (12, 13) from B. S. Blumberg (Philadelphia); albumin B (14) from L. Weitkamp (Rochester, NY). All individuals were heterozygous for the trait except for one homozygous Naskapi donor (12, 13). Purification of the albumin from serum samples was described by Takahashi *et al.* (12). S. O. Brennan (Christchurch, New Zealand) kindly provided albumin A and proalbumin Christchurch that had been purified from the serum of a heterozygous donor (the propositus) (1). As a reference standard for normal albumin A we used commercial human albumin prepared from pooled plasma (lot 102578, Calbiochem–Behring).

Previously described methods used for the characterization of the albumin variants and for structural study include the following: (*i*) $NaDodSO_4$/PAGE of the carboxymethylated albumins and CNBr digests (13), (*ii*) automated tandem HPLC mapping of tryptic peptides (12), (*iii*) HPLC mapping and isoelectric focusing of CNBr peptides (13), and (*iv*) amino acid-sequence analysis of the intact purified albumin and of tryptic and CNBr peptides with the Beckman sequencer model 890C (12), radioautography with $^{63}Ni^{2+}$ (8).

RESULTS AND DISCUSSION

Proalbumin Gainesville. Albumin Gainesville was originally reported as a slow variant in clinical electrophoresis at pH 8.6 (10). We confirmed this in a recent serum specimen and

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showed that the variant and normal albumin A are present in about equal amounts (Fig. 1). In a survey by starch gel electrophoresis at pH values of 5.0, 6.0, and 6.9 of 23 types of genetic variants from throughout the world, Weitkamp *et al.* (14) classified albumin Gainesville as the same type as the French albumin Y (serum 3433) of Fine *et al.* (3). Subsequently Brennan and Carrell (1) identified proalbumin Christchurch and established the structure of the variant propeptide as Arg-Gly-Val-Phe-Arg-*Gln* (Table 1). Fine *et al.* (3) later reported that the same propeptide was present in albumin Y, which they called Gainesville because of the prior electrophoretic typing (14). However, their study was done on the French proalbumin Y rather than on the original albumin Gainesville, which is found in a family of Irish ancestry living near Gainesville, FL. There are many more possible structural variations than there are distinguishable electrophoretic types. We therefore undertook the structural study of albumin Gainesville to test the validity of the electrophoretic typing and to ascertain whether the same structural change may occur independently in unrelated families and ethnic groups.

For the initial study the purified albumin from a heterozygous donor was submitted to amino-terminal sequence analysis for 35 cycles without separation of the variant. A predominant sequence identical to that of albumin A was obtained together with a background sequence similar to that of a proalbumin. Further structural study was done with a second serum specimen. Isoelectric focusing (Fig. 2) localized the substitution to the amino-terminal CNBr fragment (Fig. 3). The Gainesville variant albumin was then separated from the normal albumin A by use of HPLC (13). Automated sequence analysis of purified albumin Gainesville for 20 cycles gave an unambiguous propeptide sequence of Arg-Gly-Val-Phe-Arg-*Gln*- followed by the amino-terminal sequence of albumin A. This hexapeptide sequence is identical to that of proalbumins Christchurch and Y (Table 1). We concluded that the heterozygous specimen first analyzed in the sequencer was mainly a mixture of normal albumin and a degraded proalbumin in which the pentapeptide had been removed by proteolytic cleavage leaving an amino-terminal residue of glutamine that readily cyclizes and therefore is blocked in the Edman reaction in the sequencer.

Like albumin Gainesville, all previously reported proalbumins are somewhat unstable—probably because of proteolytic cleavage of the propeptide by one of the many serine proteases in serum (1–5). We found that most of the proalbumins we had been given as reference standards were

Table 1. Human proalbumin genetic variants

Proalbumin	Propeptide sequence	Cleavage by trypsin	Ref.
	−6 −1 +1		
A (normal)*	Arg–Gly–Val–Phe–Arg–Arg–Asp–	Yes	6
Christchurch	Arg–Gly–Val–Phe–Arg–*Gln*–Asp–	Yes	1
Gainesville	Arg–Gly–Val–Phe–Arg–*Gln*–Asp–	Yes	
Y	Arg–Gly–Val–Phe–Arg–*Gln*–Asp–	Yes	3
Takefu	Arg–Gly–Val–Phe–Arg–*Pro*–Asp–	No	
Lille	Arg–Gly–Val–Phe–*His*–Arg–Asp–	Yes	2
Pollibauer	Arg–Gly–Val–Phe–*His*–Arg–Asp–	Yes	4
Tokushima	Arg–Gly–Val–Phe–*His*–Arg–Asp–	ND	5
Taipei	Arg–Gly–Val–Phe–*His*–Arg–Asp–	Yes	

*Normal proalbumin A was not available for testing because it is processed proteolytically *in vivo* by a cathepsin B-like enzyme to form albumin A plus the propeptide. Proalbumins Gainesville, Taipei, and Takefu are described in the text; the others are reported in the references cited. Inheritance was not demonstrated for proalbumin Taipei. ND, not determined but cleaved by cathepsin B.

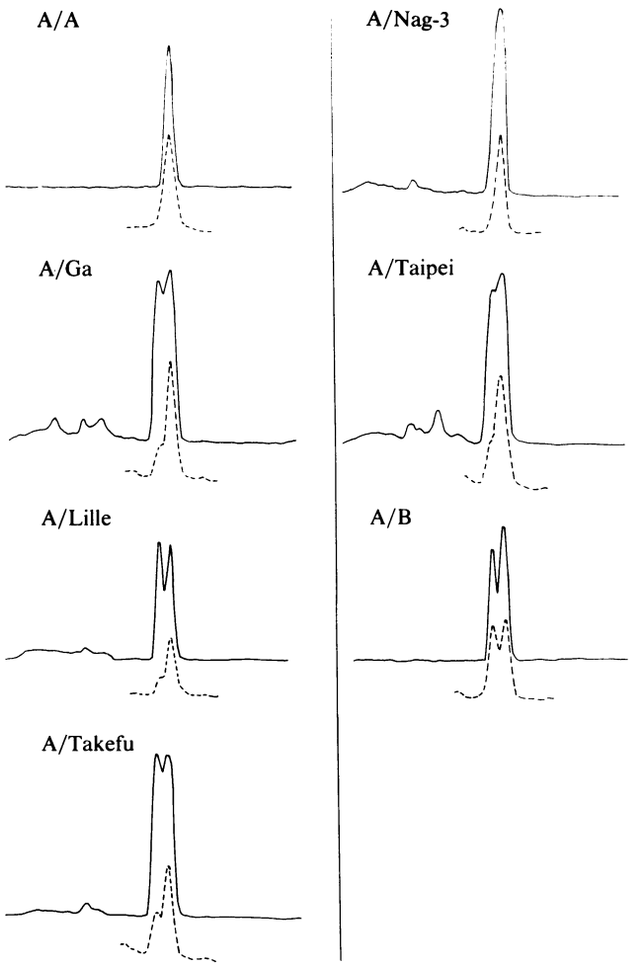

FIG. 1. Densitometric scan of the microzone electrophoretograms (solid line) and the radioautographs (dashed line) of normal albumin (A/A) and of heterozygous sera with albumin genetic variants (Ga, Gainesville; Nag-3, Nagasaki-3). All the proalbumins show decreased binding of $^{63}Ni^{2+}$ compared with albumins A and B.

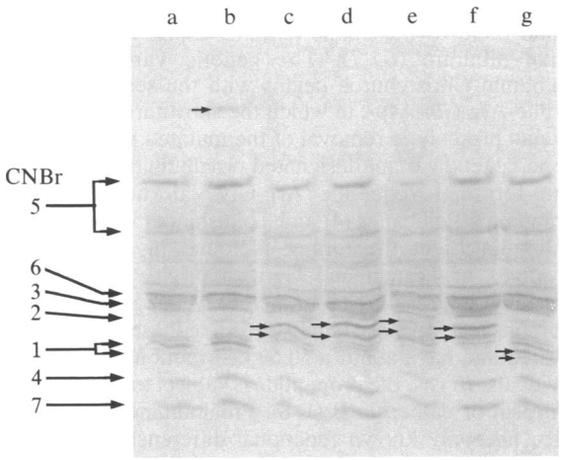

FIG. 2. Isoelectric focusing of the CNBr fragments 1–7 of normal albumin A and of genetic variants: A/A (lane a), unknown (lane b), purified proalbumin Gainesville (lane c), purified proalbumin Pollibauer (lane d), A/proalbumin Taipei (lane e), purified proalbumin Takefu (lane f), A/Nagasaki-3 (lane g). Arrows indicate bands for CNBr fragments with an amino acid substitution resulting in a change in charge. CNBr fragments (e.g., fragment 5) may have two charge forms because of the homoserine–homoserine lactone equilibrium.

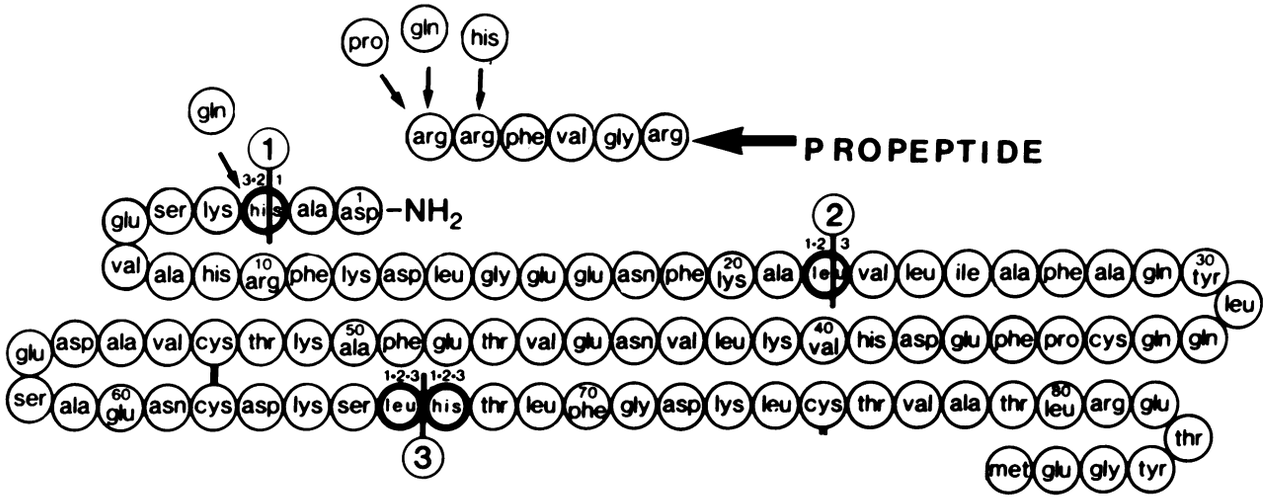

FIG. 3. Amino acid sequence of the propeptide and of the first CNBr fragment of normal serum albumin A. This figure is modified from Fig. 3 of Minghetti *et al.* (20), who determined the location of the introns 1, 2, and 3 (circled) and the codon positions at the introns. Single-amino acid substitutions in the propeptide for variant proalbumins and in the mature protein for albumin Nagasaki-3 are indicated by arrows pointing to the mutated site. Table 1 lists the variant proalbumins and gives the altered sequence of the propeptide.

somewhat degraded either during storage or in transit. Susceptibility to tryptic cleavage *in vitro* is a characteristic of proalbumins (1–8). Proalbumins Gainesville and Lille, which have different propeptides, are both decreased after incubation with trypsin (Fig. 4). The proteolytically processed product migrates close to the position of normal albumin A.

Albumin Gainesville also exhibits the decreased affinity for binding metals that is characteristic of previously reported proalbumins (2–5). The single high-affinity site for Cu^{2+} in albumins of various species is the amino-terminal tripeptide (9). In humans the normal sequence is Asp-Ala-His-, and the free amino group of the aspartic acid and the presence of histidine are obligate for strong binding. Other metals, such as nickel, are bound at the same site, so radioautography with $^{63}Ni^{2+}$ serves as a sensitive measure of metal-binding affinity (3–5, 8). In microzone electrophoresis the slower-moving proalbumin band is about equal in intensity to normal albumin for proalbumins Lille and Gainesville, but in the radioautographs the proalbumin band is reduced in relative area, indicating a lower metal-binding affinity (Fig. 1). As reported for proalbumin Tokushima (5), the binding of $^{63}Ni^{2+}$ by proalbumin Gainesville is greater at 4°C than at higher temperatures.

Proalbumin Taipei. Serum containing an electrophoretically slow, but otherwise untyped albumin variant was provided by C. Y. Chuang of National Taiwan University Hospital (Taipei). The patient was an elderly man of Chinese ancestry who had no living family, so inheritance could not be established. The first serum specimen contained some degraded and insoluble albumin, which led to an ambiguous result. A fresh lyophilized serum specimen was obtained in which the proportion of the variant compared with normal albumin was ≈40:60 (Fig. 1). The variant was purified, and the substitution was shown to be in CNBr fragment 1 by isoelectric focusing (Fig. 2). The intact protein was submitted to 12 cycles of Edman degradation in the protein sequencer. The characteristic sequence of proalbumin type Lille with the substitution $Arg^{-2} \rightarrow$ His was obtained in two independent experiments (Table 1), so the variant was designated proalbumin Taipei. Like other proalbumins of the Lille and Christchurch types, proalbumin Taipei exhibited decreased binding of $^{63}Ni^{2+}$ (Fig. 1) and was susceptible to tryptic cleavage of the propeptide (Fig. 4). Our studies of proalbumins Gainesville and Taipei showed that to avoid ambiguity from the presence of degraded products, it is essential to obtain fresh specimens of serum and to purify the proalbumin variant before structural study.

Proalbumin Takefu. Proalbumin Takefu is an electrophoretically slow albumin that occurs in a Japanese family. It has been distinguished electrophoretically from five other Japanese genetic variants by Nagata *et al.* (11), and we have differentiated it structurally from some twenty other genetic variants. Struc-

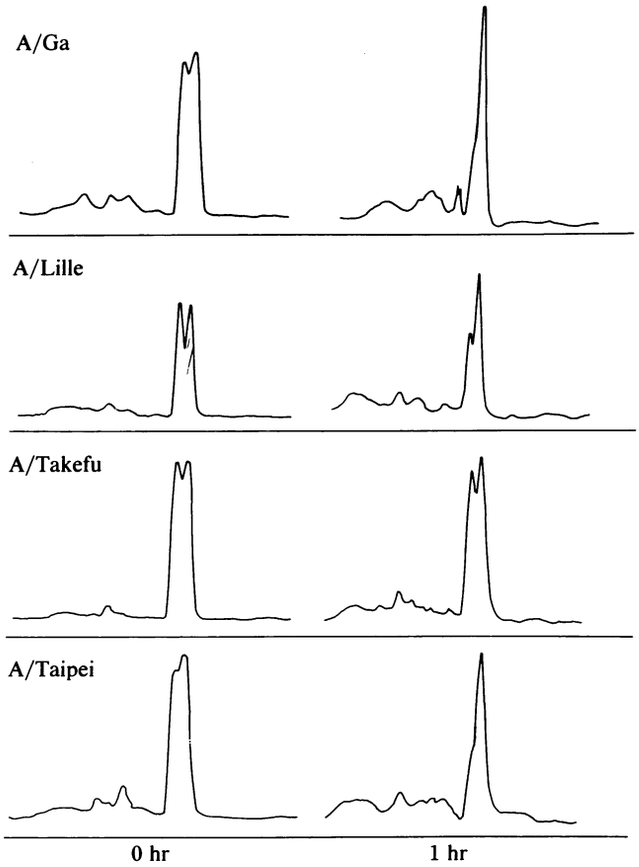

FIG. 4. Limited tryptic digestion of proalbumins Gainesville (Ga), Lille (Pollibauer), Takefu, and Taipei. Heterozygous sera (40 μl) with a proalbumin variant were digested with 20 μg of trypsin at 37°C, and digestion was stopped with trypsin inhibitor. The digests (0 hr, left; 1 hr, right) were subjected to microzone electrophoresis at pH 8.6 followed by densitometric scanning.

tural study of albumin Takefu revealed that it is a distinct type of proalbumin with the substitution $Arg^{-1} \rightarrow Pro$. By use of isoelectric focusing the structural change in albumin Takefu was located in the amino-terminal CNBr fragment 1 (Fig. 2). Amino-terminal sequence analysis of purified albumin Takefu in duplicate for 20 cycles showed the presence of the propeptide Arg-Gly-Val-Phe-Arg-*Pro*- and no secondary sequence (Table 1). Furthermore, this proalbumin variant is not susceptible to tryptic cleavage (Fig. 4); it has reduced metal-binding affinity compared with normal albumin A, but both at room temperature and at 4°C it has greater affinity than the two types of proalbumins previously described (Fig. 1).

Independent Genetic Origin of Proalbumins. Although it is to be expected that independent mutations may give rise to the same albumin genetic variant, little evidence other than electrophoretic identification has been available until recently. Proalbuminemia is an inherited trait, and the inheritance pattern has been determined for most of the proalbumin specimens for which the structural change has been identified. Proalbumin Christchurch discovered in New Zealand is the precedent name for variants having the propeptide Arg-Gly-Val-Phe-Arg-*Gln*- (1). This class includes proalbumin Gainesville present in an American family of Irish descent (10) and the French proalbumin Y (serum 3433) (3). Because these are all of Caucasian origin, it is not possible to exclude a common European founder; however, this appears unlikely because of the diversity of geographical origins. On the other hand, the Lille type of proalbumin must have arisen independently several times. It has been identified in unrelated individuals of several European nationalities; more importantly, it is present in a Chinese individual in Taiwan, and it is found both in heterozygous and homozygous states in a Japanese family in Tokushima (5). In the future as structural characterization of albumin genetic variants supersedes electrophoretic typing, more examples of independent genetic origin of identical alloalbumins will probably be discovered.

Amino Acid Substitutions, Stability, and Electrophoretic Mobility of Proalbumins. All three types of proalbumins—Christchurch, Takefu, and Lille—have a single amino acid substitution in the propeptide that prevents its proteolytic removal during normal post-translational processing (Table 1). A pair of basic residues is required for excision of the propeptide in the Golgi vesicles by an unknown protease called a convertase, which may be a calcium-dependent thiol protease (6, 7). Substitutions of $Arg^{-1} \rightarrow Gln$ or $Arg^{-1} \rightarrow Pro$ or $Arg^{-2} \rightarrow$ His block further processing *in vivo*. Cleavage after $arginine^{-2}$ occurs *in vitro* with trypsin but not with cathepsin B for the Christchurch type of proalbumin, leaving the initial sequence Gln-Asp-Ala-His. However, in proalbumin Takefu the Arg-Pro bond cannot be split by trypsin or similar proteases, so the proalbumin remains intact both *in vivo* and *in vitro* and retains the initial 50:50 ratio. In proalbumins of the Lille type the entire hexapeptide can be excised *in vitro* both by trypsin and cathepsin B to yield normal albumin (4, 5).

All proalbumins are "slow" albumins; that is, they migrate with equal mobility but more slowly than normal A in clinical electrophoresis at pH 8.6 because of the increase in net positive charge contributed by the two arginine residues in the propeptide. This shows the importance of identifying the structural change for use as the ultimate criterion for classification of albumin genetic variants.

Amino Acid Substitutions and Nucleotide Mutations. Proalbumins Christchurch and Takefu are the only examples thus far reported of alloalbumins that have different amino acid substitutions at the same position. Many such examples are known for human hemoglobin variants, so more may be expected for albumin genetic variants. In all three types of proalbumins the mutation results from a single-base change in the codon for arginine (Table 2). Four of the nine possible single-base mutations of the CGA codon for $arginine^{-1}$ would be silent, one would produce a stop codon, and four would lead to an amino acid substitution that would block processing. Two of the latter have been observed—glutamine and proline; two have not—glycine and leucine. Six of the possible single-base mutations of the CGT codon for $arginine^{-2}$ would produce amino acid substitutions that would block processing, but only histidine has been observed to date.

Amino-Terminal Sequence Analysis of Albumin from Patients with Wilson Disease. Patients with Wilson disease inherit a disturbance in copper metabolism and transport that is usually attributed to a decrease or a defect in ceruloplasmin, a protein to which about 90% of the serum copper is

Table 2. Amino acid substitutions and nucleotide mutations identified in genetic variants of human serum albumin

Genetic variant	Substitution	Codon in albumin A	Minimum change	New codon	Ref.
Proalbumins					
Christchurch type	$Arg^{-1} \rightarrow Gln$	C<u>G</u>A	G → A	C<u>A</u>A	1
Gainesville					
Y					3
Takefu type	$Arg^{-1} \rightarrow Pro$	C<u>G</u>A	G → C	C<u>C</u>A	
Lille type	$Arg^{-2} \rightarrow His$	C<u>G</u>T	G → A	C<u>A</u>T	2
Pollibauer					4
Tokushima					5
Taipei					
Albumin variants					
Nagasaki-3	$His^{3} \rightarrow Gln$	CA<u>C</u>	C → <u>R</u>	CA<u>R</u>	
Tagliacozzo	$Lys^{313} \rightarrow Asn$	AA<u>G</u>	G → <u>Y</u>	AA<u>Y</u>	15
Parklands	$Asp^{365} \rightarrow His$	<u>G</u>A<u>T</u>	G → <u>C</u>	<u>C</u>A<u>T</u>	16
Naskapi type	$Lys^{372} \rightarrow Glu$	<u>A</u>AA	A → G	<u>G</u>AA	12
Mersin					12
Mexico-2	$Asp^{550} \rightarrow Gly$	G<u>A</u>T	A → G	G<u>G</u>T	12
B (Oliphant)	$Glu^{570} \rightarrow Lys$	G<u>A</u>G	G → A	A<u>A</u>G	17
Mi/Fg (Gent)	$Lys^{573} \rightarrow Glu$	<u>A</u>AA	A → G	<u>G</u>AA	18
Chain termination					
Ge/Ct	$Gln^{580} \rightarrow Lys$	<u>C</u>AA	C → A	<u>A</u>AA	19

Albumin codons are from the genomic sequence of Minghetti *et al.* (20). Proalbumins Gainesville, Takefu, and Taipei and albumin Nagasaki-3 are described in the text; other variants are reported in the references cited. Nucleotides exchanged in the codons are underlined. R, either A or G; Y, either T or C.

bound. Species such as the dog, pig, and chicken that do not have histidine in the third position of their serum albumin lack the strong binding site for copper (9). Thus, it seemed possible that a similar substitution might occur in the albumin of Wilson patients that might account for the defect in the handling of copper. Hence, we undertook amino-terminal sequence analysis of the albumin purified from the serum of two patients, which was provided by I. H. Scheinberg (New York). In both cases a normal sequence was obtained, and histidine was present in the third position, so we concluded that the metabolic error was not attributable to a substitution in the tight copper-binding site of their albumin. Subsequently, by chromosomal mapping Frydman *et al.* (21) localized the genetic defect to human chromosome 13, rather than chromosomes 3 or 4 on which the genes for ceruloplasmin and albumin, respectively, are localized (22).

Amino-Terminal Sequence Analysis and Radioautography of Other Albumin Variants. Amino-terminal sequence analysis was done for 30 or more cycles on a number of other albumin variants in the course of determining their structural changes. These included three Amerindian variants, albumins Naskapi, Yanomama-2, and Maku, and the Japanese variant Nagasaki-3. None of these proved to be proalbumins, and the first three mentioned have the normal amino-terminal sequence. However, Nagasaki-3 has the $His^3 \rightarrow Gln$ substitution, which should affect the copper-binding site (N.T., Y.T., T. Isobe, F.W.P., M. Fujita, C. Satoh, and J. V. Neel, unpublished results). Radioautography with $^{63}Ni^{2+}$ indicated that the metal-binding affinity of Nagasaki-3 was decreased (Fig. 1). However, it was difficult to make a quantitative estimate because of the difficulty of separating this variant from normal albumin in electrophoresis at pH 8.6 or by the use of HPLC.

Apparent Clustering of Mutations in the Propeptide Sequence. Before this study more than 30 apparently different genetic variants of human serum albumin had been typed by electrophoretic analysis under various conditions (14), up to 80 had been named and described (23), and nine different types had been identified in terms of structural change (Table 2). In all instances except for a chain-termination variant (Ge/Ct), a single-nucleotide change resulting in a single-amino acid substitution had occurred. The Ge/Ct variant was apparently caused by a single-base deletion with a resultant shift in the reading frame (19). A disproportionate number of the variants have been proalbumins with an apparent clustering of the mutations in the Arg-Arg sequence of the basic hexapeptide propeptide (Table 2) (24). The structural change has now been established as a single-amino acid substitution in eight named proalbumin variants of three different types, one of which (Takefu) varies from known types. These single-point mutations are restricted to two adjacent amino acids, the Arg-Arg sequence in the propeptide just preceding the mature normal albumin A. In contrast, only eight single-point mutations have thus far been identified in the entire 585-residue sequence of the mature protein (Table 2).

The nonrandom distribution of the identified structural substitutions in albumin variants suggests a clustering of mutations in the region of the albumin gene that encodes the propeptide—i.e., the 3′ end of exon 1 (20). The Christchurch type of proalbumin has so far been identified only in Western European families of British, French, and Irish descent. Although the families are apparently unrelated, a common ancestral origin of the mutant cannot be excluded. In contrast, the Lille type of proalbumin has now been identified in Caucasians, Japanese, and Chinese, and so independent mutations must be postulated. To date, no other albumin variant has been identified in genetically diverse individuals with the exception of albumins Naskapi and Mersin, which are thought to reflect a common mid-Asiatic ancestral origin (13). Of course, the apparent clustering of mutations in the propeptide may simply reflect the relative ease of identifying proalbumin variants and of determining their change in structure. Further study of many different genetic variants will be required to establish whether the mutations cluster at hypermutable sites in the albumin gene or are associated with features of the yet unknown three-dimensional structure of the protein.

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