

A New Hemoglobin Variant, Hemoglobin Nunobiki [α 141 (HC3) Arg \rightarrow Cys]

Notable Influence of the Carboxy-terminal Cysteine upon Various Physico-chemical Characteristics of Hemoglobin

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Abstract

A new hemoglobin variant, hemoglobin (Hb) Nunobiki, was detected in a Japanese male with marginal erythrocytosis. The Hb Nunobiki component amounted to 13.1% of the total hemoglobin. Structural analysis of this variant established the substitution of a cysteine for an arginine at the carboxy terminus of the α -chain (α 141).

The oxygen equilibrium curves of Hb Nunobiki revealed extremely high oxygen affinity with a reduced Hill coefficient n , a decreased alkaline Bohr effect, and a decreased 2,3-diphosphoglyceric acid effect.

The isoelectric point of the Hb Nunobiki changed during storage, although the oxyhemoglobin state was maintained. These findings could be accounted for by the specific characteristics of a newly introduced cysteinyl residue.

Cysteinyl residue at α 141 in Hb Nunobiki did not seem to be involved in the formation of either intermolecular or intramolecular disulfide bonds under physiologic conditions.

The low proportion of Hb Nunobiki (13.1%) in the propositus was also discussed after it was verified that he exhibited four α -globin genes per diploid cell.

Introduction

Human adult hemoglobin (Hb A)¹ is a tetrameric protein consisting of two pairs of α and β polypeptide chains. Amino acid sequence anomalies, which primarily occur as a result of gene mutation in the α - and β -chains, may alter the physico-chemical characteristics of hemoglobin, such as its solubility, stability, electrophoresis, oxygen affinity, resistance to acid or alkali denaturation, auto-oxidation, and its absorption spectrum (1).

Some of these alterations cause severe pathologic conditions such as sickle cell anemia, methemoglobinemia, hemolytic anemia, and polycythemia (2).

Researchers have previously tried to explain such alterations in physico-chemical characteristics based on the detailed structure of the hemoglobin (Hb) molecule. Changes in oxygen

affinity have thus been accounted for by an amino acid substitution at the heme linkage, the heme pocket, the $\alpha_1\beta_1$ dimeric contact, the $\alpha_1\beta_1$ tetrameric contact, and a carboxy terminal end of the α or the β subunit (3).

The newly discovered abnormal hemoglobin described in this paper is the first hemoglobin variant with a cysteine at a carboxy terminus of an α chain. High oxygen affinity and slow auto-oxidation of this variant may be explained by this substitution.

Analysis of the globin gene responsible for hemoglobin production in the propositus is also described in an attempt to explain the reason for the low proportion of this abnormal hemoglobin.

Methods

A specimen of venous blood was withdrawn from the patient into a heparinized bottle. Hematologic data were obtained by the standard method of laboratory examination (4). The hemolysate was prepared by the conventional method and identification of the abnormal hemoglobin was accomplished by isoelectric focusing with a polyacrylamide gel slab containing Ampholine, pH 5–9 (LKB Instruments, Inc., Gaithersburg, MD). The abnormal hemoglobin Nunobiki (Hb Nb) was then purified from the whole hemolysate by DEAE-cellulose (DE-52, Whatman Laboratory Products, Inc., Clifton, NJ) column chromatography (5). Hemoglobin instability and heat denaturation tests were carried out by a method described elsewhere (6, 7).

The absorption spectra in several hemoglobin forms of purified Hb Nb were measured at a wavelength range of from 250 to 650 nm with a spectrophotometer (Cary type 118C, Varian Associates, Inc., Palo Alto, CA). Auto-oxidizability was examined at 37°C in 0.05 M bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane, 0.1 M NaCl buffer, pH 7.4, by measuring the increase in absorbance at 630 nm together with its absorption spectrum in the visible wave length. The oxygen equilibrium curves of the hemoglobin solution were examined according to the method of Imai et al. (8). The erythrocyte 2,3-diphosphoglyceric acid (DPG) concentration was determined with a 2,3-DPG measurement kit (Sigma Chemical Co., St. Louis, MO). Fetal Hb (Hb F) was determined by alkali denaturation according to the method of Betke et al. (9).

Globin was prepared by the method of Anson and Mirsky (10) and the chain was separated into individual components on a column of carboxymethyl-cellulose (CM-52, Whatman Laboratory Products, Inc.) with a developer of phosphate-buffered 8 M urea solution, pH = 6.7 (11). Chemical modifications and enzymatic digestions of the aberrant chain for analysis of the primary structure followed, using the methods cited in the references, i.e., p-chloromercuribenzoic acid (PCMB) treatment (12), aminoethylation (13), performic acid oxidation (14), cysteine cleavage (15, 16), and digestions with trypsin (17), chymotrypsin (18), pepsin (19), carboxypeptidase A (CP-A) (20), and carboxypeptidase B (CP-B) (20).

Fingerprinting of the peptides digested with these proteases was conducted on a silica gel plate (20 \times 20 cm, Merck Chemical Div., Merck & Co., Inc., Rahway, NJ) with a combination of electrophoresis in one direction with pyridine, acetic acid, and water (1:10:500 in vol) and ascending thin layer chromatography in a rectangular with a

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1. Abbreviations used in this paper: CP-A, carboxypeptidase A; CP-B, carboxypeptidase B; DPG, diphosphoglyceric acid; Hb, hemoglobin; Hb A, adult hemoglobin; Hb F, fetal hemoglobin; Kb, kilobase pairs; Nb, Nunobiki; PCMB, p-chloromercuribenzoic acid; PMB, p-mercuribenzoic acid.

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developer of n-butanol, pyridine, acetic acid, and water (30:30:6:12 in vol). The peptide was acid-hydrolyzed under nitrogen, and amino acid residues were estimated with an automatic amino acid analyzer (Yanagimoto L-7; Yanagimoto Co., Ltd., Kyoto, Japan).

DNA was prepared from leukocytes collected from 10 ml of peripheral blood (21). To analyze α - and non α -globin genes, 4- μ g aliquots of DNA were digested with the restriction enzymes *Sac*I and *Bgl*II, respectively, under conditions recommended by the manufacturer (Takara Shuzo Co., Ltd., Kyoto, Japan). After digestion, the cleaved DNA was phenol-extracted, ethanol-precipitated, and redissolved in 30 μ l of buffer solution, pH = 8.0. The DNA probes used in this experiment were derived from the recombinant plasmids of pJW 101, pJW 102, and pJW 151 (22), each of which respectively contains a synthetic copy of the human α -, β -, or γ -globin gene in pMB 9 (23). The plasmids were cultured and isolated under containment conditions according to the guidelines of the Ministry of Education, Science, and Culture of Japan. The DNAs obtained were then electrophoresed on 0.6% agarose gel plates, transferred onto nitrocellulose filters (Toyo Roshi Co., Ltd., Tokyo, Japan), hybridized to a [α - 32 P]deoxycytidine triphosphate-labeled probe, and autoradiographed as previously described by Southern (24).

Results

The patient was a 41-yr-old Japanese male who was admitted to the Kobe Municipal Central Hospital, Kobe, Japan, for surgery for acute ulcerative colitis. Hematological examinations before surgery indicated an increased leukocyte count and slight anemia, but no tendency toward hemolytic anemia.

After the operation, the patient demonstrated marginal erythrocytosis, as shown in Table I. This condition has persisted for over 2 yr. Other aspects in his hematologic examinations have not been remarkable.

The patient has no siblings, and the causes of his parents' deaths are unknown.

Isoelectric focusing of fresh hemolysates from the proband disclosed discrete bands of Hb Nb, Hb A, and Hb A₂ from anode to cathode in the order described (Fig. 1). An abnormal Hb A₂ was not demonstrable. Separated hemoglobins were present in the following proportions: Hb Nb, 13.1%; Hb A, 83.3%; Hb A₂, 2.6%; and Hb F, 0.95%.

Hb Nb focused at a similar position to Hb Ube-2 (α 68 Asn \rightarrow Asp) (25, 26), which possesses one more negative charge than Hb A. However, when the separated Hb Nb was stored for \sim 1 mo. at 4°C, it migrated more anodally than the freshly prepared Hb Nb, as if it had two more negative charges than Hb A (Fig. 2).

Table I. Hematologic Examinations of Hb Nb Carrier

Examinations	Surgical operation	
	Before	After
Hb (g/dl)	11.8	16.1
Erythrocytes (10^{12} /liter)	3.91	5.31
Packed cell volume (liter/liter)	36.0	46.5
Mean corpuscular volume (fl)	92	88
Mean corpuscular hemoglobin (pg)	30.2	30.3
Mean corpuscular hemoglobin concentration (g/dl)	32.8	34.6
White blood cells (10^9 /liter)	14.6	7.7
Platelet (10^9 /liter)	43.2	21.1
Reticulocytes (percent)	1.0	0.9

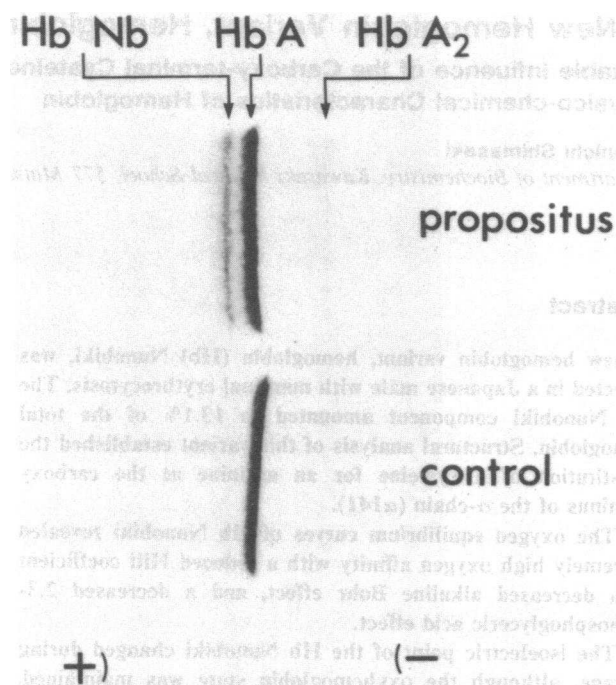


Figure 1. Isoelectric focusing of the hemolysate.

Isoelectric focusing of PCMB-treated Hb Nb demonstrated an α -chain anomaly, since a strip of the p-mercuribenzoic acid (PMB)-modified α -chain of Hb Nb (PMB- α^{Nb}) moved more anodally as compared with one of the normal PMB- α -chain (PMB- α^A), while the PMB- β -chain of Hb Nb showed the same mobility as that of Hb A. The site of focusing of the PMB- α^{Nb} seemed to have two more negative charges than the PMB- α^A , and no difference in the PMB- α^{Nb} in either the freshly prepared or the stored hemolysate was seen in their migration (Fig. 3).

Instability tests of the abnormal Hb solution both by isopropanol and by heat were negative. Absorption spectra in a wavelength range of 250–650 nm in the oxy-, met-, and deoxy-forms of Hb Nb were the same as those of isomolar Hb A. This may indicate no heme depletion, normal oxygen binding capacity, or a normal tryptophan (α -14) notch, at 289

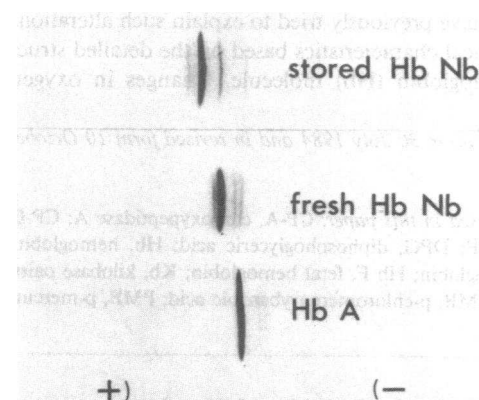


Figure 2. Isoelectric focusing of freshly prepared and stored hemolysate.

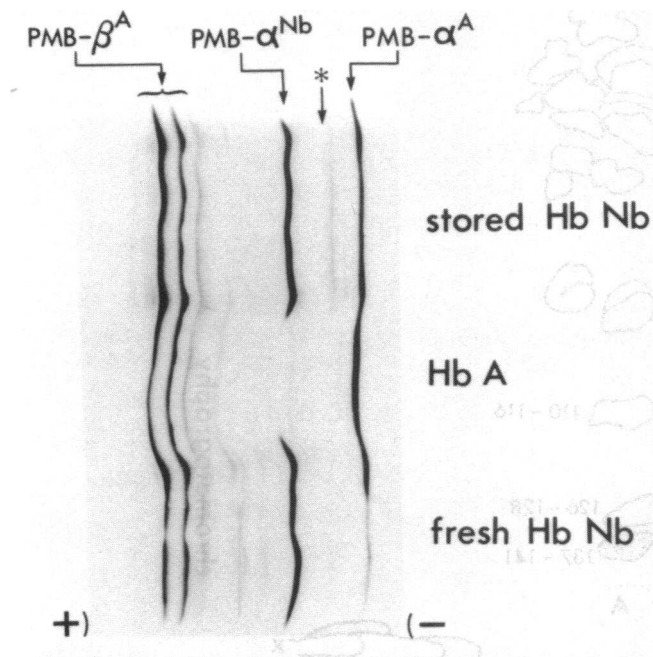


Figure 3. Isoelectric focusing of PCMB-treated hemolysate. The arrow with asterisk shows the expected migration position of abnormal PMB- α with one negative electron charge.

nm, in the hemoglobin molecule. The auto-oxidation velocity of Hb Nb was significantly slower than that of Hb A (Fig. 4).

The oxygen equilibrium curves of Hb Nb at 25°C were indicative of a high oxygen affinity: $\log P_{50} = 0.360$ (Hb A = 1.080) and 0.223 (Hb A = 0.900) at pH 7.0 and 7.4, respectively (Fig. 5). The heme-heme interaction, alkaline Bohr effect, and 2,3-DPG effect of Hb Nb were also markedly diminished as indicated by the following measurements: Hill coefficient $n = 1.25$ (Hb A = 2.88) and 1.23 (Hb A = 2.77) at pH 7.0 and 7.4, respectively; the alkaline Bohr effect = -0.34

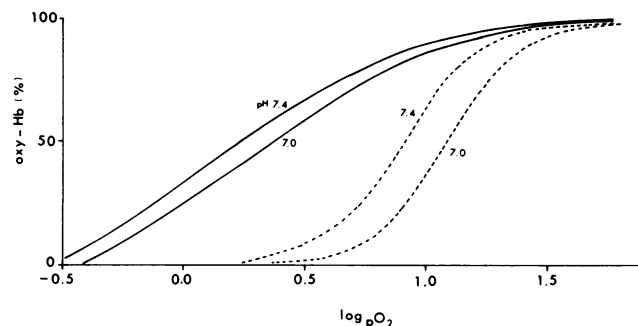


Figure 5. Oxygen equilibrium curves of Hb Nb (—) and Hb A (----).

(Hb A = -0.45) and the 2,3-DPG effect, $\Delta \log P_{50}$ from 2,3-DPG-free Hb Nb solution, = 0.060 (Hb A = 0.132) and 0.213 (Hb A = 0.355) measured in molar ratios (2,3-DPG/Hb) of 10 and 50, respectively, at pH 7.4 (Fig. 6). The concentration of 2,3-DPG in erythrocytes was $11.7 \mu\text{mol/g Hb}$, which is within normal range (12.8 ± 2.3).

Globin was prepared and an α^{Nb} -chain was isolated from other globin chains chromatographically. Then tryptic digestion was carried out. The fingerprint map of the tryptic digest of the α^{Nb} -chain showed a missing α -T-14 spot. Other spots were located at their normal positions and showed the same amino acid residues as those from the α^{A} -chain. This suggests that the α -T-14 peptide of the α^{Nb} -chain might be chemically joined with the tryptic core (α^{Nb} -T-11~13 peptide) fraction. The tryptic core peptide was then treated with performic acid to convert cysteine and cystine residues to cysteic acid for chymotrypsin digestion. Fingerprinting of the digest, however, did not provide any abnormal peptides since all the spots on the map were normally positioned and had normal amino acid compositions. This suggests that there was no superimposition of the α^{Nb} -T-14 peptide on any of the spots observed on the map. To detect the α^{Nb} -T-14 peptide, which corresponds to the C-terminal peptide, the α^{Nb} -chain was individually treated

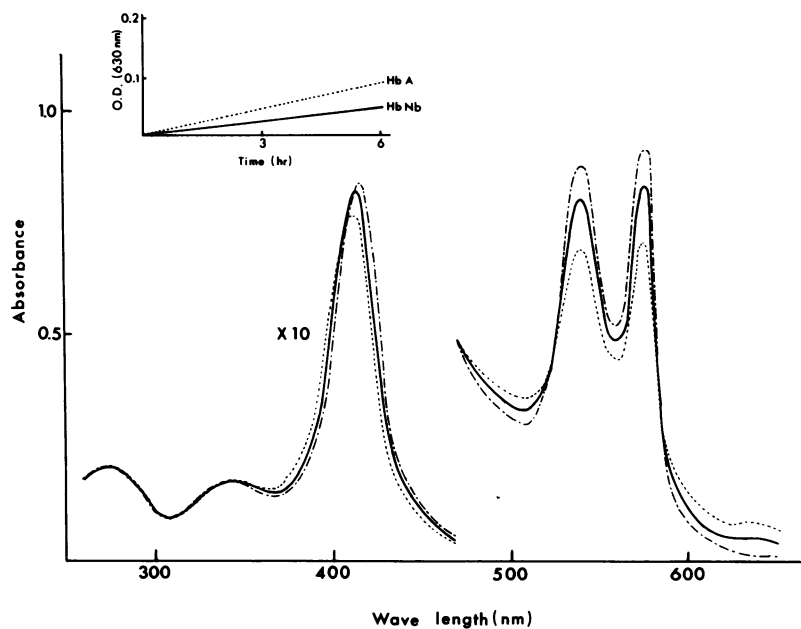


Figure 4. Absorption spectra measured before and after an auto-oxidation test of the hemolysate. ----, Hb Nb and Hb A before the test; —, Hb Nb after the test; ····, Hb A after the test. The graph in the insert shows the time course of absorbance change at 630 nm during auto-oxidation carried out for 6 h at 37°C.

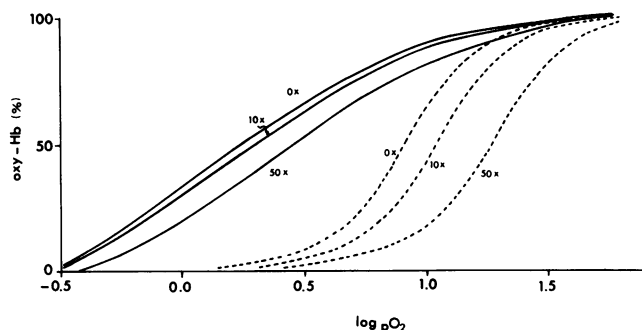


Figure 6. Oxygen equilibrium curves measured at 0-, 10-, and 50-fold molar 2,3-DPG to that of hemoglobin tetramer. —, Hb Nb; ----, Hb A.

with CP-B and CP-A. CP-B digestion of the α^{Nb} -chain demonstrated no release of amino acids from the C-terminus. This was in sharp contrast to the clear discovery of ^{141}Arg , ^{140}Tyr , and ^{139}Lys from the control α^A -chain. CP-A did not release any amino acids from the C-terminus of the α^{Nb} -chain, nor, as expected (20), did it release any from the C-terminus of the α^A -chain.

This evidence and the fact that the triplet codon CGU of Arg exists at the α^A141 of α -mRNA (27) led to the assumption that the amino acid at C-terminus of the α^{Nb} -chain was a cysteine with codon of UGU instead of the normally present Arg. This was proven by subjecting the aminoethylated α^{Nb} -chain to CP-B digestion. The amino acids from the C-terminus of the α^{Nb} -chain were detected as aminoethylated ^{141}Cys (^{AE}Cys), ^{140}Tyr , and ^{139}Lys , with amounts in the order described, and these were proportional to the incubation time course (Fig. 7). Other evidence of this substitution was provided when the fingerprint of the pepsin digest of the performic acid-treated α^{Nb} -chain was compared with that of the normal control α^A -chain. One abnormal spot was clearly seen in isolation (Fig. 8). Amino acid analysis of the acid hydrolysate of the abnormal spot showed that the residues arose from ^{137}Thr - ^{138}Ser - ^{139}Lys - ^{140}Tyr - $^{141}\text{CysSO}_3\text{H}$.

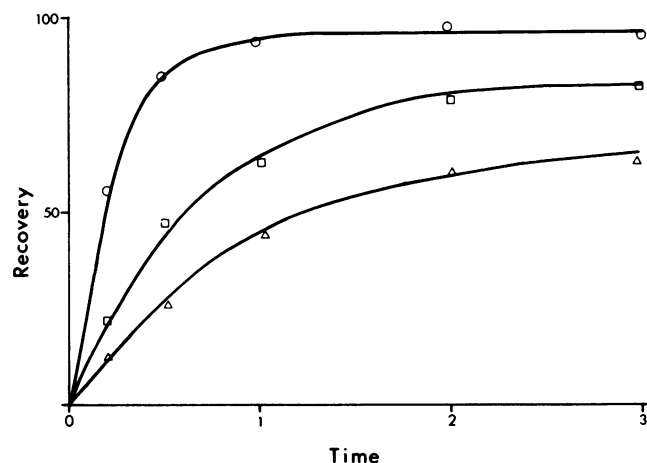


Figure 7. The recovery (%) of amino acid with time, (h) from an aminoethylated α^{Nb} -chain. The identification and the quantity of amino acids were determined by an amino acid analyser. \circ , ^{AE}Cys ; \square , Tyr; \triangle , Lys.

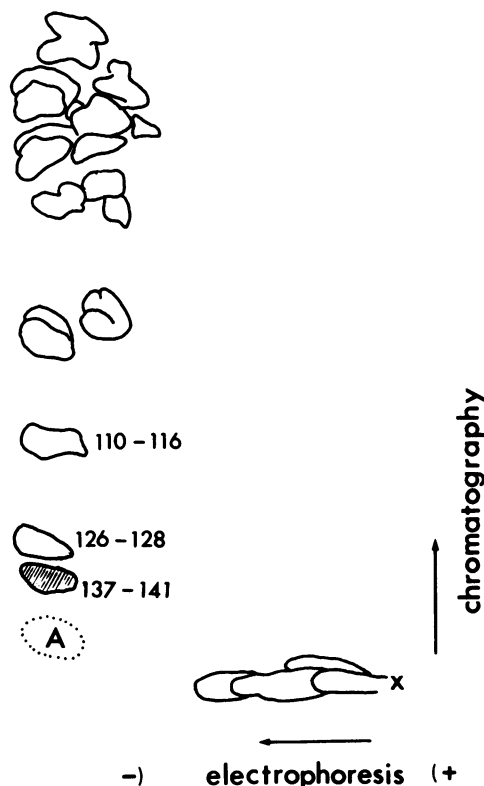


Figure 8. Fingerprint of the pepsin digest of the performic acid-treated α^{Nb} -chain. The dotted circle A is normal α^A -137-141. The shaded spot is α^{Nb} -137-141.

Other indirect but supportive findings of this substitution were made when the α^{Nb} -chain was treated with 2-nitro-5-thiocyanobenzoic acid to cyanylate the thiol group of Cys and it was consequently cleaved at amino-sites of the peptide bond by Alkali (15, 16), releasing the C-terminal thiocyanylated cysteine residue. Two segments from the α^{Nb} -chain were isolated by gel filtration in pure form and confirmed by amino acid analysis to be polypeptides composed of $\alpha^{Nb}1-103$ and of $\alpha^{Nb}104-140$ with missing cysteine residue at $\alpha141$ as expected. These findings may also support the possibility that the amino acid residue at $\alpha^{Nb}141$ is a Cys, since the reaction removed specifically a Cys residue in the sequence of the peptide.

Based on the evidence above, it was concluded that an Arg at $\alpha141$ in Hb A was replaced by a Cys in this abnormal hemoglobin. Since this mutation has not been described previously, it was designated as Hb Nunobiki with a representation formula of $\alpha_2^{141\text{Arg}\rightarrow\text{Cys}}\beta_2^A$.

As for the propositus' α -globin gene organization, DNA fragments digested with the restriction enzyme *SacI* were hybridized to a radioactive probe, pJW 101. Three bands, estimated as 17, 13, and 4.2 kilobase pairs (Kb) in length, were clearly disclosed on an autoradiogram (Fig. 9). Densitometric scanning indicated that the 4.2-Kb band occupied about twice as much space as that of the 17- and 13-Kb bands. Results demonstrated a normal duplicated α -globin gene in each haploid and the absence of more than four α -globin genes per diploid cell. An analysis of the non- α -globin genes of the propositus revealed a normal $-\gamma-\gamma-\delta-\beta$ -globin gene arrangement in each haploid.

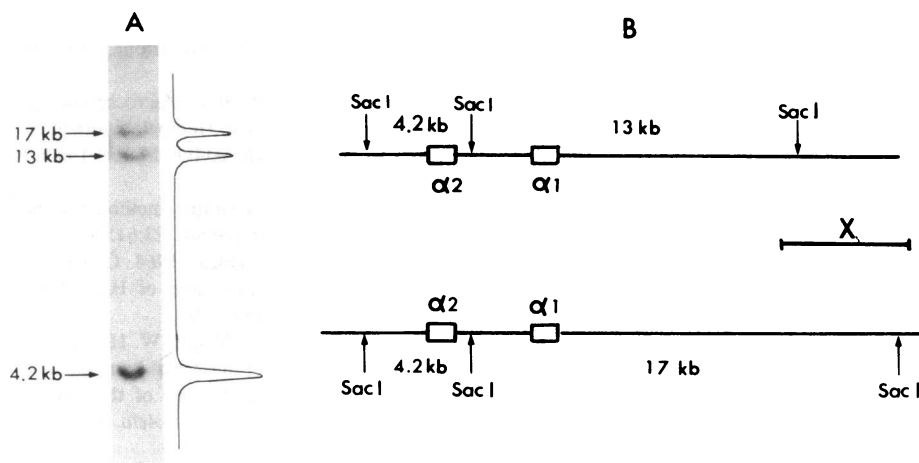


Figure 9. α -globin gene analysis of the carrier. A, An autoradiogram of α -globin specific restriction fragments. Sizes of the fragments in kilobase pairs shown with arrows were determined from the markers of λ -DNA digested with *Hind*III (28). The densitometric scanning pattern of the bands is shown on the right. B, The restriction site of *Sac*I around an α -globin gene. X represents the polymorphic restriction fragment region shown by Higgs et al. (29).

Discussion

Hb Nb is a fast-moving abnormal hemoglobin resulting from the replacement of a Cys for an Arg at α 141. The similar mobility of Hb Nunobiki to that of Hb Ube 2 (α 68 Asn \rightarrow Asp) may correspond to one electron charge difference in the residue between Cys and Arg. It is obvious that the S atom of the thiol (-SH) in cysteine is susceptible to partial oxidation leading to sulfoxide ($-\text{SO}^-$) or sulfinic acid ($-\text{SOO}^-$) ion forms and that each of these forms possesses one more negative charge than neutral Cys. This might be why the Hb Nb in the stored hemolysate, which was devoid of reducing enzymes, migrated faster than that of the freshly prepared hemolysate.

The negatively charged pigment with two additional charges found on isoelectric focusing of the PCMB-treated hemolysate is presumed to be an α^{Nb} -chain composed of both partially oxidized Cys and PMB-modified Cys at α^{Nb} 141, since both of these produce a two-electron charge difference from Arg in slightly alkaline medium. The mobility of PMB- α^{Nb} on isoelectric focusing also showed that the Cys at α^{Nb} 141 did not take part in the formation of a disulfide bond. Moreover, no polymerization of Hb Nb was observed by gel filtration as was with the non- α hemoglobin variants of Hb Port Alegre (β 9 Ser \rightarrow Cys) (30, 31) and Hb Ta-li (β 83 Gly \rightarrow Cys) (32), both of which allow polymerization of the molecule by disulfide bonding.

According to the hemoglobin molecule model of Perutz (33), the Arg at α 141 acts as one of the important amino acid residues responsible for constructing salt bridges with neighboring amino acids. These salt bridges favor stabilization of the T structure of the hemoglobin molecule. In its usual deoxy-hemoglobin form, the α -carboxyl group of the C-terminal Arg of the α -chain, α_1 , is linked to the α -amino group of the N-terminal Val of its partner α -chain, α_2 , and the guanidinium group of Arg at α_1 141 is directed toward the α -carboxyl group of Asp at α_2 126 and to the α -amino group of Val at α_2 1 via an intervening chloride ion (34, 35). The formation of these ionic linkages might be interrupted in an α^{Nb} subunit having C-terminal Cys instead of Arg. As a result, the stability of the T structure would be weakened, making the R structure favored over the T structure, as is characteristic of high oxygen affinity. The diminished 2,3-DPG effect of Hb Nb may be explained in the same manner. The predominance of the R

structure in Hb Nb may secondarily affect a pair of β subunits, so that the 2,3-DPG molecule cannot be inserted properly into its binding site between two β subunits. Thus R to T conversion of Hb Nb would be hindered.

Five Hb variants with a substituted amino acid residue at the C-terminus of the α -chain have been reported; Hb Singapore (Arg \rightarrow Pro) (36), Hb Suresnes (Arg \rightarrow His) (37), Hb J-Cubujugui (Arg \rightarrow Ser) (38), Hb Legnano (Arg \rightarrow Leu) (39), and Hb J-Camagüey (Arg \rightarrow Gly) (40). Examinations of oxygen affinity in Hb Singapore and Hb J-Camagüey have not been described, but the other variants are characterized by a high oxygen affinity that might be explained by a similar mechanism to that of the above-mentioned Hb Nb. The carriers of these variants, with the exception of Hb Singapore, have been reported to reveal slight polycythemia without any clinical symptoms. The carrier of Hb Nb has also exhibited marginal erythrocytosis. Hb Nb is thought to be a physiologically severe defect in oxygen transport, as indicated by its oxygen equilibrium curve. However, the proportion of Hb Nb to total hemoglobin is so small (13.1%) that the erythrocytosis is thought to be maintained in minimal.

It is also significant that Hb Nb exhibited a relatively slow rate of auto-oxidation. This finding might indicate that the replaced Cys at the α^{Nb} -C-terminus consumes oxidants produced during incubation and protects the Hb Nb molecule from met-Hb formation. The high oxygen affinity of this variant may also contribute to stabilize the heme Fe(II) ion as observed in Hb Creteil (β 89 Ser \rightarrow Asn) (41, 42).

The fingerprints of both the α^{Nb} tryptic soluble peptides and the chymotryptic peptides derived from the performic acid-oxidized α^{Nb} -tryptic core demonstrated disappearance of α^{Nb} -T-14 (^{140}Tyr - ^{141}Cys). One possible explanation for this observation may be that during the preparation of the native α^{Nb} -chain, a disulfide bond between the two cysteines (at 104, core, and 141) in the α^{Nb} -chain was formed in a random combination of intra- and inter- α^{Nb} -chains, thus resulting in α^{Nb} -T-14 being joined in bridged form to the insoluble tryptic core fraction, which has a Cys at 104. Therefore, free α^{Nb} -T-14 would not be produced even when cleaved off from α^{Nb} -T-13 by trypsin. Another possible explanation for the observation is that the α^{Nb} -T-14 may have been released from the core by treatment with performic acid and then was further digested by chymotrypsin, resulting in the production of two individual

amino acids of α^{Nb} -T-14, ^{140}Tyr , and $^{141}\text{cysteic acid}$. However, detection of these amino acids on the map was unsuccessful. The substitution of Cys for Arg at $\alpha 141$ was, however, successfully verified by findings on CP-B digestion of the AE- α^{Nb} -chain and on pepsin digestion of the performic acid treated α^{Nb} -chain, as already described.

The patient with Hb Nb had no hepatosplenomegaly nor any hemolytic tendencies. This might be because of the structural stability of the Hb Nb molecule. However, the production ratio of the α^{Nb} -chain to total α -chains in the propositus was small even when the sum of the proportions of Hb Nb (13.1%) and small amounts of abnormal Hb A₂ and Hb F, which could not be determined, were added up. Therefore, the low production of the α^{Nb} -chain might be a reflection of α -globin gene expression. As an approach to clarifying this point, an analysis of the α -globin gene clusters of the propositus was performed, and revealed a normal duplicated α -globin gene in each haploid and the absence of more than four α -globin genes per diploid cell. More than the four α -globin genes are frequently seen in some racial groups (43–45).

According to Leibhaber et al. (46) and Orkin et al. (47), the two α -globin genes in a haploid called $\alpha 1$ - and $\alpha 2$ -globin gene in order from 3' to 5' of the DNA sequence do not equally contribute to α -mRNA production. The transcription ratio of the $\alpha 2/\alpha 1$ -globin gene varies within a range of from 3/2 to 3/1. When this observation is considered regarding the patient of Hb Nb, and with the assumption that one of the two $\alpha 1$ -globin genes per diploid cell is converted to an α^{Nb} -globin gene, an explanation for the low proportion of Hb Nb can be provided. A definitive explanation of the low α^{Nb} -chain production requires further study.

Acknowledgments

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