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Research Article

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Hemoglobin Yoshizuka (G10(108)β Asparagine → Aspartic Acid): a New Variant with a Reduced Oxygen Affinity from a Japanese Family

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ABSTRACT During the course of a survey, a new hemoglobin, designated hemoglobin Yoshizuka, has been encountered in a Japanese family. Clinically, mild anemia was noted in five of six heterozygous individuals but no other significant abnormalities were found. Hemoglobin Yoshizuka is characterized by the substitution of aspartic acid for asparagine at the tenth residue of the G helix in the β-chain. Reduced oxygen affinity with almost normal heme-heme interaction was found to be a property of this abnormal hemoglobin.

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INTRODUCTION

The recent construction of the atomic model of the hemoglobin molecule at high resolution by Perutz and his colleagues has opened the possibility of interpretation of the detailed properties of particular hemoglobin variants in terms of the molecular structures (1–3). As Perutz points out, replacements at the region of contacts between unlike subunits of the hemoglobin molecule in the quaternary structure may affect the oxygen affinity, the pattern of interchain interactions, and the equilibrium between tetramers, dimers, and monomers on which the respiratry function of the hemoglobin molecule depends (3). Contacts between unlike subunits are of two different kinds, α_1-β_1 and α_1-β_2 (2). The evidence strongly suggests the importance of the contacts α_1-β_2 for the transmission of heme-heme interaction. In hemoglobins Chesapeake (4), J Cape Town (5, 6), Yakima (7, 8), and Kempsey (9), the oxygen affinity is raised but it is lowered in hemoglobin Kansas (10). Diminished heme-heme interactions have been reported for those five replacements which lessen the stability of the contacts α_1-β_2 (3). Hemoglobin E (11, 12) is a case of an instability resulting from disturbance of the pattern of hydrogen bonds in the neighborhood of the α_1-β_1 contacts (3). Despite the lowered oxygen affinity in this variant, heme-heme interaction has been shown to be normal. It would be interesting to know whether the interaction between heme groups is affected only in the substitution at the α_1-β_2 contacts or in any of the replacements at the contacts between unlike subunits.

In the screening survey which has been conducted with the aim of detecting hemoglobinopathies, one family was found to be heterozygous for a new hemoglobin, designated hemoglobin Yoshizuka. In this paper we describe chemical characterization of this abnormal hemoglobin and its oxygen equilibrium properties.

METHODS

Hematological studies were done by standard methods (13). Hemolysates were prepared by the toluene method (14). The clear supernatant after centrifugation at 10,000 *g* for 20 min was used for further studies. Alkali-resistant hemoglobin was estimated using the 1 min denaturation test (15) and by the method of Huisman and Meyering (16). The test for thermolabile hemoglobin was according to Dacie et al. (17).

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Electrophoresis of hemoglobins was performed on thin-layer starch gel in pH 8.6 Tris-ethylenediaminetetraacetic acid (EDTA)-borate buffer at 17 v/cm for 3 hr (18) and the gels were stained with Amido Black 10B (Merck). The hemolysate (300 mg in 5 ml 0.05 M Tris-HCl buffer, pH 8.0) was chromatographed on a 2.8 × 42 cm column of diethylaminoethyl (DEAE)-Sephadex A-50 (Pharmacia) equilibrated overnight with the starting buffer (19). A linear gradient from pH 8.0–7.6 was established with a two-chamber vessel containing 1.5 liters of pH 8.0 buffer in the first chamber and 1.5 liters of pH 7.6 buffer in the second. The 10-ml fractions corresponding to individual hemoglobins were pooled and the quantitative data were obtained by establishing the extinctions of related fractions at 415 mμ. These values were divided by the total extinctions. Hemoglobin solutions were concentrated by ultrafiltration in vacuo using cellophane tubes (Visking, size 8/32) and the purity of each peak was ascertained by thin-layer starch-gel electrophoresis. For oxygen equilibrium studies, 600 mg of hemoglobin in 7.2 ml of pH 7.95 buffer was chromatographed on a 4 × 45 cm column of DEAE-Sephadex (effluents in the center section of the peaks were used without being concentrated). Subunit hybridization of purified hemoglobins was performed by dialyzing mixtures of equal volumes of solutions of hemoglobin A with canine hemoglobin and of hemoglobin Yoshizuka with canine hemoglobin against 0.24 M acetate buffer, pH 4.7 for 5 hr at 5°C followed by dialysis overnight against the Tris–EDTA–borate gel buffer (20).

Globins were prepared for chain separation with cold acid acetone (21). 160 mg of globin was dissolved in 16 ml of starting buffer consisting of 8 M urea made 0.05 M in 2-mercaptoethanol, 0.005 M in Na_2HPO_4, and adjusted to pH 6.7 with H_3PO_4 as described by Clegg, Naughton, and Weatherall (22). After dialysis for 3 hr at 21°C, the material was applied to a 2.0 × 10 cm column of carboxymethylcellulose (CM-cellulose) (Serva, capacity 0.71 mEq/g), washed with 50 ml of the starting buffer, and eluted by means of a Na^+-ion gradient exactly as described (22). Aminoethylation of the separated chains and the removal of urea, ethyleneimine, and salts were also made without modifications (22). Trypsin (Trypsin-TCA, Worthington) digestion of the aminoethylated chains was carried out in ammonium bicarbonate volatile buffer at pH 8.6–9.0 for 4 hr at 37°C (23). A comparison of the tryptic peptides of aminoethylated β-chains from hemoglobins A and Yoshizuka was made by the peptide-mapping technique of Ingram (24) with the chromatographic solvent system of Baglioni (pyridine: isoamyl alcohol: water, 30: 30: 35 by volume) (25) followed by staining with ninhydrin (0.25% in acetone) and with the reagents specific for bivalent sulfur compounds, arginine, histidine, tyrosine, and tryptophan (26). For a peptide separation, 13–14 mg of peptides were applied along a 16 cm line on Whatman No. 3MM paper. Descending chromatography was performed with the solvent of Baglioni (25). The peptide bands were located by cutting 0.5 cm guide strips and developing them in 0.5% ninhydrin in acetone. Using the developed strips as markers, the paper areas were cut out and sewn by means of zigzag stitching to a piece of Whatman No 3MM paper 56 cm long and 23 cm wide (27). Electrophoresis at pH 6.5 (pyridine: acetic acid: water, 25: 1: 224 by volume (24) was carried out at 2000 v for 120 min, after which peptides were located as described above, eluted into a test tube with 0.1 N ammonia water, and dried in vacuo. Aliquots corresponding to one-fourth of the mate-

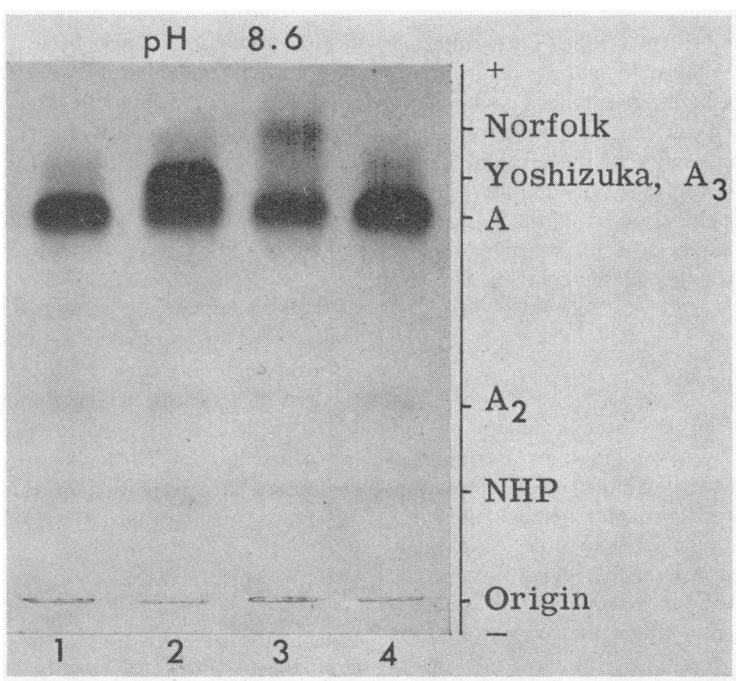

FIGURE 1 Thin-layer starch gel electrophoresis at pH 8.6 (Tris-EDTA-borate) of hemolysates from: (1) normal adult; (2) propositus of hemoglobin Yoshizuka; (3) heterozygote for hemoglobin Norfolk; (4) normal adult. Stained with Amido Black 10B. NHP, nonhemoglobin protein.

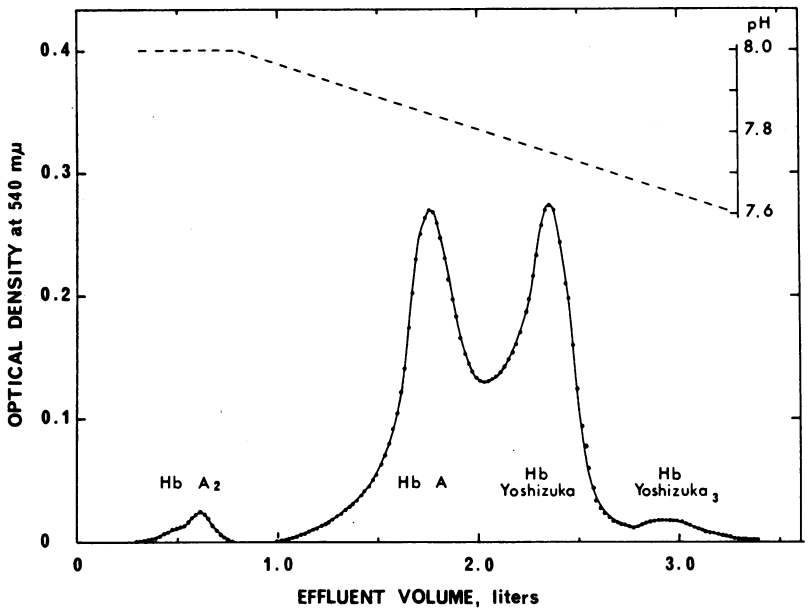

FIGURE 2 Chromatography of hemoglobins A_2, A, and Yoshizuka on a 2.8 × 43 cm column of DEAE-Sephadex using a gradient of 0.05 M Tris-HCl buffer from pH 8.0 to 7.6.

rial obtained were taken for amino acid analysis. Such samples were dried, redissolved in 6 N HCl, and hydrolyzed at 110°C for 24 hr in sealed evacuated tubes. Quantitative amino acid analyses were made on each peptide with the Hitachi model KLA-3B amino acid analyzer (28). Peptides (approximately 0.12 μmoles of β^{A}T12a and $\beta^{Yoshizuka}$T12a) were digested with leucine aminopeptidase (Sigma, aminopeptidase from hog kidney, Lot 127B-1210-1) for 3 hr at 37°C in 1 ml of 0.005 M Tris—HCl—0.005 M $MgCl_2$ buffer (29) at pH 8.5 containing 25 μg of enzyme. Amino acids derived from each peptide were analyzed as described above.

Oxygen equilibrium curves of hemoglobin were recorded automatically as successive deoxygenation curves by the method of Imai (30). The oxygen partial pressure in the sample was measured with a Beckman polarographic oxygen sensor (model 39065) and the percentage of oxygenated hemoglobin was estimated spectrophotometrically using monochromatic light at 564 mμ. The values were drawn continuously on the X-Y recorder chart. The temperature of the sample was measured by the thermister and controlled at 20°C within the variation of ±0.1°C by thermodules. The curve was reproduced very well with the standard error being maximum, about 3%, near the half saturation point. Before and after measurements, the visible absorption spectra of samples were recorded using a Beckman DK-2 self-recording spectrophotometer. Methemoglobin in the sample was estimated before measurements by the method of Evelyn and Malloy (31) and afterward calculated from extinction coefficients given by Benesch, Macduff, and Benesch (32). Methemoglobin formed during the entire procedure was below 15% except between pH 6.0 and 6.5 where about ⅓–½ of the hemoglobin was apparent in the ferric form. From the spectral curves just before and after measurements, we judged methemoglobin formation during the oxygen equilibrium studies to be only small. The percentage of saturation, *y*, was calculated by using the measured transmittance change, and subsequently the optical densities, A, by using the formula,

$$\frac{y}{100} = \frac{A_{deoxy} - A}{A_{deoxy} - A_{oxy}}$$

in which A_{deoxy} and A_{oxy} are the optical densities of deoxygenated and oxygenated hemoglobin at 564 mμ. These oxygen equilibria were studied within 1 wk after the blood collection.

Sedimentation velocity measurements on oxyhemoglobin solutions A and Yoshizuka were made with a Spinco model E ultracentrifuge with schlieren optics at 56,100 rpm. Experiments were done at a temperature of 20°C and a protein concentration of 0.1 g/100 ml of 0.1 M phosphate buffer, pH 7.4. The sedimentation coefficients were calculated from the peak positions in the schlieren patterns and are given in Svedberg units. The corrections for the density and viscosity of the solutions were not made here.

RESULTS

Family study. The propositus was a 56 yr old man who sought medical advice because of anorexia and insomnia. His past health had been good. On examination he showed no significant physical abnormalities except for hypertension. Hematological studies were disclosed as follows: hemoglobin, 14.7 g/100 ml; red cell count, 4.12 million/cmm; hematocrit, 37.0%; reticulocytes, 0.7%; white cell count, 6800/cmm (differential count normal); median corpuscular fragility, 0.44% NaCl; icterus index 5. An abnormal hemoglobin, designated hemoglobin Yoshizuka, was detected in his blood, in his four children, and in one of his siblings examined.

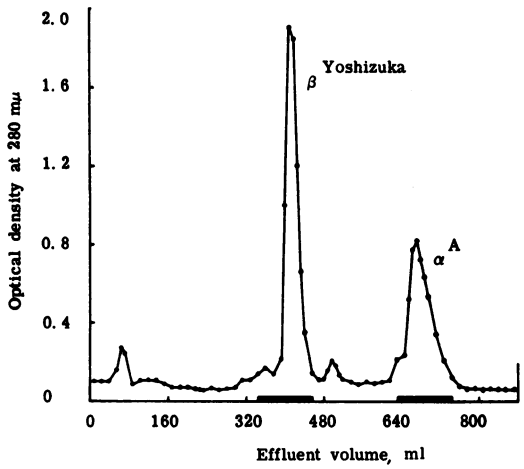

FIGURE 3 Chromatography of globin Yoshizuka (160 mg) on a 2 × 10 cm column of CM-cellulose in 8 M urea-2-mercaptoethanol buffers.

The amount of abnormal hemoglobin and the hemoglobin A_2 level were similar from one heterozygous individual to another judging from the electrophoretic pattern. The alkali-resistant hemoglobin in heterozygotes constituted between 1.20 and 2.37% of the total. Heterozygous carriers, except for the propositus, were only slightly anemic. The erythrocyte morphology was normal and no illness was associated with the presence of hemoglobin Yoshizuka.

Physicochemical characteristics of hemoglobin Yoshizuka. On starch-gel electrophoresis at pH 8.6, hemoglobin Yoshizuka migrated more rapidly than hemoglobin A. Its mobility was approximately the same as that of hemoglobin A_2 (33) and less than that of hemoglobin Norfolk (18, 34) (Fig. 1). Chromatography of the hemolysate showed that the normal and abnormal hemoglobins were present in about equal quantities (43.9% A and 50.6% Yoshizuka) (Fig. 2). The quantity of hemoglobin A_2 (2.1%) was within the normal range of 1.8–3.2% estimated from DEAE-Sephadex chromatography (13). Hemoglobin A_2 did not separate from hemoglobin Yoshizuka during chromatography and the fourth fraction (3.4%) appeared likely to be an abnormal analogue of hemoglobin A_2 (16).

Hemoglobin Yoshizuka was not alkali resistant; it was not precipitated by incubation at 50°C for 2 hr; and no inclusion bodies formed in red cells after incubation with brilliant cresyl blue. Both oxygenated and deoxygenated solutions of hemoglobin Yoshizuka had normal visible absorption spectra between 450 and 650 mμ.

Dissociation and recombination with canine hemoglobin indicated that the altered electrophoretic mobility was due to an abnormality of the β-chain. The two chains of hemoglobin Yoshizuka were separated cleanly on CM-cellulose chromatography (Fig. 3). A comparison of the tryptic peptide map of the aminoethylated β-chain from hemoglobin Yoshizuka with that from hemoglobin A showed that peptide β^AT12a (22) was missing in hemoglobin Yoshizuka and that a new peptide was present which was displaced anodally to the corresponding peptide in the normal aminoethylated β-chain peptide map (Fig. 4). By testing the maps with the reagent specific

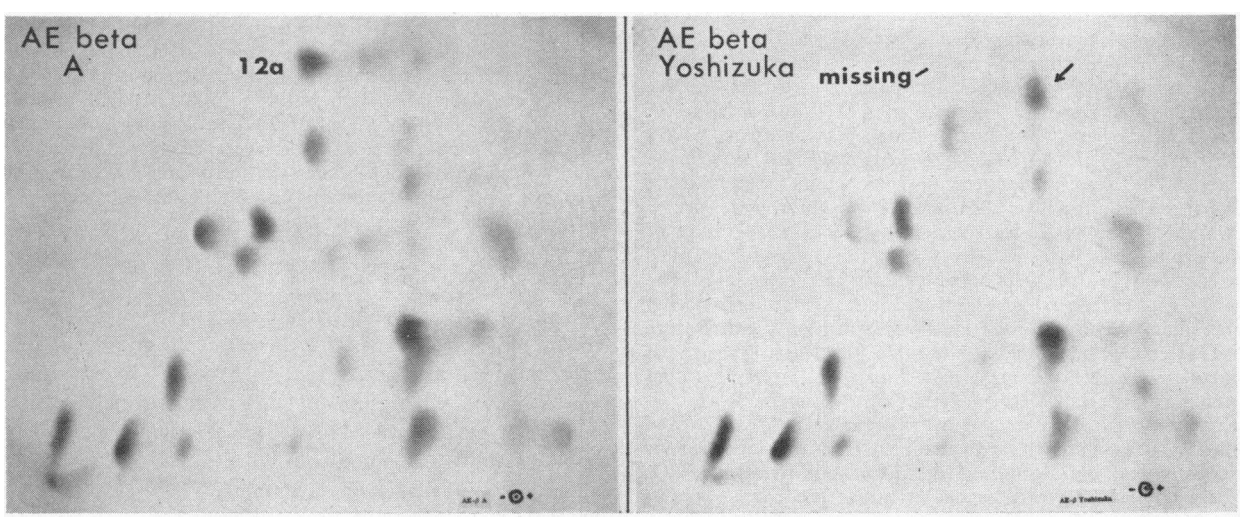

FIGURE 4 Tryptic peptide maps of aminoethylated (AE) β-chains of hemoglobins A and Yoshizuka. Chromatography was carried out first in the vertical direction in pyridine: isoamyl alcohol: water (30: 30: 35, by volume) followed by electrophoresis at pH 6.5 in the horizontal direction. The peptide spot in $\beta^{Yoshizuka}$ peptide map, which is not observed in β^A-peptide map, is indicated by an arrow.

for bivalent sulfur compounds, the new peptide, $\beta^{Yoshizuka}$ T12a, was shown to be sulfur containing. β^{A}T12a was also positive for this reagent. The presence of a small residual amount of β^{A}T12a presumably arose from traces of hemoglobin A_2 and A which remained after purification of the variant. Table I shows the results of amino acid analyses of the β^{A}T12a and $\beta^{Yoshizuka}$T12a which were purified by both preparative paper chromatography and electrophoresis. It will be seen that the two analyses after acid hydrolysis are identical which suggests that the loss of a positive charge in the abnormal peptide results from the replacement of asparagine normally found in β^{A}T12a by aspartic acid. Analyses after digestion with enzyme confirmed this conclusion. Substitution of aspartic acid for asparagine in β^{A}T12a (position 108) satisfactorily accounts for the charge difference on starch gel electrophoresis.

Oxygen equilibrium characteristics of hemoglobins. The oxygen equilibria of both unfractionated hemolysates and hemoglobins after elution from the chromatographic column were measured (Fig. 5). The oxygen affinity of the hemolysate containing about 50% of hemoglobin Yoshizuka was lower than that of the hemolysate from the normal adult. The data show that purified hemoglobin Yoshizuka has about a 1.6-fold lower oxygen affinity than hemoglobin A at 20°C, pH 7.46. The pH dependence of the oxygen equilibrium shows that hemoglobin Yoshizuka has a substantially lower oxygen affinity than hemoglobin A between pH 6.0 and 8.0 (Fig. 6). The values of n in Hill's equation (35) for hemoglobin Yoshizuka are similar to those for hemoglobin A: for hemoglobin Yoshizuka, n is 2.35–3.00; for hemoglobin A, n is 2.30–2.86. It was difficult to

TABLE I

Amino Acid Composition of β-T12a Peptides from Hemoglobins A and Yoshizuka

Amino acids	β-T12a(A)		β-T12a(Yoshizuka)	
	Acid	Enzyme	Acid	Enzyme
Leucine	3.00 (3)	3.00 (3)	3.00 (3)	3.00 (3)
Glycine	1.17 (1)	1.11 (1)	1.23 (1)	1.10 (1)
Aspartic acid	1.07 (1)		1.13 (1)	0.81 (1)
Asparagine		0.76 (1)		
Valine	1.99 (2)	1.89 (2)	2.06 (2)	1.84 (2)
S-aminoethyl-cysteine	0.77 (1)	*	0.84 (1)	*

Comparison of results after hydrolysis at 110°C in 6 N HCl and after digestion with leucine aminopeptidase. The numbers in parentheses refer to the theoretical number expected.
* Not analyzed.

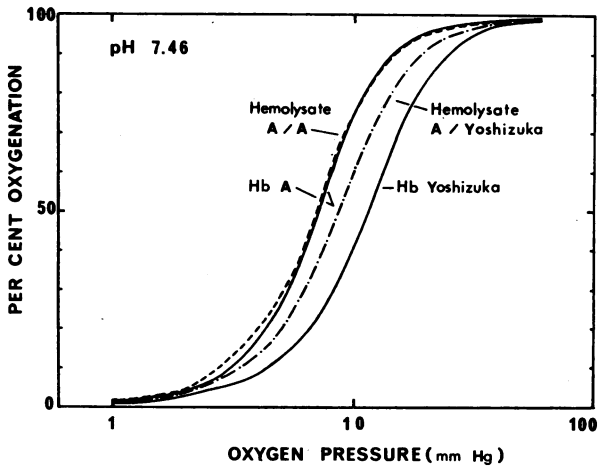

FIGURE 5 Oxygen equilibria of unfractionated hemolysates from a normal adult (A/A) and from a heterozygote for hemoglobin Yoshizuka (A/Yoshizuka) and from those of hemoglobins A and Yoshizuka isolated from the same hemolysate. The data were obtained at 20°C. Hemoglobin concentrations were 0.1% in 0.1 M phosphate, pH 7.46. Methemoglobin comprised 14–15% both of hemoglobin A and of hemoglobin Yoshizuka whereas unfractionated hemolysates A/A and A/Yoshizuka contained less than 3% ferrihemoglobin.

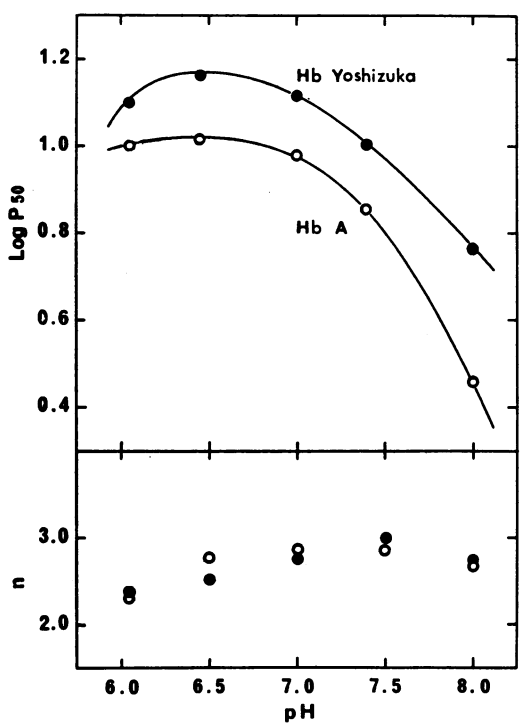

FIGURE 6 The pH dependence of the oxygen equilibrium of hemoglobins A and Yoshizuka. The values of n for hemoglobin A are 2.30–2.86 and those for hemoglobin Yoshizuka are 2.35–3.00.

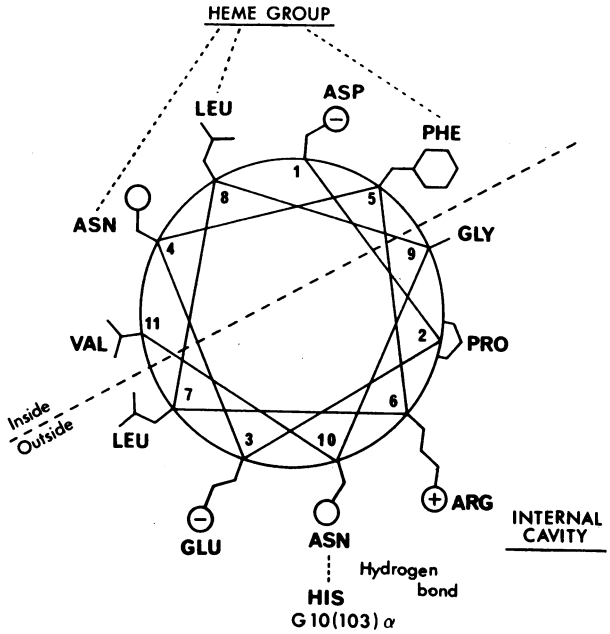

FIGURE 7 Diagram of Gβ-helix residues 1 through 11 is shown by the courtesy of the author and the publisher (10). The β-chain heme is adjacent to the inside part making contacts with residues G4β, G5β, and G8β (2). An α-chain may interact with the opposite side of the helix. G10β is thought to be hydrogen bonded to G10α to form one of the α_1-β_1 contacts.

avoid appreciable formation of methemoglobin during the purification procedures and dialysis. The tendency apparently increased in acid solutions. This is sufficient to bring about a slight increase in oxygen affinity and also a decrease in the values of n. For these reasons, the authors believe the minor variations of the n values observed are probably not significant. The curve of purified hemoglobin A which contained 14.2% methemoglobin of the total is shaped and positioned like that of the unfractionated normal hemolysate which did not contain an appreciable amount of the ferric form. This suggests that influences of the purification procedures upon oxygenation curves at this pH are only small (Fig. 5). The slope of the lines in Fig. 6, calculated by $\Delta \log P_{50}/\Delta$ pH, yields a convenient measure of the Bohr effect (36). The ratio is about 0.36 for hemoglobin Yoshizuka in the physiological pH range at 20°C as compared with 0.52 for hemoglobin A.

Ultracentrifugation. On sedimentation under the condition used (temperature 20°C, pH 7.4, protein concentration 0.1%) both hemoglobins A and Yoshizuka migrated as a single symmetrical peak with an s_{20} of 4.54S.

DISCUSSION

Hemoglobin Yoshizuka differs from normal hemoglobin in its electrophoretic mobility, its chromatographic behavior, and its oxygen equilibrium characteristics. These differences appear to be associated with a single amino acid substitution occurring in the β-chain, e.g., aspartic acid replaces asparagine at position 108. This is the tenth residue in the G helix of the β-chain according to the tentative atomic model of oxyhemoglobin molecule by Perutz (37, 38). Fig. 7 shows the approximate relative positions of residues in the G helix and in the representation described by Bonaventura and Riggs (10). Residues G4(102)β, G5(103)β, and G8(106)β are directed inward into the heme crevice making contacts with the heme group. As Perutz has described, part of the G helix of the β-chain may make contacts with the homologous segment of the α-chain. The main chain carbonyl of asparagine G10β which is replaced by aspartic acid in hemoglobin Yoshizuka is thought to be hydrogen bonded to histidine G10α. This bond involves one of the α_1-β_1 contacts by using the numbering system by Perutz (2). Two other strongly basic residues, arginine G6β and lysine G6α, are probably oriented near the side chain of G10β. The amino acid substitution in hemoglobin Yoshizuka might produce decreased oxygen affinity either through direct electrostatic interactions between the G helix and the heme group or through altering interchain relationships. The direct interionic effects analogous to those between the heme groups and the side chains found to be substituted in hemoglobins M do not seem likely since the coordinates described by Perutz would indicate that the side chain of G10β is directed away from the heme group (2). The introduction of the negatively charged carboxyl group into the basic region might cause rearrangement of these residues in the central cavity due to polar interactions near the contact α_1-β_1. A relatively small difference in electrophoretic mobility might be due to a rise in pK of histidine G10α. Nevertheless, the data at present are insufficient to explain clearly why the oxygen affinity is lowered in hemoglobin Yoshizuka.

As Perutz and his colleagues point out, the functional unit of hemoglobin is the whole tetrameric molecule rather than the dimeric (2). When one of the heme groups of a hemoglobin molecule is oxygenated, the affinity for oxygen of the three other groups is increased (39). For the expression of these interchain interactions, both contacts, α_1-β_1 and α_1-β_2 are essential. However, the crystallographic results which the Cambridge group has found suggest that the contact α_1-β_2 is of primary importance for several reasons (2). Hemoglobin Kansas has a low affinity for oxygen and reduced heme-heme interaction which is associated with the tendency to dissociate into subunits. These changes are due to the replacement of asparagine by threonine near the contact α_1-β_2 (10). The discovery of other variants, hemoglobins Chesapeake (4), J Cape Town (6), Yakima (7, 8), and Kempsey (9) has strengthened the importance of the G

TABLE II

Abnormal Hemoglobins with Altered Heme Function Other Than Hemoglobin M

Designation	Amino acid substitution	Oxygen affinity	Heme-heme interaction	Bohr effect	Contact affected	Clinical symptoms	Reference
Chesapeake	FG4(92)α Arg → Leu	Very high	n = 1.3	Normal	α_1–β_2	Polycythemia	4, 41
J Cape Town	FG4(92)α Arg → Gln	Very high*	n = 1.9*	Normal*	α_1–β_2	Polycythemia	5, 6
E	B8(26)β Glu → Lys	Low	Normal	Normal	α_1–β_1	Hemolytic anemia in homozygote	12
Yakima	G1(99)β Asp → His	Very high	n = 1.0	Normal	α_1–β_2	Polycythemia	7, 8
Kempsey	G1(99)β Asp → Asn	High*	n = 1.1*	Normal*	α_1–β_2	Polycythemia	9
Kansas	G4(102)β Asn → Thr	Low	n = 1.3	Normal	α_1–β_2	Cyanosis	10
Yoshizuka	G10(108)β Asn → Asp	Low	Normal	Reduced	α_1–β_1	None	(This paper)
Hiroshima	H21(143)β His → Asp	Very high	n = 2.0–2.6	Normal	β_1–β_2	Polycythemia	30
Rainier	HC2(145)β Tyr → His	High‡			HC2β–FG5β	Polycythemia	42
Lepore	$\alpha_2(\delta$–$\beta)_2$	High	Normal	Normal		Hemolytic anemia	43

* Data for hemolysate.
‡ Data for whole blood.

helix and the FG nonhelical segment at the contacts α_1-β_2 for the transmission of heme-heme interactions. In hemoglobin Yoshizuka, heme-heme interaction remained normal even though its oxygen affinity is abnormally low. Another variant with properties similar to those of hemoglobin Yoshizuka is that of hemoglobin E (12). This is also a case of the replacement near the contact α_1-β_1 (3). It is very interesting to note that heme-heme interactions are affected by the substitutions at the region of contacts between α_1 and β_2 chains but have not so far been shown to be impaired in the replacements at the contact α_1-β_1 (Table II).

Rosemeyer and Huehns (40) have pointed out that the binding of oxygen contributes to the tendency of hemoglobin to dissociate. This occurs across the contacts α_1-β_2 and α_2-β_1 leading to the formation of one α_1-β_1 and one α_2-β_2 dimer. It has been observed that oxygenation of hemoglobin Kansas is accompanied by a pronounced dissociation into subunits (10). These evidences support the interpretation that the regions of the α_1-β_2 (α_2-β_1) contact have a strong influence both on the subunit dissociation and on the nature of the oxygenation process. The observation of the sedimentation coefficient clearly shows that the altered oxygen affinity in hemoglobin Yoshizuka is not directly related to the degree of subunit dissociation.

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