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Hemoglobin Yakima: I. Clinical and Biochemical Studies *

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Abstract. Three members of a family who have erythrocytosis and a new hemoglobin, designated hemoglobin Yakima, are described.

The abnormal hemoglobin is characterized by the substitution of histidine for aspartic acid at residue 99 in the β-chain.

Of three possible structure-function relations which would account for the increased oxygen affinity of hemoglobin Yakima, only two seem likely. These are: (a) an intrachain shift in the normal relations between the F and G helices and the heme group, or (b) an effect of the substituted side chain at a region of contact between nonpolar residues of the α- and β-chains which favors the oxyhemoglobin quarternary structure.

Introduction

The familial occurrence of erythrocytosis has been ascribed to polycythemia rubra vera (1), benign familial erythrocytosis (2), and hemoglobinopathy due to heterozygosity for hemoglobin Chesapeake (3). A clear genetic mechanism is apparent only in the case of hemoglobin Chesapeake which is transmitted as an autosomal codominant; the abnormal hemoglobin has an increased oxygen affinity and has been characterized chemically as due to the substitution of leucine for arginine at the 92nd residue [1] of the α-chain.

We have found a new hemoglobin, designated hemoglobin Yakima, which is associated with erythrocytosis in three heterozygous individuals. Clinical studies of this family and chemical characterization of the abnormal hemoglobin are reported here. Studies showing increased oxygen affinity of hemoglobin Yakima (4) have also been done. The similar structural and functional alterations in hemoglobins Chesapeake and Yakima suggest an important role for the region between the F and G helices of both α- and β-chains in determining normal oxygen affinity.

Methods

Hematologic studies were done by standard methods. Blood volume and erythrocyte survival were measured using ^{51}Cr (5). Hemolysates were prepared by mixing 1 volume of washed, packed erythrocytes, 1 volume of water, and 0.4 volume of toluene for 10 min. The clear supernatent after centrifugation at 6000 *g* for 30 min was used for further study. Electrophoresis of samples containing 10 mg/ml of hemoglobin was done at 6 v/cm for 4 hr with starch gel in pH 8.3, Tris-EDTA-borate buffer (6), and the gels were stained with benzidine (7). DEAE-Sephadex chromatography using a gradient of Tris-HCl buffer from pH 8.0–7.1 (8) permitted separation of hemoglobin components. These were concentrated by ultrafiltration in vacuo and the fractions, which included hemoglobins A_2, Yakima, A, and A_3, were identified by electrophoresis. Subunit hybridization of chromatographically purified hemoglobins was done by dialyzing mixtures of equal volume of 20 mg/ml solutions of hemoglobin A with canine hemoglobin and of hemoglobin Yakima with canine hemoglobin for 12 hr against 0.05 M sodium acetate buffer, pH 4.65, at 4°C, followed by dialysis against the Tris-EDTA-borate gel buffer (9).

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[1] Abbreviations used for amino acid residues are: Asp, aspartic acid; Asn, asparagine; Asx, aspartic acid or asparagine; Arg, arginine; Glu, glutamic acid; His, histidine; Leu, leucine; Phe, phenylalanine; Pro, proline; Val, valine.

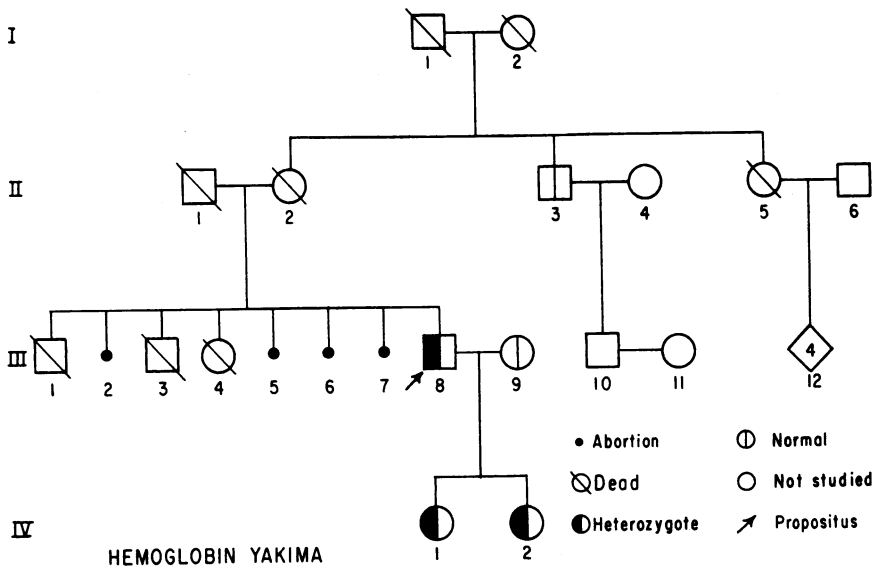

FIG. 1. PEDIGREE OF THE PROPOSITUS. ○, female; □, male; ◇, sex unknown.

A tryptic hydrolysate of the abnormal β-chain was prepared by conversion of hemoglobin Yakima to globin with cold acid-acetone (10), separation of the β-chains using a column of carboxymethyl cellulose in 8 M urea (11), aminoethylation of the β-chains with ethylenimine (12), and hydrolysis with trypsin in a solution buffered with triethylamine at pH 8–9 for 4 hr at room temperature (13).

The tryptic peptides were separated on a column of Spinco 15 A resin (0.9 × 17 cm) using a linear gradient of pyridine–acetic acid developer from pH 3.1 to pH 5.0 with continuous recording of ninhydrin color of the eluate at 570 mμ (12). The peptides were further purified by rechromatography of each zone on a column of Dowex AG50W-X2 (0.9 × 60 cm, 270–325 mesh, Bio-Rad Laboratories, Richmond, Calif.) with the same developers. After hydrolysis for 22–72 hr at 110°C in vacuo, quantitative amino acid analyses were made on each peptide with the Spinco model 120 amino acid analyzer modified to include a long-path photometer (14). The hydrolyzing agent was 6 N HCl with 9 mg/100 ml of phenol added to protect tyrosine.

Enzymic hydrolysis with carboxypeptidases A (Worthington Biochemical Corp., Freehold, N. J., DFP-treated, 3 × crystallized, 1750 U/ml) and B (585 U/ml) was carried out according to Guidotti, Hill, and Konigsberg (15). The leucine aminopeptidase (10 mg/ml), kindly provided by Dr. Robert L. Hill, was used as described by Schroeder and coworkers (16). In order to degrade the abnormal βT-11 peptide from hemoglobin Yakima and obtain the peptide His-Pro-Glu-Asn (Fig. 5) for further study, we employed all three enzymes as follows: a mixture of the peptide (approximately 1 mg in 1 ml of water), 0.1 ml of 0.025 M $MgCl_2$, and 30 μl of leucine aminopeptidase, after adjustment to pH 8.5 with Tris buffer, was placed in a 37°C oven. After 18 hr incubation, 10 μl of carboxypeptidases A and B was added and the mixture, after incubation for 4 hr at room temperature, was adjusted to pH 3.0 and applied to a column of Dowex AG50W-X2 under the same conditions as described above for rechromatographing tryptic peptides. The purified peptide His-Pro-Glu-Asn was subjected to a one-step Edman degradation procedure (17) for identification of the *N*-terminal amino acid.

Results

Family study. The propositus, III-8 in Fig. 1, was born in 1923. His father, II-1, an orphan, died at the age of 68 of a heart attack. His mother, II-2, died at the age of 72 of carcinoma of the kidney. She was reported to have had a "ruddy complexion" and severe, recurrent epistaxis. Of her eight pregnancies, three resulted in stillborn children, four in miscarriages, and only the eighth was successful. The proband's maternal uncle, II-3, is 68 yr of age and well; his maternal aunt, II-5, died at age 30 during childbirth. All of the second generation (II 1-6) were born in Sweden and nothing is known of their progenitors.

The propositus was first studied by one of us (E. E. O.) in 1954, because erythrocytosis had been found during a routine annual examination. His daughters, IV-1 and IV-2, were found to have erythrocytosis on routine laboratory studies in 1964 and the following studies were begun in 1966. All three subjects have been completely

TABLE I

Hematologic values in family with hemoglobin Yakima

Pedigree number*	Birth date	Dates studied	Hb	Rbc	Hct	Rbc Mass
			g/100 ml	*$\times 10^6$/mm^3*	*%*	*ml/kg*
III-8‡	1923	1954–66	16.5–22.9	5.0–7.3	45.5–55.2	39.7
III-9	1923	1966	12.8	3.7	37.0	
IV-1	1949	1964–66	16.1–18.2	5.3–6.0	47.0–54.5	35.3
IV-2	1951	1965–66	15.9–17.7	5.8–6.5	46.0–55.2	

Abbreviations: Hb, hemoglobin; Rbc, erythrocyte; Hct, hematocrit.
* Pedigree numbers correspond to Fig. 1.
‡ Propositus, treated with 4–10-mc doses of ^{32}P at 6–24-month intervals from 1954–65.

asymptomatic. They all showed mild rubor, but no other abnormality on physical examination. Hematologic values obtained in our laboratory [2] are presented in Table I. Red cell indexes, leukocyte, reticulocyte, and thrombocyte counts in the propositus and both daughters have been normal, and arterial gas studies (4) have shown no evidence of hypoxia. Erythrocyte survival was normal ($t_{\frac{1}{2}}$ = 28.5 days) in the propositus.

[2] Earlier blood studies from the records of their family physicians show evidence of significant erythrocytosis as early as age 3 yr in IV-1 and 6 yr in IV-2.

Starch gel electrophoresis of hemolysate from the three affected members (III-8, IV-1, and IV-2) demonstrated a new hemoglobin with a mobility just cathodal to hemoglobin A at pH 8.3, as illustrated in Fig. 2. No abnormal hemoglobin was detected in hemolysate from subjects III-9 or II-3.

Studies of the abnormal hemoglobin. A representative DEAE-Sephadex chromatogram is presented in Fig. 3. Hemolysate from the propositus and both daughters gave comparable results for hemoglobin A_2 (2.5–3.15%), hemoglobin Yakima (36.9–38.5%), and hemoglobin A (45.8–51.1%).

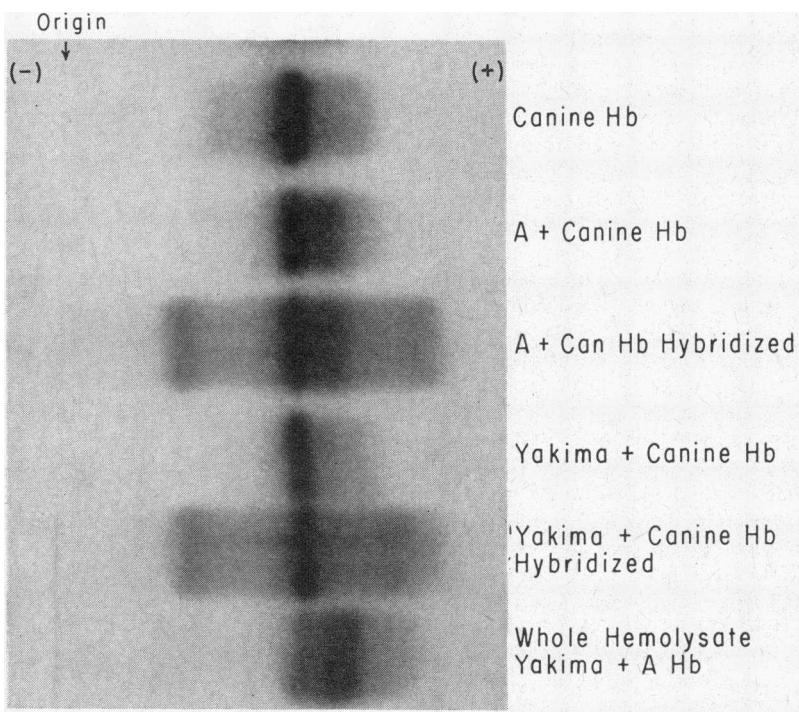

FIG. 2. HEMOGLOBIN ELECTROPHORESIS ON STARCH GEL, TRIS-EDTA-BORATE BUFFER (pH 8.3), AT 6 V/CM FOR 4 HR. Stained with benzidine.

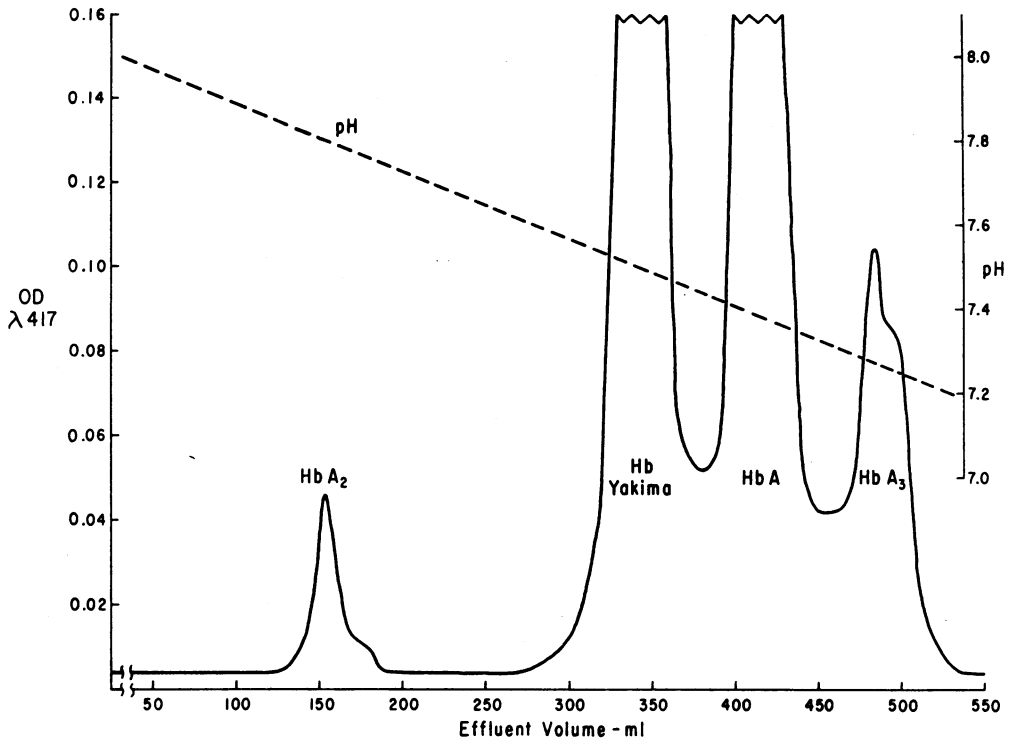

FIG. 3. DEAE-SEPHADEX CHROMATOGRAM DONE 2 WK AFTER PREPARATION OF WHOLE HEMOLYSATE FROM SUBJECT IV-2. Recovery of hemoglobin fractions: A_2, 3.15%; Yakima, 38.53%; A, 48.48%; and A_3, 9.85%.

The amount of hemoglobin A_3 varied with the age of the sample and ranged from 8.3 to 14.8%.

The results of subunit hybridization of chromatographically purified hemoglobin A and hemoglobin Yakima with canine hemoglobin are illustrated in Fig. 2. They demonstrate a normal $\alpha_2{}^A\beta_2{}^{Can}$ product and a slow $\alpha_2{}^{Can}\beta_2$ product in the hybridization of hemoglobin Yakima with hemoglobin canine. We therefore concluded that the slow electrophoretic mobility of hemoglobin Yakima was caused by a change in the β-chain.

Chromatography of the tryptic peptides from hydrolysis of the aminoethyl β-chain gave the peptide pattern illustrated in Fig. 4. Compared with the pattern for a normal β-chain from hemoglobin A, it differs in two respects: (*a*) the zone normally occupied by peptide βT-11 is missing, and (*b*) another peptide (Zone XVI) is present where normally there is none. All zones were purified by rechromatography and subjected to amino acid analysis, the results of which are presented in Table II. Comparison of the abnormal Zone XVI with the normal peptide βT-11 reveals one extra histidine residue and one less residue of aspartic acid or asparagine. Amino acid analysis of all other tryptic peptides showed compositions identical with those of the normal β-chain.

The extra histidine in the abnormal βT-11 peptide could have replaced aspartic acid residue No. 99 or asparagine residue No. 102. Since HCl hydrolysis converts asparagine to aspartic acid, we employed enzymic methods in an attempt to release asparagine as such in the hydrolysate. Leucine aminopeptidase acted on the *N*-terminal end of the abnormal βT-11 peptide, releasing equivalent amounts of leucine, histidine, and valine, but was stopped at the next residue because of the well-known blocking action of the adjacent proline. The carboxypeptidases acted on the carboxy terminal end of the peptide, removing arginine and phenylalanine, but would go no further. Guidotti and associates (15) reported the same behavior with the normal βT-11 peptide and attributed this behavior to the presence of an asparagine residue (No. 102) which sometimes blocks carboxypeptidase in other peptides.

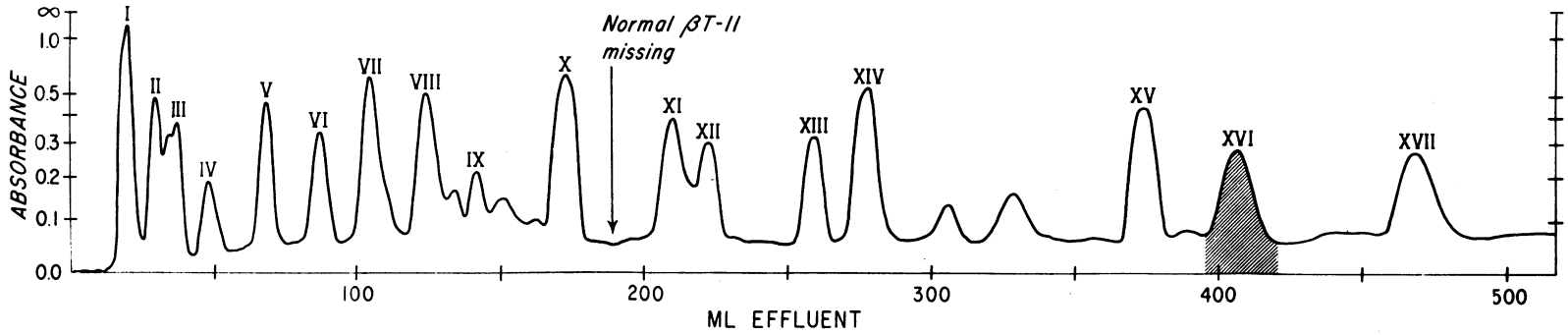

FIG. 4. PEPTIDE PATTERN OF TRYPTIC HYDROLYSATE OF AMINOETHYLATED β-CHAINS FROM HEMOGLOBIN YAKIMA. The absorbance of the reaction products of ninhydrin and peptides was measured continuously at 570 mμ. The position of the abnormal βT-11 peptide is indicated by cross-hatching.

TABLE II

*Amino acid composition of tryptic peptides of aminoethylated β-chain of hemoglobin Yakima**

Amino acid	βT-1 X‡	βT-2 VIII	βT-3 III	βT-4 VII	βT-5 I	βT-6 XIII	βT-7 XV	βT-8 X	βT-9 VI	βT-10 XII	βT-11 ABN XVI	βT-12a V	βT-12b XVII	βT-13 II	βT-14 VII	βT-15 XIV
Tryptophan		0.47		0.34												
Lysine	1.06	1.02			0.95	1.00	0.96	1.00	1.00	1.01			1.02	0.98	1.00	
S-Aminoethylcysteine										0.64		0.83				
Histidine	0.90						1.11		0.97	0.98	*2.00*		2.13		1.02	1.03
Arginine			1.04	1.02							1.04					
Aspartic acid			2.01		2.99				3.07	1.08	*1.01*	1.05			1.05	
Threonine	1.02	0.99		0.95	1.02					2.00				0.98		
Serine		0.82			1.75				0.94	0.73						
Glutamic acid	2.08		1.97	1.03	0.99					0.96	0.95			2.93	0.10	
Proline	0.88			1.04	2.08						0.94			2.09		
Glycine		1.02	2.98	0.12	2.00		0.99		2.13	1.17		1.04	1.03		1.09	
Alanine		1.94	0.98	0.10	1.00		0.94		1.92	0.97			0.96	2.00	3.93	
Valine	0.77	1.01	3.00	1.92	1.02	1.00			0.96		0.87	1.93	0.93	1.02	2.84	
Methionine					0.89											
Leucine	1.03	1.02	1.02	2.09	1.02				4.00	1.89	1.05	2.97	0.94		1.08	
Tyrosine				0.95										0.98		0.96
Phenylalanine					2.92				1.02	0.96	1.03		0.99	0.99		

* Ratios of amino acids recovered after hydrolysis in vacuo with 6 N HCl for 22 hr at 110°C. Cysteine was measured as *S*-aminoethylcysteine.
‡ Roman numerals designate zone from chromatogram shown in Fig. 4. Those which contained more than one peptide were rechromatographed.

The residual peptide formed by the combined action of leucine aminopeptidase and the carboxypeptidases (see Methods) on the normal βT-11 was eluted from the Dowex-50 column at a pH of approximately 3.1. Amino acid analysis of the peptide gave residues of Asx 1.98, Pro 1.02, and Glu 1.29. With similar treatment of abnormal βT-11 from hemoglobin Yakima, the residual peptide was eluted from the column much more slowly at a pH of approximately 3.9, indicating a greater positive charge; its amino acid analysis revealed residues of Asx 1.07, Pro 0.82, Glu 1.05, and His 1.02. After one Edman degradation, amino acid analysis of the remaining peptide showed Asx 1.00, Pro 1.01, Glu 0.99, and His 0.19 residues. Since more than 80% of the histidine, which acts on the *N*-terminal amino acid, was removed by the Edman procedure it may be concluded that a histidine has replaced the aspartic acid residue at position 99 (Fig. 5) rather than asparagine at position 102.

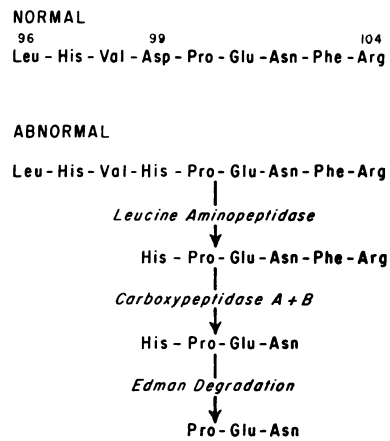

FIG. 5. COMPARISON OF AMINO ACID SEQUENCES OF NORMAL AND ABNORMAL (YAKIMA) βT-11 PEPTIDES, AND THE RESULT OF ENZYMATIC AND EDMAN DEGRADATION OF THE LATTER. *His* occurs in place of *Asp* at position 99 which corresponds to G 1 in Perutz' numbering system (20).

Discussion

Erythrocytosis due to an abnormal hemoglobin was first reported by Charache, Weatherall, and Clegg (3) in a family in which 16 members of three generations were heterozygous for hemoglobin Chesapeake. Although Charache et al. could demonstrate no elevation in serum erythropoietin in their propositus, the increased oxygen affinity, shown to be a property of the purified hemoglobin Chesapeake, would be expected to result in relative tissue hypoxia and a secondary erythrocytosis. Our family and one discovered in Australia by Reed and coworkers [3] manifested similar degrees of erythrocytosis in individuals heterozygous for hemoglobin Yakima and hemoglobin Kempsey, respectively. Preliminary studies by Adamson [4] of 24-hr urine samples from our propositus show high normal values for erythropoietin compatible with a secondary erythrocytosis.

The increased oxygen affinity shared by these three abnormal hemoglobins (3, 4)[3] may have a similar structural explanation, and may give insight into the normal structure-function relations which account for the property of reversible combination with oxygen found in normal hemoglobin.

[3] Reed, C. S., R. Hampson, B. Brimhall, R. T. Jones, M. J. Novy, M. J. Edwards, and R. D. Koler. Hemoglobin Kempsey. In preparation.

[4] Adamson, J. 1966. Personal communication.

With this goal in mind, it is interesting to compare the sites that are substituted. Using the numbering system presented by Perutz (18) that brings into register homologies in structure shared by myoglobin and the α-, β-, and γ-chains of human hemoglobin, the alterations are:

hemoglobin, Chesapeake, α-chain: arginine → leucine at position FG 4;
hemoglobin Yakima, β-chain: aspartic acid → histidine at position G 1;
hemoglobin Kempsey, β-chain: aspartic acid → asparagine at position G 1.

The clustering of all three mutations at the region between the F and G helices, and the fact that interchain contacts between the nonpolar groups of the α- and β-chain involve this region (20), suggest three possible mechanisms.

The first is analogous to the direct interionic effect between the heme group and side chains of amino acid residues found to be substituted in methemoglobins (21) and does not seem likely, since the coordinates published by Perutz (18) would indicate that the side chains of FG 4 and G 1 are directed away from the heme groups.

The second is an intrachain distortion of the normal relations between the F and G helices due to either steric hindrance that does not accommodate the substituted R group, or new bonding relations that result from a different side chain at position G 1 (β99). As Perutz, Kendrew, and

Watson (20) point out, proline occurs near the *N*-terminal portion of many helical regions, both in myoglobin (4 of 8 helices) and in the hemoglobin chains of man (14 of 23 chains), and of horse (8 of 15 chains). In most of these helices, proline occurs as the second residue, and the first position is occupied by serine, threonine, aspartic acid, or asparagine. A hydrogen bond is formed between the oxygen of the OH or the COO^- group on the β-carbon with the α-NH group of the residue at position 4 of the helix. Histidine at position G 1 in hemoglobin Yakima would not form such an intrahelical bond. The G helix may, therefore, be longer or shorter than normal, depending upon whether the CO group on the α-carbon of FG 5 forms the usual intrahelical hydrogen bond with the α-NH group at G 4. In either case, the normal relations of the heme iron to the F and G helices might be altered. This intrachain distortion would probably not apply to hemoglobin Kempsey however, because the substituted asparagine at G 1 would be expected to form an intrahelical hydrogen bond as it does in the case of the E helix in horse β-chain (18).

The third possible mechanism is an effect on quarternary structure due to interference with normal points of contact between nonpolar residues of the α- and β-chain. Perutz has illustrated a region of contact (Fig. 5 in reference 18) between the side chain of arginine at FG 4 in the normal α-chain and the indole ring of tryptophan at C 3 in the normal β-chain. As Charache and associates (3) have pointed out, the replacement of arginine by a nonpolar side group, leucine, in hemoglobin Chesapeake could alter the normal property of the tetramer which allows "the α and β chains [to] slide past each other during the spatial rearrangements of oxygenation and deoxygenation." An accordion-like increase of 7A between the heme irons of the two β-chains in the deoxygenated as compared to the oxygenated forms of hemoglobin has been described by Muirhead and Perutz (19). Any steric hindrance offered by the substituted side chains of these three abnormal hemoglobins might constrain the interchain relations in a way that would favor the quarternary form characteristic of oxyhemoglobin and thus increase oxygen affinity.[5]

Another possible effect on chain-chain interactions might be to decrease the likelihood of symmetrical dissociation of the oxygenated tetramer to $\alpha\beta$-dimers. A mechanism of exchange of $\alpha\beta$-subunits between oxygenated and deoxygenated tetramers has been postulated by Benesch, Benesch, and Tyuma (22) to account for the cooperative binding of oxygen by the four heme groups that results in the normal sigmoid oxygen dissociation curve. Such subunit exchange occurs between hemoglobin variants that have normal oxygen dissociation curves, whereas myoglobin and isolated α- or β-chains that have hyperbolic curves do not enter into exchange reactions with tetrameric hemoglobin A. Benesch and coworkers (22) interpret these data as evidence for "an exchange of subunits in such a way that the conformation of the 'attacking' oxygenated $(\alpha\beta)^0$ dimer is imposed on another $\alpha\beta$ dimer derived from a deoxygenated tetramer. The unstable intermediate $(\alpha\beta)^0$ $(\alpha\beta)^*$ has . . . high oxygen affinity" and is readily converted to a completely oxygenated tetramer. The relationship between the ratio of oxyhemoglobin to reduced hemoglobin and the partial pressure of oxygen can be used to derive the value of n in Hill's equation (3), which is usually regarded as a measure of the heme-heme or interchain interaction. It is of considerable interest that estimates of n for hemoglobins Chesapeake (3) and Yakima (4) approach 1.0; thus, no cooperative effect in oxygen binding is suggested.

Moreover, a fourth abnormal hemoglobin, hemoglobin Kansas (23), which has a low oxygen affinity, has been shown by Riggs and Bonaventura (24) to have a low heme-heme interaction as measured by n in Hill's equation and to dissociate readily into half molecules upon oxygenation. In keeping with the hypothesis of a key structure-function role of the FG and G helix regions in determining normal chain-chain interactions, hemoglobin Kansas has been characterized (24) as a β-chain substitution of threonine for asparagine acid at the fourth residue of the G helix (β102).

It would appear likely, therefore, that the side

[5] *Note added in proof.* A similar conclusion has been reached from studies of different spectra of oxyhemoglobin and deoxyhemoglobin Chesapeake at 242 mμ, reaction of SH groups with iodoacetamide and kinetic studies of the combination rate of CO with reduced Hb Chesapeake by Nagel, R. L., T. H. Gibson, and S. Charache. 1967. Relation between structure and function in hemoglobin Chesapeake. *Biochem.* **6**: 2395.

chains of amino acid residues at position FG 4 in the α-chain and G 1 and G 4 in the β-chain play an important function in normal interchain or heme-heme interactions. A decreased allosteric effect as measured by n in Hill's equation is shared by the abnormal hemoglobins that have either a decreased or an increased oxygen affinity. By analogy, it is predicted that since hemoglobin Kansas is more readily dissociated to half molecules, the hemoglobins that have an increased oxygen affinity will be less likely to undergo dissociation to $\alpha\beta$-dimers. Studies designed to test this hypothesis are underway.[6]

Acknowledgments

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