JCI The Journal of Clinical Investigation

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J Clin Invest. 1974;53(1):320-328. https://doi.org/10.1172/JCI107553.

Research Article

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ABSTRACT Examination of 13 members of a Filipino family revealed that 6 had erythrocytosis inherited as a simple autosomal dominant trait. Application of several electrophoretic and chromatographic tests failed to reveal the presence of an abnormal hemoglobin in hemolysates from affected individuals. However, measurements of oxygen dissociation curves using whole bloods, dialyzed hemolysates, and 2,3-diphosphoglyceric acid-stripped hemolysates clearly showed that affected persons had an abnormal hemoglobin characterized by a high affinity for oxygen. Compositional analyses of all tryptic peptides from the β-chains of the proband revealed a valyl-methionyl ambiguity in βT_{12a}. Blockage of lysyl residues and subsequent tryptic hydrolysis at arginyl residues permitted the isolation of fragments containing residues 105 through 146. Automatic sequence analysis of the fragments demonstrated the presence of both valine and methionine in nearly equal proportions at position β109. This new hemoglobin variant is designated Hb San Diego (β109(G11) Val → Met).

INTRODUCTION

Detection and structural characterization of the majority of abnormal human hemoglobins has been facilitated by their abnormal electrophoretic and chromatographic properties. In many cases (1–5), clinically detected erythrocytosis has provided a most important clue to the existence of an underlying molecular lesion. The discovery of Hb Olympia (5) demonstrated that abnormality of charge need not be typical of hemoglobins associated with erythrocytosis and that the presence of an abnormal hemoglobin may be deduced from the results of functional studies performed in vitro.

In this report, we describe a second electrophoretically and chromatographically silent variant which, like Hb Olympia, is associated with erythrocytosis and has a high affinity for oxygen and whose structural abnormality, the substitution of valine by methionine in its β-chains, was defined only after detailed structural analysis. This variant is designated Hb San Diego.

METHODS

Hematologic, electrophoretic, and chromatographic techniques. Routine hematologic data were obtained using standard techniques. Hemolysates were prepared according to Drabkin (6) and subjected to electrophoresis in starch gels using Tris-EDTA-borate buffers ranging in pH from 8.3 to 9.1, as well as a Tris-citrate buffer of pH 8.65. Carboxyhemoglobins were treated with *p*-chloromercuribenzoate (PCMB) [1] (7) and electrophoresed in starch gels using both phosphate (pH 7.0) and Tris-EDTA-borate (pH 8.6) buffers. Globin chains were subjected to electrophoresis in 0.1 M barbital, 6 M in urea, and 0.1 M in 2-mercaptoethanol, as described by Chernoff and Pettit (8).

Chromatography of whole hemoglobins was carried out in a 2.5 × 45 cm column of carboxymethyl (CM) Sephadex

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Received for publication 22 May 1973 and in revised form 31 July 1973.

[1] *Abbreviations used in this paper:* DPG, 2,3-diphosphoglyceric acid; PCMB, *p*-chloromercuribenzoate; TPCK, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone.

(C-50) using a starting buffer of 0.05 M phosphate, pH 6.7, and a limiting buffer of 0.05 M phosphate, pH 7.4 (9). All buffers contained 100 mg of KCN/liter.

Globin was prepared by dropwise addition of unfractionated lysates to cold HCl-acetone (10). Separation of α- and β-chains was achieved following the application of globin to 2 × 10-cm columns of Whatman CM-52 cellulose equilibrated with 8 M urea buffers made 0.05 M in 2-mercaptoethanol (11).

Peptide mapping. 73 mg of β-chains from the proband was aminoethylated as described by Jones (12), with the exception that urea was replaced by an equimolar amount of guanidine-HCl. The aminoethylated chains were dialyzed free of salts and reactants and recovered by lyophilization. 55 mg of the β-chains was then digested with trypsin (trypsin-L-(tosylamido-2-phenyl)ethyl chloromethyl ketone [TPCK], Worthington Biochemical Corp., Freehold, N. J.) according to Clegg, Naughton, and Weatherall (13). Digestion was terminated by lyophilization.

2-mg portions of the tryptic digest were subjected to paper electrophoresis in a buffer of pyridine, acetic acid, and water (25:1:224 by volume), pH 6.4 (14), followed by descending chromatography in a solvent of pyridine, isoamyl alcohol, and water (7:7:6 by volume). A second system, utilizing an electrophoretic buffer of pyridine, acetic acid, and water (1:1:78 by volume), pH 4.7 (13), and a chromatographic solvent of *n*-butanol, acetic acid, water, and pyridine (15:3:12:10 by volume) (15) was also employed. Peptides were stained with 0.2% ninhydrin in acetone and by reagents specific for tyrosine, arginine, sulfur-containing amino acids, tryptophan, and histidine in various sequences as outlined by Easley (16).

For preparative purposes, peptide maps were made using 5–6 mg of the β-chain digest. After peptides were located by staining with 0.02% ninhydrin in acetone, they were eluted from strips cut from the maps (17), hydrolyzed in 6 N HCl at 108°C in sealed capillary tubes for 24 or 72 h, and analyzed on a Beckman 120B amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.).

Reduction and S-pyridylethylation of β-chains. To prevent oxidation of the cysteinyl residues and the consequent cross-linking of the peptide chains during chemical manipulations, the thiol groups were alkylated with 4-vinylpyridine (18). 40 mg of native β-chains from the proband was dissolved in 3 ml of buffer (6 M guanidine-HCl, 0.13 M Tris, 0.1 mg/ml EDTA) and adjusted to pH 7.7 with HCl. 23 mg (0.15 mmol) of dithioerythritol was added and allowed to react at room temperature for 2 h, after which 45 μl (0.45 mmol) of 4-vinylpyridine was added and allowed to react for 1.5 h. The reaction was terminated by the addition of 1 ml of 88% formic acid, and the alkylated β-chains were separated from salts and reactants by passage through a 2 × 40 cm column of Sephadex G-25 (fine) equilibrated and developed with 9% (vol/vol) formic acid. The chains were recovered from solution by lyophilization.

Citraconylation of lysyl residues and tryptic digestion at arginyl residues of the alkylated β-chains. Specific tryptic cleavage of the β-chains at arginyl residues requires that lysine residues first be chemically modified to render them inert to tryptic digestion. This was accomplished by reaction of the lysyl residues with citraconic anhydride in mildly alkaline solution. The *S*-pyridylethyl β-chains (35 mg) were dissolved in 3 ml of 9% formic acid and titrated, using a pH-stat, to pH 8.5 with 12 N NaOH. Citraconic anhydride (400 mg) was added in aliquots of 25 μl at a rate sufficient to maintain pH in the range of 7.5–8.5 on the pH-stat, with 12 N NaOH in the syringe. Total reaction time at room temperature was approximately 30 min. The chains were dialyzed overnight against 4 liters of water adjusted to pH 8.5 with pyridine.

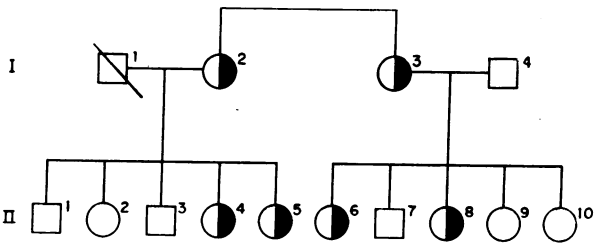

FIGURE 1 Pedigree of family with Hb San Diego, illustrating dominant mode of inheritance of erythrocytosis. □, ○, normal; ◑, carriers of Hb San Diego.

The dialyzed protein solution was warmed to 37°C on the pH-stat and adjusted to pH 8.0. The chains were then subjected to tryptic hydrolysis at arginyl residues for 10 min after the addition of 0.5 mg of trypsin (Worthington Biochemical Corp., 2× crystallized). The reaction was terminated by lowering the pH to 2.5 with 88% formic acid. The tryptic fragments were then left at room temperature at pH 2.5 for 3 h to allow acid-catalyzed cleavage of the citraconyl-blocking groups from the lysyl side chains (19).

Separation of the tryptic fragments. The mixture of tryptic fragments was lyophilized, dissolved in 3 ml of 9% formic acid, and fractionated on a 2.5 × 115 cm column of Sephadex G-50 (fine) equilibrated and developed with 9% formic acid at a flow rate of 45 ml/h. Each fraction contained 15 ml. The fractions comprising the peak containing the suspected abnormal fragment and its normal counterpart were pooled, lyophilized, and rechromatographed on the same column under the conditions described above.

Automated sequence analysis. Appropriate fragments were partially sequenced using a Beckman 890C Sequencer (Beckman Instruments, Inc.), following the method of Edman and Begg (20) as modified by Hermodson, Ericsson, Titani, Neurath, and Walsh (21). Degradation was carried out for a total of 10 cycles, establishing the sequences of both the normal and abnormal chains from positions 105 through 114.

Measurements of oxygen equilibria. Oxygen dissociation curves were obtained for whole blood at 37°C as described by Lenfant, Ways, Aucutt, and Cruz (22). Additional curves were determined using dialyzed hemolysates, employing the automated procedure of Imai et al. (23). These latter measurements were made on 0.2% solutions of freshly prepared hemoglobin in 0.1 M phosphate buffers at pH ranging from 6.15 to 7.84. Alkaline Bohr effects, defined as $\Delta \log P_{50}/\Delta pH$ from pH 7.0 to 7.4, were read from graphs relating log P_{50} to pH. Values for heme-heme, or allosteric, interaction (Hill's constant, n) were measured as the slope of the lines relating log $[Y/(1-Y)]$ to log P_{O_2} at log $[Y/(1-Y)] = 0$, where Y is the fractional saturation of hemoglobin by oxygen (24).

Removal of 2,3-diphosphoglyceric acid. 5 ml of an hemolysate (Hb concentration 2.8%) from erythrocytotic subject II-5 (see Fig. 1) and an identical volume of an hemolysate (Hb concentration 3.5%) from a normal sib (II-2) were stripped of DPG by passage through a 2.5 × 41 cm column of Sephadex G-25 (medium) equilibrated and developed with 0.1 M NaCl at a flow rate of 20 ml/h

TABLE I

Data on Hematology and Hemoglobin Function for Members of the Family with Hb San Diego

Individual	Age	Hb	Hematocrit	Hb A_2	Hb F*	P_{50}‡	Hill's n	Hb San Diego
		g/100 ml	%	%	%	*mm Hg*		
I-2§	37	18.0	52	3.25	1.46	6.81	2.24	+
II-1	15	15.8	42	2.46	0.90	11.21	2.96	−
II-2	13	15.0	40	2.65	0.75	11.12	2.89	−
II-3	11	12.8	38	2.76	0.59	11.07	2.93	−
II-4	9	16.8	47	2.00	0.84	6.49	2.06	+
II-5	5	15.8	46	2.24	2.11	6.69	2.13	+
I-3‖	35	18.1	50	2.02	1.77	7.29	2.12	+
I-4	?	14.2	42	1.75	0.53	12.00	2.95	−
II-6	15	17.2	50	2.04	1.34	6.93	2.10	+
II-7	10	14.0	40	1.91	1.03	11.50	2.92	−
II-8	9	16.8	48	1.69	1.62	6.80	2.02	+
II-9	6	13.6	40	2.45	1.17	11.30	2.95	−
II-10	3	13.2	37	1.70	1.00	11.55	3.01	−

* By alkaline denaturation.

‡ P_{50} values measured at pH 6.90, 20°C, using dialyzed hemolysates.

§ Proband.

‖ Sister of proband.

(25). Effluent fractions that contained hemoglobin were pooled and dialyzed first against 0.002 M Tris to remove NaCl and then against 0.1 M bis-Tris-HCl, pH 7.0. All dialyses were carried out at 4°C for 18–24 h. The hemoglobin samples were diluted with water and 0.1 M bis-Tris-HCl, and oxygen dissociation curves were measured at 20°C on 0.2% solutions of hemoglobin, 0.085 M in bis-Tris, pH 7.11 (26). Values for heme-heme interaction were measured as described above.

RESULTS

Family report. The proband, a 37-yr-old Filipino woman, presented to the Hematology Clinic of the San

TABLE II

Amino Acid Compositions of Tryptic Peptides from the

Peptide no.	T_1	T_2	T_3	T_4	T_5	T_{5ox}	T_6	T_7	T_{7-8}
Asp			1.86 (2)		3.00 (3)	3.14 (3)			
Thr	0.94 (1)	0.90 (1)		0.88 (1)	0.93 (1)	0.97 (1)			
Ser		0.82 (1)			1.83 (2)	1.77 (2)			
Glu	1.99 (2)		1.99 (2)	1.00 (1)	1.18 (1)	1.00 (1)			
Pro	0.89 (1)			0.96 (1)	2.07 (2)	2.03 (2)			
Gly		0.96 (1)	3.11 (3)		1.89 (2)	2.03 (2)		0.99 (1)	1.02 (1)
Ala		1.91 (2)	0.97 (1)		1.08 (1)	1.05 (1)		0.97 (1)	1.00 (1)
Val	0.83 (1)	1.00 (1)	2.94 (3)	1.91 (2)‡	0.94 (1)	1.00 (1)	0.93 (1)		
Met					0.72 (1)	0.68 (1)			
Leu	1.00 (1)	1.06 (1)	1.00 (1)	2.07 (2)	0.95 (1)	1.00 (1)			
Tyr				0.82 (1)					
Phe					2.79 (3)	2.75 (3)			
Trp§		1.00 (1)		1.00 (1)					
Lys	1.04 (1)	0.99 (1)			0.85 (1)	1.00 (1)	1.00 (1)	1.04 (1)	2.00 (2)
SAE Cys									
His	0.89 (1)							1.00 (1)	0.98 (1)
Arg			0.94 (1)	1.03 (1)					

* Values in parenthesis indicate the amino acid compositions of the corresponding peptides in the isolated β^A chain.

‡ Valine determined after 72 h of hydrolysis.

§ Tryptophan detected in eluate from short column of amino acid analyzer and on peptide maps by staining with the Ehrlich reagent.

Diego Naval Hospital in 1966 with complaints of headaches and dizziness, and was found to have an elevated hematocrit (52%) and hemoglobin level (18 g/100 ml). Physical examination was normal except for enlargement of the left lobe of the thyroid. A ^{51}Cr red cell mass was performed and was mildly elevated at 31.5 ml/kg (normal range: 24–29 ml/kg). Radioiodine uptake was normal at 24 h, and thyroid scanning demonstrated a cold nodule in the left lobe. The proband was restudied in 1968 because of persistently elevated hematocrit and hemoglobin level. At that time the ^{51}Cr red cell mass was 36 ml/kg with normal plasma and total blood volumes. Platelet and white cell counts, chest X ray, pulmonary function studies, and arterial O_2 saturation were normal, and a search for pelvic, renal, hepatic, and cerebellar tumors was negative. During the evaluation of this patient, it was independently discovered that her sister also had erythrocytosis. Investigation for underlying cardiopulmonary, renal or neoplastic disease was likewise unrevealing.

The presence of an abnormal hemoglobin in this family was suspected when a P_{50} value of 16.4 mm Hg was obtained using whole blood from the proband (normal values range from 26 to 28 mm Hg). This suspicion was confirmed by studies of oxygen binding using dialyzed and DPG-stripped hemolysates (see "functional studies"). Extension of the study to other members of the family indicated that several children of the proband and her sister also suffered from hemoglobinopathic erythrocytosis (Fig. 1). Hematologic data as well as values for functional parameters of the hemoglobins in family members are listed in Table I.

Electrophoretic and chromatographic studies of hemoglobin. Starch-gel electrophoresis of whole hemoglobins produced no signs of an abnormal hemoglobin in any member of the family under study. Furthermore, electrophoresis of globin chains after removal of heme or after treatment of hemoglobins with PCMB merely served to substantiate the electrophoretic identity of hemoglobins from normal and abnormal family members. In addition, neither chromatography of hemolysates on CM-Sephadex nor of globin chains on CM-cellulose in 8 M urea yielded any indication of the presence of an abnormal hemoglobin.

Identification of the structural abnormality. Peptide maps prepared from tryptic digests of the aminoethylated β-chains from the proband were identical in appearance to those produced by aminoethylated β^A chains (Fig. 2); no differences were apparent after the application of stains specific for tyrosine, sulfur-containing amino acids, tryptophan, histidine, and arginine. Amino acid analysis of all tryptic peptides from the β-chains of the proband produced the results summarized in Table II. With the exception of βT_{12a}, all peptides were identical in composition to those of β^A chains. Nonintegral values for valine (1.45) and methionine (0.22) were obtained for βT_{12a}. The sum of methionine and valine fell far short of the expected value of 2.0, presum-

*β-Chains of an Individual with Hb A and Hb San Diego**

T_8	T_{8-9}	T_9	T_{10}	T_{11}	T_{12a}	T_{12b}	T_{13}	T_{14}	T_{15}
	2.97 (3)	2.90 (3)	1.09 (1)	2.09 (2)	1.06 (1)			1.00 (1)	
			1.81 (2)				0.93 (1)		
	1.08(1)	0.89 (1)	0.92 (1)						
			1.02 (1)	1.04 (1)			2.93 (3)		
				0.98 (1)			2.01 (2)		
	1.99 (2)	2.00 (2)	0.93 (1)		1.09 (1)	1.04 (1)		0.99 (1)	
	2.10 (2)	2.00 (2)	1.00 (1)			1.09 (1)	1.96 (2)	4.01 (4)	
	1.13 (1)	1.06(1)		0.94 (1)	*1.45 (2)*	0.93 (1)	0.99 (1)	2.87 (3)‡	
					0.22(0)				
	3.85 (4)	3.91 (4)	1.90 (2)	0.91 (1)	3.00 (3)	1.00 (1)		0.97 (1)	
							0.91 (1)		0.88 (1)
	0.99 (1)	1.06 (1)	1.03 (1)	1.00 (1)		1.00 (1)	1.00 (1)		
1.00 (1)	1.84 (2)	1.00(1)	0.88 (1)			0.94 (1)	0.97 (1)	1.00 (1)	
			0.75 (1)		0.64 (1)				1.00 (1)
	1.14 (1)	0.93 (1)	1.01(1)	0.84 (1)		1.94 (2)		0.97 (1)	
				1.00 (1)					

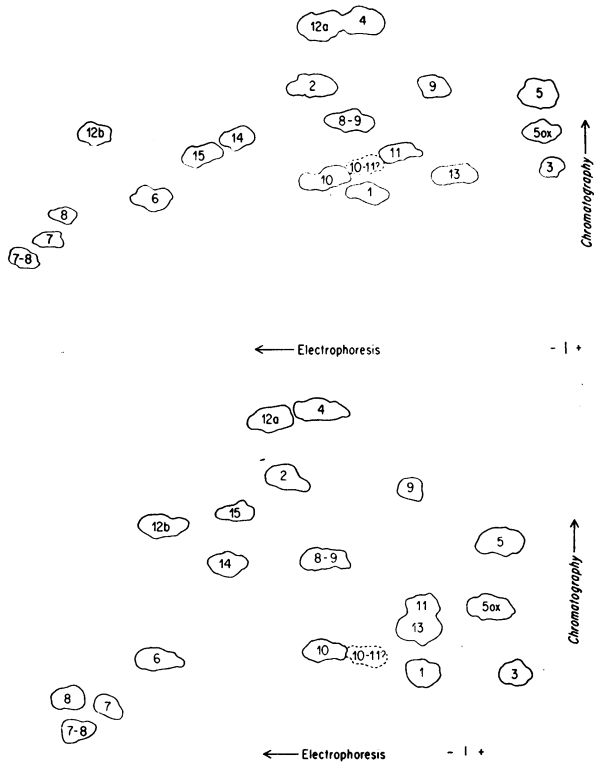

FIGURE 2 Tracings of peptide maps produced by a tryptic digest of aminoethylated β-chains from the proband. Top: electrophoresis at pH 4.7, chromatography in *n*-butanol, acetic acid, water, and pyridine. Bottom: electrophoresis at pH 6.4, chromatography in pyridine, isoamyl alcohol, and water. No differences were noted between these maps and similar maps prepared from aminoethylated β^{A} chains.

ably because hydrolyses were performed in sealed capillary tubes in the presence of small amounts of air, conditions known to favor oxidation of methionine. Additional hydrolysates prepared from other maps yielded values for valine of 1.45 and 1.41 and for methionine of 0.17 and 0.38 residues. Given the sequence of normal βT_{12a},

Leu-Leu-Gly-Asn-Val-Leu-Val-Cys^{AE},
105 109 111

the above findings are consistent with the interpretation that approximately half of the hemoglobin from the proband is abnormal and that this abnormal hemoglobin, Hb San Diego, differs from Hb A by the substitution of methionine for valine at position 109 or 111 of its β-chains. Note that the presence of aminoethylcysteine, the carboxy-terminal residue of βT_{12a}, would mask the presence of methionine on peptide maps stained for sulfur-containing amino acids, thus preventing detection of the abnormal βT_{12a} by any means short of amino acid analysis.

Sequence analysis. To facilitate sequence analysis of the region in which the structural abnormality of the $\beta^{San\ Diego}$ chain was known to reside, the β-chains of the proband were subjected to specific cleavage at arginyl residues. Cleavage at arginine in position 104 would free the amino-terminal end of βT_{12a} and thus provide a suitable starting point for automated sequence analysis.

Tryptic digestion at the arginyl residues of the mixture of normal and abnormal acylated β-chains was expected to yield fragments of four different sizes: βT-I (residues 1–30), βT-II (residues 31–40), βT-III (residues 41–104), and βT-IV (residues 105–146).

Gel filtration of the products of digestion partially separated the three major fragments (βT-I, βT-III, and βT-IV), and produced a large salt peak that was presumed to contain the smallest fragment, βT-II (Fig. 3*a*). Fractions were pooled as indicated in Fig. 3*a* and lyophilized. The material comprising pool B (and presumed to contain the βT-IV fragments from both normal and abnormal chains) produced the elution profile illustrated in Fig. 3*b* when recycled through the same column. Fractions were pooled as indicated and lyophilized, yielding a total of 6 mg of material.

At no point in the analytical or preparative procedures was there separation of normal and abnormal β-chains, peptides, or fragments. Hence the material subjected to further analysis consisted primarily of a mixture of two homologous fragments containing residues 105 through 146 from the β^{A} and $\beta^{San\ Diego}$ chains of the proband.

Analysis of the purified material produced the amino-terminal sequence Leu-Leu-Gly-Asn-, a sequence identical to that of the β^{A} chain from residues 105 through 108. A second sequence of the Phe-Phe-Glu-Ser- was also apparent but comprised only about 5% of the total amount of material present. This sequence, which corresponds to residues 41 through 44 of the β-chain, is attributable to the presence of a small amount of fragment βT-III. A trace of the βT-I fragment, with the amino-terminal

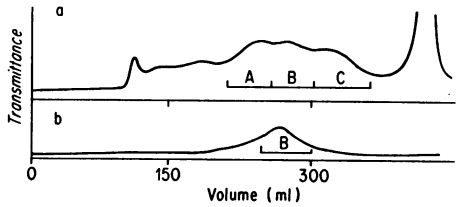

FIGURE 3 (*a*) Elution profile produced by gel filtration on Sephadex G-50 (fine) of the tryptic digest of the *N*-citraconyl-*S*-pyridylethyl β-chains after removal of the citraconyl groups (see text for details). The ordinate (transmission at 253 nm) is in arbitrary units. Fractions were pooled as indicated by the solid bars and numbered as shown. (*b*) Rechromatography of pool B (containing fragment βT-IV) on the same column; all other conditions were identical to those described for part *a*.

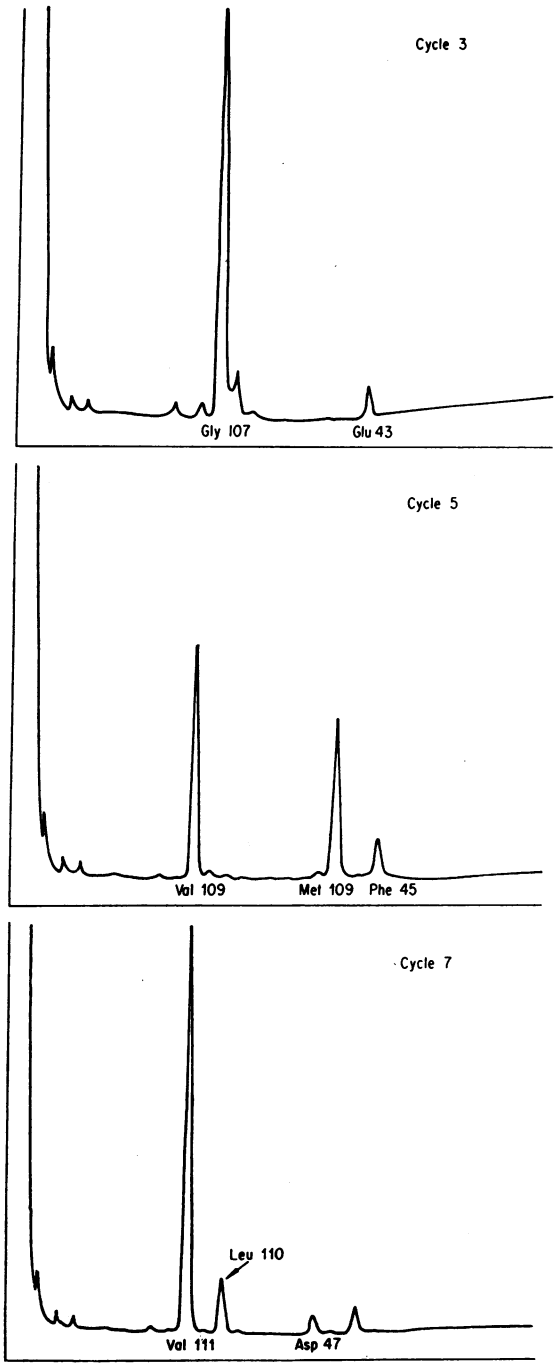

FIGURE 4 Gas chromatograms of representative cycles of the sequence analysis carried out on the material whose purification is illustrated in Fig. 3*b*. In each cycle, 6% of the total sample was chromatographed (range setting 1 $\times 10^{-9}$ amp, Packard model 7300 gas chromatograph, Packard Instrument Co., Inc., Downers Grove, Ill.). The amino acids derived from the contaminating βT-III fragment (containing residues 41 through 104) are labeled in addition to those of the major sequence. Cycle 5 shows that Val is replaced by Met at position 109 in approximately half of the βT-IV fragments. By cycle 7, some "overlap" due to incomplete reaction in previous cycles is apparent, and the leucine thus generated is indicated.

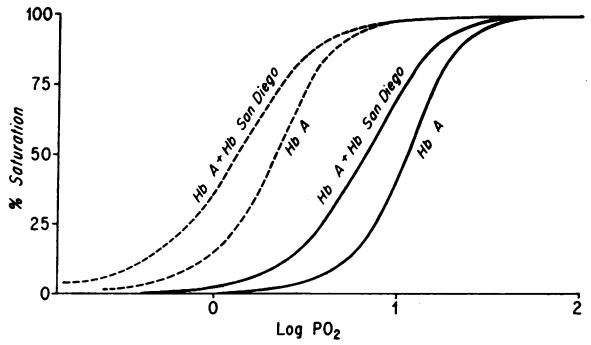

FIGURE 5 Oxygen dissociation curves determined on unfractionated hemoglobins of subjects II-5 (Hb A + Hb San Diego) and II-2 (Hb A only) both before (solid lines) and after (broken lines) removal of DPG. Curves for unstripped hemoglobins were measured at pH 6.90, 20°C; curves for stripped hemoglobins were measured at pH 7.11, 20°C. For unstripped hemoglobin from subject II-2, $P_{50} = 11.12$ mm Hg, $n = 2.89$; comparable measurements for subject II-5 yielded values of $P_{50} = 6.69$, $n = 2.13$ (see Fig. 7).

sequence of Val-His-Leu-Thr-, was also detected in some cycles. In all but the fifth cycle the sequence of the normal β-chain, from residues 105 through 114, was observed in yields approximating 95% of the expected total. On the fifth cycle, however, both valine and methionine were detected in significant amounts (Fig. 4). Comparison of yields with those produced by the phenylthiohydantoin amino acids in the other cycles (e.g., Gly 107β and Val 111β, whose relative yields are also illustrated in Fig. 4) indicates that the combined amounts of valine and methionine in cycle five equal that expected for a residue in the predominant sequence. From these data it was concluded that the β-chains of the proband are an approximately equimolar mixture of normal and abnormal chains and that the valyl residue normally occupying position 109 is replaced by a methionyl residue in the abnormal chains of Hb San Diego.

Functional studies. P_{50} values for dialyzed hemolysates, measured at pH 6.90, 20°C, ranged from 6.49 to 7.29 mm Hg for erythrocytotic individuals and from 11.07 to 12.00 mm Hg for normal family members (Table I; Fig. 5). All oxygen dissociation curves were monophasic, suggesting that hybrid tetramers ($\alpha_2^A\beta^A\beta^{SD}$) are present in solutions of Hb A plus Hb San Diego. Bohr effects for hemolysates from normal and affected individuals fell within the normal range (Fig. 6), while Hill's constant ranged from 2.02 to 2.24 among persons with Hb San Diego and from 2.89 to 3.01 in normal family members (Table I; Fig. 7).

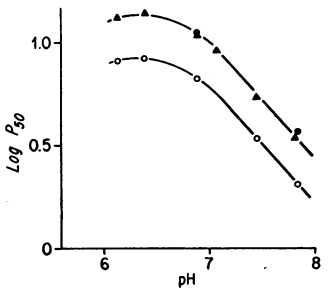

FIGURE 6 Bohr effects in hemolysates containing Hb A only (upper curve; ● subject II-2; ▲ normal subject N. E., unrelated to family under study) and Hb A + Hb San Diego (lower curve; ○ subject II-5). The Bohr effects, measured between pH 7.0 and 7.4 are −0.52 for Hb A alone and the hemolysate containing Hb A and Hb San Diego.

Oxygen dissociation curves produced by the hemoglobins of normal and abnormal individuals after removal of DPG showed the expected shifts in P_{50} values (Fig. 5). Before DPG removal, the P_{50} value for normal subject II-2 (Fig. 1) was 11.12 mm Hg, whereas that for abnormal subject II-5 was 6.69 or 60.1% of the P_{50} value for the normal, unstripped hemolysate. After DPG stripping, the P_{50} value for the hemolysate from II-2 was 2.13 mm Hg, whereas that for II-5 was only 1.34 mm Hg or 62.9% of that for the normal, stripped hemolysate. Comparisons of P_{50} values for unstripped hemolysates from all members of the family showed that those for hemolysates containing Hb San Diego ranged from 54.1 to 65.9% of those with only Hb A. Thus, the differences in oxygen affinity between normal and abnormal hemolysates stand in much the same relationship to one another both before and after removal of DPG, suggesting that there is no detectable difference in DPG effect between Hb A and Hb San Diego.

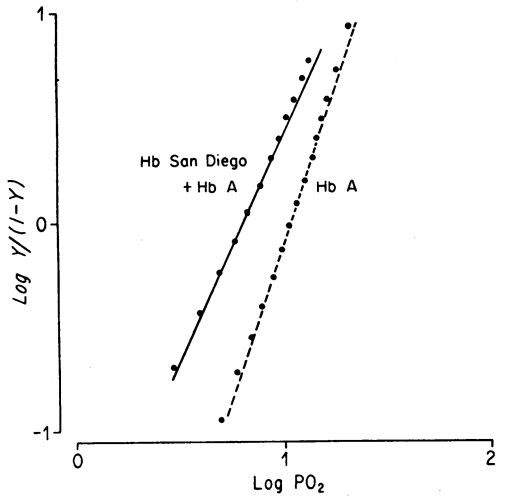

FIGURE 7 Oxygen affinities of hemolysates containing Hb A + Hb San Diego (subject II-5) and Hb A only (subject II-2). Values for Hill's coefficient (heme-heme interaction value, n) are read as the slope of the line at log $[Y/(1-Y)] = 0$. For subject II-5, $n = 2.13$; for II-2, $n = 2.89$.

DISCUSSION

The results of this study stress the need for careful analyses of both the structure and function of hemoglobins present in families with erythrocytosis. Hemoglobinopathy emerges as a likely etiologic agent when a dominant transmission of erythrocytosis is observed. Even when electrophoretic tests provide no evidence of an abnormal hemoglobin, the presence of an abnormal hemoglobin is assured when a leftward shift in the oxygen dissociation curve for whole blood persists in dialyzed and DPG-stripped hemolysates. In the case of Hb San Diego, elucidation of the nature of the structural defect required determination of the amino acid compositions of all tryptic peptides from the β-chains of an affected individual, followed by sequence analysis of that portion of the chain in which compositional ambiguity was detected. This hemoglobin thus provides another example of an electrophoretically and chromatographically silent mutant associated with erythrocytosis. The methods employed in the detection of this hemoglobin as well as of Hb Olympia (5) and Hb Brigham (4) underscore the importance of tests of hemoglobin function in the differential diagnosis of familial erythrocytosis.

The mechanism underlying the high oxygen affinity and low degree of allosteric interaction characteristic of mixtures of Hb A and Hb San Diego is not obvious at the present time. The necessity that functional parameters be measured on mixtures of Hb A and Hb San Diego adds to the interpretive difficulties. For example, the value for Hill's constant, were it measured for pure Hb San Diego, might be considerably lower than the lowest values of n which appear in Table I; however, in the absence of biphasic Hill plots and oxygen dissociation curves (Figs. 5 and 7) it is impossible to judge with accuracy the actual value of n for Hb San Diego.

The Val → Met substitution occurring at site G11 in the β-chains of Hb San Diego does not involve a residue that participates directly in α_1-β_2- or α_1-β_1-contacts. However, helix G does contain residues of importance in α_1-β_1- and α_1-β_2- interactions (27, 28). The existence of several functionally abnormal hemoglobins with substitutions in the G helix of their β-chains (4, 29–34) serves to emphasize the importance of the role this region plays in maintaining a proper equilibrium between oxy- and deoxyhemoglobin. The Met 109(G11)β-residue in Hb San Diego appears to lie close to the border of the heme crevice (33). Its proximity to Asn 108(G10)β and Cys 112(G14)β and its probable similarity of spatial orienta-

tion to His 116(G18)β, all of which participate in the α_1-β_1-contact, suggest that this contact might be altered in Hb San Diego. Movement in this contact is slight during the transitions between oxy- and deoxyhemoglobin, but Perutz (28) noted that small changes in secondary or tertiary structure in this region might be translated into larger alterations in quaternary structure. It seems equally likely that the methionyl side chain might distort spatial relationships in the interior of the β-subunit, producing the high oxygen affinity and low heme-heme interaction characteristic of Hb San Diego. The details of the stereo-chemical effects of the mutation in Hb San Diego are described in the following paper (35).

Position G11 appears to be occupied by valine in the β-chains of all mammals, with the exception of mice and the gray kangaroo (36). In the β-chains of two laboratory strains of mice, position G11 is occupied by methionine, whereas in the gray kangaroo, isoleucine is found in this position (36). The side chains of these three amino acids are nonpolar and hydrophobic, and the side chains of valine and isoleucine are structurally similar. Presumably, the presence of a nonpolar hydrophobic residue in G11β is required to maintain the functional integrity of the hemoglobin tetramer, yet with the divergence of evolutionary pathways of different mammalian taxa, more specific requirements must be met in different lineages. Hence, normal function requires that valine, as opposed to methionine, be present at position G11 in the human β-chain.

The elucidation of the structural abnormality in Hb San Diego demonstrates the efficiency and precision of techniques that couple the isolation of large fragments of chains with automated sequence analysis. The approach is flexible, in that specific cleavage of whole chains can produce fragments that are amenable to accurate automated sequencing. For example, a laborious set of analytical procedures, involving repetitive peptide mapping of tryptic digests of whole β-chains and cyanogen bromide fragments followed by compositional analysis of several peptides, was employed in the determination of the structural abnormality in the β-chains of Hb Olympia (5). The same result has since been less laboriously obtained by subjecting the fragments produced by cyanogen bromide cleavage of the β-chains of the proband to automated sequence analysis.

Hb Olympia and Hb San Diego provide another important item of information. Valine is specified by any of four mRNA codons: GUA, GUU, GUG, and GUC. methionyl codon (AUG) by a single exchange of bases. Of these four, only GUG can be converted to the Therefore, one can conclude that GUG is the normal codon in the mRNA sites corresponding to residues 20 and 109 in the human β-hemoglobin chain.

ACKNOWLEDGMENTS

This investigation was supported by grants GM 15253 and RR 00166 from the National Institutes of Health, U. S. Public Health Service.

The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the U. S. Navy Service at large.

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