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Research Article

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Hemoglobin Hiroshima (β^{143} Histidine → Aspartic Acid): a Newly Identified Fast Moving Beta Chain Variant Associated with Increased Oxygen Affinity and Compensatory Erythremia

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ABSTRACT During a survey for hemoglobinopathies in over 9000 residents of Hiroshima Prefecture, Japan, a fast moving hemoglobin was identified in eight members of three generations in a Japanese family. The abnormal hemoglobin, named Hb Hiroshima, constitutes about 50% of the total hemoglobin in hemolysates from the carriers who have a mild erythremia but are otherwise apparently clinically unaffected. All preparations of Hb Hiroshima have increased affinity for oxygen, by either tonometric or oxygen electrode determinations. At pH 7.0, the oxygen pressure, P_{50} required to half saturate an unfractionated hemolysate from a carrier was one-half that of Hb A, and the P_{50} of a purified sample containing no Hb A was one-fourth that of Hb A. The pH dependence of the oxygen equilibrium (Bohr effect) is below normal, as shown by the absolute value of the Bohr effect factor which is about half that of Hb A, in the pH range between 7.0 and 7.4. The Hill constant, *n*, for Hb Hiroshima between pH 7.0 and 7.4 is 2–2.4, compared to 2.8–3 for Hb A under the same conditions, indicating reduction of, but not complete abolition of heme-heme interaction. Urea dissociation and canine hybridization tests located the biochemical lesion in the beta chain. Fingerprints (Ingram), carboxypeptidase digestion, and amino acid analysis demonstrated that the substitution was at residue 143 in the beta chain, where histidine was replaced by aspartic acid.

In contrast to other recently described high oxygen affinity mutants that show intact Bohr effects, all three of the major characteristics of the reversible combination of hemoglobin with oxygen (oxygen equilibrium, heme-heme interaction, and pH dependence) are affected in Hb Hiroshima. A tentative interpretation of these effects, relating structure to function, is offered in terms of recently developed models of normal hemoglobin.

INTRODUCTION

In the past 15 yr, the structure of hemoglobin has been studied in detail and a great deal is known about its structural constituents and spatial organization. The oxygen transport functions of the hemoprotein have been shown to depend on interactions between heme and globin and on those between the four polypeptide chains of the molecule. The latter interactions are important, in turn, in the dissociation into symmetrical dimers and subunit exchange that occur during oxygen binding. Nevertheless, the relation between function and specific structural features is as yet but poorly understood. One approach to studying the correlation of structure with function is through abnormal hemoglobins where a

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known structural abnormality is associated with physiological malfunction of the red cell.

This report describes a recently discovered abnormal hemoglobin in several members of a Japanese family in whom there is evidence of dysfunction of the red blood cells. This hemoglobin, named Hb Hiroshima, is one of two fast moving β-chain variants discovered during a systematic survey for hemoglobinopathies among 9262 individuals who visited the outpatient clinic of the Atomic Bomb Casualty Commission (ABCC)[1] in Hiroshima, Japan between June 1963 and November 1965. The other abnormal hemoglobin, Hb Hijiyama, found during this survey has been reported elsewhere (1).

METHODS

Blood for hematologic studies was collected in ethylenediaminetetraacetic acid (EDTA). Erythrocyte counts were performed electronically,[2] and total hemoglobin was determined by the cyanomethemoglobin method (4).

Oxygen affinity of red cell suspensions and unfractionated hemolysates were determined by the spectrophotometric method of Allen, Guthe, and Wyman (5), using a modified tonometer (6). Oxygen equilibrium curves of purified preparations of hemoglobin were obtained by oxygen electrode and continuous automatic recording spectrophotometry (7). At all times during the latter determinations the methemoglobin content of the test samples was less than 3% of the total.

The abnormal hemoglobin of the index case and others in the family was first identified by agar-gel electrophoresis at pH 8.6 (8). Methods for further characterization of the hemoglobin are identical to those described in other studies from these laboratories (1, 9) and include the following: paper and starch-gel electrophoresis; column chromatography on carboxymethyl cellulose (CMC) and Amberlite IRC-50; tests of solubility of reduced hemoglobin, Hb F, and alkali resistance. The latter tests were performed on unfractionated hemolysates according to the methods of Singer, Chernoff, and Singer (10) and Betke, Marti, and Schlicht (11).

The abnormal hemoglobin was separated from Hb A by starch-block electrophoresis (9), dialyzed in Visking tubing (Union Carbide Corp., Visking Div., Chicago, Ill.) against deionized water at 5°C, and concentrated by immersion in Sephadex G25 powder. Hb A was purified in the same manner, and in tests of oxygen affinity, was eluted from the same starch block from which Hb Hiroshima was obtained.

The chain location of the anomaly was determined by urea dissociation electrophoresis and by hybridization tests between Hb Hiroshima and canine hemoglobin (1). Globin was prepared by the method of Anson and Mirsky (12); alpha and beta chains were separated by CMC column chromatography of hemolysates after treatment with p-chloromercuribenzoic acid (13). Fingerprints of tryptic digests were prepared by standard procedures (14, 15). The abnormal peptide on the fingerprints was hydrolyzed with 6 N HCl at 105°C for 22 hr and the amino acid composition determined by silica-gel thin layer chromatography (16) and automatic amino acid analysis (17).

Carboxypeptidase A and B (obtained from Sigma Chemical Co., St. Louis, Mo.) hydrolysis (18) was performed to determine the amino acid composition of the C-terminal of β-Hiroshima and β-A. The β-chain, dissolved in N/1000 HCl, was dialyzed against three changes of N/1000 HCl for 18 hr, freeze-dried, and redissolved in water to a concentration of 0.2%, and the pH, initially 3–4, was adjusted to 7.65 with N/10 NaOH. Carboxypeptidase A (10 μl, corresponding to 0.2 mg of protein: substrate enzyme ratio, 100:1) was added and hydrolysis carried out at 25°C. Carboxypeptidase B (50 μl, corresponding to the same concentration as carboxypeptidase A) was added 120 min later. The pH of the mixture was maintained at 7.6–7.7 with N/10 NaOH. During hydrolysis, 0.5 ml samples were withdrawn from the reaction mixture as follows: before addition of enzyme, 30, 120, 140, 180, and 360 min after the addition of carboxypeptidase A, the latter four intervals corresponding respectively to 0, 20, 60, and 240 min after the addition of carboxypeptidase B. These 0.5 ml hydrolysates were boiled for 5 min at 100°C, cooled, freeze-dried, and subjected first to high voltage paper electrophoresis (pyridine–acetic acid–water, pH 6.4, at 2600 v, 90 mA for 30 min) and then chromatography (butanol: acetic acid: water = 3:1:1). Ninhydrin was used to identify the locations of the amino acids on the chromatograms.

Abosoption spectra of acid and alkaline oxy- and methemoglobin, and of alkaline deoxygenated hemoglobin and cyanomethemoglobin were determined with a Hitachi-Perkin Elmer model 139 UV-VIS spectrophotometer.[3]

RESULTS

Index case and family study. The proband is a 44 yr old housewife[4] who, with the exception of hyperthyroidism treated by thyroidectomy, has been in reasonably good health during her 8 yr observation at the ABCC outpatient clinic. The only finding of note has been a persistent elevation of the erythrocyte count, hemoglobin, and hematocrit: over 5.5 million, 15.5 g/100 ml, and 50%, respectively. Erythrocyte morphology and fragility were within normal limits. Hb Hiroshima accounted for 50.7% of the total hemoglobin, Hb A_2 for 2.3%, and Hb F for 2.2%. Ferrohemoglobin solubility and alkaline resistance were normal.

Among the proband's relatives (Fig. 1), hemolysates from seven females contained about 50% of Hb Hiroshima. All are in good health, with the exception of a 41 yr old housewife (II_1) who is anemic and has recently complained of occasional chest pains, but details pertaining to her complaint are not available to us. Among those who have had children (I_1, $II_{1, 10, 12}$) there is no record of spontaneous abortions or miscarriages. Both parents and a brother (II_7) of the proband died of tuberculosis; another brother (II_{11}) died in action during the war; the only brother (II_2) of II_1 was born prematurely and died in infancy, cause unknown, but there

[1] Those seen in the clinic are voluntary participants in the ABCC Adult Health Study and In Utero Study, long-term investigations of the aftereffects of exposure to ionizing radiation. The populations have been described in detail elsewhere (2, 3).

[2] Model A Coulter Counter, Coulter Electronics, Hialeah, Fla.

[3] Nissei Sangyo Co., Ltd., Subs. of Hitachi Ltd., Kanda Tsukasa-cho, Chiyoda-Ku, Tokyo.

[4] Neither the proband nor any of her relatives were exposed to the atomic bomb in Hiroshima in 1945.

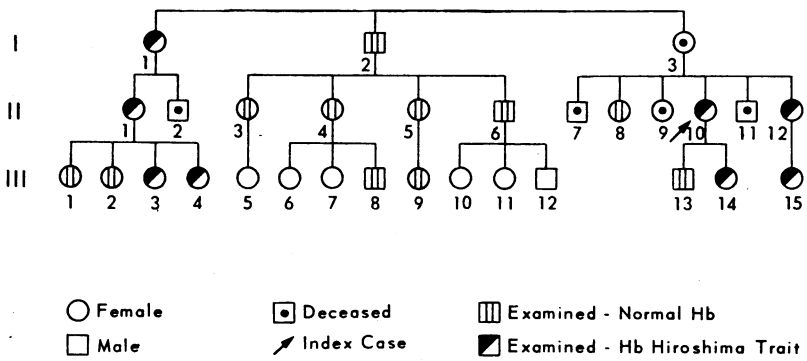

FIGURE 1 Pedigree of Hb Hiroshima kindred.

was no report of cyanosis, jaundice, or physical anomalies. It is not particularly surprising that the trait has been found only in females in this kindred, since in the two branches of the family where the gene for the abnormal hemoglobin is segregating, there is only one male (III_{13}) available for testing. The pattern of inheritance of the trait is consistent with that of a simple autosomal (codominant) gene.

Results of hematologic studies of carriers and several normal females in the kindred are shown in Table I. With the exception of II_1, the carriers of Hb Hiroshima have significantly higher hemoglobins, hematocrits, and erythrocyte counts than their noncarrier relatives ($P < 0.01$, $P < 0.01$, and $0.02 < P < 0.05$, respectively). The city and country carriers, in turn, differ significantly from one another with respect to hemoglobin and hematocrit values ($P < 0.01$), an observation that is probably explicable in terms of different socio-economic circumstances.

Oxygen equilibrium studies. Oxygen equilibria of erythrocyte suspensions and unfractionated hemolysates from two carriers (I_1 and II_1) of Hb Hiroshima were determined by tonometry and automatic recording spectrophotometry, and for either preparation the oxygen affinity was greater than normal control values and the sigmoid shape of the equilibria curves was less pronounced than usual. The P_{50} [5] for an erythrocyte suspen-

[5] Oxygen pressure required to half saturate the hemoglobin.

TABLE I

Hematologic Data for Females in Family Segregating for Hb Hiroshima Gene

Pedigree No.	Hb g/100 ml	Hct %	RBC $\times 10^6/mm^3$	Retics %	MCV μ^3	MCH $\mu\mu g$	MCHC %
Carriers of Hb Hiroshima*							
I_1	14.5	45.0	4.84	0.9	93.0	30.0	32.2
II_1‡	11.2	38.0	4.28	1.3	88.8	26.2	29.5
III_3	14.4	45.5	4.93	1.2	92.3	29.2	31.6
III_4	14.5	46.0	5.37	1.1	85.7	27.0	31.5
II_{10}	17.0	52.5	5.32	—	98.7	32.0	32.4
III_{14}	17.0	53.0	5.50	2.5	96.4	30.9	32.1
II_{12}	17.4	54.5	5.47	1.7	99.5	31.8	31.9
III_{15}	17.0	53.0	6.20	1.5	85.5	27.4	32.1
Normal complement of Hb A							
III_1	12.9	38.5	4.55	1.1	84.6	28.4	33.5
III_2	12.5	38.5	4.25	0.9	90.6	29.4	32.5
II_4	10.8	34.5	4.24	1.9	81.4	25.3	31.3
II_5	12.5	37.0	3.77	1.0	98.1	33.2	33.8
III_9	12.5	38.5	4.58	2.1	84.1	26.9	31.9

RBC = red blood cell count, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin content, MCHC = mean corpuscular hemoglobin concentration.

* The first four of the Hb Hiroshima carriers listed above and all of the normal relatives live in a small farming community whereas the remaining four carriers live in Hiroshima City.

‡ Said to be in ill-health, see text.

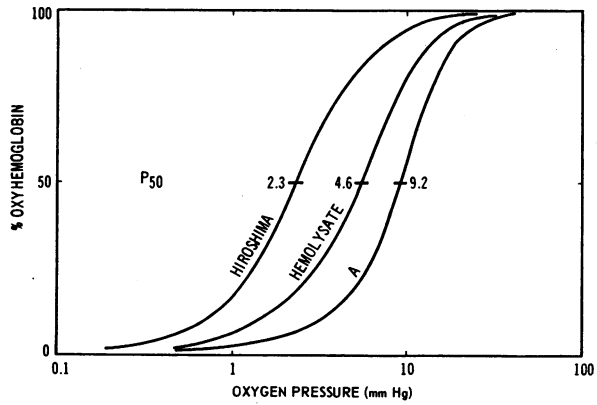

FIGURE 2 Oxygen equilibria of Hb A, Hiroshima, and an unfractionated hemolysate containing equal proportions of Hb A and Hiroshima. Purified Hb Hiroshima from III_3; unfractionated hemolysate from III_4 (see Fig. 1). Both samples, and control obtained on the same day. Purified samples prepared in the same manner at the same time, and eluted from the same starch block. Curves determined with oxygen electrode and continuous recording spectrophotometer at 564 mμ. Temperature 20°C, pH 7.0. P_{50} = oxygen pressure required to produce 50% oxyhemoglobin.

sion at pH 7.5 and 23°C was 5.0 mm Hg compared to 10.5 for Hb A and the P_{50} for an hemolysate of the mutant hemoglobin was 1.9 compared to 4.4 for Hb A.

Oxygen equilibria determined by oxygen electrode and continuous recording spectrophotometry of Hb Hiroshima showed characteristics almost identical with those for suspensions and hemolysates described above. Fig. 2 shows curves for purified Hb Hiroshima and an hemolysate from another carrier (III_3) compared with Hb A. At pH 7.0 and 20°C the P_{50} values were 2.3, 4.6, and 9.2, respectively. The effect of pH (Bohr effect) on the oxygen equilibrium of purified Hb Hiroshima is compared with Hb A in Fig. 3. At pH values decreasing from 7.8 to 6.5, though the curves shifted progressively to the right, the change was less than for Hb A. The reduction in the pH or Bohr effect is shown more clearly in the inset to Fig. 3. The Bohr effect factor ($\Delta \log P_{50}/\Delta pH$) is reduced by about one-half for Hb Hiroshima, being -0.27 over the pH range between 7.4 and 7.0, compared to -0.53 for Hb A. The Hill constant (19), n, for purified Hb Hiroshima was about 2.0 at pH 7.0, compared to 3.0 for Hb A, implying reduction in heme-heme interaction for the mutant hemoglobin. Also, as seen in the inset to Fig. 3, n tended to increase as pH increased, being 2.4 at pH 7.4, and 2.6 at pH 7.8. Imai (20) analyzed the oxygen equilibria curves for Hb Hiroshima in detail, and pointed out that at the upper ranges of oxygen saturation, the Hill plot is biphasic and the conventional interpretation of the significance of n probably does not hold.

Physical and chemical characteristics of Hb Hiroshima. The electrophoretic mobility of Hb Hiroshima at pH 8.6 was greater than that of Hb A, but at neutral or acid pH, on agar gel it migrated less rapidly than Hb A towards the anode (Fig. 4). Hb Hiroshima descended more rapidly than Hb A on either CMC or Amberlite IRC-50 column chromatography. On CMC chromatography of hemolysates treated with *p*-chloro-

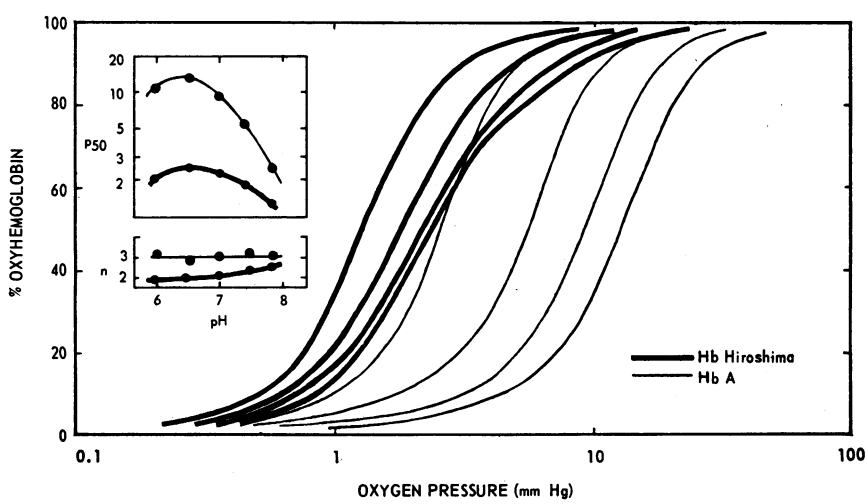

FIGURE 3 Effect of pH on oxygen equilibria of Hb A and Hiroshima, determined under the same conditions as in Fig. 2. From left to right for each set of curves, pH 7.8, 7.4, 7.0, and 6.5. Insert summarizes the pH effect. Upper panel: semi-log plot of P_{50} against pH, demonstrating that Hb Hiroshima is less sensitive to pH changes than Hb A. Lower panel: plot of the Hill constant, n, against pH. For Hb Hiroshima, n tends to increase with increase in pH (see text).

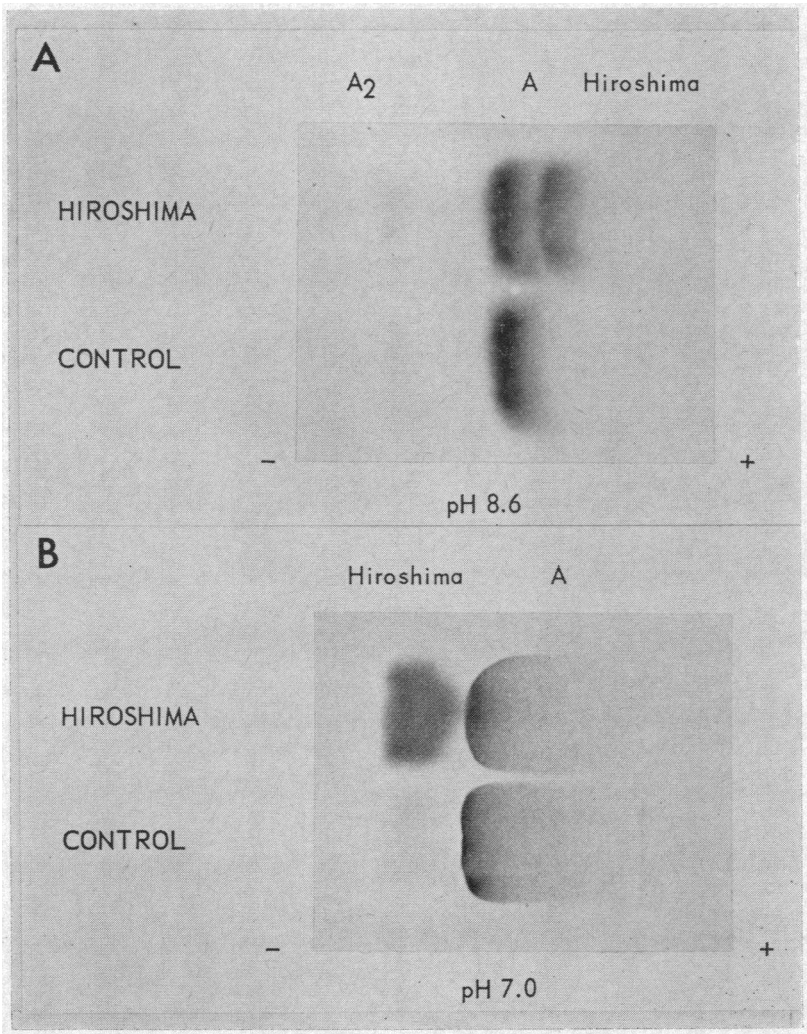

FIGURE 4 Agar-gel electrophoresis of unfractionated hemolysates from a carrier of Hb Hiroshima (II_{10}) and a normal control. A: pH 8.6; B: pH 7.0. In alkaline medium, Hb Hiroshima moves more rapidly towards the anode than Hb A, and at neutral pH, less rapidly. In B the faint band at the left in the control is Hb F.

mercuribenzoic acid to separate α- and β-chains, a third component, presumably the abnormal chain of Hb Hiroshima was eluted first, followed by β- and α-A.

Electrophoresis of Hb Hiroshima in 6 M urea demonstrated that the β-chain was abnormal and this was confirmed by hybridization tests of Hb Hiroshima chains with those of canine hemoglobin: $\alpha_2^{can}\beta_2^{Hiro}$ migrated more rapidly toward the anode than control $\alpha_2^{can}\beta_2^{A}$.

In fingerprints of Hb Hiroshima globin, spots containing β-Tp-14[6] and -15 were absent and a new spot was present immediately below α-Tp-6. In fingerprints of purified β-Hiroshima chain, β-Tp-14 and -15 were replaced by an abnormal spot immediately above and partially overlapping β-Tp-8, 9 (Fig. 5).

Table II compares the amino acid content of the abnormal spot with expected values (21) for the missing peptides. The only difference is that Asx[7] is present in the abnormal peptide in approximately double the expected amount and His is reduced to about half of normal.

The results of carboxypeptidase hydrolysis of the C-terminal of β-A and β-Hiroshima are compared in Table III. His and Tyr appeared in the hydrolysates of both β-chains 30 min after the addition of carboxypepti-

[6] β-Tp-14: beta tryptic peptide No. 14.

[7] Aspartic acid and asparagine. By this method they are indistinguishable.

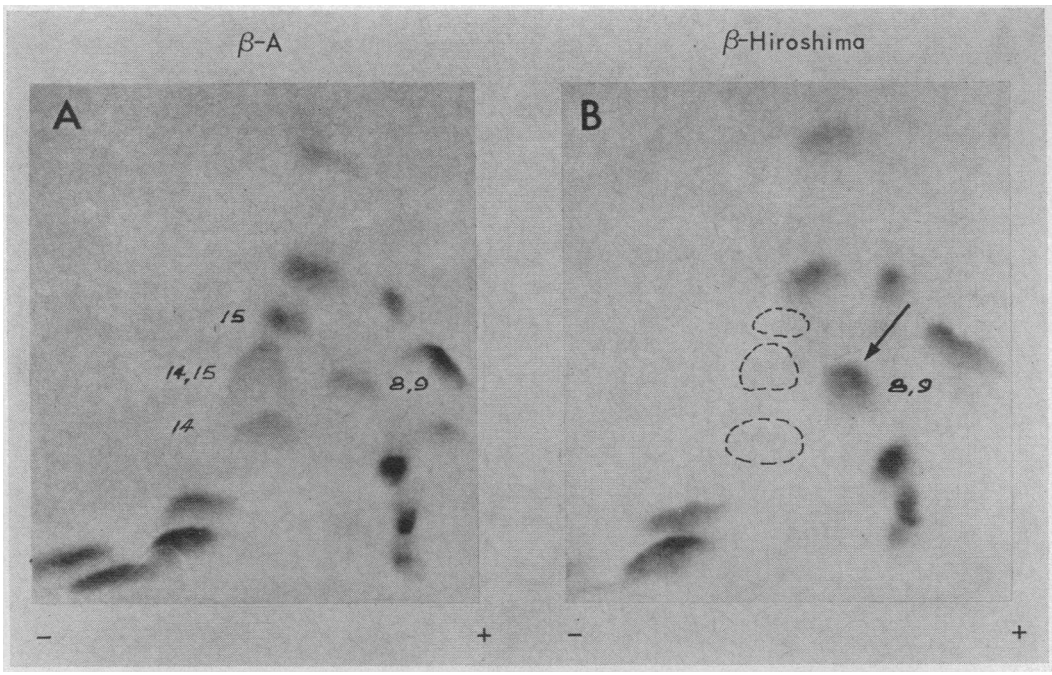

FIGURE 5 Fingerprints of tryptic digests of purified β-chains. A: β-A; B: β-Hiroshima. The areas enclosed by the dotted lines indicate the positions of the peptide spots β-Tp-14,15, and 14 and 15 that are missing from Hb Hiroshima. The arrow indicates an abnormal spot overlapping β-Tp-8,9.

dase A, evidence that the C-terminal His was intact in both chains. By 140 min, corresponding to 20 min after the addition of carboxypeptidase B, the amount of His in the β-A hydrolysate had increased, judging by the intensity of the ninhydrin reaction, but this was not apparent for β-Hiroshima. In addition, Asp had appeared in the β-Hiroshima hydrolysate, but not in that of β-A. Otherwise the amino acid content of the two mixtures was essentially the same, as it was at 360 min.

The data presented in Tables II and III, plus the evidence from electrophoretic and chromatographic studies, demonstrated clearly that a negatively charged Asp residue has been substituted for His in β-Tp-14, 15. As shown in Table IV, the two possible positions for the substitution are at β-143 or the C-terminal 146, both normally occupied by His (17). However, position 146 is excluded by the carboxypeptidase analysis and it is concluded that the histidine at β-143 in Hb A is replaced by aspartic acid in Hb Hiroshima[8]: therefore, Hb Hiroshima is designated as $\alpha_2^A\beta_2^{143\ Asp}$.

The substitution of a negatively charged Asp for His accounts for the electrophoretic characteristics of Hb Hiroshima. At alkaline and neutral pH, the positive charge of the His imidazole side chain is probably largely suppressed, so that in effect, the substitution of Asp adds at least two more negative charges per molecule of hemoglobin, or one more per beta chain, thus enhancing their respective mobilities towards the anode at pH 8.6.

That, on tryptic digestion, cleavage does not occur between β-144 Lys and β-145 Tyr in the abnormal peptide of Hb Hiroshima is apparently attributable to the presence of the acidic Asp at β-143. An analogous situation exists for two other mutant hemoglobins, J_{Oxford} and $J_{Baltimore}$ (23, 24), with substitutions, respectively, at α-15 and β-16, in homologous sections of the two chains. In each case, the substitution of Asp for Gly is in a position immediately preceding Lys. At this location in Hb A (α-16 or β-17), the lysyl bond is readily hydrolyzed, but in these mutants it is not. Other lysyl bonds in normal Hb A that are similarly resistant to tryptic hydrolysis are at α-6, α-98, and β-95, all of which are preceded by an Asp residue.

Absorption spectra of alkaline and acid oxy- and methemoglobin and of alkaline deoxy and cyanomethemoglobin derivatives of Hb Hiroshima between 450 and 650 mμ and in the Soret region were not significantly different from those of Hb A.

[8] Hb Kenwood, previously reported as having either Asp or Glu substituted at β-143 (22), was later found to be identical with Hb $N_{Baltimore}$, β-95 Lys → Glu. (Heller, personal communication.)

TABLE II

Comparison of Amino Acid Content of β-Tp-14, 15 of Hb A with the Abnormal Peptide of Hb Hiroshima

Amino acid	Hb Hiroshima abnormal peptide found	Integral	Hb A, β-Tp-14, 15 expected
		*No. of residues**	
Val	2.40	3	3
Ala	4.15	4	4
Gly	0.91	1	1
Asx	1.97	2	1
Leu	1.18	1	1
His	0.86	1	2
Lys	1.03	1	1
Tyr	++	1	1

* Expected residues according to Braunitzer et al. (21). Because of the overlap of the abnormal peptide with normal β-Tp-8, 9, the values given for the abnormal peptide were obtained by difference between the total for each acid in the combined normal and abnormal spots and those for the normal β-Tp-8, 9 known for Hb A. There was no evidence suggesting partial hydrolysis of the abnormal peptide. If hydrolysis occurred, cleavage would be expected between Lys β-144 and His β-145, giving a normal β-Tp-15 in the usual position on fingerprint, but no such spot was found. The actual value for Val is low because one residue at the N-terminal of the tryptic peptide was partially destroyed by ninhydrin. Accurate measurement of Tyr was not achieved because it is partially destroyed during acid hydrolysis but it was always present when the abnormal peptide spot was tested with α-nitroso-β-naphthol and nitric acid.

DISCUSSION

Among the large number of variant hemoglobins, a majority are evidently clinically insignificant, at least in the heterozygous state. Erythrocyte dysfunction attributable to abnormal structure of hemoglobin includes several different pathologic effects such as tactoid formation on deoxygenation (Hb S, sickle cell anemia), unstable hemoglobin molecules (Hb Köln and Zurich), increased stability of methemoglobin (Hb M diseases), and impaired oxygen affinity (Hb Kansas, Chesapeake) (25). Hb Hiroshima clearly falls into the last group.

In the face of increased oxygen affinity and compensatory erythremia, one might expect to find evidence for decreased P_{O_2} reserve in, for example, pregnancy, anemia, or prolonged exercise (26, 27). In the carriers of Hb Hiroshima there are no clinically apparent effects attributable to the mutant hemoglobin, but whether individuals homozygous for the trait would be at a disadvantage is, of course, unknown, and in view of the rarity of the gene, even in Japan where consanguinity is somewhat higher than in the West, it is unlikely that affected homozygotes will be found. Others have reported high normal values of urinary erythropoietin in carriers of high oxygen affinity hemoglobins (27, 28) and we might anticipate similar findings in our cases but so far have not been able to perform such studies.

The amino acid substitution in Hb Hiroshima has affected three of the important properties associated with the reversible combination of oxygen with hemoglobin: low oxygen affinity, heme-heme interaction, and pH dependence (29). The oxygen equilibrium curve is shifted

TABLE III

Comparison of Amino Acids Released from C-Terminal of β-A and β-Hiroshima by Carboxypeptidase A and B

Incubation time	β-chain	Amino acid									
		His	Tyr	Lys	Asp	Ala	Leu	Asn	Val	Gly	Glu
min											
Before hydrolysis	Hiroshima	−	−	−	−	−	−	−	−	−	−
	A	−	−	−	−	−	−	−	−	−	−
CPA 30	Hiroshima	+*	+	−	−	−	−	−	−	−	−
CPB 0	A	+	+	−	−	−	−	−	−	−	−
CPA 120	Hiroshima	+	+	±	±	−	−	−	−	−	−
CPB 0	A					(not tested)					
CPA 140	Hiroshima	+	+	+	+	++	+	±	+	+	−
CPB 20	A	++	+	+	−	++	+	±	++	+	−
CPA 180	Hiroshima	+	+	+	+	+++	+	+	++	+	−
CPB 60	A					(not tested)					
CPA 360	Hiroshima	+	+	+	+	+++	++	+	+++	+	−
CPB 240	A	+	+	+	−	+++	++	+	+++	+	−

* +, ++, +++: color intensity of amino acid–ninhydrin reaction.

TABLE IV

Comparison of Amino Acid Sequences of β-Tp-14, 15 of Hb A with the Abnormal Peptide of Hb Hiroshima

β-Tp-14, 15	133 134 135 136 137 138 139 140 141 142 143 144 145 146
Hb A	Val–Val–Ala–Gly–Val–Ala–Asn–Ala–Leu–Ala–HIS–Lys↑Tyr–His
Hb Hiroshima	Val–Val–Ala–Gly–Val–Ala–Asn–Ala–Leu–Ala–ASP–Lys–Tyr–His

The arrow (↑) between Lys and Tyr in Hb A marks the point of cleavage between β-Tp-14 on the left and β-Tp-15 on the right. Presumably the substitution of a negatively charged Asp for His at β-143 prevents cleavage at this point (see text). Hence in fingerprints of Hb Hiroshima, a single abnormal spot replaces the two spots normally present in fingerprints of Hb A.

towards lower pressures with respect to % oxygenation, so that at pH 7.0, the P_{50} is about one quarter that of normal. The normal sigmoid shape of the curve is somewhat altered and *n*, the slope of the curve, is reduced from 3 to about 2, indicating reduction, though not complete abolition of heme-heme interaction. Finally, the Bohr effect, though present, is less than normal as shown by a reduction of the Bohr effect factor to about half that for Hb A. All three of these factors must be taken into account in relating the structural change in Hb Hiroshima to its functional changes.

The allosteric properties of hemoglobin depend on interactions between the alpha and beta subunits and an important factor in the binding of oxygen is dissociation of the tetrameric molecule into two symmetric dimers accompanied by subunit exchange (30). Interference with interactions between unlike subunits impairs heme-heme interaction and may interfere with the Bohr (pH) effect. Though related, heme-heme interaction and the Bohr effect are separable, implying that different parts of the hemoglobin molecule participate in these two functions (31). Chemical manipulation of hemoglobin can produce this separation and several recently discovered mutant hemoglobins exhibit this property as well.

Table V compares Hb Hiroshima with several other high oxygen affinity mutant hemoglobins, all of whose abnormalities are ascribable to the substitution of a single amino acid in the alpha or beta chain (26, 28, 32–34). The following are not included: Hb M where abnormal oxygen affinity is related to stabilization of the heme iron in the ferric form; Hb Zurich (35) whose instability is clinically more important than its slightly increased oxygen affinity (36); Hb Ypsilanti, which has not yet been fully characterized with respect to the Hill constant, Bohr effect, or exact location and nature of the substitution in the beta chain (37). On the basis of the available evidence, Hb Hiroshima appears to be unique among these mutants in showing a considerable reduction of the Bohr effect.

The cluster of mutations at α-FG4 and β-G1 has been

TABLE V

Comparison of Some Hemoglobin Variants Having Increased Oxygen Affinity

Hemoglobin	Amino acid substitution	Charge change	Location of substitution			*n*‡	Bohr effect§	Mutant Hb in hetero-zygote	Ery-thremia
			Chain	Residue	Segment*				
								%	
Chesapeake	Arg → Leu	−1	α	92	FG4	1.3	normal	23–35	+
Capetown	Arg → Gln	−1	α	92	FG4	1.9	normal	35	0
Yakima	Asp → His	+2	β	99	G1	1.0	normal	37–39	+
Kempsey	Asp → Asn	+1	β	99	G1	1.1	normal	45–47	+
Hiroshima	His → Asp	−2	β	143	H21	2.0–2.6	reduced	48–52	+
Rainier	Tyr → His	+1	β	145	H23	1.2	normal	30	+

* Perutz's numbering system which permits comparison of homologous sections of myoglobin, and the alpha, beta, and gamma chains (39).

‡ Hill constant (19), normal values between 2.7 and 3.0. Though these values are probably not strictly comparable, since the conditions under which they were obtained are not identical, they at least give some indication of the reduction in heme-heme interaction. Note further that the values obtained for Chesapeake, Yakima, and Hiroshima apply to purified samples of the mutant hemoglobin, while the others refer to mixed hemolysates with varying proportions of Hb A. The value for Hiroshima varies with pH, see text.

§ The Bohr effect for Yakima is given as "near normal" for a mixed hemolysate, and the Bohr effect factor is slightly reduced; for a purified sample of Yakima, the data also suggest a slight reduction of the Bohr effect (27). Similarly, the data for Kempsey suggest some reduction of the Bohr effect (34).

commented on by others (33, 34) who suggest that the most probable explanation for high oxygen affinity of these hemoglobins is an interference with the normal contacts and interactions that occur between the α-FG interhelical region and the β-G helix, restricting the spatial rearrangements that occur during oxygenation-deoxygenation, tending to stabilize the molecule in the oxygenated conformation (26, 38).

In Hb Hiroshima, on the other hand, the substitution near the C-terminal of the beta chain suggests a somewhat different explanation for the increased oxygen affinity, namely, that there may be interference with contacts between the two sister beta chains instead of between unlike alpha and beta subunits. According to Perutz's model for horse hemoglobin (39, 40), which may also apply to human hemoglobin, the sister beta chains are thought to be cross-linked by hydrogen bonds between Asn β-H17 and the two His residues at β-H21 and β-H24. In addition, the amino and carboxy terminals of the sister beta chains may be linked between His β-H24 of one chain with Val β-NA1 of the other. Another possible contact is between His β-H24 and Lys β-H10. Muirhead, Cox, Mazzarella, and Perutz (41) showed that in human oxyhemoglobin, the amino terminal of one beta chain is 6 A away from the carboxy terminal of the other chain, whereas in the deoxy form, the distance is doubled and contacts between the sister chains are broken. Obviously, a substitution of the more negatively charged Asp for His at H21 would profoundly alter these cross-linkages. Moreover, the residue at H21 is separated by almost one helical turn from the C-terminal H24 (42), and its side chain, therefore, is probably oriented in the same direction as that of the terminal residue. Substitution of the more polar Asp in Hb Hiroshima would lead to charge rearrangements through the addition of one more negative charge in the proximity of the C-terminal, tending to strengthen the bond with the N-terminal of the sister chain. Strengthening these bonds would tend to fix the hemoglobin molecule in the contracted quaternary oxy-conformation, and interfere with dissociation into symmetrical alpha-beta dimers, leading to an increase in oxygen affinity.

The recently reported importance of 2,3-diphosphoglycerate (DPG) in hemoglobin function raises the question of its possible role in the increased oxygen affinity of Hb Hiroshima. Benesch, Benesch, and Yu (43) showed that DPG combines reversibly with normal hemoglobin in equimolar amounts, that the binding of DPG is reciprocal to that of oxygen, and that DPG reduces the affinity of hemoglobin for oxygen. Moreover, DPG is evidently bound to the beta chain of the hemoglobin molecule when it is in the expanded deoxy form, according to Perutz and Lehmann (44). These investigators also pointed out that lysyl and arginyl residues along the dyad axis of the central cavity could serve to neutralize the five negative charges of DPG and indicated that Lys β-H10, the only one of the nine invariant residues in mammalian hemoglobin with no known function (42), might be a binding site for DPG (44). Alternatively, His β-H21 has been tentatively suggested as a possible binding site for DPG on the basis of recent studies of the influence of pH on the binding of DPG to hemoglobin [9] along with the apparently favorable orientation of the histidine residue at β-H21.[10] These observations together with the stereochemical effects described in the preceding paragraph suggest that a contributing factor to the increased oxygen affinity of Hb Hiroshima may be its reduced affinity for DPG. Experiments are under way to determine whether or not this is so.

Hb Hiroshima and Rainier have substitutions in the β-H helix, close to the carboxy terminal of the chain and the substitutions are two residues apart. Though both have increased oxygen affinity, Hb Rainier has a normal Bohr effect, but increased resistance to alkali denaturation (28), whereas Hb Hiroshima has a decreased Bohr effect, but no increased resistance to alkali denaturation. One possible explanation for the reduction of the Bohr effect in Hb Hiroshima is suggested by recent studies of normal hemoglobins. The beta chains are thought to contribute more to the Bohr effect than the alpha chains (45). The most probable sources of the "alkaline" Bohr protons are considered to be the imidazole side chains of His residues, other than those involved in heme-iron linkage. The histidine residues at β-FG4 and possibly those near the carboxy terminal of the β-chain may be potential sources of Bohr protons (31, 46). Removal of the latter produces products with increased oxygen affinity and a reduced Bohr effect.

With respect to the shifts in interatomic distances that accompany the oxygenation-deoxygenation reaction (41), Rossi-Bernardi and Roughton (45) suggested that as a consequence of stereochemical effects, the two pairs of hydrogen bridges between the gamma amino Asn β-H17 and the imidazole side chains of His β-H21 and β-H24 might participate in the Bohr reaction. In Hb Hiroshima, the replacement of His by Asp at β-H21 not only alters the bonding relationships in the molecule as previously discussed, but also removes a pair of potential sources of Bohr protons, one from each subunit.

While this interpretation is no doubt an oversimplification, it is evident that the substitution of Asp for His near the beta carboxy terminal in Hb Hiroshima provides a clear illustration, along with that of Hb Rainer, that this segment of the beta chain plays an important role in several different functions of hemoglobin (47). Taken together, these recently discovered, naturally occurring, high oxygen affinity, mutant hemoglobins pro-

[9] Garby, L., G. Gerber, and C.-H. de Verdier. In preparation.

[10] Perutz, M. F., and L. Garby. Personal communication.

vide considerable support for the current picture that is developing to explain at a molecular level the complicated relationship of structure to function in hemoglobin.

In all Hb Hiroshima carriers, the mutant and normal adult hemoglobins are present in equal proportions, implying that the synthetic mechanisms for the mutant β-chain are probably as efficient as those for the normal chain, and that presumably the affinity of β-Hiroshima for heme is equivalent to that of β-A. Among the variants listed in Table V, with the exception of Hb Rainier, the β-mutants are present in higher proportion in heterozygotes than the α-variants, which is consistent with the observation that in general, the proportion of the abnormal pigment in carriers of β-variants tends to be higher than in those with α variants (23).

The amino acid substitution in Hb Hiroshima is ascribable to a single step mutation in the codon (48) for His (CAC or CAU)[11] to that for Asp (GAC or GAU). This point mutation, C to G, is the transversion type (pyrimidine to purine, or vice versa) which, according to Vogel and Röhrborn (49), is less likely to occur than the transition mutation (purine to purine, or pyrimidine to pyrimidine). Among the mutants listed in Table V, half (Capetown, Kempsey, Rainier) are transitions, which even in this small group, is reasonably consistent with other data (49) for abnormal hemoglobins showing a higher proportion of transitions than expected on the basis of random mutation alone.

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[11] Abbreviations: C = cytosine, A = adenine, U = uracil, G = guanine.

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