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Hb Providence [β82 (EF 6) Lys→Asn, Asp], Two Forms in Peripheral Blood : The First Case Report in Japan ; Structure, Function and Biosynthesis

Keiko HARANO

Department of Biochemistry, Kawasaki Medical School, Kurashiki 701-01, Japan
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ABSTRACT. Fast-moving abnormal hemoglobins with two types of β chain anomalies were discovered in a Japanese male who lived in Okayama Prefecture. Fingerprinting and amino acid analysis of the abnormal hemoglobins purified by isoelectric focusing revealed displacement of β82 (EF 6) Lys by Asn and Asp. Studies of the biosynthesis of the abnormal hemoglobins in reticulocytes demonstrated production of the β chains (the normal β^A plus the abnormal β) in slight excess over the α chain (the normal α^A) (non-α/α=1.33). Furthermore, it was found that the immediate biosynthetic product for the abnormal β chain was β82 (EF 6) Lys→Asn, rather than β82 (EF 6) Lys→Asp. Therefore, the β82 (EF 6) Lys→Asp chain was thought to be the secondary product generated in the circulating blood from β82 (EF 6) Lys→Asn. The abnormal hemoglobins showed slightly increased oxygen affinity (P_{50} 19.2 mmHg, normal control 25.4 mmHg, pH 7.4 at 37 °C) when they were in the cytosol of erythrocytes, whereas the purified specimens exhibited a decrease in oxygen affinity (Asn type P_{50} 9.43 mmHg, Asp type P_{50} 11.4 mmHg, normal A P_{50} 6.07 mmHg, pH 7.4 at 25°C) and reduced Bohr effect (Asn type 0.332, Asp type 0.269, normal A 0.423). Their oxygen affinity was affected little by addition of the organic phosphates, 2,3-DPG and IHP. Their Hill's n values (2.4 to 2.93) were within the normal range. These findings, including the results of chemical analyses, biosynthetic experiments and oxygen equilibrium studies, are in good agreement with those on Hb Providence Asn and Asp which were reported by Charache et al. and Bonaventura et al. in the United States. This is the first case of Hb Providence discovered in Japan.

The functional properties of the two forms of Hb Providence are discussed in comparison with those of Hb Rahere and Hb Helsinki with different substitutions at the same site.

For the past two years the Department of Public Health of Kawasaki Medical School Hospital has screened for diabetes mellitus in all people who were admitted to the ward for routine health examinations. The screening was done using the hemoglobin A_{Ia+b+c} (Hb A_I) level of the hemolysate as estimated by the microcolumn chromatographic method (1). In September, 1979, a hemolysate which seemed to contain an unusually high level of Hb A_I, 44%, was

原野恵子

encountered. In our experience, the Hb A_I content of hemolysate has been 5-7% in normal subjects, and even in diabetic patients it has seldom surpassed 20%. Curiously the propositus a 51 year old Japanese man, alleged that he had never suffered from diabetes mellitus. Isoelectric focusing on an Ampholine-polyacrylamide gel plate (pH range 6-9) of his hemolysate (2) revealed four hemoglobin bands, Hb A_2, Hb A, abnormal hemoglobin-1 (Hb X-1) and abnormal hemoglobin-2 (Hb X-2) in order from the cathode to the anode (Fig. 1). The results of structural analysis demonstrated that Hb X-1 was identical to Hb Providence Asn ($\alpha_2\beta_2$ 82 Lys→Asn) and Hb X-2 to Hb Providence Asp ($\alpha_2\beta_2$ 82 Lys→Asp). Both Hb Providence Asn and Hb Providence Asp had been discovered in 1976 in a young American Black woman suffering from hepatic disease (3). However, they have never encountered in Japan. Our studies on their molecular structure, oxygen affinity and biosynthetic rate have revealed several interesting new findings which are described in this paper.

MATERIALS AND METHODS

1) All blood samples for the present investigation were drawn from the antecubital vein and prevented from coagulating by heparinization.

2) Chemical and clinical laboratory tests were carried out by the standard methods.

3) The concentration of 2, 3-DPG (2, 3-diphosphoglycerate) was measured according to the instruction notes of 2,3-DPG measurement kit (Sigma Laboratory, California).

4) Oxygen affinity of whole blood samples was measured at 37°C by the method of Imai et al. after they were diluted 150 times with isotonic phosphate buffer solution (4). The hemolysates (60 μM heme concentration) were examined for oxygen dissociation curves in 0.05M bis-Tris-0.1M NaCl buffer solution of various pHs at 25°C. The effect of organic phosphate on the oxygen affinity of individual hemoglobins (Hb X-1, Hb X-2 and Hb A) was investigated after addition of 2mM 2,3-DPG or 2mM IHP (inositol hexaphosphate) to the Hb dissolved in 0.05M bis-Tris-0.1M NaCl buffer solution (pH 7.4) at 25°C.

5) The hemolysate was prepared by the standard method (5).

6) Isoelectric focusing of the hemolysate was carried out in the range of pH 6-9 by use of a polyacrylamide gel plate containing Ampholine (1.6%, mixture of two types of Ampholine with pH ranges 7-9 and 3.5-10 (mixed in proportion of 4 : 1 by vol.)) (2). Isoelectric focusing was achieved by an electric current of 30 to 1 mA at a constant voltage of 200 V in a cooling chamber (ca. 10°C) for 16 hrs.

7) The hemoglobin composition of the hemolysate was determined by measuring the absorbance (at 415 nm) of the eluates of the individual hemoglobin bands which were first isoelectrofocussed on the gel plate and then eluted with 0.1M sodium-potassium phosphate buffer (pH 7.4) containing KCN (0.01%).

8) Hemoglobins were purified by elution of isoelectrofocussed hemoglobin bands (described above) into distilled water and their concentrations were adjusted to appropriate levels by the vacuum aspiration method using collodion bags.

9) Each pI value was measured with the Corning pH Meter Model 125 after individual hemoglobins were isoelectrofocussed on an Ampholine-polyacrylamide gel plate (pH range 6-9) and extracted into distilled water (1.0 ml).

10) The content of Hb F was determined by the method of alkali denaturation (6), and that of Hb A_2 by cellulose acetate membrane electrophoresis (7).

11) Hb A_I content was determined by the microcolumn chromatography method in a chamber controlled at 23°C (NC-Roppet, Nihon Chemipher Ltd.) according to the instruction notes.

12) Hemoglobin instability was examined by Carrell's isopropanol precipitation test (8).

13) Abnormal chains of the hemoglobins (Hb X-1 and Hb X-2) were detected by urea dissociation cellulose acetate membrane electrophoresis (9).

14) Globin was prepared by the method of Anson and Mirsky (10).

Structural analysis were carried out in the following way :

15) The abnormal β chain (β^{X-1} or β^{X-2}) was separated on a CM-cellulose (CM-52, Whatman Co.) column with phosphate buffered 8M urea solution according to the method of Clegg et al. (11) with slight modification (column size : 13×250 mm ; Buf. I : 8mM Na_2HPO_4-8M urea-50mM mercaptoethanol, pH 6.8 ; Buf. II : 35mM Na_2HPO_4-8M urea-50mM mercaptoethanol, pH 6.8. A linear gradient from 8mM to 35mM was made with a two chamber-vessel containing 150 ml of Buf. I and Buf. II. Flow rate : 0.3 ml/min ; fraction size : 12 min/tube). The separated abnormal globin chains were dialyzed against water, lyophilized for adequate concentrations, gel-filtrated on a Sephadex G 25F column using 0.2M acetic acid to remove completely urea-phosphate buffer and lyophilized again.

16) The purified β chains were aminoethylated by the method of Jones (12), digested overnight with TPCK-trypsin (Worthington Co.) at room temperature while the pH was adjusted to 8.8, and the digest was subjected to fingerprinting on cellulose thin layer (Chromagram Sheet, Eastman Kodak Co.) (13). Abnormal peptide spot was cut out from the map, eluted with 10% acetic acid, lyophilized and hydrolyzed with 6N HCl at 105°C for 20 hrs. Amino acid composition of the abnormal peptide was analyzed in an automatic amino acid analyzer (Yanaco amino acid analyzer L-7).

17) Hemoglobin biosynthesis using fresh heparinized peripheral blood was carried out in the following way (14). The red cells collected by washing the peripheral blood (ca. 5 ml) with physiologic saline (5 times) were ultracentrifuged at 15,600×G in an Eppendorf Model 5412 ultracentrifuge in a cooling chamber at 10°C. 0.2 ml aliquot of reticulocyte-enriched erythrocyte layer thus separated was suspended in 1.2 ml of Lingrel-Borsook's amino acid culture medium (15) containing ^{3}H-leucine (100 μCi) and then incubated at

37°C for 2 hrs. After incubation the reticulocytes were washed with physiologic saline (5 times) to remove the incubation mixture and excess ^{3}H-leucine and lysed by adding two volumes of 0.1% saponin solution. The lysate was passed through a millipore filter (pore size : 1.2 μm) to get limpid hemolysate containing biosynthesized hemoglobins.

18) Analysis of biosynthesized hemoglobins was performed on a DEAE-cellulose (DE-52, Whatman Ltd.) column by the method of Abraham et al. (16) with slight modification. The column size was 0.6×10 cm, and the individual hemoglobins were eluted with a linear NaCl gradient buffer made from 45 ml volumes of 10mM NaCl- and 50mM NaCl-0.2M Glycine-0.01% KCN solutions. The flow rate and fraction size were adjusted to 0.2 ml/min and 1.2 ml/tube respectively. Each fraction was measured for absorbances at 415 nm. 0.5 ml aliquots of the fractions, 0.2 ml of 30% H_2O_2 and 0.4 ml of 60% $HClO_4$ were introduced into a scintillation vial, mixed and warmed in an oven at 60°C for 30 min. 8 ml of Scintisol 500 (Dojindo Laboratories) was added to this solution, and the mixture was then shaken and measured for the radioactivity (dpm) in a liquid scintillation counter (17).

19) The globin prepared from the hemolysate containing the abnormal hemoglobin (Hb X) together with Hb A was chromatographed on a CM-52 column to separate the globin chains (β^A, β^X and α^A) using the method of Clegg et al. (11) with slight modification. The column size was 0.6×10 cm, and the eluting solution was a linear Na_2HPO_4 gradient buffer made from 45 ml volumes of 8mM Na_2HPO_4-8M urea-50mM mercaptoethanol buffer (pH 6.8) and 35mM Na_2HPO_4-8M urea-50mM mercaptoethanol buffer (pH 6.8). The flow rate was adjusted to 0.2 ml/min and the eluate was fractionated in 1.2 ml/tube. After absorbance at 280 nm of the fractionated solutions was measured, one ml of each fraction was poured into a vial containing 6 ml of Scintisol followed by addition of one ml of distilled water, and mixed by shaking sufficiently. The radioactivity (dpm) of the mixtures was counted in a liquid scintillation counter. The specific and total radioactivities were calculated (18).

RESULTS

The propositus was a 51 year old Japanese male who was apparently healthy. He was admitted to the Department of Public Health of Kawasaki Medical School Hospital for a physical checkup in September, 1979. Hematological data of his peripheral blood were within normal limits except for a slight increase in reticulocyte count (WBC $6.1 \times 10^3/\mu$l, RBC $5.02 \times 10^6/\mu$l, Hb 16.1 g/dl, Ht 43.7%, MCV 88 μm^3, MCH 31.9 pg, MCHC 36.3%, reticulocyte count 3.7%). But examination of blood chemistries detected slight hepatic abnormalities (GPT 51 I.U./l, GOT 27 I.U./l), and the patient had actually had an episode of hepatitis in the past for which he had been under the care of a physician. The Hb A_I content was extremely elevated at 44% (normal range 5-7%). This was contradictory to the fasting blood sugar level

which was only 128 mg/dl. It was accordingly suspected that this unusually high value of Hb A_I might be the result of co-elution of the abnormal hemoglobin along with Hb A_I from the microcolumn.

Isoelectric focusing of the hemolysate demonstrated four discrete hemoglobin bands, Hb A_2, Hb A, Hb X-1 and Hb X-2 in order from the cathode to the anode (Fig. 1). The two abnormal hemoglobins (Hb X-1 and Hb X-2)

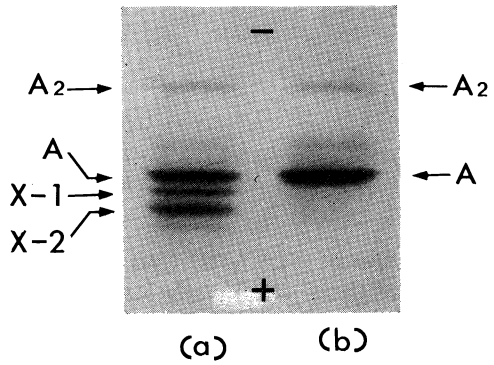


Fig. 1. Isoelectric focusing (pH range 6-9) of hemolysates.
(a) : Patient. (b) : Normal control. A_2=Hb A_2. A=Hb A. X-1=Hb X-1 (Hb Providence Asn). X-2=Hb X-2 (Hb Providence Asp).

were fast-moving. The hemoglobin composition of the hemolysate was : Hb A_2 (2.5%), Hb A (51.2%), Hb X-1 (13.2%) and Hb X-2 (33.1%). Hb F content of the hemolysate was 0.3% by Betke's method (6) and was within the normal range (0.1-1.1%). The instability test with isopropanol was negative. Isoelectric points of the hemoglobins at 24°C were Hb A_2 (7.50), Hb : A (7.14), Hb X-1 (7.09), and Hb X-2 (6.94). The 2,3-DPG content of the blood sample was 1.52 μmol/ml whole blood, 10.94 μmol/g Hb and 3.74 μmol/ml packed cells. These values were slightly subnormal. Serum iron was 89 μg/dl and TIBC (total iron binding capacity) was 326 μg/dl. Both were within the normal range.

Structural analysis :

Cellulose acetate membrane electrophoresis of the hemolysate in 5.5M urea-Tris-EDTA-borate buffer solution (pH 8.3) revealed the presence of two abnormal β chain bands (β^{X-1} and β^{X-2}) which migrated more anodally relative to the normal β chain band (Fig. 2). CM-52 column chromatography of the purified hemoglobins (Hb X-1 and Hb X-2) was employed for the preparation of abnormal globin chains (β^{X-1} and β^{X-2}). The isolated globins (β^{X-1} and β^{X-2} were dialyzed, lyophilized, aminoethylated and digested with TPCK-trypsin at room temperature for 17 hrs. The tryptic peptides thus obtained were separated by cellulose thin layer electrochromatography for fingerprinting (Fig. 3). The fingerprint showed the absence of the normal βT 9, βT 8-9 and βT 10 peptides and the appearance of a new spot near the neutral region. It was therefore

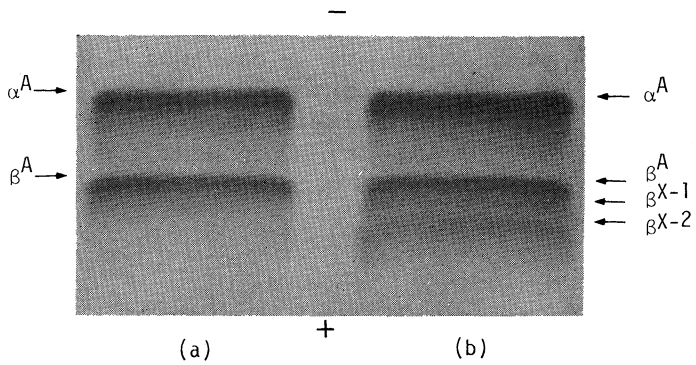


Fig. 2. Urea-dissociation cellulose acetate membrane electrophoresis of hemolysates from normal control (a) and patient (b).

conjectured that the C-terminus of βT 9 (β82 Lys) was substituted for by another amino acid. Amino acid analysis of the abnormal spot was carried out in the following way in order to avoid overlapping of the peptide spots on the map. The abnormal globin was digested with trypsin and the soluble and insoluble fractions were separated. The insoluble fraction was aminoethylated, digested again with trypsin and fingerprinted on cellulose thin layer (Fig. 4). The results indicated that the tryptic peptides βT 9 and βT 10 were united into one with loss of one molecule of lysine and addition of one molecule of aspartic acid or asparagine (Table 1). Accordingly, it was presumed that the amino acid residue of the C-terminus of βT 9 (β82 Lys) was replaced by Asx (Asp or Asn). Taking the relative electrophoretic migration into consideration, it was thought that Hb X-1 (with smaller negative charge) and Hb X-2 (with

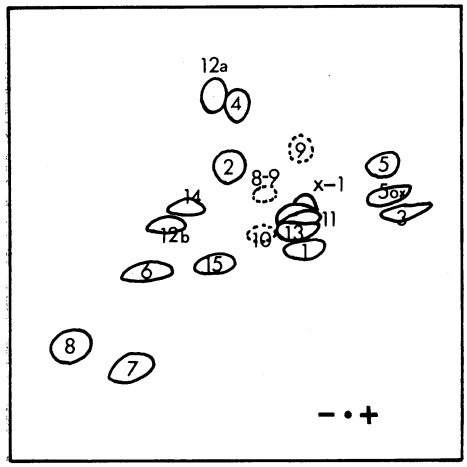
TABLE 1. Amino acid composition of the abnormal peptides, X-1 spot from the β^{X-1} chain and X-2 spot from the β^{X-2} chain, and theoretical numbers of amino acids composing βTp 9, βTp 10 and βTp 9-10 of the normal β chain.

Amino	Found (molar ratio)		Theoretical number		
acid	X-1	X-2	βTp 9	βTp 10	βTp 9-10
Lys	0.89	1.20	1	1	2
His	1.86	1.80	1	1	2
Asp	5.27	5.43	3	1	4
Thr	1.84	1.80		2	2
Ser	1.94	2.00	1	1	2
Glu	1.26	1.26		1	1
Gly	3.02	3.02	2	1	3
Ala	2.79	2.87	2	1	3
Val	1.32	0.96	1		1
Leu	5.75	5.57	4	2	6
Phe	2.03	2.09	1	1	2

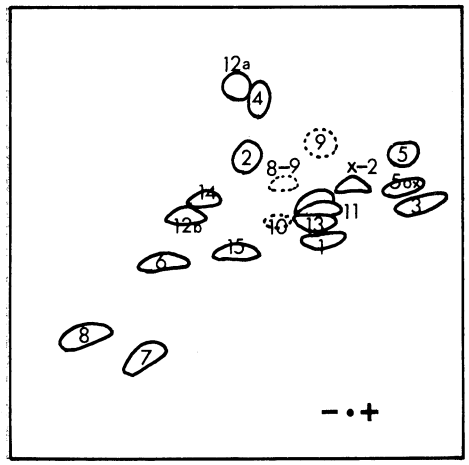
Aminoethylcysteine was recognized.

larger negative charge) were generated as the result of displacemet of Lys by Asn and Asp respectively.

Scrutiny of the list of abnormal hemoglobins recorded in the world disclosed that Hb X-1 and Hb X-2 were the same as Hb Providence which had been discovered in the United States in 1976 (3). However, this is the first case of Hb Providence that has been detected in Japan.



(a)



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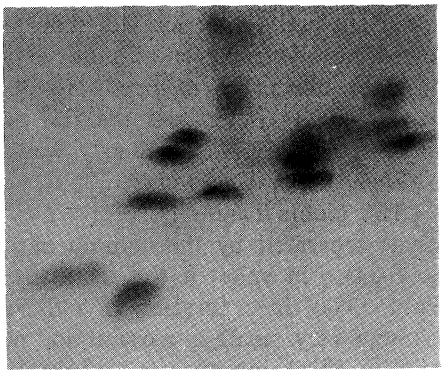

(c)

Fig. 3. Profiles of fingerprint of tryptic digest of aminoethylated abnormal β chains [β^{X-1} : (a) and β^{X-2} : (b)] and photograph of that of β^{X-2} chain (c) on cellulose thin layer. X-1 and X-2 in the figure indicate the abnormal spots. The spots encircled by dotted lines refer to the missing spots.

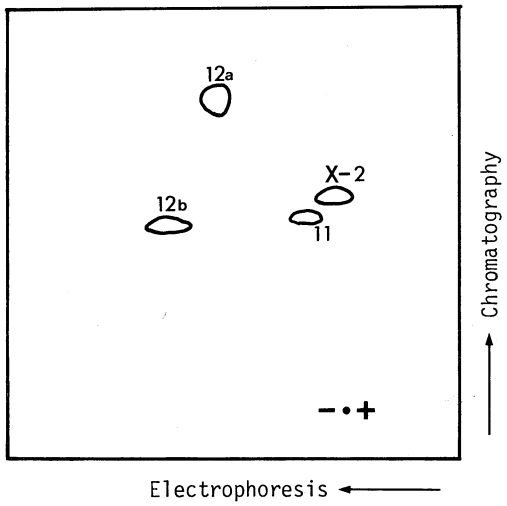

Fig. 4. Fingerprint of tryptic digest obtained from the insoluble fraction of tryptic β^{X-2} chain after aminoethylation on cellulose thin layer.

Functional analysis :

The amino acid substitution position (β82) in this abnormal hemoglobin corresponds to the 2,3-DPG binding site. It was therefore presumed that its oxygen transporting capacity might be affected. As expected, the oxygen affinity of the patient's blood sample was actually increased. The P_{50} was 19.2 mmHg at 37°C and pH 7.4 in contrast to 25.4 mmHg for the blood sample of a normal subject. The purified abnormal hemoglobins (Hb X-1 and Hb X-2) showed, however, oxygen affinities lower than that of Hb A (Table 2 and Fig. 5). The Bohr effects of stripped Hb X-1 and Hb X-2 were reduced (Teble 2). The 2,3-DPG effects and the IHP effects on the abnormal hemoglobins were greatly reduced. This tendency was more marked in Hb X-2.

Hemoglobin biosynthesis :

The globin chains (β^A, β^{X-1}, β^{X-2} and α^A) of the biosynthesized hemoglobins separated by the column chromatography on CM-52 are illustrated in Fig. 6-(a). In spite of the relatively high content of Hb X-2 in hemolysate (about 33%), incorporation of radioactive leucine into the β^{X-2} chain of Hb X-2 was very small in amount and negligible as compared with the total radioactivities (TA) incorporated (Table 3). The specific radioactivities (SA) of individual globin chains which were calculated from the chromatographic data are listed in Table 3. The ratio of the production rate of the β^A chain to that of the α^A chain (β^A/α^A) was 1.32 suggesting that the β^A chain was produced at a slightly greater rate. The β^{X-1} chain was produced 2.73 times as fast as the α^A chain, but β^{X-2} chain production was negligible. In addition, hemoglobin

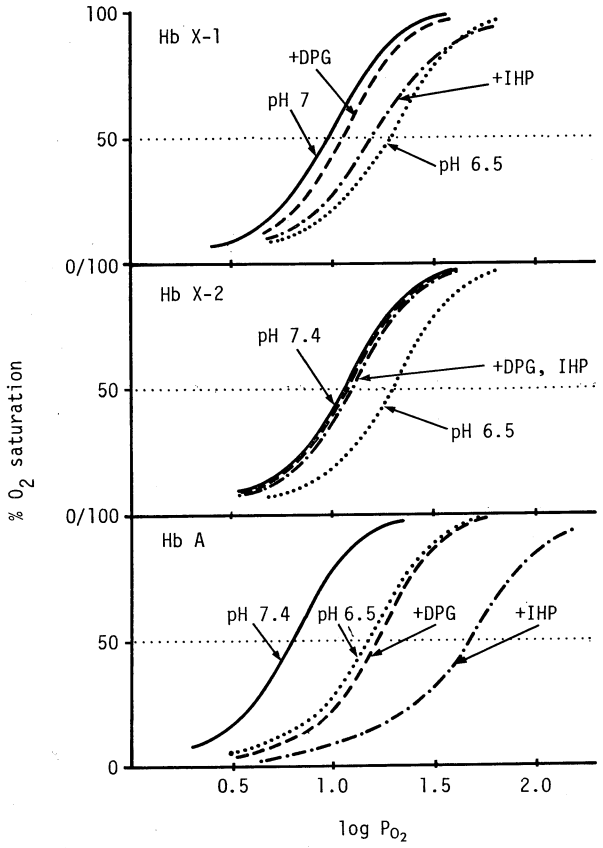

Fig. 5. Oxygen dissociation curves of purified hemoglobins. —— : pH 7.4, ····· : pH 6.5, --- : +DPG (pH 7.4), —•—•— : +IHP (pH7.4)

TABLE 2. Oxygen binding properties of purified individual hemoglobins (Hb A, Hb X-1 and Hb X-2) at various pHs and effects of organic phosphates (2, 3-DPG and IHP ; abbreviation : OP) upon them.

Cofactor	pH	Hb X-1			Hb X-2			Hb A		
		p_{50} (mmHg)	n	Bohr effect	p_{50} (mmHg)	n	Bohr effect	p_{50} (mmHg)	n	Bohr effect
	6.5	18.8	2.83	0.332	19.9	2.91	0.269	14.6	2.82	0.423
	7.4	9.43	2.89	0.270	11.4	2.93	0.210	6.07	2.96	0.266
	9.1	3.28	2.40		5.01	2.65		2.14	2.57	
organic phosphate	pH	p_{50}^{op}	n	$\frac{p_{50}^{op}}{p_{50}}$	p_{50}^{op}	n	$\frac{p_{50}^{op}}{p_{50}}$	p_{50}^{op}	n	$\frac{p_{50}^{op}}{p_{50}}$
2mM 2,3-DPG	7.4	11.1	2.98	1.18	11.9	2.98	1.04	15.6	3.06	2.57
2mM IHP	6.5	70.4	2.00	3.74	25.9	2.30	1.30	99.6	1.73	6.82
2mM IHP	7.4	16.3	2.19	1.73	11.9	2.88	1.04	47.0	2.38	7.74

analysis of whole biosynthesized hemolysate was carried out by column chromatography on DEAE-cellulose (DE-52) as shown in Fig. 7. Incorporation of ^{3}H-leucine was noted in Hb X-1 and Hb A, but was insignificant in Hb X-2, although the percentage of Hb X-2 in the total hemoglobin was maintained at a considerably high level (ca. 33%). Further chromatographic studies of the individual separated hemoglobins were carried out on CM-52. The data of SA and TA are listed in Table 3. When the globin prepared from Hb A was examined, the specific activities of β^{A} and α^{A} were 54,677 and 46,460 respectively.

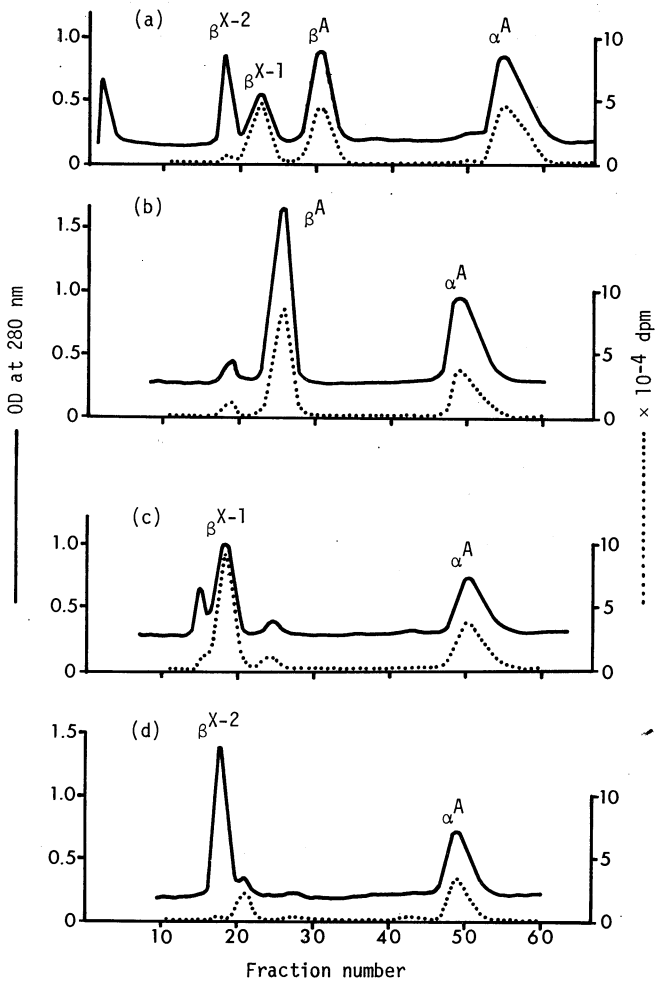

Fig. 6. Urea-CM-cellulose chromatography of globin chains obtaind from biosynthesized whole hemolysate (a) and that of globin chains of the individual hemoglobins isolated and purified from the same hemolysate by isoelectric focusing [Hb A : (b), Hb X-1 : (c) and Hb X-2 : (d)].

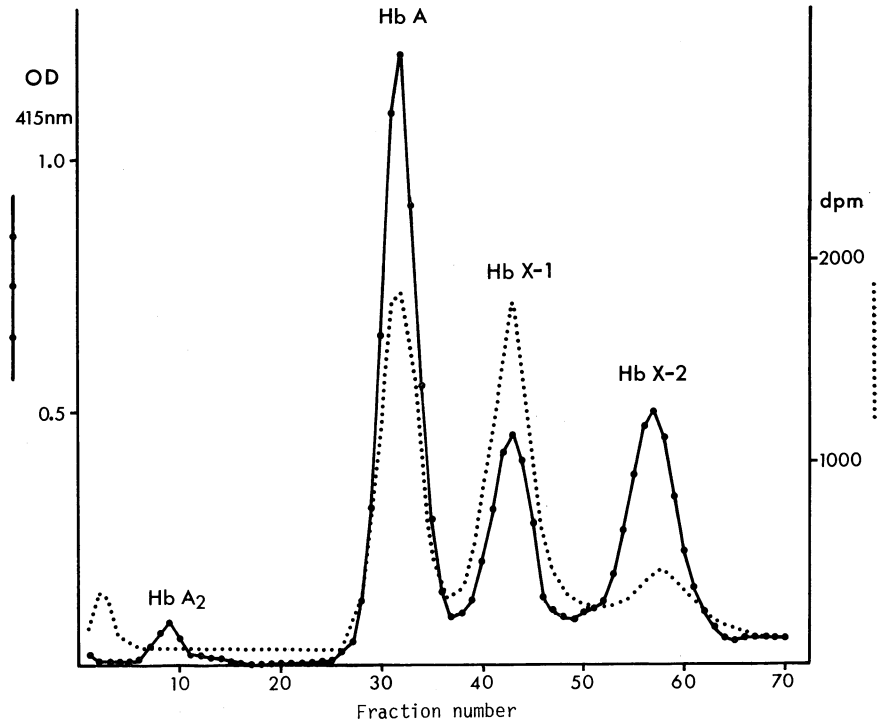

Fig. 7. DEAE-cellulose (DE-52) chromatography of biosynthesized whole hemolysate to separate the respective hemoglobins (Hb A, Hb X-1 and Hb X-2).

TABLE 3. Incorporation of radioactive leucine into the individual globin chains separated by urea-CM cellulose chromatography of the whole hemolysate freshly prepared from the erythrocytes subjected to hemoglobin biosynthesis study. Hemoglobins (Hb A, Hb X-1 and Hb X-2) are isolated and purified by isoelectric focusing. SA and TA indicate the specific and total radioactivities, respectively.

Globin		β^A	β^{X-1}	β^{X-2}	α^A	β^A/α^A	β^X/α^A
whole	SA	94148	195534	—	71417	1.32	2.74
		(98890)*	(101456)**		(71998)*	(1.37)*	(1.41)**
	TA	143716	153319		224059	$\beta^{total}/\alpha^A = 1.33$	
Hb A	SA	54677			46460	1.18	
	TA	239781			164070	1.46	
Hb X-1	SA		197395		90067		2.19
	TA		284199		168217		1.69
Hb X-2	SA			—	66668		
	TA				128152		

*Calculated from total radioisotope counts and the total absorbance (summation of the absorbances of the eluate fractions).

**Calculated from radioisotope counts and absorbances of the β^{X-1} and β^{X-2} chains.

TABLE 4. Oxygen binding properties and organic phosphate (2, 3-DPG and IHP) effect of abnormal hemoglobins possessing amino acid substitution at β82 (EF 6) site (Hb Providence, Hb Helsinki and Hb Rahere), in comparison with those of the normal Hb A.

	Hb Prov Asn		Hb Prov Asp		A		Hb Helsinki*		A*		Hb Rahere**		A**	
β82	Asn		Asp		Lys		Met		Lys		Thr		Lys	
pH	7.4		7.4		7.4		7.30		7.30		7.25		7.25	
	p_{50} mmHg	n	p_{50} mmHg	n	p_{50} mmHg	n	p_{50} mmHg	n	p_{50} mmHg	n	p_{50} mmHg	n	p_{50} mmHg	n
stripped	9.43 (1.99	2.89 nor)	11.4 (3.49	2.93 nor)	6.07 (1.00	2.96 nor)	7.03	3.08	5.01	3.07	3.76	nor	4.39	nor
2mM 2,3-DPG	11.1 (2.96)	2.98	11.9 (3.66)	2.98	15.6 (8.59)	3.06	7.94	3.09	14.29	3.05	5.96	nor	18.3	nor
2mM IHP	16.3 (10.0)	2.19	11.9 (3.83)	2.88	47.0 (27.7)	2.38	8.51	3.10	51.2	2.50	9.90	nor	63.4	nor
Bohr effect	decreased (decreased)		decreased (decreased)		normal (normal)		decreased		normal		normal		normal	

The values in parentheses and those with (**) mark were read from the figures presented in the papers reported by Bonaventura et al. (26) and Lorkin et al. (27), but those with (*) mark were quoted from Ikkala et al. (28).

The β^A/α^A ratio was 1.18, showing a balanced synthetic ratio. On the contrary, the specific activities of β^{X-1} and α^A derived from Hb X-1 were 197,395 and 90,067 respectively with a β^{X-1}/α^A ratio of 2.19. The specific activity of β^{X-2} derived from Hb X-2 was almost zero while that of α^A from Hb X-2 was 66,668.

DISCUSSION

It is apparent from the results of the structural analysis that the two different abnormal hemoglobins, Hb X-1 and Hb X-2, found in the hemolysate contain the β^{X-1} and β^{X-2} chains, and that the β82 Lys residue is substituted for by Asn in Hb X-1 and Asp in Hb X-2. Accordingly, these abnormal hemglobins are identical with Hb Providence (Hb Prov) which was discovered in a Black woman by Moo-Penn, W.F. et al. (3) in the USA in 1976. This is the first case encountered among the Japanese people. The previous authors reported two forms of Hb Providence, Hb Prov Asn (β82 Lys→ Asn) and Hb Prov Asp (β82 Lys→Asp). *In vivo*, protein is synthesized by building the amino acid sequence as encoded in its mRNA by sets of three nucleotides. The mRNA codons are AAA or AAG for lysine, GAU or GAC for aspartic acid, and AAU or AAC for asparagine. Thus, the amino acid substitution Lys→Asp in Hb Prov Asp (Hb X-2) could be explained by mutations in the first and third nucleotides of the Lys codon, and the Lys→ Asn substitution in Hb Prov Asn (Hb X-1) could be explained by a single mutation in the third nucleotide of the Lys codon. However, it is impossible for both these mutation events to occur simultaneously in the same codon as would be necessary to explain genetically the existence of Hb Prov Asn and Hb Prov Asp. Despite the much greater probability of a single one point mutaion as in the case of Hb Prov Asn over the probability of the two mutations necessary for the Hb Prov Asp, the percentage of Hb Prov Asp in the hemolysate was 33% and that of Hb Prov Asn was only about 13%. These curious phenomena may be explained by the possibility of conversion of Hb Prov Asn into Hb Prov Asp by deamidation ($-CONH_2 \rightarrow -COOH$) in the peripheral circulation subsequent to hemoglobin production in the bone marrow. It is not clear why the asparagine residue at β82 of Hb Prov Asn deamidates, while other asparagine residues in the same hemoglobin molecule do not. A plausible explanation was proposed by Shotton and Hartley (19) ; the Asn residue which is directly followed by a Gly residue is specially susceptible to non-enzymatic deamidation. Based on their conception, Lehmann suggested that Hb Singapore (20), which was initially thought to be the result of a two point mutation (α78 Asn→Asp, α79 Ala→Gly), must in fact be a one point mutation (α79 Ala→Gly) followed by deamidation. The mutation results in a new amino acid sequence (----Pro^{77}-Asn^{78}-Gly^{79}-Leu^{80}----) in the α chain and this arrangement of amino acid residues (---Asn^{78}-Gly^{79}---) is converted into (---Asp^{78}-Gly^{79}---) by the deamidation of Asn^{78}. In addition, Perutz reported that

the α72 His residue also catalyzes the deamidation of $\overset{78}{\text{Asn}}$ in cooperation with the $\overset{79}{\text{Gly}}$ residue in Hb Singapore (21). Hb Providence possesses the amino acid sequence (---$\overset{82}{\text{Asn}}$-$\overset{83}{\text{Gly}}$---) which is similar to that of Hb Singapore, and according to Perutz both the β83 Gly residue and the β143 His residue catalyze the deamidation of Asn in Hb Providence. It is interesting that the amino acid sequence ---Asn-Gly--- is one of the special sequence groups which resist the Edman degradation. The increased susceptibility of the amino acid sequence ---Asn-Gly--- to deamidation can not be applied to explain the two forms of hemoglobins (Hb Wayne Asn and Hb Wayne Asp) in Hb Wayne, since the Asn residue does not have a neighboring Gly residue in the abnormal amino acid sequence of Hb Wayne Asn (α : $\overset{137}{\text{Thr}}$-$\overset{138}{\text{Ser}}$-$\overset{139}{\text{Asn}}$-$\overset{140}{\text{Thr}}$---$\overset{145}{\text{Pro}}$-$\overset{146}{\text{Arg}}$ (frame shift product)) (22). However, the biosynthesis studies of Hb Wayne demonstrated the conversion of Hb Wayne Asn into Hb Wayne Asp by deamidation (23). Robinson et al. (24) stated that Gln or Asn residues show various rates of deamidation that are determined by the nature of the neighboring amino acid residues in both primary and tertiary structures. The percentage of Hb Wayne Asp and Hb Wayne Asn are almost equal in hemolysate. In contrast, the content of Hb Prov Asp is three times as high as Hb Prov Asn in the hemolysate. It is therefore thought that the deamidation rate of Asn is considerably affected by neighboring amino acid residues.

The biosynthesis studies which have been described in the preceding section support the theory that deamidation occurs subsequent to the primary protein synthesis of Hb Providence. The result of this process is shown in Figs. 6 and 7.

Radioactive isotope incorporation was distinctly low in Hb X-2 which was purified by column chromatography on DE-52 (Fig. 7) and the isotope incorporation into the β^{X-2} chain was almost zero, as seen in Fig. 6. These findings are in good agreement with those obtained by Charache et al. (25) who purified hemoglobins (normal and abnormal) by column chromatography on DEAE-Sephadex, and separated the globin chains by 8M urea-CM-cellulose column chromatography, while we purified them by isoelectric focusing. We can therefore presume that the primary synthesized globin chain is the β^{X-1} chain and not the β^{X-2} chain, although hemolysate prepared from the patient's peripheral blood contained less Hb X-1 than Hb X-2. The specific radioactivities of the β^{A}, the β^{X-1} and the α^{A} chains are 94,148, 195,534 and 71,417, respectively, and the ratio of β^{A} : β^{X-1} : α^{A} is 1 : 2.08 : 0.76. The specific activity of the β^{X-1} chain is 2.7 times as large as that of the α^{A} chain indicating that the β^{X-1} chain is produced with greater speed than the α^{A} chain. Despite a two fold greater synthesis rate for the β^{X-1} chain over the β^{A} chain, the β^{A} and the β^{X-1} chains are eventually equal in total isotope incorporation β^{A} : β^{-X1} = (143,716 : 153,318 = 1 : 1.07) and exhibit the similar SA values each other (β^{A} : β^{X} = 98,890

: 101,456=1.00 : 1.03) when they were calculated from total isotope counts and the total absorbance (Table 3). It is accordingly presumed that the high specific activity of the β^{X-1} chain in the face of the small percentage of the β^{X-1} chain is a result of gradual conversion of β^{X-1} into β^{X-2} after the β^{X-1} chain has been synthesized Our biosynthesis study *in vitro* shows that the β^{X-2} chain is not translated from the mRNA of the β chain of Hb Providence, but rather the secondary modification product of β^{X-1} chain. The ratio of β^{total}/α^{A} (297,035/224,059=1.33) suggests a slightly excessive synthesis of β chain over the α^{A} chain, as has been pointed out by Charache et al. (25). The cause of overproduction of the β chains remains to be explored. It seems, however, that overproduction of the β chains may compensate for the diminution of the abnormal β chains, since the patient shows a slight reticulocytosis which might be derived from the instabilities of the abnormal hemoglobins. Hb Wayne, a frame shift α chain mutant possessing Asn at the α139 site, similarly presents two forms (Asn and Asp) of hemoglobins. It has been pointed out that Hb Wayne Asp is converted from Hb Wayne Asn by deamidation but its conversion rate was very small in bone marrow, unlike in peripheral blood (22, 23). This is also thought to be the case in Hb Providence.

The purified hemoglobins (Hb Prov Asn and Hb Prov Asp) stripped of 2,3-DPG showed low oxygen affinity, however, the whole blood sample of the Hb Providence carrier which contained 2,3-DPG showed an elevated oxygen affinity. Similar results were reported with purified and stripped hemoglobin specimens by Bonaventura et al. (26) and with whole blood by Charache et al. (25).

They interpreted the results in the following way. The β82 (EF 6) Lys residue of normal hemoglobin, which is positively charged, plays an important role in binding 2,3-DPG. This substance stabilizes the hemoglobin molecule in the deoxy (or T) conformation and reduces its oxygen affinity. The mutation Lys→Asn in Hb Providence Asn results in the loss of one positive charge and impairs 2,3-DPG binding, and the Lys→Asp mutation in Hb Providence Asp causes an alteration of the charge from positive to negative which prevents 2,3-DPG binding. These phenomena decrease the stability of the deoxy conformation. The oxygen affinity of the whole blood is, therefore, increased. Observations of analogous phenomena were made by other investigators on Hb Rahere (β82 Lys→Thr) (27) and Hb Helsinki (β82 Lys→Met) (28), both of which possess an amino acid substitution in the same site as Hb Providence. However, the oxygen affinity of purified Hb Rahere deprived of 2,3-DPG is increased. Hb Helsinki resembles more closely Hb Providence, as there is a slight decrease in oxygen affinity in the purified hemoglobin, and whole blood with erythrocytes containing 2,3-DPG shows increased oxygen affinity. Hb Providence and Hb Helsinki are thought to tend towards the T state in molecular conformation more than Hb Rahere.

The stripped Hb Providence shows reduced Bohr effect and normal cooperativity (Hill's n value). It has been said by various authors (25,26) that

release of protons from this hemoglobin at various pHs is diminished. Generally, the Bohr effect is decreased if conformational isomerization, which breaks the salt bridge between the N-terminus and the C-terminus of the α chains as well as the one between the β146 His and the β94 Asp, takes place on account of amino acid substitution. Unfortunately, this hypothesis does not seem to hold in Hb Providence, because the Hill's n values are within the normal range. The Bohr effect and the Hill's n value are normal in Hb Rahere, but the Bohr effect is decreased and the Hill's n value is normal in Hb Helsinki.

The carriers of Hb Helsinki and Hb Rahere were reported to be polycythemic (blood Hb, 16–19.6 g/dl) (27,28) and those of Hb Providence in the USA showed mild polycythemia (3). In the Japanese carrier of Hb Providence the blood hemoglobin concentration was 16.1 g/dl at the time of the first examination, but during the second examination which was made two years afterwards his Hb concentration was 17.5 g/dl and hematocrit was 50%. A polycythemic tendency cannot be ruled out in this patient, although it is not as apparent as in the carriers of Hb Rahere and Hb Helsinki (27,28). Accordingly, it can be said that patients with abnormal hemoglobins possessing an amino acid substitution at β82, which is one of the 2,3-DPG binding sites, are prone to be polycythemic.

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