Functional and Physicochemical Studies of Hemoglobin St. Louis β28 (B10) Leu → Gln

A VARIANT WITH FERRIC β HEME IRON

JOELLE THILLET, MICHEL COHEN-SOLAL, MAXIME SELIGMANN, and JEAN ROSA

From the Unité de Recherches sur les Anèmies, INSERM U.91, Hôpital Henri Mondor, 94010 Creteil, and the Institut de Recherches sur les Maladies du Sang, Hôpital Saint Louis, 75010 Paris, France

ABSTRACT Studies have been performed on a 20-yr-old man exhibiting methemoglobinemia and a severe hemolytic anemia involving formation of Heinz bodies. This condition was due to an abnormal Hb present in the red cells of the proband: Hb St. Louis, β28 (B10) Leu → Gln, whose structural characteristics have been previously reported. This unstable Hb represented 30% of the total and was isolated by starch block electrophoresis at pH 8.6. Electrophoretic and spectral studies showed Hb St. Louis to be a valency hybrid, $\alpha_2 \beta_2^+$. The presence of hemichrome in this Hb was detected by electron paramagnetic resonance studies. During this study, an electrophoretic technique was developed that allows study of the mobility of hemichrome.

Oxygen equilibria performed on purified Hb St. Louis revealed a high oxygen affinity and a markedly reduced cooperativity. The Bohr effect was normal, but the interaction of this hemoglobin with 2,3-diphosphoglycerate was decreased. The oxidation rate of Hb St. Louis was normal. Hb St. Louis was completely reduced by dithionite and ferrous citrate, and the functional properties of this reduced form were normal. In contrast, Hb St. Louis was only partially reduced by diaphorase.

The mechanism of the oxidation of Hb St. Louis therefore appears to differ markedly from that postulated for other Hbs M.

INTRODUCTION

Hemoglobins M are abnormal hemoglobins in which the heme iron of the mutated chain is stabilized in a high-spin, ferric state. Five distinct types have been described to date: Hb Boston (1), Hb Saskatoon (1), Hb Iwate (2), Hb Hyde Park (3), and Hb Milwaukee (4). The stereochemical effects of these mutations have been extensively studied (5–7). We have recently characterized an additional variant in which the leucyl residue in position β28 (B10) is substituted by a glutaminyl residue (8). The β heme group of this variant, termed Hb St. Louis, is permanently in a ferric state. In the present report, we describe functional and physicochemical studies of this hemoglobin and the clinical manifestations of its presence. Hb St. Louis is the second example of a ferri Hb involving neither the histidine in position E7 nor that in position F8 in its substitution, the first such being Hb Milwaukee. Hb St. Louis is further distinguished from others Hbs M by its greater instability and its ability to form hemichrome.

METHODS

Handling of blood samples. Routine hematologic examinations by standard methods (9) and enzyme assays were performed on freshly drawn, heparinized, venous blood specimens. Other studies were done on acid citrate dextrose samples. The hemolysate was prepared without toluene (10) by centrifugation at 20,000 *g* and hemoglobin was determined as cyanmet hemoglobin according to Drabkin (10). By simultaneous amino acid analysis and measurement of absorption, it was shown that the molar extinction coefficient of Hb St. Louis was the same as Hb A in its cyanmet form at 546 nm.

Electrophoretic studies. Hb electrophoresis on cellulose acetate was performed with Tris-EDTA-borate buffer, at pH 8.6. In the case of electrophoresis of the hemichrome, imidazole (0.2 M) was added to the Tris buffer. Isoelectric focusing was carried out on polyacrylamide gels according to Drysdale et al. (11). The pH gradient employed was in the range 6–8. Isolation of the abnormal Hb was performed by starch block electrophresis as previously described (12).

Nomenclature of different forms of Hb St. Louis. The

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abnormal Hb isolated from the fresh hemolysate without further manipulation is termed Hb St. Louis I. As will be shown hereafter, the fully oxidized hemoglobin, obtained by reaction with an excess of ferricyanide, presented some specific properties: this oxidized form is designated as Hb St. Louis II. The form obtained by complete reduction of Hb St. Louis with dithionite is referred to as Hb St. Louis III.

Spectral studies. The spectra of the various liganded forms of Hb A and Hb St. Louis were recorded on a Cary 14 spectrophotometer (Cary Instruments, Fairfield, N. J.). For spectral studies, reagent (ferricyanide) and ligands (imidazole and cyanide) were used in a molar ratio of ligand to heme of 2 to 1.

Electron paramagnetic resonance (EPR).[1] EPR studies were performed as described elsewhere.[2]

Oxygen Hb equilibria. Oxygen equilibrium curves were determined on red blood cells and on solutions of Hb by the spectrophotometric method of Benesch et al. (13). Equilibrium curves of the red blood cells were determined in phosphate buffers (0.15 M) in the pH range 6.45–7.45. Oxygen equilibria of isolated Hb A and Hb St. Louis were determined with 2,3-diphosphoglycerate (DPG)-free Hb as previously described (14). Assays were performed in 0.05 M bis-Tris buffer in the pH range 6.45–7.15 and 0.05 M Tris-HCl buffers in the pH range 7.45–9.0. The amount of methemoglobin was measured before and after each determination according to Benesch et al. (15). All these experiments were performed at 37°C unless otherwise stated. The Bohr effect was calculated from the formula (Δ logP50/Δ pH).

DPG effect studies. The effect of DPG on Hb was investigated by two methods: (*a*) The P_{50} of Hb A and of Hb St. Louis was determined in the presence of a molar ratio DPG/Hb = 2.5, with 0.05 M Tris-HCl buffer at pH 7.15 (16). (*b*) The binding of DPG to Hb was also directly measured by ultrafiltration at pH 7.2 in bis-Tris buffer (50 mM, 0.1 M Cl^-) as described by Bauer et al. (17), with a high-vacuum apparatus (Sartorius Balances, Brinkmann Instruments; Westbury, N. Y.) with membranes of nitrate cellulose and highly purified nitrogen. The binding experiments were carried out on 50 μM deoxyHb with a concentration of free DPG of approximately 50 μM. DPG was used in its free acid form after conversion from the cyclohexylammonium salt (18) and titration to neutrality. The concentrations of DPG in the DPG-Hb mixture and in the ultrafiltrate were determined by the enzymatic method of Rose and Leibowitz (19).

Oxidation and reduction studies. Oxidation of Hb St. Louis by ferricyanide was performed according to Hayashi et al. (20) with a molar ratio of ferricyanide to ferrous heme of 100:1.

Several methods of reduction were used: (*a*) To obtain the ferrous form of Hb St. Louis, the following technique was used: a sample of purified Hb St. Louis was chromatographed through a band of 0.1 M dithionite solution, on a column of Sephadex G 25 previously equilibrated with phosphate buffer (0.1 M) at pH 7.0. (b) The kinetics of reduction of Hb St. Louis samples (forms I and II) by sodium dithionite ($Na_2S_2O_4$) were determined as previously described (20). (*c*) Reduction with ferrous citrate was performed according to Perutz et al. (5). (*d*) The enzymatic reduction was carried out with pig heart diaphorase by the technique of Suzuki et al. (21), modified as follows: the mixture contained 1.3 μmol NADH, 0.1 μmol methylene blue, and 20 μl diaphorase per milliliter of solution.

Dissociation equilibrium studies. Measurement of dissociation equilibria were performed on Sephadex G100 according to Bunn (22) and by ultracentrifugation. Sedimentation analyses were carried out with a Spinco model E analytical ultracentrifuge at 50,000 rpm and 25°C (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

Sulfhydryl determination. The reactive sulfhydryl groups of Hb were measured spectrophotometrically by titration with *p*-hydroxymercuribenzoate according to the method of Boyer (23), as modified by Benesch and Benesch (24).

Binding of cyanide. Experiments to determine the extent of cyanide binding to hemoglobin were performed according to Hayashi et al. (25). Assays were performed at room temperature and pH 7.0.

Other studies. Methemoglobin was determined on fresh samples according to the method of Benesch (15). The heat instability of hemoglobin was tested according to the procedure of Grimes et al. (26).

Materials. Potassium ferricyanide, potassium cyanide, and sodium dithionite were supplied by Merck AG (Darmstadt, W. Germany). *p*-Hydroxy-mercury benzoate and imidazole were obtained from Sigma. Chemical Co. (St. Louis, Mo.). Cellulose acetate was provided by Chemotron (Milano, Italy), maïze starch by BDH Chemicals Ltd. (Poole, England), ampholine by LKB Produkter (Bromma, Sweden), diaphorase by Boehringer Mannheim Corp. (New York) and Sephadex by Pharmacia. Fine Chemicals, Uppsala, Sweden.

RESULTS

Clinical data and case report

Hb St. Louis, α_2^A $\beta_2$28 (B10) Leu → Gln, was detected in a patient who had originated from Soisson in northern France. At 6 yr of age, he exhibited an hemolytic anemia and was referred to the department of hematology at Hôpital St. Louis in Paris. Examination revealed the association of pallor, jaundice, and cyanosis of his lips and fingernails. The spleen was enlarged to a breadth of three fingers below the costal margin. The liver was of normal size. Laboratory findings (Table I) showed a severe hemolytic anemia and 15% methemoglobin. Mesobilifuscinuria was absent. Splenectomy resulted in a permanent improvement in his clinical and hematological status (Table I). At 15 yr of age, cholecystectomy was performed because of cholecystitis and gallstone formation. Since then, he has exhibited a variable degree of jaundice and anemia. However, no blood transfusions have been required, and he had led a normal life.

The abnormal hemoglobin was not present in either of his parents or his 10 siblings. The propositus was the last of the children. The patient was born when his father was 41 and his mother 40 yr old.

Electrophoretic and spectral studies

Hb St. Louis I. Preliminary studies demonstrated that the fresh hemolysate contained 70% Hb A and

[1] *Abbreviations used in this paper*: DPG, 2,3-diphosphoglycerate; EPR, electron paramagnetic resonance.

[2] Hyafil, F., J. Thillet, and F. Gueron. In preparation.

TABLE I
Hematological Data

	Hb	Erythrocytes	Packed cell volume	Mean corpuscular volume	Mean corpuscular hemoglobin	Mean corpuscular hemoglobin concentration	Reticulocytes	Heinz bodies	Methemoglobin	Leucocytes	Platelets	Serum bilirubin
	g/dl	*$10^6/\mu l$*	%	*μm^3*	*pg*	%	%	%	%	*$10^3/\mu l$*	*$10^5/\mu l$*	*mg/dl*
Before splenectomy	10	2.9	28.3	97.5	34.5	35.3	20	20	15	20	4–6	4.0
After splenectomy	12.3	3.13	35	115	40	35.5	15	60	15	10–20	3–4	4.0

30% Hb St. Louis; the latter was spontaneously partially oxidized (8). Nevertheless, the electrophoretic pattern of Hb St. Louis on cellulose acetate was markedly different from those of ferrihemoglobin A and various M hemoglobins. To determine precisely the state of oxidation of the iron in Hb St. Louis, further studies were performed.

Hemolysate from the patient was submitted to starch block electrophoresis. The spectra of the two eluted bands were determined in the visible range. The spectrum obtained from the Hb A fraction was typical of that of oxyhemoglobin. It contained less than 5% of ferrihemoglobin, thus demonstrating the low rate of autoxidation during the isolation procedure. In contrast, the spectrum of Hb St. Louis I (see Methods section for nomenclature) was analogous to that of a mixture of 50% oxyhemoglobin and 50% ferrihemoglobin (Fig. 1). It is notable that the absorption maximum at 630 nm observed in the spectrum of Hb St. Louis I was identical to that seen in the spectrum of ferri Hb A; this peak is always displaced in the previously described cases of Hbs. M.

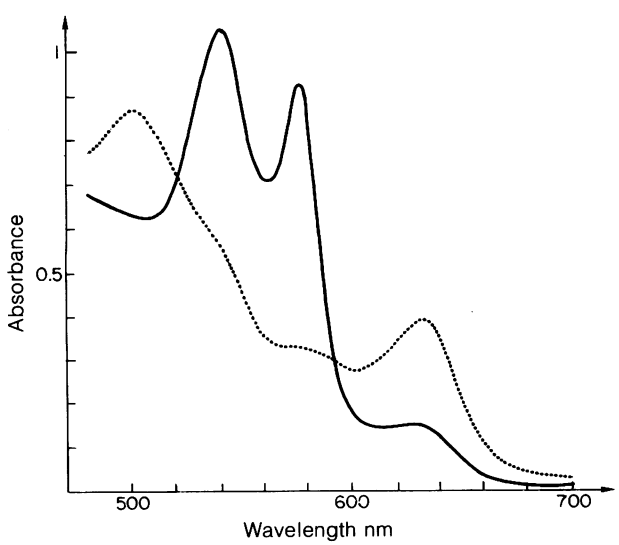

FIGURE 1 Visible spectra of ferri Hb A (· · · ·) and of Hb St. Louis I purified by starch block electrophoresis (———) in 0.15 M sodium phosphate buffer at pH 6.5.

The pattern obtained upon isoelectrofocusing of the patient's hemolysate (Fig. 2*a*) was compared to that of a mixture of equal part of oxyhemoglobin and ferrihemoglobin A (Fig. 2*b*). As described by Bunn and Drysdale (27), the control sample (Fig. 2*b*) contained four major components: two hybrids IBI and IBII were observed midway between the bands corresponding to oxy and to ferrihemoglobin. These intermediate bands were assumed to be valency hybrids $(Fe^{3+})_2$ $(Fe^{2+})_2$. The hemolysate from the patient (Fig. 2*a*) contained two major bands, readily identified on the basis of their spectra as Hb A and Hb St. Louis I. The latter focused slightly more cathodically than IBII in the control.

Hb St. Louis II. The isoelectrofocusing pattern of the hemolysate from the patient oxidized by ferricyanide is shown in Fig. 2*c*. The sample contained two major bands, one of ferrihemoglobin A and another that focused more cathodically.

The same band was also obtained from a sample of pure Hb St. Louis I reacted with ferricyanide. This band was designated Hb St. Louis II (see Methods section). A visible spectrum of this latter sample was performed (Fig. 3) and exhibited four maxima

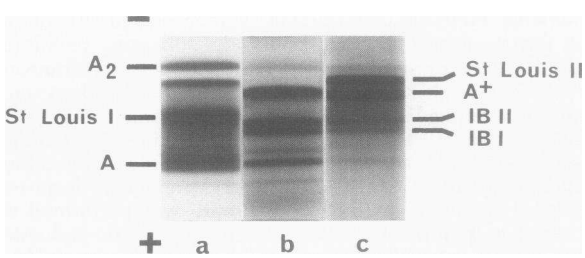

FIGURE 2 Isoelectric fractionation of several samples in the pH range 6–8. (*a*) Hemolysate from the propositus. (*b*) A mixture of equal parts of Hb A and Hb A oxidized by addition of ferricyanide. (*c*) Propositus's hemolysate after addition of ferricyanide.

at 500, 535, 565, and 630 nm. Rachmilewitz (28) has demonstrated that this kind of spectrum is due to a mixture of ferrihemoglobin and hemichrome, a low-spin component in which the sixth coordinance of the iron is bound to an imidazole group. To determine the percentage of hemichrome present in Hb St. Louis II, the following method was used: a differential spectrum was recorded with a sample of Hb St. Louis II in the assay cuvette and a sample of ferrihemoglobin A in the reference cell. The concentration of Hb St. Louis II was maintained constant while that of ferrihemoglobin A was progressively increased until the spectrum of a pure hemichrome was obtained. At this moment, the concentration of ferrihemoglobin A in the reference cuvette was found to be 75% of the concentration of the total hemoglobin in the sample of Hb St. Louis II. This result demonstrated the presence of approximately 25% hemichrome in Hb St. Louis II.

Up to now, no electrophoretic data have been published concerning hemichromes. An attempt was therefore made to compare the electrophoretic mobility of Hb St. Louis II, which contains hemichrome (supposedly due to the binding of iron to an imidazole group in the chain), with the electrophoretic mobility of artificial hemichromes. These latter were prepared by reacting ferrihemoglobins with an (0.2 M) imidazole-buffered solution. Since the affinity constant of imidazole for ferrihemoglobin was low, electrophoresis was performed in the presence of excess imidazole. These conditions precluded the use of isoelectrofocusing, since it was not possible to introduce imidazole in the polyacrylamide gel without destruction of the pH gradient.

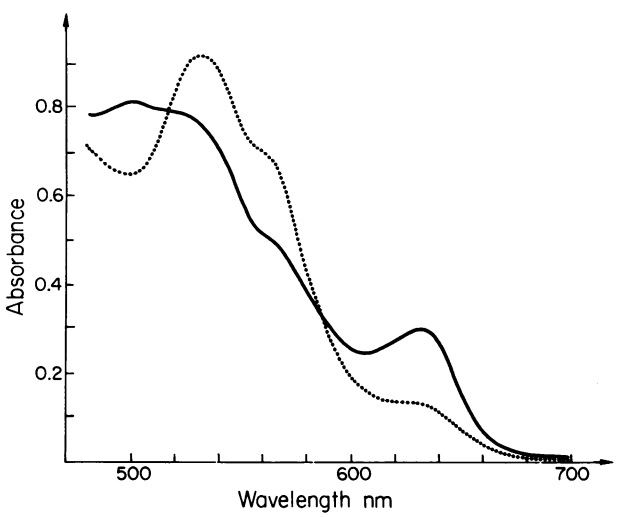

FIGURE 3 Visible spectra of hemichrome obtained after treatment of ferri Hb A with imidazole (· · · ·) and of purified Hb St. Louis II (———). The spectra were determined in phosphate buffer (0.15 M) at pH 6.5.

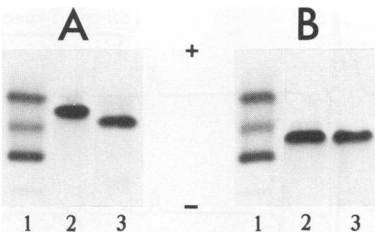

FIGURE 4 A: Electrophoretic patterns on cellulose acetate. A buffer of Tris-EDTA borate at pH 8.6 was employed: (*1*) Mixture of oxy Hb A, S, and C. (*2*) Ferri Hb A. (*3*) Hb St. Louis II. B: The same samples as in A. A buffer containing Tris-EDTA borate imidazole at pH 8.6 was employed.

In Fig. 4, the electrophoretic mobilities on strips of cellulose acetate of various samples of hemoglobins are compared in Tris-EDTA-borate buffer without (A) and with (B) 0.2 M imidazole. Sample 1 contained a mixture of oxyHb A, S, and C. These hemoglobins exhibited the same mobilities in the two respective buffer systems. Sample 2 contained ferrihemoglobin A in buffer A and hemichrome in buffer B. This latter form demonstrated a greater cathodic mobility than that of ferrihemoglobin A. Sample 3 contained Hb St. Louis II in buffer A and imidazole-reacted St. Louis II in buffer B. Hb St. Louis II demonstrated a mobility different from that of an imidazole hemichrome. The hemichromes obtained from ferrihemoglobin A (B2) and from Hb St. Louis II (B3) were identical in their migration.

EPR studies

The main results of the EPR studies are presented in Fig. 5.[3] The EPR spectrum of oxygenated Hb St. Louis (Fig. 5*a*) exhibits the low-field ($g \simeq 6$) peak characteristic of high-spin ferric iron, which arises from the β chains. A splitting of this peak was resolved at the temperature of liquid helium, indicating a distortion of the iron site as compared to Hb A (29). At pH 6.2, a low-spin signal was also observed, ascribable to a hemichrome. Thus Hb St. Louis I is partly present in a hemichrome form. At pH 9 the hemichrome signal remained, and another low-spin signal appeared that corresponded to the expected hydroxy ferric compound.

The spectrum of whole red blood cells from the patient (Fig. 5*b*) also shows the high-spin signal, together with the low-spin signals of hemichrome and the hydroxy ferric compound. In particular, the presence of the hemichrome signal shows that the hemichrome is not an artifact.

In Hb St. Louis II (Fig. 5*c*) both the α and β chains

[3] Detailed EPR studies will be presented elsewhere (see footnote 2).

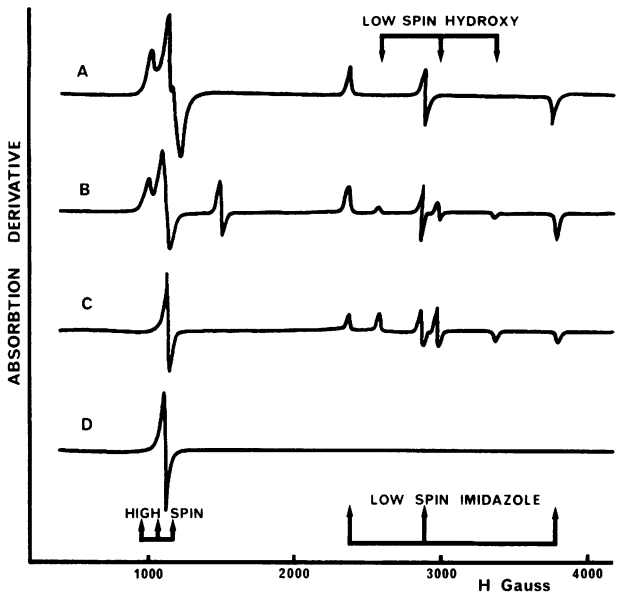

FIGURE 5 EPR spectra of different forms of Hb St. Louis. A. Oxygenated Hb St. Louis I at pH 6.2. B. Whole red blood cells from the proband at pH 7.0. C. Hb St. Louis II at pH 6.2. D. Control ferri Hb A at pH 6.0.

contribute to the EPR spectrum. The spectrum contained both the low-spin hemichrome signal, probably due to the β chain, and high-spin signals, probably originating from both α and β chains. A striking feature was the occurrence, even at pH 6.2 of a hydroxyferric low-spin signal. The absence of any EPR signal in dithionite-treated Hb St. Louis (Hb St. Louis III) provides evidence that the iron has been effectively reduced.

These results distinguish Hb St. Louis from the $\alpha_2^{2+}\beta_2^{3+}$ valency hybrid that exhibits no distortion in its high-spin form (29). Hb St. Louis also differs from the Hbs M that do not form hemichromes. Furthermore, the pH-dependent spin equilibrium observed in Hb St. Louis has not as yet been observed in the Hbs M (30, 31).

Functional studies

Oxygen saturation curves for intact red cells from the propositus and from a normal control are shown in Fig. 6. The right shift of the patient's curves is indicative of a significant decrease in oxygen affinity. The P_{50} values at pH 6.45, 7.15, and 7.45 were 57, 36, and 29 mm Hg, respectively (normal values: 52±2, 30±1, and 22±1 mm Hg). The Hill coefficient "*n*" was found to be 2.2 (normal 2.6), indicating a decreased cooperativity. The patient's curve was not biphasic as shown in the Hill plot.

In contrast, oxygen equilibria of purified Hb St. Louis I at different pH's show a high oxygen affinity (Fig. 7). The *n* value 1.5 for Hb St. Louis I is small, as in the red cells, and indicates a poor cooperativity between subunits (normal control 2.7). The amount of methemoglobin remained constant before and after the assays. The alkaline Bohr effect appeared to be normal: (Δ log P50/Δ pH) = −0.44, as compared to −0.45±0.05 for normal Hb.

The level of DPG of 14 μmol/g Hb, measured on whole cells, is normal. However, since the whole cells contain large amounts of denatured hemoglobin in Heinz bodies, it appeared most efficient to express the DPG level relative to the functional hemoglobin. Thus the DPG concentration was determined on the clear supernatant hemolysate: it was found to be 28 μmol/g hemoglobin in the case of Hb St. Louis, whereas the normal value was not changed. This high DPG/hemoglobin ratio provides an explanation for the apparent discrepancy between the decreased O_2 affinity in the red cells and the increased affinity of purified hemoglobin. This discrepancy could become greater if abnormal hemoglobin does not bind DPG normally.

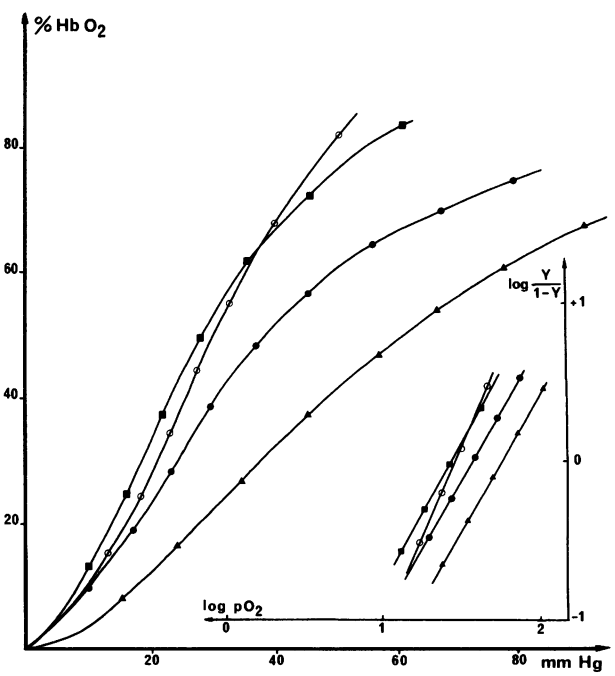

FIGURE 6 Oxygen dissociation curves of intact red blood cells. Measurements were performed in phosphate buffer (0.15 M) at 37°C. Normal at pH 7.15 (-○-○-○-) P_{50} = 30 mm Hg; propositus at pH 7.15 (-●-●-●-) P_{50} = 36 mm Hg; propositus at pH 7.45 (-■-■-■-) P_{50} = 29 mm Hg; propositus at pH 6.45 (-▲-▲-▲-) P_{50} = 57 mm Hg. The St. Louis red cells contained approximately 70% of Hb A. The right section of the figure shows the Hill plot obtained from these curves.

DPG effect

The shift towards the right of the oxygen dissociation curve of Hb St. Louis I in the presence of DPG is not as marked as that of Hb A (Table II).

This result was the first indication of an abnormal binding of DPG by Hb St. Louis I. Direct measurement of the DPG/deoxyhemoglobin constant of dissociation of Hb St. Louis I by the ultrafiltration technique allowed identification of the cause of this abnormal reaction.

The apparent dissociation constant K' was found to be 60 μM for Hb St. Louis I. Under the same experimental conditions K' was 15 μM for normal Hb A and 0.16 mM for ferrihemoglobin A. The K' of deoxy Hb St. Louis I was intermediate between those of deoxy Hb A and ferriHb A.

Oxidation studies

Studies of Hb A and Hb St. Louis I showed them to be oxidized at similar rates: 50% of each was oxidized in 135 s. The time-course of the oxidation was of the first order. It is notable that the rates of oxidation of Hbs M were faster than that of Hb A, especially in the case of Hb M Boston and M Iwate (20).

Reduction studies

Reduction by sodium dithionite. The reduction of Hbs St. Louis I and II by sodium dithionite under CO gas resulted in the formation of Hb CO St. Louis, the absorption spectrum of which was identical to that of CO Hb A. The rates of reduction of metHb A, Hb St. Louis I, and Hb St. Louis II to Hb Co under CO gas were too rapid to be measured with accuracy. In contrast, the reduction of Hbs M was relatively slow and could be measured (20).

Properties of $\alpha_2^{++}\beta_2^{++}$ St. Louis (St. Louis III). After reduction by dithionite, the oxy form of ferrous Hb St. Louis was stable for several hours. On cellulose acetate, the presence of two bands was demonstrated in this form (i.e. Hb St. Louis III): one band migrated as Hb A, and the other migrated as Hb St. Louis I, the latter probably being formed by an oxidative process during electrophoresis. This demonstrates that in the absence of methemoglobinization, Hb St. Louis has the same charge as Hb A. Moreover, the oxygen equilibrium at 10°C of freshly prepared Hb St. Louis III exhibited a normal P_{50} and n compared with those of Hb A under the same conditions (Table II). In contrast, the same experiment performed on Hb M Iwate (32) showed a normal P_{50}, but this hemoglobin did not recover a normal cooperativity. The dissociation constant between Hb St. Louis III and DPG was normal (14 μM). Similar results were obtained by reduction with ferrous citrate, namely, Hb St. Louis III demonstrated functional properties like those of a normal Hb A.

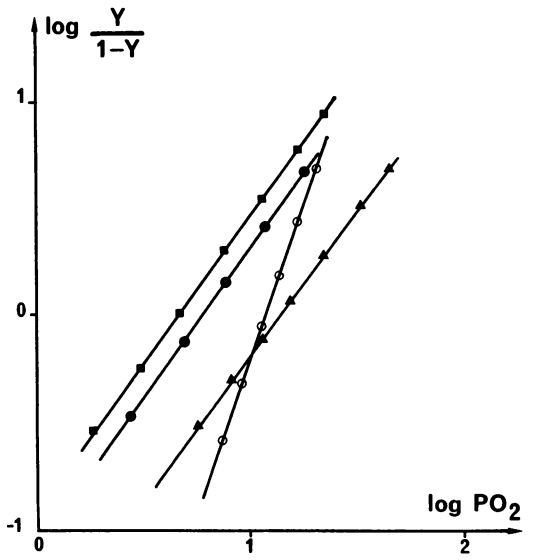

FIGURE 7 Hill plot of oxygen dissociation curves of phosphate-free hemoglobins A at pH 7.15 (-○-○-○-), Hb St. Louis at pH 7.15 (-●-●-●-), 7.45 (-■-■-■-), and 6.45 (-▲-▲-▲-). These hemoglobins were studied at a concentration of 20 μM in 0.05 M chloride, 0.05 M bis-Tris buffers at pH 6.45 and 7.15, and 0.05 M Tris buffer at pH 7.45, 37°C.

Reduction by diaphorase. In four of the five different abnormal hemoglobins that cause methemoglobinemia (except Hb Milwaukee), methemoglobin reductase is ineffective. In the patient's red cells, methemoglobin reductase could not maintain Hb St. Louis in a ferrous form.

A ferricyanide treatment has been described that

TABLE II
Effects of 2,3-DPG and Dithionite on the Oxygen Equilibria of Hb A and Hb St. Louis

	Hb St. Louis		Hb A	
	P_{50}	n	P_{50}	n
	mm Hg		*mm Hg*	
None, 37°C	6	1.5	12	2.7
DPG/Hb: 2.5, 37°C	10	1.5	22	2.9
After treatment with dithionite at 10°C*	4	2.7	5.5	2.5

Experimental conditions as stated in legend to Fig. 7. Equilibria were determined at pH 7.15.
* See Methods section.

results in a great enhancement of the activity of methemoglobin reductase (33). Unfortunately, this system cannot be applied to Hb St. Louis, since ferricyanide converts it into hemichrome. Therefore, we have compared the reduction of ferriHb A, ferriHb Saskatoon, and Hb St. Louis I, in the system described by Suzuki et al. (21) with pig heart diaphorase. This diaphorase reduced ferriHb A but was unable to reduce Hb M Saskatoon. Hb St. Louis I was only partially reduced. 15–17% of unreduced Hb St. Louis remained at the end of the kinetic study.

Dissociation equilibrium

The pattern of elution of Hb St. Louis I on Sephadex G100 was similar to that of Hb A, as was its coefficient of sedimentation on analytical ultracentrifugation (4.2 $S_{w20°C}$) indicating that it exhibits a normal dissociation equilibrium. Hb St. Louis II displayed a dissociation constant identical to that of ferriHb A. The number of titrable SH groups in Hb St. Louis was found to be normal, as in the case of Hb Milwaukee (34).

Binding of cyanide

The binding of cyanide to metHb A and to Hb St. Louis II was similar. The cyanide dissociation curve was monophasic, in contrast to that of other Hbs M; the apparent K' value was found to be 50 μM for metHb A and for Hb St. Louis II.

Stability

When heated to 50°C, Hb St. Louis I precipitated. Such instability may explain the low percentage of the abnormal Hb present in the red cells, since its synthesis was found to be normal (35).

DISCUSSION

Up to the present time, two groups of abnormal hemoglobins have been found to cause methemoglobinemia (36). The first is the Hbs M. In this case, the mechanism of the methemoglobinemia may be explained by the existence of a covalent bond between a side-chain of an amino acid and the fifth or the sixth iron coordinance of the heme of the mutated chain. This has been recently demonstrated in Hbs Boston (7), Iwate (6), and Hyde Park (6). The iron of their mutated chains is bound to the phenol group of tyrosine F8 or E7. In Hb Milwaukee (5), the heme iron is covalently bound to the carboxylic group of glutamic E10. The second group is several unstable Hbs that undergo spontaneous methemoglobinization (37). The mechanism that permits the formation of methemoglobin in these cases was uncertain: it could be a structural modification of the heme pocket that would allow water to enter this normally hydrophobic site (38). The present observations on the physicochemical properties of Hb St. Louis and the stereochemical ones in the following report could contribute to the elucidation of the molecular basis for the formation of methemoglobin.

The major structural properties of Hb St. Louis I are: (*a*) A migration on isoelectrofocusing near that of the valency hybrids $(Fe^{++})_2$ $(Fe^{+++})_2$ of Hb A. (*b*) An EPR spectrum demonstrating the presence of a high-spin signal characteristic of the ferri form. (*c*) An optical spectrum that demonstrated that this ferri form constituted 50% of Hb St. Louis I.

These results lead us to conclude that Hb St. Louis I is a valency hybrid. Since the substitution in Hb St. Louis is localized at the β28 residue, it seems reasonable to assume that the β chains are present in the ferri form: $\alpha_2{}^{A}\beta_2{}^{+\ St.\ Louis}$. The small difference between the pI's of Hb St. Louis I and the valency hybrids of Hb A could indicate some conformational differences between them, since the replacement of leucine 28 by a glutamine does not induce a difference of charge between normal and mutated β chains.

These properties permit us to consider Hb St. Louis as a new Hb M, since the iron atoms of the mutated chains in both Hb St. Louis and the Hbs M are permanently in a ferric state. Nevertheless, the mechanism of oxidation of Hb St. Louis I resembles that hypothesized for the unstable hemoglobins that exhibit methemoglobinization (see following paper). Such a mechanism of oxidation could explain the observed differences between the functional properties of Hb St. Louis I (oxygen affinity, cooperativity, and Bohr effect) and those of the Hbs M.

Another aspect of our functional studies concerns the effect of DPG on hemoglobin. The results obtained for Hb St. Louis I correspond closely to those obtained by Bauer et al. (17) for the binding of DPG to cyanide derivatives of hemoglobin valency hybrids $(\alpha^{++}\beta^{+++CN})_2$. It was clearly demonstrated that the β met chains of Hb St. Louis I were structurally in the oxy form (see following paper), even under deoxygenation. Consequently, their fixation of DPG was decreased since the deoxy form has the greatest affinity for DPG.

A strict correlation is generally found between the P_{50} of the erythrocyte and the DPG level (39), but in some cases the amount of total hemoglobin and that of hemoglobin reacting normally with DPG differ. In these instances, the abnormal hemoglobin may either be unstable and precipitate in the erythrocytes, or it may not bind DPG normally; alternatively, fetal hemoglobin, which does not bind DPG

(40), may be present in large amounts. In such cases, a discrepancy may be found between the P_{50} of the total erythrocytes and the DPG level. This observation can be explained if the DPG level is expressed relative to the total hemoglobin, while the DPG itself only reacts with normal hemoglobin. In the case of Hb St. Louis, both the first and second conditions are possible: Hb St. Louis is unstable and does not react normally with DPG.

The properties of Hb St. Louis I on reduction are different from those of Hbs M. When reduced chemically (e.g. by dithionite), both Hb St. Louis I and Hb M were completely reduced. However, all the functional parameters of Hb St. Louis were restored in this reduced form, in contrast to Hb M Iwate (32), in which cooperativity remains decreased after reduction under the same conditions. This fact could be considered as additional evidence for the different stereochemical mechanism involved in the oxidation of Hb St. Louis and Hb M. Upon enzymatic reduction with diaphorase, Hb St. Louis I was partially reduced while Hb M are not (5). Nevertheless in case of Hb M Milwaukee, in which the mutation is at the E11-residue, the enzymatic reduction is possible (5). This difference may result from dissimilarities between the liganded state of the iron in these various hemoglobins. In Hb St. Louis I the affinity of the displaced His E7 for the iron could be intermediate between that of His E7 in normal ferriHb A and that of Tyr E7 in Hb Boston or Saskatoon (7).

The structural properties of Hb St. Louis II are as follows: (*a*) an optical spectrum that demonstrated the simultaneous presence of 75% of the ferri form of iron and 25% hemichrome (*b*) an EPR spectrum that confirmed the presence of hemichrome with its characteristic low-spin signals. (*c*) an electrophoretic migration distinct from that of ferriHb A and of an hemichrome produced by reaction with imidazole (*d*) a normal dissociation constant as compared to ferri Hb A.

This last property led us to assume that the presence of a stable asymetrical hybrid containing ferri and hemichrome chains was not possible. Thus, during electrophoresis molecular species (with and without hemichrome) would give more than one electrophoretic band if they had different charges. Since Hb St. Louis II focused as a single band, it demonstrates that the hemichrome in Hb St. Louis II has the same charge as the ferri form of Hb St. Louis.

The liganded forms of Hb St. Louis II with KCN (8) or imidazole (Fig. 4) had the same electrophoretic mobilities as the corresponding liganded forms of ferriHb A. It is notable that these ligands have a higher affinity constant for the ferric iron than H_2O or OH^-. Consequently it could be hypothesized that the slight modification of charge observed in Hb St. Louis II was due to a conformational change of its β chains in their weakest liganded form.

According to Rachmilewitz (37), hemichrome formation requires dissociation into dimers in addition to the oxidation of Hb. The present observations concerning Hb St. Louis II differed from those reported for several unstable hemoglobins, Freiburg (41), Riverdale Bronx (42), Köln (37), and Hammersmith (43) that form hemichromes after their oxidation. In each case, Rachmilewitz observed that the characteristic spectrum of hemichrome was recorded only after the transient appearance of ferrihemoglobin (37). In contrast, irrespective of the procedure employed for oxidation of Hb St. Louis I, it was not possible to detect a form of Hb St. Louis II without hemichrome; the percentage of such hemichrome was invariable. Moreover, the increased dissociation of hemoglobin into dimers postulated in case of these unstable hemoglobins was not observed in Hb St. Louis II. According to these results, it could be hypothesized that the mechanism of the hemichrome formation in Hb St. Louis II occurs only as a local phenomenon at the level of the heme pocket. This hypothesis is supported by the finding presented in the following paper and differs markedly from that postulated for the other unstable Hbs, which implied an entire change in the tertiary structure of the mutated β chains (37).

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