Hemoglobin Syracuse ($\alpha_2\beta_2^{143(H21)His\rightarrow Pro}$), a New High-Affinity Variant Detected by Special Electrophoretic Methods

OBSERVATIONS ON THE AUTO-OXIDATION OF NORMAL AND VARIANT HEMOGLOBINS

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ABSTRACT Family members from four generations were found to have polycythemia and increased whole blood O_2 affinity (P_{50}; 11 mm Hg; normal, 27 mm Hg). No abnormal hemoglobin bands were seen after electrophoresis on starch gel at pH 8.6 or agar gel at pH 6.0. Analysis of the oxygenated hemolysate by isoelectric focusing on polyacrylamide gel revealed two closely spaced bands. When deoxygenated hemolysate was analyzed in oxygen-free gels, the two components were more widely separated. About 40% of the patient's hemoglobin focused at a more acid pH than hemoglobin A, indicating a hemoglobin variant with impaired Bohr effect. Chromatography of globin in 8 M urea revealed two β-chain peaks, the first of which was eluted at a lower buffer molarity than normal β chain. Fingerprints of tryptic digests of the aminoethylated chains were done on silica gel thin-layer plates. Tp 14 from the abnormal β chain had slower electrophoretic mobility and a greater R_f value. Amino acid analyses of this peptide gave values identical with those of βTp 14, except that it contained one proline residue and no histidine. Since the one His in βTp 14 is in position 143, hemoglobin Syracuse is $\alpha_2\beta_2^{143\,His\rightarrow Pro}$. Native Hb Syracuse could be separated from hemoglobin A on a carboxymethylcellulose column. The inclusion of 0.1 mM EDTA in the preparative buffers proved very useful in reducing the formation of methemoglobin. Oxygen equilibria of purified hemoglobin Syracuse showed high oxygen affinity (P_{50} value 12% that of hemoglobin A) and lack of cooperativity between subunits (Hill's $n = 1.1$). The alkaline Bohr effect was about half that of hemoglobin A. The proline substitution at βH21 disrupts the helical configuration and probably prevents the formation of salt bonds that are important in stabilizing the deoxy structure and contribute to the alkaline Bohr effect. Since β143 His is a binding site for 2,3-diphosphoglycerate (2,3-DPG), it is not surprising that hemoglobin Syracuse had markedly impaired reactivity with 2,3-DPG. Hemoglobin Syracuse auto-oxidized more slowly than hemoglobin A, probably reflecting a slower rate of dissociation of oxygen from fully liganded hemoglobin.

INTRODUCTION

After the discovery of hemoglobin Chesapeake in 1966 (1), 16 other human hemoglobin variants have been reported in association with familial erythrocytosis. Because these mutant hemoglobins have increased affinity for oxygen, the release of oxygen to tissues is impaired, and secondary erythrocytosis ensues. About one third of these variants are electrophoretically silent (2–6). Accordingly, the diagnosis must be established by the demonstration of a "shift to the left" of the oxygen dissociation curve. In this report we describe a new high-affinity variant, hemoglobin Syracuse ($\alpha_2\beta_2^{143\,(H21)\,His\rightarrow Pro}$),

Dr. Jensen was a fellow of the Deutsche Forschungsgemeinshaft.

Received for publication 3 September 1974 and in revised form 20 November 1974.

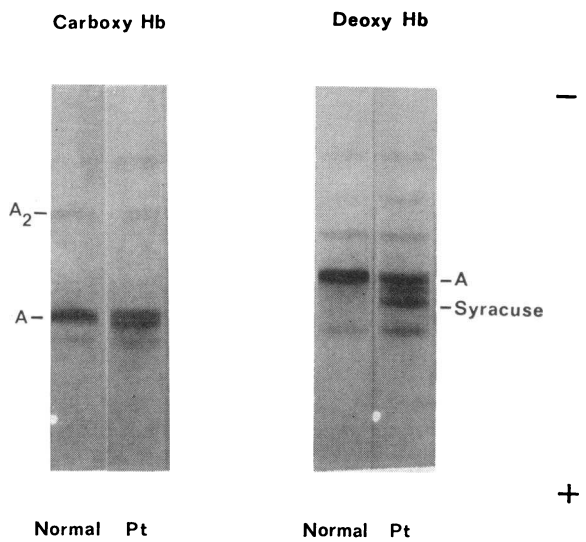

FIGURE 1 Comparison of isoelectric focusing patterns of normal and proband's (patient's) hemolysates. Left, hemoglobin saturated with CO. Right, completely deoxygenated hemoglobin. 20-μl samples containing 200 μg hemoglobin were applied anaerobically to cylindrical gels (3.5 × 100 mm) containing 6–8 Ampholine (LKB Produkter AB, Stockholm, Sweden).

which could not be separated from hemoglobin A by conventional electrophoretic methods. However, isoelectric focusing on polyacrylamide gel proved useful in demonstrating the hemoglobin variant and studying its properties. The site and nature of the amino acid substitution in hemoglobin Syracuse is of additional interest since β143 histidine is a binding site for 2,3-diphosphoglycerate (2,3-DPG) (7). Functional studies of hemoglobin Syracuse were facilitated by the adoption of conditions that minimized the formation of methemoglobin.

METHODS

A 34-yr-old Caucasian woman was found to have polycythemia with an hematocrit of 60. She was in good health although she tired easily. Five other family members from four generations were also found to have erythrocytosis. Their hemoglobin values ranged from 19.2 to 23.8 g/100 ml. Physical examinations of affected individuals revealed no relevant abnormalities.

Routine hematologic examinations were performed on family members. Red cell 2,3,-DPG was measured by the method of Krimski (8), as modified by Schröter and von Heyden (9). Whole blood oxygen affinity was measured on fresh blood specimens by the mixing method of Edwards and Martin (10).

Blood was collected in sterile tubes containing acid citrate dextrose and mailed in refrigerated containers. Hemolysates were prepared by lysis of washed erythrocytes with distilled water and toluene.

Hemoglobin electrophoresis was done in the following media and buffers: cellulose acetate (Tris-EDTA-borate, pH 8.6), starch gel (Tris-EDTA-borate, pH 8.6), and agar (citrate, pH 6.0). In addition, hemolysates and purified hemoglobin solutions were analysed by isoelectric focusing on polyacrylamide gel (11). In some experiments, deoxygenated samples were applied to gels anaerobically in an oxygen-free glove box, as described by Bunn and McDonough (12).

Structural studies. Globin was prepared from hemolysates by acid-acetone precipitation at −20° C, and then separated into α and β chains by carboxymethylcellulose chromatography in 8 M urea (13). The fractions corresponding to the individual chains were aminoethylated and digested with trypsin (TRTPCK, Worthington Biochemical Corp., Freehold, N. J., globin-trypsin ratio 50:1). 2 mg of tryptic digest was applied to 20 × 40-cm silica gel thin-layer plates (MN Polygram Sil, Brinkmann Instruments, Inc., Westbury, N. Y.) and subjected to electrophoresis on a cooling plate (Savant Instruments, Inc., Hicksville, N. Y.) for 100 min at 1,000 V with pyridine-acetic acid-water (5:1:94) buffer, pH 6.5. Ascending chromatography was then done in a butanol-1-acetic acid-pyridine-water (68-14-40-25) mixture. Peptide maps were stained by spraying with a 0.1% ninhydrin solution in butanol-1-acetic acid-water (100:3:1). Silica gel powder containing individual peptide spots was scraped off the plates and transferred into small columns prepared from Pasteur pipets. The peptides were eluted with hot (about 60°C) 5% acetic acid (800 μl). After the acetic acid was evaporated overnight in a vacuum desiccator, the dry peptides were redissolved in 6 N HCl and the solution transferred into 100-μl capillary tubes, which were flame-sealed on both ends to minimize contact with air. After hydrolysis at 110°C for 16 h, the tubes were cut open and the contents were blown out and dried

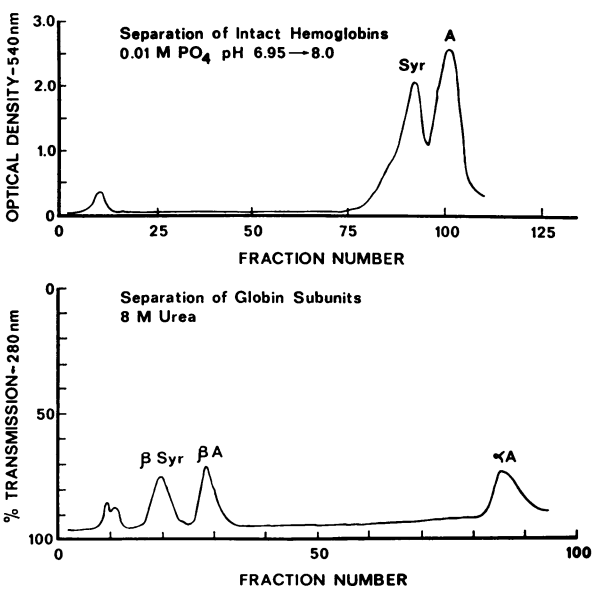

FIGURE 2 Bottom: Elution profile of proband's globin on urea-carboxymethylcellulose column. Tracing was obtained from an ultraviolet recorder (LKB Produkter AB). Separation of normal and abnormal β chains is shown. Top: Elution profile of proband's hemolysate on carboxymethylcellulose column. The partial separation of the intact hemoglobins is shown.

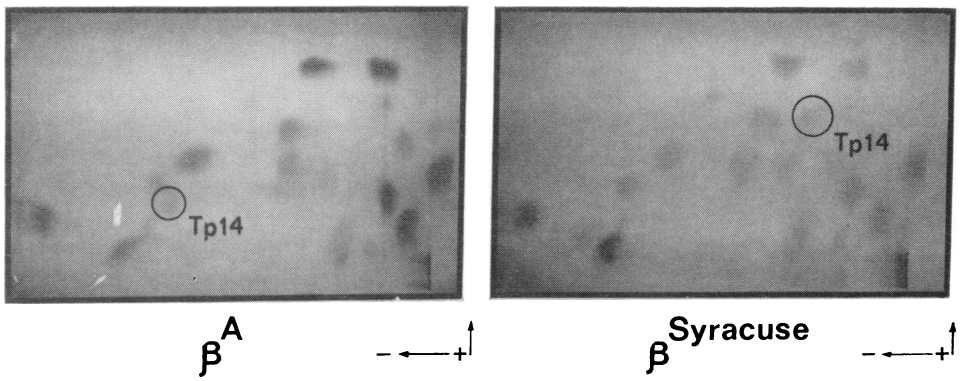

FIGURE 3 Maps of tryptic peptides of isolated β^A and $\beta^{Syracuse}$ subunits. 2 mg of tryptic digest was applied per plate. Silica gel thin-layer plates (MN Polygram SIL). Electrophoresis: pyridine-acetic acid-water (5:1:94) buffer, pH 6.5, 100 min, 1,000 V. Chromatography in butanol-1-pyridine-acetic-acid-water (68:14:40:25). Stain: 0.1% ninhydrin in butanol-acetic-acid-water (100:3:1).

in a vacuum desiccator. Amino acid analyses were performed by Dr. Ezio Merler in a modified Phoenix amino acid analyzer (Phoenix Precision Instrument Div., Virtis Co., Inc., Gardiner, N. Y.).

Functional studies. Hemoglobins Syracuse and A were separated as follows: 400 mg of hemolysate was dialyzed against 0.01 M phosphate buffer, pH 6.75, containing 0.1 mM EDTA. A 2.5 × 20 cm carboxymethylcellulose column (Whatman CM-52) was equilibrated with the same buffer. The hemoglobins were eluted in the cold room over a 120-h period with a linear gradient (starting buffer: 0.01 M phosphate, pH 6.95, 0.1 mM EDTA (1,000 ml); finishing buffer: 0.01 M phosphate pH 8.0 (1,000 ml). Hemoglobin-rich fractions were pooled, concentrated by pressure filtration, and then dialyzed against 0.1 M NaCl, 0.005 M Tris, pH 8.1, and 0.1 mM EDTA. Oxygen equilibria were determined spectrophotometrically as described previously (14, 15) on solutions containing 0.1 mM hemoglobin (tetramer), 0.1 M total chloride, 0.05 M bis-Tris, or Tris (pH 6–8). The data were plotted on a double logarithmic scale according to the Hill equation: $\log (Y/(1-Y)) = n \log P_{O_2} - n \log P_{50}$, where Y is the fractional saturation of hemoglobin with oxygen and P_{50} is the partial pressure of oxygen at which hemoglobin is half saturated, ($Y = 0.5$). The slope of this plot, n, is an index of cooperativity between hemoglobin subunits.

Solutions of 2,3-DPG were prepared and analyzed as described previously (14). Methemoglobin was determined spectrophotometrically by the method of Evelyn and Malloy (16).

RESULTS

The oxygen affinity of the proband's blood was markedly increased: P_{50} was 11 mm Hg compared to a normal value of 27 mm Hg. Affected family members had normal levels of red cell 2,3-DPG. These results indicated the presence of a functionally abnormal hemoglobin.

Electrophoretic experiments. Conventional zone electrophoresis of the proband's hemolysate was performed on different media and at different pH values as described in the Methods section. In all cases, a single band was found, which had the same mobility as hemoglobin A.

In contrast, separation of the normal and variant hemoglobins could be achieved by isoelectric focusing on polyacrylamide gel. This high-resolution system has been very effective in analyzing proteins difficult to separate by other electrophoretic methods. For example, this system has proven very useful in separating deoxyhemoglobin from liganded hemoglobin (17). If hemoglobin A is first deoxygenated and then treated with sufficient carbon monoxide to make it approximately 50% saturated before electrofocusing under anaerobic conditions, the purple deoxyhemoglobin band is clearly separated from the red carboxyhemoglobin band, with isoelectric points of 7.07 and 6.95, respectively (12). This result is a graphic demonstration of the alkaline Bohr effect, reflecting the fact that deoxyhemoglobin has a higher affinity for protons than the liganded forms of hemoglobin and therefore a higher isoelectric point.

Fig. 1 shows two gel focusing patterns of the proband's hemolysate, each side by side with a normal

TABLE I
Amino Acid Analysis of βTp 14

Residue	Found	Expected
Asp	1.1	1
Pro	0.9	0
Gly	1.0	1
Ala	3.9	4
Val	2.6	3
Leu	1.0	1
Lys	1.0	1
His	0	1

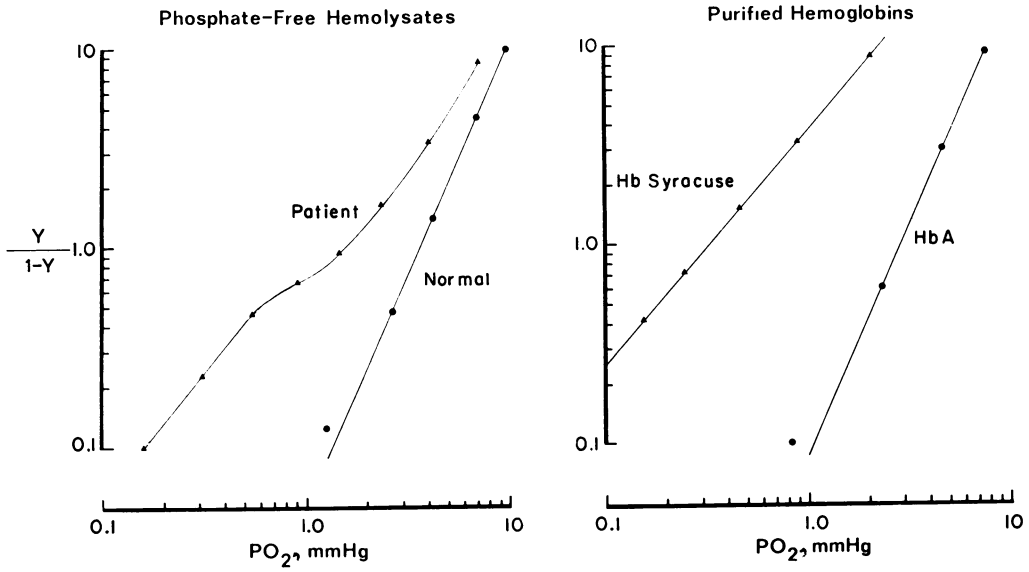

FIGURE 4 Left: Hill plots of phosphate-free hemolysates from the proband (▲) and a normal control (●). Right: Hill plots of hemoglobin Syracuse (▲) and of hemoglobin A (●) purified from the same chromatography column. Oxygen equilibria were done on 0.1 mM hemoglobin solutions in 0.1 M Cl^- and 0.05 M *bis* Tris-HCL buffer, pH 7.2, 20°C.

hemolysate, all run under anaerobic conditions. In the carboxy form (shown on the left) the proband's hemoglobin was barely separated into two very closely spaced bands. Identical results were obtained when the oxygenated hemolysate was analyzed under aerobic conditions. When deoxyhemoglobin was analyzed, the separation of the normal and abnormal hemoglobin bands was greatly enhanced, while the normal hemolysate still showed one single major component (Fig. 1, right). The widening between the normal and abnormal bands in the deoxy sample is due to the lesser increase in isoelectric point (and therefore less change in banding position) upon deoxygenation of the variant hemoglobin compared to hemoglobin A. These patterns prove the existence of two hemoglobin species in the proband's hemolysate, one of which has an impaired Bohr effect and comprises about 50% of the total hemoglobin. In the deoxy pattern, a faint intermediate band was consistently observed, which is the asymmetrical hemoglobin hybrid $\alpha_2\beta^A\beta^{Syracuse}$. Normally the hybrid comprises 50% of the total when a mixture of two hemoglobins of unlike charge are deoxygenated and analyzed as described above (12). However, mixed hybrids between hemoglobin A and high-affinity variants are less stable in the deoxy form than a hybrid derived from two functionally normal hemoglobins (18). This reflects the relative instability of the deoxy or "T" conformation of many of the high-affinity variants.

TABLE II
Effect of EDTA on Oxygenation of Hemoglobin

EDTA	P_{50}	n
mM	*mm Hg*	
0	3.4	2.7
0.1	3.5	2.8
1.0	4.0	2.8

Normal phosphate-free hemolysate: 0.1 mM hemoglobin (tetramer) in 0.1 M Cl^-, 0.05 M *bis* Tris, pH 7.2, 20°C.

Structural analysis. Carboxymethylcellulose-urea chromatography of the proband's globin gave the elution profile shown in Fig. 2 (bottom). Two β-chain peaks were obtained in approximately equal amounts. The abnormal chain was eluted before the normal one, at lower ionic strength. When peptide maps of tryptic digests of the normal and abnormal β chains are compared (Fig. 3), it is evident that Tp XIV has changed its position in the latter, having a slower electrophorectic mobility and a higher R_f value. The amino acid composition of the aberrant peptide is shown in Table I. The relative amounts of amino acids in the analysis correspond closely to those expected in β^A Tp XIV, except that histidine is missing and a proline residue is found instead. The only histidine in this peptide is at residue 143, at the COOH-terminal end of the H-helix. Thus hemoglobin Syracuse is β143 (H21) histidine→proline, corresponding to a transversion of adenine to cytosine in the RNA genetic code.

Functional studies. Oxygen equilibria of phosphate-free hemolysates are shown in Fig. 4 (left). The bi-

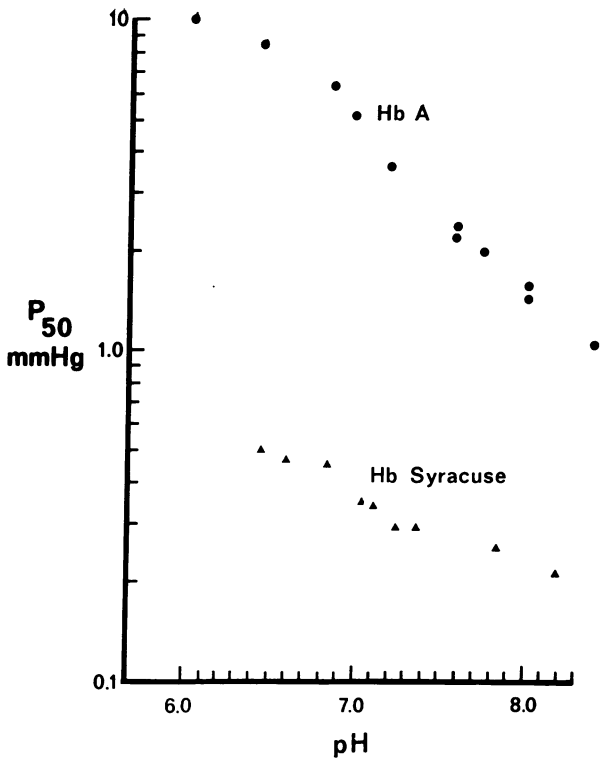

FIGURE 5 Alkaline Bohr effect of purified hemoglobins Syracuse (▲) and A (●). Experimental conditions are stated in the legend of Fig. 4. For pH values above 7.6, 0.05 M Tris was used instead of bis Tris. P_{50} values are plotted on a logarithmic scale against pH. The slope of this plot ($\Delta \log P_{50}/\Delta$ pH) is a quantitative measure of the Bohr effect.

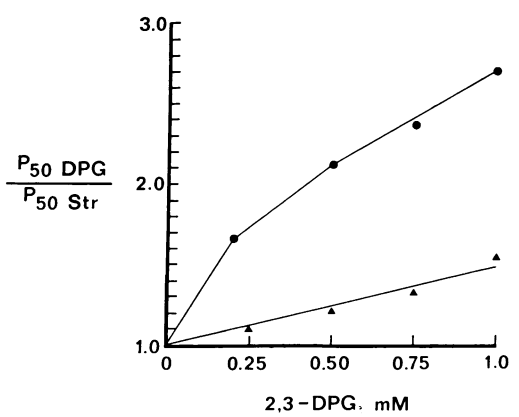

FIGURE 6 The effect of 2,3-DPG on the oxygen affinity of purified hemoglobins Syracuse (▲) and A (●). Experimental conditions are stated in the legend of Fig. 4. Because of the large difference in oxygen affinities of the two hemoglobins, results are expressed as the ratio of P_{50} values in the presence and absence of 2,3-DPG. $P_{50\ Str} = P_{50}$ in absence of 2,3-DPG.

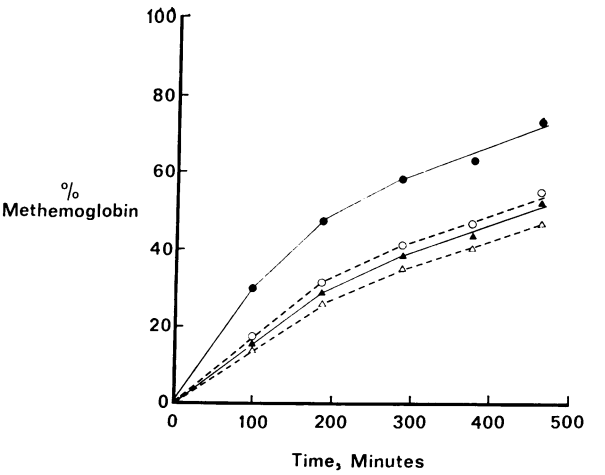

FIGURE 7 Rate of auto-oxidation of purified hemoglobins. Hemoglobin A (●), hemoglobin Syracuse (▲), hemoglobin A + 0.1 mM EDTA (○), hemoglobin Syracuse + 0.1 mM EDTA (△). Solutions of oxyhemoglobin (0.05 mM tetramer) were incubated at 37°C in 0.1 M PO_4, pH 6.6.

phasic Hill plot of the proband's hemolysate is independent evidence that a high affinity hemoglobin is present in approximately equal amounts with hemoglobin A. To study the functional properties of hemoglobin Syracuse, it had to be isolated, in preparative amounts, free of hemoglobin A. Because of difficulties in resolving the oxygenated forms of the normal and variant hemoglobins electrophoretically, we anticipated that chromatographic preparation of pure hemoglobin Syracuse would be difficult. It was necessary to apply relatively small amounts of hemoglobin to the carboxymethylcellulose column and elute slowly to achieve an adequate separation (Fig. 2, top). During the long time required for preparation (6 days), some of the hemoglobin autooxidized to methemoglobin. In view of the report of Kellett and Schachman (19) that small amounts of

TABLE III
Effect of pH on the Auto-Oxidation of Hemoglobins A and Syracuse

pH	EDTA (0.1 mM)	Methemoglobin*	
		Hb A	Hb Syr
		%	
6.6	0	40	23
	+	24	20
7.3	0	24	14
	+	13	10

* Specimens containing oxyhemoglobin (0.05 mM tetramer) were incubated in 0.1 M PO_4, pH 6.6 and 7.3, for 200 min at 37°C.

EDTA enhanced the stability of low concentrations of hemoglobin, we added 0.1 mM EDTA to the elution buffers. This resulted in a significant inhibition of auto-oxidation during the preparative isolation of hemoglobin Syracuse (see Fig. 7 below). The final concentration of EDTA was 0.02 mM in solutions on which oxygen equilibria were measured. As shown in Table II, at this concentration EDTA has no detectable effect on the oxygen binding of hemoglobin. The purity of specimens of hemoglobin Syracuse was estimated in three ways: (*a*) the elution pattern of the globin on urea carboxymethyl-cellulose chromatography; (*b*) gel electrofocusing of the deoxygenated hemoglobin; (*c*) Hill plot of the oxygen binding curve. The latter proved to be a sensitive assay of purity since contamination with A hemoglobin resulted in an abrupt deviation of the Hill plot from linearity at high fractional saturation. Results were discarded if the amount of contaminating A hemoglobin exceeded 10%.

Hill plots for hemoglobins Syracuse and A, purified on the same column, are shown on the right in Fig. 4. At pH 7.2, 20°C, the P_{50} for phosphate-free hemoglobin Syracuse was 0.33 mm Hg, about 12% that of hemoglobin A, indicating a marked increase in oxygen affinity. The *n* value for hemoglobin Syracuse was about 1.1, signifying almost total absence of subunit cooperativity.

Fig. 5 shows a plot of P_{50} values for hemoglobins Syracuse and A, measured at selected pH values. The slope of this plot (Δ log P_{50}/Δ pH) provides a direct measure of the alkaline Bohr effect. Values of -0.24 and -0.57 were obtained for hemoglobins Syracuse and A, respectively. The marked reduction in the Bohr effect of hemoglobin Syracuse is in good agreement with the isoelectric focusing results reported above.

Since β143 (H21) histidine is known to be one of the residues responsible for the specific binding of 2,3-DPG to hemoglobin (7), it was important to determine the effect of added 2,3-DPG to the oxygen affinity of hemoglobin Syracuse. As Fig. 6 shows, hemoglobin Syracuse had markedly impaired reactivity with 2,3-DPG.

Fig. 7 compares the rates of auto-oxidation of hemoglobin Syracuse and A prepared from the same column. No EDTA was used in the purification of these specimens. Hemoglobin solutions (0.05 mM tetramer) were incubated in 0.1 M phosphate, pH 6.6, at 37°C. In the absence of EDTA, hemoglobin A autooxidized about twice as rapidly as hemoglobin Syracuse. The addition of 0.1 mM EDTA slowed the rate of auto-oxidation of hemoglobin A considerably but had less effect on the rate of auto-oxidation of hemoglobin Syracuse. As Table III shows, the rate of auto-oxidation of both hemoglobins was strongly pH-dependent, both in the absence and presence of EDTA.

TABLE IV

Electrophoretic Properties of High-Affinity Hemoglobin Variants

Hemoglobin variant	Electrophoretic separation from Hb A: Starch gel, pH 8.6	Agar gel, pH 6	Gel electrofocusing	Reference
Chesapeake (α92 Arg → Leu)	Yes	Yes		1
J-Capetown (α92 Arg → Gln)	Yes			20
Yakima (β99 Asp → His)	Yes			21
Kempsey (β99 Asp → Asn)	Yes		Yes	22
Ypsilanti (β99 Asp → Tyr)	Yes			23
Hiroshima (β146 His → Asp)	Yes	Yes	Yes	24
Andrew-Minneapolis (β144 Lys → Asn)	Yes		Yes	25
Abruzzo (β143 His → Arg)	Yes			26
Rainier (β145 Tyr → Cys)	No	Yes		27
Bethesda (β145 Tyr → His)	No	Yes	No	15, 28
Little Rock (β143 His → Gln)	No	Yes		29
Malmo (β97 His → Gln)	No	No	Yes	3, 30
Syracuse (β143 His → Pro)	No	No	Yes	This report
Olympia (β20 Val → Met)	No	No	No	2
Brigham (β100 Pro → Leu)	No	No	No	4
San Diego (β109 Val → Met)	No	No		5
Heathrow (β103 Phe → Leu)	No	No	No	6
Creteil (β89 Ser → Asn)	No	No		31

DISCUSSION

The increased oxygen affinity in the proband's whole blood and phosphate-free hemolysate suggested that a hemoglobin variant was responsible for her erythrocytosis. However, routine electrophoretic techniques failed to reveal any abnormal bands. Isoelectric focusing on polyacrylamide gel was required for the demonstration of the variant hemoglobin. As Fig. 1 shows, the separation of hemoglobin Syracuse from hemoglobin A was further enhanced by analysis of the deoxygenated hemolysate under anaerobic conditions. The electrophoretic properties of the 16 "high-affinity" variants reported to date are summarized in Table IV. Seven of these can be separated from hemoglobin A by routine electrophoresis on starch gel or cellulose acetate at pH 8.6. Three require agar gel electrophoresis at a low pH to demonstrate the abnormal variant. In two, hemoglobins Malmö and Syracuse, neither of these techniques achieved a separation, but gel focusing was successful. This comparison indicates that all of these techniques should be applied before a variant can be considered to be "electrophoretically silent."

Recent advances in the understanding of structure-function relationships in hemoglobin (32) permit a

tentative explanation of the functional properties of hemoglobin Syracuse.

Bohr effect. The decreased alkaline Bohr effect of hemoglobin Syracuse was demonstrated in two ways. As conventionally determined, the plot of log P_{50} vs. pH gave a slope less than half that of hemoglobin A (Fig. 5). In addition, the gel focusing patterns shown in Fig. 1 demonstrate that the increase in isoelectric point after deoxygenation is smaller in hemoglobin Syracuse than in hemoglobin A. By taking advantage of the abnormal functional properties of hemoglobin Syracuse, its separation from hemoglobin A was enhanced. This approach may prove useful in the study of other hemoglobins.

β143 histidine is not one of the residues that contribute directly to the alkaline Bohr effect (*32, 33*). Thus, the decreased Bohr effect of hemoglobin Syracuse indicates conformational changes that go beyond the site of the amino acid substitution. Since proline is an imino rather than an amino acid, it cannot participate in an α helix (except as one of the three initial residues). Thus, the H helix in hemoglobin Syracuse terminates at the 20th residue rather than the 21st. As a result, the remaining part of the β chain including the COOH-terminal histidine (β146) has an altered and perhaps less ordered spatial orientation. The imidazole group of β146 histidine is responsible for about half of the alkaline Bohr effect. In deoxyhemoglobin, an intrasubunit salt bond is formed between this positively charged group and the carboxyl group of β94 aspartic acid (*32*). Upon oxygenation, this bond is broken and a proton is released. This interaction requires a precise stereochemical fit, which is probably hindered by the structural alteration in hemoglobin Syracuse.

High oxygen affinity and decreased subunit cooperativity. The salt bridges at the COOH-terminal histidine residue of the β chain not only contribute to the alkaline Bohr effect (as mentioned above) but also help to stabilize the low-affinity (deoxy) quarternary structure of the hemoglobin molecule (*32*). In addition, the deoxy structure is further stabilized by the insertion of the penultimate tyrosine residue (β145 and α140) into a pocket between the F and H helices of the same subunit. If these interactions are impaired by a conformational alteration at the COOH-terminal end of the β chain, the high affinity (oxy) structure would be favored, resulting in increased oxygen affinity. Likewise, if the allosteric equilibrium between the two structures is shifted towards the high-affinity form, cooperativity between subunits would be reduced, since this phenomenon depends on the transition from the deoxy to oxy structure, triggered by oxygenation of individual subunits. The 10-fold increase in oxygen affinity of hemoglobin Syracuse and decreased cooperativity between subunits ($n = 1.1$) are consistent with this interpretation.

Interaction with 2,3-DPG. β143 histidine is one of the residues responsible for the binding of 2,3-DPG to deoxyhemoglobin (*7*). Since it is replaced by a nonpolar proline residue in hemoglobin Syracuse, it is not surprising that this variant has impaired interaction with 2,3-DPG (see Fig. 6). Hemoglobin F, which has an uncharged serine residue at this position, and hemoglobin Little Rock ($\alpha_2\beta_2^{143\,His\rightarrow Gln}$) also have impaired binding with 2,3-DPG. However, unlike Syracuse, these two hemoglobins have normal subunit cooperativity and Bohr effect. The other variant reported to have a substitution at this site is hemoglobin Abruzzo ($\alpha_2\beta_2^{143\,His\rightarrow Arg}$) (*26*). It has an increased oxygen affinity but interacts strongly with 2,3-DPG. Perhaps the positively charged guanidinium group serves as an acceptable binding site for 2,3-DPG.

If a hemoglobin variant cannot form a stable deoxy quarternary structure, its interaction with 2,3-DPG will be impaired. This appears to be true for hemoglobin Bethesda (*15*) and hemoglobin Kempsey (*18*). The amino acid substitutions in these two variants do not involve 2,3-DPG binding sites. Thus, the failure of hemoglobin Syracuse to interact with 2,3-DPG is likely due not only to lack of one of the specific binding sites, but also to the alterations in conformation discussed above.

Auto-oxidation of hemoglobin. When these studies were initiated, we found that during the lengthy period required for the separation of hemoglobin Syracuse and hemoglobin A there was some auto-oxidation. Even more methemoglobin formation occurred during the deoxygenation of samples in the tonometers. The presence of 10–20% methemoglobin precluded any meaningful functional studies. The addition of EDTA to the column chromatography and dialysis buffers reduced the total formation of methemoglobin to less than 5%. As Fig. 7 shows, during the incubation of the column-purified hemoglobins at 37°C, pH 6.6 (conditions known to favor autooxidation), the formation of methemoglobin A was greatly curtailed in the presence of EDTA. Hemoglobin Syracuse autooxidized less readily than hemoglobin A and this rate was only slightly lessened in the presence of EDTA.

Recently, studies by Rifkind (*34*) have provided valuable new information on the mechanism of autooxidation of hemoglobin. He showed that trace amounts of cupric ion (Cu^{2+}) greatly enhance the rate of autooxidation of horse and donkey hemoglobin. Cu^{2+} can oxidize deoxyhemoglobin stoichiometrically as follows:

$$Cu^{2+} + Fe^{2+}\text{globin} \rightarrow Cu^{1+} + Fe^{3+}\text{globin} \qquad (1)$$

This reaction probably requires that the oxidized heme group be free of oxygen. Cu^{2+} can be regenerated by the following pH-dependent reaction:

$$4\ Cu^{1+} + O_2 + 4H^+ \rightarrow 4\ Cu^{2+} + 2\ H_2O. \quad (2)$$

Thus, hemoglobin can be oxidized by trace amounts of copper ion in catalytic fashion, particularly at low pH.

However, hemoglobin auto-oxidizes in the absence of free copper ion (12, Fig. 7). There is experimental evidence indicating that this reaction involves the generation of the superoxide anion (35):

$$Fe^{2+}(O_2)\text{globin} \rightarrow Fe^{3+}\text{globin} + O_2^- \quad (3)$$

Recent experiments of Wallace, Maxwell, and Caughey (36) indicate that the superoxide anion is displaced from Fe^{3+} globin by anionic nucleophiles such as Cl^-. This reaction is also facilitated by protons (36). We found that the auto-oxidation of both A and Syracuse hemoglobins was enhanced at low pH even in the presence of EDTA, which eliminates the contribution of reactions 1 and 2 to auto-oxidation (See Table III).

Normally, of course, the vast majority of dissociations of oxygen from fully liganded hemoglobin occur as follows:

$$Fe^{2+}(O_2)\text{globin} \xrightarrow{k_4} Fe^{2+}\text{globin} + O_2. \quad (4)$$

Because of the difference in oxygen affinity, reaction 4 (k_4) may proceed more slowly with hemoglobin Syracuse than hemoglobin A. Recent kinetic measurements by MacDonald and Gibson[1] indicate this to be true: k_4 of hemoglobin Syracuse was half that of hemoglobin A. Accordingly, hemoglobin Syracuse should be less vulnerable to attack by Cu^{2+} ion, and EDTA should have less effect on the auto-oxidation of hemoglobin Syracuse than hemoglobin A. The data in Fig. 7 are consistent with this interpretation.

In view of these results, it seems advisable to add a low concentration of a chelating agent such as EDTA (or triethylenetetraamine [34]) to mutant or chemically modified hemoglobins that require prolonged periods for isolation. Only trace amounts are required, since these agents have very high binding affinities for divalent cations. As Table II shows, concentrations of EDTA ranging from 0.01 to 0.1 mM had no effect on the oxygen affinity of phosphate-free hemoglobin A. Furthermore, Rifkind showed that EDTA does not bind to hemoglobin (34).

It would be of considerable interest to measure the rate of autooxidation of other hemoglobin variants having abnormal oxygen affinity. Hemoglobin Kansas has a low oxygen affinity (37), an enhanced k_4 (reaction 4) (38) and an increased rate of auto-oxidation (37). In contrast, hemoglobin Chesapeake has a high oxygen affinity (1, 39), normal k_4 (39) and, like hemoglobin Syracuse, a decreased rate of auto-oxidation.[2] However, hemoglobin Kempsey, another high-affinity variant, has a normal rate of auto-oxidation[2] and a k_4 value identical to that of hemoglobin A (18).

[1] Unpublished data.

[2] Bunn, H. F. Unpublished results.

ACKNOWLEDGMENTS

We are indebted to Margaret McDonough and Geoffrey Schmidt for their able technical assistance.

This work was supported by U. S. Public Health Service Grants HL-15670, AM-15322, and 5T01-AM-05581, National Heart and Lung Institute Contract 72-2992-B, and the Nehemias Gorin Foundation. Some of these studies were carried out in a Clinical Research Center supported by the United States Public Health Service Grant FR-00128.

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