FUNCTIONAL STUDIES OF THE DOUBLE MUTANT HEMOGLOBIN STANLEYVILLE II/S $\alpha_2^{78\ Lys}\ \beta_2^{6\ Val}$: IDENTIFICATION OF A SITE OF INTERMOLECULAR CONTACT ON THE α CHAIN

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SUMMARY: A double mutant hemoglobin, composed of the β^S chains ($\beta^{6\ Val}$) and the α Stanleyville II chains ($\alpha^{78\ Lys}$), has been isolated from the red blood cell hemolysate of a patient with the genotype $\alpha^A/\alpha^{Stanleyville\ II}/\beta^A/\beta^S$. The gelling and salting out properties of the double mutant Hb Stanleyville II/S were markedly modified with respect to Hb S. The observed reduction in the polymerization propensity indicated the involvement of the α^{78} site in intermolecular interactions. The lysine which replaces asparagine at the 78th position of the mutant α chain is situated on the surface of the molecule at the corner between the EF segment and the F helix. The increased positive charge in this region resulted also in the alteration of the functional properties of the molecule, endowing it with a slightly increased oxygen affinity and reduced Bohr effect.

At the present time, there is considerable interest in the zones on the surface of the hemoglobin molecule which are involved in intermolecular contacts (1, 2). Study of surface-substituted sickle hemoglobins has provided important information in this regard (2, 3, 4). The effect of the second substitution (other than that of $\beta^{6\ Val}$), on the gelation properties of the molecule and on its solubility in phosphate buffers of high ionic strength have facilitated localization of the sites of intermolecular contact (2, 3, 4). Such data may serve to identify the disposition and orientation of molecules in the fibers constituting gels of deoxy Hb S. Thus, study of the naturally occuring variants, Hb C Harlem $\alpha_2^A\ \beta_2^{6\ Val,\ 73\ Asn}$ (3) and Hb Memphis/S $\alpha^A\ \alpha^{23\ Gln}\ \beta_2^{6\ Val}$ (5), has demonstrated the contributions of residues β^{73} and α^{23} to sites of intermolecular contact. Furthermore, similar studies of Hb C Ziguinchor $\alpha_2^A\ \beta_2^{6\ Val}$,

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$^{58\ Arg}$ have eliminated the involvement of the β58 site (4, 6). Additional data have been provided by BENESCH et al. (2), who have used artificially prepared sickle variants to evaluate the contributions of sites α16, α47, α48, α54 and α68. We have recently isolated the double mutant hemoglobin Stanleyville II/S ($\alpha_2^{78\ Lys}\ \beta_2^{6\ Val}$) from the RBCs of a patient with the genotype $\alpha^A/\alpha^{Sta.II}/\beta^A/\beta^S$. In this report we describe the effect of the surface substitution characteristic of Hb Stanleyville II, i.e $\alpha^{78\ Lys}$ (7, 8) on the intermolecular interactions in this double mutant sickle variant. In addition, we report the effect of this substitution on the functional properties of the double mutant Hb Sta. II/S.

MATERIALS AND METHODS

The propositus was a 24 year old woman from the Zaïr Republic who was doubly heterozygous for both Stanleyville II and the sickle trait as described elsewhere (8). Blood was drawn into EDTA (9) and hemolysates were prepared according to DRABKIN (10). Identification of the various hemoglobins present in the propositus RBCs was performed according to methods currently used in this laboratory (11). Hb Sta. II/S was prepared by chromatography of the whole hemolysate on a column of DEAE-Sephadex A50 in 0.05 M Tris-HCl buffer (12) and was eluted at pH 8.2. Gelation studies were carried out according to BOOKCHIN and NAGEL (13) on the double mutant sickle variant Sta. II/S (containing less than 10 % of Hb A_2 contaminants), on Hb S (from S cells containing about 10 % of hemoglobins F and A_2) and on mixtures of S/double mutant, S/A and A/double mutant. The salting out properties of Hb S and of the double mutant $\alpha_2^{Sta.\ II}\ \beta_2^S$ were studied at room temperature (22° C) in concentrated potassium phosphate buffers (pH 6.8) in the final concentration range of 1.5 - 2.5 M . Experiments were performed in the absence and presence of dithionite (57 mM) using the method described by ITANO (14) with slight modifications.
Oxygen equilibrium curves were determined according to the spectrophotometric method of BENESCH et al. (15) at 37° C. Determinations on red blood cells were carried out in 0.15 M phosphate buffer (pH 7.15), while those on isolated, 2,3-DPG free hemoglobins were performed in 0.05 M Tris or bis Tris buffers containing 0.02 or 0.1 M NaCl in the pH range 6.45-7.45. In some experiments carbamoyl hemoglobin derivatives were used for oxygen equilibria

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Abbreviations

RBCs	:	Red blood cells
$\alpha^{Sta.\ II}$	:	α chain of Hb Stanleyville II
Hb Sta. II/S	:	Double mutant hemoglobin Stanleyville II/S
2,3 - DPG	:	2,3 Diphosphoglycerate

measurements. These derivatives were prepared according to NIGEN et al. (16) by incubation of liganded hemoglobin (0.5 mM) with KCNO (1 mM) for 1 hour at pH 6.2 and 37° C. The Bohr effect was calculated from the formula (Δ log P50/Δ pH). The interaction of 2,3-DPG with the double mutant Stanleyville II/S was studied by measurement of the P50 of stripped hemoglobins to which different concentrations of 2,3-DPG had been added. Erythrocyte 2,3-DPG levels were determined by the method of ROSE and LIEBOWITZ (17).

RESULTS

The propositus whose RBCs contained the double mutant hemoglobin Sta. II/S had the genotype $\alpha^{A}/\alpha^{Sta.II}/\beta^{A}/\beta^{S}$. This case is similar to that previously reported (8). Red cell hemolysates from the propositus therefore contained six hemoglobin types which were structurally identified as : Hb A : $\alpha_2^{A}\ \beta_2^{A}$ (39%) Hb Sta. II : $\alpha_2^{Sta.\ II}\ \beta_2^{A}$ ($\simeq$ 14 %), Hb S : $\alpha_2^{A}\ \beta_2^{S}$ ($\simeq$ 30 %), double mutant Sta. II/S : $\alpha_2^{Sta.\ II}\ \beta_2^{S}$ ($\simeq$ 15 %), Hb A_2 : $\alpha_2\ \delta_2$ ($\simeq$ 2 %) and a further hemoglobin fraction present in trace amounts representing the mutant Hb A_2 ($\alpha_2^{Sta.\ II}\ \delta_2^{A2}$). Quantitation was made possible by globin chain electrophoresis of Hb fractions separated by starch block electrophoresis where Hb S and Hb Sta. II migrated together as did Hb A_2 and the double mutant Hb Sta. II/S.

The double mutant fraction Sta. II/S could be isolated and freed from most of the contaminating Hb A_2 by chromatography on a DEAE-Sephadex A50 column in 0.05 M Tris HCl buffer at pH 8.2 (Fig. 1).

Two different methods were used to study the effect of the $\alpha^{78\ Asn \rightarrow Lys}$ substitution on the intermolecular interactions in the sickle variant Sta. II/S ($\alpha_2^{78\ Lys}\ \beta_2^{6\ Val}$). The first involved the determination of the minimal hemoglobin concentration necessary for deoxy Hb gelation (M.G.C.), which is a direct index of polymerization. The second method evaluates the salting out properties of hemoglobins bearing the sickle substitution (2, 3, 4, 14) and reflects their poor solubility in media of high ionic strength.

The effect of the $\alpha^{78\ Asn \rightarrow Lys}$ substitution on the gelling

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Abbreviations

KCNO : Potassium cyanate

P50 : The partial pressure of oxygen at which hemoglobin is half saturated with oxygen.

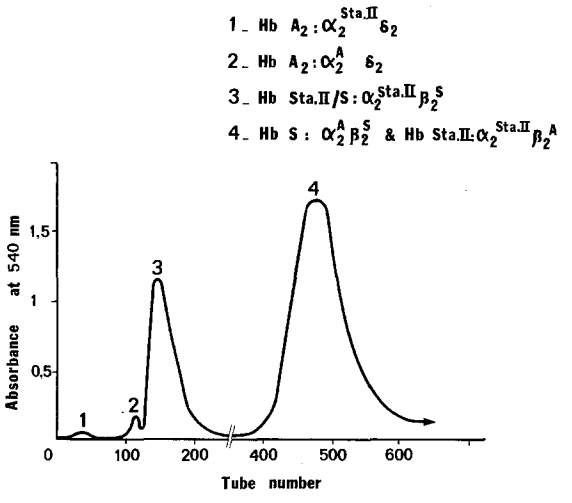

Figure 1. Elution profile of the different hemoglobin fractions present in the propositus red cell hemolysate upon chromatography on DEAE Sephadex A 50 in 0.05 M Tris-HCl buffer.

TABLE I.

Effect of the α^{78} Lys substitution on the intermolecular interactions expressed in terms of minimal hemoglobin concentrations necessary for deoxy hemoglobin gelation (M.G.C.) in 0.15 M potassium phosphate buffer (pH 7.35) at 25° C.

Hb S $\alpha_2 \beta_2^6$ Val	double mutant α_2^{78} Lys β_2^6 Val	Hb A $\alpha_2 \beta_2$	Hybrids present	M.G.C.	% Met Hb after gelation
100 %		—		23.8	0 %
—	100 %	—		30	4 %
50 %	50 %	—	$\alpha^A \alpha^{Sta.II} \beta_2^S$	27.2	2 %
40 %	—	60 %	$\alpha_2^A \beta^A \beta^S$	32.7	2 %
—	40 %	60 %	$\alpha^A \alpha^{Sta.II} \beta^A \beta^S$	37.8	3 %

behaviour of hemoglobin S is demonstrated in Table I. The deoxygenated double mutant sickle variant Sta. II/S required a higher M.G.C. (30 g/dl) than that required for deoxy Hb S (23.8 g/dl).

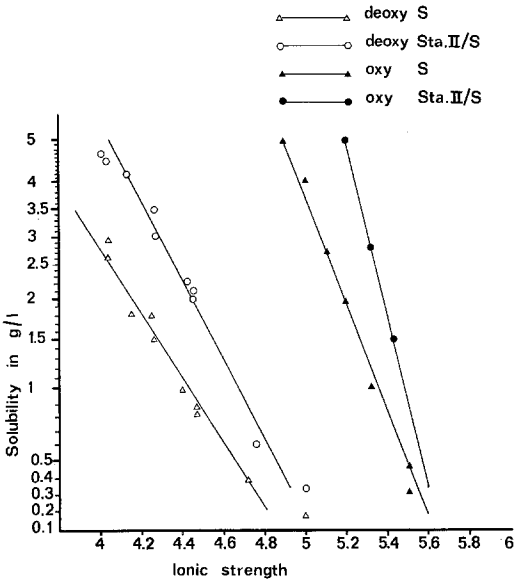

Figure 2. Comparison of the salting out curve of the double mutant hemoglobin Stanleyville II/S with that of Hb S (hemolysates from homozygotes). The solubilities of 5 mg of each hemoglobin were determined in final volumes of 1 ml phosphate buffer (pH 6.8) and expressed in g/l. The ionic strength of sodium dithionite is included in the total ionic strength for the deoxy hemoglobins.

Mixtures of both S/double mutant in equal proportions gelled at an intermediate concentration (27.2 g/dl). In addition, the M.G.C. of mixtures of the double mutant hemoglobin and Hb A (40 : 60) were higher than those of comparable S/A mixtures. It is noteworthy that concentrations of Met Hb did not exceed 4 % in any experiment.

The α^{78} Asn → Lys substitution also modified the salting out properties of both oxy and deoxy forms of the sickle variant Sta. II/S. As demonstrated in Fig. 2, the solubility of both forms in concentrated phosphate buffers was increased with respect to both the oxy and deoxy forms of Hb S.

Measurement of the oxygen affinity of red blood cells from the propositus in 0.15 M phosphate buffer at pH 7.15 (Table II.a) showed a shift to the right of the oxygen dissociation curve with a P50 of 47 mmHg (normal 31.5 $\pm$ 1 mmHg). The erythrocyte 2,3 - DPG level was particularly high (27 µmol/g Hb) as compared to that in normal cells (14 $\pm$ 1.5 µmol/g Hb). Although the level of such organic phosphate was markedly elevated, the P50 of the

TABLE II

a. Data on the oxygen affinity of cells from the propositus at 37° C.

	AA	Propositus	AS
P 50 (mmHg) in phosphate buffer (0.15 M ph 7.15)	31.5 ± 1	47	45 - 52
2,3 - DPG (µmol/g Hb)	14 ± 1.5	27	17 ± 2

b. Measurements of oxygen affinity and of the interaction with 2,3 - DPG of stripped hemoglobins A or S and of the double mutant Sta. II/S at 37° C.

Buffer (containing 0.02 M NaCl)	2,3 - DPG/Hb Molar ratio	P 50 (mmHg)	
		A or S	Double mutant Sta. II/S
bis Tris HCl 0.05 M pH 6.45	0	32 ± 1	19
Tris HCl 0.05 M pH 7.45	0	11 ± 0.5	9.5
Tris HCl 0.05 M pH 7.15	0	17 ± 1	12.5
	2.5	28 ± 1	27
	5	31 ± 1	30

c. Effect of salt concentration (NaCl) and of carbamoylation on the oxygen affinity of Hb A and Hb Sta. II/S

Buffer at pH 6.45	P 50 (mmHg)			
	Hb A	Carbamoyl Hb A	Hb Sta. II/S	Carbamoyl Hb Sta. II/S
0.05 M bis Tris HCl-0.02 M NaCl	32 ± 1	20	19 - 20	18
0.05 M bis Tris HCl-0.1 M NaCl	33	27	33	24.5

cells was comparable to the low values currently found in this laboratory for AS cells (45 - 52 mmHg) with an erythrocyte 2,3-DPG level of 17 µmol/g Hb. The intracellular copolymerization of Hb A with the sickle hemoglobin was shown by MAY and HUEHNS (18) to be primarily responsible for the low oxygen affinity of AS cells. In the presence of such 2,3 - DPG level , the oxygen affinity of the propositus'cells was rather higher than expected. A possible explanation for such an increased oxygen affinity is an inhibition of polymerization due to the substitution present on some of the α chains.

The oxygen affinity of the purified and stripped double mutant Hb Sta. II/S in 0.05 M Tris HCl or bis Tris HCl 0.02 M NaCl buffers presented some alteration (Table II.b). Thus, it was markedly increased at acid pH (pH 6.45) in the presence of such low concentration of chloride ions (0.02 M NaCl) thereby, decreasing the Bohr effect to − 0.30 (normal − 0,50 $\pm$ 0.05). Such an increase in oxygen affinity was completely abolished upon increase of the NaCl concentration to 0.1 M (Table II.c). However, cooperativity and the effect of 2,3 - DPG were normal. This abnormal functional behaviour is evidently related to the $\alpha^{78\ Asn \rightarrow Lys}$ substitution ; a supposition which was confirmed by the study of the functional properties of Hb Stanleyville II itself $\alpha_2^{78\ Asn \rightarrow Lys}\ \beta_2^{A}$. This hemoglobin was obtained from another subject heterozygous for the $\alpha^{Sta.\ II}$ trait, since Hb Sta. II could not be chromatographically separated from the propositus'hemolysate. The results obtained indicate an identical functional alteration in both Sta. II ($\alpha_2^{78\ Lys}\ \beta_2^{A}$) and Sta. II/S ($\alpha_2^{78\ Lys}\ \beta_2^{6\ Val}$)(manuscript in preparation).

As will be subsequently discussed, stereochemical considerations led us to postulate that the strong positively charged lysine in position α^{78} might exhibit an inhibitory effect on the ionization of the $\alpha\text{-}NH_2$ group of valine 1 α. Such a group is known to be involved in the Bohr effect (19). In order to test this hypothesis, we used carbamoyl derivatives of Hb A and Hb Sta. II/S. It is known that carbamoyl Hb A derivatives ($\alpha_2^{C}\ \beta_2$ or $\alpha_2^{C}\ \beta_2^{C}$)exhibit an increased oxygen affinity and a

Abbreviations

α^{C} : α Carbamoyl chain

β^{C} : β Carbamoyl chain

decreased Bohr effect, and that this is due to the blockade of the α-NH_2 group of valine at the N terminus of the α chain (16).

The data presented in Table II.c indicate that the oxygen affinity of carbamoyl Hb A at pH 6.45 was increased to the same extent as the oxygen affinity of Hb Sta. II/S, whereas similar carbamoylation of Hb Sta. II/S did not further increase its oxygen affinity at this pH. These results suggest that the mechanism of alteration of the Bohr effect in Hb Sta. II (or Sta. II/S) may involve the valine 1 α which lies in close proximity to lysine at position α^{78}.

DISCUSSION

The present study of the double mutant hemoglobin Stanleyville II/S ($\alpha^{78\ Lys}\ \beta_2^{6\ Val}$) provides information on the modification of its intermolecular interactions and on its functional properties. The first modification resulting from the $\alpha^{78\ Asn \rightarrow Lys}$ substitution was a marked inhibition of molecular aggregation as demonstrated by the gelling and solubility experiments. This substitution lies on the surface of the molecule at the corner between the EF segment and the F helix of the α chain, a region which is not implicated in subunit contact and which does not affect the stability of the molecule in a manner which might explain the inhibition of molecular aggregation. The inhibition of gelation and the enhanced solubility of the double mutant Sta. II/S indicate that the presence of the positively charged lysine, instead of the neutral asparagine residue inhibits an intermolecular contact in this zone. These data conflict with those obtained by HALL-CRAGGS et al. (7) on the basis of clinical observations. The theoretical model of LEVINTHAL et al. (20) predicts that this is a site of intermolecular interaction in which the asparagine 78α of Hb A or Hb S may be linked to the NA2 histidine residue in the β^S chain of a neighboring molecule by a hydrogen bond. The substitution of the uncharged asparagine by the charged lysine residue would inhibit the formation of such a bond. Moreover a charge repulsion would occur, thereby inhibiting intermolecular interaction in this region. In mixtures of the double mutant hemoglobin Sta. II/S and Hb S, the propensity to polymerize was also reduced. It is known that in mixtures of oxyhemoglobins, tetramers are in a ra-

pid dissociation equilibrium with their corresponding αβdimers (21). Therefore in mixtures of Hb S ($\alpha_2^A \beta_2^S$) and the double mutant Hb Sta. II/S, a hybrid tetramer containing only one $\alpha^{Sta.\ II}$ chain may form. The M.G.C. at which gelling of such a mixture occured (27.2 g/l) was intermediate between the M.G.C. of Hb S (23.8 g/dl) and that of the double mutant Sta. II/S (30 g/dl). These data suggest that both α^{78} positions in the same molecule are implicated in intermolecular contacts in the gel and consequently both α^{78} sites are active. The same conclusion may also be reached by comparison of the M.G.C. of S/A and of the double mutant/A mixtures. Recently studies on the crystal structure of deoxy Hb S demonstrated that both α^{78} sites are involved in intermolecular crystal contact (22). Thus, our present data shed additional light on the resemblance between fiber and crystal contacts (23).

The modified oxygen affinity and Bohr effect of the double mutant Sta. II/S could be directly attributed to the α^{78} Lys substitution. These alterations may be explained on the basis of the three-dimensional model of hemoglobin. Thus position α^{78} which is occupied by asparagine in Hb A, is close to the NA1 valine 1 α . According to PERUTZ (19), a salt bridge is formed in the deoxy [T] structure between this valine and the carboxyl group of arginine 141 in the partner α chain. Upon the T → R transition, this bridge breaks with a concomitant release of Bohr protons. The presence of lysine in the α^{78} position would therefore inhibit the ionization of the amino group of the valine 1 α residue, thereby explaining the decreased Bohr effect. It would also weaken the salt bridge which links the valine 1 α to the arginine 141 of the partner α chain. This effect could account for the slightly increased oxygen affinity by destabilising the T state. These alterations are relatively weak at alkaline pH where the positive charge of lysine α^{78} is inhibited but become significant at acid pH and low anion concentrations (0.02 M NaCl) where the positive charge of the lysine residue is fully expressed. This hypothesis is further supported by data on the oxygen affinity of carbamoyl Hb Sta. II/S which showed only a little increase in oxygen affinity relative to the unmodified Hb Sta. II/S.

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