Vol. 111, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1983 Pages 8-13

SICKLE CELL HEMOGLOBIN FIBER FORMATION STRONGLY INHIBITED BY THE STANLEYVILLE II MUTATION (α78 Asn → Lys)

Marie-Dominique Rhoda, Josiane Martin, Yves Blouquit, Marie-Claude Garel, Stuart J. Edelstein[+] and Jean Rosa

Unité INSERM U.91, Hôpital Henri Mondor. 94010 Créteil, France

Received December 17, 1982

A double mutant hemoglobin possessing both the Hb S (β6 Glu → Val) and the Hb Stanleyville II (α78 Asn → Lys) mutations has been purified from the blood of a donor heterozygous for both of the mutations. The purification required two chromatography steps, with the second permitting resolution of the double mutant from Hb A_2 remaining after the first step. Measurement of the competence for fiber formation by the double mutant hemoglobin was carried out by the centrifugation of gels to obtain C_{sat}. The double mutant was found to have a greatly elevated C_{sat}, 26.4 gm/dl, compared to 15.2 gm/dl for the Hb S control. The concentration of the pellet of the centrifuged gel for the double mutant was in the range 48-50 gm/dl, suggesting that no major rearrangement in the structure of the fibers had been induced by the Stanleyville II mutation. Electron microscopic observations on the fibers of the double mutant confirmed that a normal appearance was maintained.

Formation of fibers in the red cells of individuals with sickle cell disease is triggered by the single amino acid change, β6 Glu → Val, but many portions of the surface of the Hb S molecules appear to participate in the intermolecular contacts of the fibers (1). The multiplicity of surface residues involved in contacts is related to the complexity of the fiber structure which places Hb S molecules in inequivalent positions. The structure of the fibers, as deduced from computer reconstructions of electron microscope images, involves a 14-filament helix organized in seven sets of double filaments (2). A number of lines of evidence suggests a close structural relationship between these helical double filaments and the linear double filaments observed in crystals of Hb S (3). In particular, β chain mutations at positions that occur at contacts in the crystals have been found to influence fiber formation in combination with Hb S (4). A number of α chain mutations have been investigated as well, in terms of their effects of fiber formation (5, 6, 7). Certain α chain mutations occur at positions identified with contacts that occur in the crystals of Hb S, while others occur at contacts between double filaments that are unique to the fibers (1). In this

[+]Permanent address : Section of Biochemistry, Molecular and Cell Biology, Wing Hall, Cornell University. Ithaca, N.Y. 14853, USA

0006-291X/83/040008-06$01.50/0

latter class of mutants is hemoglobin Stanleyville II (α78 Asn → Lys) which has been found in combination with the Hb S mutation in individuals of Sudanese ancestry (8) and has an inhibitory effect on fiber formation (7). In this report we describe further studies on a fully purified double mutant hemoglobin Stanleyville-S isolated from an individual heterozygous for both mutations. Fiber formation by the double mutant has been measured by the C_{sat} centrifugation assay. In addition, structural analysis of the fibers formed by the double mutant have been carried out by electron microscopy, in order to determine if there is a change in the structure, such as has been found for the Sealy (Hasharon)-S double mutant (9).

MATERIALS AND METHODS

The double mutant Hb Stanleyville II-S (α_2 78 Asn → Lys β_2 6 Glu → Val) is one of the hemoglobin forms that occurs naturally in our donor who is heterozygous for both mutations. The double mutant, hereafter referred to as Hb Stan./S, was purified in two steps by column chromatography. The first step involved DEAE cellulose chromatography along the lines described by Abraham et al. (10). The hemolysate containing 1.5 gm of hemoglobin in the CO form was dialysed against buffer A (0.2 M glycine, pH 7.8) and applied to 2.5 x 45 cm column equilibrated with the same buffer at 4°C. Passage of buffer A containing 8 mM NaCl resulted in the formation of three distinct bands composed respectively of Hb A, a mixture of Hb S and Hb Stanleyville II and a mixture of Hb A2 and Hb Stan./S. The band containing Hb A2 and Hb Stan./S was removed from the column and the hemoglobins were eluted with a small volume of buffer A with 0.2 M NaCl. In order to separate Hb A2 and Hb Stan./S a second chromatography step was carried out on Biorex 70 (Bio-rad 200-400 mesh) along the lines described by Trivelli et al. (11) and modified by Blouquit et al. (12). The sample, equilibrated with a 0.12 M sodium phosphate buffer, pH 6.75, was applied to a 2.5 x 18 cm column at room temperature and developed initially with the same buffer to elute Hb A2. The column was then developed with 0.3 M NaCl added to the buffer to elute the Hb Stan./S. Following isolation, the samples were converted to the oxy form by photolysis under oxygen atmosphere and then to the deoxy form in a nitrogen atmosphere and concentrated by vacuum dialysis and ultracentrifugation.

Solubility (C_{sat}) measurements were performed by the ultracentrifuge assay under the conditions described by Benesch et al. (6), in order to permit comparison of the results on Hb Stan./S with their results for other double mutants. Electron microscopic observations on the fibers of Hb Stan./S and Hb S controls were carried out on embedded samples following procedures previously published (13, 14).

RESULTS AND DISCUSSION

The hemolysate containing the double mutant Hb Stan./S also had various other molecular species that necessitated two column chromatography steps in order to obtain purification. The first column (DEAE cellulose) resulted in a fraction containing Hb Stan./S with Hb A2. These two components were cleanly separated by the second (Biorex) column. The elution profile for this latter step is presented in Fig. 1, along with electrophoresis of the two fractions and various controls. The electrophoresis, carried out at pH 9 in 6 M urea buffer in order to examine the components in the form of

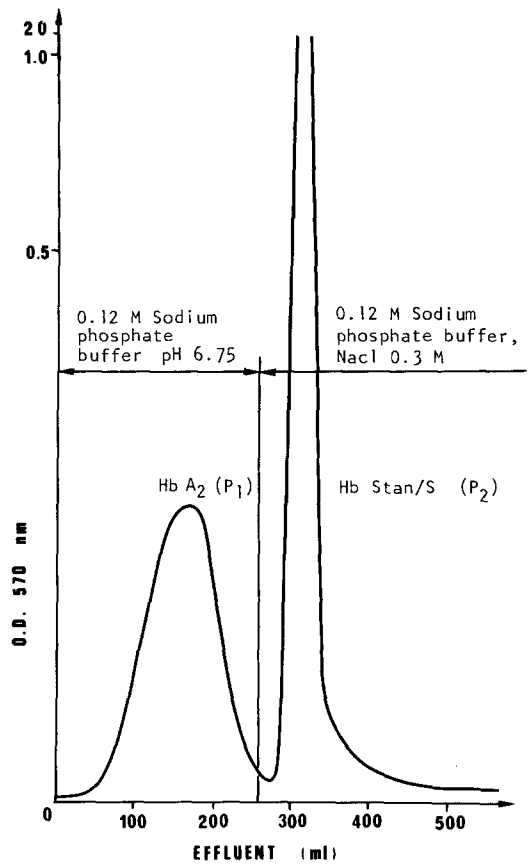

Fig. 1. Chromatographic separation of Hb Stan./S from Hb A_2 on Biorex column at 4°C.

chains, revealed only the presence of the chains expected for the various purified components (Fig. 2).

The C_{sat} of the Hb Stan./S and of the mixture Hb Stan. + Hb S were measured by ultracentrifuge assay and compared respectively to a Hb S control and a mixture of Hb A + Hb S. The control Hb S had been subjected to DEAE and Biorex chromatography in the CO form and then converted to the deoxy form by the same procedure as Hb Stan./S. The C_{sat} values obtained for the Hb S control, 15.2 gm/dl agrees very well with the values reported by Benesch et al. (6), indicating that the chromatography and CO-removal procedures have not altered the hemoglobin properties. The C_{sat} value for the mixture of Hb Stan. + Hb S is identical to the C_{sat} value of the control mixture Hb A + Hb S. For Hb Stan./S, the C_{sat} value obtained is markedly higher, 26.4 gm/dl placing it among the double mutants with the most elevated solubilities. Repetition of these measurements indicated a reproducibility to

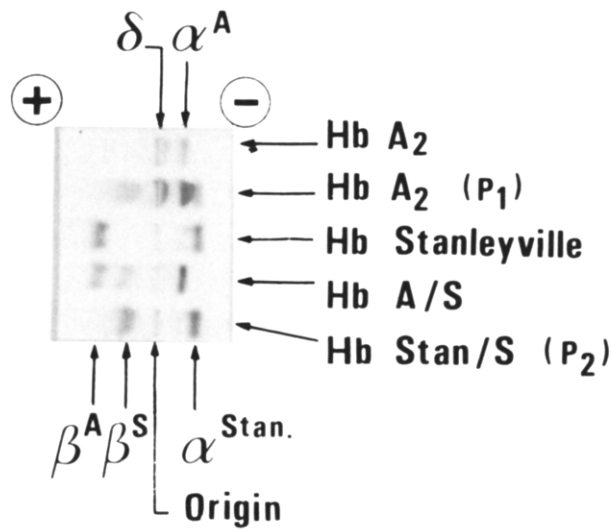

Fig. 2. Globin chain electrophoresis on cellulose acetate strips in 6 M urea buffer pH 9.

within 0.3 gm/dl. The pellet concentration of the centrifuged gel was also measured in the four experiments. This parameter cannot be measured as accurately as C_{sat}, but values of the pellet concentration in the usual range, 48-50 gm/dl were obtained in each case. Therefore, it appears that a "normal" fiber structure prevails for Hb Stan./S, in contrast to the case of the double mutant Hb Sealy/S, where this value is reduced to 43 gm/dl, reflecting an altered fiber structure lacking filaments at certain positions (9)(Table 1).

Table 1

Hb mixtures	Tetramer composition in the mixtures	Hemoglobin concentration g/dl		
		Initial	Supernatant (C_{sat})	Pellet
Hb S (isolated from SS blood)	$\alpha\beta^S$ $\beta^S\alpha$	28.2 28	15.2 15.4	51.8 50.3
Hybrid tetramer α_2^{Stan} II β_2^S	$\alpha^{Stan}\beta^S$ "cis + trans" $\beta^S\alpha^{Stan}$ tetramer"	35.1 35.1	26.4 26.4	48.1 47.2
Hb A + Hb S 50% 50%	$\alpha\beta$ $\alpha\beta$ $\alpha\beta^S$ $\beta\alpha$ $\beta^S\alpha$ $\beta^S\alpha$	33.3 33.3	26.2 26.2	49 50.4
Hb Stan II + Hb S 50% 50%	$\alpha^{Stan}\beta$ | $\alpha^{Stan}\beta$ | $\alpha\beta^S$ $\beta\alpha^{Stan}$ | $\beta^S\alpha$ | $\beta^S\alpha$ "trans" tetramer	31.6 31.6	27 27.3	49.1 49.3

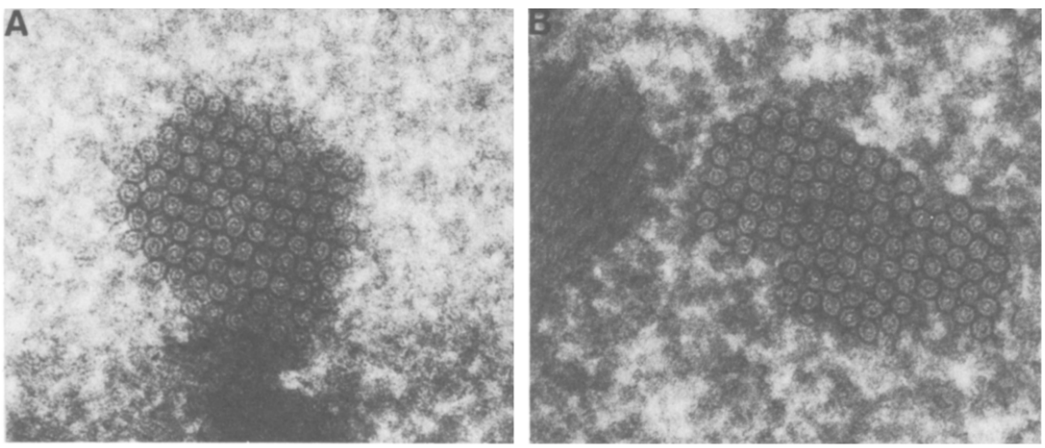

Fig. 3. Electron microscopy of embedded fiber preparations.
A : Hb S control
B : Hb Stan./S
X : 200 000

In spite of the normal pellet density for the centrifuged Hb Stan./S fibers, a structural alteration which retained a normal packing density could have occurred. Therefore fibers were prepared for electron microscopic for both Hb Stan./S and the control Hb S . As seen in Fig. 3, fibers with a typical appearance and a normal size (diameter of 20 $\pm$ 1 nm) were obtained in both cases. Various detailed patterns were found in the cross-sectional views, that have been interpreted previously in terms of the superposition patterns arising from finite thicknesses of the 14-filament helices and slight deviations from perfect perpendicularity of the cross sections (13).

The observations reported here indicate that the location of the Stanleyville II mutation, the α78 position, is likely to participate in a contact that plays a major role in the stabilization of the fibers, with considerably less stabilization possible when the Asn is replaced by a Lys residue. As visualized in the surface maps of Hb S, the α78 position is on the opposite side of the molecule from the β6 region that participates in the primary contact within double filaments ; the α chain region around α78 has been suggested to participate in several of the contacts between helical double filaments in the fibers (1). This site thus represents an interesting possible target for anti-sickling compounds, since an increase in C_{sat} comparable to the increase observed for Hb Stan./S compared to Hb S would effectively convert a homozygous condition to a state approximating the heterozygous condition. While it may be difficult to find a compound that would react specifically at this site, the various pyridoxal compounds specific for the β chains of hemoglobin (15) probably bind to Hb at or near this region and may result in inhibition of fiber formation.

ACKNOWLEDGEMENTS

We thank R. Crepeau and M. Szalay for their help with the electron microscopy results reported here. This work was supported in part by INSERM grant PRC 121 038 and NIH grant HL-13591-09.

REFERENCES

1. Edelstein, S.J. (1981) J. Mol. Biol. 150, 557-575.
2. Dykes, G., Crepeau, R.H. and Edelstein, S.J. (1979) J. Mol. Biol. 130, 451-472.
3. Wishner, B.C., Ward, K.B., Lattman, E.E. and Love, W.E. (1975) J. Mol. Biol. 98, 179-194.
4. Nagel, R.L., Johnson, J., Bookchin, R.M., Garel, M.C., Rosa, J., Schilivo, G., Wajcman, H., Moo-Penn, W. and Castro, O. (1980) Nature 283, 832-834.
5. Benesch, R.E., Kwong, S., Benesch, R. and Edalji, R. (1977) Nature 269, 772-776.
6. Benesch, R.E., Kwong, S., Edalji, R. and Benesch, R. (1979) J. Biol. Chem. 254, 8169-8172.
7. Hassan, W., Beuzard, Y., North, M.L. and Rosa, J. (1977) Biochem. Biophys. Res. Commun. 77, 1108-1117.
8. Van Ros, G., Wiltshire, B., Renoirte-Monjoie, A.M., Vervoort, T. and Lehmann, H. (1973) Biochimie 55, 1107-1119.
9. Crepeau, R.H., Edelstein, S.J., Szalay, M., Benesch, R.E., Benesch, R., Kwong, S. and Edalji, R. (1981) Proc. Natl. Acad. Sci. USA, 78, 1406-1410.
10. Abraham, E.C., Reese, A., Stallings, M. and Huisman, T.H.J. (1976) Hemoglobin 1, 27-44.
11. Trivelli, L.A., Ranney, H.M. and Lai, H. (1971) New Engl. J. Med. 284, 353-357.
12. Blouquit, Y., Senan, C., Rosa, J. J. Chromatogr. (In press)
13. Garrell, R.L., Crepeau, R.H. and Edelstein, S.J. (1979) Proc. Natl. Acad. Sci. USA, 76, 1140-1144.
14. Edelstein, S.J. and Crepeau, R.H. (1979) J. Mol. Biol. 134, 851-855.
15. Benesch, R.E., Yung, S., Suzuki, T., Bauer, C. and Benesch, R. (1973) Proc. Natl. Acad. Sci. USA, 70, 2595-2599.