Proc. Natl. Acad. Sci. USA
Vol. 76, No. 1, pp. 223–226, January 1979
Biochemistry

X-ray diffraction studies of fibers and crystals of deoxygenated sickle cell hemoglobin

(polymer transformation into crystals/crystal model for polymer structure/pairs of staggered filaments/stabilization of double filaments by Val β6 contacts)

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Communicated by Max F. Perutz, October 30, 1978

ABSTRACT **Paracrystalline fibers of deoxygenated sickle hemoglobin in erythrocytes or concentrated solutions exhibit a phase transformation to a fully crystalline state. X-ray diffraction patterns of the fiber and crystallites are similar except in two respects: the equatorial spacings of the fibers suggest that they pack into a square lattice with $a = 220$ Å, whereas those of the crystals can be indexed on the basis of a net of 187 Å by 54 Å, and the second-order near-meridional reflections are strong on the fiber pattern but weak on that of the crystallites. The crystallites are isomorphous with single crystals grown in polyethylene glycol solution at pH 4.5 whose structure has been determined at near-atomic resolution (Wishner, B. C., Ward, K. B., Lattmen, E. E. & Love, W. E. (1975) *J. Mol. Biol.* 98, 179–194). Double filaments of molecules with an axial repeat of 64 Å comprise the basic unit of both the crystal and fiber structures. Each filament of the pair is translated with respect to its neighbor by half a molecular diameter along the fiber axis. The two filaments are held together by contacts made by Val 6β in the molecules of one strand with hydrophobic side chains of the molecule in the neighboring strand. This interaction is probably the cause of the aggregation of filaments into fibers that leads to the sickling of erythrocytes.**

X-ray diffraction patterns (1) of polymers found in sickled erythrocytes of individuals homozygous for sickle cell hemoglobin (Hb S) and in concentrated solutions of deoxygenated Hb S show a series of sharp meridional reflections arising from a 64-Å repeat along the fiber. Potentially, these diffraction patterns contain information about intermolecular contact regions within the polymer, but, because of the poor resolution inherent in cylindrically averaged fiber diagrams and because insufficient parallel alignment of fibers further decreases the resolution, the surface lattice of the polymer has not as yet been established from these patterns; without it the intermolecular interactions cannot be determined.

The packing of molecules into the polymers should be evident from electron micrographs, but these show several polymorphic forms, including a 6-stranded microtubular structure (2) and 14-stranded solid elliptical cylinders (3). Occasional sheetlike structures (4), and six-membered discs (5) have also been observed. Such a wide range of polymorphism is difficult to reconcile with our diffraction patterns, all of which appear similar except for differences in resolution. The patterns most likely arise from a single diffracting system.

On re-examination of deoxygenated sickled erythrocytes sealed in capillaries several years ago, we have discovered strongly birefringent ribbonlike structures as well as bundles of needles of about 3-μm diameter and 1-cm length. These were in equilibrium with a water clear solution, which showed that they could not be fibers because the critical equilibrium concentration of the polymer–monomer system of deoxy-Hb S is about 0.22 g·cm^{-3} (6) under these experimental conditions. Thus the needles must be a crystalline phase that arises from transformation of the polymeric phase. In this report, the diffraction patterns from such a capillary are compared with those from oriented fibers of deoxy-Hb S and from a single crystal of deoxy-Hb S grown at pH 4.5 in the presence of polyethylene glycol.

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EXPERIMENTAL PROCEDURES

Venous blood, anticoagulated with EDTA, was obtained from patients with sickle cell anemia. The cells were washed three times with phosphate-buffered saline, pH 7.2. The packed erythrocytes were lysed with toluene according to the procedure of Drabkin (7) modified to omit the dilution of the packed cells. The final hemoglobin concentration varied between 0.23 and 0.28 g·cm^{-3}. Deoxygenation was achieved at 4°C with a flow of humidified N_2, balanced with 5% CO_2, over a solution of Hb S that was kept in continuous oscillation for several hours. This solution was poured into a cellulose nitrate centrifuge tube, overlaid with degassed mineral oil, and allowed to equilibrate for 1 hr at 25°C. After being centrifuged for 30 min at 180,000 × g in an SW 50.1 rotor in a Beckman L3-50 ultracentrifuge, the pelleted polymer was introduced anaerobically by shear into a thin-walled quartz capillary which was then sealed with hot wax.

Crystals of deoxy-Hb S were grown according to Wishner and Love (8). A 50% (wt/vol) solution of polyethylene glycol (6000 molecular weight) was added to citrate-buffered (0.2 M, pH 4.5) Hb S to give final concentrations of about 6% Hb S and 11% polyethene glycol. The solution was deoxygenated by the addition of 1.5 mol of dithionite per mol of heme and remained at about 25°C for approximately 1 month. Large aggregates of single crystals were observed within a heavy precipitate of denatured deoxy-Hb S. Single crystals were extracted from the aggregates and mounted in the usual way in thin-walled capillaries for recording x-ray diffraction patterns.

Nickel-filtered Cu radiation from a Jarrel Ash microfocus generator, operated at 45 kV and 6 mA for 15–80 hr depending upon the distance of the specimen from the film, was used for obtaining diffraction patterns. The x-ray beam was collimated with a defining pinhole of 100 μm and was of small divergence.

RESULTS

A representative diffraction pattern from a capillary prepared 4 years ago that originally contained sickled erythrocytes is shown in Fig. 1 *left*. Patterns recorded over a range of 180° in azimuthal angles with respect to the x-ray beam were all virtually identical, showing that each is equivalent to a rotation diagram of a single crystal, although, in fact, it was obtained

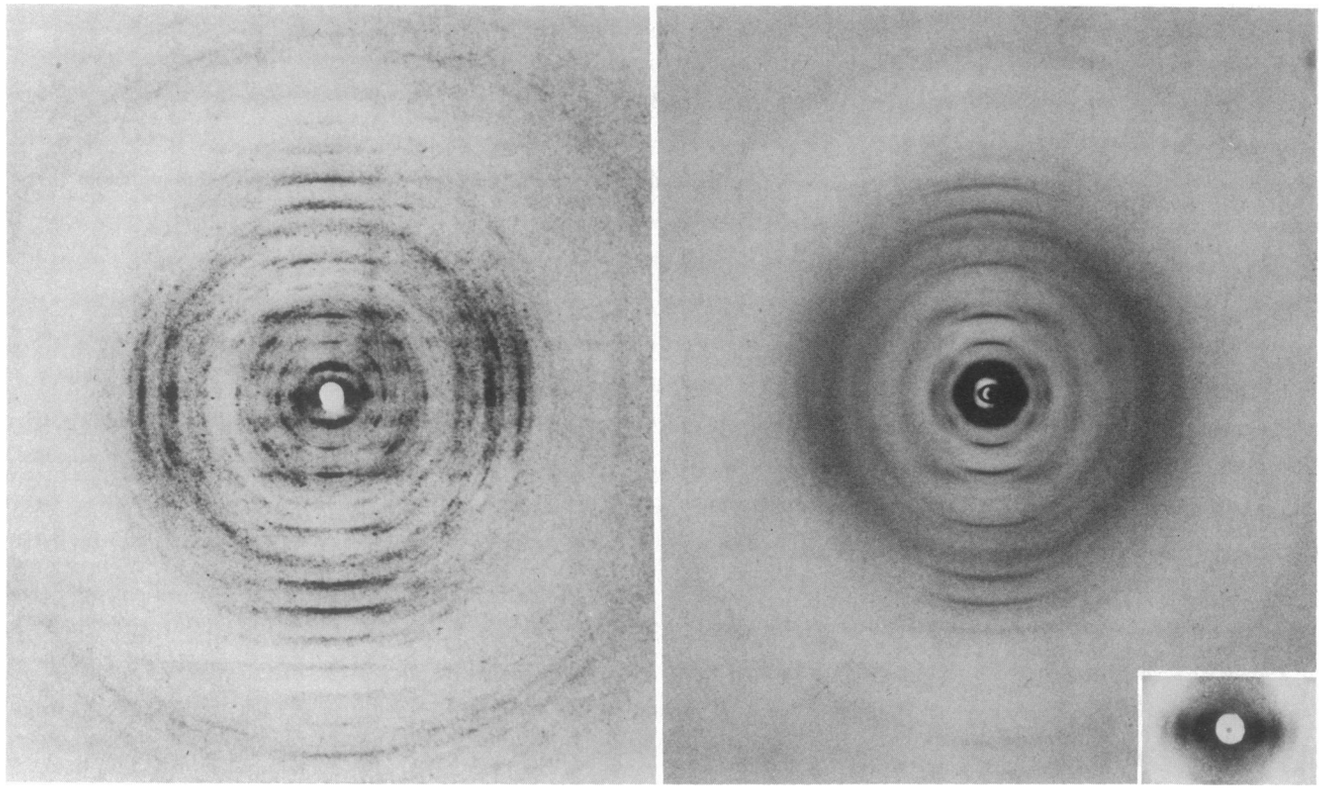

FIG. 1. X-ray diffraction patterns, (*Left*) Packets of crystallites found in a 4-year-old capillary that originally contained sickled erythrocytes; (*Right*) polymers obtained from high concentration of deoxy-Hb S. Equatorial spacings (see *Inset*) are at (220, 110, 73, and 52 Å)$^{-1}$. The axial repeat on both patterns is (64 Å)$^{-1}$.

from a packet of crystallites whose almost parallel needle axes are cylindrically averaged. Similar rotation diagrams from capillaries of sickled erythrocytes as well as from capillaries of deoxy-Hb S lysates can now be obtained reproducibly by incubation at 37°C after 2 weeks to 2 months. Fig. 1 *right* shows a fiber pattern of a lysate with the same repeat, (64 Å)$^{-1}$, taken shortly after preparation. The patterns (Fig. 1) are similar in their spacings and intensity distributions except in two regions: on the equator, where the spacings of the diffraction maxima are markedly different in the two patterns, and in the second-order meridional reflection, which is strong on the fiber pattern and weak on the crystalline patterns.

The space group of the crystallites cannot be determined from Fig. 1 *left* because of the nonparallellism of the needles and the overlap of diffraction maxima, but the equatorial reflections of the crystalline bundles can be indexed (Table 1) on the basis of an orthogonal net of dimensions 187 Å by 54 Å. On the other hand, the small angle equatorial reflections on the fiber diagram, shown enlarged in the *inset* on Fig. 1 *right*, are at spacings of (220, 110, 73, and 52 Å)$^{-1}$. Whether the polymers pack into a square or hexagonal lattice cannot be determined, because only the first three orders of the 220-Å spacing are observed. Finch *et al.* (2), however, recorded an 11 reflection from a square lattice with a = 225 Å. The diffraction maximum at (52 Å)$^{-1}$ is probably associated with the molecular packing in the polymeric structure and is independent of the packing of polymers into regular arrays. It corresponds to the diameter of hemoglobin along the molecular z axis (2).

Table 1. Equatorial spacings (in Å) and indices of crystalline diffraction patterns

Indices	Spacings	
	Observed	Calculated
020	96.0	93.5
040	48.6	46.8
041	35.0	35.1
060	31.2	31.2
002, 012, 061	27.5	27.0, 26.7, 27.0
003, 013, 082, 0,10,1	17.9	18.0, 17.9, 17.7, 17.7

A rotation diagram about the a axis of a deoxy-Hb S crystal is shown in Fig. 2 *left*. The similarity with Fig. 1 *left* is striking and becomes even more evident upon the optical smearing of Fig. 2 *left* by rotating it through ±7° (Fig. 2 *right*) to simulate the approximate extent of nonparallel alignment of the crystallites (Fig. 1 *left*) and of the paracrystalline arrays in the fibers of deoxy-Hb S (Fig. 1 *right*). Absences of the 4th and 6th orders and the strong 13th-order reflection corresponding to a spacing of about 5 Å are conspicuous. The meridional reflections (Fig. 2 *right*) in fact are the strong 110 and 120 reflections on the first layer line; 310 on the third; 740 on the seventh; and 800, 820, and 830 on the eighth. The same apparent meridional reflections occur on the pattern of the rotationally averaged needles (Fig. 1 *left*) and can be attributed to the same *hk*0 reflections smeared by the nonparallel alignment of the needle axes. It seems likely that, in the fiber pattern also, the apparent meridional intensity on these layer lines is really off-meridional as in the crystal patterns, but is smeared over the meridian by disorientation of the fibers.

From the unit cell dimensions of the deoxy-Hb S crystals, a = 64 Å, b = 187 Å, c = 54 Å, and β = 92.5°, obtained from measurements of 5° precession films of the *hk*0 (Fig. 3) and *h*0*l* zones, indices are assigned to the equatorial reflections (Fig. 2). The spacings and indices are the same as given in Table 1 for the 0*kl* reflections of the crystallites that had grown from the fibers. These values are within 1% of the cell dimensions reported by Wishner *et al.* (9) for deoxy-Hb S crystals. The arcing of maxima on the equator (Fig. 2 *left*) is generated by the

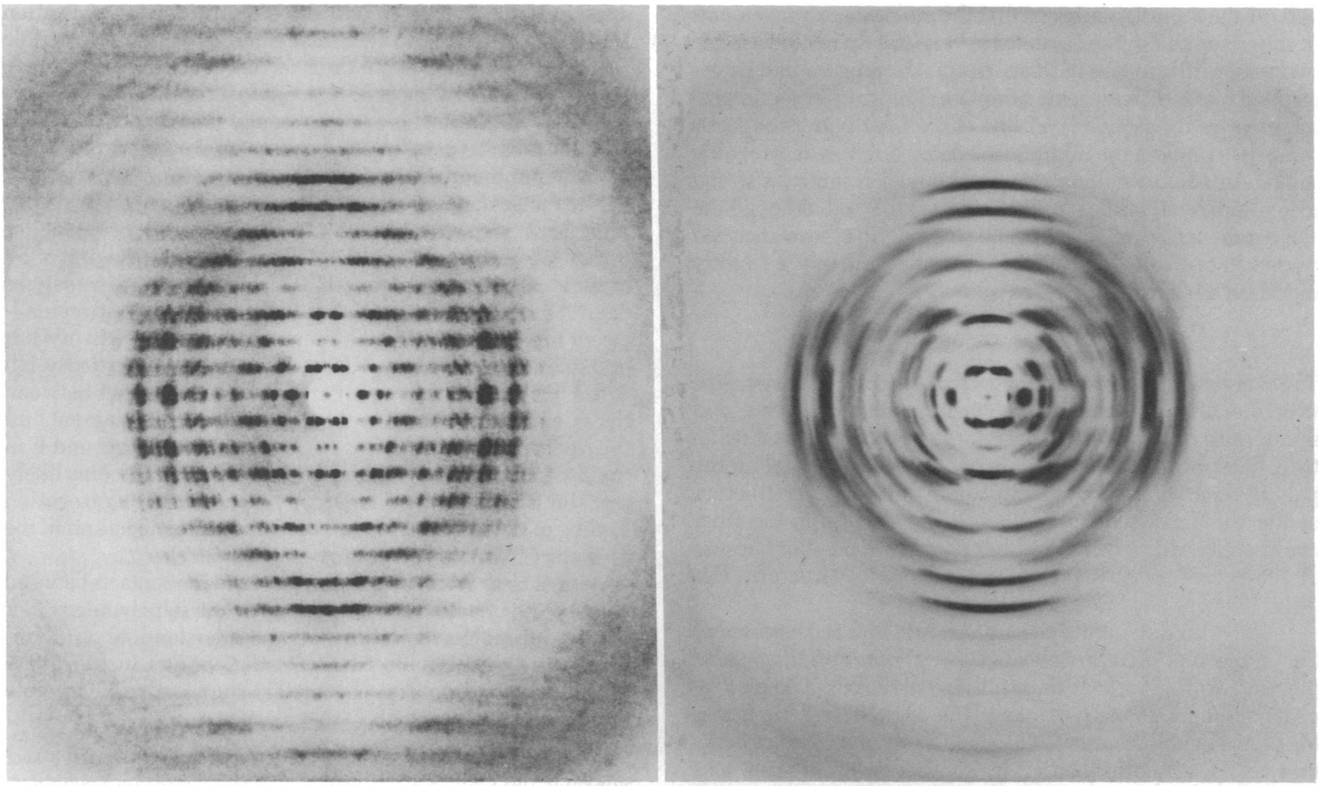

FIG. 2. (*Left*) A rotation diagram of a single crystal of deoxy-Hb S grown in the presence of polyethylene glycol at pH 4.5. The unit cell dimensions are $a = 64$ Å, $b = 187$ Å, $c = 54$ Å, and $\beta = 92.5°$. (*Right*) Rotation diagram as in *Left* optically smeared about an axis parallel to the incident x-ray beam by ±7°.

misalignment of the rotation axis of the crystal similar to that which occurs in the patterns of fiber and of packets of crystallites (Fig. 1).

The fiber diffraction pattern (Fig. 1 *right*), the pattern of the packets of crystallites (Fig. 1 *left*), and the optically smeared rotation diagram of a single crystal (Fig. 2 *right*) are characterized by off-meridional diffuse scattering on the layer lines. Nonparallel alignment of fibers and crystals are responsible for these diffuse regions. On the third layer line, for example, the diffuse region extends approximately from $(35 \text{ Å})^{-1}$ to $(21 \text{ Å})^{-1}$ off the meridian. In conjunction with the strong equatorial reflections in the region of $(18 \text{ Å})^{-1}$, the diffuse scattering produces an appearance of a distorted hexagonal ring in reciprocal space, the vertices of which are on the third layer lines and on the equator. The reflections that comprise the diffuse region and the observed distances from the meridian in reciprocal space, as measured on the rotation diagram, as well as reciprocal distances calculated from lattice dimensions are shown in Table 2. No other reflections fall within this range of reciprocal spacings. From the precession film (Fig. 3) it is obvious that the strong 360 and 380 and the medium 390 reflections contribute to the diffuse region. Because of the overlap of reflections on the rotation diagram, however, only five maxima appear, even though each is composed of reflections from several planes. Comparison of these reflections with the observed structure factors of the deoxy-Hb S crystal at 3.5 Å resolution, a listing of which was provided to us by J. C. Hanson, facilitated the identification. Similarly, other diffuse regions can be related to crystalline reflections.

The striking similarity of the diffraction patterns both near

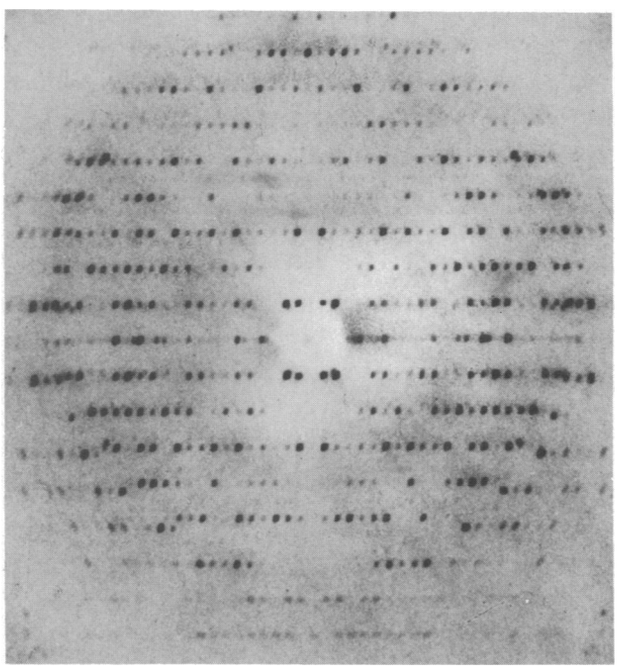

FIG. 3. A 5° precession photograph without layer line screen of the *hk*0 zone of a single crystal of deoxy-Hb S grown in buffered polyethylene glycol at pH 4.5.

Table 2. Reflections in the diffuse region on the third layer line

Indices of groups of reflections	Distance from meridian × 10^2 $(\text{Å})^{-1}$	
	Observed	Calculated
$34\bar{1}$, 341	2.88	2.63, 3.03
$35\bar{1}$, 351, 360	3.27	3.05, 3.45, 3.21
$36\bar{1}$, 361, $31\bar{2}$, 312	3.75	3.50, 3.90, 3.54, 3.94
$34\bar{2}$, 342, 380	4.31	4.08, 4.48, 4.28
$38\bar{1}$, 381, 390	4.77	4.46, 4.86, 4.81

and off the meridian suggests that the molecular arrangements in the fibers and in the crystals are very closely related. Of the two major differences in the patterns, the equator can be accounted for by differences in sampling. The stronger meridional intensity on the second layer line of the fiber pattern suggests some rearrangement of the molecules but this is probably minor. In one crystalline sample obtained recently, a strong second-order meridional reflection was observed although the cell parameters were close to those of the normal form analyzed by Wishner *et al.* (9, 10) and was thus probably a slightly modified version of that form.

DISCUSSION

The x-ray diffraction patterns show that the fiber and crystalline structures of deoxy-Hb S are sufficiently similar in spacings and in intensity distributions to permit the use of the crystal structure, determined at near-atomic resolution, as a model for the polymeric structure. The identification of the atomic structure of the contacts between neighboring molecules in the fiber could not be made from the fiber patterns alone, but because of the correlation between fiber and crystal structures this important problem can now be resolved.

In the crystal structure the asymmetric unit is composed of two molecules that are related by a *noncrystallographic* pseudo-two-fold screw axis parallel to the *a* axis. The pair repeats at intervals of 64 Å along the crystallographic *a* axis forming a double filament. The second pair of molecules in the unit cell is related to the first by another two-fold screw axis which is *crystallographic* and parallel to the *b* axis. As a result of this symmetry operation the second pair of double filaments is antiparallel to the first. Wishner *et al.* (10) investigated whether the relative intensities of the near-meridional reflections in the fiber pattern could be accounted for by the transform of a single or double filament or pairs of double filaments as arranged in the crystal structure. Strong resemblances between observed and calculated intensities were found for the double and the quadruple filaments. The agreement is not perfect, indicating that the transform of the structure is that of a double filament modulated by an interference function that is determined by the displacement of the neighboring double filament along the fiber axis by an amount as yet unknown.

Our direct comparisons of crystalline and fiber patterns suggest that not only is the double filament a part of the polymeric structure but that two such double filaments may comprise the basic unit of the polymer. The restriction of antiparallelism could be relaxed because the two-fold *molecular* symmetry axis is inclined only by approximately 9° to the crystallographic screw axis (9, 10). However, one would expect that the relative displacements along the fiber axis of neighboring double filaments in the fibers be close to that in the crystal. From these considerations alone we cannot unequivocally determine the packing of the double filaments in the fiber, but the model in which six filaments are in register (2) can be eliminated. The proposed 14-stranded model (3) may be an approximation to the fiber structure, providing it consists of double filaments staggered in a manner such that calculated transforms are in agreement with our observed diffraction patterns.

The mechanism of the rearrangement of neighboring double filaments in a fiber that packs into a square or hexagonal lattice with a reciprocal spacing of $(220\ \text{Å})^{-1}$ to a crystal in which two antiparallel double strands form a lattice of cross-sectional dimensions of 187 Å by 54 Å remains inexplicable at present. It appears that polymer growth and alignment after nucleation leads to a disordered metastable state in which the double filaments are rotated about the fiber axis to form a helix of long period. With annealing at 37°C, double filaments condense into a continuous lattice of lower free energy than that of the fibers, and sheaves of needle-like crystals grow and rupture the erythrocyte membranes.

The molecular interactions that stabilize the crystalline lattice must also, in part, stabilize the fibers. Single filaments of deoxy-Hb with a repeat of 64 Å form the structural unit of all crystals of vertebrate deoxy-Hb (11); detailed x-ray analyses have shown the atomic structure of the contacts between adjacent molecules along the filaments to be the same in crystals of human deoxy-Hb S, A, F, and C (9, 10, 12–14). In deoxy-Hb S, the two filaments of a pair are joined by a contact between the side chain of Val 6β in the molecules of one filament and the hydrophobic side chains lying between helices E and F in the β-chains of the neighboring filaments (9). It seems likely that this is the specific interaction that causes the aggregation leading to sickling and that it is the same in the fibers and in the crystals.

It is not clear what the differences are in the contacts between neighboring double filaments in crystals and in polymers. Fiber patterns of higher resolution and an understanding of the rearrangement of double filaments that occurs in the phase transformation from fiber to crystal would lead to a complete mapping of such contacts.

We are indebted to Dr. John F. Bertles for his encouragement and support of this study, and we thank Drs. Max F. Perutz for suggestions and critical reading of the manuscript, Aaron Klug for helpful discussions, and J. C. Hanson of the Thomas C. Jenkins Department of Biophysics, Johns Hopkins University, for a listing of structure factors of the deoxy-Hb S crystals. This work was supported by National Institutes of Health Grant HL 15293.

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