

Electron microscopy of fibers and discs of hemoglobin S having sixfold symmetry

(deoxyhemoglobin S/image reconstruction/mutant hemoglobins/optical diffraction/sickle cell anemia)

M. OHTSUKI*^{†‡}, S. L. WHITE*, E. ZEITLER*^{†‡§}, T. E. WELLEMS*, S. D. FULLER*[¶], M. ZWICK*[¶],
M. W. MAKINEN*, AND P. B. SIGLER*^{††}

* Department of Biophysics and Theoretical Biology, † Department of Physics, and ‡ The Enrico Fermi Institute, The University of Chicago, Chicago, Illinois 60637

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ABSTRACT Aggregated forms of deoxyhemoglobin S were examined with a field emission transmission electron microscope. Images of isolated helical fibers were obtained from sickled cell lysates stained directly on the electron microscope grid. Optical and digital analyses of the electron micrographs showed that the fibers are similar to those characterized by J. T. Finch, M. F. Perutz, J. F. Bertles, and J. Döbler [(1973) *Proc. Natl. Acad. Sci. USA* 70, 718-722] in that they consist of stacked discs each composed of six hemoglobin molecules. The fibers exhibit an outer diameter of 160-170 Å and an inner diameter of about 60 Å with an axial spacing of 58 Å per disc. The fiber can be described as a helix consisting of 56 discs per helical turn. We observed discs of six hemoglobin molecules, which may be stable substructural components of the fibers. They were observed in preparations of hemoglobin fibers and exhibited 6-fold symmetry by power spectrum analysis. A reconstructed image of a disc digitally filtered for 6-fold symmetry has a maximum external diameter of ~170 Å and a central hole of 60 Å diameter and is similar to the axial projection of a single disc from a low-resolution, three-dimensional reconstructed model of a fiber.

The erythrocyte sickling phenomenon of sickle cell anemia is associated with aggregation of hemoglobin S (HbS) molecules into linear arrays or fibers (1-9). These aggregates form long bundles of fibers within the erythrocyte and are believed to be responsible for deformation of the cell into abnormal shapes with the consequent acute physiologic manifestations known as sickle cell crisis. The molecular basis of sickle cell anemia is a mutation in the β -globin gene causing replacement of Glu A3(6) β with valine in each of the β subunits of hemoglobin (11).

It is clearly desirable to relate the structural alteration of the hemoglobin molecule to the mechanism responsible for the formation of the characteristic linear aggregates. Structural features of aggregates of deoxyhemoglobin S (deoxyHbS) molecules have been characterized by electron microscopy (1-5), x-ray diffraction (6), and physical chemical studies (7-10). On the basis of these studies, models have been presented that are consistent with the $\beta 6$ site at the points of intermolecular contact (12); however, direct structural determination of the molecular orientation of the deoxyHbS molecule in the fiber is required to resolve the role of the substituted valine residue.

Correlating the features of the fibers with the chemical and physical properties of the deoxyHbS molecule has been complicated by the fact that at least two distinct types of fibers have been described by electron microscope studies: a six-stranded helix (1, 2) having 6-fold axial symmetry and two types of

eight-stranded helices in which pairs of strands are related by either 4-fold (2) or 8-fold axial symmetry (5). The models deduced from electron micrographs are difficult to substantiate with the x-ray fiber diffraction patterns of sickled cells or of gels obtained from deoxyHbS solutions (6) because of poor orientation. Furthermore, the ultrastructural properties of linear aggregates of deoxyHbS molecules formed from purified solutions are not necessarily identical to those observed directly from freshly lysed sickle cells. In short, polymorphism appears to characterize the aggregation of deoxyHbS (1-6). Any convincing structural mechanism should, therefore, account for the varied modes of interaction.

In this communication we report a preliminary characterization by electron microscopy of one type of ordered aggregate of deoxyHbS molecules, the 6-fold symmetric helix similar to that initially described by Finch *et al.* (1). Our electron micrographs also reveal many disc-like structures which appear to be rings of six hemoglobin molecules with apparent 6-fold symmetry and could represent a substructural component of the fibers. The results of these studies, by both direct observation of single discs and image reconstruction techniques, demonstrate that the fiber is a hollow, tubular structure.

METHODS AND MATERIALS

Specimen Preparation. Citrated blood was obtained from a single patient with homozygous sickle cell disease. The HbS content was quantitated electrophoretically to be at least 95%. Red blood cells were washed five times successively with 1% NaCl and centrifuged upon each washing at $1000 \times g$ for 10 min. Four volumes of the packed cells were then mixed with one volume of a solution containing 10 mM 2,3-diphosphoglycerate and 1.0 M NaCl at pH 7, and the mixture was deoxygenated by passing high purity nitrogen over the surface of the cell suspension for approximately 2 hr. All further manipulations were carried out in a nitrogen-filled glove box. Light microscopic examination of a droplet of the cell suspension placed between a sealed slide and coverslip demonstrated a high proportion of sickled forms (see ref. 4).

The sickled cells were lysed with distilled water on a glass slide. From the upper layer of the lysate, approximately 10- to 20- μ l aliquots were transferred onto grids with a 20 Å thick

Abbreviations: HbS, sickle hemoglobin; deoxyHbS, sickle deoxyhemoglobin.

[§] Present address: Institute for Electron Microscopy, Max Planck Institute, Berlin-Dahlem, West Germany.

[¶] Present address: Department of Chemistry, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403.

[¶] Present address: Systems Science Program, Portland State University, Portland, OR 97207.

^{††} To whom reprint requests should be addressed.

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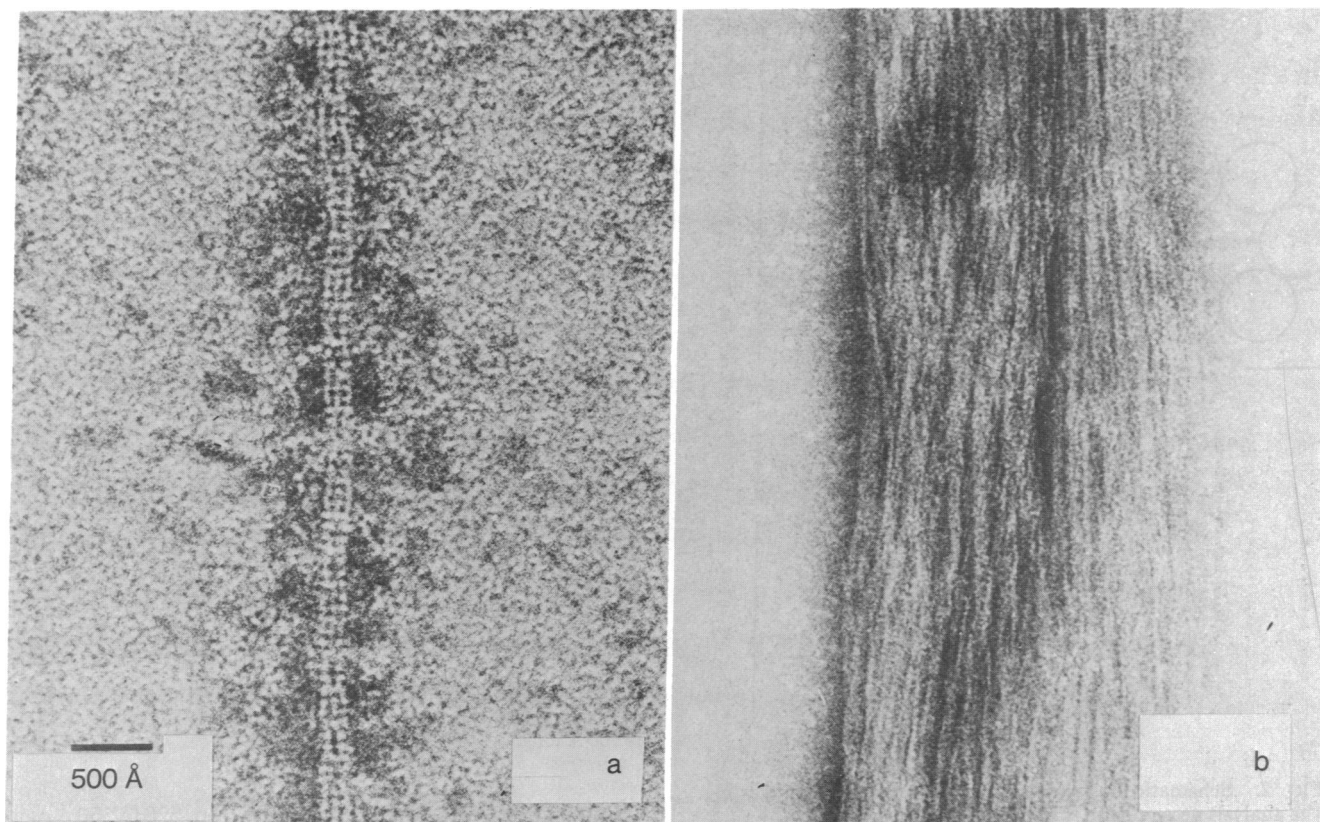


FIG. 1. Electron micrographs of HbS fibers negatively stained with sodium phosphotungstate. (a) Single six-stranded fiber from a lysed sickled cell. (b) Aggregates prepared from gels of purified deoxyHbS.

carbon film supported on a carbon-coated fenestrated plastic film. After 2 min, excess solution was removed from the grid and the adherent lysate was stained with 2% (wt/vol) sodium phosphotungstate for 2 min. In some cases lysates were fixed with 1% (wt/vol) glutaraldehyde on the grid before staining.

HbS was purified chromatographically from blood of heterozygous patients (8) and concentrated by ultrafiltration. Gels were prepared at room temperature from deoxygenated solutions that contained 3.4 mM HbS, 3.4 mM inositol hexaphosphate, and 65 mM phosphate buffer (pH 6.4). The gel was partially teased out on a glass slide in 0.04 mM inositol hexaphosphate (pH 6.4), deposited on a grid, promptly fixed for 2 min with 2% glutaraldehyde, and stained with 2% sodium phosphotungstate.

Electron Microscopy. Specimens were examined with a field emission transmission electron microscope. The conventional thermionic emission gun of a Hitachi HU-12 electron microscope was replaced with a field emission gun as described by Munch (13). To minimize radiation damage, we used the minimal beam exposure method described previously (14), and the microscope was operated at 50 kV. Electron micrographs were taken at a magnification of 38,000 ($\pm 1\%$). Calibration of the magnification was checked with a grating replica of 4630 Å spacing (E. F. Fullam, Inc.), and a single crystal of Cu-phthalocyanine. Electron microscopy of deoxygenated purified gels of deoxyHbS was carried out with a Hitachi HU-11A microscope operated at 75 kV.

Image Analysis. Optical filtering and reconstruction were carried out with diffraction patterns obtained with a 1 mW Spectra-Physics He-Ne laser, as outlined by Horne and Markham (15). For computer analyses and reconstructions, selected images were photographically enlarged on Kodak 7302 Pro-

fessional Film and the optical density was measured on a 50- μm grid with a 50- μm aperture of an Optronics Drum Scanner, controlled on line by a PDP 11/40. The enlargement chosen was such that the scan sampled the image at 4-Å intervals.

Three-dimensional reconstructions of the fiber images were generated as proposed by DeRosier and Moore (16). Rotational analyses of the discs were performed by a method formally equivalent to that of Crowther and Amos (17).

RESULTS

Fig. 1 compares micrographs of the deoxyHbS aggregates obtained by two different methods of preparation. Isolated helical strands characteristically dominated specimens (Fig. 1a) that were prepared by promptly depositing the lysate of sickled cells on grids and staining them. On the other hand, a wide and inconsistent spectrum of aggregated and confluent forms was observed when preparations were made from gels of deoxyHbS solutions (Fig. 1b). Optical transforms of images like those in Fig. 1b revealed no periodic structures. The straight helical fibers in Fig. 1a consist of discs stacked roughly normal to the helix axis with a gentle, helical twist. A schematic diagram of these structures is shown in Fig. 2. This mode of aggregation resembles the structure described by Finch *et al.* (1) and does not resemble the eight-stranded structure described by either Josephs *et al.* (2) or by Crepeau *et al.* (5).

Fig. 3 is an optical diffraction pattern taken of the image of the fiber in Fig. 1a. A digital transform prepared in connection with the three-dimensional reconstruction of the fiber (M. Zwick and T. Wellems, unpublished results) shows that the maximum observed on the first observable layer line is consistent with the first maximum of a J_6 Bessel function and the phase distribution indicates a fiber of even symmetry. Similar

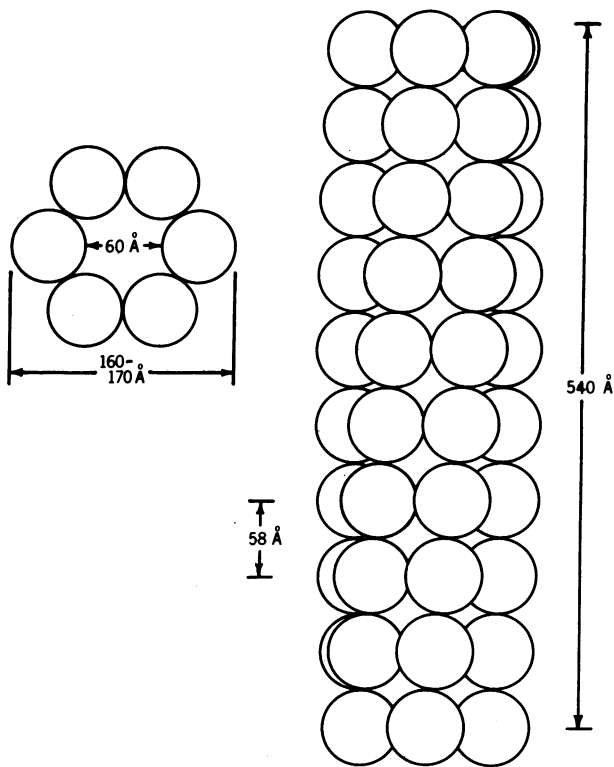


FIG. 2. Schematic illustration of HbS fibers and discs according to the analysis presented in the *text*. An average rise of 9.3 discs per one-sixth turn of the helix is indicated on the diagram on the right.

diffraction patterns are obtained from other micrographs of this type of preparation. As shown in Fig. 2, the structure can be viewed as six equivalent longitudinal strands of hemoglobin molecules in horizontal register helically wrapped around a central cavity. The first observable layer line should therefore represent the axial spacing at which one longitudinal strand completes one-sixth of a helical turn. Transforms calculated from a digital representation of the best two electron micro-

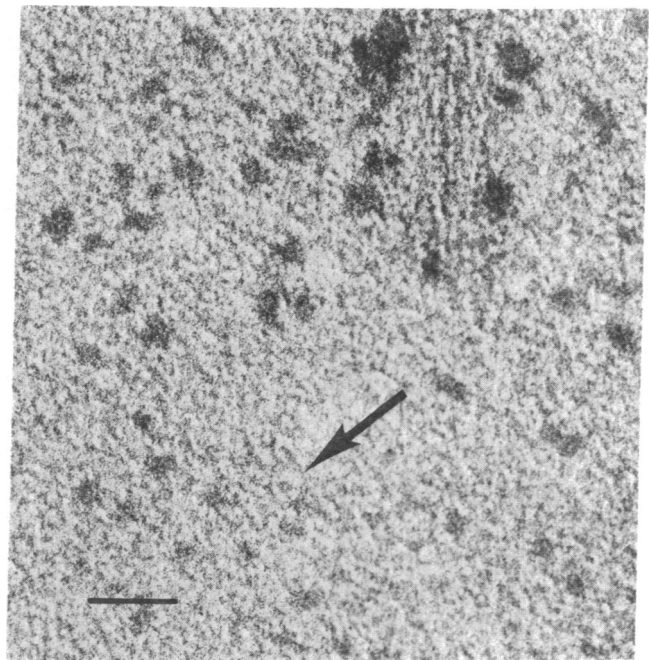


FIG. 4. Electron micrographs of a disc (arrow) in the presence of fibers negatively stained with sodium phosphotungstate. The bar is 500 Å long.

graphs indicate that this distance ranges from 520 to 550 Å, with a most probable value of 540 Å, and that the meridional reflection corresponds to an axial rise per disc of $58 (\pm 1)$ Å. Direct measurement of the axial disc-spacings in 66 specimens indicates an axial rise per disc in the range of 54–62 Å. This distance was the same whether or not the lysate was fixed with glutaraldehyde. This repeat distance is in agreement with 58-Å spacing obtained by digital analysis. The average outer fiber diameter is estimated at 170 Å by direct measurement of the fiber images and is again in agreement with the 160-Å outer diameter obtained by reconstruction studies.

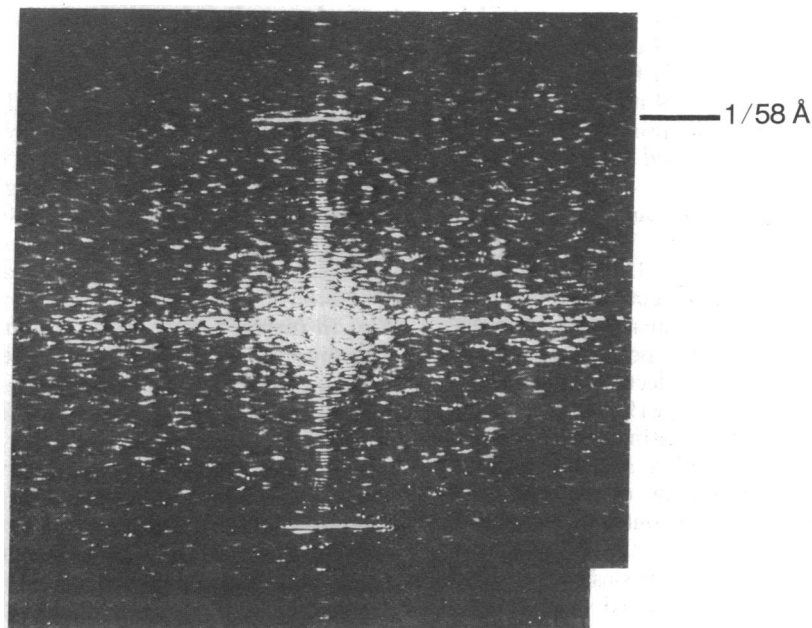


FIG. 3. Optical diffraction pattern of the fiber shown in Fig. 1*a*. Spacings for the meridional and first layer reflections are derived from digital analysis.

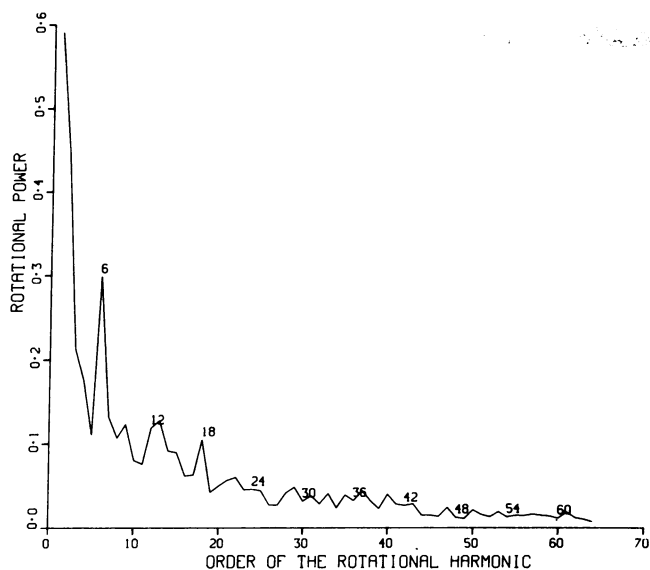


FIG. 5. Rotational power spectrum of the disc in Fig. 4 demonstrating 6-fold rotational symmetry. The spectrum is normalized to unity for the zero-order term.

A most interesting feature of our electron micrographs is the presence of discs or rings together with fibers in almost every field (Fig. 4). Fig. 5 shows the rotational power spectrum analysis (17) of the ring in Fig. 4, which clearly demonstrates 6-fold symmetry to an estimated resolution of 32 Å. An image of this ring, digitally filtered for 6-fold symmetry, is shown in Fig. 6.

We have not been able to demonstrate clear 6-fold symmetry in all discs—probably because of the ease with which the analysis is disrupted by minor distortions from circularity; however, R. Josephs and H. S. Jarosch (personal communication) have confirmed our observation on re-examination of their electron micrographs of lysed sickled cells.

A preliminary three-dimensional image reconstruction of a fiber has been prepared for structure analysis (T. Wellemans and M. Zwick, unpublished results). An axial projection of a disc from this reconstruction is presented in Fig. 7 in order to

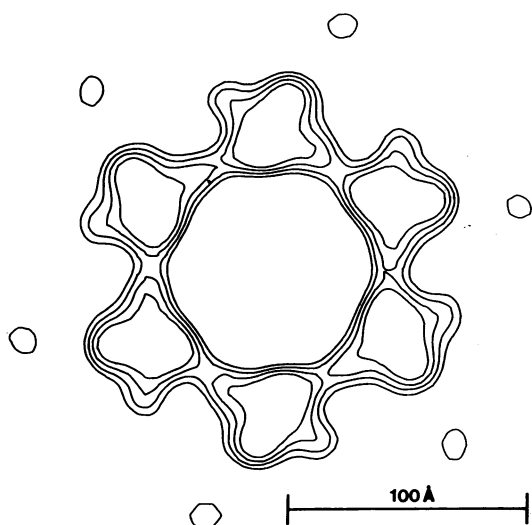


FIG. 6. A computer-generated contour map of the disc in Fig. 4 digitally filtered for 6-fold symmetry. The reconstruction was generated from harmonics of order 0, 6, 12, and 18, corresponding to a resolution of $d > 25$ Å.

compare its appearance with that of the rotationally filtered disc structure (Fig. 6).

DISCUSSION

The dimensions and structural parameters of the helical deoxyHbS fibers presented here are slightly different from those reported by Finch *et al.* (1) for a comparable type of fiber. They reported an outer fiber diameter of 170 Å for negatively stained fibers in sickled cell lysates and 230 Å in fixed gels. The axial rise per residue in the two types of preparation was 62 and 64 Å, respectively. Fibers of deoxyHbS analyzed in this study exhibit an outer diameter of 160–170 Å, an inner diameter of about 60 Å, and an axial disc-repeat distance of 58 Å. The analysis of helical parameters indicates that there are 56 discs per turn stacked normal to the helical axis rather than 48 as suggested by Finch *et al.* (1) and that there are consequently 9.3 rather than 8.0 deoxyHbS molecules per one-sixth of a turn along a helical strand of molecules. These differences in dimensions may result from differences in sample preparation and do not necessarily imply a fundamental difference between the structures. Indeed, it is likely that the fibers reported here are essentially the same as those reported by Finch *et al.* (1).

The ring-like structures of 6-fold rotational symmetry are of considerable interest. Whereas the inner and outer diameters of the rings are difficult to measure accurately because of the unevenness of the negative strain, the digitally filtered image of the rings gives rotationally averaged dimensions that are consistent with our assessments of the fiber dimensions. The general appearance of the axial projection of a disc from the three-dimensional reconstructed fiber in Fig. 7 is similar to that of the digitally filtered image of the ring in Fig. 6. On this basis it is reasonable to suggest that the rings represent stable substructures that are identical to the discs of the intact deoxyHbS fiber. The observation of the isolated discs coupled with the axial view from the reconstructed fiber demonstrates directly that the deoxyHbS fiber is a hollow, tubular structure.

The demonstration of structurally discrete intermediates in the polymerization of helical aggregates of deoxyHbS is an important objective. There is therefore a considerable temptation to assign a role in the sickling process to structures ob-

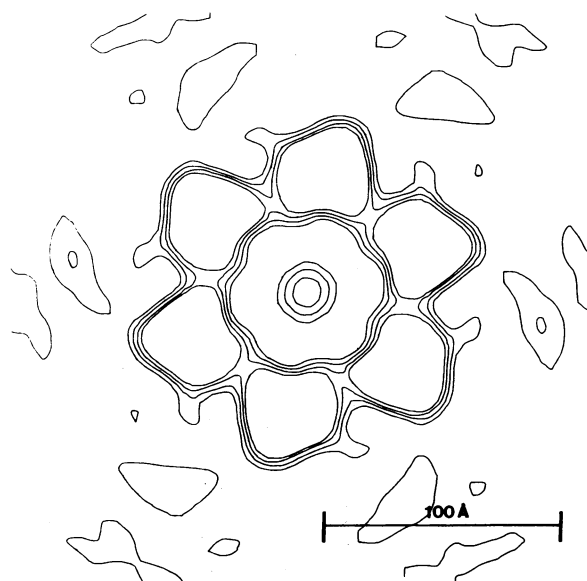


FIG. 7. A contour map of the axial projection of a section corresponding to a single disc from a low-resolution, three-dimensional reconstruction of a fiber.

served in conjunction with the fibers. For instance, Finch *et al.* (1) have stated that although they could not detect isolated discs of deoxyHbS molecules, they could observe aggregates of single-stranded filaments in cell-free lysates similar to those illustrated to Fig. 1*b*. They consequently have concluded that polymerization of molecules to form these linear aggregates may represent a primary step of kinetic importance in fiber formation. At the present time the role that filaments or discs play in the assembly or disassembly of the fibers is not clear. Considering the large size of the nucleating aggregate (8, 9, 18), either single filaments or discs could initiate fiber formation.

Although the observation of the isolated discs in the presence of fibers is not sufficient to assign them a precise role as a kinetic intermediate, the demonstration of the discs does indicate that stable circular arrays of deoxyHbS can be formed through unique intermolecular interactions. Since the discs are clearly 6-fold symmetric, rather than a trigonal arrangement of molecular pairs, we can conclude that the paired interactions involving the $\beta 6$ site in the crystal of deoxyHbS (19) and invoked to explain the molecular contact sites of the 4-fold symmetric eight-stranded helix (2) cannot provide the intermolecular contact sites for the helix of 6-fold symmetry described here. Therefore, in order to explain the detailed structural basis of fiber formation in terms of the stereochemical relationships of amino acid residues on the surface of the hemoglobin molecule, the structure of several types of helical fiber will have to be analyzed.

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