STRUCTURE OF THE FIBERS OF SICKLE CELL HEMOGLOBIN IN THE PRESENCE OF 2,3-DIPHOSPHOGLYCERATE

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SUMMARY: Bundles of fibers of hemoglobin S in the presence of 2,3-diphosphoglycerate or inositol hexaphosphate have been prepared from stirred solutions and examined by electron microscopy. The bundles are found to contain ordered arrays of fibers in an eight-stranded form, with strands staggered by 37 Å, as characterized by optical and computer transforms. The characteristic reflections observed for individual fibers are also seen for large areas of the bundles, indicating the presence of the same form of the fibers throughout the bundles. Since 2,3-diphosphoglycerate is the natural hemoglobin cofactor, the structure observed in these studies is likely to bear a close correspondence to the structure in vivo.

An important element in elucidating the details of the molecular basis of sickle cell disease concerns the precise arrangement of the individual molecules in the fibers of hemoglobin S (Hb S) which characterize the disease. Following the reports of fiber structures with aligned (1) and staggered (2) strands, an eight-stranded structure with staggered strands was identified as the predominant form in sickled cells lysed directly on electron microscope grids with solutions of negative stain (3). In these eight-stranded fibers adjacent strands are staggered by half a molecule (or about 30 Å). A similar pairing of strands occurs in crystals of Hb S prepared from polyethylene glycol (4). This similarity has given support to the view that the contacts between molecules of Hb S in the crystal may have a close correspondence to the contacts in the fibers (3-5), and would thus provide information potentially of value in designing anti-sickling agents. However, we had also obtained preliminary indications that in the presence of 2,3-diphosphogycerate (DPG) or inositol-hexaphosphate (IHP), the lattice structure of the fibers is significantly altered (6,7). Since DPG is the effector in erythrocytes, the form of the fibers in the presence of DPG or IHP, is likely to correspond to the structure in circulating sickled cells. Studies on the structure of the fibers in the

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presence of known amounts of DPG or IHP are most conveniently carried out with fibers prepared from hemolysates rather than with sickled cells. It is now possible to obtain large quantities of IHP-Hb S fibers from hemolysates that are stirred, which prevents gelation and leads to formation of large bundles of fibers (8). We have found that bundles of DPG-Hb S fibers can also be obtained with this technique and studies on the detailed structure of both the DPG-fibers and the IHP-fibers have now been carried out. The results indicate an eight-stranded form of the fibers with adjacent strands staggered by 37 Å. Since the arrangement of molecules in this structure differs significantly from the arrangement in the crystals (4), a re-examination of the question of the amino acid residues at the contacts between molecules in the fibers will be required.

MATERIALS AND METHODS

Hb S was obtained from washed erythrocytes by lysis with distilled water and purified by centrifugation in the presence of NaCl to remove membrane fragments. Hemolysates were concentrated by vacuum dialysis to 15-20% and adjusted to pH 7, 0.01 M phosphate and 5 mM IHP or DPG. Aliquots were deoxygenated in an ice bath by stirring slowly to prevent bubble formation in the presence of pre-purified nitrogen. The samples were then transferred to a 35°C water bath and stirring continued until a color change due to light scattering indicated the onset of the formation of bundles of filaments.

For electron microscopy, grids coated with either carbon or, for high resolution, formvar-backed holey films were used. A solution of 2% phosphotungtic acid (PTA) and 5 mM IHP or DPG were bubbled with nitrogen and used as a negative stain. In a nitrogen atmosphere one drop of PTA was applied to a grid and a pipet with the Hb solution was touched to the drop of PTA briefly. Subsequent washing with additional PTA was used to remove free Hb S molecules, although care had to be taken as this also reduced the number of fibers present on the grid. The excess stain was removed and the grid quickly dried with a stream of nitrogen. A Philips EM 301 was used throughout this investigation.

Optical diffraction patterns of the electron microscope plates were examined and photographed using an optical diffractometer from Lansing Research Corp., Ithaca, New York. Fibers with good optical transforms were digitized on a Syntex densitometer and Fourier transforms were calculated and displayed by a Nova 1200 minicomputer and Tektronix display screen.

RESULTS AND DISCUSSION

When samples of deoxy-Hb S in the presence of IHP were gently stirred, we observed the rapid formation of a stable suspension of large aggregates with a rise in turbidity, but no formation of a semi-solid gel, in agreement with the

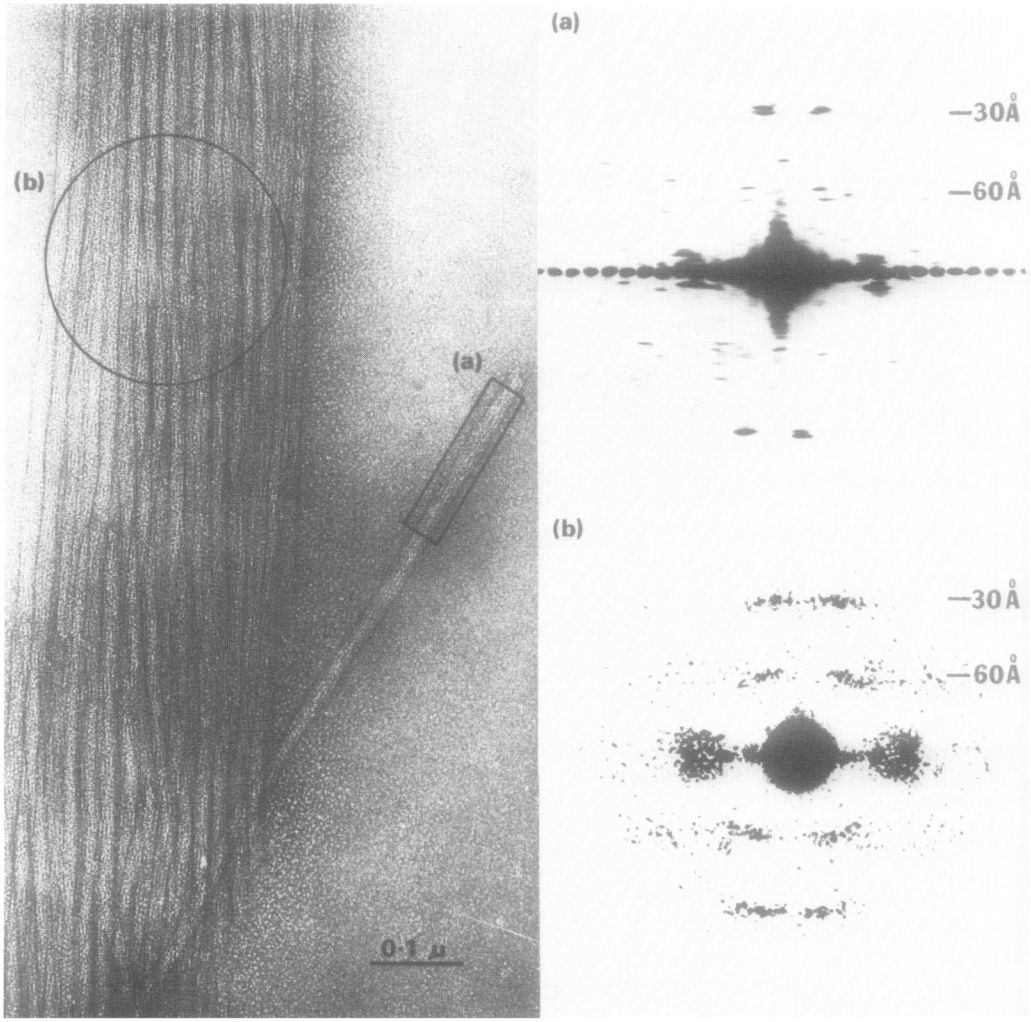

Figure 1. Electron micrograph of Hb S fiber bundle and optical transforms. A micrograph of a bundle of Hb S fibers and a single fiber is shown on the left, with the areas outlined used to produce the optical transforms shown on the right. The transforms are (upper) from a single fiber and (lower) from a region of the bundle. The latter was taken without slits on the optical diffractometer and therefore does not show the reflections along the equator and meridian resulting from the transform of the slits, as seen with the single fiber.

findings of Pumphrey and Steinhardt (8). Similar results were obtained when DPG was substituted for IHP. In order to examine these structures more closely, samples were prepared as above and applied to electron microscope grids. At low magnification, many bundles are observed, with an average length of 5-6 microns. Similar aggregates were reported by Pumphrey and Steinhardt, but

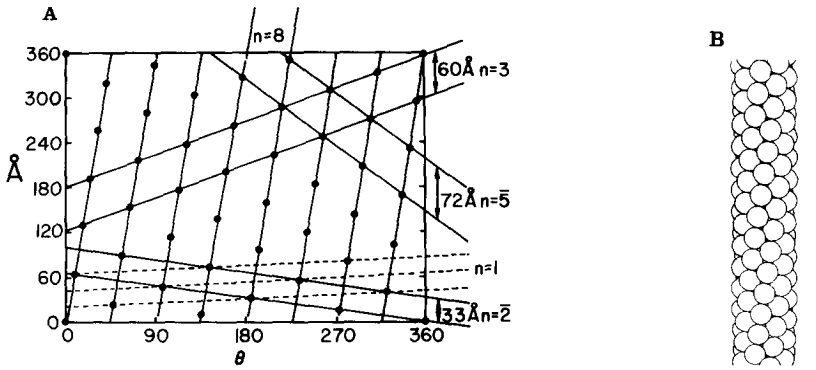

Figure 2. Structure of fibers of Hb S in bundles. A. Surface lattice for model of Hb S fibers showing planes giving rise to observed reflections of the optical transforms. The number n associated with each set of planes refers to its Bessel order. The basic one-start helix is illustrated with dashed lines. B. Model of fiber, with Hb S molecules represented by spheres, in arrangement corresponding to surface lattice presented in A. The fiber is presented in a right-handed orientation, in terms of the long strands of individual Hb S molecules, as indicated by preliminary results using the tilting stage of the electron microscope.

since their structure at higher resolution had not been reported, we have extended the electron microscopic studies to higher magnifications, as illustrated in Fig. 1. This figure shows a typical bundle, which can be seen at this magnification to be composed of many individual fibers about 200 Å in diameter. In addition, the micrograph also contains a single fiber that occurred in the same field. Optical transforms were recorded for the indicated regions on the single fiber and the larger area in the center of the bundle. The single fiber displays paired, near-meridional reflections at positions corresponding to spacings of close to 30 Å and 60 Å along the fiber axis and near-equatorial reflections that are relatively far from the meridian. A similar diffraction pattern is seen for the larger area of the bundle, with the reflections at the positions corresponding to a spacing of 60 Å somewhat more intense than for the single fiber. However, overall the reflections on the optical transform of the bundle are more diffuse and arc-like than the reflections on the transform of the single fiber, due to the fact that the individual fibers in the bundle are not in perfect parallel alignment.

Optical transforms with the paired reflections at 30 Å and 60 Å have been reported earlier for individual fibers from gels of Hb S prepared in the presence of organic phosphates (6,7). In those studies large bundles of fibers similar to the one shown in Fig. 1 were also encountered, but their yield was much lower than in the stirred solutions. The reflections on the optical transforms can be related to the surface lattice for the fibers shown in Fig. 2. The structure can be described by a one-start helix with 45 Hb S molecules in 17 turns. In addition there are families of lattice lines, as indicated in Fig. 2, corresponding to (a) the reflections at 33 Å which arise for a second-order Bessel function (n = 2); (b) the reflections at 60 Å which arise from a third-order Bessel function (n = 3); and (c) near-equatorial reflections at about 360 Å which arise from an eight-order Bessel function (n = 8), with this family of lattice lines composed of the individual strands of Hb S molecules. The assignment of Bessel orders was performed by relating the positions of the reflections to the spacings predicted by the proposed lattice and confirmed by computer Fourier transform of several selected fibers. The computer transforms provide the phases of the reflections as well as the amplitudes and as expected, the pairs of reflections assigned to even-order Bessel functions were found to have the same phase and the pairs of reflections assigned to odd-order Bessel functions were found to have opposite phase. A slight inequivalence in the positions of the individual Hb S molecules accounts for the splitting of the left and right 30 Å reflections into closely spaced upper and lower components which may be seen in Fig. 1 on the transform of the single fiber (a). A more detailed description of this inequivalence and of the computer transforms will be presented elsewhere (Dykes, G., Crepeau, R.H. and Edelstein, S.J., in preparation). The computer transforms also reveal more clearly than the optical transforms another pair of reflections near the 60 Å layer line corresponding to a fifth-order Bessel function (n = 5). The family of lattice lines that gives rise to this feature is also shown in Fig. 2, but the low amplitude for this reflection on the optical transform indicates that the stain penetration is poor in the grooves responsible for this feature. The surface lattice shown

in Fig. 2 is characterized by a repeat distance of 360 Å, although some variation in this parameter has been observed for different fiber preparations. In general, the fiber can be described as an eight-stranded structure with adjacent strands staggered by 37 Å.

The structure of the fibers in the bundles reported here and summarized in Fig. 2, differs from the eight-stranded structure with staggered strands obtained by lysis of sickled cells with solutions of negative strain (3) in two respects. First, the optical transforms of the fibers from lysed cells differed from the transforms reported here principally in terms of a meridional reflection found on the 30 Å layer line, rather than the paired reflections found in the presence of organic phosphates (Fig. 1). Second, the phase of the pairs of spots corresponding to spacings along the fiber axis of 60 Å and 72 Å are observed in computer transforms to correspond to odd-order Bessel functions. The two forms of the fibers could be interconverted by a 7 Å translation of the individual strands of the fiber with respect to one another. Since the sickled cells used in the earlier study (3) had been stored for varying lengths of time, it is probable that portions of the DPG originally present were depleted, since DPG has a half-life of only several days in stored cells (9). In fact, fibers with optical transforms corresponding to those seen in Fig. 1 have been found in freshly withdrawn sickled cells in our more recent studies. Thus the form of the fibers described in this report would appear to be the major structure in sickled cells. Details of the structure at higher resolution will be required to obtain information on questions concerning the orientation of individual Hb S molecules and the stereochemistry of the contacts between adjacent molecules. For work on such questions the stirred solution method for preparation of bundles of fibers is a convenient procedure for obtaining large amounts of fibers in a relatively stable form and should facilitate future studies.

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