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Glutamine synthetase forms three- and seven-stranded helical cables

(protein structure/optical diffraction/three-dimensional reconstruction)

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ABSTRACT When cobaltous ion is bound to glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2], the two-layered hexagonal molecules polymerize face-to-face, to form long strands. The strands then wind round each other to form three- and seven-stranded cables. The structures of these cables are not immediately evident from electron micrographs because of the confusing superposition of front and back portions of the cables. But optical diffraction and filtering by the procedure of Klug and DeRosier leads to interpretable images of the cables.

Because a micrograph of the seven-stranded cable contains 24 views of the glutamine synthetase molecule, it is possible to reconstruct the three-dimensional electron density of a cable and its constituent molecules at a resolution of 30–50 Å. This reconstruction confirms that the symmetry of a glutamine synthetase molecule is D_6. It suggests that the single subunit is an oblate ellipsoid with its minor axis (about 48 Å) roughly parallel to the 6-fold axis of the molecule and its major axis (about 63 Å) perpendicular to the 6-fold axis of the molecule. The subunits of the two hexagonal layers of a molecule are eclipsed. Neighboring molecules along a strand also have their hexagonal faces together, but they are rotated about the strand axis by about 7° with respect to one another, rather than being eclipsed. Six outer strands are coiled about a straight central strand, and each forms identical contacts with the central strand. Moreover, these contacts between central and outer strands are apparently similar to the contacts between neighboring outer strands.

Nearly 25 years ago in these PROCEEDINGS, Pauling and Corey proposed structures for the α-helix and β-sheets. They later suggested (1) that some protein fibers might consist of cable-like structures, built up from three or seven α-helical strands wound round each other (Fig. 1). About the same time, Crick (2) explained the formation of cables from strands in terms of intermolecular forces. He noted that helical strands tend to coil into cables, because a favorable interstrand contact can be repeated along the length of the strands only if the strands coil about each other.

In studying the effect of cobaltous ion on glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming); EC 6.3.1.2], we have observed three- and seven-stranded cables reminiscent of those suggested by Pauling and Corey. Though our cables contain the same number of strands as theirs, they differ greatly from theirs in scale: in our cables the repeating unit is a glutamine synthetase molecule of molecular weight 600,000, whereas in theirs it is several amino-acid residues. Nevertheless Crick's idea about cable formation applies to the glutamine synthetase cables as well as to coiled α-helices. Both types of cables form because there is a contact between strands of substantial binding energy. This contact can be repeated along the length of the cable only by introducing a twist into the single strands so that they can wrap around each other. Our interest in the glutamine synthetase cables is that they allow us to examine gross features of such protein–protein interactions in several polymorphs, and that they also permit us to determine the three-dimensional structure of the glutamine synthetase molecule to very low resolution.

Glutamine synthetase is a regulatory enzyme of great complexity, both in structure and function (3). A single molecule contains 12 virtually identical polypeptide chains of molecular weight 50,000, arranged like the carbon atoms in two benzene rings face-to-face. When the enzyme is dialyzed against EDTA and then against a divalent cation, the molecules polymerize face-to-face to form a strand (4). Our basic discovery is that if Co^{2+} is added to glutamine synthetase, either in the native state or after treatment with EDTA, face-to-face strands form and then wind round each other to form three- and seven-stranded cables.

MATERIALS AND METHODS

(These will be described more fully in a forthcoming paper.)

Bacteria Growth and Enzyme Purification. *Escherichia coli* strain W was grown at 37°C in the glycerol–glutamate medium described by Shapiro and Stadtman (5). The cells were harvested after remaining 2–3 hr in stationary phase. Glutamine synthetase isolated from bacteria grown under these conditions is expected to have nearly its full complement of covalently bound AMP. Two purification procedures were used: the Woolfolk–Stadtman procedure (5) and the Zn^{2+} precipitation procedure (6). Final specific activities

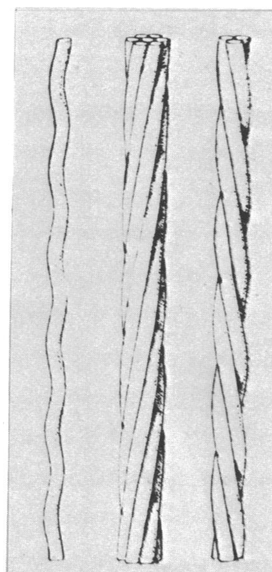

FIG. 1. A proposal by Pauling and Corey (1) of how α-helices might interact to form cables.

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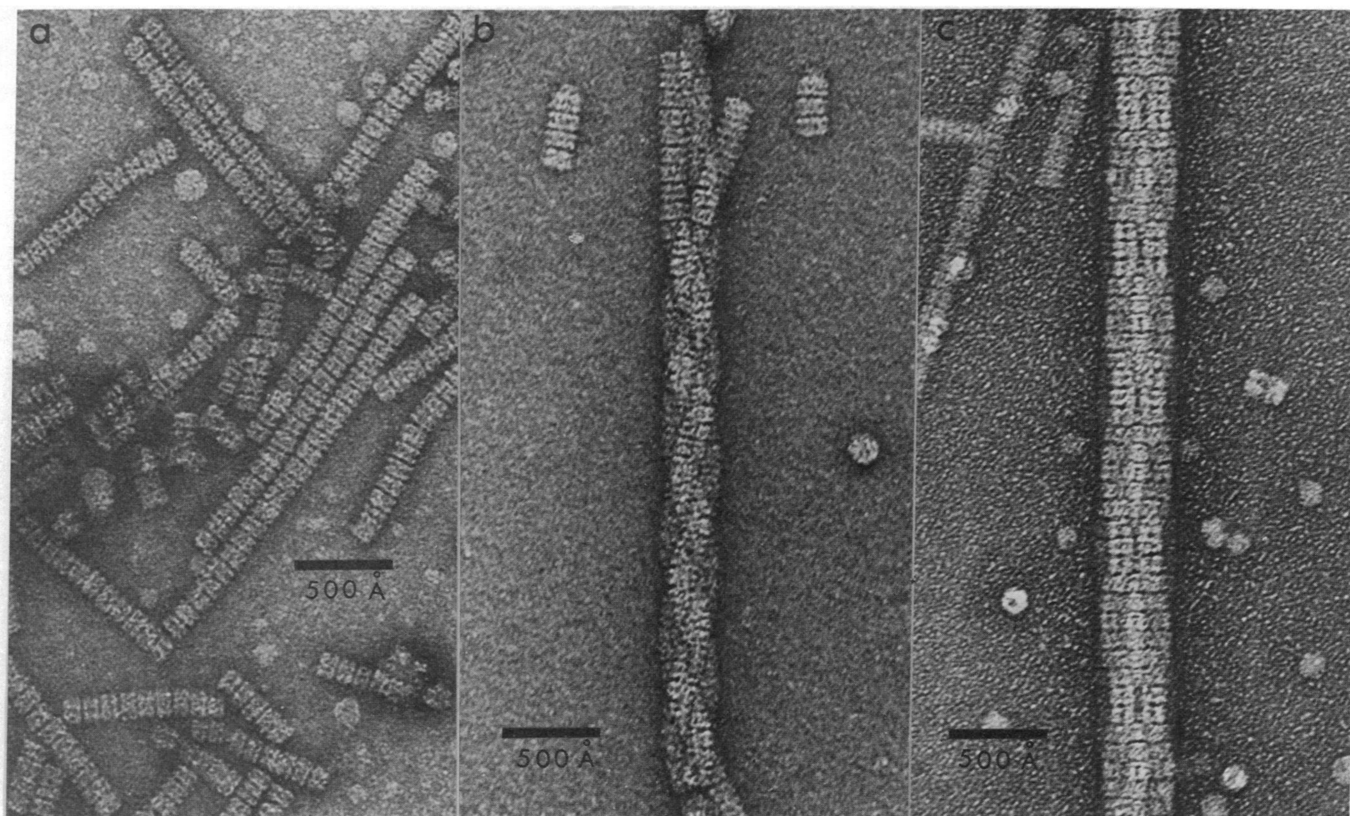

Fig. 2. The three helical aggregates of glutamine synthetase: (a) single strands formed by face-to-face aggregation of individual molecules; (b) three-stranded cables; (c) six strands around a central strand forming a 6+1-stranded cable.

were found to be 84 units/mg for enzyme isolated by the Woolfolk-Stadtman technique and 70 units/mg for enzyme isolated by Zn^{2+} precipitation. States of adenylylation estimated from the $A_{260}:A_{290}$ ratio according to Shapiro and Stadtman (5) were found to be 9–10 for both methods of isolation.

Preparation of Samples for Microscopy. Glutamine synthetase was diluted to 100 μg/ml with 100 mM EDTA–10 mM imidazole (pH 7.0) then dialyzed at 4°C against the same buffer for 2 hr. These samples were then transferred to 10 mM Co^{2+}–10 mM imidazole (pH 7.0) and allowed to dialyze for 20 hr. Other samples were diluted with 10 mM imidazole (pH 7.0) but were not treated with EDTA prior to dialysis against Co^{2+}.

Electron Microscopy and Optical Image Analysis. A drop of sample was applied to a 400 mesh grid, previously prepared by coating with parlodion and carbon film, and the excess protein was washed off after 2 min with distilled water. The samples were then negatively stained with 1% uranyl acetate. Excess stain was removed with filter paper, and the grids were allowed to dry. Optical filtering and reconstruction were carried out on a folded optical diffractometer constructed from a design obtained from Dr. Kevin Leonard of Oxford University (see ref. 7).

Three-Dimensional Reconstruction. Four micrographs from a total of about 60 were selected by optical diffraction for computational analysis. Optical densities on the micrographs were recorded using an Optronics P1000 film scanner controlled by a PDP-8 computer. The scan rasters and apertures were set at 50 μm which corresponds to 9.2 Å at 5.5×10^5 magnification. The procedure of reconstruction was essentially that described by DeRosier and Klug (8) and DeRosier and Moore (9). All but the final steps were carried out by Fortran IV programs written at UCLA. For the inverse Fourier-Bessel transformation (Fourier synthesis of electron density in three dimensions) the actual program described by DeRosier and Moore was used. Two-dimensional sections of electron density were calculated and traced onto Mylar sheets which were stacked to form a three-dimensional map.

RESULTS

Formation of cables

Valentine *et al.* (4) discovered that glutamine synthetase when dialyzed against EDTA and then against $MnCl_2$ or $CoCl_2$ forms cylindrical strands with the faces of the double hexagonal molecules against one another. At 20°C the strands rapidly form lateral associations, and within 30 min grow into microcrystalline bundles, in some cases resembling wheat sheaves. In repeating these experiments at 4°C we find that the aggregation proceeds much more slowly, and with Co^{2+} as the added ion, the lateral associations of strands leads to ordered cables. Seven hours after exposure to Co^{2+} at 4°C, glutamine synthetase is mainly in the form of single, cylindrical strands (Fig. 2a). After 20 hr at 4°C, the strands have associated into the thick cables of the sort shown in Fig. 2c, and occasional thinner cables such as those of Fig. 2b. Further investigation showed that cables form upon exposure to Co^{2+}, whether or not the enzyme has first been treated with EDTA.

Of the three forms of aggregate shown in Fig. 2, we know least about the single strands. We have examined all three aggregates by optical diffraction (8), in which the electron micrograph is used as a grating for diffraction of visible light. The diffraction patterns for the cables shown in Figs.

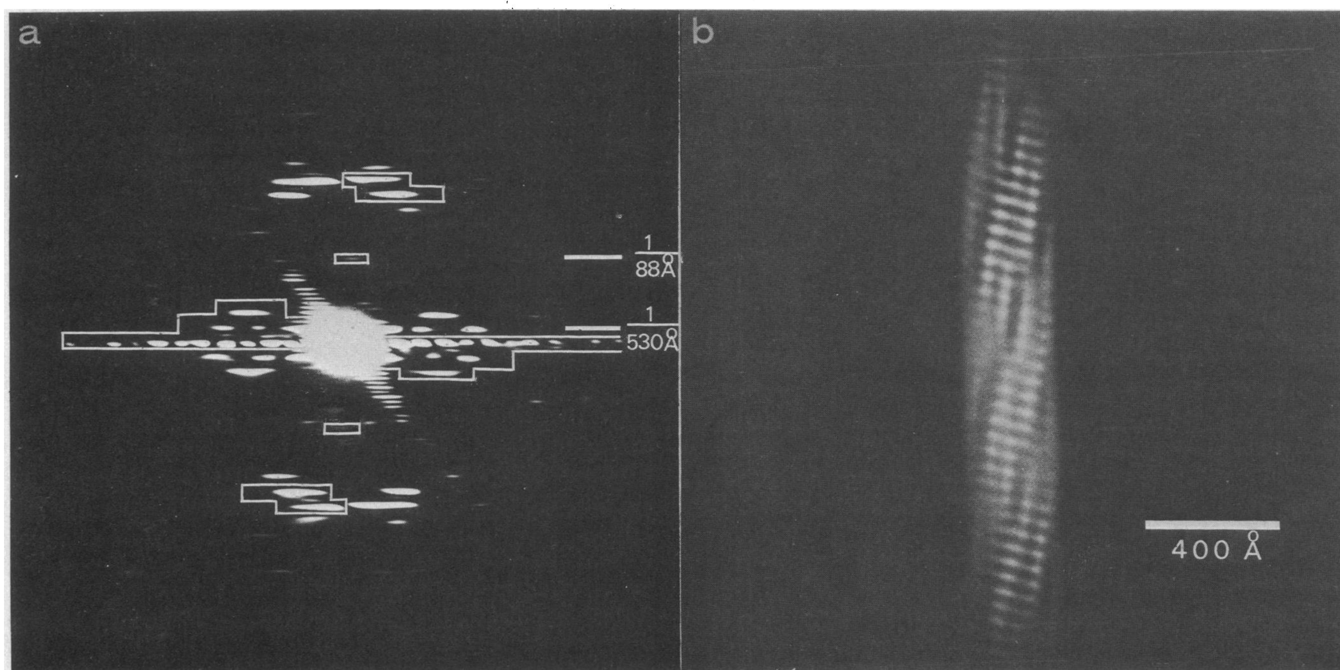

FIG. 3. (a) Optical diffraction pattern of a three-stranded cable. (b) One-sided filtered image produced from the reflections in (a) enclosed in rectangles.

2b and c contain numerous reflections, and lead to information on the arrangement of molecules in these helical strands (see below). In contrast, the diffraction patterns of single strands display diffraction only on the meridian of the pattern with strong reflections at 1/(100 Å) and 1/(50 Å). These show only that the separation of molecules along the strand is 100 Å ± 10 Å, and that each molecule consists of two layers, each about 50 Å high. This, of course, is evident from the micrographs themselves. Analysis of the cables suggests that there is a definite helical twist to the single strands within them. The lack of sufficiently strong off-meridional diffraction from the isolated single strands prevents us from determining the relative angular displacement of each molecule with respect to its neighbors. Presumably there is a regular arrangement of the single strand, but it is slightly disrupted during application of the heavy atom stain or during drying.

The three-stranded cable

Inspection of the narrow cable of Fig. 2b reveals a good deal about the arrangement of molecules within it. Three strands are visible at the frayed end of this cable. This is a common observation with the narrow cables. The number of strands that form the cable cannot be 2, 4, or any even number, because an even number of strands would give rise to a mirror line running down the center of the cable. Just such a line is evident in the thicker cable of Fig. 2c, but is not present in the thinner cable, which has instead the glide line characteristic of an odd number of strands (10). The only odd number of strands consistent with the width of the cable (300 Å) is three. Viewing the cable at a glancing angle perpendicular to the cable axis, we find that the structure repeats after every six annuli, each made up of three molecules. This repeat distance is 560 ± 30 Å.

For further information about the helical symmetry of the thin cable we studied the optical diffraction patterns of several of these structures. Fig. 3a shows a typical example. As with all helical structures, the reflections fall on layer lines. The first layer line lies 1/(530 ± 30 Å) from the equator, indicating that the repeating distance of the structure is about that measured directly from the micrograph. A weak reflection at the intersection of the sixth layer line with the meridian tends to confirm that the structure repeats every six annular units.

Klug and DeRosier (11) demonstrated that it is possible to produce a filtered image of a helical structure, showing only the side nearer the carbon substrate of the grid, or the side away. This is possible because the diffraction patterns from the two sides are distinct, and one of the patterns can be removed by an opaque filtering mask, leaving only the other to be retransformed by a lens into a "filtered image." Our assignment of the diffraction spots for the three-stranded cable is summarized in Fig. 3a, where reflections assigned to one side are enclosed in rectangles. Note that reflections on the meridian contain contributions from both sides. Since proper attenuation of these spots is difficult or impossible, they contribute to the filtered image with their full weights, rather than the correct partial weight. Fig. 3b shows the filtered image produced by transforming the indicated reflections from Fig. 3a. We have not yet established if this filtered image represents the side of the cable nearer to the carbon substrate or farther from it, so that we are still uncertain as to the sense of the twist of the strands in the helix. Nevertheless, the filtered image shows the path of the strands and the orientation of individual glutamine synthetase molecules within them.

The 6+1-stranded cable

The thick cable of Fig. 2c displays a mirror line running the length of the structure. This suggests that the cable is formed from an even number of strands. Frayed ends are less helpful with this structure than with the three-stranded one, but in several micrographs we have counted five to seven strands. The diameter of 400 ± 30 Å is consistent with a six-stranded structure. A six-stranded structure formed from circular glutamine synthetase molecules 140 Å in diameter

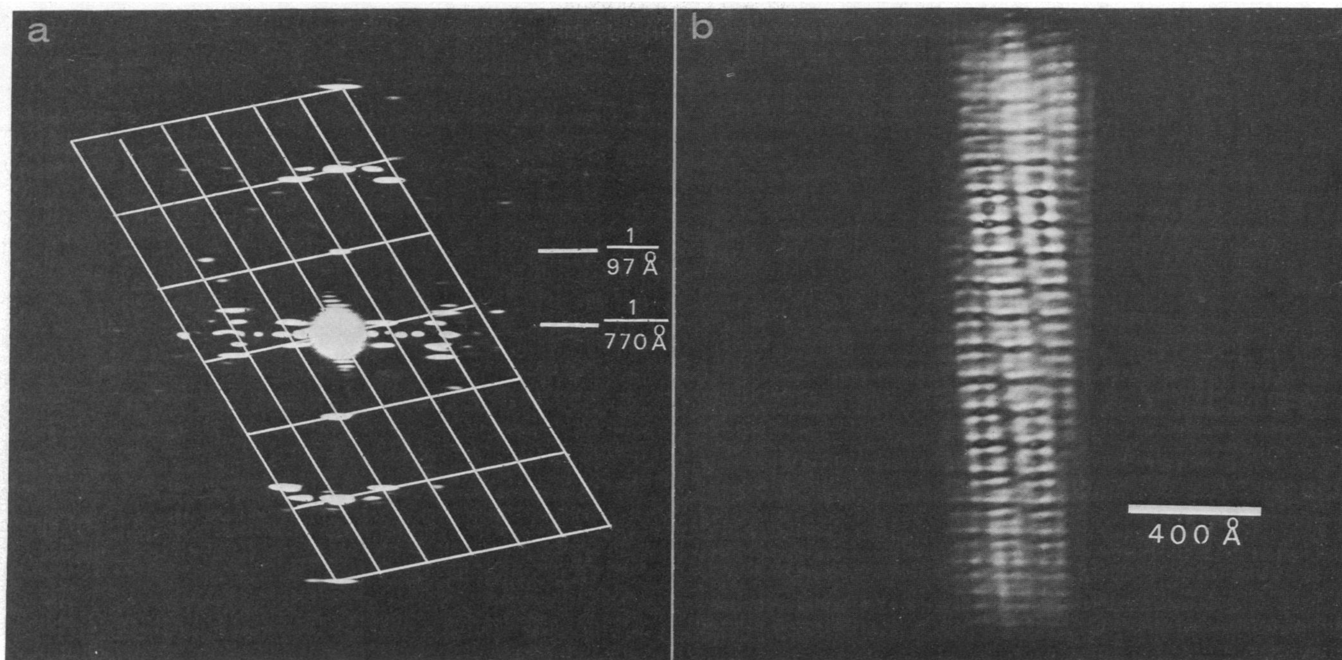

FIG. 4. (a) Optical diffraction pattern of a 6+1-stranded cable. The lattice identifies spots arising from one side of the cable. The lattice for the other side is the mirror image of the one shown. (b) One-sided filtered image produced from only those reflections in (a) that lie near lattice points.

(4) would have a diameter of 400–435 Å. But this structure would also contain a cylindrical channel about 140 Å in diameter that might be expected to fill up with the uranyl acetate stain. Since none of the dozens of thick cables we have examined display a dark strip running along the center of the structure, we must conclude either that stain cannot penetrate into the channel or that the channel is occupied by a seventh strand that excludes stain. Given the uniform penetration of stain into the outer six strands, the presence of an additional inner strand is a more likely explanation. In fact this seventh strand is visible in the three-dimensional reconstruction. We thus refer to this cable as the 6+1-stranded helix.

The helical parameters of the 6+1-stranded helix were determined by inspection of both micrographs and their diffraction patterns. Viewing the helix at a glancing angle perpendicular to its axis shows that it is composed of annuli of glutamine synthetase molecules. The structure repeats after every eight of these annuli (780 ± 30 Å). These tentative conclusions are confirmed by the diffraction pattern (Fig. 4a). The first layer-line lies (750 ± 30) $Å^{-1}$ from the equator. The conclusion that the structure repeats after every eight annular units is confirmed by reflections on the meridian at the intersection of the 8th, 16th, 24th, and 32nd layer-lines. The strong reflection at the 16th layer-line arises from the two-layered molecules in the annuli. The presence of a reflection on the 32nd layer-line shows that at least some features in the structure are ordered to a resolution of 23 Å.

The assignment of reflections in the diffraction pattern to one or the other of two lattices again allows filtering of the image free of one or the other side of the helix; Fig. 4b is such an image.

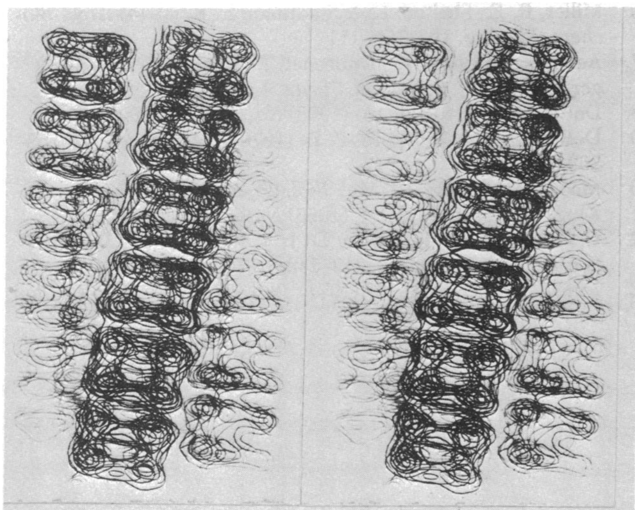

FIG. 5. A stereo pair photograph of a 6+1 cable reconstructed in three dimensions.

Three-dimensional reconstruction of the 6+1-stranded helix

Because a micrograph of the 6+1 cable contains 24 views of the glutamine synthetase molecule (6 outer strands × 8 molecules per repeat divided by 2 for the mirror line of symmetry), it is possible to reconstruct the three-dimensional structure of the cable to low resolution. The expected resolution d for a structure of diameter D containing n views of the molecule is $d = \pi D/n$ (12). Because the width of the structure is about D = 400 Å, the reconstruction will have lateral resolution of about 50 Å. The resolution parallel to the helix axis is expected to be finer, approaching 30 Å, owing to the higher diffraction orders that are available along this direction.

A stereo-pair photograph of a portion of the reconstructed cable is shown in Fig. 5, where the electron density has been sectioned parallel to the helical axis. An important feature of the structure, only marginally visible in this photograph, is the central strand of glutamine synthetase molecules. Though this strand is less well defined than the outer strands, it appears to have the same helical parameters as the

group of outer six strands. That is, it appears to repeat every eight molecules, each molecule being rotated about the helix axis by $360°/(6 \times 8) = 7.5°$ with respect to the molecule below.

Because the central strand occupies the near axial position portion of the reconstruction, where the errors in the reconstruction are greatest, we have exercised skepticism in accepting this strand as real rather than artifact. Three lines of evidence suggest that it is in fact real: (*1*) the reconstruction shows pockets of stain around the position of this central strand, which must be held in place by protein in the central cavity; (*2*) the phase correlation of layer-lines (13), a measure of error in reconstructions, is very good out to a resolution of 1/(50 Å), suggesting that detail as close as 50 Å to the axis should be valid; and (*3*) the central strand, while less clearly defined than the outer strands, shows molecules with partially resolved subunits of approximately correct dimensions.

DISCUSSION

The Glutamine Synthetase Molecule. Valentine *et al.* (4) concluded from electron micrographs of individual molecules that the twelve subunits of glutamine synthetase are arranged in two eclipsed hexagonal layers, that the aggregate has dihedral symmetry, that the maximum profile of the hexagonal faces is 140 Å and that the subunit extent along the hexagonal axis is about 45 Å. The three-dimensional reconstruction supports all these conclusions, and yields some additional information about the molecule. Fig. 5 shows that the molecules are "dished-in" along the direction of the 6-fold molecular axes. In other words, adjacent molecules along a strand have a discus-shaped void between their hexagonal faces. It is also apparent that the molecule contains 2-fold axes perpendicular to their 6-fold axes. This is another way of saying that they have D_6 symmetry, rather than the C_6 symmetry that would lead naturally to face-to-face polymerization. Thus polymerization must be the result of activation of some new bond when Co^{2+} is added.

Though the reconstruction is characterized by no more than 30 Å resolution in the axial direction and 50 Å resolution in the lateral direction, it is possible to say a little about the subunit shape. Both the filtered images and the three-dimensional reconstruction show that the subunits are elliptical when viewed perpendicular to the helix axis. But individual molecules viewed down their 6-fold axes show subunits with circular profiles. Together these observations imply that the subunit at low resolution is an oblate ellipsoid of revolution. Measurement on contoured output indicates that the minor axis of a subunit (parallel to the 6-fold axes) is about 48 Å in length, and the major axis (perpendicular to the 6-fold axes) is about 63 Å in length, yielding an axial ratio for the subunit of 1.3.

Structure of the 6+1 Cable. Examination of the three-dimensional reconstruction suggests that interactions between glutamine synthetase molecules in the 6+1 cable are of only two types. The first type is the interaction of molecules at their hexagonal faces that produces the strands. The second type is the lateral interaction that binds strands into cables.

Within the 6+1 cable, the face-to-face interactions are very similar for the central and the outer strands. In the outer strands, glutamine synthetase molecules are rotated about the cable axis by 7.5° with respect to neighboring molecules. This 7.5° rotation is visible in the three-dimensional reconstruction (Fig. 5, especially in the strand on the left). Molecules in the central strand also appear to be rotated by 7.5°, but this must be confirmed by refinement. If it is true that center and outer molecules are rotated by the same amount, the contacts between them must be the same in each seven-molecule annulus of the structure. Then, since six outer strands surround a central strand with 6-fold symmetry, every center-outer contact in the cable would be of the same type.

Examination of the three-dimensional reconstruction suggests that interaction between two outer strands is closely similar to the interaction between an inner and an outer strand. In both classes of contact, the 6-fold axes of interacting molecules are inclined at about 11° with respect to each other. Further details of these interactions and comparisons with the three-stranded cable will be given in a forthcoming paper.

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