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Cross-β Protein Structures. I. Insulin Fibrils†

Michael J. Burke‡ and Malcolm A. Rougvie*

ABSTRACT: Structural studies have been carried out upon the fibrils which are produced when insulin is heated in acid. Electron microscopy and low-angle X-ray diffraction data suggest that the fibrils have a uniform cross-section with dimensions of about 29 by 47 Å. Infrared dichroism and wide-angle X-ray diffraction clearly demonstrate the cross-β structure of the fibrils. Ultraviolet circular dichroism and optical rotatory dispersion spectra are consistent with a β conformation. A detailed structure is suggested in which individual insulin molecules are stacked in layers 4.7 Å thick in the direction of the fibril axis, with one molecule per layer. Interaction of the extended chains in adjacent layers is believed to be of the parallel pleated-sheet type.

Under appropriate solvent conditions, the globular protein insulin polymerizes to form submicroscopic fibrils. The chemistry of the polymerization process has been studied extensively by Waugh (*e.g.*, 1957) who found that it could be reversed yielding biologically active, crystallizable insulin. Because of the reversibility of the process he suggested as early as 1944 (Waugh, 1944) that the fibrils are arrays of only slightly distorted globular insulin molecules. Koltun *et al.* (1954) supported this proposal on the basis of X-ray diffraction analysis. Their analysis has been accepted by Reithel (1963) in a review of protein association, and more recently by other authors (Beaven *et al.*, 1969). However, primarily on the basis of infrared dichroism measurements, Ambrose and Elliott (1951) concluded that the fibrils are composed of polypeptide chains which are in the β conformation and which extend transverse to the fibril axis (*i.e.*, cross-β structure).

Other evidence related to the question is discussed briefly in the review by Klostermeyer and Humbel (1966). In this paper we provide evidence which affirms the essential correct-

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‡ Present address: Department of Chemistry, University of Minnesota, Minneapolis, Minn. 55455.

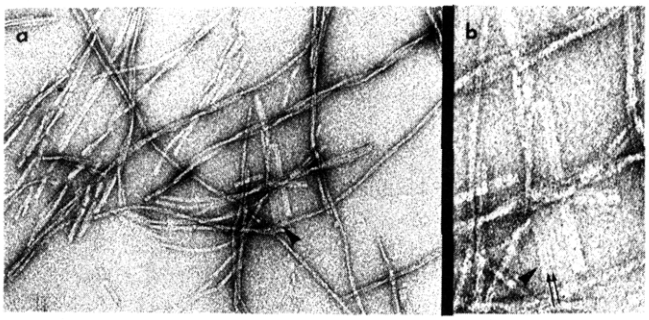

FIGURE 1: Electron micrograph of insulin fibrils negatively stained with uranyl formate. (a) × 150,000 (b) × 359,000. The arrows indicate fibrils showing lateral structure of small size.

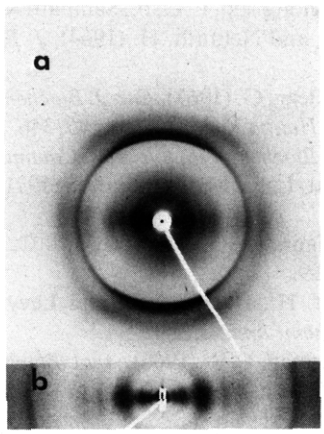

FIGURE 2: X-Ray diffraction patterns of a rod specimen of insulin fibrils, rod axis vertical. (a) Wide-angle pattern, with prominent 4.76-Å meridional diffraction. (b) Low-angle equatorial diffractions are shown.

ness of the Ambrose and Elliott proposal and extends it to somewhat greater detail.

Materials and Methods

Insulin Fibrils. Samples of crystalline insulin were obtained from Calbiochem. A modification of the method of Waugh (1944) as used for the conversion of native to fibrillar insulin. Typically, a 1% solution of insulin in water was adjusted to pH 2.0 with hydrochloric acid and about 10 ml was placed in a sealed-glass test tube. The tube was heated in a water bath to between 80 and 100° until a clear gel was formed. The time required for gelling was between 2 and 10 min depending on the exact temperature and pH. The sample was then cooled and frozen rapidly by imersion in a Dry Ice–acetone bath, thawed under running tap water, and reheated (80–100°) for approximately 2 min. The process of freezing and reheating was repeated three or four times until a firm gel formed. Total conversion of native to fibrillar insulin was possible by this procedure and sedimentation velocity experiments with the fibrillar solution showed no slowly sedimenting components.

Electron Microscopy. Copper grids were coated with Formvar and carbon films. A dilute solution of the fibrils was deposited on the grids and then removed. The above procedure was then repeated substituting for the fibril solution 1% uranyl formate in distilled water. Specimens were examined with an Hitachi HU-11 E electron microscope.

X-Ray Diffraction. Fibrils were studied as rod and disk specimens. Rod specimens were prepared by suspending a drop of an aqueous fibril solution between the ends of two 1-mm diameter glass rods separated by 2 mm. After drying, rod specimens 2 mm long and about 0.1 mm in diameter were obtained, in which the fibrils were oriented parallel to the main axis of the rod. These specimens were generally mounted perpendicular to the X-ray beam. Disk specimens were prepared in cylindrical capillary tubes of about 1 mm diameter, by filling a short segment of the capillary with an aqueous fibril solution and allowing several days for drying. After the drying period, disks about 0.1 mm thick and 1 mm in diameter were obtained. The fibrils were oriented in the plane of the disks which were mounted for examination with the X-ray beam in their plane. Nickel-filtered Cu Kα radiation was used (λ = 1.54 Å).

During the course of the investigation, it was found that addition of small amounts of glycerol (2–10% by weight) to the gel samples improved specimen formation. Less breakage of rods occurred during drying and although wide-angle diffraction patterns were unaffected, improved fibril packing was evidenced by increased sharpness of low-angle equatorial diffractions. The data reported below were taken with such specimens. In all cases, excess glycerol was removed from the formed specimens by exposure to vacuum at room temperature for at least 24 hr.

Optical Measurements. Infrared and infrared dichroism spectra were obtained on a Beckman IR4 recording spectrophotometer which was modified for dichroism measurements with a beam condenser and polarizer. Fibrils were oriented by stroking fibril solutions on calcium fluoride windows with a fine brush.

Optical rotatory dispersion (ORD) and circular dichroism (CD) spectra were measured with a Cary 60 spectropolarimeter modified for the measurement of CD. The ORD and CD spectra were made with a 1-mm path-length quartz cuvet. The solvent was dilute hydrochloric acid with a pH between 2 and 4. The concentration was generally determined by dry weight measurement.

Results

In electron micrographs such as that of Figure 1, the insulin fibrils are shown to be very long and have an irregular appearance which on close inspection seems to be due to varying degrees of lateral aggregation and to twisting of a basic unit of uniform cross-section. The dimensions of the basic unit cannot be evaluated with great precision, but at the points indicated lateral spacings of about 40 Å are observed. In this picture, as in others taken at higher mangification, there is no consistent evidence for any large axial periodicity.

The X-ray diffraction results extend the same concept of fibrillar structure. This is shown by the patterns of rod specimens (Figure 2 and Table I). A strong, sharp meridional arc at 4.76 Å is the most prominent diffraction seen, suggesting cross-β structure. Diffractions are confined to layer lines of this fundamental spacing seven on greatly overexposed patterns, a point which will be of some consequence in the discussion which follows. Other prominent characteristics include the low-angle equatorial diffractions at 47 and 29 Å (Figure 2 insert) which are taken to represent the lateral packing parameters of the fibril.

The X-ray data obtained in this study are compared to those presented by Koltun *et al.* (1954) in Table I. Aside

TABLE I: X-Ray Diffraction Data for Insulin Fibrils.

Meridional and Near Meridional Reflections (Å)		Equatorial Reflections (Å)	
This Study	Koltun[a]	This Study	Koltun[a]
	16.1	47	55
	12.0	29	30.8
	8.2	15.5	14.0
4.76[b]	4.79		11.8
	4.12	9.6	9.5
3.92	3.80	7.8	8.0
3.48	3.54		6.9
2.39[b]	2.40	5.3	5.5
2.230	2.25		
		4.09	
		3.50	3.08
		2.84	2.77

[a] Koltun *et al.* (1954). [b] Meridional reflections.

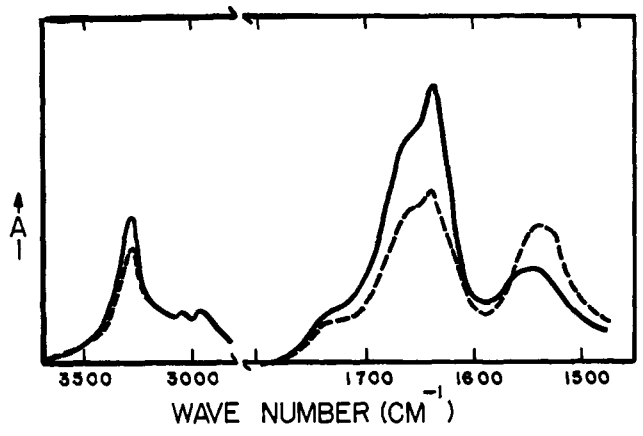

FIGURE 3: Infrared dichroism of insulin fibrils. Solid line, electric vector parallel to fibril axis; broken line, electric vector perpendicular to fibril axis.

from some minor differences, the equatorial results are in reasonably good agreement at low and intermediate angles. The spacings which we report as 15.5 and 7.8 Å are very broad and have apparently been interpreted by Koltun *et al.* as pairs of spacings of 14 and 11.8 Å, and 6.9 and 8 Å, respectively. Disagreement at higher angles involves weak, diffuse reflections which were apparently severly obscured by liquid glycerol diffraction in the Koltun specimens. On the meridian the discrepancies are of greater significance. In addition to the 4.76- and 2.39-Å layer lines, Koltun *et al.* reported diffractions at lower angles which led them to conclude that a 48.0-Å axial repeat was present. We find no evidence for such diffractions and conclude that they are artifacts. With disk specimens, which resemble in fibril orientation the film pack specimens of Koltun *et al.*, spacings related to transverse fibril order can and do contribute intensity to the meridian. This may partially explain their results. The disk specimens did not provide any evidence of double orientation of the fibrils which would be useful in indexing the equatorial diffractions.

No detailed analysis of the equatorial intensity distribution has been made, but we find that the low- and intermediate-angle diffractions may be accounted for on the basis of an orthogonal lattice of 29 × 47 Å. The limited number of diffractions are fit somewhat less well by a hexagonal arrangement of fibrils with centers separated by about 50 Å.

Infrared dichroism spectra of solid samples of oriented insulin fibrils clearly indicate, by the absorption at 1632 cm^{-1} for the amide I band, the presence of β structure (Figure 3). The presence of a shoulder at 1690 cm^{-1} having opposite dichroism would indicate antiparallel β structure, however, no such component at 1690 cm^{-1} could be detected possibly due to an overlapping absorption band at 1740 cm^{-1} (Miyazawa and Blout 1961). This 1740-cm^{-1} band, which is also present in the infrared spectrum of native insulin films, may originate from the many primary amides present in insulin. The shoulder at 1660 cm^{-1} could originate from α-structure nonhydrogen-bound insulin or from native insulin incorporated in the fibrils. Although these data cannot be used to prove the absence of a weak 1690-cm^{-1} absorption band, it can be used to show that a 1690-cm^{-1} band of moderate strength is absent. If the 1690-cm^{-1} component of the amide I band of the fibrils were nearly as strong as it is in the antiparallel β structure of silk (*e.g.*, Iizuka and Yang, 1966) this infrared spectrum would have uncovered it without difficulty. The dichroism of the amide I and II absorption bands indicate that the hydrogen bonds are parallel to the long axis of the fibrils. This implies that the peptide chains are normal to the long axis of the fibrils and, therefore, in a cross-β structure. Infrared spectra of solid noncrystalline samples of native insulin have an absorption maximum at 1660 cm^{-1} and do not show the presence of β structure.

In Table II the CD and ORD of fibrillar and native insulin are reported. The data for fibrillar insulin are similar to that found with other β structures (Townend *et al.*, 1966; Sarker and Doty, 1966; Timashelf *et al.*, 1967; Iizuka and Yang, 1966; Davidson *et al.*, 1966). The differences in the CD spectra of fibrillar and native insulin suggest that major conformational changes have taken place in the conversion of native to fibrous insulin (Figure 4).

Discussion

As the evidence presented above makes clear, fibrous insulin has a cross-β structure as first proposed by Ambrose and Elliott (1951) and is not the linear aggregate of slightly modified native insulin suggested by Koltun *et al.* (1954). The substantial change in conformation which must accom-

TABLE II: CD and ORD of Insulin Fibrils and Native Insulin.

Material	CD Bands		ORD Bands	
	λ (nm)	$\Delta\epsilon$[a]	λ (nm)	$[m']$[b]
Insulin fibrils	217.5	−4.4	232	−5,900
	193.5	+6.0 ± 3.0	208	+14,000
Native Insulin	222	−2.4	232	−3,800
	208	−3.0		
	195	+6.0 ± 3.0		

[a] $\Delta\epsilon$ is the mean residue circular dichroism and is not corrected for solvent refractive index. [b] $[m']$ is the mean residue rotation corrected for solvent refractive index.

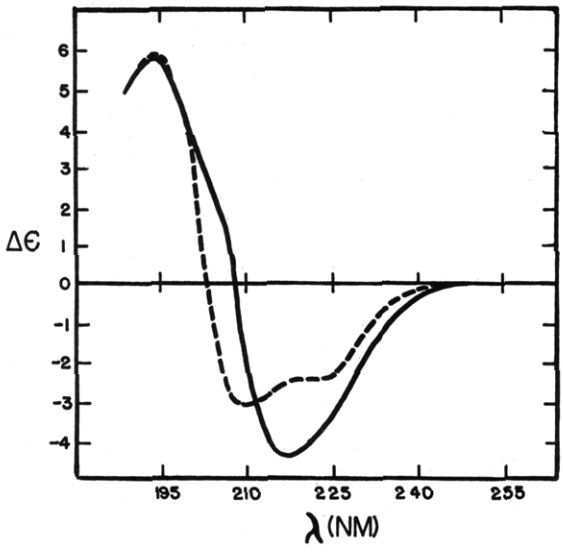

FIGURE 4: Circular dichroism of insulin. Solid line insulin fibrils; broken line, native insulin. $\Delta\epsilon$ is the mean residue circular dichroism and is not corrected for solvent refractive index.

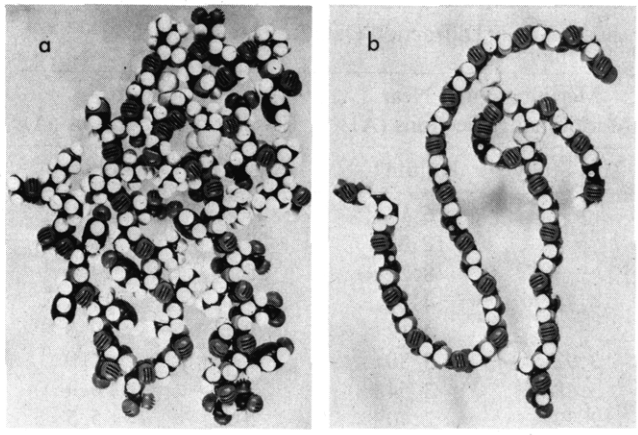

FIGURE 5: Proposed structure of an insulin molecule within a fibril. The model with side chains (a), and without side chains (b), is viewed looking down the fibril axis. The model as pictured represents an insulin molecule confined to a rectangular solid of about 4.8 Å depth, 30 Å width, and 50 Å length.

pany formation of cross-β fibrils from the native insulin structure (Adams *et al.*, 1969) is well illustrated by the changes which occur in the infrared and rotatory properties of the protein.

We believe that the insulin fibrils differ in significant ways from the cross-β structures proposed for such naturally occurring materials as the egg stalk of *Crysopa flava* (Parker and Rudall, 1957; Geddes *et al.*, 1968) and for denatured proteins (Astbury *et al.*, 1935; Senti *et al.*, 1943) and synthetic polypeptides (Bradbury *et al.*, 1960). In those cases the folds occurring in the extended polypeptide chains are believed to lie in planes parallel to the fibril axis with the resulting intrachain hydrogen bonds lying in the same plane forming antiparallel pleated sheets. Evidence for the antiparallel nature of the structures comes from X-ray diffraction studies (a 9.6-Å layer line) and is additionally provided in some instances by the fact that stretching of the cross-β fibril yields a parallel-β structure.

The size and uniformity of the basic insulin fibril means that the association process must be such as to limit the lateral growth of the fibril. This, taken with the fibril dimensions derived from diffraction experiments, suggests that each β-type insulin molecule occupies a layer approximately 4.76 $\times$ 29 $\times$ 47 Å. A rectangular solid of these dimensions and an assumed density of 1.4 g/cm^3 would have a weight of 5500, in reasonable agreement with the molecular weight of insulin 5700. Such a model, which is shown in Figure 5, requires that chain folds lie in planes approximately at right angles to the fibril axis, that is essentially in the plane of the side chains. The model indicates that this can be done while maintaining interchain hydrogen bonding. The chain fold is now, in fact, a portion of a helical turn since for best side-chain packing, chain segments adjacent to one another in the side-chain direction are expected to be displaced by 4.76/2 Å in the hydrogen-bond direction. The model permits but does not require that chains in successive layers be hydrogen bonded in antiparallel fashion. In fact we suggest that these interactions are of the parallel type, citing as evidence the infrared data and, the absence of diffraction intensity on odd-layer lines of a 9.5-Å spacing, especially, since such intensity is found for those cross-β materials cited above. This suggestion is supported by model building studies which indicate that the intrachain disulfide loop will be much less disruptive of intermolecular hydrogen bonding for a regular, parallel stacking of molecules than if arrangements which permit antiparallel interaction are used. Consideration of the alternative hexagonal arrangement of fibrils mentioned above, indicates that two molecules of insulin per unit cell would be required but that our conclusions about the nature of the interactions between molecules along the fibril axis would not be materially altered.

We also wish to consider the proposed structure in terms of the intermolecular interactions involved. The importance of hydrophobic types of interaction in insulin fibrils has been emphasized previously (Waugh, 1957). It is clear that the extensive hydrogen bonding in the cross-β structure must be of considerable importance to the strength and stability of the fiber. That the fibrils behave as if hydrophobically bonded with respect to solvent effects, suggests that extensive interactions involving nonpolar residues on the surface of the fibrils serve to protect the interior hydrogen bonding from disruptive effects of polar solvents.

We cannot predict the degree to which this type of intermolecular interaction may be responsible for the apparently hydrophobically mediated interactions found in other molecular aggregates such as, for example, bacterial flagella (Gerber and Noguchi, 1967), but we have found that structures essentially similar to that described are formed by glucagon and soluble derivatives of feather keratin. Details on these systems will be communicated at a later date.

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An X-Ray Crystallographic Study of the Binding of Peptide Chloromethyl Ketone Inhibitors to Subtilisin BPN'†

Jon D. Robertus, Richard A. Alden, Jens J. Birktoft, Joseph Kraut,*
James C. Powers,‡ and Philip E. Wilcox§

ABSTRACT: The difference-Fourier method was used to elucidate the mode of binding of polypeptide chloromethyl ketone inhibitors to the proteolytic enzyme subtilisin BPN'. Four inhibitors with an L-phenylalanine chloromethyl ketone group at their C termini were investigated: benzyloxycarbonyl-L-alanyl-L-phenylalanine chloromethyl ketone, benzyloxycarbonyl-L-alanylglycyl-L-phenylalanine chloromethyl ketone, benzyloxycarbonylglycylglycyl-L-phenylalanine chloromethyl ketone, and acetyl-L-alanylglycyl-L-phenylalanine chloromethyl ketone. All were found to alkylate the catalytic site His-64 with their phenylalanine side chain fitting snugly into a hydrophobic crevice. In each case the inhibitor polypeptide chain forms a system of hydrogen bonds of the antiparallel β sheet type with an extended segment of backbone chain in the enzyme consisting of residues 125–127. Furthermore, this extended backbone segment comprises one wall of the phenylalanine side-chain-binding crevice. The assumption that this geometry is a valid model for binding of polypeptide substrates is supported by the convincing way in which it explains the observed specificity of the enzyme toward the first, second, and third residues preceding the cleaved peptide bond in such substrates. When the corresponding catalytic and binding sites, considered as a single entity, are compared between subtilisin and chymotrypsin, it is found that they are sterically indistinguishable at current levels of resolution. Since the overall folding of the two molecules is otherwise entirely different, it is surmized that the two classes of serine proteases represent an example of convergent molecular evolution.

With determination of the crystal structure of the bacterial protease subtilisin BPN' now well advanced (Wright *et al.*, 1969; Alden *et al.*, 1971), it becomes possible to apply the powerful difference-Fourier technique of X-ray crystallography to the problem of how this enzyme interacts with its substrates. There is, however, a fundamental limitation inherent in any attempt to study enzymic mechanisms by X-ray crystallographic methods. Enzyme reactions occur on a time scale in the neighborhood of 10^{-8} sec, whereas several days are required to collect adequately informative X-ray data. It would therefore seem to be beyond present capabilities to "look" directly at any individual step in an enzymic reaction by X-ray techniques. Nevertheless a number of obvious stratagems are available that permit the crystallographer to obtain detailed structural information relevant to substrate binding, information which is unobtainable by any other means and which at least provides a basis for construction of reasonable hypotheses regarding the actual enzymic mechanisms.

For purposes of brief discussion these stratagems can be

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‡ Department of Chemistry, Georgia Institute of Technology, Atlanta, Ga. 30332.
§ Department of Biochemistry, University of Washington, Seattle, Wash. 98105. Deceased Nov 2, 1971.