An Electron-Microscope Study of β -Glucuronidase Crystals

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β -Glucuronidase from rat preputial glands was crystallized as thin sheets having $p6$ symmetry in projection with $a = 20.2$ nm. A filtered image was produced by Fourier methods to a resolution of 2.2 nm by averaging information from six areas. This suggests an approximately triangular molecular outline in projection, and this is taken to indicate a probable tetrahedral arrangement of the four subunits of the β -glucuronidase molecule.

β -Glucuronidase (β -D-glucuronide glucuronohydrolase, EC 3.2.1.31) is widely distributed in vertebrate tissue and is often found in high concentration in lysosomes (Szego *et al.*, 1971), where it may be involved in polysaccharide degradation. The enzyme is a glycoprotein of mol.wt. about 300 000 and is composed of four subunits of mol.wt. 75 000 that appear to be identical as assessed by gel electrophoresis and *N*-terminal amino acid determination (Hawley, 1973; Hawley & Marsh, 1970; Stahl & Touster, 1971).

In this present paper we describe the crystallization of this enzyme, and also the results of an investigation of its crystal structure, based on electron microscopy supplemented with computer image-processing techniques.

Experimental

Proteins

Crude β -glucuronidase was prepared from the preputial glands of Sprague-Dawley rats as described by Levvy *et al.* (1958), and purified by gel filtration on Sephadex G-100 by the method of Snaith & Levvy (1967) followed by crystallization as described below. The crystalline enzyme was shown to be homogeneous by displaying a single band on polyacrylamide-gel disc electrophoresis (by the method of Davis, 1964) and by exhibiting a single symmetrical sedimentable peak by ultracentrifugation with schlieren optics. This preparation had a specific activity of 7.3×10^5 Fishman units/mg of protein, which is comparable with the activity reported by Ohtsuka & Wakabayashi (1970).

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Catalase crystals (used for electron-microscope calibration) were generously given by Dr. N. G. Wrigley, National Institute of Medical Research, London NW7 1AA, U.K.

Assay procedure

β -Glucuronidase activity was assayed as described by Levvy *et al.* (1958), by measuring the rate of liberation of phenolphthalein from phenolphthalein β -glucuronide at pH 4.5 at 37°C. Activity was expressed in Fishman units, with 1 Fishman unit corresponding to the liberation of 1 μ g of phenolphthalein/h at a substrate concentration of 1 mM (1 Fishman unit = 8.73×10^{-13} kat).

Protein concentration was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard (once crystallized, 6.2% water; Sigma Chemical Co., St. Louis, MO, U.S.A.).

Electron microscopy

For electron-microscope observation, samples of the crystalline enzyme were negatively stained with uranyl formate or a 1:1 uranyl formate/aluminium formate mixture (Unwin, 1972) essentially as described elsewhere (Valentine *et al.*, 1968; Dickson, 1974). Specimens were examined under standard conditions (80 kV; double-condenser illumination; 0.03 mm thin gold-foil objective aperture; liquid-N₂-cooled anti-contamination device) on a Philips EM 300 electron microscope. The microscope was left for at least 60 min with the high tension on and anti-contamination device fully charged to ensure thermal stability. Images were recorded on either Gevaert Scientia 23D56 plates or Kodak 35 mm fine-grain release positive film and were developed in Kodak D19.

High-dispersion electron-diffraction patterns were recorded by using standard techniques (Dickson,

1974). Camera lengths of the order of 11 m were used and the patterns were calibrated by reference to the electron-diffraction pattern of crystalline catalase.

Image processing

Optical diffraction patterns were recorded on the apparatus described by Beaton & Filshie (1976).

Images selected for computer processing were scanned on either an Optronics P-1000 Photoscan or a Perkin-Elmer PDS microdensitometer with a raster spacing corresponding to approx. 0.5 nm on the original object. Image processing was carried out on the CDC CYBER 7600 computer of the C.S.I.R.O. Division of Computing Research by using methods analogous to those commonly used in optical filtering [for reviews see Amos (1974) and Crowther & Klug (1975)].

Areas of 512×512 density points were selected from the input density arrays, and their Fourier transforms were computed by using the optimized Fast Fourier Transform routine described by Fraser (1979). Separate phase and amplitude matrices of this transform were displayed on a line printer to enable identification of the peaks in the Fourier transform corresponding to the lattice structure of the crystal (these peaks correspond to the spots seen on the optical diffraction pattern). Filtered images were then produced by Fourier inversion of the data contained in small circular windows centred on the peaks. The diameter of these holes was chosen so that averaging took place over approximately 50 unit cells, which would be expected to increase the signal-to-noise ratio of the image by about 7-fold (see Fraser & Millward, 1970). This procedure is analogous to the use of an opaque mask with holes in optical filtering. Filtered images were output as grey-scale images (on line printer or as photographic negatives on an ILL COMp80 microfilm device) or contour plots as required.

It is often difficult to determine the exact amplitude and phase of many of the reflexions in the Fourier transform because the lattice points seldom correspond to the sampling points in the transform. This problem can be overcome to a large extent by interpolating the original image on to a different raster (Aebi *et al.*, 1973; Stewart & McLachlan, 1976), but this becomes very difficult with objects having hexagonal symmetry. To overcome this problem, we took advantage of the distinctive shape of the unit cell of these crystals. It is relatively easy to locate the four vertices of the unit cell (illustrated in Fig. 1) in filtered images, and so, for each image, an average unit cell was obtained by adding six to nine unit cells in real space after first interpolating each on to a 64×64 point raster skewed so that it corresponded to the two unit-cell vectors. (The averaging was carried out more to minimize errors in determining

the exact location of the vertices of the unit cells, rather than to increase the signal-to-noise ratio.) This average unit cell was then transformed to yield the desired amplitudes and phases, which now correspond to the sampling grid of the transform.

A final average image was obtained by averaging the symmetry-related reflexions (equivalent to sixfold rotational averaging of the unit cell) from six different areas.

Results

Crystals

When 0.2–0.5 mg/ml solutions of β -glucuronidase in 100 mM-NaCl/50 mM-sodium acetate buffer, pH 4.5, were dialysed against 2 mM-sodium phosphate buffer, pH 7.5, at 3°C, thin plate-like crystals formed over a period of several days. Plate 1(a) shows a typical phase-contrast micrograph of such a preparation, which is seen to consist predominantly of very thin hexagons. Himeno *et al.* (1975) have reported a similar crystalline form of this enzyme. Incorporation into crystals did not permanently affect enzyme activity, as no loss of activity was noted in assays of enzyme from redissolved crystals. No information is available on whether active sites are blocked when the enzyme molecules are incorporated into the crystal lattice. Crystals that were extended in a plane perpendicular to the hexagon were not observed, and this precluded a structural investigation based on X-ray-diffraction techniques. However, the crystal plates were often sufficiently thin to allow the structure to be investigated by electron microscopy, at least to the extent of determining the projection of the structure in the plane of the sheets.

When viewed negatively stained under the electron microscope, a large proportion of the sheets were sufficiently thin to allow structural details to be observed. All such images displayed a clearly hexagonal arrangement of particles, although there were some differences in detail observed between different patterns. The most commonly observed pattern is shown in Plate 1(b) and is characterized by a hexagonal arrangement of small and large areas of high stain density of approximately circular outline. These areas are surrounded by rings of less-densely staining material (presumed to represent protein), and closer inspection suggests that each ring is composed of six approximately circular lightly staining particles of about 8 nm diameter. Each of these particles probably represents an individual β -glucuronidase molecule. The crystal lattice appears, by eye, to have $p6$ hexagonal plane symmetry, although, because the detailed outline of the particles is somewhat obscured by noise, it is not possible to rule out the possibility of $p3$ symmetry.

EXPLANATION OF PLATE 1

β -Glucuronidase crystals with electron and optical diffraction patterns

(a) Phase-contrast micrograph of crystals. Magnification $\times 220$ (bar = $50\ \mu\text{m}$). (b) Electron micrograph of crystal negatively stained with uranyl formate. Magnification $\times 445\,000$ (bar = $100\ \text{nm}$). (c) Pattern seen occasionally, in which the difference in size between the small and the large dense areas is slight. Magnification $\times 455\,000$ (bar = $100\ \text{nm}$). (d) Electron-diffraction pattern of catalase and β -glucuronidase crystals recorded simultaneously: $\square$, reflexions from catalase; $\circ$, reflexions from β -glucuronidase. The β -glucuronidase pattern can be indexed on a hexagonal lattice with $a = 20.2\ \text{nm}$. (e) Optical diffraction pattern from a micrograph of a negatively stained crystal.

In a few instances the pattern was much simpler (see Plate 1c) owing to the difference in size of the small and the large areas of high stain density becoming very slight. These patterns were usually associated with crystals that appeared to be more densely stained, and this pattern was thought to be due to either a difference in stain penetration or possibly to dynamic effects produced by multiple scattering in the thicker objects.

The lattice spacings were determined by electron diffraction, with the use of glutaraldehyde-fixed negatively stained catalase crystals (Wrigley, 1968) for calibration purposes. To eliminate systematic errors, diffraction patterns were recorded with both catalase and β -glucuronidase crystals included in the field (see Plate 1d). By taking the lattice parameters of catalase as $a = 6.85\text{ nm}$ and $c = 17.5\text{ nm}$ (Wrigley, 1968), the β -glucuronidase pattern can be indexed on either a $p3$ or a $p6$ lattice with $a = 20.2\text{ nm}$.

The crystalline order was well preserved in the sheets as assessed by optical diffraction (Plate 1e). The outermost reflexion that could be consistently observed was the (63) order, which corresponds to a resolution of 2.2 nm . This is about the limit of resolution commonly observed in crystals negatively stained with uranyl salts (Unwin, 1975).

Computer image processing

The high degree of order present in the β -glucuronidase crystals made Fourier-based image processing seem attractive. By computing the phases of the lattice reflexions, it was expected that a decision could be made between $p3$ and $p6$ symmetry, and it was also expected that one could obtain more detailed information on the shape of the individual molecules by enhancing the signal-to-noise ratio of the images.

To select images for computer processing, negatives were first examined by eye to identify areas in which the crystal lattice appeared to be well preserved over a considerable area and did not contain dislocations nor appear to be visibly deformed in any way (e.g. by bending). These areas were then examined by optical diffraction to select those in which the structure was sufficiently well preserved to enable all six (63) order spots to be clearly visible (this indicated that the structure was well preserved to a resolution of at least 2.2 nm in all directions). Only the areas (six in all) that passed this rigorous and objective criterion were scanned in the densitometer and used for computer processing.

Fig. 1 is a contour plot of a typical image obtained by Fourier filtering of a single area. Only the central 128×128 density values have been included in order to avoid edge effects. Inspection reveals that all of the symmetry elements required for a $p6$ plane group (Henry & Lonsdale, 1969) are present, and these are

shown on Fig. 1. The alternative group ($p3$) lacks the 6-fold axis (where it has a 3-fold axis) and the six 2-fold axes. The assignment of $p6$ symmetry was confirmed by inspecting the phases of the reflexions in the Fourier transform. When referred to the presumed 6-fold axis as phase origin, these phases were all close to 0° or 180° (the amplitude weighted divergence from 0° or 180° was 7.1°), which indicates that the transform is entirely real. This is the expected result for the centrosymmetric $p6$ plane group, but would not be expected for $p3$ symmetry, because this group lacks a centre of symmetry.

The amplitude and phase information from reflexions related by $p6$ symmetry was then combined (this corresponds to a 6-fold rotational averaging about the 6-fold axis at the origin of the unit cell) and averaged from the six areas investigated. These values were then used to produce the final average filtered image shown in Fig. 2 by Fourier inversion.

Discussion

These studies establish that the projected structure of β -glucuronidase crystals can be indexed as having

Fig. 1. *Computed equidensity map of image*
A single area has been Fourier-filtered. The $p6$ unit cell is indicated, together with the symmetry elements required by this group (see Henry & Lonsdale, 1969).

Fig. 2. *Equidensity map of an averaged filtered image*
Fourier inversion of the amplitudes obtained from six areas was used to produce this map.

$p6$ symmetry with $a = 20.2\text{nm}$. The alternative $p3$ symmetry is ruled out on the basis of the symmetry elements that can be observed in filtered images (particularly the six 2-fold axes) and on the basis of the phases of the reflexions in the computed Fourier transforms of the images. It is important to establish the symmetry of the projection, because the 6-fold rotational averaging procedure used to obtain the final averaged image is valid only if it possesses $p6$ symmetry ($p3$ symmetry would allow only a 3-fold rotational averaging).

A $p6$ symmetry implies that there are $6N$ molecules per unit cell, and a value of 6 is generally consistent with the general appearance of the filtered image. Each 6-fold axis is surrounded by a structure that can be likened to a flower with six petals, and the most likely interpretation of this structure is that each petal corresponds to an individual β -glucuronidase molecule (reference to Fig. 2 illustrates that each unit cell also contains six petals). Fig. 3 shows a possible model for the structure, in which the molecule is assumed to be a tetrahedral arrangement of subunits of approximately 5nm diameter (broadly consistent with a subunit mol.wt. of 75000). We stress that this is an idealized representation and is only one of many related possibilities. It does, however, yield a reasonable approximation to the observed structure and is consistent with the molecular-weight data for β -glucuronidase. A rotational polarity is also evident, in that each petal does not point directly towards a 6-fold axis. This is quite a real effect, as can be seen, for example, by comparing the relative intensity of the (21) and (12) reflexions.

Studies using ultracentrifugation, gel filtration and polyacrylamide-gel electrophoresis have estab-

lished a tetrameric structure for rat tissue β -glucuronidase (Hawley & Marsh, 1970), and there is also evidence suggesting that, at least to a first approximation, the four subunits are identical. It is therefore likely that they are arranged in the molecule in such a way as to place each subunit in an equivalent environment. Only a tetrahedral or a square planar arrangement satisfies this criterion. Studies on negatively stained isolated β -glucuronidase particles (Dickson, 1974) did not allow a decision to be made between these alternatives. Although some details of the molecular structure are visible in the final averaged image, it is not possible, at the resolution used in this present study, to identify clearly either the outline or the position of the constituent subunits of the β -glucuronidase molecule. However, it is comparatively easy to accommodate particles having an approximately triangular outline (which would be consistent with a tetrahedral arrangement of subunits) with the appearance of the average filtered projected structure of these crystals (Fig. 3 shows one of the many possible ways this can be done). Square or oblong particle shapes (expected from a square planar configuration), on the other hand, cannot be so simply reconciled, and so we propose that a tetrahedral molecular structure is more likely for β -glucuronidase. However, a final decision on the molecular symmetry properties must await the solution of the structure at higher resolution or possibly in three dimensions.

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Fig. 3. Possible interpretation of the image
An idealized β -glucuronidase molecule is represented by a tetrahedron of four subunits of approximately 5nm diameter. This is only one of many related models that are consistent with the image and molecular-weight data.

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