Architecture of Peroxisomal Alcohol Oxidase Crystals from the Methylotrophic Yeast *Hansenula polymorpha* as Deduced by Electron Microscopy

JANET VONCK* and ERNST F. J. VAN BRUGGEN

BIOSON Research Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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The architecture of alcohol oxidase crystalloids occurring in vivo in the peroxisomes of methylotrophic yeasts was deduced from electron micrographs of similar crystals of the *Hansenula polymorpha* enzyme grown in vitro. Three characteristic views of the crystal are observed, as well as single layers in the very early stages of crystal formation. The crystal is concluded to be cubical, with every octameric molecule making the same contacts with four neighbors in one plane, at right angles to its fourfold axis. The unit cell contains six octamers, in three mutually orthogonal orientations, and two large holes, which can accommodate other peroxisomal proteins involved in methanol metabolism. The crystal contains channels, connecting the holes, which allow the diffusion of relatively large molecules through the crystal. Crystal formation depends on just one contact per subunit, which may explain the fragility of the crystals.

Methanol-utilizing yeasts like *Hansenula polymorpha* and *Kloeckera* sp. contain crystalline inclusions in their peroxisomes when they are grown on methanol as the carbon and energy source (15, 16, 26, 27). These crystals have been shown to consist of alcohol oxidase (AOX), the enzyme that converts methanol and oxygen to formaldehyde and hydrogen peroxide (25), although other enzymes, notably catalase and dihydroxyacetone synthase (the enzymes responsible for the degradation of H$_2$O$_2$ and the assimilation of formaldehyde, respectively), are also located inside the peroxisomes (5, 6, 25).

The AOX molecule is an octamer with a total molecular weight of approximately 600,000. Each subunit contains a flavin adenine dinucleotide as a noncovalently bound prosthetic group (12, 13, 18). Electron microscopy and image analysis of isolated protein have shown that the molecule has a more or less cubical shape and 422 point symmetry (12, 29).

AOX is synthesized in the cytosol as monomers and is transported without posttranslational modifications into the peroxisome, where it is assembled into active, flavin adenine dinucleotide-containing octamers (7, 13, 17). Aspects of the biosynthesis, transport, and assembly of AOX have recently been reviewed (21).

The three-dimensional structure of the molecule is not known, despite several attempts to crystallize the protein. Most crystallization trials have yielded crystals similar to those found inside the peroxisomes (19, 22), which do not diffract beyond 6 Å (0.6 nm). *Pichia pastoris* AOX has also yielded a crystal form of space group P2$_1$ (1), which has a completely different arrangement, as shown by electron microscopy (30). So far, this crystal form seems the most promising for a structure determination.

The peroxisomal crystals in all yeast species studied have an identical appearance. Several models have been suggested for their architecture. Osumi et al. (15) and Kanaya et al. (11) analyzed the crystallloid from *Kloeckera* peroxisomes by electron microscopy. By performing tilting experiments, they showed that the crystals have a cubical structure (15). Their model contains AOX and catalase in equal molar amounts as structural units. In *H. polymorpha*, catalase can be removed from the peroxisomes by an osmotic shock without damage to the crystal structure (25). Also, the fact that the same crystals can be grown from pure AOX indicates that catalase forms no structural part of the peroxisomal crystals of *H. polymorpha* and *P. pastoris*.

Veenhuis et al. (25) proposed a very open structure for the *H. polymorpha* crystal in which the molecules in alternate planes are rotated 45° relative to each other. This model contains large channels and holes through which reaction products and even protein molecules might diffuse. It does not, however, account for the observed alternating low and high protein densities of the crystal.

Tykarska et al. (19) suggested an alternative arrangement, based on X-ray diffraction data of their cubical crystals from *P. pastoris* AOX, which does account for the observed protein densities and contains fewer holes. They did, however, assume that the molecules in the crystals have two possible orientations in alternate planes, as in the previous model, which implies a tetragonal structure.

In this article we propose a new, highly symmetrical model for the crystal architecture, based on electron microscopical studies of *H. polymorpha* AOX crystals grown in vitro.

**MATERIALS AND METHODS**

**Protein.** AOX from *H. polymorpha* CBS 4732 isolated according to Van der Klei et al. (20) was a gift from Ida van der Klei.

**Crystall growth.** Small crystals were grown by mixing a small drop (maximum, 50 μl) containing 1 to 2 mg of protein per ml in 50 mM Tris-HCl (pH 7.0) with 1 to 4% polyethylene glycol 6000 (PEG 6000) on a piece of Parafilm and keeping it at room temperature for several minutes to 2 h. A drop of 5 μl was applied to a carbon-coated Formvar film glow discharged in pentylamine. After 2 min, the excess liquid was

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* Corresponding author.
removed with a piece of filter paper and the specimen was negatively stained with 1% (wt/vol) uranyl acetate.

Alternatively, a small piece of carbon film was stripped off from mica onto a drop of AOX-PEG 6000 mixture and picked up with a copper grid covered with a Formvar film after 2 min to 1 h and negatively stained as described above.

Electron microscopy. Electron micrographs were taken with a Philips EM 300 or EM 400 or JEOL JEM 1200EX electron microscope on Kodak SO-163 at several electron optical magnifications.

Computer model building. Projection images were calculated on a Convex C1-XP superminicomputer using FORTRAN 77 programs and further processed within the framework of IMagic (24). Surface representations were made on a Commodore Amiga 2000 using the Sculpt-Animate-3D program (Byte by Byte Corp., Austin, Tex.).

RESULTS

Electron microscopy. AOX very rapidly forms three-dimensional crystals in vitro upon incubation with PEG 6000. Within 15 min after addition of the precipitant, small crystals can be seen with the electron microscope (Fig. 1). Most crystals show the same face, which consists of a very characteristic square pattern of alternating light and dark units; this view is identical to the crystals seen inside peroxisomes (25). (Longer incubation under comparable conditions yields large, though poorly diffracting, crystals [22].) They have a square two-dimensional unit cell with unit-cell axes of 16 nm, containing a total of one light unit and one dark unit. The crystals are negatively stained, which means that the protein is embedded in an electron-dense layer of a heavy metal salt which replaces the water, so that a projection is darker where there is more stain; therefore, a light area indicates the presence of much protein, whereas a dark area indicates a hole filled with the stain. Stain accumulates in and around the crystals, which makes large ones in general appear darker than smaller crystals, as can be seen in Fig. 1a.

Occasionally, other orientations are present, most often when groups of crystals lie closely together, preventing each other from lying flat on the support film. Many of these crystals do not show a recognizable pattern, which means they are not viewed along a crystal axis. Often broad (approximately 16-nm) white lines separated by narrower lines of stain can be seen, which are also visible in images of tilted ultrathin sections of peroxisomal crystals (compare, e.g., images in references 16, 23, and 25). There are, however, two more characteristic views in which separate molecules can be recognized, which means that molecules in different planes are exactly aligned in the viewing direction. One view shows a rectangular pattern with a repeat of 11 nm in one direction and 16 nm in the other. The other view displays threec fold symmetry; it consists of very dark stain patches forming a hexagonal pattern, surrounded by light protein. The stain patches, which are approximately 19 nm apart, are connected by thin lines of stain. These same views were also observed by Osumi et al. (15) in sections of peroxisomal crystals. By tilting the specimens, the three views could be transformed into each other. Osumi et al. concluded that the hexagonal pattern is a [111] view and the rectangular pattern is a [110] view of the crystal. Crystals showing any of these two views are always quite small as a consequence of the cubical morphology of the crystals: in [111] views, crystals are standing on a vertex, and in [110] views, crystals are on an edge. This small size makes it difficult to determine their unit-cell dimensions accurately. In Fig. 1b and c, examples of groups of crystals are shown, where all three patterns are present.

The size of the crystals is dependent on the incubation time with PEG 6000. After about 15 min, small linear or branched aggregates are present, as well as very small crystals. These aggregates become very rare in samples incubated longer. One two-dimensional aggregate can be seen in Fig. 1a, and more examples are shown in Fig. 1d. It is likely that these represent very early stages of crystal growth. With longer incubation times, the crystals become progressively larger and also their shape becomes more square. After incubations of more than 1.5 or 2 h, most of the crystals are too thick to penetrate with the electron beam, although the structures described before can still be recognized along the edges of these crystals. An example of many large crystals at low magnification is shown in Fig. 1e.

When an unsupported carbon film is floated on the protein solution, large but very thin crystals are often found, mostly near irregularities in the support film, e.g., folds. The time during which the film is left floating on the protein solution or the total time during which PEG 6000 is present does not influence the yield of these large crystals. Probably they grow very rapidly on the carbon film during the drying process, when the protein concentration rises because of evaporation of water. The same views are present here as in the small crystals; because the crystals are often bent, different views are sometimes present in a single crystal. However, here as well as in the small crystals, most of them show the square-lattice view (Fig. 2a and b). Many lattice defects can be seen, which may be due to the very fast growth of these crystals. The [110] and [111] views are relatively rare; examples of these two are shown in Fig. 2f and g, respectively. In some tetragonal-view crystals, the density distribution is slightly different from the simple alternating pattern: the light units are all equal, but half of the dark positions show an intermediate density, forming a characteristic pattern where light and dark lines alternate with light and intermediate lines (Fig. 2c to e). This pattern occurs too often to be explained as an artifact caused by the staining process and thus must be due to real differences in the crystal on alternating lines.

A model for the crystal architecture. In the square pattern, the alternating light and dark units have the right size for an AOX molecule (12, 29). The alternating high and low density of the different positions suggests that for half of the positions there is a molecule present in each layer, giving rise to

FIG. 1. Small H. polymorpha AOX crystals grown in solution. They were grown in vitro in the presence of 2.5% PEG 6000 and negatively stained with 1% uranyl acetate. (a) Most crystals in this field show a tetragonal view. A single layer can be recognized in the lower portion of the picture (arrowhead). The incubation time was 15 min. (b and c) Examples of groups of crystals. Apart from general views characterized by broad lines, crystals of the views with square, rectangular, and hexagonal lattices are present. The incubation time was 30 min. (d) Two-dimensional aggregates, representing the very early stages of crystal formation. A molecule can have either four neighbors or two neighbors on opposite sides. The incubation time was 15 min. (e) Larger AOX crystals at low magnification. The incubation time was 105 min.

(a to d) Bar, 50 nm; (e) bar, 1 μm.
FIG. 2. AOX crystals grown on a carbon film in the presence of 1% PEG 6000, negatively stained with uranyl acetate. (a to e) Tetragonal views (along [001]). (a and b) Light and dark molecules alternate in the directions indicated by arrowheads. This projection has \textit{p4mm} symmetry; the square unit cell contains a total of one light molecule and one dark molecule. (c to e) A different density distribution can be seen. Rows containing alternating light molecules and intermediate-density molecules are indicated by arrowheads. The rows in between consist of light molecules (adjacent to the intermediate ones) alternating with dark molecules (adjacent to the light ones in the indicated rows). (f) Rectangular view (along [011]). The unit-cell axes are 16 and 11 nm. (g) Part of the crystal shows a hexagonal view (along [111]). The unit-cell axes are approximately 19 nm. Bar, 50 nm.
very light projections, whereas the positions adjacent to these molecules are occupied in only one of two layers. Consequently, there is a large hole completely filled with stain, which obscures the molecule above or below it. From the pattern shown in Fig. 2c to e, more conclusions about the distribution of molecules and holes can be drawn. The three different densities suggest that the structure is made up of three layers and that there are one, two, or three molecules superimposed on each position. An arrangement in which each layer of the crystal is made up of a square "net", as shown in Fig. 3a, fits the data. A layer is shifted relative to the layer below it along a diagonal (compare Fig. 3a and b), thus ensuring that each empty position becomes covered with a molecule and holes appear above continuous rows. This results in a structure with alternately one and two molecules in a vertical column (Fig. 3c). The third layer would be equal to the first, adding a third molecule to the double positions and a second to half of the single ones, yielding the pattern of Fig. 2c to e. This arrangement is illustrated in Fig. 3d. All crystals consisting of an even number (2n) of layers have only two different densities, like a two-layered structure (Fig. 3c), with n and 2n molecules on top of each other, respectively. Those with an odd number of layers (2n+1), however, possess three different densities; the number of molecules stacked are in the ratio 2n+1: n+1:n. Nevertheless, it is not to be expected that this difference would be visible when n ≥ 2, since the number of stain-filled holes has much more effect on the electron image than the number of protein molecules. For a three-layered structure, the number of holes in the different columns are in the ratio 0:1:2, but for five layers it is already 0:2:3, and the difference between two and three holes (that is, 25 and 37.5 nm of stain) is probably hardly visible, since two holes already prevent most electrons from reaching the film.

The small twodimensional aggregates shown in Fig. 1d are consistent with this interpretation: some molecules have four neighbors, but these neighbors in turn can have only two neighbors. The aggregates are similar to the pattern shown in the single layers of Fig. 3.

Having deduced a model for the crystal packing which fits all observations, the next question is the orientation of the molecules, which are octamers with 422 point symmetry, in the crystal lattice. Within one layer, there are molecules in contact with four others and molecules in contact with two others. The former class, however, is over a hole in the layer below, as can be seen in Fig. 3, so its total number of neighbors is four, whereas the second class is in the vertically continuous columns and consequently also has four neighbors, two in the same layer, one above and one below.

This means that all molecules in the crystal are surrounded by four others, lying in one plane, where this plane can be in each of three mutually orthogonal directions. Because of the 422 point symmetry of the molecules, it is probable that a molecule is oriented with its fourfold axis perpendicular to the plane in which its neighbors lie; this is the only possible arrangement in which it makes the same contact with each of its four neighbors. This leads to a structure in which all molecules have identical surroundings. They form continuous rows in three perpendicular directions, each of which has a 42 screw axis. The fourfold axis of each molecule points toward a hole. The molecules contact each other via their twofold symmetrical sides. Another consequence is that the crystal is identical in three directions; i.e., the views along [001], [010], and [100] are identical. The cubical unit cell is of space group 432 with a side of approximately 22 nm (8). The centers of the holes are the sites with the highest point symmetry, 432, and they are located on the corners of the unit cell and in the center. There are octamers on the edges with their fourfold axis along the edges and on the faces with the fourfold axis perpendicular to the face. Crystal packing is shown schematically in Fig. 4.

The cubical structure of the crystal explains the three characteristic views shown in Fig. 1 and 2. The tetragonal views (Fig. 1a and 2a and b) represent views along one of the faces of the cubical unit cell ([001], [010], or [100]). The
FIG. 4. Schematic representation of crystal packing. The octameric AOX molecules are shown as cubes, and a cross indicates the fourfold axis. One unit cell of space group I432 is shown as a cube; the parts of the molecules inside the cube are grey; the parts that do not belong to this unit cell are shown in a different shade (white [in front of the cube] and black [behind the cube]). Note that the unit cell is larger than that of the projection shown in Fig. 2a; the three-dimensional unit cell is rotated over 45° relative to the projection along [001]. The edge of the cube is 22 nm long.

rectangular view (Fig. 1c and 2f) is a view along one of the diagonals of the cube ([110], [101], or [011]); consequently, the lengths of the unit-cell axes are in the ratio 1:√2. (The symmetry of the structure leads to a smaller unit cell in this orientation: the projections of two adjacent layers are identical, so the height of the unit cell is one layer instead of two.) In the hexagonal view of Fig. 1b and c and 2g, the crystal is viewed along a body diagonal of the unit cell, resulting in threefold symmetry (a view along [111]). The unit-cell axis of the hexagonal view is approximately 19 nm, which is the right size for a view along a body diagonal of a cubical unit cell with sides of approximately 22 nm (8).

The very dark stain-filled pits on the threefold positions can now be explained: they originate from the alignment of the holes in the structure, which are located in the center and at the corners of the unit cell (Fig. 4); the crystals are viewed exactly down the channels connecting the holes, as is illustrated in Fig. 5. The presence of these channels along the diagonals is a clear confirmation for our crystal packing model.

If the crystal did not have cubical symmetry but had tetragonal symmetry, the views along [100] and [010] would not be identical to the (tetragonal) view along [001] and thus another special projection would be expected, with a rectangular unit cell and unit-cell dimensions corresponding to the height and width of a molecule, which are almost the same (12, 29). Like the tetragonal view, this view would arise when a crystal was lying flat and so this would be expected to be at least as numerous as the tetragonal view. The fact that it is never observed gives another argument for a cubical structure. Furthermore, the [111] projection of a tetragonal structure does not display threefold symmetry.

The obvious fourfold symmetry of the projections of the high-protein-density columns in the [001] view has led other groups to the assumption that all molecules are viewed along their fourfold axis in the tetragonal view (11, 15, 19, 25). The model proposed here implies that this is not the case; the molecules on the light positions are viewed along a twofold axis, with a rotation over 90° in the plane of projection for consecutive layers (there is a 4, screw axis in the direction of projection). The low-density columns, however, do exist solely as molecules viewed along the fourfold axis, alternating with holes. The views of the molecule in the [001] projection (Fig. 1a and 2a and b) can be compared with the images of single molecules and single-layered crystals and the model derived from them (29). The two-dimensional crystals that have been grown from *H. polymorpha* AOX are of plane group p4 and all molecules are viewed along their fourfold axis. Their internal density distribution is very smooth, but they show a darker center, like the dark projections in the [001] view. Single molecules have a preferential orientation on their side (with the fourfold axis almost parallel to the support film). Although they are not imaged along a twofold axis, a subdivision in two halves is very distinctive. A model for the AOX molecule has been proposed on the basis of these views. With this model it can be shown that superimposing two molecules viewed along a twofold axis with a 90° difference in rotation (i.e., their fourfold axes perpendicular to each other) does indeed yield a projection showing four masses at the corners (Fig. 6), like the light projections in the tetragonal view.

In samples which have been incubated for a very short time, single layers of the three-dimensional crystals are observed in the very early stages of the crystallization process (Fig. 1d). Here also, molecules which have more than two neighbors (and would in three dimensions be bounded by holes below and above them) have no distinctive features, apart from a slightly darker center, whereas those with only one or two neighbors (in two opposite directions) are bisected by a line pointing toward the neighboring molecule(s). This observation gives a more direct indication that the model is correct.

In Fig. 7, six molecules (representing the volume of a unit cell) are shown, viewed from several directions, to give an impression of the spatial relationships of the molecules relative to each other. In order to show whole molecules, the unit cell has been shifted in the direction of [111] with respect to the conventional crystallographic unit cell in Fig. 4.
crystalloids as consisting of AOX and catalase in equal molar amounts (9, 11, 15, 16) is unlikely, because similar crystals can be formed of isolated AOX free of catalase. Therefore, neither of the two models based on the assumption that the light units are AOX and the darker regions represent the smaller catalase molecules (11, 15) can be correct. However, these researchers also published micrographs of ultrathin sections through peroxisomal crystals showing hexagonal [111] and rectangular [110] views, similar to those presented here, and by tilting the specimens, showed how the views relate to each other (15). This indicated that the crystal has a cubical structure, which is consistent with our model. Veenhuis et al. (25) interpreted the darker regions in the crystals not as a smaller catalase molecule, but as an AOX molecule in a deeper layer, rotated over 45° relative to those in the light regions. Their model does not explain the observed staining pattern. Veenhuis et al. showed a micrograph of a very thin cryosection, which was interpreted as a three-layered structure. However, this pattern has a striking resemblance to a single layer in our model (compare Fig. 1d and Fig. 3).

Although several crystal forms of AOX have been grown in vitro, the peroxisomal type seems to be the one most easily formed under most conditions. The fragility and poor diffracting power of crystals of the in vivo form have been mentioned by several groups (19, 22). Our model shows a possible cause for this: molecules make only two contacts with each neighbor, as can be seen in Fig. 7, and it is conceivable that they have some rotational freedom around the line connecting these two contact areas. This might cause the crystals not to diffract beyond 6 Å (0.6 nm). A different crystal form (30) of P. pastoris AOX grown in the presence of azide diffracts to much higher resolution (1). This may be due to their much more extended protein contacts (31). The presence of azide to reduce all flavin adenine dinucleotide groups is not sufficient for forming well-ordered crystals, as the crystals formed by Tykarska et al. (19) were also grown with azide.

The two-dimensional crystals of H. polymorpha AOX (29) and P. pastoris AOX (28) are grown from solutions which can also yield the three-dimensional crystals described here (using longer incubation times and higher PEG 6000 concentrations). However, their crystal packing is different and the two crystal forms have no relation to each other. The two-dimensional crystals consist of a tetragonal array of AOX molecules with their fourfold axis perpendicular to the support film on which they were grown. The conditions which have to be fulfilled for the formation of two-dimensional crystals are much more restrictive than those for three-dimensional crystals: two-dimensional crystals are formed only in the presence of phosphate, on specially treated support films, and after staining with sodium sili
tungstate. Clearly, the two-dimensional crystals grow directly on the support film because of the local conditions, while in solution the circumstances still favor growth of three-dimensional crystals.

Although AOX can form several different crystals, depending on the crystallization conditions and the yeast species from which it was isolated, growth of the same crystal form in vivo is widely conserved across species, which is remarkable. This fact raises questions regarding the physiological significance of the crystal architecture, as several researchers have shown an association of other enzymes involved in methanol metabolism with the peroxi
somal crystals (5, 6, 23, 25).

The most striking feature of the crystals is the large holes
and the channels connecting them. The holes are slightly larger than an AOX octamer, cubical with a side of approximately 13 nm, and they may accommodate the other enzymes that have been shown to be localized in the peroxisomal matrix of methanol-grown yeast, catalase (25) and dihydroxyacetone synthase (5, 6). Dihydroxyacetone synthase from methylotrophic yeasts has a subunit molecular weight of 77,000 (slightly more than that of the AOX monomer) and forms dimers (2, 10). Beef liver catalase, on the other hand, is a tetramer of subunits consisting of 506 amino acids (AOX has 664 [13]; it has 222 symmetry and the dimensions 105 by 105 by 60 Å (10.5 by 10.5 by 6 nm) (14). Catalase from the yeast Saccharomyces cerevisiae has approximately the same size (3). Both catalase and dihydroxyacetone synthase molecules are smaller than AOX, and consequently, they can easily fit in the holes. It is imaginable that they become trapped inside the AOX crystal during its formation. Whether they can diffuse through the crystals, as suggested by Veenhuis et al. (25), is a different matter. The sizes of the channels, which are seen most clearly in the [111] projection, cannot be determined accurately without a high-resolution model of the AOX molecule, but from the crystal model (Fig. 7), it can be deduced that they are probably not more than 4 to 5 nm across. While this may be wide enough for a dihydroxyacetone synthase molecule, catalase in its tetrameric form is almost certainly too large. However, there is no doubt that smaller molecules (substrates and reaction products) can pass through the channels.

A peroxisome-deficient mutant of H. polymorpha has been isolated (4) which, when induced to synthesize AOX, forms crystals of the normal architecture in the cytosol (23). This mutant appears to be impaired in the translocation of proteins (or possibly just the assembly factor) over the peroxisomal membrane. By immune labelling it has been shown that dihydroxyacetone synthase and other enzymes are still specifically located in the crystals instead of randomly in the cytosol. This finding indicates that these enzymes have a specific affinity for AOX and are not just associated with it in vivo because of their peroxisomal localization. The crystal architecture shows that this putative affinity must be directed towards the fourfold faces of AOX, which line the holes and are not in contact with other AOX molecules.

The very unusual open architecture of the AOX crystals, with free space for other enzymes catalyzing the next steps in methanol assimilation and large channels for the diffusion of substrates and reaction products, and their conservation across methylotrophic yeast species show that they have a physiological function in the metabolism of methanol and do not serve merely for storage of protein material.

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