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Electron microscopic studies of methanol oxidizing enzymes

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Methanol occurs widely in nature as a product of e.g. the hydrolysis of lignin from wood or pectin in fruit. Many microorganisms able to utilize methanol as sole carbon and energy source have been isolated; these include prokaryotic as well as eukaryotic species. All these species possess an enzyme that catalyzes the first step in the metabolism of methanol: the conversion to formaldehyde. These enzymes differ considerably in structure as well as in catalytic mechanism, among the different groups of organisms. This thesis focuses on two groups of methanol converting enzymes, the first from a recently discovered group of Gram-positive bacteria, the second from methylotrophic yeasts. These enzymes were studied by electron microscopy in order to get a better understanding of their structure.

In chapter 1, an overview is given of the current knowledge of methanol oxidizing enzymes, as well as a description of the techniques used in electron microscopy and computer image processing of proteins.

Several strains of thermotolerant methanol utilizing Bacilli were isolated in 1988. These strains contain a novel, NAD-dependent methanol dehydrogenase with a subunit molecular weight of 43,000. Biochemical experiments indicated that this enzyme contained more than the two or four identical subunits that most alcohol dehydrogenases have; conflicting evidence existed as to the exact number. In chapter 2, an electron microscopical and biochemical characterization of the enzyme from the strain *Bacillus* C1 is presented. Electron micrographs clearly showed that the number of subunits was a multiple of five: many projections showed rings of five identical units. There were also rectangular views present, which had the right dimensions for a side view of the five-fold rings. By image processing the side views could be divided into two distinct classes, each consisting of two almost identical halves. From this it is concluded that the MDH molecule consists of ten subunits, organized in two rings of five, stacked face to face. A computer model is presented that explains the occurrence of the three distinct views. Further, it was shown that MDH contains two zinc and one magnesium ions per subunit, which is unusual for alcohol dehydrogenases. The amino acid composition and the N-terminal sequence were determined. The sequence shows no homology with the known alcohol dehydrogenases of the horse liver or the *Drosophila* type, but it does with the recently discovered third type of alcohol dehydrogenases.

In chapter 3, the methanol/formaldehyde oxidoreductases (MFO's) of two other Gram-positive bacteria are discussed: *Amycolatopsis methanolica* and *Mycobacterium gastri*. These enzymes have larger subunits than *Bacillus* sp. C1 MDH, with molecular weights of 50,000 and 49,000, respectively, and they contain NADP rather than NAD. Their metal contents is most likely similar to the *Bacillus* enzyme. Electron microscopy shows that the enzymes are also decamers with a structure similar to *Bacillus* MDH. However, after image processing a side view is seen which was not observed in the

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Bacillus enzyme and the most frequently occurring *Bacillus* side view is missing. The enzymes must have a difference on the outer rim which causes them to assume different stable positions on the support film, probably due to the extra mass. Amino acid sequence analysis of the N-termini and several internal peptides revealed homology between the *A. methanolica* and *M. gastri* enzymes and *Bacillus* MDH. The two MFO's have an eleven amino acid long N-terminal extension relative to MDH. This is not enough to explain the difference in molecular weight between the enzymes, so there must be extra amino acids internally as well.

Both MFO's have homologies with the third type alcohol dehydrogenases. Whether other members of this type also have a decameric structure remains to be established. Recently, a fourth decameric alcohol dehydrogenase was found in a non-methanol utilizing, Gram-negative sulphur bacterium, showing that the decamer may be a common structural theme among alcohol dehydrogenases.

Methanol-grown yeasts contain very large peroxisomes, often with a completely crystalline core. The crystals have been shown to consist of the enzyme alcohol oxidase (AOX), which oxidizes methanol to formaldehyde, using molecular oxygen. The byproduct of the reaction, hydrogen peroxide, is degraded by the enzyme catalase which is also located in the peroxisomes. AOX consists of eight identical subunits of approximately 660 amino acids, each containing a non-covalently bound FAD. The subunits are produced in the cytoplasm, transported as monomers into the peroxisomes, and inside the peroxisomes active, FAD containing octamers are formed. Details of this process are still largely unknown. In order to get more insight in the transport, assembly and activation of AOX, knowledge of the atomic structure is essential. Because of the large size of the molecule, this can be a very big project for x-ray crystallography. Electron microscopy was chosen as a means to get information about the structure of AOX at intermediate resolution. In chapter 4, the electron microscopical work on AOX from the yeast *Hansenula polymorpha* is described. In order to get the highest possible resolution in electron microscopy, it is important to have two-dimensional (2-D) crystals. Therefore, a method was developed to grow 2-D crystals of *H. polymorpha* AOX. Large crystals were found which diffracted to 18 Å in negative stain. By image processing the projected structure was calculated, which exhibited fourfold symmetry. Image processing of single molecules showed a different profile. This was concluded to be a side view of the molecule as seen in the 2-D crystals. A model for the 3-D structure of the AOX molecule is proposed, which explains the two views.

No crystals suitable for x-ray crystallography had been found for *H. polymorpha* AOX. However, crystallization had been successful with AOX form the related yeast *Pichia pastoris*, which has 85% sequence homology with *H. polymorpha* AOX. For that reason, the electron microscopical work was repeated with *P. pastoris* AOX. This work is

described in chapter 5. By an adaptation of the 2-D crystallization method, *P. pastoris* AOX could also be induced to form 2-D crystals, which were even better ordered than the *H. polymorpha* crystals. Their calculated structure was not much different. Image analysis of single molecules also gave similar results, so it was concluded that the model proposed for *H. polymorpha* AOX could also be used for the *P. pastoris* enzyme.

In chapter 6, an electron microscopical study of the *P. pastoris* AOX crystals grown for x-ray crystallography is presented. X-ray crystallography had shown that the crystals are of space group $P2_1$. The unit cell volume suggested that the asymmetric unit consisted most likely of two octamers, with a total molecular weight of more than 1,300,000. It was possible to make thin fragments of the crystals suitable for electron microscopy. Image analysis showed that some fragments were a few and others just one layer of molecules thick. In the single layer fragments, individual molecules could be recognized and their orientation was established by comparison to the model. The orientation of the electron microscopical view in relation to the 3-D unit cell could also be found by comparing the unit cell parameters in both cases. Thus it was possible to confirm the presence of two octamers in the asymmetric unit and to indicate the orientation of the four octamers in the unit cell.

In chapter 7, the peroxisomal AOX crystals are discussed. These have an entirely different architecture than the *in vitro* crystals in the previous chapter. Most attempts to crystallize AOX either in three or in two dimensions yield crystals similar to the peroxisomal form, but they are very fragile and do not diffract to high resolution. Several models have been proposed in the past for the architecture of these crystals, mainly based on thin sections of whole cells, but for different reasons all these models were unconvincing. The model presented here is based on studies of small, *in vitro* grown crystals in different stages of development. Eventually, the crystals attain a cubic shape, but in the very early stages of growth some single layers can be seen. Also thin crystals with a staining pattern characteristic for an odd number of layers were found. Finally, some crystals were not viewed parallel to the cube edges but at several other angles, and in a few cases other characteristic patterns emerged. The proposed model explains all these views as well as the fragile nature and the cubic shape of the mature crystals. All molecules are surrounded by four others lying in one plane, perpendicular to the fourfold axis of the central molecule: the neighbors are rotated 90°, as a result of which they are surrounded in a different plane and the crystal is identical in three perpendicular directions. This arrangement leaves large holes in the structure, above and below each molecule. These holes are aligned in a view along the vertex of the cube, and large channels are visible in that orientation. A model of this arrangement is shown on the front cover.