Purification of Marginal Plates from Bovine Renal Peroxisomes: Identification with L-α-Hydroxyacid Oxidase B

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Abstract. The matrix of mammalian peroxisomes frequently contains crystalline inclusions. The most common inclusions are membrane associated plate-like "marginal plates" of hitherto unknown nature in renal peroxisomes and central polytubular "cores" composed of urate oxidase in hepatic peroxisomes. In bovine kidney, peroxisomes of proximal tubules exhibit peculiar angular shapes that are caused by multiple marginal plates (Zaar, K., and H. D. Fahimi. 1990. *Cell Tissue Res.* 260:409–414). Enriched or highly purified peroxisome preparations from this source were used to purify and characterize marginal plates. By SDS-PAGE, one major polypeptide of M_r 33,500 was observed that corresponded to the marginal plate protein. This polypeptide was identified by its enzymatic activity as well as by immunoblotting and preembedding immunocytochemistry as the isozyme B of L-α-hydroxyacid oxidase (EC 1.4.3.2). Morphologically, marginal plates were revealed to consist of rectangular straight-edged sheets, exhibiting a defined crystalline lattice structure. The sheets apparently are composed of a single layer of protomers which associate laterally to form a plate-like structure. As deduced from the negative staining results and the additional information of the thickness of marginal plates, each protomer seems to consist of eight subunits forming a cube-like array. The tendency of L-α-hydroxyacid oxidase B to self-associate in vitro (Philips, D. R., J. A. Duley, D. J. Fennell, and R. S. Holmes. 1976. *Biochim. Biophys. Acta.* 427:679–687) corresponds to the mode of association of cubical protomers to form the so-called marginal plates in renal peroxisomes.

PEROXISOMES are ubiquitous organelles that are morphologically characterized by a finely granular matrix surrounded by a single limiting membrane. Crystalline inclusions of different types are often found in the peroxisomal matrix (Hruban and Rechcigl, 1969; Böck et al., 1980). The most regularly occurring inclusions in mammalian peroxisomes are the central polytubular "cores" in hepatic peroxisomes and the plate-like "marginal plates" in renal peroxisomes, which are closely associated with the peroxisomal membrane (for review and nomenclature see Hruban and Rechcigl, 1969). Whereas the cores have been well characterized and are known to be composed mainly of the peroxisomal marker enzyme urate oxidase (Tsukada et al., 1966; DeDuve and Baudhuin, 1967; Angermüller and Fahimi, 1986; Völkl et al., 1988; Usuda et al., 1988*a*), nothing is known about the nature of the marginal plates.

Marginal plates are defined as plate-like straight or slightly curved electron-dense inclusions directly underlying the peroxisomal membrane but separated from it by a narrow electron-lucent gap. They were first described by Van Breemen and Montgomery (1960) in the proximal tubules of dog and rabbit kidney cortex and since have been found in peroxisomes in kidneys of most other species (Hruban and Rechcigl, 1969) including primates and humans (Tisher et al., 1966, 1968). However, they have also been described, e.g., in liver peroxisomes of humans (Sternlieb and Quintana, 1977) as well as in peroxisomes of lipid synthesizing epithelia (Gorgas and Zaar, 1984).

In the course of our recent studies on renal cortex peroxisomes from several mammalian species, a population of large peroxisomes with peculiar angular shapes was observed in the proximal tubules of the bovine kidneys which contained both types of inclusions: the typical membrane-associated marginal plates as well as the polytubular cores in the central portion of the matrix (Zaar et al., 1986, 1987). The unusual shape of these peroxisomes is obviously due to the presence of multiple marginal plates (Zaar and Fahimi, 1990).

A procedure is now presented for the isolation and purification of marginal plates from enriched or highly purified peroxisome fractions of bovine kidney cortex. Moreover, this marginal plate fraction was characterized enzymatically, as well as by SDS-PAGE, immunoblotting, immunocytochemistry, and EM. The results indicate that marginal plates in bovine kidney consist of isozyme B of L-α-hydroxyacid oxidase.

Materials and Methods

Morphological Studies

Tissue Processing. Bovine kidneys obtained from the local slaughterhouse

were excised shortly after exsanguination of animals and fixed for 5 min by perfusion through the renal artery. First physiological (0.9%) saline was introduced followed by 2.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.6) containing 4% polyvinylpyrrolidone (PVP). Alternatively the tissue was perfused transparenchymally for 5 min with the same fixative (Sandström, 1970). Adequately fixed kidney cortex tissue was identified and dissected under a binocular and immersed for an additional hour in freshly prepared fixative. Sections of 50–100 μm obtained with an Oxford Vibratome were postfixed with the reduced osmium procedure (Angermüller et al., 1986), dehydrated and embedded in Epon 812. Sections were viewed in a Philips EM 301 microscope.

Processing of Isolated Fractions. Fractions obtained after gradient centrifugation were fixed by mixing with an equal volume of 5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.6) and either filtered onto Millipore filters according to the method of Baudhuin et al. (1967) as modified by Völkl and Fahimi (1985) or centrifuged at 25,000 *g* for 30 min to form a pellet. Pellets were enclosed in 3% (wt/vol) agar, sliced, dehydrated, and embedded in Epon 812.

Negative Staining. Samples were diluted to ~0.2 mg protein/ml. 5 μl were applied to a thin carbon film over a 400-mesh grid coated with Formvar. Grids were hydrophilized shortly before use by exposure to a glow discharge in a Balzers CTA 010 Beglimmungsgerät (Balzers GmbH, Lichtenstein). Excess sample was removed by touching with a filter paper, and one drop of either 1% (wt/vol) sodium phosphotungstate (pH 7.0) or 1% (wt/vol) uranyl formiate (pH 5.0) were applied to the grid. After 20 s the grid was washed with several drops of stain. Excess stain was removed and the grids were air dried and examined as soon as possible. Magnifications were calibrated either with a diffraction grating replica (E. F. Fullam, Inc., Schenectady, NY) or by using the periodicities of negatively stained crystalline catalase (Wrigley, 1968).

Biochemical Studies

Isolation of Peroxisomes. Highly purified peroxisome preparations from beef kidney cortex were obtained as described (Zaar et al., 1986, 1987). Briefly, a light mitochondrial fraction (fraction D) (Zaar et al., 1986) was prepared by differential centrifugation from tissue homogenates and top-loaded on gradients of metrizamide (1.12–1.30 g/cm^3). Peroxisomes (PO)[1] were separated using short-path, density-dependent banding in a vertical rotor (VTI 50; Beckman Instruments, Fullerton, CA) at an integrated force of 1.25×10^6 *g* × min. Alternatively, light mitochondrial fractions were suspended in 47% (wt/wt) sucrose in 5 mM MOPS buffer (pH 7.4). Centrifugation for 5 h in a fixed angle rotor (45 Ti; Beckman Instruments) at 45,000 rpm resulted in pelleting of a fraction enriched in peroxisomes referred to as sucrose peroxisomes (SPO). SPO-fractions, which could be obtained at high yield, were used as the starting material for the isolation of marginal-plates.

Isolation of Marginal Plates. Triton X-100 (0.5% wt/vol) was added to SPO fractions under gentle stirring in an ice bath and the mixture was incubated for 30 min. Nonsolubilized components were pelleted by centrifugation and this procedure was repeated twice. The pellets obtained after detergent treatment were suspended in 5 mM MOPS buffer (pH 7.4). Samples of 2 ml (~10 mg protein/ml) were layered on 10-ml stepped sucrose gradients (5 ml 52% (wt/wt) and 5 ml 54.2% (wt/wt) sucrose in 5 mM MOPS buffer, pH 7.4). After centrifugation for 14 h at 39,000 rpm using a SW 40 swing out rotor (Beckman Instruments), the interface fractions were collected and submitted to analytical procedures. If necessary several interface fractions were pooled and recentrifuged on the discontinuous gradient.

Biochemical assays were performed according to standard procedures as described recently (Zaar et al., 1986, 1987, 1989). Activities of isozymes A and B of L-α-hydroxyacid oxidase were determined with either 10 mM glycolate (L-HAOX A) or 10 mM L-α-hydroxy butyrate (L-HAOX B) as substrates. The production of H_2O_2 was measured by the method of Osumi and Hashimoto (1978).

Preparation of Antibodies. Antibodies against the peroxisomal enzymes catalase (CAT), urate oxidase (UOX), L-α-hydroxyacid oxidase A and B (L-α-HAOX A and B) and D-amino acid oxidase (D-AAOX) were raised in rabbits. The enzymes from rat liver and kidney were isolated by means of established methods as given in the following references and purified to homogeneity as evidenced by SDS-PAGE: catalase (Price et al., 1962); urate oxidase (Mahler et al., 1955; Völkl et al., 1988) L-α-hydroxy acid oxidase A and B (Yokota et al., 1985); D-amino acid oxidase (Yagi, 1971). Immunization of rabbit was performed according to the procedure of Louvard et al. (1982) and IgG fractions were separated from total anti-serum (Mayer and Walker, 1980). The monospecificity of antibodies was tested with highly purified peroxisome fractions and isolated peroxisomal enzymes using immunoblotting (Burnette, 1981). The antibodies raised against the isozyme A of L-α-hydroxy acid oxidase from rat liver and isozyme B from rat kidney were highly specific for each enzyme and showed no cross reactivity.

Preparation of Protein A–Gold (PAG) Complex. Gold sols with a particle size of 12 nm were prepared (Mühlpfordt, 1982) and conjugated to protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) at pH 5.9 using the method of Slot and Geuze (1981). The PAG complex was isolated by sucrose density centrifugation (10–30% wt/wt) and stored in 25% (vol/vol) glycerol at −20°C. The diluted complex (1:50) exhibited an optical density of 0.45 at 528 nm.

SDS-PAGE and Immunoblotting. SDS-PAGE was performed using a microslab electrophoresis apparatus (KS 8010 MSE; Marysol Industry Co., Ltd., Tokyo). Samples were applied to gels (9 × 5 × 0.1 cm, 10–12.5% resolving, 3% stacking gel), stacked at 15 mA and resolved at 25 mA (total time ~1 h). Proteins were stained with Auro Dye solution (Moeremans et al., 1987).

For immunoblotting proteins were electrotransferred onto nitrocellulose sheets (30 V/60 min). Nonspecific binding sites were blocked with 0.15 M newborn calf serum/PBS, pH 7.2, containing 0.05% Tween 20 (NCS/PBS/T) for 1 h with gentle rocking. Nitrocellulose sheets were incubated overnight with the appropriate antibodies diluted 200 times with NCS/PBS/T, followed by a fourfold wash. Antigen–antibody complexes were visualized by PAG (Brada and Roth, 1984) followed by an anti-protein A step (Bendayan and Duhr, 1986) and a second treatment with PAG.

Preembedding Immunolabeling of Marginal Plate Fractions. The isolated marginal plates were resuspended in 20 mM TBS, pH 7.2, 1% BSA (TBS/BSA) and incubated overnight with anti-HAOX B (400 ng/ml) or anti-UOX (200 ng/ml) antibodies. After incubation the marginal plate fraction was washed by centrifugation through a layer of 20% (wt/wt) sucrose, 0.1% BSA in TBS onto a cushion of 52% (wt/wt) sucrose/TBS. Washed marginal plates were incubated for 1 h in PAG (dilution 1:50) and centrifuged through a layer of 30% (wt/wt) sucrose in TBS. Pellets were fixed with 2.5% glutaraldehyde, enclosed in agar, sliced, dehydrated, and embedded in Epon 812. Ultrathin sections were stained with lead citrate.

Results

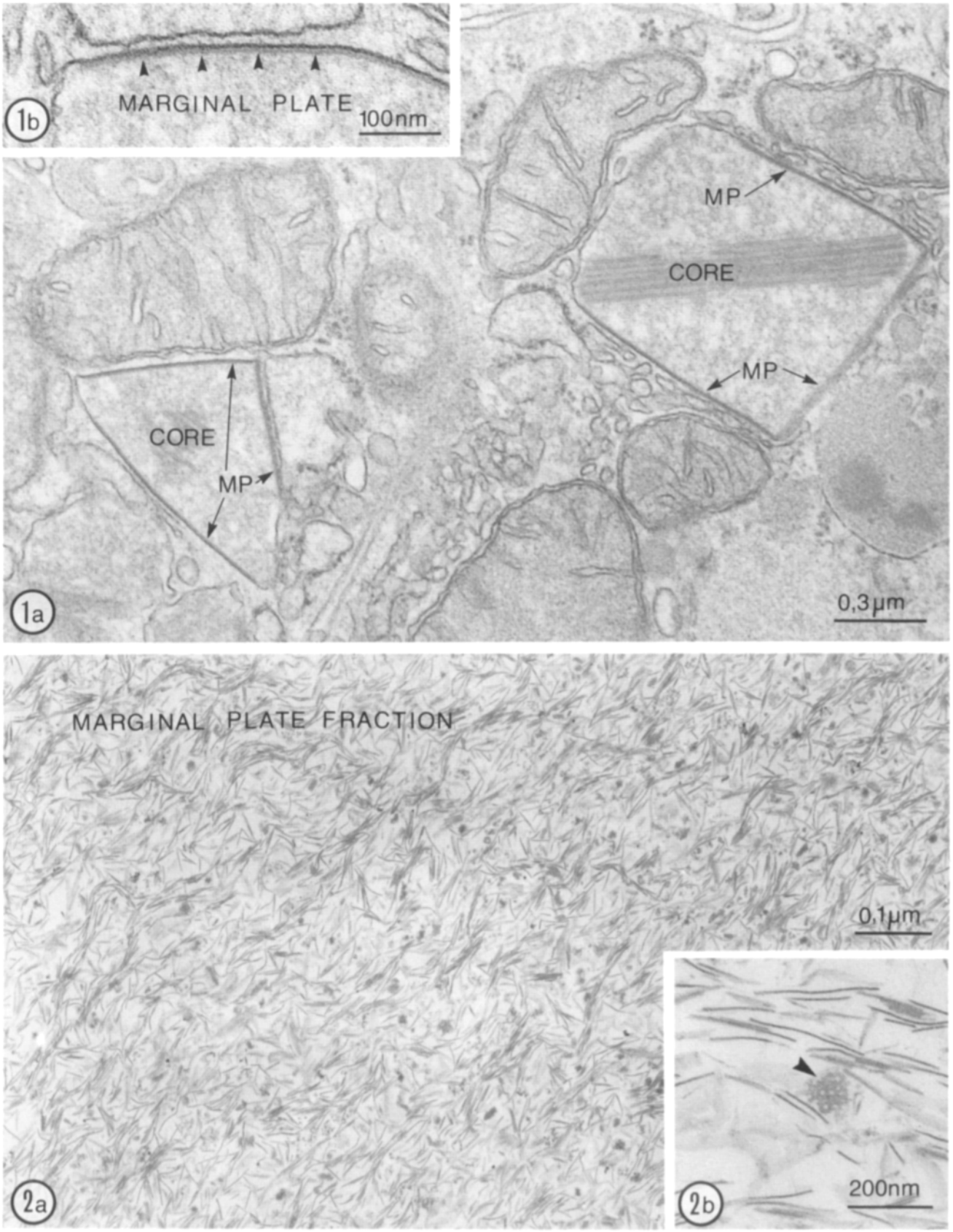
Angular Peroxisomes in the Epithelium of Proximal Tubules

Peroxisomes are abundant in the epithelial cells lining the proximal tubules of bovine kidney cortex. They are usually located in the perinuclear and basal cytoplasm and exhibit a peculiar angular shape (Fig. 1). Particularly in the straight P-3 segment of tubules, the organelles are very large (up to 1.5 μm in diameter). In all other nephron segments peroxisomes are small and inconspicuous (average diameter 0.2–0.3 μm). Most peroxisomes of the proximal tubule appear unusually shaped probably due to the occurrence of multiple marginal plates, electron-dense plate-like inclusions located directly beneath the peroxisome membranes (Fig. 1, *a* and *b*). Indeed, peroxisomal membranes in such regions adopt the straight appearance of the marginal plate (Fig. 1 *a*). Marginal plates have a thickness of ~7.5 nm with a length of up to 1.5 μm. Membranes and marginal plates are separated by an even electron-lucent narrow space of 2–3 nm (Fig. 1 *b*). In addition, the bovine kidney peroxisomes contain a second type of inclusion that is the polytubular cores located in the central portion of the matrix (Fig. 1 *a*). They consist of 5 nm primary tubules arranged in a circular fashion forming secondary tubules.

Isolation of Bovine Kidney Cortex Peroxisome Fractions

Recently, we described a method for the isolation of highly purified intact peroxisomes from kidney cortex of different

1. *Abbreviations used in this paper*: PAG, protein A–gold complex; PO, peroxisome; RSA, relative specific activity; SPO, sucrose peroxisomes.



Figures 1 and 2. (Figure 1) (*a*) Two peroxisomes in an epithelial cell of bovine kidney cortex proximal tubule. The organelles show peculiar angular outlines apparently due to the occurrence of multiple marginal plates, straight plate-like matrical inclusions (MP). Another type of inclusion is the polytubular core (*core*) in the center of the matrix. (*b*) Part of a peroxisome. A marginal plate (*arrowheads*) is apposed to the inner aspect of the peroxisomal membrane, being separated from it by a small electron lucent gap. Bars: (*a*) 0.3 µm; (*b*) 100 nm. (*Figure 2*) (*a*) Overview showing a section through the pellet of a purified marginal plate preparation from bovine kidney cortex peroxisomes. It consists mainly of straight plate-like structures with different lengths that are sectioned in different planes. (*b*) A minor component found in such preparations is made up of the fragments of the polytubular cores (*arrowhead*). Bars: (*a*) 0.1 µm; (*b*) 200 nm.

Table I. Composition and Enzyme Activities of Sugar Peroxisome Fractions and Purified Marginal Plate Fractions from Beef Kidney

	Homogenate		Sugar peroxisome fractions				Marginal plate fractions			
	n	*mU/mg protein*	*n*	*mU/mg protein*	*Yield (%)*	*RSA‡*	*n*	*mU/mg protein*	*Yield (%)*	*RSA*
Catalase	15	197.5 ± 6.1	4	1,072 ± 378	7.1 ± 3.4	5.3	6	1.72 ± 1.64	§	0.008
Urate oxidase	9	0.57 ± 0.22	4	18.9 ± 12.1	42.7 ± 21	32	7	62.2 ± 32.9	1.6 ± 1	109
L-α-Hydroxyacid oxidase A	5	0.145 ± 0.076	5	2.5 ± 1.3	22.7 ± 11	17.1	7	2.56 ± 1.23	0.26 ± 0.17	17.2
L-α-Hydroxyacid oxidase B	5	0.085 ± 0.049	7	1.2 ± 0.49	19 ± 9	14.3	7	9.89 ± 2.96	1.8 ± 1.2	117
D-Aminoacid oxidase	10	2.96 ± 0.83	3	31.4 ± 19.3	14.4 ± 7	10.8	5	0.47 ± 0.29	§	0.17
Cytochrome *c* oxidase	15	128 ± 37.6	4	33.2 ± 22.9	0.4 ± 0.2	0.3	6	1.23 ± 1.06	§	0.02
Esterase	16	447 ± 226	4	1,683 ± 1,166	4.8 ± 2.3	3.6	6	42.6 ± 28.6	§	0.1
Arylsulfatase	5	1.19 ± 0.35	4	8.4 ± 6.6	9.3 ± 5	7	6	0.34 ± 0.28	§	0.3
Protein (%)	15	100	7		1.33 ± 0.65		7		0.015 ± 0.01	

Values are means of *n* experiments ± SD.
* Yield = recovery (%) of the homogenate value.
‡ RSA = relative specific activity (percentage recovered enzyme activity/percentage recovered protein; homogenate = 100%).
§ Yield <0.005%.

mammalian species using a metrizamide gradient and a vertical type rotor with a short path (Zaar et al., 1986, 1989). Highly purified peroxisome preparations isolated this way were used for the immunological characterization of matrical inclusions in the present study. The relatively low yield of 0.12% of homogenate protein, of the latter procedure (Zaar et al., 1987) as well as the high cost of the gradient medium metrizamide, however, renders this method impractical for the large scale preparation of peroxisomal matrix inclusions. In the alternative method designed to obtain peroxisomes at higher yield sucrose was used as gradient medium instead of metrizamide. Moreover, organelles present in light mitochondrial fractions were separated by floatation in a sucrose solution with density of 1.21 g/cm³. A crude peroxisome preparation referred to as sucrose peroxisomes (SPO) and constituting on the average 1.3% of the homogenate protein was recovered in the pellet after centrifugation. The distribution of organellar marker enzymes in SPO fractions as well as their yield from the homogenate are given in Table I. SPO fractions contain about 40% of the peroxisomal marker enzyme urate oxidase. Other peroxisomal enzymes are recovered at rates between 7 (catalase) and 23% (L-HAOX A). The preparation is mainly contaminated by lysosomes (relative specific activity [RSA] of lysosomal arylsulfatase = 7) and microsomes (RSA of microsomal esterase = 3.6) with only a small contribution of mitochondria (RSA of the mitochondrial marker enzyme cytochrome *c* oxidase = 0.4). SPO fractions were used for the isolation of marginal plates.

Isolation of Highly Purified Marginal Plate Fractions

Matrical inclusions of peroxisomes were released by solubilizing of organelle membranes with Triton X-100 and were purified by density gradient centrifugation using a two-step sucrose gradient of 52 and 54.2% (wt/wt) respectively. Marginal plates accumulated at the sucrose interface in a distinct yellow-colored fraction. Morphologically this fraction is composed to a high degree of straight or slightly curved plate-like structures of varying length (up to 1 μm) and an average thickness of 7.5 ± 0.8 nm (Fig. 2, *a* and *b*). In addition to marginal plates, the fraction also contained varying amounts of the polytubular core fragments (Fig. 2 *b*).

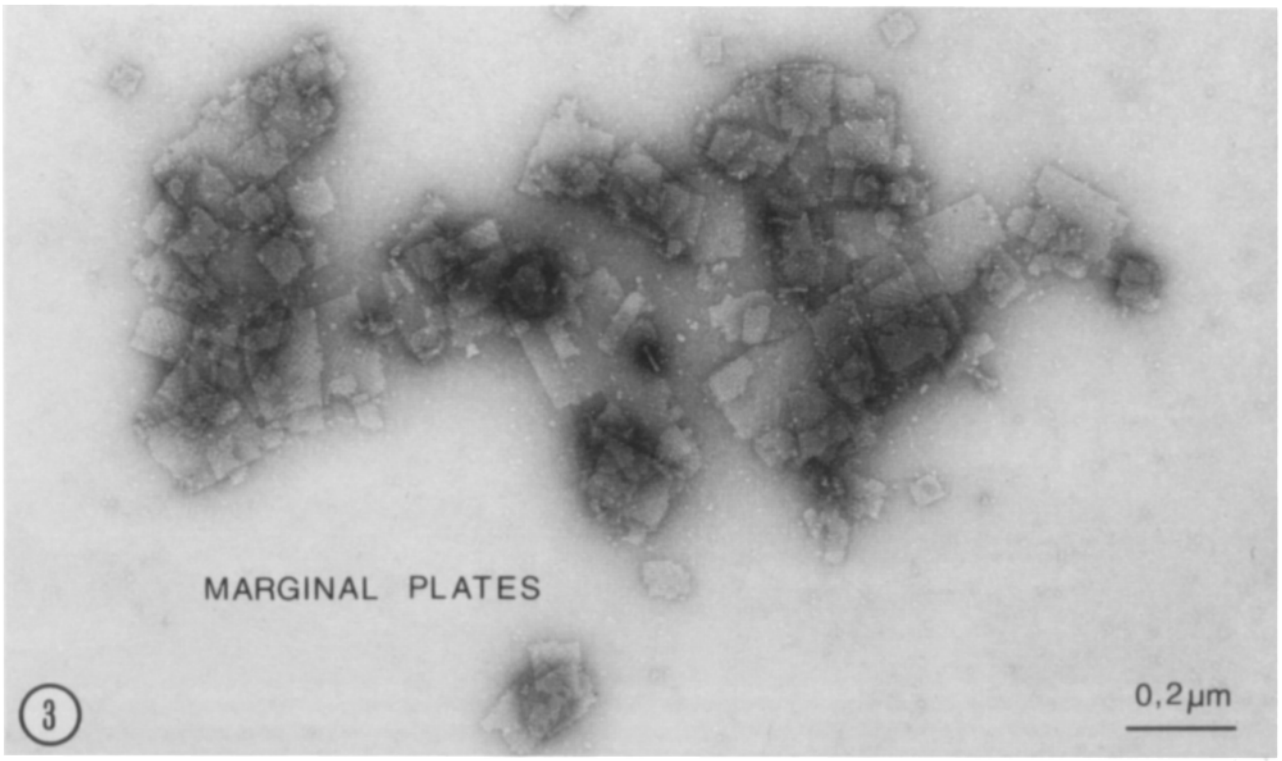
Morphological Characterization of Marginal Plates

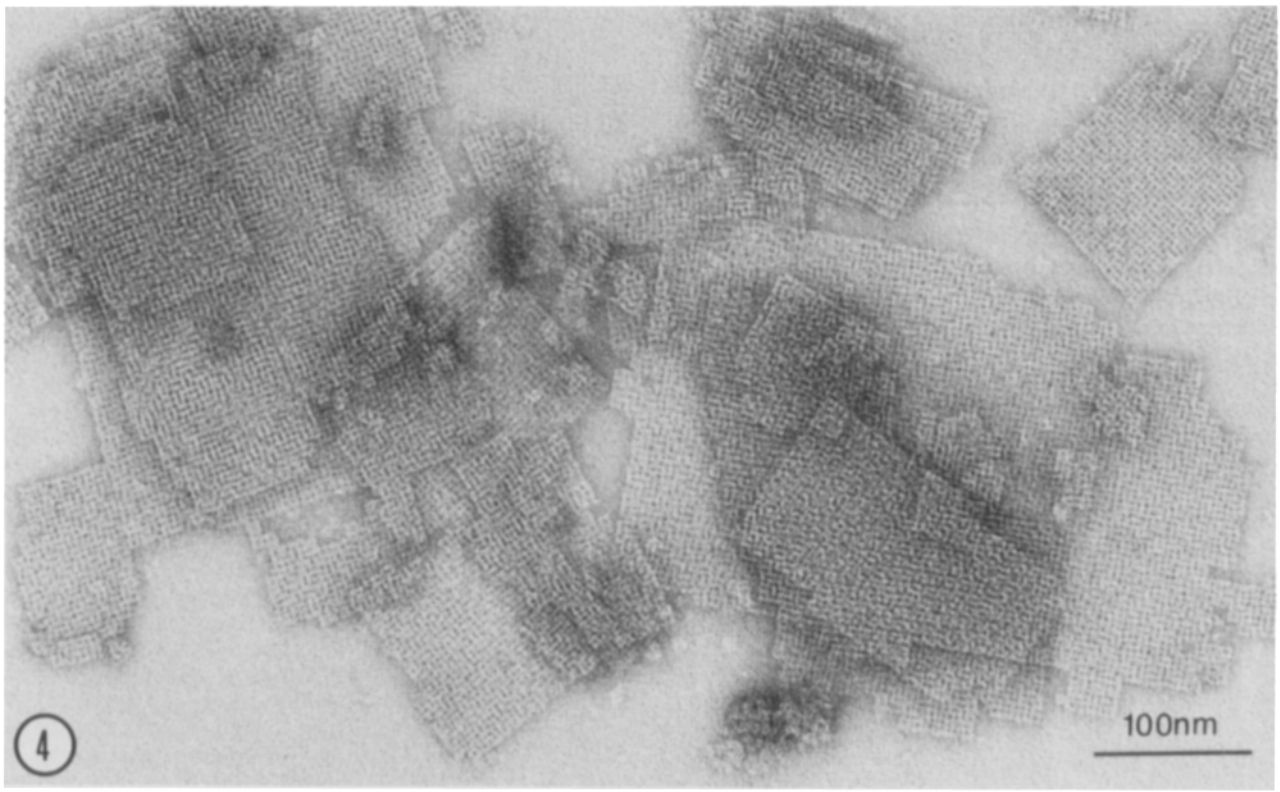
The morphology of marginal plates was further studied with the negative staining technique. Fig. 3 shows that the marginal-plate fraction consists of rectangular straight-edged plate-like structures of different sizes. Higher magnification reveals that marginal plates are sheets exhibiting a crystalline lattice structure (Fig. 4). Measurements of the sheet substructure in 40 plates revealed average periodicities of 7.4 ± 0.5 nm and 8.0 ± 0.3 nm in perpendicular axes of the plane. At high magnification the sheets sometimes showed, particularly at their edges, evidence of disintegration revealing their composition of rectangular or square units (Fig. 5). Each unit consists of four globular subunits with one globule at each corner, connected at right angles by thinner strands (Fig. 5). Because in thin-sectioned preparations the marginal plates have a thickness of 7.5 ± 0.8 nm (see above), the square units seen in negative staining preparations should be either tetragonal or cube-like structures. Measurements of the size of such units in 20 different preparations of disintegrating crystals revealed consistently square-shaped aspects with dimensions of 6.5 ± 0.7 nm. This is consistent with the assumption that the units are protomers formed by eight globular subunits in a cube-like array with a mean side length of 6.5 nm. Thus, marginal plates probably are composed of a single layer of laterally associating cube-like protomers.

Biochemical Characterization of Marginal Plates

Purified marginal plate fractions typically exhibited a yellow color. To elicit the chromophore, absorption spectra were run of fractions solubilized in 6 M guanidinium HCl. The absorption maxima observed at 443, 370, and 271 nm pointed to a flavin moiety. It is well known that most peroxisomal oxidases are flavin enzymes with either FAD or FMN as coenzymes (Masters and Holmes, 1977). For this reason particular attention was dedicated to this class of enzymes during further investigation.

The biochemical composition of marginal-plate fractions is summarized in Table I. On the average, 0.015% of the homogenate protein was recovered in this fraction. The only significant *enriched* enzyme activities found corresponded to L-α-hydroxyacid oxidase (HAOX) and urate oxidase (UOX). By far the highest RSA was observed when using L-hydroxybutyrate as substrate (RSA 117), which is known to be the preferential substrate for isozym B of HAOX. Glycolate, which is the substrate preferred by L-α-HAOX A was also oxidized, but to a much lesser extent. Its RSA 17.2 was





Figures 3 and 4. (*Figure 3*) Negative staining preparation of a purified marginal plate fraction stained with uranyl formiate. Marginal plates appear as straight-edged, rectangular sheets of different size which exhibit a well defined substructure. Bar, 0.2 µm. (*Figure 4*) Higher magnification reveals that the sheets show a highly regular lattice structure with defined periodicities and a composition of subunits. Bar, 100 nm.

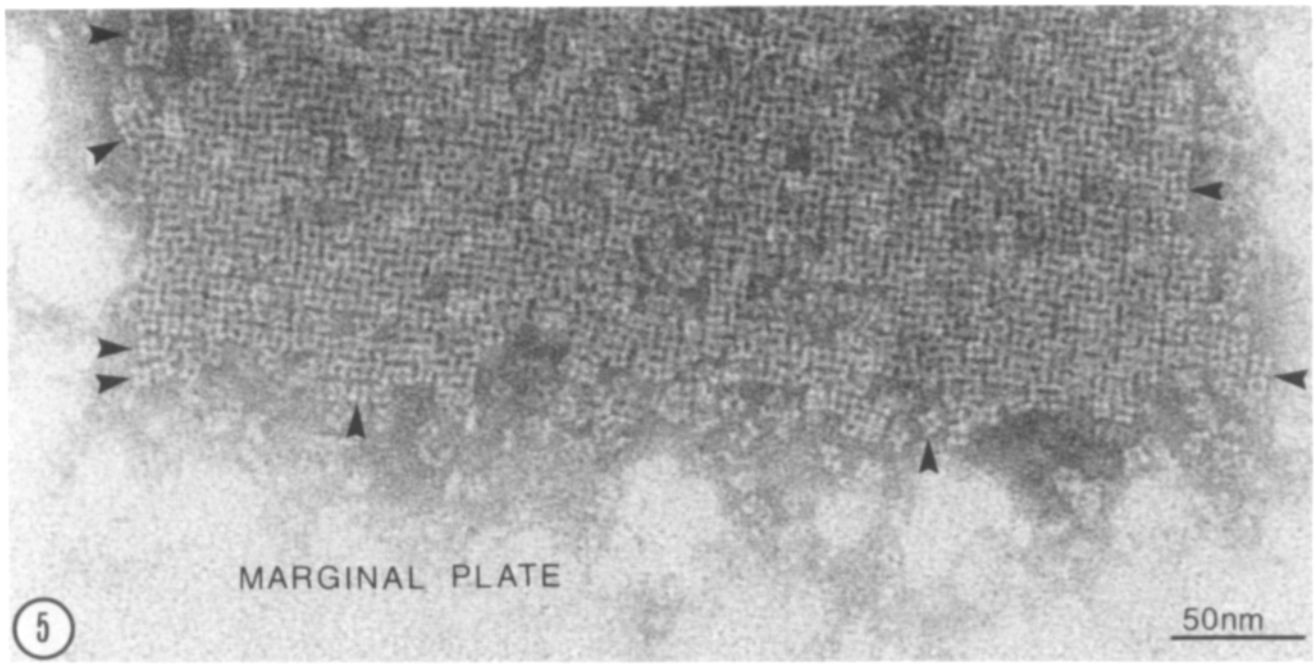


Figure 5. Focal disintegration of the marginal plates, particularly at their edges, reveals a composition of tetrameric square-like units of ~6.5 nm side length (*arrowheads*). Each unit consists of four globular subunits, one globule at each corner. Globules seem to be connected at right angles by thinner strands. Essentially the units in the surface view appear as squares suggesting that three-dimensionally they must represent cube-like protomers with one globular subunit at each corner. Bar, 50 nm.

slightly less than that found in the SPO fraction, which means that L-α-HAOX A was not enriched in the marginal plate fraction. A second enzyme activity that is significantly enhanced over the homogenate was urate oxidase (RSA 109).

SDS-PAGE Analysis of Marginal Plate Fractions

In Fig. 6 *A* the polypeptide pattern as revealed by SDS-PAGE of a highly purified peroxisome preparation (PO) and a marginal plate fraction (MP) is compared. Two polypeptide bands are most prominent in the marginal plate fraction, the major band corresponding to a M_r of 33.5 kD and a minor one to a M_r of 32 kD. Both polypeptide bands are also present in the total peroxisome fraction (Fig. 6 *A*).

Immunoblotting. Polypeptides of highly purified peroxisomes (PO) and marginal plate fractions (MP) separated by SDS-PAGE were blotted and the blots incubated with monospecific polyclonal antibodies raised against peroxisomal oxidases, e.g., L-α-hydroxyacid oxidase isozymes A and B (HAOX A and B), urate oxidase (UOX), D-amino acid oxidase, acyl-CoA oxidase as well as against peroxisomal

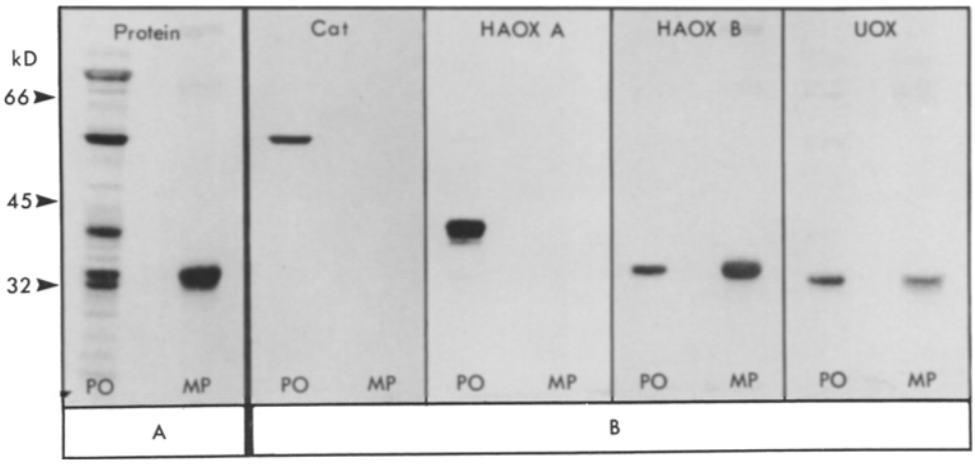
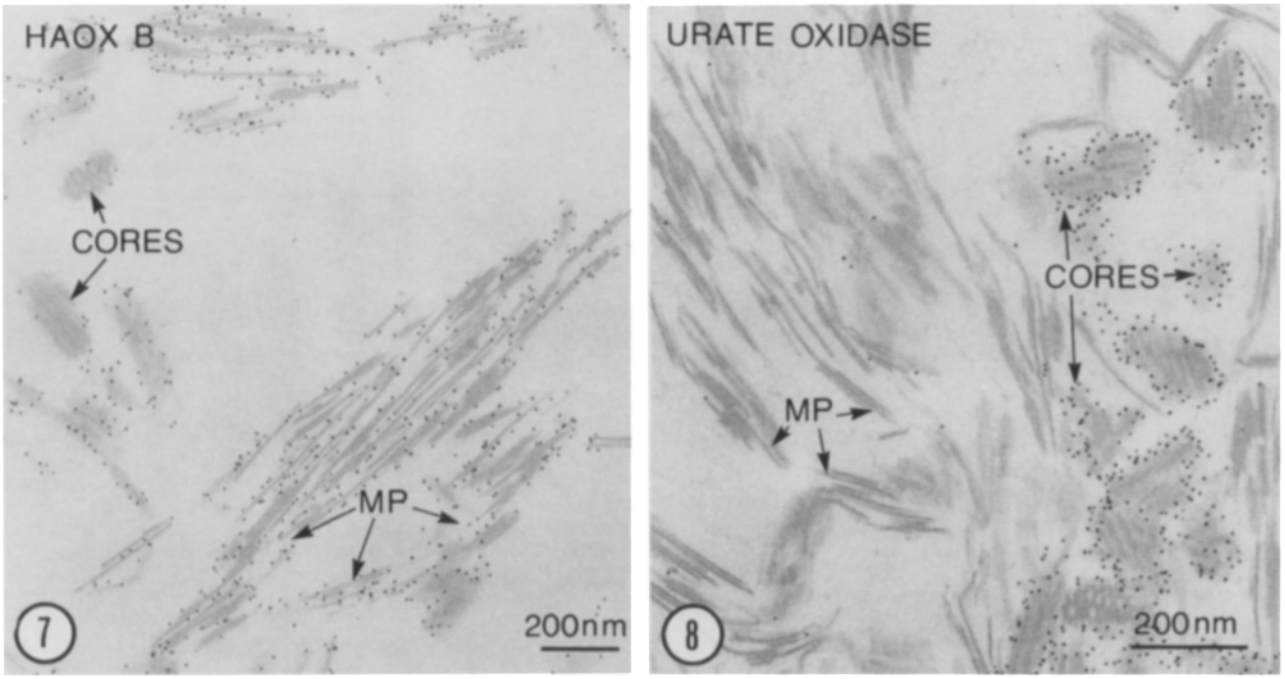


Figure 6. (*A*) SDS-PAGE of highly purified bovine kidney cortex peroxisomes (*PO*) (1.5 μg protein/lane) and purified marginal plates (*MP*) (1.3 μg protein/lane). Proteins were blotted to nitrocellulose and stained with Auro Dye. The marginal plate fraction consists of one major (M_r 33.5 kD) and a second minor (M_r 32 kD) polypeptide component with slightly lower molecular weight, which is not well separated. Both polypeptides are also present in the peroxisome fraction. (*B*) Immunoblots of highly purified bovine kidney cortex peroxisomes (*PO*) (1.3 μg protein/lane) and purified marginal plates (*MP*) (1.0 μg protein/lane). Blots were incubated with antibodies to catalase (*Cat*), hydroxyacid oxidase A and B (*HAOX A* and *HAOX B*) and urate oxidase (*UOX*). The antigen-antibody complexes were visualized with the PAG complex. Note that anti-HAOX B shows reactivity with the major and anti-UOX with the minor component of the marginal plates. All other antibodies tested (see Materials and Methods) showed no reactivity. Standards used were: BSA, 66 kD; ovalbumin, 45 kD; urate oxidase, 32 kD; trypsinogen, 24 kD.



Figures 7 and 8. Preembedding localization of HAOX B and UOX. Figs. 7 and 8 show the localization of gold particles after preembedding incubation of a slightly cross contaminated marginal plate fraction with anti-HAOX B (Fig. 7) and anti-UOX (Fig. 8) and visualization of the antigen-antibody complexes with the PAG. Note that after incubation with anti-HAOX B significant gold labeling occurs only on marginal plates. In contrast after incubation with anti-UOX only the polytubular cores are labeled. Bars, 200 nm.

thiolase, enoyl-CoA hydratase, and catalase (CAT). As is demonstrated by Fig. 6 *B*, the major polypeptide of the marginal-plate fraction (M_r 33.5 kD) clearly exhibits strong reactivity with the anti-HAOX B and the minor one (M_r 32 kD) with the anti-UOX antibody. Anti-catalase and anti-HAOX A (Fig. 6 *B*) as well as all other antibodies tested (not shown) did not bind to marginal plate fraction proteins. These results confirm the biochemical observations that only HAOX B and UOX activities are significantly enriched in marginal plate fractions. Furthermore, these results are in agreement with the morphological findings that core fragments that are known to be composed of UOX are the major contaminants of marginal plate fractions.

Immunocytochemistry. To verify that UOX is only a core constituent and is not associated with the marginal plates, unfixed freshly prepared marginal plate fractions were incubated either with anti-HAOX B or anti-UOX. Marginal plate fractions with relative high cross-contamination rates were chosen to assure that both structural components, marginal plates as well as polytubular cores were present in sufficient amounts. After PAG visualization of bound antibodies, with anti-HAOX B gold labeling is restricted to marginal plates, whereas the polytubular cores are not labeled (Fig. 7). On the other hand, cores and core fragments are exclusively labeled with anti-UOX, with no label observed on marginal plates (Fig. 8).

Discussion

The results of the present study indicate that marginal plates in bovine kidney cortex peroxisomes are plate-like two-dimensional crystals of L-α-hydroxyacid oxidase B. The polytubular cores, the second inclusion, present in bovine renal peroxisomes, on the other hand, have been shown to contain mainly urate oxidase (see also Zaar et al., 1986; Usuda et al., 1988*b*; Angermüller, 1989).

Hydroxyacid Oxidase B and its Tendency to Self-Association

Hydroxyacid oxidase activity has been well known as a peroxisomal marker in mammalian hepatic and renal tissues (for review see DeDuve and Baudhuin, 1966; Hruban and Rechcigl, 1969; Masters and Holmes, 1977; Böck et al., 1980). The enzyme exists in two isozymic forms, L-α-hydroxyacid oxidase A (HAOX A) and B (HAOX B) which in mice have been traced back to different genetic loci (Duley and Holmes, 1974). Both FMN-containing flavoproteins catalyze the oxidation of L-α-hydroxyacids to corresponding α-ketoacids. HAOX A is most active with glycolate or other short-chain L-α-hydroxyacids and is therefore referred to as glycolate oxidase (glycolate: O_2 oxidoreductase EC 1.1.3.1) or short-chain L-α-hydroxyacid oxidase. HAOX B preferentially oxidizes long chain or even aromatic L-α-hydroxyacids and therefore has been termed long chain L-α-hydroxyacid oxidase (L-α-hydroxyacid: O_2 oxidoreductase EC 1.4.3.2) (for review see Masters and Holmes, 1977). Rat kidney HAOX B also metabolizes L-amino acids at a quite low rate and indeed was first described as L-amino acid oxidase (Blanchard, 1945). In mammals HAOX A is predominantly localized in liver but has also been found in kidneys of pig (Robinson et al., 1962; Saga et al., 1969) and marsupials (Duley, 1976). HAOX B is mainly found in the kidney tissue. The HAOX isozymes also differ in the apparent molecular weights of their monomers (HAOX A 36.8 kD; HAOX B 33.5 kD) (Yokota et al., 1985). For the rat HAOX B, in early

studies apparent molecular weights between 40,000 (Duley and Holmes, 1976) and in excess of 430,000 (Blanchard et al., 1945) have been reported. This controversy was shown to be related to the tendency of this isozyme to self-association in vitro dependent on the protein concentration (Philips et al., 1976). According to these authors rat HAOX B, at infinite dilution, forms a protomer of four subunits (M_r 150,000). These protomers show self-association into dimers (8 subunits), tetramers (16 subunits) and multiples thereof. In the present study we have shown that HAOX B is the main component of the marginal plates, the two-dimensional plate-like crystal in the matrix of bovine renal peroxisomes. Negative staining of marginal plates revealed a composition of tetrameric structures which with the additional information of the thickness of the marginal plates are inferred to be of octameric nature. The subunit composition as well as the mode of association into plate-like crystals thus, seem to correspond to the self-association behavior described for rat HAOX B by Philips et al. (1976). It is of interest that in freeze-etch preparations of angular beef kidney cortex peroxisomes containing marginal-plates a similar lattice structure has been observed on the straight E-fracture faces of membranes that are underlied by marginal plates (Zaar and Fahimi, 1990).

Matrical Inclusions in Peroxisomes

Crystalline matrical inclusions are a common feature of peroxisomes from many different sources (for reviews see Hruban and Rechcigl, 1969; Böck et al., 1980). Inclusions occur either genuinely, or are induced (Veenhuis and Harder, 1987; Gorgas and Krisans, 1989). Hitherto only two types of inclusions have been isolated and studied in detail. (*a*) The complex polytubular cores that are common in peroxisomes of mammalian hepatocytes, except hominoids and humans (for review see Usuda et al., 1988*a*; Völkl et al., 1988), are composed mainly of urate oxidase, a cuproprotein which forms protomers of four subunits (Pitts et al., 1974). (*b*) In addition, in certain methylotropic yeasts, growth on methanol-containing nutrient media induces very large cubical crystals in peroxisomes, which are composed of alcohol oxidase (Veenhuis et al., 1981) a flavoprotein (FAD) which forms protomers of 8 identical subunits (Kato et al., 1976; Goodman et al., 1984). With the isolation of marginal plates in the present study, a third type of common peroxisomal crystalline inclusion now has been characterized. As noted above, marginal plates are plate-like crystals consisting of HAOX B protein, a FMN-containing flavoprotein that forms protomers of four identical subunits (Philips et al., 1976). In all three cases an oligomerization of the oxidase protomers apparently generates the typical morphologically recognizable crystalline inclusions. Such oligomerization of oxidases could give rise to the known subcompartmentation of the peroxisomal matrix (Alexson et al., 1985; Yokota et al., 1987). The functional significance of such a subcompartmentation is not fully understood. One aspect could be to achieve a high focal concentration for a particular enzyme in the matrix containing otherwise only soluble enzymes (Alexson et al., 1985). To our knowledge nothing is known about the mechanisms underlying the association of protomers into crystalline structures. Further studies using isolated peroxisomes from beef kidney cortex and the isolation procedure described in this study for marginal plates should be helpful in elucidating the mechanisms of marginal plate assembly.

The excellent technical assistance of Karin Mertzsch, Heribert Mohr, Gabi Krämer,and Inge Frommer is gratefully acknowledged. We wish to thank Prof. Dr. H. Schirmer and PD Dr. L. Krauth-Siegel Institute for Biochemistry II, University of Heidelberg for help in spectral characterization of marginal plate chromophore.

This study was supported by grants Fa 146/1-3 and Vo 317/3-2 from Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, Germany.

Received for publication 26 September 1990 and in revised form 13 December 1990.

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