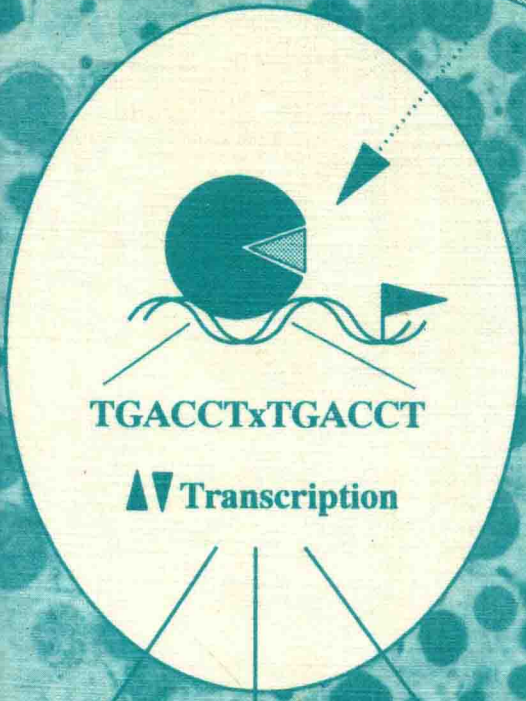
PEROXISOMES

Biology and Importance in Toxicology and Medicine



Edited by Gordon Gibson
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Preface

Peroxisomes (or 'microbodies') are single membrane-limited cytoplasmic organelles present in cells of animals, plants, fungi and protozoa. Studies by De Duve and coworkers demonstrated that rat liver peroxisomes contain both hydrogen peroxide generating oxidase enzymes and catalase. Later studies by Lazarow and De Duve demonstrated that liver peroxisomes, like mitochondria, contain a complete fatty acid β-oxidation cycle, whereas Hess and coworkers first reported that clofibrate, a hypolipidaemic drug, could produce hepatic peroxisome proliferation in the rat. Since these pioneering studies, much effort has been devoted to elucidating the biochemical properties of these organelles and their response to chemicals and other stimuli.

The various chapters contained in this volume cover all aspects of the structure, function, regulation and response to chemicals of mammalian peroxisomes. The biogenesis of peroxisomes in various species is considered by Small, whereas the metabolic role of peroxisomes is described by Mannaerts and Van Veldhoven. Peroxisome disorders in man are reviewed by Wanders and coworkers.

Several chapters consider the mechanism of initiation of peroxisome proliferation in hepatocytes together with aspects of the molecular biology of peroxisomes and peroxisomal enzymes. Green and coworkers describe the molecular mechanism of peroxisome proliferator action which appears to involve one or more peroxisome proliferator-activated receptors. The mechanism of initiation of peroxisome proliferation, together with the interaction with lipid metabolism and receptors is covered by Bell and Elcombe. Rodent liver peroxisome proliferators are known to induce cytochrome P450 isoenzymes in the CYP4A subfamily and the relationship of this phenomenon to the mechanism of peroxisome proliferation is discussed by Gibson and coworkers. The molecular basis of gene regulation by peroxisome proliferators is also reviewed by Latruffe and coworkers, whereas Osumi and coworkers describe in-depth studies on the regulatory sequence of the acyl-CoA oxidase gene. The molecular evolution and functional significance of the urate oxidase gene is reviewed by Yeldandi and Yeldandi. Many peroxisome proliferators or their subsequent metabolites contain a free carboxyl group and hence may form coenzyme A esters. The synthesis of such esters and their possible role in peroxisome proliferation and the hepatic effects of peroxisome proliferation is

described by Bronfman. Apart from particular chemicals various dietary factors and metabolic states can modulate peroxisome biogenesis in rodent liver and this is reviewed by Flatmark and Christiansen. The use of sulphur substituted fatty acid analogues to study peroxisome proliferation and effects on lipid metabolism is described by Berge and colleagues.

Apart from studies in the intact animal, peroxisome proliferation can readily be demonstrated in rat and mouse hepatocyte cultures. The use of primary hepatocyte cultures as an *in vitro* model system to investigate various aspects of peroxisome proliferation is reviewed by Bieri. Some studies have demonstrated structure–activity relationships for peroxisome proliferation in rodent hepatocytes. An overview of structure–activity relationships is presented by Lake and Lewis, whereas Eacho and coworkers describe studies with a number of tetrazole substituted acetophenones. The structural requirements for peroxisome proliferation in rat hepatocyte cultures by some phenoxyacetic acid and fatty acid analogues is covered by Feller and Intrasuksri.

Much effort has been devoted to investigating differences in hepatic peroxisome proliferation. While rats and mice may be considered responsive species to peroxisome proliferators, other species such as the Syrian hamster are less responsive and species such as the guinea-pig appear to be essentially refractory. Species differences in the hepatic effects of bezafibrate are presented by Fahimi and coworkers, whereas species differences in both the metabolism of and effects of di-(2-ethylhexyl)phthalate and di-(2-ethylhexyl)adipate are reported by Lhuguenot and Cornu. The effects of clofibrate and some analogues in the rat and primates are described in the chapter by Holloway and Thorp and that by Tucker and Orton. The chapter by Holloway and Thorp also presents information on differences between two enantiomers.

Apart from the liver, peroxisome proliferation may also be observed in other tissues. The effect of rodent liver peroxisome proliferators in extrahepatic tissues including the kidney, intestine, testis and adipose tissue is reviewed by Hinton and Price.

Many peroxisome proliferators have been shown to increase the incidence of liver tumours in rats and mice. Clearly the elucidation of the mechanisms of peroxisome proliferator-induced rodent liver tumour formation together with species differences in response is of great importance in assessing the hazard of these chemicals to man. Rao and Reddy describe the pathology of peroxisome proliferator-induced rodent liver tumours and provide an overview of possible mechanisms of hepatocarcinogenesis. Grasso reviews both early and late changes produced by peroxisome proliferators in rodent liver including the production of altered hepatic foci.

Peroxisome proliferators are considered to be non-genotoxic carcinogens and the various genotoxicity studies with hypolipidaemic agents and phthalate esters are reviewed by Budroe and Williams. Takagi and coworkers report data on the ability of peroxisome proliferators to produce oxidative DNA damage, as assessed by levels of 8-hydroxydeoxyguanosine, in rodent liver. The hyperplastic effects of peroxisome proliferation in rodent liver is reviewed by

Styles, whereas Lake considers the respective roles of peroxisome proliferator-induced oxidative stress and cell replication in the mechanism of liver tumour formation. The ability of peroxisome proliferators to act as either initiators or promoters of rodent hepatocarcinogenesis is considered in the chapter by Popp and that by Cattley and Grasl-Kraupp and coworkers. While there is little evidence for peroxisome proliferators being initiators of hepatocarcinogenesis, they do appear to promote the growth of preneoplastic lesions and the conversion of such lesions into tumours. Finally, Stott and Hawkins review aspects of the risk assessment of peroxisome proliferators.

During the final stages of editing this work, we were made aware of the untimely death of Dr Jeff Thorp, formerly of ICI Pharmaceuticals Division, Safety of Medicines Department, Macclesfield, England. In the field of peroxisome proliferation, Jeff will best be remembered as being 'Mr Clofibrate', as he was primarily responsible for the development of this drug in the 1960s at ICI. As clofibrate is the 'classical' peroxisome proliferator and one of the first to be identified, Jeff's contribution then clearly makes him one of the forefathers of peroxisome proliferation. After his recent retirement from ICI, Jeff still maintained his interest in the area and still made a significant impact at both national and international meetings. His presence will be dearly missed and we respectfully dedicate this work to his contributions to the field.

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Contents

Chapter 1

Peroxisome biogenesis

G. M. Small

Introduction

Peroxisomes are single membrane-bound organelles that are present in most eukaryote cells. They contain a variety of enzymes that have functions in cell respiration and fatty acid metabolism. The number per cell, size and specific enzyme composition of peroxisomes varies between organisms and between tissues. Peroxisomes contain no DNA (Leighton *et al.*, 1968; Kamiryo *et al.*, 1982), therefore all of their proteins are encoded by nuclear genes. Consequently, these proteins must be directed from the cell cytosol to the organelle. Our current understanding of this process, and the mechanisms involved in the formation of new peroxisomes is the subject of this chapter.

Early postulations regarding the biogenesis of peroxisomes were largely based on morphological studies. Peroxisomes were often shown to be in close association with the endoplasmic reticulum, and connections between these two organelles were described (Novikoff and Shin, 1964). Thus it was envisaged that peroxisomal proteins were synthesized in the endoplasmic reticulum, and that segments of this organelle would pinch off to form peroxisomes (see de Duve, 1973). A multitude of data, generated in the last 10 years, have not supported this theory. There is now compelling evidence that peroxisomal proteins are synthesized on free polyribosomes in the cell cytosol, and that they are imported into pre-existing peroxisomes post-translationally. New peroxisomes are then thought to form by fission from pre-existing peroxisomes. The evidence for this was reviewed in detail in 1985 (Lazarow and Fujiki, 1985) and will not be reiterated here. In this chapter I present more recent data that have emerged over the last few years to update our knowledge and ideas of peroxisome biogenesis.

Peroxisome assembly

It is now established that peroxisome biogenesis occurs by the import of both matrix and membrane proteins into pre-existing peroxisomes (Lazarow and Fujiki, 1985; Borst, 1989). Thus every cell must contain at least one peroxisome, and there must be a rapid synthesis of peroxisomal membranes to accommodate

the process of peroxisome proliferation. Certainly, membranes are often described to be associated with peroxisomes. While early reports identified these membranes as smooth endoplasmic reticulum, Lazarow *et al.* (1980) suggested that peroxisomes themselves may be interconnected either temporarily or permanently, thus forming a peroxisome reticulum. Three-dimensional reconstructions of sections of regenerating mouse and rat liver peroxisomes (Gorgas, 1985; Yamamoto and Fahimi, 1987) have confirmed that there are interconnections between peroxisomes. In the model of regenerating rat liver after partial hepatectomy, two to five peroxisomes were found to be interconnected via a membrane reticulum (Yamamoto and Fahimi, 1987). This reticulum would presumably break apart on homogenization, and the membranes would reseal to form individual isolated peroxisomes. Proliferation of rat liver peroxisomes, following treatment with the hypocholesterolaemic drug BM 15766 (Baumgart *et al.*, 1987), is accompanied by a proliferation of membranous structures associated with peroxisomes. Using antibodies to peroxisomal integral membrane proteins Baumgart *et al.* (1989) demonstrated that these membrane loops are part of the peroxisome membrane system. The membranes label with an antibody to the peroxisomal integral membrane protein PMP70 and do not stain for glucose 6-phosphatase, an endoplasmic reticulum marker. Thus, it is likely that the first step which occurs during peroxisome proliferation is an increase in the peroxisomal membrane system, and that this is necessary for the subsequent import of matrix proteins.

Peroxisome assembly has been studied morphologically and biochemically in the methylotrophic yeast *Candida boidinii* (Veenhuis and Goodman, 1990). This study monitored the formation of new peroxisomes in cells precultured in glucose containing medium and then diluted into methanol containing medium. The formation of new peroxisomes appeared to occur in three stages. Within the first hour of the switch to induction medium the small peroxisomes that are present when the cells are grown in glucose became elongated. During this stage there was an increase in catalase activity and in the amount of the integral membrane protein PMP47 in the peroxisome membranes. This was followed by division of the peroxisomes during the next 2–3 h to form a peroxisome cluster. Three peroxisomal proteins, alcohol oxidase, dihydroxyacetone synthase and a peripheral membrane protein PMP20 were all induced during this period. Finally, as the peroxisomes enlarged there was a decrease in their number per cell. These studies suggest that there is a defined pathway for peroxisome growth and maturation during induction. The events are similar to those described for proliferating peroxisomes of rat liver in that the first detectable event is an induction of peroxisomal membrane proteins, which is presumably followed by induction and import of matrix proteins as the peroxisomes increase in size. Finally, the peroxisomes divide to form daughter organelles.

Organization of the peroxisome matrix

Peroxisomes were classically described as single membrane-bound organelles with a homogenous non-compartmentalized matrix that may contain a single electron-dense core (de Duve and Baudhuin, 1966). Urate oxidase is present in liver peroxisomes of most mammals and is localized exclusively in the crystalloid cores (Hruban and Swift, 1964; Leighton *et al.*, 1969). This enzyme is lacking in hepatic peroxisomes of man, and these peroxisomes do not contain cores (Goldfischer and Reddy, 1984). However, it should not be taken for granted that peroxisomes lacking cores also lack uricase as this enzyme is present in soybean nodule peroxisomes which lack cores (Nguyen *et al.*, 1985).

The question of protein organization or compartmentalization of the peroxisomal matrix enzymes is also under investigation. Alexson *et al.* (1985) demonstrated that enzymes leaked out of peroxisomes in a differential manner when the organelles were subjected to a variety of treatments such as freeze–thawing or mild sonication. They found that catalase and thiolase leaked out of peroxisomes most readily, while acyl-CoA oxidase was more resistant, especialy to mechanical treatments such as sonication. The bifunctional (or trifunctional) protein of the β-oxidation cycle, enoyl-CoA hydratase/β-hydroxyacyl-CoA dehydrogenase (HD), exhibited a tendency to remain associated with the peroxisome particles and urate oxidase was not released in any of the experiments, but rather remained with the membrane fragments. The results of these experiments are suggestive of some sub compartmentalization within the peroxisome matrix. Results of Triton X-114 fractionation of mouse liver proteins are in agreement with those of Alexson *et al.* (1985). Triton X-114 binds proteins that have hydrophobic binding sites, and such proteins are recovered in the detergent phase, whereas non-hydrophobic proteins fractionate with the aqueous phase. Of the mouse liver peroxisomal proteins examined, the membrane protein PMP70 partitioned in the detergent phase and catalase and acyl-CoA oxidase were recovered in the aqueous phase (Poole and Crane, 1992). In this case the bifunctional protein was in the detergent phase in control animals, but in livers from animals treated with clofibrate there was more HD in the aqueous phase. This suggests that HD may be tightly associated with the peroxisome membrane, but that this association may be disrupted in clofibrate treated animals.

Other peroxisomal enzymes, not measured in the experiments outlined above, have been shown by morphological studies to have specific intraperoxisomal localizations. Histological and immunoelectron microscopy studies revealed that D-amino acid oxidase is localized to a small area in the matrix of rat liver peroxisomes, that appears to be devoid of other peroxisomal proteins (Usuda *et al.*, 1991). Using similar methods, Zaar and Fahimi (1991) studied the localization of the two isozymes of L-α-hydroxyacid oxidase (A and B) in beef and sheep kidney cortex. They demonstrated that the A form (which is found exclusively in rat liver peroxisomes) was diffuse in the peroxisome matrix, while the B form (the isozyme found normally in renal peroxisomes) was localized to

crystalline structures termed 'marginal plates' that are commonly found in kidney peroxisomes, but also occasionally in peroxisomes from other tissues.

Assembly of peroxisomal proteins

The majority of peroxisomal proteins are oligomers, many requiring cofactors to enable them to be enzymatically active (Lazarow and Fujiki, 1985). Catalase is a tetrameric haemoprotein which is synthesized as a haeme-less precursor (Lazarow and de Duve, 1971). The majority of haeme addition, and oligomerization takes place within the peroxisome (Lazarow and de Duve, 1973). This is consistent with the idea that the protein is transported into peroxisomes in an unfolded state, and that the functional tertiary structure is formed within the organelle.

The assembly of alcohol oxidase in yeast has been studied extensively. This protein is an octomer in its native form. Octomerization occurs with a half-life of 20 min and the process takes place within the peroxisomes (Goodman *et al.*, 1984). Alcohol oxidase of *Hansenula polymorpha* has been expressed in *Saccharomyces cerevisiae* (Distel *et al.*, 1987). This protein was imported into peroxisomes, but failed to oligomerize, and was enzymatically inactive. In contrast, when an extra copy of the alcohol oxidase gene was introduced into *H. polymorpha* itself, the enzyme octomerized and was enzymatically active (Distel *et al.*, 1988). In this case the peroxisomes were enlarged, indicating that there was also an increase in peroxisome membranes, and the enzyme was located within a single large peroxisome in each cell (Distel *et al.*, 1988). The fact that new peroxisomes were not formed in *Hansenula* may reflect differences in peroxisome formation and compartmentalization in this yeast compared with *S. cerevisiae*. Crystalloid structures are not observed in *S. cerevisiae* when it is grown in peroxisome-inducing medium, nor when alcohol oxidase is expressed in this yeast (Distel *et al.*, 1987).

Targeting signals

Proteins that are synthesized in the cytosol and then transported to their host organelle contain information within their primary sequence that plays a role in targeting them to their respective organelles. Such topogenic sequences have been demonstrated in mitochondrial, chloroplast and nuclear proteins (for reviews see Pfanner and Neupert (1989), Smeekens *et al.* (1990) and Silver (1991)). We now have strong evidence, gained by a variety of experimental approaches both *in vitro* and *in vivo*, that such signals exist in peroxisomal proteins.

The finding that firefly luciferase is a peroxisomal protein (Keller *et al.*, 1987) has provided a useful model for studying peroxisome targeting signals. Keller *et al.* (1987) expressed the cloned firefly luciferase gene in CV-1 cells and localized

the expressed product by immunofluorescence and by immunoelectron microscopy. This study demonstrated that luciferase is targeted to peroxisomes of mammalian cells. By the same experimental approach and the use of deletions, linker insertions and gene fusions, this group identified a tripeptide (serine-lysine-leucine) at the carboxy terminus of luciferase, that is involved in directing this protein to peroxisomes (Gould *et al.*, 1987). Subsequent searches revealed that this tripeptide, or conservative variants thereof, exists at the carboxy termini of a number of peroxisomal proteins (Table 1.1), and is one class of peroxisomal targeting signal (Gould *et al.*, 1988, 1989, 1990a).

Table 1.1. Comparison of the carboxy termini of several peroxisomal matrix proteins

Protein	Organism		C-terminus	+/−*	Reference
Luciferase	Firefly		S-K-L	+	de Wet *et al.* (1987)
Catalase	Rat		A-N-L	?	Furuta *et al.* (1986)
	S. cerevisiae		S-K-F	−	Cohen *et al.* (1988)
	Human		A-N-L	?	Korneluk *et al.* (1984)
	C. tropicalis		P-R-K	−	Murray and Rachubinski (1987), Okada *et al.* (1987)
Acyl-CoA oxidase	Rat		S-K-L	+	Miyazawa *et al.* (1987)
	S. cerevisiae		I-N-K	−	Dmochowska *et al.* (1990)
	C. tropicalis	POX4	L-S-K	−	Okazaki *et al.* (1986)
		POX5	L-S-S	−	Okazaki *et al* (1986)
		POX2	L-S-K	−	Okazaki *et al.* (1987)
	C. maltosa		L-S-K	−	Hill *et al.* (1988)
Hydratase–dehydrogenase +epimerase thiolase	Rat		S-K-L	+	Osumi *et al.* (1985)
	C. tropicalis		A-K-I	†	Nuttley *et al.* (1988)
	Rat		P-G-N	−	Hijikata *et al.* (1987), Hijikata *et al.* (1990), Bodnar and Rachubinski (1990)
	S. cerevisiae		I-K-E	−	Igual *et al.* (1991)
D-Amino acid oxidase	Pig		S-H-L	+	Fukui *et al.* (1987)
Alanine glyoxylate aminotransferase	Rat		N-K-L	−	Oda *et al.* (1987)
	Human		K-K-L	?	Takada *et al.* (1990)
Urate oxidase	Rat		S-R-L	+	Motojima *et al.* (1988)
	Rabbit		S-R-L	+	Motojima and Goto (1989)
	Soybean		S-K-L	+	Nguyen *et al.* (1985)
Amine oxidase	*H. polymorpha*		C-G-K	−	Bruinenberg *et al* (1989)
Alcohol oxidase	*H. polymorpha*		A-R-F	−	Ledeboer *et al.* (1985)
Dihydroxyacetone synthase	*H. polymorpha*		G-K-A	?	Janowiez *et al.* (1985)
Citrate synthase	*S. cerevisiae*		S-K-L	+	Rosenkrantz *et al* (1986)

* Predicted to act as a peroxisomal targeting signal according to Gould *et al.* (1989).
† This tripeptide does appear to act as a peroxisomal targeting signal in *Candida tropicalis* (Aitchison *et al.*, 1991).