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REVIEW ARTICLE

Import of proteins into peroxisomes and other microbodies

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INTRODUCTION

A characteristic feature of eukaryotic cells is the presence of different membrane-enclosed compartments or organelles. Compartmentalization apparently enables cells to separate, regulate and optimize different metabolic pathways and functions. Based on morphology and function, several types of organelle can be distinguished, each containing their own set of proteins and other molecular tools. To fully appreciate how a eukaryotic cell works, it is necessary to understand how organelles function, how they arise and are maintained. Proteins play an essential role in these processes; they catalyse the reactions performed by the organelles, they regulate transport of substrates and products and are involved in the delivery of proteins into the organelles. Almost all proteins are synthesized in the cytosol, yet many have their destination inside one of the intracellular compartments (Fig. 1). Therefore, specific routing systems must exist that direct intracellular protein traffic.

This review focuses on a particular type of organelle, the microbody. Since its discovery, about four decades ago, it has become clear that it is involved in a variety of metabolic processes. Microbodies are known under different names, peroxisomes, glyoxysomes and glycosomes, reflecting their widely different functions. Because microbodies belong to the last class of organelle discovered, knowledge of their function and biogenesis lags behind that of other organelles. However, aided by the growing number of sequences of genes encoding microbody proteins, the molecular details of microbody protein import are becoming unravelled fast. A brief description of the various microbody subclasses and their functions is presented in the next section, followed by sections on the current knowledge of microbody biogenesis, in particular of protein routing into peroxisomes, topogenic signals and components of the protein import machinery.

DISCOVERY AND DEFINITION OF MEMBERS OF THE MICROBODY FAMILY

The name **microbody** was used first in 1954 by Rhodin to describe a new type of organelle which he observed by electron microscopy in mouse-kidney cells (Rhodin, 1954). These microbodies are small vesicular compartments with a diameter of about 0.5 μm, bounded by a single membrane and filled with a dense granular matrix, sometimes containing a crystalline core. For many years the function of microbodies was an enigma, until de Duve and coworkers discovered that they contain urate oxidase (uricase), D-amino acid oxidase and catalase (de Duve & Baudhuin, 1966), which were previously thought to be located in lysosomes. Since both oxidases produce hydrogen peroxide, which is subsequently degraded by catalase, this type of microbody was called a **peroxisome**.

In 1967, Breidenbach and Beevers demonstrated that the two key enzymes of the glyoxylate cycle in germinating seeds, malate synthase and isocitrate lyase, previously believed to be mitochondrial, are located in a distinct particle which they named the **glyoxysome**. Thereafter, it was found that these glyoxysomes may not only contain all enzymes of the glyoxylate cycle (see Tolbert, 1981, for a review), but also the enzymes needed for the β-oxidation of fatty acids (Cooper & Beevers, 1969; Trelease, 1984) enabling germinating seeds to convert lipids into carbohydrates. The glyoxysomal β-oxidation enzymes catalyse the same reactions as their mitochondrial counterparts, with one exception: the first enzyme of the glyoxysomal β-oxidation is an acyl-CoA oxidase instead of a dehydrogenase. To degrade the peroxide produced, glyoxysomes also contain catalase and therefore can be considered as true peroxisomes, equipped with catalase and a hydrogen peroxide-producing oxidase (Tolbert, 1981). The term glyoxysome is now generally used for peroxisomes that hold at least malate synthase and isocitrate lyase. These two enzymes of the glyoxylate cycle are unique for glyoxysomes, whereas malate dehydrogenase, aconitase and citrate synthase also occur as mitochondrial isoenzymes.

In 1976, β-oxidation enzymes were also discovered in mammalian peroxisomes (Lazarow & de Duve, 1976) which again proved the relationship between glyoxysomes and peroxisomes. Moreover, plant and yeast glyoxysomes may lose their glyoxylate-cycle enzymes while keeping peroxisomal enzymes (Burke & Trelease, 1975; Schopfer *et al.*, 1976; Zwart, 1983; Titus & Becker, 1985). Microbodies with glyoxylate-cycle enzymes have also been discovered in organisms other than plants, such as (aerobic) protozoa (Müller, 1975, 1988), yeast (Szabo & Avers, 1969), amphibia (Goodman *et al.*, 1980) and in birds (Davis *et al.*, 1988). The glyoxylate enzymes malate synthase and isocitrate lyase activities have been found in mammals, but their peroxisomal localization has not yet been established (Davis *et al.*, 1989, 1990).

Another type of microbody, the **glycosome**, was discovered in parasitic protozoa, in particular the Trypanosomatidae (Opperdoes & Borst, 1977). Although glycosomes morphologically resemble peroxisomes, their enzymic content is aberrant; they mainly contain enzymes of the glycolytic pathway (Opperdoes, 1988). Their true peroxisomal nature is still a matter of debate; but in glycosomes of certain species 'authentic peroxisomal' enzymes like catalase and enzymes from the β-oxidation system or the ether-lipid biosynthesis pathway are present (Opperdoes, 1988; Opperdoes *et al.*, 1988), indicating that glycosomes are not only morphologically related to peroxisomes. This means that the best biochemical description for a microbody in general (not including hydrogenosomes) is a single-membrane-bound particle containing catalase and some enzymes of the β-oxidation system.

Finally, organelles called **hydrogenosomes** are found in anaerobic protozoa that lack mitochondria (Müller, 1975, 1988). These organelles have a completely different enzymic composition, devoid of typical peroxisomal enzymes such as catalase or enzymes of the β-oxidation pathway but containing an oxygen-sensitive hydrogenase as characteristic enzyme (Müller, 1975; Yarlett *et al.*, 1981). Most hydrogenosomal enzymes participate in the metabolism of pyruvate into acetate, CO_2 and H_2. Originally, hydrogenosomes were described as microbody-like organelles surrounded by a single membrane and having an

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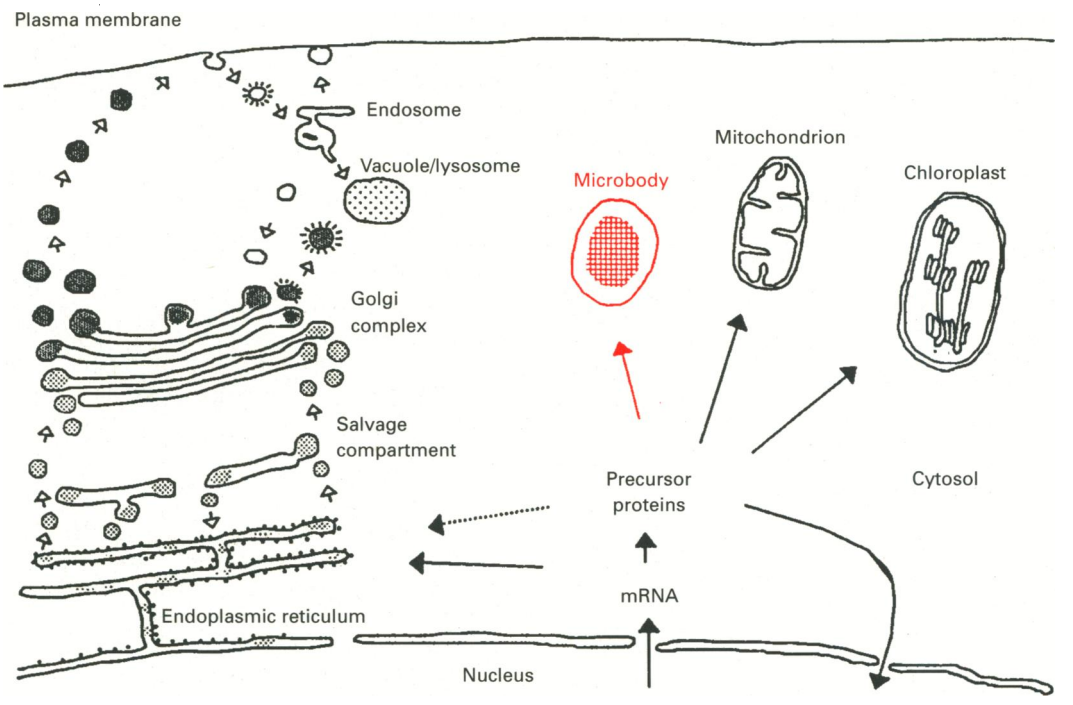

Fig. 1. Protein traffic inside a eukaryotic cell

Co-translational protein import is indicated by a dotted arrow, post-translational import by closed arrowheads, protein transport that occurs via vesicular traffic by open arrowheads. Copied and modified (with permission) from Verner & Schatz (1988).

equilibrium density comparable to other microbodies (Müller, 1975). More recent data indicate that hydrogenosomes are enclosed by two membranes and have a mitochondria-like morphology with extensive folding of the inner membrane (Finlay & Fenchel, 1989; Yarlett *et al.*, 1981). The first sequences of hydrogenosomal proteins show the presence of a mitochondria-like presequence, indicating once more that hydrogenosomes have erroneously been called microbodies (Johnson *et al.*, 1990; Müller, personal communication). Table 1 summarizes common and unique properties of organelles that import proteins post-translationally and is intended to show the unique features of microbodies. Details of their protein import will be discussed below.

FUNCTION OF MICROBODIES

Microbodies are distinguished from other organelles above all by the amazing variety of metabolic processes they can harbour. Until 1982, already 40 enzymic functions had been found to reside in peroxisomes (Tolbert, 1981). Some metabolic routes are present in both higher and lower eukaryotes; the more specialized reactions are species-specific. Metabolic pathways that are generally peroxisome-bound include (*a*) the hydrogen-peroxide producing oxidation of various compounds like D-amino acids, L-α-hydroxy acids, (poly)amines, methanol, oxalate, phytanic acid, L-pipecolic acid, urate, xanthine or glyoxylate (Yokata *et al.*, 1985; Hölttä, 1977; Zwart *et al.*, 1980; Beard *et al.*, 1985; Wanders *et al.*, 1989; Beard & Holtzman, 1987; Tolbert, 1981) and (*b*) the β-oxidation of alkanes, (very) long-chain and unsaturated fatty acids (Fukui & Tanaka, 1979; Dommes *et al.*, 1981). Other, more specialized metabolic routes occurring in microbodies have been observed in certain organisms; e.g. bioluminescence in algae (Nicolas *et al.*, 1987; Morse *et al.*, 1990) and insects (Keller *et al.*, 1987), penicillin biosynthesis in *Penicillium chrysogenum* (Müller *et al.*, 1991), biosynthesis of ether-phospholipids (plasmalogens and alkyl-glycerophospholipids) (Schutgens *et al.*, 1986), bile acids (Östlund Farrants *et al.*, 1989) and cholesterol in mammals (Keller *et al.*, 1986; Thompson *et al.*, 1987).

Many peroxisomal enzymes are induced under specific environmental conditions. Extreme examples have been encountered in the methylotrophic yeast *Hansenula polymorpha*. During growth on glucose or glycerol its peroxisomes have no apparent function and are therefore called 'unspecialized microbodies' (Veenhuis & Harder, 1987). Switching *H. polymorpha* from glucose to methanol as the sole source of carbon and energy leads to the synthesis of large amounts of the peroxisomal enzyme alcohol (or methanol) oxidase. Concomitant with the induction of alcohol oxidase, peroxisomes proliferate from one or two small microbodies per cell to over 20 large, cubic peroxisomes filled with crystalline alcohol oxidase. In extreme cases, these 'micro'-bodies occupy up to 80% of the cell volume (Veenhuis *et al.*, 1978, 1981). Similarly, growth on oleate (Veenhuis *et al.*, 1987) or alkanes (Fukui & Tanaka, 1979) increases the size and number of peroxisomes in other yeasts. Proliferation of microbodies has been observed in other organisms too, for example plant glyoxysomes proliferate during germination of seeds (Lazarow & Fujiki, 1985). Peroxisome proliferation in mammalian cells can be induced by hypolipidaemic drugs like clofibrate (Reddy *et al.*, 1980; Bremer *et al.*, 1981), plasticizers or chlorinated hydrocarbons (Lock *et al.*, 1989; Bremer *et al.*, 1981), thyroid hormones (Fringes & Reith, 1982; Just & Hartl, 1983), high-fat diets (Ishii *et al.*, 1980) and diabetes (Horie *et al.*, 1981).

BIOGENESIS OF MICROBODIES

For many years it was believed that microbodies originate from the endoplasmic reticulum by budding (for a review see Lazarow & Fujiki, 1985). This observation was based on direct

Table 1. Some properties of organelles that import proteins post-translationally

	Microbodies						
	Peroxisomes	Glyoxysomes[a]	Glycosomes	Hydrogenosomes	Mitochondria	Chloroplasts	Nuclei
Membranes	Single	Single	Single	Double[b]	Double	Double	Double
DNA	Absent	Absent	Absent	Absent	Present	Present	Present
Crystalline inclusions	Sometimes	Sometimes	Sometimes	Sometimes	Absent	Absent	Absent
Average density (g/ml)[c]	1.23[d]	1.23–1.25[e]	1.23[f]	1.18[g]	1.16–1.18[h]	1.22–1.25[h]	Not applicable
Characteristic enzymes or pathways	Catalase, H_2O_2-producing oxidases, β-oxidation	Malate synthase, isocitrate lyase, malate synthase, aconitase, citrate synthase, β-oxidation, catalase	Glycolysis (catalase)[i], (β-oxidation)[i]	Hydrogenase, H_2- and acetate-producing enzymes	Tricarboxylic acid cycle, oxidative phosphorylation	Photosynthesis	DNA and RNA synthesis
Import signal							
Location	*C*-terminal (tripeptide)[j] *N*-terminal (thiolase)	*C*-terminal (tripeptide)[j]	*C*-terminal (tripeptide)[j], *N*-terminal (aldolase) *C*-terminal (PGK[l])	*N*-terminal[k]	*N*-terminal	*N*-terminal	Internal
Number of amino acids	3, 11 (rat thiolase B)	3	3, 20 (PGK[l])	8 (ferrodoxin)[k], 9 (succinate thiokinase)[k]	20–70	20–70	4 and up[m]
Cleavage of signal	Exceptional	Exceptional	No	Yes	Yes	Yes	No

[a]Glyoxysomes are a special class of peroxisomes.
[b]Some publications mention one membrane (see text).
[c]In sucrose gradient.
[d]Baudhuin (1974).
[e]Huang (1981).
[f]Opperdoes & Borst (1977).
[g]Yarlett *et al.* (1985); density of 1.24–1.26 reported by Müller (1975).
[h]Tolbert (1974).
[i]In a minority of glycosomes only (Opperdoes, 1988).
[j]SKL-like tripeptides.
[k]Not yet proven to be the import signal.
[l]Phosphoglycerate kinase.
[m]Nath & Nayak (1990).

membrane contacts between endoplasmic reticulum and microbodies observed by electron microscopy in liver tissue (see Zaar *et al.*, 1987), which had been misinterpreted as luminal continuity. Several observations show that new microbodies do not arise from the endoplasmic reticulum. Ultrastructural analysis for example of the yeast *H. polymorpha* showed that the number of peroxisomes increases by division (Veenhuis *et al.*, 1978). Biochemical analysis proved that, with a few exceptions (see below), most matrix proteins are made on free ribosomes and subsequently imported without proteolytic processing (Goldman & Blobel, 1978; Zimmerman & Neupert, 1980; Roa & Blobel, 1983; Miura *et al.*, 1984; Fujiki & Lazarow, 1985; Fujiki *et al.*, 1986). Transport of proteins into the microbodies via the endoplasmic reticulum would have required that they contain a signal sequence which is removed upon passage through the microsomal membrane. However, no evidence for such a scheme was found. Moreover, microbody proteins do not show the asparagine-linked oligosaccharide modifications that take place in the endoplasmic reticulum (Lazarow & Fujiki, 1985). This makes it unlikely that microbody matrix proteins are delivered to microbodies via the endoplasmic reticulum.

Still, the possibility remained that the microbody membrane buds as a vesicle from the endoplasmic reticulum and that the matrix proteins are imported post-translationally. When it was proven, however, that microbody membrane proteins are also synthesized on free ribosomes in their mature size and thus incorporated post-translationally (Fujiki *et al.*, 1984; Köster *et al.*, 1986), the idea that microbodies originate from the endoplasmic reticulum was abandoned. The differences in phospholipid composition between microbody membranes and the endoplasmic reticulum membrane (Fujiki *et al.*, 1982; Opperdoes *et al.*, 1984; Zinser *et al.*, 1991) contribute to the current idea that microbodies originate by division, as do mitochondria and chloroplasts, and thus do not arise *de novo*.

PROTEIN IMPORT: TOPOGENIC SIGNALS

The *C*-terminal microbody targeting signal

Microbody proteins are generally imported without proteolytic processing, implying that the import signal must be present in the polypeptide sequence of the mature protein. The identification of a microbody targeting signal was accelerated by the discovery that the enzyme luciferase from firefly (*Photinus pyralis*) is imported into peroxisomes when expressed in mammalian cells (Keller *et al.*, 1987). The enzyme turned out to have a peroxisomal localization in its homologous host as well (Keller *et al.*, 1987). By way of gene-deletion, gene-fusion and linker-insertion experiments, the twelve *C*-terminal amino acids of luciferase were shown to be essential for peroxisomal targeting. When this peptide was fused to the *C*-terminus of the cytosolic proteins chloramphenicol acetyltransferase or dihydrofolate reductase, these reporter proteins were efficiently transported into peroxisomes of mammalian cells (Gould *et al.*, 1987). Detailed mutagenesis showed that the luciferase targeting signal consisted of the *C*-terminal tripeptide SKL, and that this sequence is sufficient to direct different cytosolic reporter proteins to peroxisomes. A number of conservative changes can be made in this tripeptide without destroying its activity. In this way a minimal peroxisomal targeting signal could be defined, which has the consensus sequence: S/C/A-K/H/R-L (Gould *et al.*, 1989).

If this tripeptide motif is the only microbody targeting signal, it should also be present at the *C*-terminus of other microbody proteins. Table 2 lists the *C*-terminal sequences of microbody proteins from higher and lower eukaryotes (most data are from Swiss protein sequence database, release 21, and EMBL nucleotide sequence database, release 30, March 1992). Two aspects can be noted about the occurrence of the *C*-terminal tripeptide motif (red in Table 2) in microbody proteins: (1) it is present in many but not all microbody proteins, and (2) it is predominantly found in peroxisomal proteins from mammals, whereas it is less frequently used by lower eukaryotes, especially yeasts. Since microbody proteins with the *C*-terminal tripeptide motif are present in many eukaryotes examined (Table 2), it is not surprising that this tripeptide motif is recognized as a genuine microbody targeting signal by mammals, yeast and plants, as was found in expression studies with luciferase (Gould *et al.*, 1990*a*). Further evidence is provided by the observation that the *C*-terminal 12 amino acid sequence of the PMP20 protein from the yeast *Candida boidinii* (see Table 2), containing the microbody tripeptide motif, is indeed also identified by mammalian cells as a targeting signal (Keller *et al.*, 1987; Gould *et al.*, 1990*a*). Recently, it was shown that the tripeptide signal is also recognized as an import signal by glycosomes (Fung & Clayton, 1991; Blattner *et al.*, 1991). In a different approach, antibodies were raised against a peptide ending in SKL. These antibodies were able to detect matrix proteins of mammalian and yeast peroxisomes, glyoxysomal proteins from germinating seeds and glycosomal proteins from *Trypanosoma brucei* but were unable to recognize hydrogenosomal proteins from *Trichomonas vaginalis* (Gould *et al.*, 1990*b*; Keller *et al.*, 1991). This again illustrates the universal nature of the *C*-terminal tripeptide motif for all microbodies.

The presence of a *C*-terminal tripeptide motif is not a guarantee for microbody import. This conclusion must be drawn from several experiments. Linker-scanning mutagenesis of the *N*-terminal half of luciferase abolished its peroxisomal import, despite the presence of an intact microbody targeting signal (Gould *et al.*, 1987). Similarly, the glycosomal glyceraldehyde-phosphate dehydrogenase from *T. brucei*, ending in AKL (see Table 2), was cytosolic when expressed in mammalian CV-1 cells (Borst, 1989). Analogous results were obtained in the yeast *Saccharomyces cerevisiae* where some fusion proteins ending in a microbody targeting signal were not imported into the peroxisomes (Distel *et al.*, 1992). In another case, attaching a SKL or SRL signal *C*-terminally to a cytosolic protein directed only about 10% of the protein into peroxisomes of *S. cerevisiae* (de Hoop *et al.*, 1992*a*). These data imply that microbody import is influenced by the protein attached to the *C*-terminal targeting signal.

The microbody targeting signal defined so far, namely a *C*-terminal tripeptide conforming to the consensus sequence S/A/C-K/R/H-L, does not apply to all microbody proteins. This can be inferred from the fact that many microbody proteins lack such a sequence (Table 2). One possible explanation is that the consensus sequence may be more degenerate than proposed by Gould *et al.* (1989). This is supported by the following results. Glycosomes appear to recognize a *C*-terminal SKM as an import signal in addition to other sequences (C. E. Clayton, personal communication). The *C*-terminal sequences ARF and NKL of *H. polymorpha* alcohol oxidase and dihydroxyacetone synthase, respectively, appear to comprise the targeting signal (T. Didion, personal communication). The motif NKL is also present at the *C*-terminus of rat alanine:glyoxylate aminotransferase (Oda *et al.*, 1990). The *C*-terminal sequence AKI of the trifunctional enzyme hydratase:dehydrogenase:epimerase from the yeast *Candida tropicalis* (Aitchison *et al.*, 1991) fulfils the function of targeting signal as well. These recent data imply that the third position of the signal can be occupied by a Met, Phe or Ile. This suggests that the essential sequence of the signal may be a small amino acid at the first, a basic residue at the penultimate and a large non-polar residue at the *C*-terminal position. This would

Table 2. Comparison of *C*-termini of microbody proteins

Protein	Species	*C*-terminal sequence	Reference
Mammals			
Acyl-CoA oxidase	Rat	KKSPLNKTEVHESYHKHLKPLQSKL	Miyazawa *et al.*, 1987
Alanine: glyoxylate aminotransferase	Human	ATRENVDRVTEALRAALQHCPKKKL	Purdue *et al.*, 1990
	Rat	ATTENADRVAEALREALQHCPKNKL	Oda *et al.*, 1990
D-Amino acid oxidase	Human	ALEAAKLFGRILEEKKLSRMPPSHL	Momoi *et al.*, 1988
	Mouse	AMEAANLFGKILEEKKLSRLPPSHL	Tada *et al.*, 1990
	Pig	ALEVAKLFGKVLEERNLLTMPPSHL	Ronchi *et al.*, 1982
	Rabbit	ALEAAKLFGKILEEKKSSRMPPSHL	Momoi *et al.*, 1990
Carnitine octanoyltransferase	Rat	TVEAVSGASPCRILLPVSLNVSKRC	Chatterjee *et al.*, 1988
Catalase	Human	EKPKNAIHTFVQSGSHLAAREKANL	Quan *et al.*, 1986
	Bovine	SDVHPEYGSRIQALLDKYNEEKPKN	Schroeder *et al.*, 1982
	Rat	QKPKNAIHTYVQAGSHIAAKGKANL	Furuta *et al.*, 1986
Hydroxyacid oxidase	Rat	HRCMTLSGCQSVAEISPDLIQFSRL	Osumi *et al.*, 1985
Hydratase: dehydrogenase	Rat	RRLVAQGSPPLKEWQSLAGPHGSKL	Diep-Le *et al.*, 1991
Peroxisomal membrane protein 70	Rat	YYLHMDGRGNYEFKKITEDTVEFGS	Kamijo *et al.*, 1990
Peroxisomal assembly factor 1 (=pmp35)	Human	PKCGTEVHSLQPLKSGIEMSEVNAL	Shimozawa *et al.*, 1992
	Rat	PKCGTEVHSVQPLKSGIEMSEVNAL	Tsukamoto *et al.*, 1991
Sterol carrier protein 2	Human	LKITGNMGLAMKLQNLQLQPGNAKL	Yamamoto *et al.*, 1991
	Mouse	LKIAGNMGLAMKLQNLQLQPGKAKL	Moncecchi *et al.*, 1991
	Rat	LKIAGNMGLAMKLQSLQLQPDKAKL	Yamamoto *et al.*, 1991
Thiolase*	Human	RAYGVVSMCIGTGMGAAAVFEYPGN	Bout *et al.*, 1988
Thiolase A and B*	Rat	RAYGVVSMCIGTGMGAAAVFEYPGN	Hijikata *et al.*, 1990
Urate oxidase	Baboon	IKEVATSVQLTLSSKKDYLHGSSRL	Wu *et al.*, 1989
	Mouse	IKEVATSVQLTLRSKKDYLHGSSRL	Wu *et al.*, 1989
	Pig	IKEVATSVQLTLSSKKDYLHGSSRL	Wu *et al.*, 1989
	Rabbit	EVLLPLDNPYGKITGTVKRKLSSRL	Motojima *et al.*, 1989
	Rat	EVLLPLDNPYGKITGTVRRKLPSRL	Reddy *et al.*, 1988
Xanthine dehydrogenase	Rat	ACVDQFTTLCVTGVPENCKSWSVRI	Amaya *et al.*, 1990
Plants			
Catalase	Castor bean	ISYWIQCDKSLGQKLATRLNVKPSI	Gonzales, 1991
	Cotton	ISYWSQADKSVGQKLASLLNVRPSI	Ni *et al.*, 1990
(1)	Maize	ISYWSQCDAALGQKLPSRLNLKPSM	Redinbaugh *et al.*, 1988
(2)	Maize	NHGHSCFLCEIVIRSQFHTTYEPEA	Redinbaugh *et al.*, 1988
	Sweet potato	ISYLTQADRSLGQKVASRLNIRPTM	Sakajo *et al.*, 1987
Glycerate dehydrogenase	Cucumber	PFLDENVSPPAASPSIVNAKALGNA	Greenler *et al.*, 1989
Glycolate oxidase	Spinach	SLKEISRSHIAADWDGPSSRAVARL	Volokita *et al.*, 1987
Isocitrate lyase	Castor bean	KETWTRTGAMEMGSAGSEVVAKARM	Beeching *et al.*, 1987
	Cotton	KETWTRPGAGNIGSEGNLVVAKARM	Turley *et al.*, 1990*a*
Malate dehydrogenase*	Watermelon	ERIGLEKAKKELAGSIEKGVSFIRS	Gietl, 1990
Malate synthase	Castor bean	TLDDFLTLDAYNNIVIHYPKGSSRL	Rodriguez *et al.*, 1990
	Cucumber	NLDDFLTLDAYNYIVIHHPRELSKL	Graham *et al.*, 1989
	Cotton	NLDDFLTLDAYNYIVIHHPRELSKL	Turley *et al.*, 1990*b*
	Pumpkin	TLDDFLTLDAYNHIVIHHPRELSRL	Mori *et al.*, 1991
	Rape	ELDDFLTLAVYDHIVAHYPINASRL	Comai *et al.*, 1989
Urate oxidase	Soybean	DDVYLPTDEPHGSIQASLSRLWSKL	Nguyen *et al.*, 1985
Insects			
Catalase	Fruitfly	NFTQVHADFGRMLTEELNLAKSSKP	Orr *et al.*, 1990
Luciferase	Click beetle	IPRNVTGKITRKELLKQLLEKSSKL	Wood *et al.*, 1989
	Firefly	VPKGLTGKIDGRAIREILKKPVAKM	Masuda *et al.*, 1989
	Firefly	LTGKLDARKIREILIKAKKGGKSKL	de Wet *et al.*, 1987
Urate oxidase	Fruitfly	VFIPVDKPHGTIYAQLARKNINSHL	Wallrath *et al.*, 1990
	Fruitfly	VFIPTDKPHGTIYAQLSRKSLKSHL	EMBL X57114
	Fruitfly	VFIPVDKPHGTIYAQLARKNISSHL	EMBL X57113
Xanthine dehydrogenase	Fruitfly	ACQDKFTELLEIPEPGSFTPWNIVP	Beard *et al.*, 1987
	Calliphora vicina	ACQDEFTNLIEQPPAGSYVPWNIVP	Houde *et al.*, 1989

Table 2. continued

Protein	Species	*C*-terminal sequence	Reference
Micro-organisms			
Acyl-CoA oxidase (*POX4*)	*Candida maltosa*	ILNRPSLEARERYEKSDETAAILSK	Hill *et al.*, 1988
Acyl-CoA oxidase (*POX5*)	*C. tropicalis*	MLNRPDLEVRERGEKSEEAAEILSS	Okazaki *et al.*, 1986
Acyl-CoA oxidase 2 (*POX4*)	*C. tropicalis*	MLNRPTLDERERFEKSDETAAILSK	Okazaki *et al.*, 1986
Acyl-CoA oxidase (*POX2*)	*C. tropicalis*	MLNRSALENRERSERGKAAADILSK	Okazaki *et al.*, 1987
Acyl-CoA oxidase	*Saccharomyces cerevisiae*	LGRGFEFDQKLGGAANAEILSKINK	Dmochowska *et al.*, 1990
Alcohol oxidase (1 and 2)	*Pichia pastoris*	SGEALDMTVPNFKLGTYEKTGLARF	Koutz *et al.*, 1989
Alcohol oxidase	*Hansenula polymorpha*	SGSDLDMTIPNFRLGTYEETGLARF	Ledeboer *et al.*, 1985
6-aminopenicillin acyltransferase	*Penicillium chrysogenum*	PTNPDEMFVMRFDEEDERSALNARL	Barredo *et al.*, 1989
Amino oxidase	*H. polymorpha*	AKRAVHKETKDKTSRLAFEGSCCGK	Bruinenberg *et al.*, 1989
Catalase A	*S. cerevisiae*	KGLSEAIKKVAEAKHASELSSWSKF	Cohen *et al.*, 1988
Catalase	*C. tropicalis*	AYFTKVHPDLGDLIKKEILELSPRK	Okada *et al.*, 1987
Citrate synthase	*S. cerevisiae*	ASIERPKSYSTEKYKELVKNIESKL	Rosenkrantz *et al.*, 1990
Dihydroxyacetone synthase	*H. polymorpha*	PLLLHDFLDLKEKPNHDKVNKLNKL	Didion, personal communication
Hydratase: dehydrogenase: epimerase	*C. tropicalis*	IQTHVVDRGTIAINNAAIKGDKAKI	Nuttley *et al.*, 1988
Isocitrate lyase	*C. tropicalis*	GGVTSTAAMGAGVTEDQFKETKAKV	Atomi *et al.*, 1990
Malate dehydrogenase	*S. cerevisiae*	PICVSQLKKNIDKGLEFVASRSASS	Minard *et al.*, 1991
Malate synthase	*H. polymorpha*	AEFLTTLLYDDIVTIGPEVDISSLK	Bruinenberg *et al.*, 1990
Peroxisomal membrane protein 20	*C. boidinii*	IVEYAAIENGGEVDVSTAQKIIAKL	Garrard *et al.*, 1989
Peroxisomal membrane protein 47	*C. boidinii*	HSFEKALSMRSPRTRTTTVASSAKE	McCammon *et al.*, 1990
Pas3p (48 kDa integral membrane protein)	*S. cerevisiae*	LSSASSSVYSSSSNFGVSSSFSFKP	Höhfeld *et al.*, 1991
Sterol carrier protein (*POX18*)	*C. tropicalis*	KVKGNMMKATAIEAVFKKLDPRPKL	Tan *et al.*, 1990
Thiolase	*S. cerevisiae*	KKDQIGVVSMCIGTGMGAAAIFIKE	EMBL NSCPOT1

Trypanosomatidae (both glycosomal proteins and cytosolic isoenzymes)

Protein	Location	Species	Sequence	Reference
ALDO[a]	Glycosomal	*Trypanosoma brucei*	AQLGKYNRADDDKDSQSLYVAGNTY	Clayton, 1985
PEPCK[b]	Glycosomal	*T. brucei*	QADYEVYPGWAFTFLRGLRTCPQAC	Parsons & Smith, 1989
GAPDH[c]	Glycosomal	*T. cruzi*	NEWGYSHRVVDLVRHMASKDRSARL	Kendall *et al.*, 1990
	Glycosomal	*T. brucei*	NEWGYSHRVVDLVRHMAARDRAAKL	Michels *et al.*, 1986
	Cytosolic	*T. brucei*	KLVSWYDNETGYSNKVLDLIAHITK	P. Michels *et al.*, personal communication
	Glycosomal	*Leishmania mexicana*	EWAYSHRVVDLVRYMAAKDAASSKM	P. Michels *et al.*, personal communication
GPI[d]	Glycosomal	*T. brucei*	PGMRVNNHDSSTNGLINMFNELSHL	Marchand *et al.*, 1989
PGK[e]	Glycosomal	*Crithidia fasciculata*	CPCGSGCAAVPAAATATVSMVLASP	Swinkels *et al.*, 1988
	Cytosolic	*C. fasciculata*	TGGGASLELLEGKSLPGVTVLTNKE	Swinkels *et al.*, 1988
	Glycosomal	*T. brucei*	LDEKSAVVSYASAGTGTLSNRWSSL	Osinga *et al.*, 1985
	Cytosolic	*T. brucei*	TGGGASLELLEGKTLPGVTVLDDKE	Osinga *et al.*, 1985
TIM[f]	Glycosomal	*T. brucei*	RDVNGFLVGGASLKPEFVDIIKATQ	Swinkels *et al.*, 1986

* Protein made with an *N*-terminal precursor peptide.
[a]Aldolase.
[b]Phosphoenolpyruvate carboxykinase.
[c]Glyceraldehyde-phosphate dehydrogenase.
[d]Glucose-phosphate isomerase.
[e]Phosphoglycerate kinase.
[f]Triose-phosphate isomerase.

bring the *C*-terminal sequence of some other microbody enzymes into the consensus (underlined in red in Table 2). There are indications that the actual signal allowed depends on the particular protein and/or species, as is supported by the following findings. Mutating the ultimate leucine of luciferase into a Phe or Ile abolished its ability to be imported by peroxisomes of mammalian cells (Gould *et al.*, 1989). Moreover, for *S. cerevisiae* catalase A it has been claimed that the last 15 amino acids ending in SKF do not form the targeting signal (Hartig *et al.*, 1990). Replacing the AKI of the *C. tropicalis* trifunctional enzyme by GKI or AQI is without effect on the targeting to peroxisomes of the related yeast *Candida albicans*, but abolishes peroxisomal uptake in *S. cerevisiae* (Aitchison *et al.*, 1991).

As another possibility, the *C*-terminal position of the signal may not be an absolute requirement. This will be discussed in the sections below.

Putative internal microbody targeting signals

Gould *et al.* (1988, 1989) have suggested that the tripeptide motif may also function at an internal position near the *C*-terminus. The suggestion was based on two observations. Firstly, internal tripeptide motifs are often found close to the *C*-terminal

Table 3. Comparison of *N*-terminal microbody peptide sequences

*Proteolytic cleavage site. References: 1, Gietl (1990); 2, Hijikata *et al.* (1990); 3, Bout *et al.* (1988); 4, Swinkels *et al.* (1991); 5, Bruinenberg *et al.* (1989); 6, Clayton (1985); 7, Blattner (1991); for other references see Swinkels *et al.* (1991). Box indicates thiolase B targeting signal; see the text.

Species	Protein	N-terminal sequence		Proven targeting signal
Watermelon	Malate dehydrogenase	MQPIPDVNQRIARISAHLHPPKSQMEESSALRRANCR*	[1]	No
Rat	Thiolase A	MSESVGRTSAM-HRLQVVLGHLAGRPESSSALQAAPC*	[2]	Yes [4]
Rat	Thiolase B	M-HRLQVVLGHLAGRSESSSALQAAPC*	[2]	Yes [4]
Man	Thiolase	M-QRLQVVLGHLRGPADSGWMPQAAPC*	[3]	No
Saccharomyces cerevisiae	Thiolase	MSQRLQSIKDHLVLSAMGLGESKRKNSLLEK	[4]	No
Candida tropicalis	Thiolase	M-DRLNQLSGQLKPNAKQSILQKNPDDVVIV	[4]	No
Yarrowia lipolytica	Thiolase	M-DRLNNLATQLEQNPAKGLDAITSKNPDDV	[4]	No
Hansenula polymorpha	Amine oxidase	M-ERLRQIASQATAASAAPARPAHPLDPLST	[5]	No
Trypanosoma brucei	Aldolase	MSKRVEVLLTQLPAYNRLKTPYEAELIETAK	[6]	Yes [7]
Putative consensus		RL QL H		

end of microbody proteins (boxed in red in Table 2). Secondly, the 27-amino-acid *C*-terminal peptide of human catalase, which does not end in a tripeptide motif but contains an internal SHL sequence, acts as a peroxisomal signal in mammalian cells. However, the actual involvement of this SHL or any other internal tripeptide has not been proven. Whatever the signal in human catalase is, it is also recognized by other species, as heterologous expression in *S. cerevisiae* has shown (de Hoop *et al.*, 1992*b*), indicating that this mechanism of import has been conserved during evolution as well. We also found (Faber *et al.*, unpublished work) that the *C*-terminal sequence of amine oxidase, including the unique internal SRL sequence at 9 amino acids from the end (see Table 2), is not involved in targeting. Moreover, Gould *et al.* (1989) showed that an extension of the *C*-terminal SKL sequence of firefly luciferase with one or two amino acids abolishes its targeting activity. However, it cannot be excluded that in some instances the signal is located internally. If the tripeptide sequence acts at an internal position, one has to assume that its activity is context-dependent to account for the inactivity of similar sequences in cytosolic proteins. As an additional possibility, (internal) microbody targeting signals may be overruled by other topogenic signals; Afalo (1990) has shown that a mitochondrial signal attached to the peroxisomal luciferase directs the protein to mitochondria. It seems reasonable to assume that in order to be recognized by the import machinery, a targeting sequence has to be exposed. The *C*-terminus may be the preferred but not the only position at which exposure can be achieved. For the nuclear localization signal, a position-dependent functioning has been well-documented (Roberts *et al.*, 1987; Nelson & Silver, 1989).

An internal microbody targeting signal has been identified by Small *et al.* (1988). They showed that an *N*-terminal region (amino acids 1–118) and an internal region (amino acids 309–427) of acyl-CoA oxidase of *C. tropicalis* (*POX4* gene product) contain information that specifically targets fragments of acyl-CoA oxidase or a fused reporter (dihydrofolate reductase) protein to peroxisomes of yeast. Their data, obtained with an *in vitro* import system, were confirmed by Kamiryo *et al.* (1989) who expressed truncated versions of the enzyme in *Candida maltosa*. However, the peroxisomal localization of the proteins has not been established unequivocally, since the protease resistance, taken as a criterion for import, could also have been caused by association with membranes or protein aggregation (Borst, 1989). Despite these reservations, the existence of internal microbody import signals cannot be excluded.

N-Terminal microbody targeting signals

A few microbody proteins are synthesized as precursor proteins, e.g. thiolase of higher eukaryotes which is synthesized with an *N*-terminal presequence (Table 3) (Miura *et al.*, 1984; Fujiki *et al.*, 1985; Hijikata *et al.*, 1990). The presequence is split off during import, but this is not an obligatory step in the import process (Balfe *et al.*, 1990; Swinkels *et al.*, 1991; van Roermund *et al.*, 1991). Swinkels *et al.* (1990, 1991) reported that the presequences of rat thiolase A and B contain a microbody targeting signal. This became clear from the following observations: when part of the sequence is removed, the protein becomes cytosolic and fusion of the presequence to chloramphenicol acetyl transferase renders this cytosolic reporter protein peroxisomal. Via deletion mutagenesis, the topogenic information of rat thiolase B could be restricted to the first 11 amino acids: MHRLQVVLGHL (boxed in red in Table 3). An analogous sequence is found in other thiolases, including the yeast thiolases, which are not subjected to processing upon import (Swinkels *et al.*, 1991). Interestingly, the first 11 amino acids of amine oxidase from *H. polymorpha* share a considerable similarity with the thiolase sequences of *C. tropicalis* and *Yarrowia lipolytica* (Table 3), suggesting that the use of *N*-terminal targeting signals may not be restricted to thiolases. As argued earlier, the targeting signal of amine oxidase does not reside in the last 9 amino acids, including the only internal SRL (see above). Almost all proteins listed in Table 3 contain the sequence RLxxxxxQ/HL (in which x denotes an arbitrary amino acid, red in Table 3). This consensus is partly present in the *N*-terminus of glycosomal aldolase from *Trypanosoma brucei*. Interestingly, it has recently been shown that this *N*-terminus is sufficient to direct a cytosolic protein into glycosomes (Blattner *et al.*, 1991).

Another microbody protein which is synthesized with an *N*-terminal presequence and does not have the *C*-terminal tripeptide motif is malate dehydrogenase from watermelon. This presequence is 37 amino acids long and is cleaved off upon import into glyoxysomes (Gietl, 1990). Comparison of the presequence of malate dehydrogenase with those of rat and human thiolases (Table 3) reveals some similarities. Presently, it is not known whether the topogenic signal of malate dehydrogenase resides in the presequence.

Unique import signals for glycosomal proteins?

Like most microbody proteins, the import of glycosomal proteins occurs without proteolytic processing or other post-

translational modifications (Opperdoes, 1988). For a long time, the elucidation of the glycosomal import signal has been hampered by the absence of an *in vitro* import system and a suitable *in vivo* expression system, but the existence of highly similar isoenzymes has given interesting clues. Swinkels *et al.* (1988) compared the amino acids sequences of glycosomal and cytosolic phosphoglycerate kinase from *Crithidia fasciculata*. These two enzymes are virtually identical except for a *C*-terminal extension of 38 amino acids present in the glycosomal enzyme. A similar difference between the phosphoglycerate kinase isoenzymes exists in *T. brucei*, where the *C*-terminal extension of the glycosomal form is 20 amino acids long. Both extensions are rich in small, hydrophobic and hydroxyl amino acids, but they do not share obvious sequence similarities (Swinkels *et al.*, 1988). Recently, transformation systems for Trypanosomatidae have been established (Cruz & Beverley 1990; ten Asbroek *et al.*, 1990). Using *in vivo* import, Blattner *et al.* (1991) showed that the 20-amino-acid *C*-terminal extension of phosphoglycerate kinase from *T. brucei* contains indeed all the information to target an attached reporter protein into the glycosome. The *N*-terminus of glycosomal aldolase from *T. brucei* can fulfil a similar function (Blattner *et al.*, 1991; see above). No peroxisomal import was obtained when the glycosomal phosphoglycerate kinase was expressed in *S. cerevisiae* (Swinkels, 1989), or when glycosomal aldolase was expressed in monkey kidney cells (Fung & Clayton, 1991), implying that these import signals are specific for glycosomes.

Proteins with multiple intracellular locations

Eukaryotic cells contain several isoenzymes that perform the same catalytic function but differ in their intracellular location. These isoenzymes are useful tools to investigate the signals determining their different localization, since they mostly share a considerable sequence similarity. Several microbody enzymes have isoenzymes located in other subcellular compartments. Unfortunately, for most of them insufficient sequence data are available, with the exception of glycosomal enzymes which have already been discussed.

The enzyme alanine:glyoxylate aminotransferase has a species-dependent intracellular location. It is exclusively peroxisomal in rabbit and baboon liver, predominantly mitochondrial (about 90%) in cat and both mitochondrial and peroxisomal in rat and marmoset (New World Monkey). Livers of pigs and cattle appear to be devoid of this enzyme (Danpure *et al.*, 1990). In normal human liver alanine:glyoxylate aminotransferase is peroxisomal. No tripeptide motif is present at its *C*-terminal end; the sequence ends in -PKKKL (see Table 2). Compared to the rat mitochondrial enzyme, the human peroxisomal enzyme appears to have lost the mitochondrial targeting signal by a mutation in the initiation codon (Takada *et al.*, 1990). Although alanine:glyoxylate aminotransferase is exclusively peroxisomal in livers of healthy humans, it is mainly (> 90%) mitochondrial in a subset of patients suffering from the autosomal recessive disease primary hyperoxaluria type 1 (Danpure *et al.*, 1989). Three point mutations associated with this disease have been identified, causing amino acid substitutions at position 11 (Pro → Leu), 170 (Gly → Arg) and 340 (Ile → Met). Not all three mutations have to be present to induce the mitochondrial rerouting. It has been suggested that the mutation at amino acid 11 generates a mitochondrial targeting sequence. The presence of the mutation in amino acid number 170 makes the mitochondrial rerouting more efficient. This mutation might induce a defect in a putative internal peroxisomal targeting signal. The sequence around amino acid 170 does not resemble a known peroxisomal targeting signal, but has some similarity to an internal (amino acids 388–394) fragment of the peroxisomal firefly luciferase (Purdue *et al.*, 1990). If the mutation at amino acid 11 occurs in combination with the mutation at amino acid 340, only part of the enzyme is rerouted to mitochondria, indicating that this combination induces only a weak mitochondrial import signal. The mutations at amino acids 11 and 340 separately do not cause rerouting (Purdue *et al.*, 1990). It will be interesting to elucidate the precise targeting signal in alanine:glyoxylate aminotransferase.

OTHER COMPONENTS OF THE IMPORT MACHINERY

Cytosolic requirements

Proteins cannot be translocated across membranes in a tightly-folded state (Randall & Hardy, 1986; Eilers *et al.*, 1988; Kumamoto, 1991; Gething & Sambrook, 1992). The import of proteins into organelles such as endoplasmic reticulum (Chiroco *et al.*, 1988; Deshaies *et al.*, 1988), lysosomes (Chiang *et al.*, 1989), nucleus (Shi & Thomas, 1992), chloroplasts (Keegstra, 1989), mitochondria (Deshaies *et al.*, 1988; Manning-Krieg *et al.*, 1991) and the transport of proteins through the secretory pathway (Pelham, 1989), appears to require the help of unfolding proteins (unfoldases). These unfoldases, that keep the proteins to be imported in a translocation competent state, are members of the HSP70 family. It would be interesting to know whether members of the HSP70 family are also involved in microbody import. A *C*-terminal position of the microbody import signal implies that microbody proteins will have become folded before their import signal emerges from the ribosome. Therefore, involvement of helper proteins, to prevent folding of newly-synthesized microbody proteins into a translocation-incompetent state, is likely. Interestingly, a 72-kDa protein which binds to clofibrate, a compound that induces proliferation of peroxisomes in hepatic parenchymal cells, is a member of the HSP70 family (Alvares *et al.*, 1990).

Since microbodies arise by fission of pre-existing microbodies only, a 'seed' microbody (or other import-competent microbody structure) has to be present as a cytosolic requirement for microbody biogenesis. Cell fusion experiments (Allen *et al.*, 1989) indicate that a wild-type nucleus is not sufficient for peroxisomal biogenesis, but that a cytosolic component, most likely a peroxisome itself, is necessary as well.

Membrane proteins

In chloroplasts, a 36-kDa integral membrane protein has been identified as a receptor for protein import (Pain *et al.*, 1988; Schnell *et al.*, 1990). Mitochondria contain at least two membrane proteins (MOM19 and MOM72) that act as receptors for import of precursor proteins (Pfanner *et al.*, 1991). By analogy, the microbody membrane is likely to contain protein-import receptors. In view of the great variety and the inducibility of matrix proteins, it would not be surprising if the microbody membrane contains different receptors, some constitutive, others inducible. Analysis of peroxisome-deficient mutants, currently performed in several laboratories (Zoeller & Raetz, 1986; Zoeller *et al.*, 1989; Erdmann *et al.*, 1989, 1991; Cregg *et al.*, 1990; Balfe *et al.*, 1990; Tsukamoto *et al.*, 1990, 1991; Shimozawa *et al.*, 1992), will probably shed light on many of these questions. In peroxisome-deficient cells protein import is generally impaired, resulting mostly in the accumulation but sometimes in the degradation of peroxisomal matrix proteins in the cytosol (Schram *et al.*, 1986; Erdmann *et al.*, 1989; Cregg *et al.*, 1990; Tsukamoto *et al.*, 1990). Up till now, four genes have been isolated and sequenced that encode peroxisome-restoring activities. Tsukamoto *et al.* (1991) and Shimozawa *et al.* (1992) isolated a gene encoding a 35-kDa peroxisomal membrane

protein (PAF1) from rat and man which complements peroxisome deficiency in CHO cells and human cells. A 48-kDa integral peroxisomal-membrane protein (Pas3p) is essential for peroxisome biogenesis in *S. cerevisiae* (Höhfeld *et al.*, 1991). All three proteins are very likely part of the import machinery of peroxisomal membranes, but no direct proof is available yet. Erdmann *et al.* (1991) isolated a gene encoding a 117-kDa protein (Pas1p) which is able to restore peroxisome formation in *S. cerevisiae*. The hydropathy profile indicates that Pas1p is a soluble protein. Pas1p contains two putative ATP-binding sites, but no other clues about its function are available so far.

Pmp-70 is a membrane protein of rat peroxisomes. It has an ATP-binding site and may have a function in the active transport across the membrane (Kamijo *et al.*, 1990). The pmp-70 protein is, in contrast to other major membrane proteins, markedly induced by clofibrate and thyroxine, drugs that cause peroxisome proliferation and induce the β-oxidation pathway (Hartl & Just, 1987). Two peroxisomal membrane proteins of *Candida boidinii*, pmp-20 and 47, are induced by growth on methanol, a condition that results in the massive induction of alcohol oxidase-containing peroxisomes. This indicates that these two membrane proteins may have functions specifically related to methanol metabolism (Goodman *et al.*, 1986).

How many different import systems may we expect?

Since at least two different microbody import signals have been discovered (see above), at least two different import receptors are envisioned. Indeed, differences have been observed between the import of thiolase and other matrix proteins in fibroblasts of patients without functional peroxisomes (Zellweger disease) (Balfe *et al.*, 1990). Walton *et al.* (1992) showed that two Zellweger cell lines are unable to import proteins carrying the *C*-terminal SKL tripeptide signal.

In a large number of Zellweger cells, vesicles containing peroxisomal membrane proteins (peroxisomal ghosts) are present (Santos *et al.*, 1988*a*,*b*; Suzuki *et al.*, 1989; Cregg *et al.*, 1990; Tsukamoto *et al.*, 1990). Even several matrix proteins can be found inside peroxisomal-ghost vesicles (van Roermund *et al.*, 1991). The presence of membrane proteins in the peroxisomal ghosts (Lazarow *et al.*, 1986; Santos *et al.*, 1988*a*,*b*) indicates that the import mechanism for (most) membrane proteins differs from the majority of matrix proteins. So far, five peroxisomal membrane proteins have been sequenced [rat pmp-70, PAF-1 (= pmp-35) from CHO cells, pmp-20 and pmp-47 of *C. boidinii* and Pas3p; see Table 2]. Only pmp-20 contains the *C*-terminal microbody targeting tripeptide signal.

Energy requirements

Import of proteins into mitochondria (Gasser *et al.*, 1982), chloroplasts (Grossman *et al.*, 1980), nuclei (Adam *et al.*, 1990), endoplasmic reticulum (Waters & Blobel, 1986) and export of proteins from prokaryotes (Enequist *et al.*, 1981; Chen & Tai, 1987) require energy. The energy can be provided by ATP and/or a protonmotive force across the organellar membrane. There is ample evidence that a pH gradient is present across the peroxisomal membrane (Nicolay *et al.*, 1987; Waterham *et al.*, 1990). Also, the presence of a peroxisomal ATPase has been demonstrated in yeast (Douma *et al.*, 1987, 1989) and rat liver peroxisomes (Cuezva *et al.*, 1990). The latter ATPase belongs to the subclass of V-ATPases, a new class of proton-translocating ATPases, specifically associated with the endomembrane system of eukaryotes (Cuezva *et al.*, 1990). Bellion & Goodman (1987) showed that a proton ionophore disturbs import of peroxisomal proteins *in vivo*. This effect might be caused by the dissipation of the peroxisomal pH gradient, but could also be due to the depletion of intracellular ATP. Using an *in vitro* import system, Imanaka *et al.* (1987) showed that import of acyl-CoA oxidase requires ATP but not a membrane potential. The latter conclusion is not surprising because isolated peroxisomes are permeable for small molecules (van Veldhoven *et al.*, 1983; Beaufay *et al.*, 1964) and therefore probably not capable of generating a membrane potential.

Intra-microbody assembly factors

Newly-synthesized proteins have to adopt an 'unfolded, import-competent' conformation to be translocated across a membrane. This implies that refolding and often also assembly into oligomeric complexes (Kindl & Kruse, 1983; Lazarow & Fujiki, 1985) have to occur inside microbodies. In mitochondria and chloroplasts, the assembly of oligomeric proteins is dependent on intraorganellar heat-shock proteins (Cheng *et al.*, 1989; Amir-Shapira *et al.*, 1990; Manning-Krieg *et al.*, 1991; Gething & Sambrook, 1992). There is indirect evidence that the octameric protein alcohol oxidase of methanol-cultured methylotrophic yeast requires an intraperoxisomal factor for octamerization and/or activation (Cregg *et al.*, 1990). This assembly factor might be unique for methylotrophic yeasts because alcohol oxidase does not assemble into octamers inside peroxisomes of *S. cerevisiae* (Distel *et al.*, 1987). Also dihydroxyacetone synthase, another peroxisomal matrix protein from methylotrophic yeasts, does not acquire its normal activity inside peroxisomes of *S. cerevisiae* (Gödecke *et al.*, 1989).

EVOLUTIONARY SPECULATIONS AND FUTURE PERSPECTIVES

Because microbodies do not bud from the endoplasmic reticulum and cannot arise *de novo*, the origin of the first microbody is still a matter of speculation. Do all members of the microbody family have the same ancestor? Their presence in almost all eukaryotic cells indicates that they appeared very early in the evolution, as did chloroplasts and mitochondria (Gray, 1989). For mitochondria and chloroplasts it is generally assumed that they originated via endosymbiosis. Their double membranes may still be a silent witness of this event (Blobel, 1980). Moreover, they contain their own DNA and protein-synthesizing machinery, equipped with prokaryotic 70 S ribosomes. Microbodies, in contrast, do not contain DNA or ribosomes and are surrounded by a single membrane. Does this mean that microbodies have an autogenous origin, and are derived via invagination of (plasma) membranes, like probably the endoplasmic reticulum, the vacuole/lysosome and the nucleus (Blobel, 1980; Sabatini *et al.*, 1982)? One can also argue that mitochondria and chloroplasts had to retain their double membranes, since they need an intraorganellar acidic compartment to generate a proton gradient. As soon as a DNA-containing organelle has lost its double-membrane system, it is much more sensitive to damage, which might gradually result in the transfer of its DNA to the nucleus.

Several facts argue in favour of an endosymbiotic origin, firstly; rat peroxisomal thiolases have more (42%) similarity to prokaryotic (*Escherichia coli*) thiolase than to rat mitochondrial thiolase (37%; Yang *et al.*, 1990). Besides, both peroxisomal and prokaryotic β-oxidation pathways contain a multienzyme complex; *E. coli* has a tetrafunctional enzyme carrying enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyacyl-CoA epimerase as well as enoyl-CoA isomerase activity (Yang *et al.*, 1990). The peroxisomal β-oxidation pathway of, for example, the yeast *C. tropicalis* contains a trifunctional enzyme exhibiting hydratase, dehydrogenase and epimerase activity. All these

activities are present on separate enzymes in mitochondrial β-oxidation (DiRusso, 1990; Imamura *et al.*, 1990; Ishii *et al.*, 1987). Secondly, two kinds of microbody are present in *Neurospora crassa* (Kionka & Kunau, 1985). One type has the 'normal' microbody density of 1.21 g/cm^3 in sucrose gradient and contains β-oxidation enzymes equipped with an acyl-CoA dehydrogenase instead of the hydrogen peroxide-producing oxidase normally present. The other type of microbody bands at a higher density (1.26 g/cm^3) and contains catalase. Unfortunately, no sequence information about proteins resident in these microbodies is available yet. Finally, the endosymbiont theory gives an appealing explanation of how a complete pathway, like β-oxidation or glycolysis, could become compartmentalized in a single step. The alternative, a step-by-step transfer of these enzymes from the cytosol into a microbody, is less attractive, although this scenario may explain the large variation of microbody matrix enzymes between different species. The acquisition of a *C*-terminal tripeptide targeting signal might have been relatively easy; the signal is very short compared to the large *N*-terminal signals (Table 1) and the *C*-terminal addition of a tripeptide probably does not interfere with folding. The amino acids serine, arginine and leucine can each be made from six codons, so are relatively easy to get attached to a protein sequence. Once this import system was established in the early eukaryotic cell, one can imagine that later, via individual evolutionary events, many proteins were relocated to the microbodies using the same import receptor and their newly-acquired *C*-terminal tripeptide signal. So, microbodies might have been generated by one or more early (endosymbiotic) events, followed by the step-by-step transfer of many separate proteins.

Definitive proof for the evolutionary origin of microbodies will probably come from additional sequence data of microbody enzymes and their cytosolic (prokaryotic and/or eukaryotic) counterparts. The observation that all microbodies are able to import proteins equipped with a *C*-terminal SKL tripeptide may point towards a common ancestor for microbodies. The analysis of import signals and other components of the protein import machinery will provide data about the relationship between the different members of the microbody family. This will lead to detailed knowledge of organelle biogenesis and will give insight in the origin and treatment of diseases caused by peroxisome-assembly defects.

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