Volume 291, number 2, 299–302 FEBS 10262 October 1991

ADONIS 001457939100971X

Overexpression of alcohol oxidase in *Pichia pastoris*

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Received 8 July 1991

The protein import capacity of peroxisomes in methylotrophic yeasts was studied using *Pichia pastoris* containing one or two extra copies of the gene encoding the peroxisomal protein alcohol oxidase The alcohol oxidase overproduced in this strain was only partially imported and assembled into the active, octameric form of the protein The excess remained in the cytosol as protein aggregates composed of monomers These results are discussed in view of the possible application of peroxisomes as storage compartments for heterologous proteins

Alcohol oxidase, Methylotrophic yeast, Peroxisome

1. INTRODUCTION

Peroxisomes are single-membrane bounded organelles containing a matrix of proteins, generally including catalase and one or more hydrogen peroxide-producing oxidases They are versatile organelles that can vary in number, size and protein content [1,2] In yeast, proliferation of peroxisomes is dependent on growth conditions; some of the enzymes involved in the metabolism of particular C- and N-sources in the medium accumulate in the organelle Therefore, the composition of peroxisomes can be easily manipulated in yeast [3–6]. Strong proliferation of peroxisomes occurs for example when methylotrophic yeasts are shifted from glucose to methanol as the sole source of carbon and energy Under certain conditions, these peroxisomes can occupy up to 80% of the cytosolic volume [7] These peroxisomes have a crystalline matrix composed of alcohol oxidase octamers, which is the active form of this protein [8,9] Also present in the peroxisomes, although not as abundant as alcohol oxidase, are 2 other key enzymes of methanol metabolism, namely catalase and dihydroxyacetone synthase [10]

The import capacity of peroxisomes present in methanol-cultured yeast may offer the possibility to store heterologously expressed proteins, equipped with the appropriate targetting signals, into peroxisomes [11]. Peroxisomal localization could have the advantage that the proteins are kept separate from proteolytic activities in the cytosol This system may have a potential value for storage of heterologously expressed proteins which are susceptible to proteolysis. In a first attempt to analyze the import capacity of peroxisomes present in methanol-cultured cells, we overexpressed alcohol oxidase and analyzed to what extent the enzyme was imported.

Abbreviation Hp-AOX, the alcohol oxidase gene from *Hansenula polymorpha*

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2 MATERIALS AND METHODS

2 1 *Plasmid construction, yeast transformation and genetic analysis*

A 2 5-kb fragment containing the coding sequence of the alcohol oxidase gene from *Hansenula polymorpha (Hp-AOX)* [12], flanked by 10 bp 5′ and 250 bp 3′ untranslated sequences, was inserted into the *Bam*HI site of vector pAOBam This is an expression vector derived from pAO804 [13] by insertion of a *Bam*HI linker (AATTGGATCC) into the unique *Eco*RI site In the final construct, the *Hp-AOX* gene was located between the promoter and terminator of the *AOX1* gene of *Pichia pastoris*

A transformation system of the related methylotrophic yeast *Pichia pastoris* [14] was used for overexpression of alcohol oxidase Transformation of *Pichia pastoris* GS115 (*his4*) was performed as described by Cregg et al [14], and was based on complementation of histidine auxotrophy To force homologous integration into the defective *his4* gene, the plasmid DNA was linearizedwith *Stu*I, which cuts approximately in the middle of the *HIS4* gene present in pAOBam (Fig 1A) Transformants were screened for proper integration of the plasmid by Southern blotting The blot was screened with random-primed [α-^{32}P]dCTP-labelled [15] plasmid pYM4, which is pBR322 containing the *HIS4* gene of *P pastoris* [14]

2 2 *Preparation of cell extracts, measurement of activity and octamerization*

A culture of transformed cells, grown on Difco's Yeast Nitrogen Base without amino acids and with 0 5% glycerol to an OD_{600} of 2–3, was 10-fold diluted with medium containing 1% methanol as the sole carbon source After 16 h, cells were harvested (OD_{600}=2–2 5) and extracts were prepared [8] Alcohol oxidase activity was assayed as described [16] Monomeric and octameric alcohol oxidase were separated by sucrose gradient centrifugation [8] and visualised by Western blotting [17] The antibodies used were either a polyclonal antibody raised against denatured alcohol oxidase of *Hansenula polymorpha* or a monoclonal antibody (OAO11) The polyclonal antibody recognized both alcohol oxidase of *P pastoris* and *H polymorpha*, while the monoclonal antibody was specific for alcohol oxidase from *H polymorpha* (M de Hoop, unpublished results)

2 3 *Immuno-electronmicroscopy*

The intracellular localization of alcohol oxidase and dihydroxyacetone synthase was determined by immunogold labelling on thin sections of Lowicryl-embedded cells [10]

3 RESULTS

3 1. *Multi-copy integrations in* Pichia pastoris *GS115*

To generate a strain that overproduces alcohol oxidase when cultured on methanol, *P pastoris* GS115 was equipped with additional copies of the alcohol oxidase gene from the related yeast *H polymorpha* The alcohol oxidase gene was placed between the promoter and the terminator of the *P pastoris* alcohol oxidase gene The recombinant plasmid was targeted into the defective *his4* gene of the host strain GS115. To favour homologous recombination, the plasmid was linearized in the *HIS4* gene prior to transformation (Fig 1A). DNA from histidine prototrophic transformants was digested with *Bgl*II and analyzed by Southern blotting using pYM4 as a probe. This plasmid contained the *HIS4* gene and the *E coli* gene encoding β-lactamase [14] Host DNA yielded one band of 3 kb representing the defective *his4* gene (Fig 1A and Fig. 2, lane 1) Single-copy integrations in the *his4* locus resulted in the replacement of the 3-kb band by a number of new bands (Fig 1B and Fig. 2, lane 2). Knowing the positions of the *Bgl*II sites, we could identify a band of 2 4 kb as the fragment containing the β-lactamase gene The bands of 4.0 and 4 5 kb hybridize because they contain *HIS4* sequences plus different parts of the plasmid An additional band of 5 5 kb and an intensified 2.4 kb band indicated a double-copy integration of the plasmid in the defective *his4* gene (Fig. 2, lane 3)

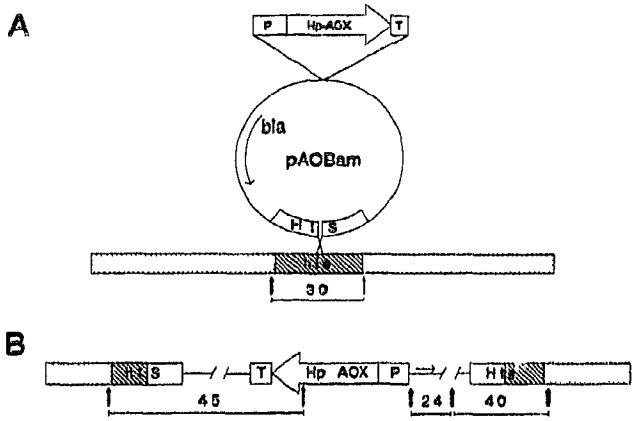

Fig 1 Gene targeting of the *H polymorpha* alcohol oxidase gene into the *his*4 locus of *P pastoris* Panel (A) depicts the transformation vector (pAOBam) containing the *Hp-AOX* gene (arrow) between the promoter (P) and terminator (T) of the *P pastoris AOX1* gene. Homologous recombination of the plasmid, linearized in the *HIS* sequence, with the *his* locus (dashed) of the *P pastoris* genome (stippled) is indicated Panel (B) shows the gene configuration after integration in the *his4* locus The positions (arrows) and lenght of *Bgl*II fragments (B) are indicated

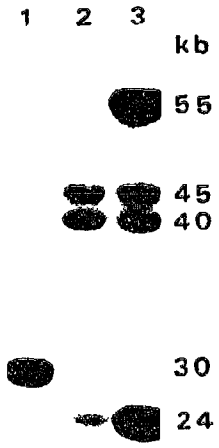

Fig 2 Southern analysis of *Pichia pastoris* GS115 cells containing no (1), one (2) or two copies (3) of the *Hp-AOX* gene integrated in the *his4* gene The *Bgl*II fragments hybridizing with plasmid pYM4 are shown

3 2 *Overexpression of alcohol oxidase*

Providing *P pastoris* with additional copies of the *Hp-AOX* gene resulted in higher alcohol oxidase expression levels upon growth on methanol Whereas the increase in activity with the first additional copy was 73%, that with a second copy was considerably smaller (Table I) To examine the localization of the alcohol oxidase, we performed immunogold labeling on Lowicryl-embedded cell material Fig 3A shows the localization of endogenous alcohol oxidase in *P pastoris* GS115 In these methanol-grown cells, the protein was exclusively located in the peroxisomes; no alcohol oxidase could be found outside the peroxisomes This is in contrast with GS115 cells containing copies of the *AOX* gene of *H polymorpha*. In these cells, alcohol oxidase-immunoreactive material was also found outside the peroxisomes, often in irregular, electron-dense structures not surrounded by a membrane (Fig. 3B,C and D)

Extracts of wild-type GS115 and GS115 containing a double-copy integration of the *Hp-AOX* gene, cultured on methanol, were analyzed by sucrose gradient centrifugation In GS115, only octameric alcohol oxidase could be detected (Fig 4A) The strain, equipped

Table I

Alcohol oxidase activities in *Pichia pastoris* GS115 containing additional copies of *Hp-AOX*

Yeast strain	*Hp AOX* copies	AO activity*
GS115	0	0 85 ± 0 10
GS115 sc**	1	1 47 ± 0 12
Gs115 dc**	2	1.89 ± 0 06

*Activity in μmol H_2O_2 produced per mg protein per min at 37°C, **sc means single copy, dc means double copy of the *Hp-AOX* gene

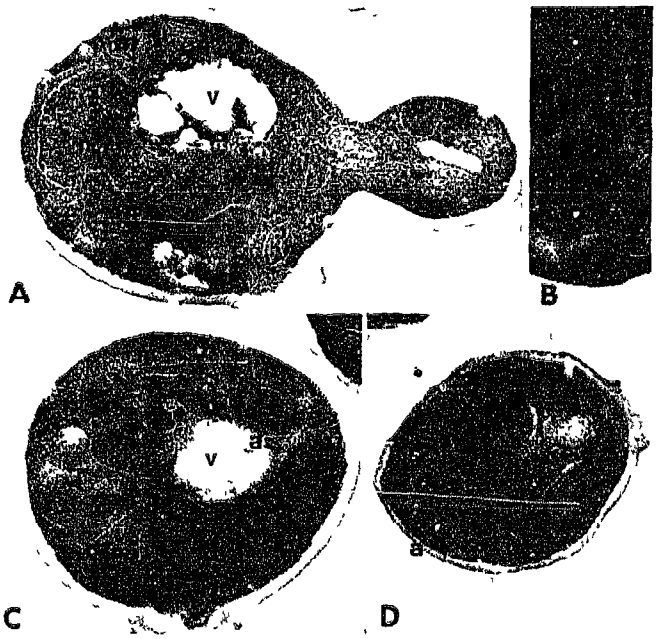

Fig 3 Immuno-electronmicroscopy of *Pichia pastoris* GS115 (A, 15 400×) and a transformant containing two copies of the *Hp-AOX* gene (B 31 000 ×, C, 16 800×, and D, 12 600×) The localization of alcohol oxidase on thin sections of methanol-induced transformants is marked by 10-nm gold particles Organelles are indicated as follows, m = mitochondrion, n = nucleus, p = peroxisome and v = vacuole a = cytosolic aggregate of alcohol oxidase

with two copies of the *Hp-AOX* gene, contained octameric alcohol oxidase as well as alcohol oxidase-immunoreactive material sedimenting at a rate roughly similar to that of bovine serum albumin run in a parallel

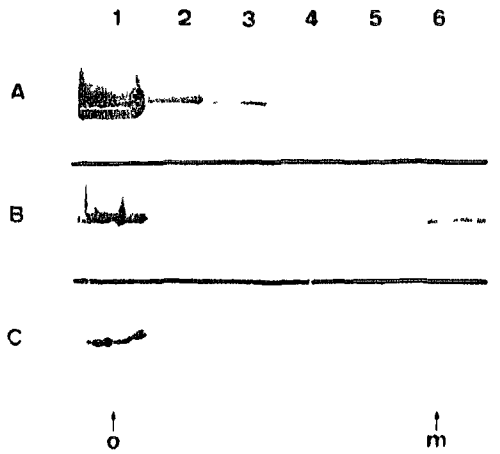

Fig 4 Oligomerization of alcohol oxidase in *Pichia pastoris* GS115 equipped with two copies of the *Hp-AOX* gene Western blots of sucrose gradient fractions (lane 1 bottom, lane 6 top) of cell lysates are shown A Western blot of a cell lysate (200 μg) of non-transformed *Pichia pastoris* GS115 probed with polyclonal antibody against alcohol oxidase B Western blot containing cell lysate (100 μg) of transformant GS115 overexpressing alcohol oxidase probed with polyclonal antibody against alcohol oxidase C, identical to (B) but probed with monoclonal antibodies which preferentially recognized alcohol oxidase from *Hansenula polymorpha* 'o' indicates position of octameric molecules while 'm' represents position of monomeric alcohol oxidase Only the relevant parts of the blots are shown

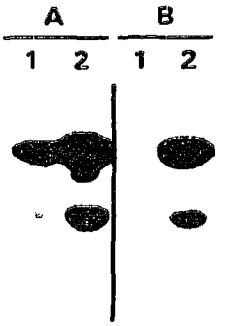

Fig 5 Western blots of methanol-induced cell lysates from *Pichia pastoris* (lane 1, 25 μg) or *Hansenula polymorpha* (lane 2, 25 μg) probed with polyclonal antibodies made against denatured alcohol oxidase from *Hansenula polymorpha* (A) or the monoclonal antibody OAO11 which preferentially recognized *Hansenula polymorpha* alcohol oxidase (B)

gradient (data not shown) suggesting that it represented monomeric alcohol oxidase (Fig. 4B) The amount of the monomeric alcohol oxidase was estimated to be 20–30% of the total alcohol oxidase. Discrimination of the import system against heterologous alcohol oxidase did not occur This can be concluded from a Western blot analysis using a monoclonal antibody which is specific for alcohol oxidase from *Hansenula* and does not react with alcohol oxidase from *Pichia* (Fig 5). Clearly the alcohol oxidase of *Hansenula* assembled into octamers (Fig 4C) as did the endogenous alcohol oxidase from *P. pastoris* (Fig. 4A). Moreover, we have found earlier that peroxisomes of *P pastoris* are able to recognize and accumulate alcohol oxidase from *H polymorpha* When the *Hp-AOX* gene was expressed in a *Pichia* strain in which both endogenous alcohol oxidase genes were disrupted, all alcohol oxidase was localized exclusively inside peroxisomes and was in the active octameric form (M de Hoop, unpublished results). Fig 4C shows that the alcohol oxidase of *Hansenula* was able to assemble into octamers in *P pastoris* Since octamers are normally present inside peroxisomes only [8,16], we conclude that the octameric alcohol oxidase from *Hansenula* is localized inside the organelle The overexpression of alcohol oxidase had no effect on import of other matrix proteins, e.g dihydroxyacetone synthase After immunogold-labelling with antibodies against dihydroxyacetone synthase, gold particles were exclusively confined to the peroxisomal matrix (data not shown)

4 DISCUSSION

Methylotrophic yeasts are an attractive host for the expression of heterologous proteins for several reasons Firstly, methanol is a cheap C-source Secondly, methylotrophic yeasts can be grown to high cell densities (130 g dry cell weight/l) in continuous culture [18]. Thirdly, the promoter of the highly expressed and tightly regulated alcohol oxidase gene can be used to drive synthesis of a foreign protein. Finally, integrating expression vectors yielding stable transformants have been designed

[13]. The peroxisomes present in methanol-cultured yeast might add yet another advantage to this expression system, because the organelles may be used for storage of heterologously expressed proteins known to be susceptible to proteolytic cleavage.

The results presented here show that alcohol oxidase expressed from several gene copies in *P pastoris* is not fully imported and assembled into peroxisomes Obviously, the balance between alcohol oxidase synthesis and its post-translational import is disturbed in these cells, suggesting that saturation of either the import capacity of the peroxisomal space or of the peroxisomal import machinery had occurred Both situations should result in a cytosolic accumulation of all peroxisomal matrix proteins. A similar situation exists in peroxisome-deficient cells, where peroxisomal matrix enzymes are located in the cytosol. In these cells cytosolic crystals composed of active alcohol oxidase are observed [19]. Such alcohol oxidase crystalloids were never observed in the cytosol of strains described in this study and also dihydroxyacetone synthase was still only detectable in the matrix and was not observed in the cytosol. Therefore, we believe that the rate of alcohol oxidase import versus synthesis is limiting, rather than the capacity of peroxisomes for protein storage The fact that peroxisomes can occupy 80% of cell volume in methanol-limited continuous cultures [20] demonstrates that peroxisomes should have a larger storage capacity than seen in the multi-copy gene experiments described here Even under the conditions where peroxisomes do not proliferate such as glycerol [16] or glucose [21], single organelles appear to have the ability to internalize the high amounts of alcohol oxidase synthesized in transformants containing multi-copy plasmids Because in our experiments alcohol oxidase is being expressed from multiple copies of the strong alcohol oxidase promoter, it is reasonable to assume that the rate of alcohol oxidase synthesis is exceptionally high in these strains Why would a rapid rate of synthesis cause saturation of the import system and result in aggregation in the cytosol? Too high an expression rate might oversaturate the capacity of cytosolic unfoldases [22] in their task of holding alcohol oxidase monomers in an import-competent conformation. This might result in the formation of alcohol oxidase aggregates as observed in our studies Once aggregated, the alcohol oxidase is probably no longer suitable for import

Concerning the prospects to compartmentalize heterologous proteins in peroxisomes, one has to take into account that peroxisomes have a large storage capacity, but that the efficiency of import may be inversely related to rate of synthesis of peroxisomal proteins Furthermore, it is also likely that different heterologous proteins are imported at different rates and therefore, it may be necessary to adjust expression rates for optimal compartmentalization of each protein To improve import of heterologous proteins into peroxisomes, either the expression of endogenous peroxisomal proteins has to be reduced, or the import machinery must be further increased by e.g. overexpression of chaperones, like members of the HSP70 family [22,23]

Acknowledgements We thank Dr M A G Gleeson for his interest and stimulating discussions and are grateful to Prof Dr W Harder for critically reading the manuscript A plasmid containing the *Hp-AOX* gene was kindly provided by Drs B Distel and A Ledeboer Part of this work was carried out at the Salk Institute Biotechnology and Industrial Associates, Inc (SIBIA), La Jolla, CA, USA in the group of Dr G Thill We would like to thank him for giving M de Hoop this opportunity This was supported by a Wood/Whelan Research Fellowship from the International Union of Biochemistry and the 'Groninger Universiteitsfonds'

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