Gene regulation and its applied aspects of peroxisomal enzymes in the methylotrophic yeast *Candida boidinii*

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Contents

Chapter III Establishment of high-level production of oxidases in the

peroxisome of *Candida boidinii*

Introduction

The methylotrophic yeast is able to use methanol as a sole carbon and energy source. Methanol is a cheap and high-purity industrial chemical that is prepared in large quantities from natural-occurring methane. Because of growth characteristics on this cheap substrate, methylotrophs are considered to have a great potential in biotechnology. Since the first isolation of the methylotrophic yeast *Candida boidinii* (initially identified as *Kloechera* sp.) in 1969 (59), this yeast has been studied intensively both in the physiological activities and their application. In the early 1970s, the methylotrophic yeasts were extensively studied as the good candidates for the production of single-cell protein (SCP) using the cheap carbon source. In the 1970s, the metabolic pathways for methanol assimilation and dissimilation were elucidated (44), and from the late 1980s to the 1990s, a variety of genes of the enzymes involving in methanol-metabolism were cloned (25). The unique metabolic functions of the methylotrophic yeast have been applied to the development of production of several useful compounds (87). Furthermore, several methylotrophic yeast strains have been developed and attracted much attention as a eukaryotic host for the efficient heterologous gene expression system in both academic and industrial fields. The merits of the system are; i) the gene expression can be tightly regulated by the growing carbon source; ii) the production is highly efficient both in intracellular and secretory protein production; iii) the growth medium is cheap and cells can be grown up to high-cell density; iv) the system has advantages for the foreign proteins from eukaryotic cells because the methylotrophic yeast have an essentially similar intracellular structure necessary for protein folding. By these systems, many kinds of useful proteins have been produced, which include plant, bacterial and human proteins, enzymes, antibodies, cytokines, plasma proteins, and hormones (25).

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The initial reactions of methanol metabolism take place in specialized organelle, peroxisomes, followed by subsequent metabolic steps in the cytosol. Methanol is oxidized by alcohol oxidase (AOD) to generate formaldehyde and hydrogen peroxide, which is decomposed to water and molecular oxygen by catalase (CTA). Although formaldehyde is a toxic chemical to all living cells, it is a central intermediate of the methanol metabolism, in which it enters both the assimilatory pathways to synthesize cell constituents and the cytosolic dissimilatory pathway to yield energy. The initial reaction of the former pathway is catalyzed by dihydroxyacetone synthase (DAS), generating dihydroxyacetone and glyceraldehyde-3-phosphate in a transketolase reaction between formaldehyde and xylulose-5-phosphate. These three key enzymes, AOD, DAS, and CTA, in methanol metabolism are localized in peroxisomes. In the dissimilatory pathway, formaldehyde is oxidized to carbon dioxide via formate by two subsequent dehydrogenases, glutathione-dependent form-aldehyde dehydrogenase (FLD) and formate dehydrogenase (FDH) (Fig. 0).

The key enzymes of methanol metabolism are strongly induced and accumulated in large amounts during growth on methanol. Especially, AOD content reaches up to 30% of the total soluble proteins of the methanol-grown cells. Accompanied with the development of the genetical methods, the high-level gene expression systems using the strong promoters of AOD genes, *AOD1* (*C. boidinii*), *AOX1* (*Pichia pastoris*), *MOX* (*Hansenula polymorpha*), and *AUG1* (*P. methanolica*), have been established (9, 26, 63, 76). In *C. boidinii* system, protein productions under the control of their localization in the cell have been established; for example, adenylate kinase from *Saccharomyces cerevisiae* as an intracellular protein and glucoamylase from *Rhizopus oryzae* as a secreted (64, 71). In spite of the application of methanol-inducible genes, the molecular basis of the methanol-induction has not been studied in detail.

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Peroxisomes

CH_3OH

O_2

H_2O_2 ← AOD

CTA

$\frac{1}{2}O_2 + H_2O$

HCHO

DAS

Xu5P

DHA   GAP

DHAP   GAP   Xu5P

FBP → F6P

Cytosol

HCHO → HCOOH
FLD
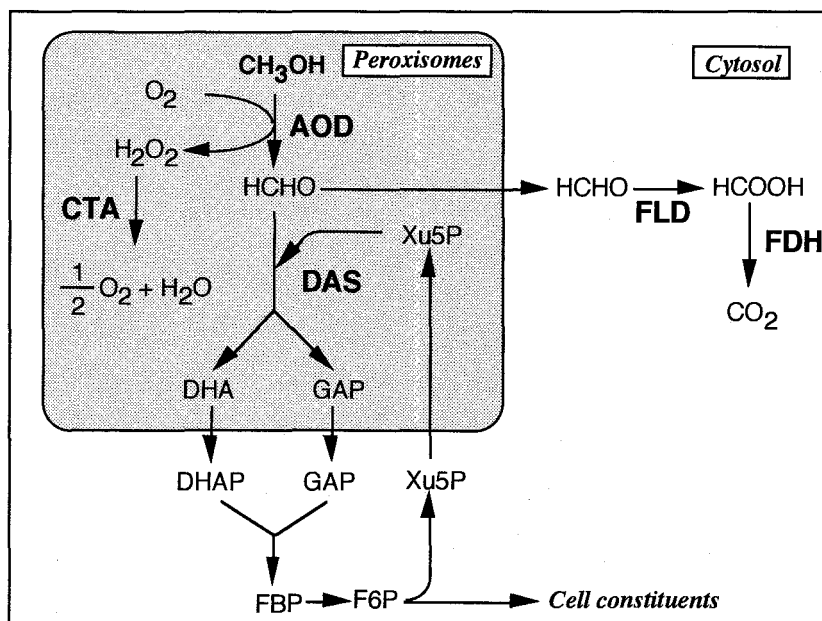
FDH

CO_2

Cell constituents

Fig. 0.   Methanol metabolism in the methylotrophic yeast.   AOD, alcohol oxidase; DAS, dihydroxyacetone synthase; CTA, catalase; FLD, glutathione-dependent formaldehyde dehydrogenase; FDH, formate dehydrogenase; DHA, dihydroxyacetone; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; Xu5P, xylulose-5-phosphate; FBP, fructose-1,6-biphosphate; F6P, fructose-6-phosphate.

A methylotrophic yeast C. boidinii can grow not only on methanol but also on oleate or D-alanine as a sole carbon source (29).   These carbon sources induce peroxisome proliferation, and the initial key enzymes metabolizing these compounds are localized in this organelle, i.e., acyl-CoA oxidase in oleate metabolism and D-amino acid oxidase in D-alanine metabolism.   Since the growth on different carbon sources resulted in distinct patterns of intracellular protein and the morphology of peroxisomes, C. boidinii, whose molecular breeding methods had been established, has been used as a useful model organism to analyze the mechanism of peroxisome biogenesis and degradation.

This thesis concerns the gene regulation and its applied aspects of peroxisomal enzymes in C. boidinii.   In Chapter I, the regulation of the

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expression of peroxisomal proteins and organelle proliferation by peroxisome-inducing carbon sources are described. The author cloned and disrupted the gene encoding D-amino acid oxidase, which was induced by D-alanine, in order to clarify the physiological role of this enzyme. In Chapter II, the author evaluates five methanol-inducible promoters, and clarifies that the gene regulation of methanol-inducible genes is closely related to the flow of methanol metabolism. Finally in Chapter III, based on the obtained results in Chapter I and II, the author describes successful strategy for production of useful oxidases in the peroxisomes, which are developed up to 80% of intracellular volume during methanol-induction.

Chapter I

Regulation of peroxisome proliferation and peroxisomal proteins by carbon and nitrogen sources

Section 1   Regulation of peroxisomal proteins and organelle proliferation by multiple carbon sources in the methylotrophic yeast *Candida boidinii*

Introduction

Peroxisomes are single-membrane-bound organelles that are ubiquitously found in eukaryotic cells. In mammalian cells, peroxisomes are involved in various metabolic processes, such as the β-oxidation of fatty acids, cholesterol synthesis, and D-amino acid metabolism (96). Therefore, peroxisomal proliferation and this metabolism should be strictly controlled at various levels of regulation corresponding to cellular demand. In the last decade, proteins involved in peroxisome biogenesis have been identified in various organisms, and recently their nomenclature has been unified, they being designated as peroxines (12). The sequence similarity of several *PEX* genes in yeasts and mammalian cells indicates that the basic molecular mechanism for peroxisome biogenesis has been conserved during evolution.

In yeasts, peroxisomes generally develop in response to environmental stimuli. For example, a methylotrophic yeast, *Candida boidinii*, can grow not only on methanol but also on oleate or D-alanine as a single carbon source concomitant with peroxisomal proliferation (29). Peroxisomes play an indispensable role in this growth of cells on these peroxisome-inducing carbon sources (PICs), since *C. boidinii pex* mutants showed impaired growth on all of

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these PICs (68). However, the protein composition of peroxisomes depends on the PIC in the medium, reflecting that peroxisomal metabolism differs among three PICs. So far, the protein composition, and regulation of peroxisomal matrix and membrane proteins in cells grown on a single PIC have been studied in *C. boidinii* ATCC32195 (29). However, in the strain used, all of the analyzed peroxisomal membrane proteins (PMPs), Pmp20, Pmp47, and CbPex11p (Pmp30), are encoded by two different loci (24, 46, 52).

In this study, the author analyzed the regulation of peroxisomal proteins and organelle proliferation in *C. boidinii* strain S2 grown on multiple PICs to study the induction of peroxisomal proteins and organelle proliferation (68, 73). The strain used was a haploid strain of *C. boidinii* with which a heterologous protein can be produced (64, 71). To follow peroxisomal proliferation *in vivo*, a *C. boidinii* transformant producing a green fluorescent protein (GFP) tagged with peroxisomal targeting signal 1 (PTS1), -AKL, at the carboxyl terminal, was observed under a fluorescence microscope (51). In this section, the study was conducted to determine i) whether there is any "priority rule" in the utilization of multiple PICs (*i.e.*, do cells synthesize peroxisomal proteins characteristic of all PICs present in the medium or specific PICs?); ii) whether peroxisomal proliferation is sensitive to glucose repression for all PICs; and iii) whether the observed regulation occurs at the mRNA level.

Materials and Methods

Strains, media, and cultivation

C. boidinii S2 was used in all experiments (88). The organism was grown on the synthetic MI medium described previously (66). As carbon sources, 2% glucose (w/v) , 1% methanol (v/v), 0.5% oleate (v/v), and 0.6% D-alanine

(w/v), were used. Tween 80 was added to the oleate-medium at a concentration of 0.05% (v/v). When D-alanine was used as both nitrogen and carbon sources, NH_4Cl was omitted from the MI medium. The initial pH of the medium was adjusted to 6.0. Cultivation was aerobic at 28°C with reciprocal shaking, and growth was followed by measuring the optical density at 610 nm.

C. boidinii strain TK62 (ura3 (66)) and strain pex5Δ (ura3) were used as hosts for transformation. The strain pex5Δ was derived from strain TK62 as PEX5 gene disruptant (Sakai, Y., unpublished results). Escherichia coli JM109 (78) was used for plasmid propagation.

Enzyme assays

Cells were harvested by centrifugation at 500 x g for 10 min at 4°C, washed twice with ice-cold distilled water, resuspended in 0.1 M potassium phosphate buffer, pH 7.5, and then subjected to disruption with a KUBOTA Insonator Model 201M (2 MHz for 35 min). The cell debris was removed by centrifugation at 16,000 x g for 5 min at 4°C. The resultant supernatant was immediately subjected to enzyme activity assays. The activities of catalase (CTA), alcohol oxidase (AOD), D-amino acid oxidase (DAO), and acyl-CoA oxidase (ACO) were assayed by the methods of Bergmeyer (2), Tani et al. (89), Goodman et al. (29), and Shimizu et al. (81), respectively. Protein was determined by the method of Bradford (4) with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum albumin was used as the standard.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western analysis

Standard 9% Laemmli gels (43), with separating gels of pH 9.2, were employed. A cell-free extract containing 50 μg protein was loaded per each lane. Western analysis was performed as described by Towbin (93) using an Amersham ECL detection kit (Arlington Heights, IL, USA). The VA9 monoclonal anti-Pmp20 antibody, IVA7 monoclonal anti-Pmp47 antibody, and G358 polyclonal anti-AOD antibody were kindly provided by Dr. J. M. Goodman (University of Texas, Southwestern Medical Center at Dallas).

Northern analysis

Total RNAs were extracted from cells using an ISOGEN RNA extraction kit (Nippon Gene Co., Ltd., Tokyo, Japan), and electrophoresed on a 1.1% agarose gel made with 20 mM MOPS buffer containing 1 mM EDTA and 2.2 M formaldehyde. Fractionated total RNA was blotted onto membrane filter (Gene Screen Plus; Biotechnology Systems NEN Research Products, Boston, MA, USA). Hybridization was performed under highly stringent conditions as previously described (65). Labeling was performed by the random primer extension method of Feinberg and Vogelstein (19). The ^{32}P-labeled probes were a 0.7-kb *Bgl* II-*Sal* I-fragment derived from pMOX33 harboring the coding region of *AOD1* (74), a 0.4-kb *Sty* I-*Hinc* II fragment harboring *C. boidinii CTA1* (catalase) (Sakai, Y., unpublished results), a 1.9-kb *Pst* I fragment harboring *C. boidinii PMP20* (Horiguchi, H., unpublished results), a 1.6-kb *Hinc* II-*Hind* III-fragment derived from pMP471 harboring the coding region of *PMP47* (73), a 0.4-kb *Sty* I-*Hind* III fragment of pDA7 coding for DAO (Chapter I, Section 2), a 0.7-kb fragment coding for ACO (Yurimoto, H., unpublished results) and a 0.9-kb *Cla* I-*Hind* III fragment harboring *C. boidinii ACT1* DNA (coding for actin) (73).

Construction of *C. boidinii* GFP-AKL

The *GFP* gene expression cassette consisted of the *C. boidinii* actin promoter, the modified *GFP* gene and the *C. boidinii* actin terminator. The modified *GFP* genes were constructed through the use of PCR. The *GFP* gene was tagged with Gly-Gly (STOP) or Gly-Gly-Ala-Lys-Leu (AKL) at the carboxyl terminus. These modified *GFP* genes were ligated into pACT1 that containing pBR322, actin promoter, actin terminator, and *URA3*). The resulting plasmids (pGFP-STOP and pGFP-AKL) were integrated into the chromosomal DNA of *C. boidinii* TK62 or *C. boidinii pex5Δ* by the modified lithium acetate method (65). Transformants were termed GFP-AKL/wt, GFP-STOP/wt, GFP-AKL/*pex5Δ*, and GFP-STOP/*pex5Δ*.

Fluorescence microscopy

C. boidinii strain GFP-AKL was used to observe peroxisomal proliferation *in vivo*. The cell suspension was placed on a microscope slide and examined using the FITC channel of a Axioplan 2 fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with a Plan-NEOFLUAR 100x/1.30 (oil) objective and Nomarski attachments. Cells were photographed on Fuji NEOPAN SS 135 black-and-white film. The number of peroxisomes per cell was determined for 40 to 80 cells in a randomly selected field.

Results

Regulation of peroxisomal proteins by PIC(s)

i) Regulation by a single PIC

In *C. boidinii* strain S2, three PMPs (Pmp47, CbPex11p, and Pmp20) and at least seven other proteins are encoded by a single locus (67, 73). At first, the

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author studied the regulation of these PMPs and several peroxisomal enzymes by a single PIC in C. *boidinii* S2.
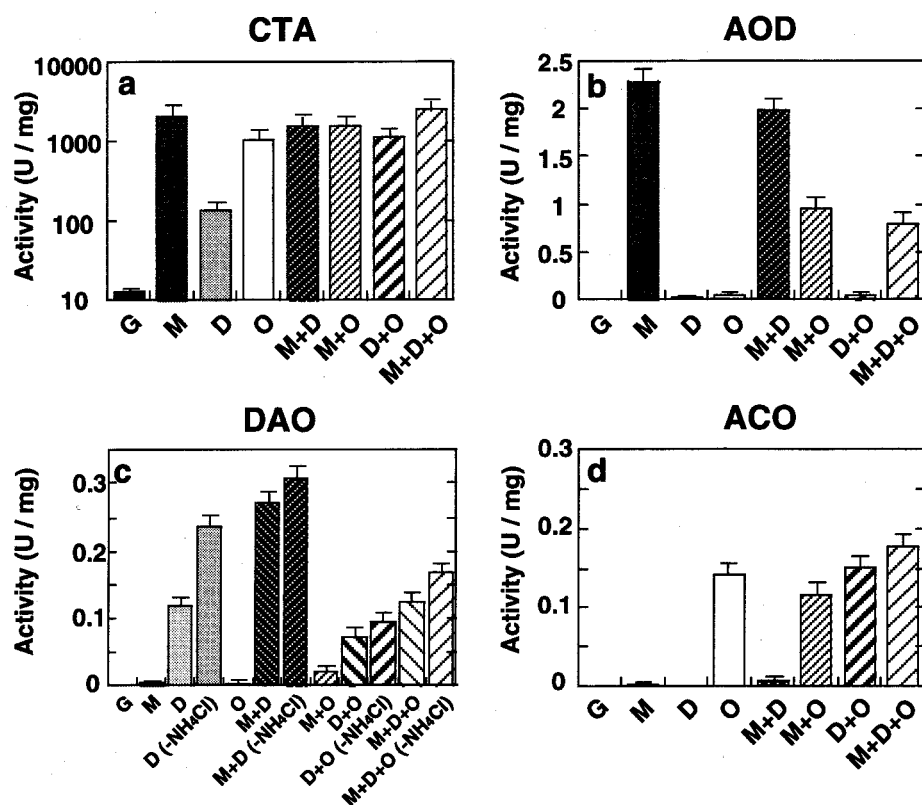

Fig. 1-1. Peroxisomal enzyme activities of C. *boidinii* S2 grown on a single PIC or various combinations of PICs. Cells were grown to the early-log phase on the indicated carbon source(s), and then enzyme activities (a, catalase (CTA); b, alcohol oxidase (AOD); c, D-amino acid oxidase (DAO); d, acyl-CoA oxidase (ACO)) were measured as described under Materials and Methods. The enzyme activities are expressed as units/mg protein in the crude cell-free extracts. The results from three independent experiments are given. The carbon sources were: G, glucose; M, methanol; D, D-alanine; O, oleate.

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C. boidinii S2 was grown on glucose or a peroxisome-inducing carbon source (PIC), *i.e.*, methanol, D-alanine, or oleate, and then peroxisomal enzyme activities were determined (Fig. 1-1a-d, columns G, M, D, and O). The regulation of PMPs was also followed by Western analysis (Fig. 1-2). Catalase (CTA) activity and Pmp47 were significantly induced by all PICs when compared to those in glucose-grown cells. (The amount of Pmp47 in oleate- and methanol-grown cells was higher than that in D-alanine-grown cells.) In contrast to CTA activity and Pmp47, other proteins were induced by a specific PIC: the activities of alcohol oxidase (AOD) and Pmp20 were induced by methanol, the activities of acyl-CoA oxidase (ACO) was induced by oleate, and the activity of D-amino acid oxidase (DAO) was induced by D-alanine. When D-alanine was used as the single carbon and nitrogen source (Fig. 1-1c, D (-NH_4Cl)), DAO activity was about twice higher than when it was used as the single carbon source (Fig.1-1c, D).

Next, Northern analysis was performed to confirm that the observed regulation occurred at the mRNA level. As shown in Fig. 1-3, mRNAs of *CTA1* and *PMP47* were detected in cells grown on all PICs. In contrast, mRNAs of *AOD1* and *PMP20* were observed only in methanol-grown cells and mRNA of ACO was detected only in oleate-grown cells (Fig. 1-5b, lanes G, M, D, and O). These mRNAs were not detected in glucose-grown cells. All hybridizing bands were detected at the expected sizes (data not shown), and *ACT1* mRNA was almost constant. These band intensities precisely reflected the regulatory profile estimated from the enzyme activities and the results of Western analysis.

From the results obtained, a haploid strain of *C. boidinii*, strain S2, was shown to have a similar profile of peroxisomal protein regulation by a single PIC to that of *C. boidinii* ATCC 32195 (29, 52), *i.e.*, peroxisomal proteins could be classified into i) metabolism-specific proteins (AOD and Pmp20 for methanol,

ACO for oleate, and DAO for D-alanine), and ii) metabolism non-specific proteins (CTA and Pmp47).
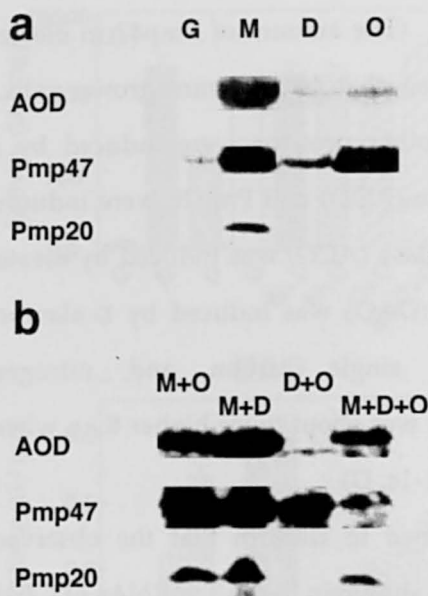

Fig. 1-2.   Western blots of crude extracts of cells of   C. boidinii S2   grown on (a) a single PIC   or   (b)   various combinations of PICs. Cells were grown on G, glucose; M, methanol; D, D-alanine; or O, oleate, or on combinations of them.   Each lane was loaded with   50 µg protein.
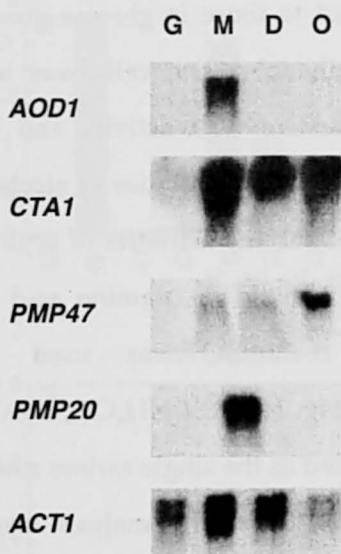
Fig. 1-3.   Northern analysis of peroxisomal matrix   and   membrane   proteins   in C. boidinii S2.   Total RNAs   (20 µg) extracted from   cells   grown on   M,   methanol; O, oleate;   D, D-alanine; or   G, glucose, as a single carbon source   were loaded on each lane,   and then probed with the indicated DNA   fragment   as   described   under Materials and Methods.   The   C. boidinii ACT1   DNA coding for actin was used as a control for constitutive expression.

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ii) Regulation by multiple PICs

To determine whether or not there is a priority rule among PICs for peroxisomal metabolism, cells were grown on various combinations of PICs., *i.e.*, methanol (M), D-alanine (D), and oleate (O). Enzyme activities (Fig. 1-1) and induced PMPs (Fig. 1-2b) were analyzed.

The activities of a peroxisomal marker enzyme, CTA, and Pmp47, were induced when cells were grown on any combination of PICs (Fig. 1-1a for CTA; and Fig. 1-2b for Pmp47). Methanol-specific proteins, AOD and Pmp20, were induced when methanol was present in the medium (Fig. 1-1b; and Fig. 1-2b), and the induction of these methanol-specific proteins was not affected by the presence of other PICs, *i.e.*, oleate and/or D-alanine. In the case of M+O and M+D+O (Fig. 1-2b), the AOD activities seemed to be reduced by the presence of oleate. However, in this case, the peroxisome metabolism was diverse, and consequently, the specific activities of each enzyme decreased. In fact, compared from the repressed level by glucose as described below, these activities were still at the induced level.

Similarly, the activities of oleate-inducible enzymes, ACO was induced when oleate was present in the medium (Fig. 1-1d), and the induction was not repressed in the presence of methanol and/or D-alanine. DAO activity was induced when D-alanine was present in the medium (Fig. 1-1c), and the induction was not repressed by the coexistence of methanol and/or oleate. When D-alanine was used as both the carbon and nitrogen source (Fig. 1-1c, denoted as (-NH$_4$Cl)), DAO activity was higher than that when D-alanine was used as the carbon source and NH$_4$Cl was used as the nitrogen source.

Thus, the three PICs induced peroxisomal proteins independently, and there was no priority rule among the PICs, *i.e.*, *C. boidinii* seems to utilize any

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combination of PICs simultaneously when multiple PICs are present in the medium.
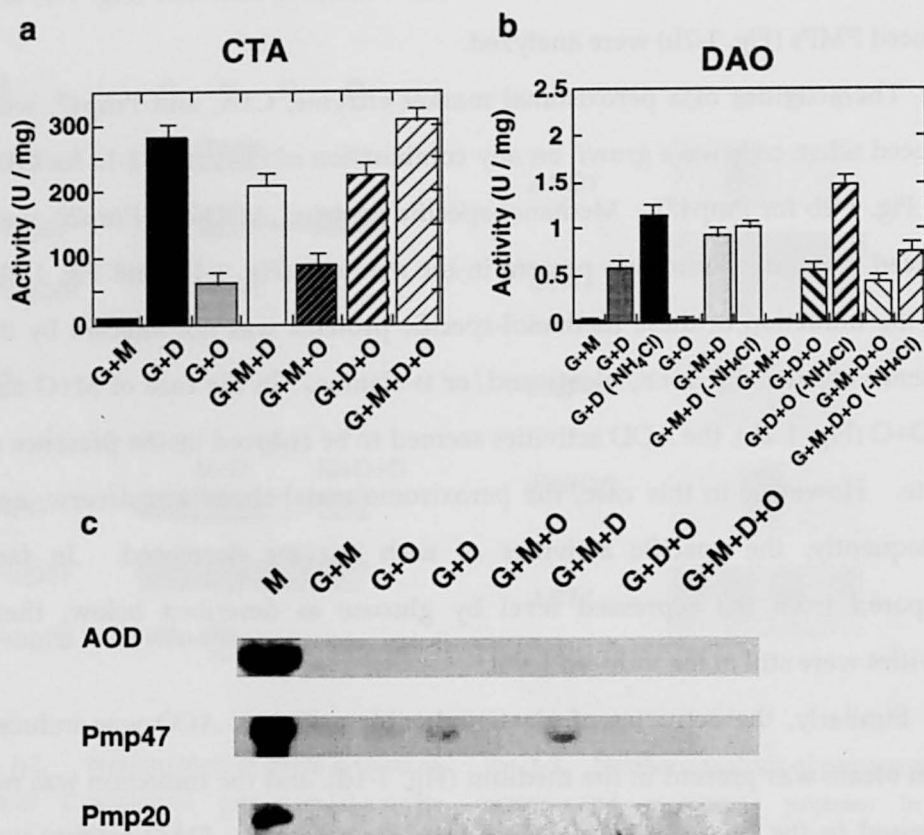

Fig. 1-4. (a-b) Enzyme activities and (c) Western analysis of *C. boidinii* S2 grown on various combinations of PICs and glucose. Cells were grown to the early-log phase on the indicated carbon sources, and then enzyme activities were measured as described under Materials and Methods. a, catalase; b, D-amino acid oxidase. The enzyme activities are expressed as units/mg protein in the crude cell-free extracts. The results from three independent experiments are given. The carbon sources were: G, glucose; M, methanol; D, D-alanine; O, oleate. (c) Each lane was loaded with 50 μg protein. Western analysis was performed as described under Materials and Methods.

iii) Sensitivity to glucose

In yeasts, methanol- and oleate-inducible peroxisomal enzymes and peroxisomes have been reported to be sensitive to glucose repression. Furthermore, glucose triggers an autophagic process to degrade methanol- and oleate-inducible peroxisomes (5, 94, 95).

First, the effect of glucose on the formation of peroxisomal proteins was studied by growing cells on glucose-containing medium in combination with various PICs (Fig. 1-4 a-b). When glucose was present in the medium, the induction of AOD- and ACO-activities was repressed in all cases tested (data not shown). On the contrary, DAO activity was highly induced in all media containing D-alanine, i.e., G+D, G+M+D, G+D+O, and G+M+D+O (Fig. 1-4b). And CTA activities in D-alanine-containing media (G+D, G+M+D, G+D+O, or G+M+D+O) was higher than that in media without D-alanine (G+M, , G+O, or G+M+O) (Fig. 1-4a). Thus, methanol- and oleate-induced peroxisomal enzymes were repressed by glucose, but the induction of DAO- and CTA-activities was not repressed in the presence of glucose. When glucose was present together with various PICs in the medium, the AOD and Pmp20 proteins were not detected on Western analysis (Fig. 1-4c). However, in spite of the presence of glucose, Pmp47 could be detected when D-alanine was present as a PIC (Fig. 1-4c, lanes G+D and G+M+D). Thus, although AOD and Pmp20, which were massively induced by methanol, were completely repressed by glucose, D-alanine-induced Pmp47 was not.

To confirm that the DAO1 gene (coding for DAO) is insensitive to glucose repression at the mRNA level, cells were grown on G, M, D, G+M, G+D, or M+D as carbon source(s), and Northern blot analysis was performed using DAO1- or AOD1-DNA as probe. As shown in Fig. 1-5a, the DAO1 mRNA was detected not only in D and M+D but also in G+D. In contrast, the AOD1

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mRNA was detected in M and M+D, but not in G+M. Similarly, the *ACO1* (coding for ACO) mRNAs were detected in O, M+O, and D+O (Fig. 1-5b). These results suggested that while the expression of *AOD1* and *ACO1* are repressed by glucose, that of the *DAO1* is not, and that their expression is regulated at the mRNA level.
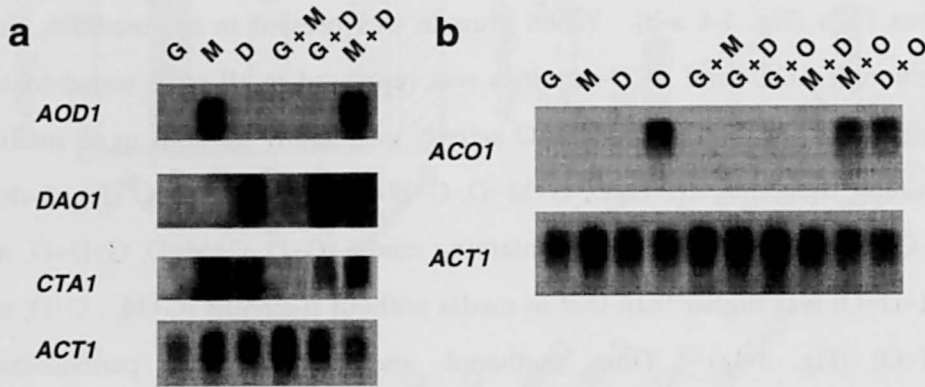

Fig. 1-5. Induction of *DAO1* (D-amino acid oxidase) was insensitive to glucose repression in *C. boidinii* S2. Total RNAs (20 µg) were extracted from cells grown on the indicated carbon sources, loaded on each lane, and then probed with *AOD1*-, *DAO1* -, *CTA1*- (a) or *ACO1*- (b) DNA. G, glucose; M, methanol; D, D-alanine. The *C. boidinii ACT1* DNA coding for actin was used as a control for constitutive expression.

Peroxisomal proliferation by *C. boidinii* strain GFP-AKL

The fact that the induction of three peroxisomal proteins (DAO, catalase, and Pmp47) by D-alanine was not repressed by glucose strongly suggested that the proliferation of D-alanine-induced peroxisomes is also insensitive to glucose repression. To confirm this, the author introduced a GFP-AKL-expression plasmid into *C. boidinii* to visualize peroxisomes and to follow peroxisomal

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proliferation *in vivo* (strain GFP-AKL/wt). GFP-AKL is GFP tagged with -Ala-Lys-Leu (-AKL) at the C-terminal. It was constitutively expressed in *C. boidinii* S2 under the *C. boidinii* ACT1 (actin) promoter. The C-terminal 3 amino acid residues, -AKL, comprise a typical motif of peroxisomal targeting signal 1 (PTS1) (30, 45), and is sufficient for peroxisomal transport in *C. boidinii*. This GFP analysis enabled us to identify peroxisomes even when they were low in number, as in D-alanine- or glucose-grown cells.

The author constructed four GFP expressing *C. boidinii* strains (GFP-AKL/wt, GFP-STOP/wt, GFP-AKL/*pex5Δ*, and GFP-STOP/*pex5Δ*). These four strains induced on methanol-containing medium were observed under the fluorescent microscope. GFP-AKL/wt cells contained intrinsic green fluorescent punctate structures (Fig. 1-6a). In contrast, cells of GFP-AKL/*pex5Δ*, GFP-STOP/wt and GFP-STOP/*pex5Δ* did not have any punctate structures, but green fluorescence was diffused in the whole cytosol (Fig. 1-6b, c, and d). The punctate structures observed in GFP-AKL/wt cells were identified as peroxisomes (see Discussion).

When cells were grown on glucose as a single carbon source, the cells contained 1 to 2 (1.42 ± 0.0019 (S. D.)) very small peroxisomes (Fig. 1-7a). On the other hand, cells grown on PICs contained peroxisomes corresponding to each carbon source; methanol-grown cells had 3 to 6 large peroxisomes in a cluster (Fig. 1-6a), oleate-grown cells had 8 to 12 (10.3 ± 0.85) small peroxisomes (Fig. 1-7d), and D-alanine-grown cells had 2 to 3 (2.28 ± 0.17) small peroxisomes (Fig. 1-7b). When D-alanine was used as both the carbon and nitrogen source, cells had 2 to 5 (3.17 ± 0.13) small peroxisomes (Fig. 1-7c).
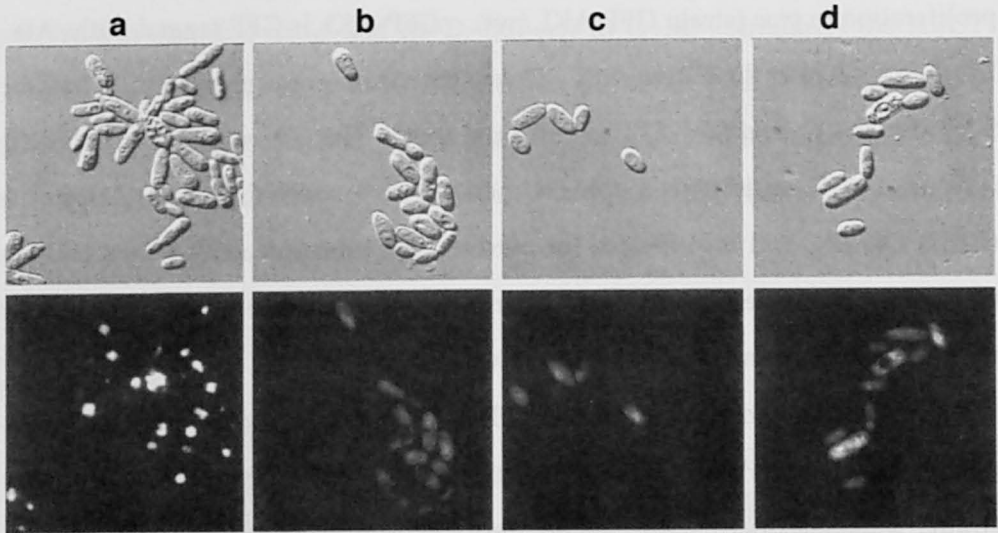
Fig. 1-6. Observation of peroxisome proliferation in vivo by use of GFP. *C. boidinii* strains (a, GFP-AKL/wt; b, GFP-STOP/wt; c, GFP-AKL/*pex5Δ*; d, GFP-STOP/*pex5Δ*) were placed on methanol-containing medium and observed under the fluorescent microscope. Pictures were obtained using Nomarski (upper) and fluorescence optics (lower).

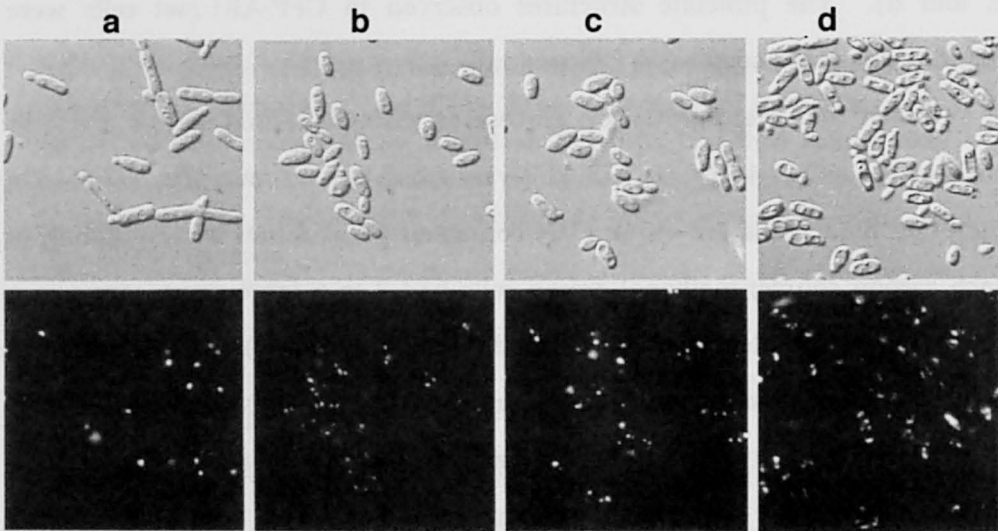
Fig. 1-7. Peroxisomes of the cells grown on various carbon sources labeled with GFP. *C. boidinii* strain GFP-AKL was grown on various carbon sources (a, glucose; b, D-alanine; c, D-alanine (- NH₄Cl); d, oleate). Cell suspensions were subjected to fluorescent microscopic observation. Pictures were obtained using Nomarski (upper) and fluorescence optics (lower).

.

Fig. 1-8. Peroxisomes of the cells grown on various combination of carbon sources labeled with GFP. *C. boidinii* strain GFP-AKL was grown on various carbon sources (a, glucose + methanol; b, glucose + D-alanine; c, glucose + D-alanine (- NH_4Cl); d, glucose + oleate; e, methanol + oleate.) Cell suspensions were subjected to fluorescent microscopic observation. Pictures were obtained using Nomarski (upper) and fluorescence optics (lower).
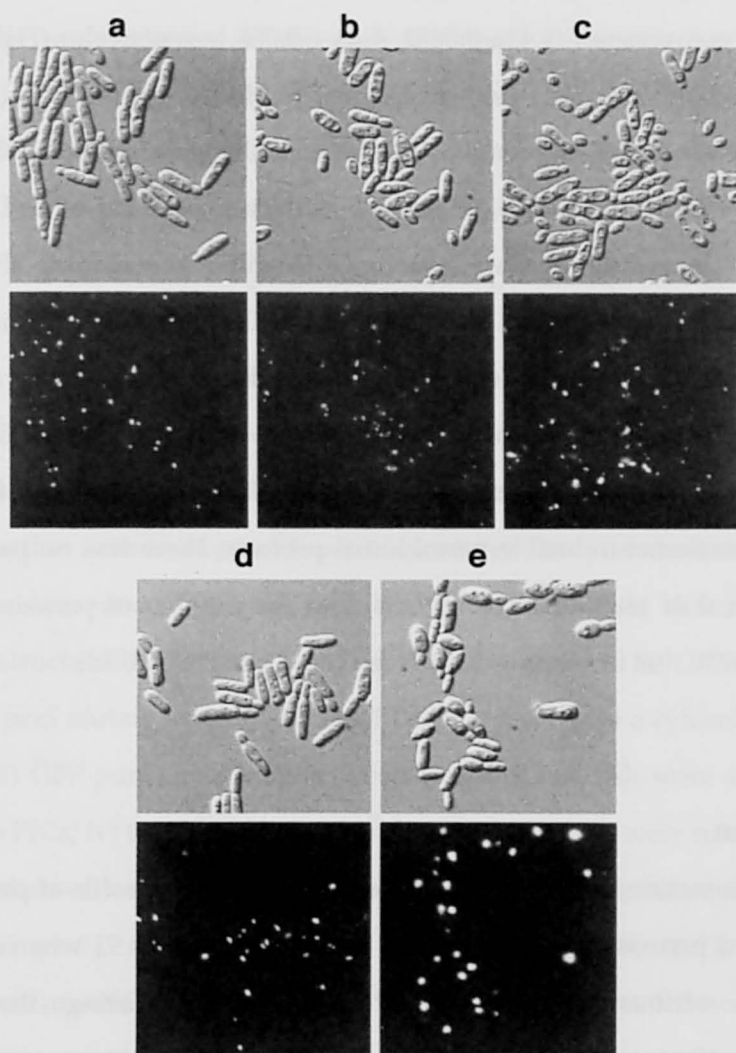
When cells were grown on G+M or G+O, the cells contained one or two very small peroxisomes (1.41± 0.0082, 1.43 ± 0.058, respectively) (Fig. 1-8a and d), which is similar to the number observed in glucose-grown cells (Fig. 1-7a). But cells grown on G+D contained 2 to 3 small peroxisomes (2.03 ± 0.042) (Fig. 1-8b), which is similar to the number in D-alanine-grown cells (2.28 ± 0.17) (Fig. 1-7b). In addition, cells grown on G+D (-NH$_4$Cl) contained 2 to 5 small peroxisomes (2.27 ± 0.12) (Fig. 1-8c), which is similar to the number in D-alanine (-NH$_4$Cl) (2.28 ± 0.17) (Fig. 1-7c). Thus, glucose did not inhibit the proliferation of D-alanine-induced peroxisomes. When cells were grown on M+O, the cells contained 5 to 10 peroxisomes (8.2 ± 0.30) (Fig. 1-8e). Although these cells contained methanol-inducible peroxisomal proteins, there was no peroxisomal cluster typical of methanol-grown cells, and the number of peroxisomes was more similar to that in oleate-grown cells (10.3 ± 0.85) (Fig. 1-7d).

Discussion

In this section, the author examined the regulatory profile of peroxisomal proteins and peroxisomal proliferation in *C. boidinii* strain S2 when cells were grown on various combinations of metabolically distinguishable PICs (methanol, oleate, and D-alanine), and glucose, a potent repressor of peroxisomal proliferation.

Waterham *et al.* reported that *C. boidinii* peroxisomes could have two metabolically different enzymes, *i.e.*, AOD and ACO, in one compartment, when cells are grown in oleate-methanol limited continuous cultures or when methanol is added at the stationary phase of oleate-grown cells (99). However, under their conditions, the concentration of oleate in the medium was supposedly insufficient for any repression of methanol-inducible proteins, and

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it was still unclear as to the presence of a priority rule among PICs. In this study, cells were collected at the early-log phase to ensure that all carbon sources were still present in the medium. The author's results show that there is no priority rule among PICs as to the induction of peroxisomes, and that *C. boidinii* is able to utilize multiple PICs simultaneously.

While peroxisomal induction by methanol or oleate was completely repressed by the coexistence of glucose, that by D-alanine was not. This was proved in terms of i) induction of Pmp47, DAO- and CTA-activities, ii) *DAO1* expression, and iii) organelle proliferation. The author followed peroxisomal proliferation using a *C. boidinii* strain producing GFP-AKL. The punctate structures the author observed were identified as peroxisomes from the following observations: i) wild type GFP (without PTS1) did not show a punctate structure but rather a cytosolic diffusion pattern; ii) GFP-AKL in the *C. boidinii pex5* mutant (defective in the PTS1 receptor) gave a cytosolic diffusion pattern; iii) GFP-punctate structures proliferated when cells were shifted from glucose to PICs; iv) their morphology and number on PICs were consistent with previous observations on electron microscopy; and v) oleate-induced cells of the *C. boidinii pex11* mutant contained large peroxisomes ((67), Sakai, Y., unpublished results). Therefore, peroxisomal proliferation followed by GFP-AKL was not an artifact but was indeed a reflection of peroxisomal proliferation in *C. boidinii* cells. Since peroxisomal assembly and proliferation require many peroxine gene products, and matrix and membrane proteins, their regulation needs to be properly coordinated. So far, peroxisomal proliferation in yeast has been believed to be sensitive to glucose repression. However, this study firstly showed that all peroxisomal proliferation may not necessarily be sensitive to glucose repression, at least in the case of D-alanine-induced peroxisomes in *C. boidinii*.

It is also suggested herein that the synthesis of peroxisomal matrix enzymes and PMPs is mainly regulated at the mRNA level by PICs and glucose. These peroxisomal genes have been cloned, and their regulation is being studied at the transcriptional level by placing the reporter genes under their promoters (Chapter II, Section 1). Through such studies the author hopes i) to clarify the unique regulatory profile of D-alanine-induced peroxisomes; ii) to determine the strength of each promoter; and iii) to answer the question of whether or not the precedence of PMP synthesis to matrix enzymes is indeed controlled at the transcriptional level (73, 97).

Summary

A methylotrophic yeast, *C. boidinii,* was grown on various combinations of peroxisome-inducing carbon source(s) (PIC(s)), *i.e.,* methanol, oleate, and D-alanine, and the regulation of peroxisomal proteins (both matrix and membrane ones) and organelle proliferation were studied.   This regulation was followed i) at the protein or enzyme level by means of the peroxisomal enzyme activity and Western analysis; ii) at the mRNA level by Northern analysis; and iii) at the organelle level by direct observation of peroxisomes under a fluorescent microscope.   Peroxisomal proliferation was followed *in vivo* by using a *C. boidinii* strain producing a green fluorescent protein (GFP) having peroxisomal targeting signal 1 (PTS1).   When multiple PICs were used for cell growth, *C. boidinii* induced specific peroxisomal proteins characteristic of all PIC(s) present in the medium, responding to all PIC(s) simultaneously. Thus, these PICs were considered to induce peroxisomal proliferation independently and not to repress peroxisomes induced by other PICs.   Next, the sensitivity of the peroxisomal induction to glucose repression was studied. While the peroxisomal induction by methanol or oleate was completely repressed by glucose, the D-alanine-induced activities of D-amino acid oxidase and catalase, Pmp47, and the organelle proliferation were not.   These results indicate that peroxisomal proliferation in yeasts is not necessarily sensitive to glucose repression.   Lastly, this regulation was shown to occur at the mRNA level.

Section 2   Physiological role of the D-amino acid oxidase gene, *DAO1*, in

carbon and nitrogen metabolism in the methylotrophic yeast

Candida boidinii

Introduction

D-Amino acid oxidase (DAO, EC 1.4.3.3) is a flavoprotein that catalyzes the oxidation of D-amino acids to the corresponding 2-imino acids and hydrogen peroxide. Each imino acid is nonenzymatically hydrolyzed to α-keto acid and ammonia (34). Almost all mammals have this enzyme in their kidneys, livers, and brains. Although its molecular properties and kinetic mechanism have been elucidated in detail (23, 50), its physiological role is unclear. Recent several lines of evidence suggested that DAO is involved in the catabolism of endogeneous D-serine in the brain (36).

On the other hand, several DAO-encoding genes have been identified in some yeasts and fungi, e.g., *Trigonopsis variabilis* (28), *Fusarium solani* (38), and *Rhodotorula gracilis* (62). The enzymes from these lower eukaryotes have been used for the enzymatic determination of D-amino acids (92), the industrial production of α-keto acids (21), and the production of 7-aminocepharosporanic acid, a key raw material for semisynthetic cephalosporin antibiotic production (27). In spite of this practical importance in biotechnology, the physiological role of DAO in these lower eukaryotes has not been studied.

The methylotrophic yeast *Candida boidinii* is unique in its ability to grow on D-alanine as a carbon and/or nitrogen source. DAO was shown to be peroxisomal in *C. boidinii* (84). Studies on the regulation of peroxisomal proteins in *C. boidinii* revealed that DAO is distinct from other peroxisomal oxidases (acyl-CoA oxidase and alcohol oxidase) in its regulatory profile , *i.e.*, the expression of DAO was induced by D-alanine but not repressed in the presence of glucose (Chapter I, Section 1).

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In this section, the author cloned the *DAO1* gene from *C. boidinii* and disrupted the *DAO1* gene better to understand the physiological role of DAO and D-amino acid metabolism in *C. boidinii*. The signal for targeting to peroxisomes in the *DAO1*-encoded protein was also elucidated.

Materials and Methods

Yeast and bacterial strains, media, and cultivation

C. boidinii S2 (88) was the origin of chromosomal DNA and was used as the wild-type strain. *C. boidinii* TK62 (*ura3* (66)) was used as the host for transformation. The yeast strain was grown on the synthetic MI medium. *Escherichia coli* JM109 was used for plasmid propagation and for the construction of a *C. boidinii* S2 genomic library.

DNA isolation and transformation

Yeast DNA was isolated by the method of Cryer *et al.* (10) or Davis *et al.* (11). Plasmid DNAs from *E. coli* transformants were isolated by the method of Birnboim and Doly (3). Transformation of *E. coli* was performed by the method of Hanahan (35). Transformation of *C. boidinii* was performed by the modified lithium acetate method, as described previously (65).

Cloning of the *C. boidinii DAO1* gene

Based on the amino acid sequences of highly conserved regions in several DAO-encoding genes and the preferred codon usage in *C. boidinii*, two mixed primers, primer 1 and primer 2 were designed (Fig. 1-11): primer 1 (30-mer), 5'-CGCggatccATGKMTCCARYTMGWGGWCAR-3; primer 2 (27-mer), 5'-CGCggatccWGCRKSWCCRTARTTRTG-3'. Both primers had an additional

Bam HI site at the 5' end (in lowercase letters) for subcloning of the PCR fragment. The PCR reaction mixture consisted of 0.35 mg *C. boidinii* S2 genomic DNA as the template, 0.5 mg of each mixed primer, 0.2 mM dNTPs, 50 mM KCl, 10 mM Tris/HCl buffer (pH 8.3), 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin and 2.5 U ExTaq^{TM} DNA Polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan) in a total volume of 100 ml. PCR was performed with a Perkin Elmer Model 480 DNA thermal cycler (Norwalk, CT, USA) under the following temperature profile conditions ([denaturation: 95°C, 1 min; annealing: 37°C, 1 min; extension: 72°C, 3 min] for 3 cycles, and [denaturation: 95°C, 1 min; annealing: 55°C, 1 min; extension: 72°C, 3 min] for 30 cycles). The amplified 350-bp fragment was digested with *Bam* HI and then subcloned into the *Bam* HI site of pBluescript II KS+ (Stratagene Ltd., La Jolla, CA, USA). Nucleotide sequence analysis showed that the amino acid sequence deduced from the nucleotide sequence of the 350-bp insert was highly similar to those of other DAOs. The propagated recombinant plasmid was then *Bam* HI-digested, and the resultant 350-bp fragment was gel-purified and used as a probe for hybridization experiments.

The genomic DNA from *C. boidinii* S2 was digested with nine different restriction enzymes and then subjected to electrophoresis on a 0.7% agarose gel. The separated DNA was blotted onto a Biodyne nylon membrane (Pall Bio Support, New York, NY, USA), and then hybridized to the 350-bp ^{32}P-labeled probe under high stringency conditions. A 6.0-kb *Xba* I fragment hybridized to the probe. Also, the *Xba* I-digested chromosomal DNA corresponding to the size of ca. 6.0 kb was ligated into the *Xba* I site of pBluescript II KS+, and then transformed into *E. coli* JM109. Transformants were transferred to Biodyne nylon membranes. After the lysis of bacteria and binding of the liberated DNA to the nylon membrane, the blots were used for the colony hybridization

experiment with the 32P-labeled probe in Church buffer [7% SDS, 0.25 M NaPO$_4$ (pH 7.2), 1 mM EDTA, 0.25 M NaCl and 1% BSA] (6). Clones that showed strong signals were picked up from the original plates and used for further studies.

DNA sequencing

From the *DAO1*-harboring plasmid pDA7, nest-deleted plasmids were derived using a Kilosequence deletion kit (Takara Shuzo Co., Ltd.). The nucleotide sequences were determined with a Dye deoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA, USA) and an ABI 373A DNA sequencer (Applied Biosystems). The nucleotide sequences of *DAO1* will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB042032.

Enzyme assays and protein methods

The enzyme activity of DAO was measured spectrophotometrically at 30°C by the peroxidase method. The reaction mixture comprised 33 mM potassium phosphate buffer (KPB, pH 8.0), 0.67 mM 2, 2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid), 2.0 U/ml peroxidase (from horseradish), and 66.7 mM D-alanine. Enzyme activity was determined by measuring the increase in absorbance at 420 nm. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 μmol of hydrogen peroxide per min.

Anti-DAO polyclonal antibodies from rabbits were prepared by Sawady Technology Co. Ltd. (Tokyo, Japan).

Construction of the *DAO1* gene disruption cassette and one-step gene disruption

The 1.5-kb *Xba* I-*Acc* I fragment derived from pDA7 (harboring the entire coding sequence of *DAO1*) was gel-purified and then introduced into *Xba* I-*Acc* I digested pBluescript II SK+. The plasmid obtained was digested with *Sty* I and *Hind* III to remove a ca. 360-bp fragment including most of the coding sequence of *DAO1*. The remaining linearized plasmid and the 4.6-kb *Sac* I-*Xho* I fragment derived from pSPR, which had the *C. boidinii URA3* gene with repeated flanking sequences (75), were gel purified, blunt-ended, and then subjected to ligation. The resulting plasmid (disruption vector) was digested with *Sac* I and *Xho* I, and then used for the transformation of *C. boidinii* strain TK62. The disruption of the *DAO1* gene (yielding the *dao1Δ* strain) and popping out of the *URA3* gene (yielding the *dao1Δura3* strain) were confirmed by genomic Southern analysis of *Bgl* II-digested DNA from transformants using the 360-bp *Hind* III-*Acc* I fragment from pDA7 as a probe (Fig. 1-9).

Introduction of *DAO1* and C-terminal AKL-deleted *DAO1* into the *dao1Δura3* strain

The *DAO1* fragment was amplified by PCR using pDA7 as a template and two primers, DAO-N (ACGCGTCGACAAAATGGGTGATCAAATTGTTG) and DAO-C (AACTGCAGCTAAAGTTTAGCTTTAACTTTTTGGT). Similarly, the *DAO1* fragment lacking the C-terminal AKL sequence (DAO1ΔAKL) was amplified by PCR using two primers, DAO-N and DAKL-C (AACTGCAGCT-ATTTAACTTTTTGGTTATCAACTAA). The amplified fragments were gel-purified, digested with *Sal* I and *Pst* I, and then inserted into pACT1. pACT1 harbored the *C. boidinii ACT1* promoter and terminator sequences with *Sal* I and *Pst* I sites to insert coding sequences for expression, and the *C. boidinii URA3*

gene as the selectable marker . The resulting plasmids were linearized with *Bam* HI and then introduced into strain *dao1Δura3*, yielding strain ExDAO and strain ExDAOΔAKL.

Subcellular fractionation

Strain ExDAO and ExDAOΔAKL cells were grown on oleate, spheroplasted, and then gently disrupted by osmotic changes (73). Unlysed cells, large organelles, and other cell debris were removed from the lysate by centrifugation at 500 x *g*. The resulting supernatant was divided into two equal portions. To one, Triton X-100 was added to a final concentration of 1%, and then both were centrifuged at 20,000 x *g* to obtain supernatant and pellet fractions. The fractions were assayed for DAO and catalase.

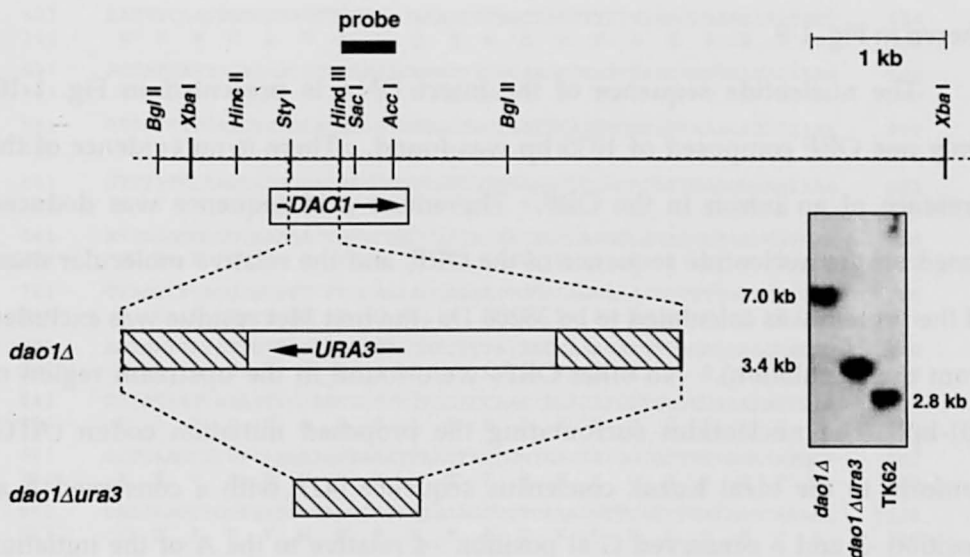
Fig. 1-9. Physical map of the cloned *DAO1* gene, gene disruption, and Southern analysis The shaded boxes at the ends of the *URA3* represent repeated sequences for homologous recombination to remove the *URA3* gene after gene disruption. The arrows indicate coding regions. *Bgl* II-digested total DNAs from the *dao1Δ* and *dao1Δura3* strains, and the host strain TK62 were probed with the ^{32}P-labeled 360-bp *Hind* III-*Acc* I fragment from pDA7.

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Results

Cloning of C. *boidinii* DAO1 and its primary structure

Synthetic mixed primers, primer 1 and primer 2, were designed based on amino acid sequences which are highly conserved regions in DAO-encoding genes (Fig. 1-11). The PCR reaction with primer 1, primer 2 and the C. *boidinii* genomic DNA, as a template, amplified a 350-bp fragment. Also, the DNA sequence of the amplified fragment could code for an open reading frame (ORF) showing high similarity to other DAO amino acid sequences. On genomic Southern analysis with this 350-bp PCR-amplified fragment as a probe, a single 6.0-kb band was observed with *Xba* I-digested genomic DNA. The corresponding DNA of this size was gel-purified and a gene library was constructed on pBluescript II KS+. Colony hybridization selection gave three independent positive clones exhibiting identical physical maps, as shown in Fig. 1-9.

The nucleotide sequence of the insert DNA is presented in Fig. 1-10. Only one ORF composed of 1035 bp was found. There is no evidence of the presence of an intron in the ORF. The amino acid sequence was deduced based on the nucleotide sequence of the ORF, and the relative molecular mass of the protein was calculated to be 38206 Da (the first Met residue was excluded from the calculation). No other ORFs were found in the upstream region of 601-bp. The nucleotides surrounding the proposed initiation codon (ATG) conform to the ideal Kozak consensus sequence (42), with a conserved A at position -3 and a conserved G at position +4 relative to the A of the initiation codon.

30

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-600    CTAGAGTTGTATCAATCAATACCCTGCCCTTAACGTTATTTGAGTCAGTCACAGGATATG    -541
-540    CGACAAATTAAGCGCTAACTTGATCTTAACAGGCGGATAAAATGCCGTATTCGCTCATAT    -481
-480    TTTTCTCACAGACAGAAATTCATGTTTCCCCATACTAAAAACTCGGATCGCTAAAAATTC    -421
-420    CATTGCGATAAGGGTAAATTAGTATGGGAATTTGTGGGAGAAATAATTAGTTGAAACTGG    -361
-360    CTGGAGAAGAAACTCCGTTAAGAATCCCTGTTTTTTTATTTCATGTTGTTTCCCTCATGT    -301
-300    GAAGAAAGGTAACCGTTGACACCATCTGAACCTCTTTAGTAGCAATGCCCTATCTGTCAT    -241
-240    CACTATCAATTACTGTCTTTCAAGAAATCGTACCACTCTTTGAAAAGATGTTTGTTTTAT    -181
-180    TCTCTTCATTTCTCTTTAAATGTTTTAACAAAAAGGAATAAAATTAAGTACTATTTAAAG    -121
-120    ATAAGAGAATCACTCTTTTTTTAAAAAAAATTATTTGTATTTGAATAAAATTAATTCTTTTC    -61
 -60    ATCATTAGTTTCTTTGTTAAGAAAAAATAATTAAATATCAAATTATTAAAATAAAACAAA     -1
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   1    ATGGGTGATCAAATTGTTGTTCTTGGTTCCGGTATTATTGGTTTATATACTACATACTGT     60
   1     M  G  D  Q  I  V  V  L  G  S  G  I  I  G  L  Y  T  T  Y  C     20

  61    TTAATCTATGAGGCTGGATGTGCTCCAGCTAAAATTACTATTGTTGCTGAATTTTTACCA    120
  21     L  I  Y  E  A  G  C  A  P  A  K  I  T  I  V  A  E  F  L  P     40

 121    GGTGATCAATCTACATTATATACATCTCCATGGGCAGGTGGTAATTTTTCTTGTATTTCA    180
  41     G  D  Q  S  T  L  Y  T  S  P  W  A  G  G  N  F  S  C  I  S     60

 181    CCAGCTGATGATACAACATTGGCTTATGATAAATTCACATATCTTAATTTATTCAAGATT    240
  61     P  A  D  D  T  T  L  A  Y  D  K  F  T  Y  L  N  L  F  K  I     80

 241    CACAAAAAATTAGGTGGACCAGAATGTGGATTAGATAATAAGCCAAGTACTGAATATTGG    300
  81     H  K  K  L  G  G  P  E  C  G  L  D  N  K  P  S  T  E  Y  W    100

 301    GATTTTTATCCTGGTGATGAAAAAGTCAATTCTTTAAAACAATATCTTAAAGATTTTAAA    360
 101     D  F  Y  P  G  D  E  K  V  N  S  L  K  Q  Y  L  K  D  F  K    120

 361    GTTATTCCAAAATCAGAATTACCAGAAGGTGTTGAATATGGTATTAGTTATACTACATGG    420
 121     V  I  P  K  S  E  L  P  E  G  V  E  Y  G  I  S  Y  T  T  W    140

 421    AATTTCAACTGTCCTGTTTTCTTACAAAATATGGCTAATTTTTTAAATAAAAGAAATGTT    480
 141     N  F  N  C  P  V  F  L  Q  N  M  A  N  F  L  N  K  R  N  V    160

 481    ACCATTATTAGAAAACATTTAACACATATTTCTCAAGCTTATTTAACAGTTAATACAAAA    540
 161     T  I  I  R  K  H  L  T  H  I  S  Q  A  Y  L  T  V  N  T  K    180

 541    GTTGTTTTCAACTGTACAGGTATTGGTGCTGCTGATTTAGGTGGTGTTAAAGATGAAAAA    600
 181     V  V  F  N  C  T  G  I  G  A  A  D  L  G  G  V  K  D  E  K    200

 601    GTTTATCCAACTAGAGGACAAGTTGTTGTTGTTAGAGCTCCACATATTCAAGAAAATAAA    660
 201     V  Y  P  T  R  G  Q  V  V  V  R  A  P  H  I  Q  E  N  K    220

 661    ATGAGATGGGGTAAAGACTATGCTACTTATATTATTCCAAGACCATATTCTAATGGTGAA    720
 221     M  R  W  G  K  D  Y  A  T  Y  I  I  P  R  P  Y  S  N  G  E    240

 721    TTAGTCTTAGGTGGTTTCTTACAAAAGGATAATTGGACAGGTAATACTTTTGGTTTTGAA    780
 241     L  V  L  G  G  F  L  Q  K  D  N  W  T  G  N  T  F  G  F  E    260

 781    ACTGATGATATTGTTAGTAGAACTACATCTTTATTACCAAAGATTTTAGATGAACCACTT    840
 261     T  D  D  I  V  S  R  T  T  S  L  L  P  K  I  L  D  E  P  L    280

 841    CATATTATTAGAGTTGCAGCTGGTTTAAGACCAAGTAGACATGGTGGTCCAAGAATTGAA    900
 281     H  I  I  R  V  A  A  G  L  R  P  S  R  H  G  G  P  R  I  E    300

 901    GCTGAAGTTTGTGAAGAAGGTAAATTAACTATTCATAATTATGGTGCTTCTGGATATGGT    960
 301     A  E  V  C  E  E  G  K  L  T  I  H  N  Y  G  A  S  G  Y  G    320

 961    TATCAAGCTGGTTATGGTATGTCTTATGAAGCTGTCAAACTTTTAGTTGATAACCAAAAA   1020
 321     Y  Q  A  G  Y  G  M  S  Y  E  A  V  K  L  L  V  D  N  Q  K    340

1021    GTTAAAGCTAAACTTTAGATTGATGTTTTTTCACATCATTATTAAACACAATATTACAAT   1080
 341     V  K  A  K  L  *

1081    TAATAGATATTATTATTTTCATTTTTAAACTTTTCTTTTACTAAATTTCTATTTTATTCT   1140

1141    CCTTATATATATAA
```

Fig. 1-10. Nucleotide and deduced amino acid sequences of *DAO1* from *C. boidinii*. The deduced amino acid sequence is shown below the nucleotide sequence. The first nucleotide, A, of the ATG initiation codon is referred to as position 1. The asterisk indicates a stop codon.

```
                       *       **    *  *   **        *
C. boidinii      1 MGDQ--IVVL GSGIIGLYTT YCLIYEAGCA PAKITIVAEF LPGDQSTL-Y   47
T. variabilis    1 M-AK--IVVI GAGVAGLTTA LQLLR-KG-- -HEVTIVSEF TPGDLSIG-Y   42
R. gracilis      1 MHSQKRVVVL GSGVIGLSSA LILAR-KGYS ---VHILARD LPEDVSSQTF   46
F. solani        1 MSNT--IVVV GAGVIGLTSA LLLSKNKG-- -NKITVVAKH MPGDYDVE-Y   44
porcine kidney   1 M----RVVVI GAGVIGLSTA --L-----CI HERYHSVLQP LDVKVYADRF   39


                       *                                          *
C. boidinii     48 TSPWAGGN-- ---FSCISPA DDTTLA--YD KFTYLNLFKI HKKLGGPECG   90
T. variabilis   43 TSPWAGAN-- ---WLTFYDG GKLA-D--YD AVSYPILREL AR--SSPEAG   82
R. gracilis     47 ASPWAGAN-- ---WTPFMTL TDGPRQAKWE ESTFKKWVEL V-----PTGH   86
F. solani       45 ASPFAGAN-- ---HSPM-AT EESS-E--WE RRTWYEFKRL VE--EVPEAG   83
porcine kidney  40 T-PFTTTDVA AGLWQPYTSE PSNPQEANWN QQTFNY---L LSHIGSPNAA   85


                                                   *
C. boidinii     91 L---DNKPST EYWDFYPGDE KVNSL----- ------KQYL KDFKVIPKSE  126
T. variabilis   83 IRLINQRSHV LKRDLPKLEG AMSAICQ--- -RNPWFKNTV DSFEIIEDRS  128
R. gracilis     87 AMWLKGTRRF AQNE------ ------DGLL GH--WYKDIT PNYRPLPSSE  122
F. solani       84 VHF--QKSRI QRRNVDTEKA QRSGFPDALF SKEPWFKNMF EDFREQHPSE  131
porcine kidney  86 NMGLTPVSGY ---------- --NLFREAV- -PDPYWKDMV LGFRKLTPRE  121


C. boidinii    127 LPEGVE--YG ISYTTWNFNC PVFLQNMANF LNKRNVTIIR KHLTHISQAY  174
T. variabilis  129 RIVHDDEAYL VEFRSVCIHT GVYLNWLMSQ CLSLGATVVK RRVNHIKDAN  178
R. gracilis    123 CPPG---AIG VTYDTLSVHA PKYCQYLARE LQKLGATFER RTVTSLEQAF  169
F. solani      132 VIPGYDSGC- -EFTSVCINT AIYLPWLLGQ CIKNGVIVKR AILNDISEAK  179
porcine kidney 122 LDMFPDYRYG WFNTSLILEG RKYLQWLTER LTERGVKFFL RKVESFEEV-  170


                        *  *            *      * ***      *
C. boidinii    175 LTVNT----K VVFNCTGIGA ADLGGVKDEK VYPTRGQVVV VRA-----PH  215
T. variabilis  179 LLHSSGSRPD IVNCSGLFA RFLGGVEDKK MYPIRGQVVL VRNSLPFMAS  228
R. gracilis    170 -----DGAD LVVNATGLGA KSIAGIDDQA AEPIRGQTVL VKS-----PC  208
F. solani      180 KLSHAGKTPN IIVNATGLGS YKLGGVEDKT MAPARGQIVV VRNESSPM--  227
porcine kidney 171 ----ARGGAD VIINCTGVWA GVLQ--PDPL LQPGRGQIIK VDAPW-LKNF  213
                                                  primer 1
                          ●          **        *                    *
C. boidinii    216 IQENKMRWGK DYATYIIPRP YSNGELVLGG FLQKDNWTGN TFGFETDDIV  265
T. variabilis  229 FSSTPEKENE DEALYIMTR- F-DGTSIIGG CFQPNNWSSE PDPSLTHRIL  276
R. gracilis    209 KRCTMDSSDP ASPAYIIPRP --GGEVICGG TYGVGDWDLS VNPETVQRIL  256
F. solani      228 LLTSGVEDGG ADVMYLMQR- AAGGGTILGG TYDVGNWESQ PDPNIANRIM  276
porcine kidney 214 IITHDLERGI YNSPYIIP-- -GLQAVTLGG TFQVGNWNEI NNIQDHNTIW  260


                       *              * ●*   *    *
C. boidinii    266 SRTTSLLPKI LDEP----LH IIRVAAGLRP SRHGGPRIEA EVC-------  304
T. variabilis  277 SRALDRFPEL TKDGP---LD IVRECVGHRP GREGGPRVEL EKI-------  316
R. gracilis    257 KHCLRLDPTI SSDGTIEGIE VLRHNVGLRP ARRGGPRVEA ERIVLPLDRT  306
F. solani      277 QRIVEVRPEI ANGKGVKGLS VIRHAVGMRP WRKDGVRIEE EKL-------  323
porcine kidney 261 EGCCRLEPTL ------KDAK IVGEYTGFRP VRPQ-VR--- ------LERE  294


                               * **  ●      *      *      *
C. boidinii    305 ---------- -----EEGKL TIHNYGASGY GYQAGYGMSY EAVKLLVDN-  338
T. variabilis  317 ---------- -----PGVGF VVHNYGAAGA GYQSSYGMAD EAVSYVERA-  350
R. gracilis    307 KSPLSLGRGS ARAAKEKEVT LVHAYGFSSA GYQQSWGAA- EDVAQLVDEA  356
F. solani      324 ---------- -----DDETW IVHNYGHSGW GYQGSYGCA- ENVVQLVDKV  357
porcine kidney 295 Q--LRFGSSN TE-------- VIHNYGHGGY GLTIHWGCAL EVAKLFGKVL  334
                                      primer 2
                           *
C. boidinii    339 -----QKV-K AKL.......  .......... .......... ..........  345
T. variabilis  351 -------LTR PNL.......  .......... .......... ..........  356
R. gracilis    357 FQRYHGAARE SKL.......  .......... .......... ..........  368
F. solani      358 -----GKAAK SKL.......  .......... .......... ..........  365
porcine kidney 335 EERNLLTMPP SHL.......  .......... .......... ..........  347
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Fig. 1-11. Alignment of the deduced amino acid sequence of the C. boidinii DAO1 gene product with the sequences of DAOs from T. variabilis, R. gracilis, F. solani, and porcine kidney. Identical amino acid residues are gray-shadowed. Asterisks indicate the amino acid residues common to all five sequences. Closed circles indicate the amino acid residues that are thought to be residues in the active site in DAO from porcine kidney. Arrows indicate the locations of the PCR primers (primer 1 and primer 2) used for cloning.

Figure 1-11 shows the alignment of the deduced amino acid sequences of the DAOs compared. The deduced amino acid sequence of the identified ORF showed 56.8%, 50.1%, 53.3%, and 42.2% similarity to those of *T. variabilis, R. gracilis, F. solani,* and porcine kidney DAO, respectively. On biochemical and 3D structural analyses of DAO from porcine kidney (47, 98), Tyr-228, Arg-283, and Gly-313 (indicated by closed circles in Fig. 1-11) were shown to be residues in the active site, and these catalytic residues are also conserved in this ORF. The amino acid sequence of G-X-G-X-X-G near the N-terminus (amino acid residues 9 to 14) of the ORF may be the binding site for the adenine of FAD (100). Also, the deduced amino acid sequence of DAO contained a D-amino acid oxidase signature (amino acid residues 310 to 328) specific to DAO [PROSITE, PS00677 (48)].

In addition, the overexpression of this ORF in *C. boidinii* caused much higher DAO activity than in the wild-type strain (Chapter III, Section1). From these observations and the results of the following gene disruption analyses, the author concluded that this ORF encodes the gene for DAO in *C. boidinii, DAO1.* The *S. cerevisiae* genome database did not contain a DAO ortholog.

Disruption of *C. boidinii DAO1*

The *DAO1*-disruption vector was used to transform *C. boidinii* TK62 to uracil prototrophy. Since the integrated plasmid had tandem repeated sequences, the *URA3* gene of the *dao1Δ* strain was expected to pop out from the chromosome through site-specific recombination at these repeated sequences at a high frequency. The *dao1Δura3* strain was isolated as showing resistance to 5-fluoroorotic acid, as described previously (75). The disruption of *DAO1* and popping out of *URA3* were confirmed by genomic Southern analysis (Fig. 1-9). The DNA from the wild-type strain gave a single band of 2.8 kb; this band

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shifted to 7.0 and 3.4 kb for the *dao1Δ* and *dao1Δura3* strains, respectively, as expected for disruption of the *DAO1* gene and deletion of the *URA3* sequence caused by homologous recombination, respectively. In addition, cell-free extracts of D-alanine-induced cells of these *dao1Δ* strains did not exhibit any DAO activity with D-alanine as a substrate (data not shown). These results confirmed that *C. boidinii* S2 contains only one gene coding for *DAO1*.

Growth characteristics of the *dao1Δ* strain

Next, the author compared the growth of the wild-type and the *dao1Δ*strain on various carbon and nitrogen sources. The growth of both strains on glucose, glycerol, methanol, and oleate as a sole carbon source was similar (data not shown). But the *dao1Δ* strain could not grow on D-alanine as a sole carbon and nitrogen source or on D-alanine as a sole carbon source (with NH_4Cl as a nitrogen source) (Fig. 1-12). Therefore, DAO is essential for *C. boidinii* to grow on D-alanine as a carbon source. In addition, the growth of the wild-type strain on D-alanine plus NH_4Cl was slower than that on D-alanine without NH_4Cl, especially at the early growth phase. The enzyme activity as well as the mRNA level of DAO in the cells grown on D-alanine plus NH_4Cl was lower than that with D-alanine without NH_4Cl (Chapter I, Section 1). These results suggested that the NH_4Cl repressed the expression of *DAO1*.

To determine whether or not *DAO1*-expression is the only rate-limiting factor when cells are grown on D-alanine plus NH_4Cl at an early growth phase, the author constructed a *DAO1*-expression plasmid under the actin (*ACT1*)-promoter and introduced it into the *dao1Δura3* strain, yielding strain ExDAO. The *ACT1* promoter is a constitutive promoter and not repressed by NH_4Cl. If *DAO1* expression is not repressed by NH_4Cl and is the only rate-limiting factor for growth on D-alanine plus NH_4Cl at an early growth phase, the author

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expected that strain ExDAO would grow at a growth rate comparable to that of the respective cells grown on D-alanine as a sole carbon and nitrogen source. However, the growth rate of strain ExDAO on D-alanine plus NH_4Cl at an early growth phase was still lower than that for D-alanine as a sole carbon and nitrogen source. Therefore, it was suggested that NH_4Cl repressed not only the expression of *DAO1* but also the expression of some other genes involved in the metabolism of D-alanine. In *S. cerevisiae*, the expression of *GAP1* gene encoding the general amino acid permease was reported to be repressed by the presence of ammonium ion (40). Similar regulatory mechanism might be present in *C. boidinii*.
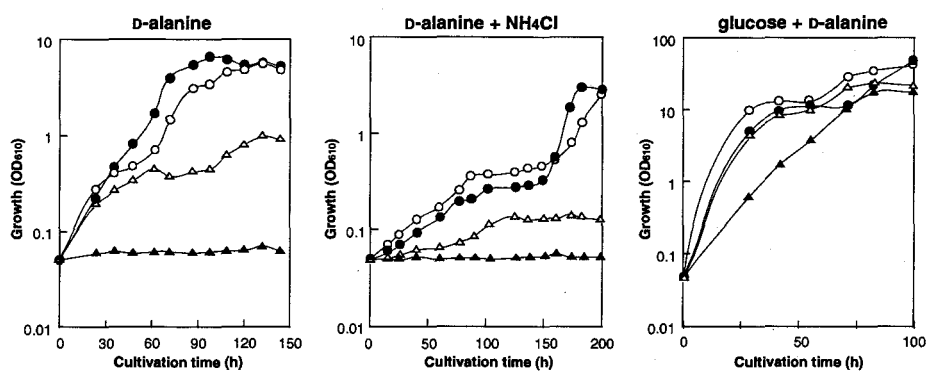
Fig. 1-12. Growth curves of strains grown on D-alanine as a carbon source or a nitrogen source. Symbols: ●, the wild-type strain; ○, strain ExDAO; △, strain ExDAO△AKL; ▲, the *dao1△* strain.

When D-alanine was used as a nitrogen source and glucose was used as a carbon source, the *dao1△* strain retained growth ability and could assimilate D-alanine as a nitrogen source. These results indicated that an alternative metabolic system is involved in the utilization of D-alanine as a nitrogen source. This is supported by the observation that wild-type cells showed a diauxic

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growth curve on glucose plus D-alanine while the *dao1Δ* strain did not. Although DAO was not essential for the growth on D-alanine as a nitrogen source, the growth rate of the *dao1Δ* strain was slower than that of the wild-type strain, especially at an early growth phase. Therefore, DAO is assumed to contribute to D-alanine utilization as a nitrogen source, especially at an early phase of growth.

In spite of extensive analyses, the author could not find any other mutant phenotype in the *dao1Δ* strain, e.g., osmotic sensitivity, temperature sensitivity, and so on.

Subcellular localization of DAO in *C. boidinii*

The peroxisomal localization of *C. boidinii* DAO has been well demonstrated through subcellular fractionation experiments (84). The C-terminal three amino acid sequences of DAOs from other sources include -SKL (*R. gracilis* and *F. solani*), -PNL (*T. variabilis*), and -SHL (porcine kidney). These sequences are thought to function as peroxisome targeting signals (PTS1), however, there has been no direct experiment showing the necessity of these target signals for DAO localization to peroxisomes.

To determine whether or not the last three carboxyl terminal amino acid residues of Dao1p, -AKL, is necessary for peroxisomal targeting, a truncated *DAO1* gene missing the last three amino acids, -AKL (*DAO1ΔAKL*), was constructed and placed under the *C. boidinii* actin promoter, and then integrated into the *URA3* locus in strain the *dao1Δura3* strain (resulting in strain ExDAOΔAKL).

To determine the localization of the AKL-deleted DAO, biochemical analysis was performed. Strain ExDAO and strain ExDAOΔAKL were grown on oleate, and the harvested cells were osmotically disrupted. After removal
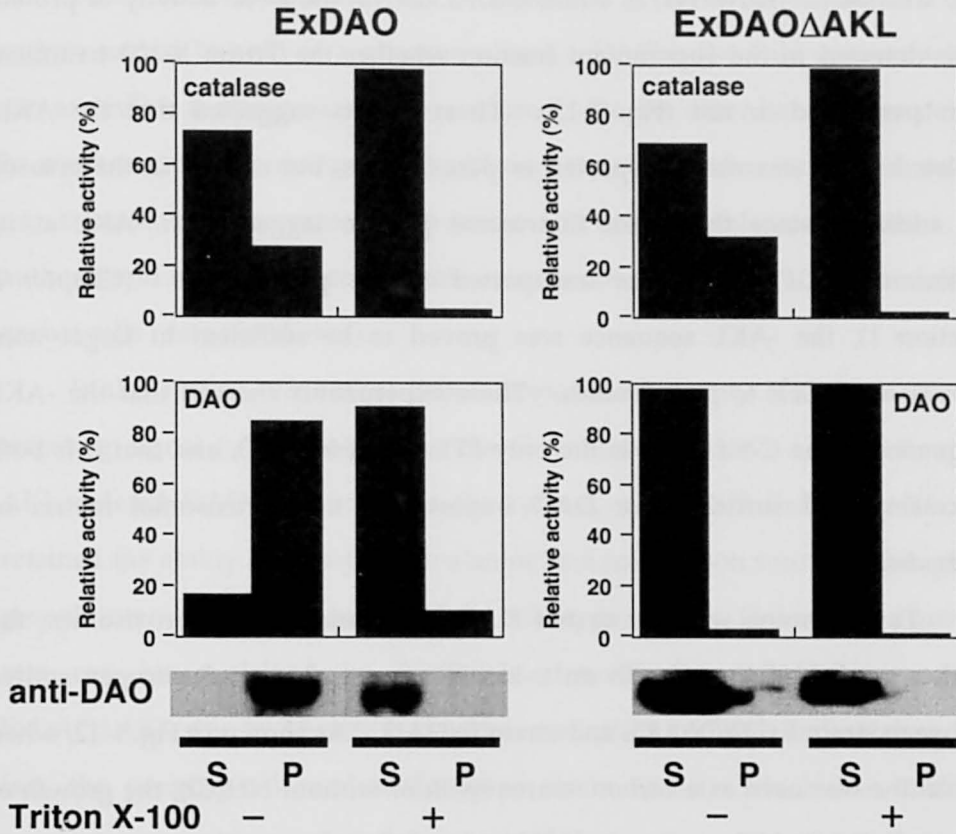
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Fig. 1-13. Subcellular distribution of DAO (in strain ExDAO) and AKL-deleted DAO (in strain ExDAOΔAKL). After osmotic lysis of each strain, a sample was treated (+) or not treated (-) with Triton X-100, and then centrifuged at 20,000 x g. The activities of catalase and DAO were assayed in the supernatant (S) and pellet (P) fractions. The samples were subjected to SDS-PAGE and then immunoblot analysis with anti-DAO. Total activities (U) of each experiment (the activity in the supernatant fraction plus the activity in the pellet fraction) were as follows: DAO; 0.611 for strain ExDAO (- Triton X-100) (-T), 0.567 for (+ Triton X-100) (+T), 2.03 for strain ExDAOΔAKL (-T), 2.11 for (+T). Catalase; 847 for strain ExDAO (-T), 1050 for (+ T), 522 for strain ExDAOΔAKL (- T), 596 for (+ T).

of cell debris and nuclei, a sample treated with or without Triton X-100 was centrifugated at 20,000 x g for 20 min. In strain ExDAO, the DAO activity and protein shifted from the pellet fraction to the supernatant fraction on Triton X-

100 treatment. However, in strain ExDAOΔAKL, the DAO activity or protein was detected in the supernatant fraction whether the Triton X-100 treatment was performed or not (Fig. 1-13). These results suggested that the AKL-deleted DAO was not transported to peroxisomes, but existed in the cytosol. In addition, since the green fluorescent protein tagged with -AKL at its C-terminus (GFP-AKL) was transported to the peroxisomes (Chapter I, Section 1), the -AKL sequence was proved to be sufficient to target non-peroxisomal GFP to peroxisomes. These experiments showed that the -AKL sequence at the C-terminus is the only PTS found in DAO, and that it is both necessary and sufficient for DAO import into the peroxisomal matrix of *C. boidinii*.

To determine whether or not DAO only functions in peroxisomes, the author compared the growth on D-alanine as a carbon and nitrogen source between strain ExDAOΔAKL and strain ExDAO. As shown in Fig. 1-12, when D-alanine was used as a carbon source (with or without NH_4Cl), the growth of strain ExDAOΔAKL was remarkably retarded, but it still retained growth ability on D-alanine as a carbon source when compared with the *dao1Δ* strain. This suggested that the AKL-deleted DAO is able to perform its metabolic function to a certain degree in the cytosol. When D-alanine was used as a nitrogen source, a significant difference in growth was not observed between these strains.

Discussion

In this section, the author describe the cloning of *DAO1* from the *C. boidinii* genome, and determination of its primary structure. The author constructed and analyzed a *DAO1*-disrupted strain of a lower eukaryote for the

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first time. DAO is necessary for *C. boidinii* to grow on D-alanine as a sole carbon source, but not as a nitrogen source. DAO is the only enzyme which can assimilate D-alanine as a carbon source. On the other hand, there should be another metabolic system which utilizes a D-amino acid as a nitrogen source, e.g., D-amino acid racemase and D-amino acid aminotransferase. However, the author could not detect these enzyme activities in cell-free extracts of D-alanine-grown cells (data not shown).

In this section, the author demonstrated the necessity of the peroxisomal targeting signal (-AKL) located at the C-terminus of DAO. The C-terminal AKL deleted DAO was active in the cytosol, and strain ExDAOΔAKL still retained the ability of growth on D-alanine as a sole carbon source, although its growth rate was slower than that of the wild-type strain. This retarded growth rate can be explained by inefficient detoxification of H_2O_2 in strain ExDAOΔAKL due to discrete compartmentalization of DAO and catalase. In fact, the peroxisomal catalase-disrupted strain (*cta1Δ*), which has DAO in peroxisomes, could grow but showed retarded growth on D-alanine as a carbon source (Horiguchi, H. and Sakai, Y. unpublished results). Sulter *et al.* reported that the peroxisome-deficient mutant strains of *Hansenula polymorpha* grew well on glucose plus D-alanine associated with enhanced DAO activity in the cells, and also showed that the DAO was localized in the cytosol and partially aggregated (83). It is thought that most DAO proteins can be correctly folded to the active form in the cytosol.

In contrast to strain ExDAOΔAKL strain, the *pex5Δ* strain (the gene disruptant of the PTS1 receptor, *PEX5*), which is a peroxisome-deficient mutant, completely lost the ability of growth on D-alanine (Sakai, Y., unpublished results). Both the *pex5Δ* strain and strain ExDAOΔAKL have cytosolic DAO and catalase activity. Then what causes the severe phenotype observed in

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strain *pex5Δ* than in strain ExDAOΔAKL?   One possible explanation is that proper peroxisome assembly is necessary for normal D-alanine metabolism, e.g., transport of a reaction product of DAO, pyruvate, from peroxisomes to mitochondria.   In addition, the *pex5Δ* strain could grow on glucose plus D-alanine as well as the *dao1Δ* strain.   These results suggest that peroxisomal D-alanine metabolism is not required for the assimilation of D-alanine as a nitrogen source, but is necessary for the assimilation of D-alanine as a carbon source.

DAOs from *T. variabilis*, *F. solani*, and *R. gracilis* have been shown to be able to convert cephalosporin C to glutaryl-7-ACA, an intermediate for the production of 7-amino cepharosporanic acid, which is the key starting material for semi-synthetic cephem antibiotics.   For the applied utilization of DAO, the author tried to construct a high-level DAO production system in *C. boidinii* (Chapter III, Section 1).

Summary

A methylotrophic yeast, *C. boidinii*, exhibits D-amino acid oxidase activity (DAO, EC 1.4.3.3) during its growth on D-alanine as a sole carbon or a nitrogen source. The structural gene (*DAO1*) encoding DAO was cloned from a genomic library of *C. boidinii*. The 1035-bp gene encoded 345 amino acids and the predicted amino acid sequence showed significant similarity to those of DAOs from other organisms. The *DAO1* gene was disrupted in the *C. boidinii* genome by one-step gene disruption. The *DAO1*-deleted strain did not grow on D-alanine as a carbon source but did on D-alanine as a sole nitrogen source (with glucose as the carbon source). These results suggested that while DAO is critically involved in growth on D-alanine as a carbon source, there should be another enzyme system which metabolizes D-alanine as a nitrogen source in *C. boidinii*. The author also showed that the three C-terminal amino acid sequence of DAO, -AKL, was necessary and sufficient for the import of DAO into peroxisomes.

Chapter II

Gene regulation of methanol-inducible genes
coupled with methanol metabolism

Section 1 Regulation and evaluation of five methanol-inducible promoters
in the methylotrophic yeast *Candida boidinii*

Introduction

Detailed knowledge of the physiology of the expression host would help in setting the optimal conditions for maximal production of foreign proteins. Of key importance is transcriptional control driven by a regulatable promoter. By using a regulatable promoter, proteins that are toxic to the host organisms can be produced, because the protein production can be separated into two phases, *i.e.*, the growth phase and the production phase.

Since the methylotrophic yeasts, *Pichia pastoris*, *Hansenula polymorpha*, and *Candida boidinii*, can grow on methanol, a cheap and pure substance, as the carbon source, these yeasts are widely used for the production of foreign proteins in both the academic and industrial fields (9, 26, 76). Gene expression in the methylotrophic yeast can be tightly controlled by regulated methanol-inducible promoters, and several methanol-inducible genes have been identified (39, 69, 70, 80).

In preceding studies, it was reported that the efficient heterologous protein production using *C. boidinii* in collaboration with several companies (64, 72). And recently, the author developed a novel system for toxic protein production where a membrane-bound organelle, peroxisome, was utilized to enable accumulation of toxic proteins (Chapter III, Section 2). In the study, the

author had to overcome the inefficient induction level of alcohol oxidase promoter observed in *C. boidinii* strain *aod1Δ*. This problem prompted the author to conduct a detailed study on the nature of methanol-inducible promoters in *C. boidinii*. In the methylotrophic yeast gene expression systems, most researchers use the alcohol oxidase promoter, and other methanol-inducible promoters have not been well-characterized. Since the biochemical pathway for methanol metabolism and the molecular mechanism for peroxisome assembly are well-conserved among the methylotrophic yeast strains, the present study with *C. boidinii* would facilitate better understanding and reliable use of the methylotrophic yeast gene expression systems, not only with *C. boidinii* but also with *P. pastoris* and *H. polymorpha*.

On the SDS-PAGE gel of methanol-induced *C. boidinii* cells, three proteins involved in methanol metabolism, *i.e.*, alcohol oxidase (Aod1p), dihydroxyacetone synthase (Das1p), and formate dehydrogenase (Fdh1p), are detected as the three major bands. This indicates that the promoters of the genes encoding these three enzymes are strong under methanol. In addition to the methanol-inducible *AOD1*, *DAS1*, and *FDH1* genes, two other methanol-inducible genes that encode two peroxisome membrane proteins (PMPs), *PMP20* and *PMP47*, had been cloned, and their gene regulation in yeast grown on various carbon and nitrogen sources was analyzed at the mRNA level (Chapter I, Section 1, (69, 70)).

To properly evaluate the regulation of these methanol-inducible promoters, a reliable reporter system in *C. boidinii* had to be established. In the previous study on the transcriptional control of the *AOD1* promoter (P*AOD1*), the *Saccharomyces cerevisiae* adenylate kinase gene (*ScADK1*) was used as the reporter (71). In this study, in order to assess the level of methanol-inducible gene expression in *C. boidinii*, the author employed the *ScPHO5* gene as the

reporter. The *ScPHO5* gene encodes periplasmic acid phosphatase in *S. cerevisiae* and is commonly used in studies on the budding yeast (22, 56). The advantages of using the *ScPHO5* gene as the reporter are: i) since acid phosphatase accumulates in the periplasmic space, the enzyme activity showed a linearity with the expression level even under the strongest promoter; ii) the enzyme assay is easy; iii) the enzyme activity can be visualized on colonies; iv) enzyme activity is optimal and stable at rather acidic pHs of 4 to 6, which are suitable for yeast growth.

In this section, the author studied the regulation of P_{AOD1}, the *DAS1* promoter (P_{DAS1}), the *FDH1* promoter (P_{FDH1}), the *PMP47* promoter (P_{PMP47}) and the *PMP20* promoter (P_{PMP20}) in detail. The aims of this study were as follows: i) to evaluate the strength of each methanol-inducible promoter under the same experimental conditions (*i.e.*, the same copy number of *ScPHO5*-expression cassette and the same location in the chromosome); ii) to analyze the expression of these promoters controlled by various carbon and nitrogen sources. For example, which promoter can be used for expression under growth on glucose as an additional carbon source to methanol? Also, how strong is the induction of the promoters of the PMP genes on methanol and on other carbon and nitrogen sources? The results obtained in this study will facilitate detailed analysis on induction by methanol, and the establishment of a new type of gene expression system based on novel promoters.

Materials and Methods

Strains, media, and cultivation conditions

C. *boidinii* S2 was used as the wild-type strain (88). C. *boidinii* strain TK62 (*ura3* (66)) was used as the host for transformation. *Escherichia coli* JM109 and

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SA116 (66) were used for plasmid propagation.

The yeast strains were grown on the synthetic MI medium. One or more of the following were used as the carbon source: 2% glucose (w/v), 2% glycerol (v/v), 1% methanol (v/v), 0.5% oleate (v/v), 1% sodium formate (w/v), and 0.6% D-alanine (w/v).

DNA methods

Polymerase chain reaction (PCR) was performed using ExTaq™ DNA Polymerase (Takara Shuzo Co. Ltd., Kyoto, Japan) on a Perkin Elmer Model 480 DNA thermal cycler (Norwalk, CT, USA). DNA was sequenced using a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and a Shimadzu DSQ-1000L DNA sequencer (Kyoto, Japan).

Isolation of the upstream regions of *AOD1*, *DAS1*, *FDH1*, *PMP20*, and *PMP47*

The oligonucleotide primers used in this study are summarized in Table 2-1. *PAOD1* was amplified by PCR with the 5'-primer AODP5-1 and 3'-primer AODP3-1 using pMOX33 that contains a 3.3-kb *Eco* RI-*Sal* I fragment from CL701 (74) as the template. The 1.7-kb amplified fragment was subcloned to pT7Blue(R) (Novagen, Madison, WI, USA) to produce pAOD1P. *PDAS1* was amplified by PCR with the 5'-primer 5'-DAS1 and 3'-primer 3'-DAS1 using pDAS1 (70) as the template. The 1.8-kb amplified fragment was subcloned to pT7Blue(R) to produce pDAS1P. *PPMP20* was amplified by PCR with the 5'-primer NP20 (M13 primer 3) and 3'-primer PRPMP201 using pMP201 (Horiguchi, H., unpublished data) as the template. The 1.4-kb amplified fragment was subcloned into the *Bam* HI-*Sac* I site of pBluescript II SK+ to produce pYP201.

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Table 2-1. Oligonucleotide primers used for PCR in this study

Name	Sequence
NP20	5'-GTAAAACGACGGCCAGT-3'
SPETERM	5'-GGA<u>ACTAGT</u>TAATTCAACAAGTTGTATCT-3' *Spe* I
PRPMP201	5'-CGC<u>GGATCC</u>TTTCTTAATATATACTGTATTATA-3' *Bam* HI
PR47S2	5'-TCC<u>GAGCTC</u>AACCTATAACGTGGTTCGGAT-3' *Sac* I
47ProN	5'-CGC<u>GGATCC</u>ATTGATGTGAGTTGTTTCTTG-3' *Bam* HI
5'-DAS1	5'-TCC<u>GAGCTC</u>GAATTCAAAATGTGGAGAAAAGA-3' *Sac* I
3'-DAS1	5'-CG<u>GGATCC</u>TTTTGTAATTTTTTTTAATAATTTATTAT-3' *Bam* HI
PfdhP5	5'-TAAG<u>GTATAC</u>TACATTTTATCATAC-3' *Acc* I
PfdhP3	5'-<u>GCGGCCGC</u>TTTGTAAAGTCAATAATATAATATATAATC-3' *Not* I
PfdhT5	5'-<u>GCGGCCGC</u> <u>CCCGGG</u>ATTTTCTTAACTTGAAAACTATAATTG-3' *Not* I *Sma* I
PfdhT3	5'-AATGAGTATGCT<u>GAGCTC</u>AGAGATTG-3' *Sac* I
PPHO5	5'-<u>GCGGCCGC</u>ATGTTTAAATCTGTTGTTTATTCA-3' *Not* I
PPHO3	5'-<u>CCCGGG</u>TTATTGTTTTAATAGGGTATCATT-3' *Sma* I
AODP5-1	5'-CTG<u>GAGCTC</u>GGAGTATACGTAAATATATAA-3' *Sac* I
AODP3-1	5'-CG<u>GGATCC</u>TATTGAAAAATAATTTTGTTTTTTT-3' *Bam* HI

The underlined nucleotide sequences are additional restriction enzyme recognition
sequences of the indicated enzymes.

The upstream regions of *FDH1* and *PMP47* that could function as
promoters were newly isolated in this study.   The genomic library that carried
the ca. 5-kb *Hin*d III-digested genomic library of *C. boidinii* S2 was used for
colony hybridization, with the ^{32}P-labeled 0.9-kb PCR-amplified fragment
which had previously been used for cloning of *FDH1* (69), as the probe. The
clone that showed a strong positive hybridization signal was isolated, and the
2-kb *Hin*c II-*Hin*d III fragment was subcloned into pBluescript II SK- to form

pFdh2. A 0.6-kb fragment was amplified by PCR with the 5'-primer PfdhP5 and 3'-primer PfdhP3 using pFdh2 as the template, and then subcloned into pT7Blue(R) to form pFP1. The 0.9-kb fragment from *Xho* I-*Acc* I-digested pFdh2 and the 0.6-kb fragment from *Acc* I-*Not* I-digested pFP1 were ligated into the *Xho* I-*Not* I site of pBluescript II SK- to form pFdhP.

The genomic library constructed with the *Sau* 3AI-partially-digested genomic library of *C. boidinii* was used for colony hybridization, with the 0.8-kb *Eco* RV-*Ssp* I fragment from pMP471 harboring *PMP47* (73) as the probe. Five clones that showed strong positive signals were found to harbor a 9-kb *Bam* HI fragment and contained *C. boidinii PMP47* and its upstream region (pYR47pro). *PPMP47* was amplified by PCR with the 5'-primer PR47S2 and 3'-primer 47ProN using pYR47pro as the template. The 1.9-kb amplified fragment was subcloned to pT7Blue(R) to form pYP471.

The nucleotide sequences of *PDAS1* (1843 bp), *PFDH1* (1478 bp), *PPMP20* (1375 bp) and *PPMP47* (1921 bp) will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers, AB035094, AB035095, AB035096, and AB035097, respectively.

Construction of the *ScPHO5*-expression cassette and its integration into the *ura3* locus of *C. boidinii* chromosomal DNA

A DNA fragment encoding the *AOD1* terminator was generated by PCR with the 5'-primer SPETERM and 3'-primer NP20 using pMOX620 DNA as the template. The amplified 0.5-kb fragment was digested with *Spe* I and *Hin* dIII, and subcloned into pBluescript II SK+ to form pATM. The resulting 0.5-kb *Spe* I-*Hin* dIII fragment harboring the *AOD1* terminator was purified on a gel. pSH39 (a YIp5 derivative harboring the 1592-bp *Bam* HI-*Bgl* II fragment of the coding region of *S. cerevisiae PHO5* ; a kind gift from Dr. S. Harashima (53)) was

double-digested with *Bam* HI and *Spe* I, and the 1.5-kb fragment containing the *ScPHO5* structural gene was purified on a gel. These two gel-purified fragments were ligated to the *Bam* HI-*Hin* dIII site of pBluescript II SK+ to form pPAT.

pAOD1P, pDAS1P, pYP201, and pYP471 were digested with *Bam* HI and *Sac* I. Then, each fragment encoding P*AOD1*, P*DAS1*, P*PMP20*, or P*PMP47*, respectively, was purified on a gel. pPAT was digested with *Bam* HI and *Hin* dIII; then, the 2.0-kb fragment containing the *ScPHO5* structure gene and *AOD1* terminator was purified on a gel. The fragments containing the promoter region of *AOD1*, *DAS1*, *PMP20*, or *PMP47*, and the *ScPHO5-AOD1* terminator cassette were ligated to the *Sac* I-*Hin* dIII site of pBluescript II SK+ to form pAP, pDP, p20P, and p47P, respectively. pAP was digested with *Xho* I and flush-ended, and then ligated with the 1.9-kb *Bam* HI-*Pst* I-flush-ended fragment from pBARCU1 harboring the *C. boidinii URA3* (65) to form pAPU (Fig. 2-1). pDP, p20P, and p47P were digested with *Xho* I, flush-ended, and then ligated with the 3.6-kb flush-ended *Sal* I fragment from pBARCU1 to form pDPU, p20PU, and p47PU, respectively (Fig. 2-1).

The P*FDH1*-*ScPHO5* expression cassette was constructed by a different procedure. The 2.5-kb *Eco* RI-*Xba* I fragment from pFDH1 harboring *C. boidinii FDH1* (69) was subcloned into pBluescript II SK- to form pFdEX. A 0.15-kb fragment was PCR-amplified with the 5'-primer PfdhT5 and 3'-primer PfdhT3 using pFdEX as the template, and then subcloned into pT7Blue(R) to form pFT1. The 0.85-kb fragment from *Sac* I-*Xba* I-digested pFdEX and the 0.15-kb fragment from *Not* I-*Sac* I-digested pFT1 were ligated at the *Not* I-*Xba* I site of pBluescript II SK- to form pFdhT. This insert was used as the *FDH1* terminator. The P*FDH1* fragment from *Xho* I-*Not* I-digested pFdhP and *FDH1* terminator from *Not* I-*Xba* I-digested pFdhT were ligated to the *Xho* I-*Xba* I site of pBluescript II

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KS+ to form pFdhPT. *ScPHO5* was PCR-amplified with the 5'-primer PPHO5 and 3'-primer PPHO3 using pSH39 as the template, and then digested with *Sma* I and *Not* I. This fragment was ligated with *Not* I-*Sma* I-digested pFdhPT to form pFP. pFP was *Xho* I-digested, flush-ended, and then ligated with the 3.6-kb flush-ended *Sal* I fragment from pBURCU1 to form pFPU (Fig. 2-1).
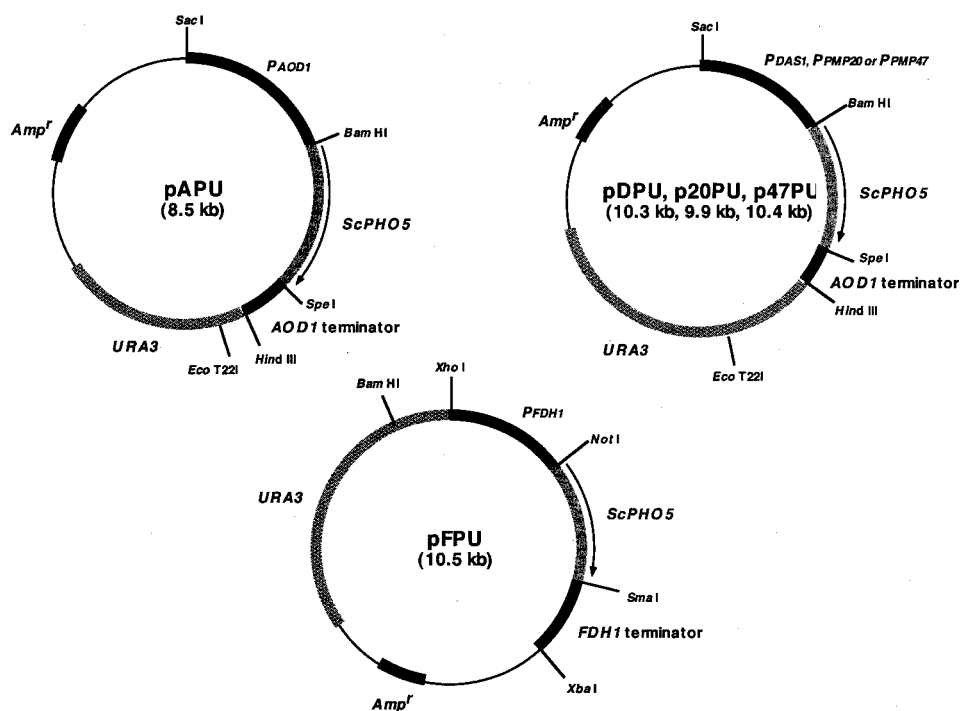

Fig. 2-1. Structure of the *ScPHO5*-expression vectors under the control of *PAOD1*, *PDAS1*, *PFDH1*, *PPMP20*, or *PPMP47*. The plasmids were constructed as described in the Materials and Methods. The exact sizes of *Sac* I-*Bam* HI-fragments of *PAOD1*, *PDAS1*, *PPMP20*, and *PPMP47* were 1672 bp, 1855 bp, 1417 bp and 1935 bp, respectively. The exact size of *Xho* I-*Not* I-fragment of *PFDH1* was 1492 bp. These plasmids were linearized by the appropriate restriction enzymes indicated within the *URA3* fragment of the respective structure, and integrated into the *ura3*-locus of *C. boidinii* TK62 chromosomal DNA by homologous recombination.

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The *Eco* T22I-linearized pAPU, pDPU, p20PU, and p47PU , and *Bam* HI-linearized pFPU, were each transferred into *C. boidinii* strain TK62 (*ura3*), and Ura+ transformants were isolated.   The integrative events of the transformants were analyzed by Southern analysis with *Eco* RI-digested chromosomal DNA, using the 32P-labeled *Bam* HI-*Pst* I-digested derivative harboring *C. boidinii* *URA3* as the probe (data not shown).   Transformants which showed a single integrate event of pAPU, pDPU, pFPU, p20U, or p47U at the *ura3* locus of the chromosomal DNA in *C. boidinii* TK62 were isolated, and named strain AP, strain DP, strain FP, strain 20P, and strain 47P, respectively.

Histochemical staining

C. boidinii transformants were placed on YNB plates in a medium containing one of the following as the carbon source: 1.5% methanol (v/v), 3% glycerol (v/v), or 2% glucose (w/v).   After a 24- to 48-hour incubation at 28° C, each plate was overlaid with melted soft agar (1% ; 50° C) containing a staining solution consisting of 0.25 mg of α-naphthyl-phosphate per ml, and 10 mg of Fast blue salt B (MERCK, Darmstadt, Germany) per ml in 50 mM acetate buffer (pH 4.0).   The colonies showing acid phosphatase activity stained dark red within 5 to 60 minutes at 28°C.   This diazo-coupling method was described by Dorn (14).

Enzyme assays

The *C. boidinii* transformant strains were grown on glucose medium containing 0.5% yeast extract, and then transferred to a synthetic MI medium containing the described carbon and nitrogen source and 0.5% yeast extract at a cell density OD_{610} of 0.1.   Cells were harvested at the middle-log phase, washed twice with 50 mM acetate buffer, pH 4.0, and then suspended in an

appropriate amount of 50 mM acetate buffer, pH 4.0. Acid phosphatase activity was assayed according to the method described by Torriani (91). One unit of enzyme was defined as the amount of enzyme which liberated 1 nmole of *p*-nitrophenol per min. The specific acid phosphatase activity of a cell suspension (units per unit of optical density at 660 nm) was determined according to the method of Toh-e *et al.* (90).

Results

ScPHO5 as the reporter to evaluate gene expression in *C. boidinii*

From the previous studies, P_{AOD1} shows a maximum level of expression in cells grown on methanol or methanol plus glycerol as the carbon source(s); an intermediate level of expression in cells grown on glycerol; and a repressed level in cells grown on glucose or ethanol (71). To test the applicability of *ScPHO5* as the reporter in *C. boidinii*, strain AP which had the P_{AOD1}-*ScPHO5* cassette at the *ura3* locus in one copy, was constructed to evaluate the regulated expression of *ScPHO5* under P_{AOD1}. The level of acid phosphatase activity in AP cells grown on methanol was 38 times higher than the level in AP cells grown on glycerol (Table 2-2). In contrast, the previous results using the *ScADK1* gene as the reporter showed that the level of adenylate kinase activity in methanol-grown cells was only 8 times higher than that in glycerol-grown cells. In addition, the level of acid phosphatase activity in the strain which harbored two copies of the P_{DAS1}-*ScPHO5*-expression cassette grown on methanol, was twice the level in strain DP grown on the same medium (data not shown). These results indicated that the level of acid phosphatase activity showed linearity with the gene expression level and that there was no rate-limiting step within the tested range of the secretory pathway. Therefore, the

ScPHO5 gene is suggested to be a better reporter than the *ScADK1* gene for the evaluating methanol-inducible promoters in *C. boidinii*.

Next, the author performed histochemical staining of acid phosphatase activity in *C. boidinii* colonies. In the wild-type strain, acid phosphatase activity was completely repressed by the high concentration of inorganic phosphate ion (>1 g/l) in the medium (data not shown), and the level of background acid phosphatase activity was negligible; therefore, the wild-type strain was not stained regardless of whether methanol, glycerol or glucose was used as the carbon source in the medium (Fig. 2-2). When strain AP was grown on methanol, the colonies were stained dark red, indicating high acid phosphatase activity. However, AP cells grown on glycerol were stained light red, indicating lower acid phosphatase activity. In contrast, when strain AP was grown on glucose, the colonies were not stained. The depth of color in these histochemical experiments varied according to the strength of P_{AOD1} allowing visual evaluation of gene expression on the plates.
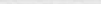
carbon source / strain	methanol	glycerol	glucose
AP			
wild type			

Fig. 2-2. Histochemical staining showing the level of acid phosphatase activity in the wild-type strain and strain AP on various carbon sources. *C. boidinii* wild-type strain and strain AP were cultured on the indicated carbon source [methanol, 1.5% (v/v); glycerol, 3% (v/v); or glucose, 2% (w/v)] for 24-48 hours. Then, each colony was overlaid with melted soft agar containing a staining solution consisting of 0.25 mg of α-naphthyl-phosphate per ml and 10 mg of Fast blue salt B per ml in 0.05 M acetate buffer (pH 4.0).

Isolation of the promoters of *DAS1, FDH1, PMP20* and *PMP47*

The author newly isolated the upstream regions of the *DAS1, FDH1, PMP20,* and *PMP47* genes. *ScPHO5* expression vectors (Fig. 2-1) using each of these upstream regions as the promoter were constructed and integrated into the chromosomal *ura3* locus of *C. boidinii* TK62. By Southern analysis using the *Bam* HI-*Pst* I fragment from the *CbURA3*-fragment as the probe, transformants which had a single copy of a plasmid were selected. Colonies of the transformed strains, strain DP, strain FP, strain 20P, and strain 47P, were all stained red when grown on a methanol plate, but were not stained when grown on a glucose plate (data not shown). Therefore, these newly obtained upstream regions, P_{DAS1}, P_{FDH1}, P_{PMP20}, and P_{PMP47}, were shown to function as methanol-inducible promoters.

Strength of the isolated promoters

Since the efficiency of transcription is crucial to the efficient production of heterologous proteins, the author first examined the strength of the newly isolated methanol-inducible promoters. As shown in Table 2-2, in methanol-grown cells, the level of *ScPHO5* expression as assessed by the level of acid phosphatase activity, was highest when placed under P_{DAS1}, and the level of *ScPHO5* expression decreased in the order of P_{AOD1}, P_{FDH1}, P_{PMP20}, and P_{PMP47}. The level of acid phosphatase activity under P_{DAS1} was approximately 50% higher than that under P_{AOD1}. The level of acid phosphatase activity under P_{FDH1} and P_{PMP20} was approximately 18% and 12%, respectively, of that under P_{DAS1}.

Table 2-2. Level of acid phosphatase activity under the control of various methanol-inducible promoters on various carbon sources

Carbon Source	Enzyme activity[a]					
	P_{AOD1}	P_{DAS1}	P_{FDH1}	P_{PMP20}	P_{PMP47}	Background[b]
methanol	304 ± 7.12 (100)	473 ± 18.4 (100)	87.3 ± 5.17 (100)	58.6 ± 6.19 (100)	14.2 ± 0.974 (100)	1.33 ± 0.645
glycerol	8.03 ± 4.56 (2.64)	8.13 ± 0.547 (1.71)	2.16 ± 0.348 (2.47)	0.504 ± 0.193 (0.860)	0.422 ± 0.0627 (2.97)	0.662 ± 0.217
oleate	14.8 ± 3.42 (4.87)	1.69 ± 0.324 (0.357)	7.63 ± 1.13 (8.74)	0.578 ± 0.180 (0.986)	54.5 ± 5.38 (384)	1.38 ± 0.364
D-alanine	4.08 ± 0.110 (1.34)	1.20 ± 0.379 (0.254)	6.09 ± 0.417 (6.87)	0.716 ± 0.141 (1.22)	1.75 ± 0.0368 (12.3)	0.629 ± 0.257
glucose	1.05 ± 0.237 (0.345)	1.64 ± 0.259 (0.346)	1.85 ± 0.596 (2.12)	0.873 ± 0.322 (1.49)	0.794 ± 0.280 (5.59)	0.910 ± 0.234
glucose + methanol	0.416 ± 0.106 (0.137)	0.659 ± 0.167 (0.139)	0.470 ± 0.0993 (0.538)	0.375 ± 0.0230 (0.640)	0.378 ± 0.0486 (2.66)	0.444 ± 0.198
glucose + sodium formate	0.181 ± 0.0838 (0.0596)	2.07 ± 0.284 (0.438)	19.1 ± 2.74 (21.9)	0.127 ± 0.0280 (0.217)	0.158 ± 0.0441 (1.11)	0.523 ± 0.108

[a] The level of acid phosphatase activity is expressed as U/OD_{660} and, in parentheses, as a percentage of the activity of the respective strain grown on methanol. The mean ± standard deviation of three independent experiments is presented.

[b] Level of acid phosphatase activity in *C. boidinii* wild-type strain.

Regulation by various carbon and nitrogen sources

In the previous studies, the regulated expression of *AOD1*, *DAS1*, *FDH1*, *PMP20*, and *PMP47* by various carbon and nitrogen sources, has been analyzed by enzyme assay, Western analysis and Northern analysis (Chapter I, Section 1, (69, 70)).   To clarify the regulatory profile at the transcriptional level (*i.e.*, at the level of activation of each promoter), the author determined the level of *ScPHO5* expression as assessed by the level of acid phosphatase activity, under P*AOD1*, P*DAS1*, P*FDH1*, P*PMP20*, and P*PMP47* on various carbon sources (Table 2-2).

P*AOD1*, P*DAS1*, P*FDH1*, and P*PMP20* were maximally induced in the methanol medium.   In contrast, P*PMP47* was induced by all three peroxisome-inducing carbon sources, *i.e.*, methanol, oleate, and D-alanine, and was maximally induced by oleate.   P*AOD1* and P*FDH1* were slightly induced when grown on an oleate- or D-alanine-containing medium.   On the other hand, P*DAS1* and P*PMP20* were induced only by methanol, and were not induced by oleate or D-alanine as observed in the case of P*AOD1* and P*FDH1*; that is, P*DAS1* and P*PMP20* seem to be more tightly regulated than P*AOD1* and P*FDH1* in regard to the carbon source.

The presence of glucose completely repressed methanol-induced expression of all of the tested promoters.   However, formate-induced expression under P*FDH1* was not repressed by the presence of glucose.   This may be due to the fact that the main physiological role of Fdh1p is detoxification of formate, while the main roles of the other genes are more directly related to methanol metabolism (69).

These regulatory profiles that were obtained by expressing the *ScPHO5* gene are in agreement with the previous results obtained at the mRNA level. Therefore, the author conclude that the upstream regions of the five methanol-inducible genes used in this study functioned like the original promoter and that the cloned promoter fragments contained sufficient information in their *cis-*elements to be regulated by the carbon source on which the yeast was grown.
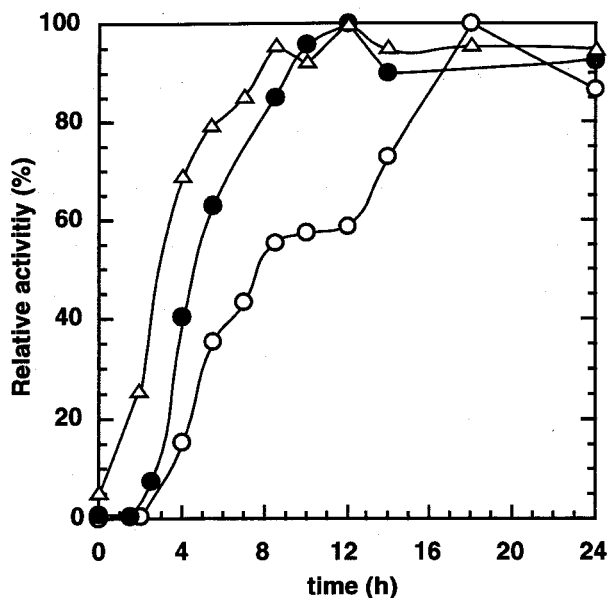
Fig. 2-3. Time course of methanol induction of acid phosphatase activity under the control of PDAS1, PFDH1, or PPMP47. C. boidinii strain DP, strain FP, and strain 47P were cultured on glucose-containing media, and then transferred to methanol-containing media at 0 h. The level of acid phosphatase activity in the three strains was measured at the indicated time points. The strains shown are: strain DP (○); strain FP (●); and strain 47P (△). In each strain, the maximal level of acid phosphatase activity that was measured, was set to 100%.

Induction Kinetics of PDAS1, PFDH1, and PPMP47

When C. boidinii cells are transferred from a glucose to a methanol medium, peroxisomal membrane proliferation precedes the induction of peroxisomal matrix proteins (97). Furthermore, since Pmp47 is necessary for the transport/folding process of the matrix enzyme, Das1p (73), Pmp47 should already be present when Das1p is transported to peroxisomes. Next, the author compared the induction kinetics of PDAS1, PPMP47, and PFDH1 at the early stage of methanol induction. (Fdh1p is a cytosolic methanol-inducible enzyme). As shown in Fig. 2-3, upon introducing cells to the methanol

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medium, P_{PMP47} was maximally induced the earliest, followed by P_{FDH1} and then P_{DAS1}. These results suggest that each promoter contains sufficient information that determines the order of gene expression during induction by methanol.

Discussion

A major objective of this study was to evaluate the strength of methanol-inducible promoters. P_{DAS1}, P_{FDH1}, P_{PMP20}, and P_{PMP47} were studied along with the previously cloned P_{AOD1}. The author's results clarified the differences in the strength and regulation of the five methanol-inducible promoters, P_{AOD1}, and P_{DAS1}, P_{FDH1}, P_{PMP20}, and P_{PMP47}, and these results were consistent with the previous results obtained at the mRNA level (Chapter I, Section 1, (69-71, 74)). Of these five promoters, P_{DAS1} is tightly regulated, was the strongest methanol-inducible promoter of those tested, and has the potential of producing heterologous proteins at levels higher than P_{AOD1}. In addition, the expression under P_{DAS1} was more strictly regulated than expression under P_{AOD1} by the various peroxisome-inducing carbon sources.

In the production of secretory proteins in yeasts using methanol-inducible promoters, the fermentation process is usually divided into two phases, the growth phase (during which cells are grown on glucose or glycerol to high cell density) and the production phase (during which gene expression is induced by transferring the cells to methanol). Since formate-induced gene expression under P_{FDH1} was not repressed by the addition of glucose, glucose can be added as an additional carbon source in the case to support efficient cell growth. Therefore, in the case of gene expression under P_{FDH1} in a formate medium, the desired protein can be produced not only during the production phase but also

during the growth phase, and the yield of protein is expected to be higher than that in the usual two-phase production system.   Recently, an enhanced version of *PFDH1* (m*PFDH1*) was developed to optimize the expression in such fermentation system.

In these five methanol-inducible promoters from *C. boidinii*, the author searched the sequence similar to the regulatory elements reported in other methanol-inducible genes, *P. pastoris AOX2* and *H. polymorpha MOX* (33, 60). All five promoters had the similar sequence to the consensus sequences in these elements raising the possibility that the conserved regions may work as a methanol-inducible element also in *C. boidinii*. (Fig. 2-4A and B).   Of five promoters, *PPMP47* was an oleate-inducible promoter, and contained the oleate response element including a inverted repeat sequence which was observed in *S. cerevisiae* oleate-inducible genes (15).

Methanol induction is closely related with peroxisome development in *C. boidinii*.   Methanol induces the expression of methanol-metabolizing enzymes, as well as some peroxines involved in peroxisome biogenesis, and peroxisome proliferation occurs.   The kinetic analysis of the early stage of methanol induction revealed that *PMP47* expression precedes *DAS1* expression, and the timing of expression is regulated by the promoter of the respective protein at the transcriptional level.   Further studies on the determination of the *cis*-elements in each promoter, that are involved in gene activation or repression will clarify the molecular mechanism of gene activation during adaptation of the methylotrophic yeast from a glucose to a methanol medium.

```
(A)
Consensus        GATAG-GCTA-TTT---T---G-AT--AAT
                 A       CGC                A

CbAOD1    -474   GATAGTGATAACTTTAAAATAGTAT--ACT  (complement, 16/19)
CbDAS1   -1048   CATAG-GACA-GTCGGCT---GAAATGCAA  (complement, 13/19)
CbFDH1    -627   GATAGTGGTAAAGA-TATA-ATTAT-AACA  (complement, 14/19)
CbPMP20   -269   GATAGTAATA-CGGAGATGCTCTCTTACTT  (complement, 11/19)
CbPMP47   -508   GATAG-GCTA-AAAA--TC--ACATTCATA  (complement, 13/19)
```

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(B)                                        (C)
HpMOX     -484   TCCTTGCACCGCAA              ScFOX1   -275
CbAOD1    -580   TACTTACATACCTA  (8/14)      ScFOX2   -272
CbDAS1   -1606   TCCGTGGAAAGCAG  (9/14)      ScFOX3   -188
CbFDH1    -299   TCTTTGTACCATAA  (10/14)     ScCTA1   -209
CbPMP20   -674   TCAGTTCAGTGCAA  (9/14)      ScPEX1   -158
CbPMP47   -694   GTATTTCAGCGCAA  (9/14)      CbPMP47  -597
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Fig. 2-4. Sequence alignment of similar regions in methanol-inducible promoters (A and B), and in oleate-inducible promoters (C). The sequences are positioned so that maximal sequence similarity is obtained. The nucleotides are numbered relative to the translation initiation codon of all genes. (A) Sequence alignment with consensus sequences between methanol-inducible genes reported by Ohi *et al.* (60). Matches to the consensus sequence are indicated by grey boxes, and the number of matches are indicated at the right side. (B) Sequence alignment with the MOX binding motif in *H. polymorpha MOX* (*HpMOX*) reported by Gödecke *et al.* (33). Matches to the motif are indicated by grey boxes, and the number of matches are indicated at the right side. (C) Sequence alignment with oleate response elements observed in oleate-inducible genes from *S. cerevisiae* (Sc). Sequences are: *FOX1* gene, encoding acyl-CoA oxidase (13); *FOX2* gene, encoding the multifunctional enzyme (37); *FOX3* gene, encoding thiolase (16); *CTA1* gene, encoding the peroxisomal catalase (8); *PEX1* gene, encoding a protein involved in peroxisomal assembly (17). The arrows and boxes indicate the imperfect inverted repeats.

Summary

The author isolated the promoter regions of five methanol-inducible genes (P*AOD1*, alcohol oxidase; P*DAS1*, dihydroxyacetone synthase; P*FDH1*, formate dehydrogenase; P*PMP20*, Pmp20; and P*PMP47*, Pmp47) from the *C. boidinii* genome, and evaluated their strength and studied their regulation using the acid phosphatase gene of *S. cerevisiae* (*ScPHO5*) as the reporter. Of the five promoters, P*DAS1* was the strongest methanol-inducible promoter whose strength was approximately 1.5 times higher than that of the commonly used P*AOD1* in methanol-induced cells. Although the expression of P*AOD1* and P*DAS1* was completely repressed by the presence of glucose, formate-induced expression of P*FDH1* was not repressed by glucose. Expression under P*PMP47*, another methanol-inducible promoter, was highly induced by oleate. The induction kinetics of P*PMP47* and P*DAS1* revealed that methanol induces the expression of peroxisome membrane protein Pmp47, earlier than the expression of matrix enzyme dihydroxyacetone synthase (Das1p), and that this information is contained in the promoter region of the respective gene. This is the first study which evaluates several methanol-inducible promoters in parallel in the methylotrophic yeast.

Section 2   Timing of the alcohol oxidase induction is regulated by the flow of C1-metabolism

Introduction

In the methylotrophic yeast *Candida boidinii*, methanol-metabolizing enzymes, alcohol oxidase (AOD), dihydroxyacetone synthase (DAS), and formate dehydrogenase (FDH) are massively produced in the cells grown on methanol as a sole carbon and energy source. · The promoters of the genes encoding these enzymes are useful for the heterologous gene expression in *C. boidinii* (76). Not only in *C. boidinii* but also in other methylotrophic yeasts, *Pichia pastoris*, *Pichia methanolica*, and *Hansenula polymorpha*, the heterologous expression system using strong methanol-inducible promoters have been established (9, 26, 63). However, the molecular mechanism of methanol-induction has been still unclear. The author isolated the promoter regions of five methanol-inducible genes and evaluated their strength and studied their regulation using acid phosphatase gene of *Saccharomyces cerevisiae* (*ScPHO5*) as a reporter (Chapter II, Section 1). Of the five promoters, the promoter of DAS-encoding gene (P_{DAS1}) was the strongest methanol-inducible promoter whose strength was approximately 1.5 times higher than that of the promoter of AOD-encoding gene (P_{AOD1}) in methanol-induced cells.

In this section, the author compared the induction kinetics of two methanol-inducible promoters, P_{AOD1} and P_{DAS1}. In order to identify the true inducer of *AOD1* and *DAS1*, the effects of methanol and its metabolites on both promoters were analyzed by using the $aod1\Delta das1\Delta$ strain. With this strain, the effects of each intermediate on the gene induction could be evaluated respectively, which could not be dissected with the wild-type strain. As a result, the author clarified that the methanol-inducible gene expression was not

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only induced by methanol itself but also coupled with the methanol-metabolic pathway.

Materials and Methods

Strains and media

C. *boidinii* S2 was used as the wild-type strain (88). C. *boidinii* strain AP had the P_{AOD1}-ScPHO5 cassette at the *ura3* locus in one copy, and strain DP had the P_{DAS1}-ScPHO5 cassette at the *ura3* locus in one copy (Chapter II, Section 1). The double disruptant of *AOD1* and *DAS1* (70) was reverted to uracil auxotrophy after 5-fluoroorotidic acid selection, yielding the *aod1Δdas1Δura3* strain (strain *aΔdΔU*) as described previously (75). The *Eco* T22I-linearlized pAPU and pDPU (Fig. 2-1) were each transferred into C. *boidinii* strain *aΔdΔU*. Transformants which had a single copy of pAPU or pDPU were isolated, and named strain APaΔdΔ and strain DPaΔdΔ, respectively. The yeast strains were grown on the synthetic MI medium. One or more of the following were used as the carbon source or the inducer of P_{AOD1}: 2% glucose (w/v), 0.7% methanol (v/v), 1 mM formaldehyde, 0.5% (w/v) dihydroxyacetone, and 0.5% (w/v) glyceraldehyde.

Enzyme assays

The C. *boidinii* transformant strains were grown on glucose medium containing 0.5% yeast extract, and then transferred to a synthetic MI medium containing the described carbon and nitrogen source and 0.5% yeast extract at a optical density at 610 nm of 1. After 24 h (for methanol- or glyceraldehyde-containing media) or 8 h (for formaldehyde- or dihydroxyacetone- containing media), cells were harvested and washed twice with 50 mM acetate buffer,

pH 4.0, and then resuspended in an appropriate amount of 50 mM acetate buffer, pH 4.0. Acid phosphatase activity was assayed as described in Chapter II, Section 1.

Results

The expression of *DAS1* preceded that of *AOD1*

In the previous study on the evaluation of five methanol-inducible promoters, P*DAS1* was found to be the strongest promoter and seemed to be more tightly regulated than P*AOD1* (Chapter II, Section 1). Comparison of the induction kinetics for P*DAS1*, P*PMP47*, and P*FDH1* at the early stage of methanol-induction suggested that the order of gene expression was defined by each promoter. The regulation and differences in the expression of two major methanol-inducible genes, *AOD1* and *DAS1*, were further analyzed by determining the induction kinetics of *AOD1* and *DAS1* at the protein level (Western analysis), at the mRNA level (Northern analysis), and at the promoter level (promoter-reporter assay).

As shown in Fig. 2-5A, DAS protein was detected after 4 h of methanol induction and AOD protein was detected only after 6h. At mRNA levels, *AOD1*-and *DAS1*-mRNA were both detected after 2 h of induction (Fig. 2-5B). While the quantity of *DAS1*-mRNA reached almost 100% of the maximum after 4 h, that of *AOD1*-mRNA reached maximum after 6h. Furthermore, the induction kinetics of P*AOD1* and P*DAS1* using ScPHO5 as a reporter gene revealed that the activation of P*DAS1* was significantly earlier than that of P*AOD1* (Fig. 2-5C). Thus, the expression of *DAS1* preceded that of *AOD1* and these regulation were controlled at the transcriptional level. AOD catalyzes the first step of the methanol metabolism, nevertheless, why does the induction of P*DAS1*

precede that of P_{AOD1}? Moreover, how do cells make difference in the induction speed between AOD and DAS?
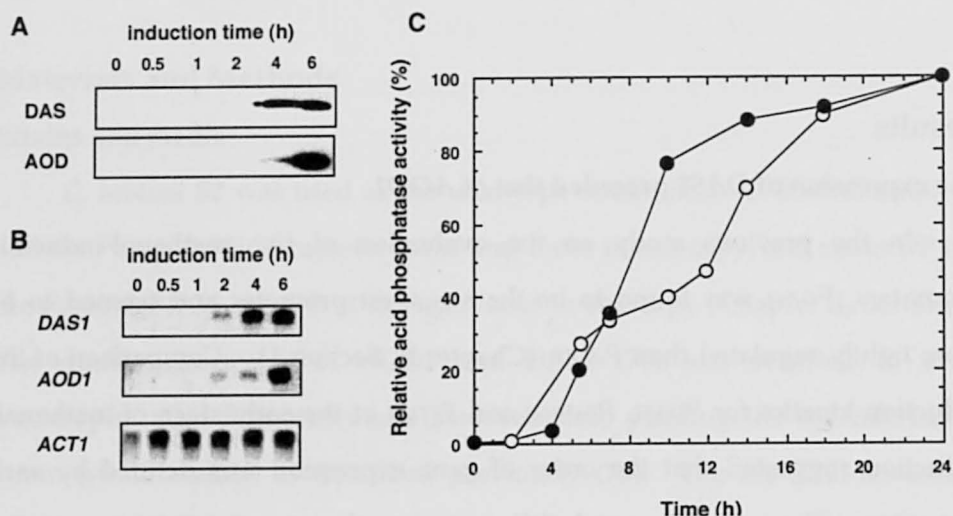

Fig. 2-5. Induction of AOD and DAS during the early stage of methanol induction. (A) Western analysis. (B) Northern analysis. Proteins (3.0 µg) and mRNAs (1.0 µg) extracted from each time point after transfer the wild-type strain to the methanol medium were loaded on to each lane. (C) Promoter-reporter assay. Acid phosphatase activities at each time point after transfer strain AP (○) and strain DP (●) to the methanol medium were measured.

P_{AOD1} is activated by anabolites

The author speculated that some metabolites in methanol metabolism might be concerned with the activation of P_{AOD1} and/or P_{DAS1}. In order to identify the true inducer of *AOD1* and *DAS1*, the effect of some metabolites on both promoters were analyzed by using the *aod1Δdas1Δ* strain as a host strain for promoter-reporter assay. In the wild-type strain, methanol is oxidized to formaldehyde by AOD, and formaldehyde is assimilated to dihydroxyacetone and glyceraldehyde-3-phosphate by DAS. On the other hand, in the *aod1Δdas1Δ* strain, since methanol is not oxidized and formaldehyde is not

assimilated, the author could dissect the effects of methanol itself, its catabolites (formaldehyde and downstream compounds), and their anabolites including dihydroxyacetone and glyceraldehyde.

Strain APaΔdΔ and strain DPaΔdΔ were incubated on MI medium containing methanol, formaldehyde, dihydroxyacetone, or glyceraldehyde for 8 h, and acid phosphatase activities were measured (Fig. 2-6). P_{DAS1} was activated by methanol and formaldehyde, which is the substrate of DAS, at the same level. Dihydroxyacetone and glyceraldehyde did not activate P_{DAS1}. P_{AOD1} was also activated by methanol and formaldehyde, and formaldehyde activated P_{AOD1} at the higher level than methanol. Furthermore, P_{AOD1} was activated not only by methanol and formaldehyde but also by dihydroxyacetone and glyceraldehyde, although the level of activation was lower than by formaldehyde. As a result, P_{AOD1} was activated not only by methanol but also by its metabolites.
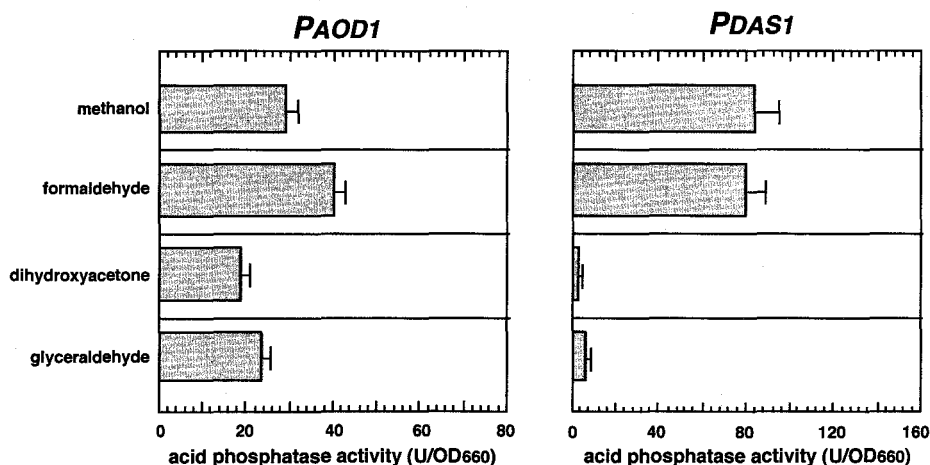

Fig. 2-6. Effect of methanol and its metabolites on the activity of P_{AOD1} and P_{DAS1} in aod1Δdas1Δ strain. C. boidinii strain APaΔdΔ (P_{AOD1}) and strain DPaΔdΔ (P_{DAS1}) were incubated for 8h on the media containing 1% methanol, 1 mM formaldehyde, 0.5% dihydroxyacetone, or 0.5% glyceraldehyde. Acid phosphatase activities are presented as mean ± S.E. of three independent experiments.

Discussion

When the methylotrophic yeasts utilize methanol as a carbon sources, it is a important problem, for physiological significance and the industrial use of the yeast, how the yeasts avoid the toxicity of formaldehyde which is a central metabolic intermediate of methanol metabolism. Formaldehyde is a highly reactive chemical that exhibits a toxic effect on all organisms through its nonspecific reactivity with proteins and nucleic acids (20, 31). It was reported that *C. boidinii* have several enzymes which concerned with degradation and detoxification of formaldehyde such as formaldehyde dehydrogenase and methylformate synthase (54, 69). Since formaldehyde is generated in peroxisomes and oxidation of formaldehyde occurs in cytosol, some mechanism which avoid the accumulation of formaldehyde in peroxisomes should exist.

At this point, the author found two phenomena: i) at the early stage of induction by methanol, P_{DAS1} was activated at the maximum level earlier than P_{AOD1}, and DAS which catalyze the second reaction of methanol metabolism was induced earlier than AOD which catalyze the first reaction of methanol metabolism. ii) While P_{DAS1} was activated only by methanol and formaldehyde which is a substrate of DAS reaction, P_{AOD1} was activated by methanol and formaldehyde but also by dihydroxyacetone and glyceraldehyde which are the products of DAS reaction. From these results, *C. boidinii* is thought to have a clever transcriptional regulation system to avoid the accumulation of formaldehyde. If AOD was induced strongly by methanol at the early stage of methanol induction, a large amount of formaldehyde would be accumulated in peroxisomes. To minimize the accumulation of formaldehyde, DAS which consumes formaldehyde as a substrate should be induced earlier than AOD. In fact *C. boidinii* firstly induces DAS and guarantees the metabolic pathway of formaldehyde. That is, *C. boidinii*

seemed to minimized the formaldehyde toxicity by regulating the timing of AOD- and DAS- induction during methanol induction. Then, how do cells make difference in the induction kinetics between AOD and DAS? Judging from the author's results, P_{AOD1} recognizes that the metabolic pathway of formaldehyde, *i.e.*, detoxification pathway of formaldehyde, are ready to work by responding to dihydroxyacetone and glyceraldehyde. Therefore, in the early stage of methanol induction, P_{DAS1} is activated by methanol at the earlier stage than P_{AOD1}, and the assimilation of formaldehyde catalyzed by DAS begins, and then, P_{AOD1} is fully activated by their anabolites (Fig. 2-7).
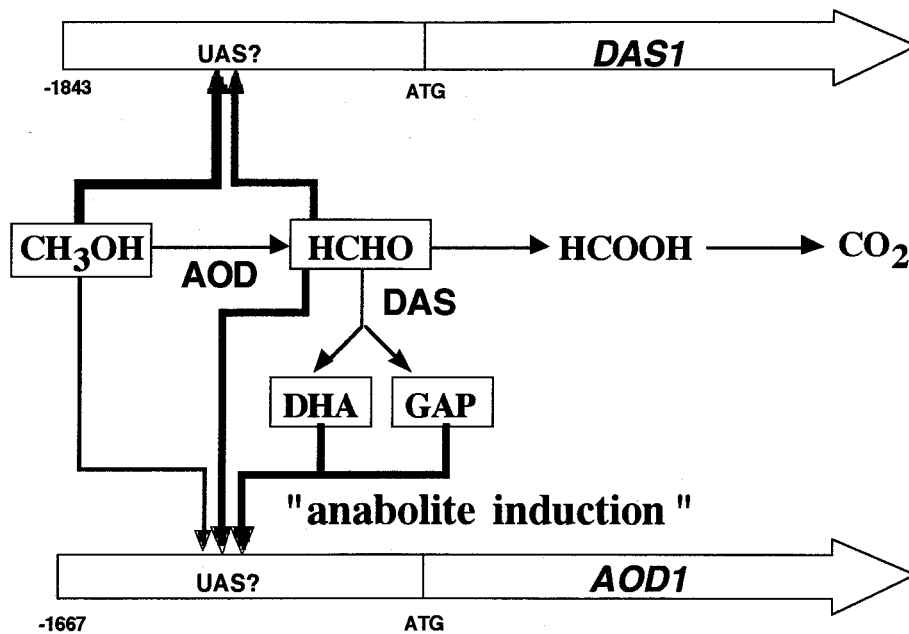

Fig. 2-7. Anabolite induction in the *C. boidinii*. DHA, dihydroxyacetone; GAP, glyceraldehyde-3-phosphate.

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Thus, the transcriptional regulation of *AOD1* is assumed to be coupled with the C_1-metabolic flow in order to protect the cells from the toxicity of formaldehyde. This regulation is a novel transcriptional regulation that some anabolites which exist in downstream of metabolic pathway induce the enzyme which catalyzes the first-step reaction. The author named this regulatory system, "anabolite induction". Identification of *cis*-elements which response to methanol and its metabolites in P_{AOD1} will elucidate the molecular mechanism of this anabolite induction.

Summary

In the methylotrophic yeast *C. boidinii*, at the early stage of induction of methanol, the kinetics of the expression of *AOD1* and *DAS1* was compared by Western analysis, Northern analysis, and promoter-reporter assay using ScPHO5 as a reporter. The Das1p and *DAS1*-mRNA were detected earlier than the Aod1p and *AOD1*-mRNA. Also, the induction kinetics of P_{AOD1} and P_{DAS1} revealed that the activation of P_{DAS1} preceded that of P_{AOD1}. By the experiments using the *aod1Δdas1Δ* strain, both promoters were activated by methanol and its metabolite, formaldehyde, however, P_{AOD1} was activated by some anabolites, such as dihydroxyacetone or glyceraldehyde, while P_{DAS1} was not. Therefore, it was suggested that P_{AOD1} is regulated by the unique transcriptional regulation coupled with the C_1-metabolic flow, in order to avoid the accumulation of formaldehyde which is toxic to the cell.

Chapter III

Establishment of high-level production of oxidases
in the peroxisome of *Candida boidinii*

Section 1   Characterization and high-level production of D-amino acid
oxidase in *Candida boidinii*

Introduction

D-Amino acid oxidase (DAO, EC 1.4.3.3) is a flavoprotein that catalyzes the streospecific oxidative deamination of D-amino acids, producing the corresponding α–keto acids and ammonia with the simultaneous reduction of molecular oxygen to hydrogen peroxide (34).

DAO reactions have considerable importance not only in basic research but also in biotechnology. The DAOs from porcine kidney and several fungi have been used for the enzymatic measurement of D-amino acids (92) and the production of α-keto acids (21). Also, the DAOs from several fungal sources, e.g., *Trigonopsis variabilis* and *Rhodotorula gracilis*, have been used for the bioconversion of cephalosporin C (CPC) to glutaryl-7-aminocephalosporanic acid, an intermediate for the production of 7-amino cepharosporanic acid (7-ACA), which is the starting key material for semi-synthetic cephem antibiotics (27). The enzymatic-synthetic route has some advantages over the conventional chemical methods used to prepare 7-ACA. Therefore, many efforts have been made to obtain DAO in large amounts from microorganisms (1, 7, 28, 49).

A methylotrophic yeast, *Candida boidinii*, is a promising host for the high-level production of DAO, since a high-level heterologous gene expression system involving the alcohol oxidase gene (*AOD1*) promoter has been

established (76). Especially, peroxisomes of the alcohol oxidase-depleted strain of the methylotrophic yeast *Candida boidinii* (55) (strain *aod1Δ*) were seems to be the optimum place for enzyme production. The rationale and the merits for our strategy are as follows; i) Since many kinds of oxidases are compartmentalized together with catalase in peroxisomes (96), the toxicity of heterologous oxidase was expected to be minimized when produced in a membrane-bound compartment. ii) Methanol-grown *C. boidinii* has huge peroxisomes which contain two major matrix proteins, alcohol oxidase and dihydroxyacetone synthase (73). Oxidase can be transported by the peroxin molecules (machinery proteins involved in protein transport to peroxisomes) which originally transported alcohol oxidase (82), and can occupy the intraperoxisomal space in strain *aod1Δ*. Therefore, the author could expect efficient peroxisomal transport and folding of the peroxisomal proteins. iii) Many oxidases contain FAD as a coenzyme, and overproduction of oxidases could lead to a depletion of FAD. Alcohol oxidase, which is a FAD octameric enzyme (41), makes up 10-20% of the total soluble protein in methylotrophic yeast under batch-culture conditions (88). Therefore, in strain *aod1Δ*, the author could expect that FAD, which was originally used in the production of alcohol oxidase, would be available in sufficient amounts for production of the putative protein.

The author cloned the *DAO1* gene encoding DAO from *C. boidinii*. By gene disruption analysis, the author showed that DAO is necessary for *C. boidinii* to grow on D-alanine as a sole carbon source, but not as a nitrogen source (Chapter I, Section 2). For the applied use of DAO from *C. boidinii*, the author overexpressed *DAO1* in *C. boidinii*, and then purified and characterized the DAO. Furthermore, the author have shown that the *C. boidinii aod1Δ* strain is a suitable host for the high-level production of DAO.

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Materials and Methods

Yeast strains, media, and cultivation

C. boidinii S2 was used as the wild-type strain. *C. boidinii* strain TK62 (*ura3* (66)) and strain *aod1Δ-U* (*ura3, aod1Δ* (55)) were used as hosts for transformation.

The yeast strain was grown on the synthetic MI medium. The concentrations of the carbon and nitrogen sources were 2% glucose (w/v), 3% glycerol, 1% methanol (v/v), 0.6% D-alanine (w/v), and 0.5% (w/v) methylamine. When D-alanine was used as both the carbon and nitrogen source, and when methylamine was used as the nitrogen source, NH_4Cl was omitted from the MI medium.

Enzyme assays

The activity of DAO was measured as described in Chapter I, Section 2. In some experiments, the DAO activity was assayed by polarography with a digital oxygen electrode (Rank Brothers, Cambridge, UK). The activity of orotidylate decarboxylase (ODC) encoded by *C. boidinii URA3* was measured by the method of Yoshimoto *et al.* (103). The enzyme activities of AOD and cytochrome *c* oxidase were assayed as described previously (73).

Construction of a *DAO1*-expression *C. boidinii* strain

The *DAO1* open reading frame was PCR-amplified to generate a *Not* I site at both the 5' and 3' ends, with pDA7 harboring the DAO-encoding DNA as the template. The oligos used were the 5' primer for the N-terminus (5'-ATAA-GAATgcggccgcAAAATGGGTGATCAAATTGTTGTT-3') and the 3' primer for the C-terminus (5'-ATAAGAATgcggccgcTAAAGTTTAGCTTTAACTTTTTG-3'). Both primers had an additional *Not* I site at the 5' end (in lowercase letters).
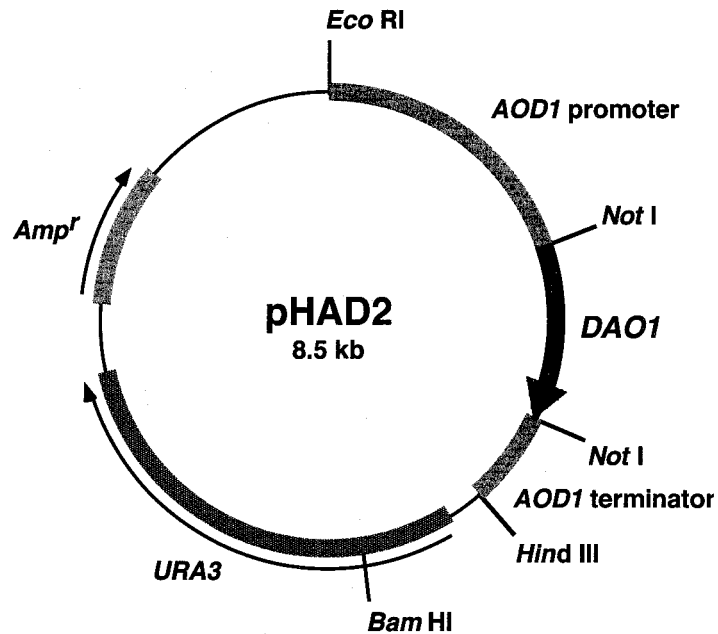
Fig. 3-1. Physical map of *C. boidinii* *DAO1* expression vector pHAD2. The PCR-amplified DAO-coding gene was inserted into the *Not* I site of pNOTeI. pHAD2 was linearized with *Bam* HI and then integrated into the *ura3*-locus of *C. boidinii* chromosomal DNA by homologous recombination.

The amplified fragment was digested with *Not* I, gel-purified, and then introduced into the *Not* I site of pNOTeI, which is an *AOD1*-promoter driven expression vector (64). The resulting *DAO1*-expression vector was named pHAD2 (Fig. 3-1). The direction of the inserted *DAO1* was confirmed by physical mapping of the plasmid. pHAD2 was linearized with *Bam* HI within the *C. boidinii* *URA3* sequence and then used to transform *C. boidinii* strain TK62. By genomic Southern analysis of *Eco* RI-digested total DNA from the transformants, a single-copy integrant strain (strain DOS) and a multiple integrant strain (strain DOM) were isolated using the 1.9-kb *Bam* HI-*Pst* I fragment of the *C. boidinii* *URA3* gene as a probe. In same way, linearized

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pHAD2 was used to transform strain *aod1Δ-U*, and then a single-copy integrant strain (strain DOSaΔ) and a multiple integrant strain (strain DOMaΔ) were isolated.

Purification of DAO

Methanol-grown cells of *C. boidinii* strain DOM were harvested and then washed twice with 0.1 M potassium phosphate buffer (KPB), pH 8.0, containing 5 mM dithiothreitol (DTT). The cells were disrupted with a 200M Insonator (Kubota, Tokyo, Japan), and the cell debris was removed by centrifugation at 20,000 x *g*. The resultant supernatant was put on a DEAE-Toyoperl 650M column equilibrated with 0.4 M KPB, pH 8.0, containing 5 mM DTT. After the column was washed with the same buffer, the active fractions that passed through the column were pooled, and then dialyzed against 10 mM Tris-HCl buffer, pH 7.6, containing 5 mM DTT. The enzyme was then put on a DEAE-Sepharose column equilibrated with the same buffer. After washing of the column with the same buffer, elution was done with a gradient of 0 to 0.2 M NaCl. The active fractions were pooled and saturated with 25% ammonium sulfate. The enzyme was then put on a Butyl-Sepharose column equilibrated with 25% ammonium sulfate in 10 mM Tris-HCl buffer, pH 7.6, containing 5 mM DTT. After washing of the column with the same buffer, elution was done with a gradient of 20 to 0% ammonium sulfate. The active fractions were pooled and then put on a Mono Q column equilibrated with 10 mM Tris-HCl buffer, pH 7.6, containing 5 mM DTT. After washing of the column with the same buffer, elution was done with 0 to 0.2 M NaCl. The active fractions were pooled and used as the purified enzyme. To measure the molecular mass of the native enzyme, the purified enzyme was put on a Superose 12 column equilibrated with 0.1 M KPB, pH 7.0, containing 0.2 M NaCl.

Subcellular fractionation

Subcellular fractionation of strain DOM was done as described previously (73). An organellar suspension (~2.0 ml) was layered on top of a 38-ml semicontinuous gradient [2.0 ml of 35, 37, 40, and 43%, 4.0 ml of 45, 46, 48, 50, 51, and 52%, and 2.0 ml of 54, 56, and 60% (w/w) sucrose], followed by centrifugation at 4°C for 5 h at 100,000 x g. The gradient was devided, by pipetting, into 18 fractions from the bottom (fraction 1) to the top (fraction 18). Fractions were assayed for DAO, AOD, and cytochrome c oxidase activities.

Results and Discussion

Expression of $DAO1$ in $C.$ $boidinii$

First, the $DAO1$ gene was expressed in the wild-type $C.$ $boidinii$ strain. The $C.$ $boidinii$ $AOD1$ promoter is methanol-inducible, and suitable for high-level expression of both heterologous and endogeneous genes (74, 76). The PCR-amplified $DAO1$ gene was ligated to pNOTeI under the $AOD1$ promoter, yielding pHAD2. After transformation of $C.$ $boidinii$ strain TK62 with pHAD2, a single-copy transformant, strain DOS, and a double-copy transformant, strain DOM, integrated at the $ura3$ locus, were selected. The integration event and the copy number for each transformant were tested by Southern analysis, and also the level of ODC activity, which is encoded by the $URA3$ gene (data not shown). The DAO activities of these transformants grown on methanol were compared. Strain DOM expressed DAO (13.7 U/mg, Table 3-1) at a higher level than strain DOS (11.7 U/mg, Table 3-1), and approximately 9 times as high as the glucose-D-Ala grown wild-type strain (1.5 U/mg, Chapter I, Section 1).

Table 3-1. DAO production by various transformants of C. boidinii[a]

Host	Strain	Copy number[b]	DAO activity (U/mg)	
			C-source: methanol N-source: NH$_4$Cl	methanol + glycerol methylamine
wild type	DOS	1	11.5	9.79
	DOM	2	13.7	11.6
aod1Δ	DOSaΔ	1	9.83	14.9
	DOMaΔ	8	26.5	41.6

[a] Each transformant was incubated for 24 h in medium containing methanol and NH$_4$Cl, or grown on methanol/glycerol/methylamine medium for 48 h.

After centrifugation at 20,000 x g, the supernatants were used as cell-free extracts.

[b] The copy number of the integrated plasmid was estimated from the activity of ODC and by Southern analysis.

Table 3-2. Purification of DAO from C. boidinii strain DOM

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell-free extract	414	5230	12.6	1.00	100
DEAE-Toyopearl	100	3530	35.1	2.78	67.5
DEAE-Sepharose	37.5	3300	88.1	6.98	63.1
Butyl-Sepharose	8.4	1220	146	11.5	23.3
Mono Q	3.9	540	139	11.1	10.3

Purification and characterization of C. boidinii DAO

Next, DAO was purified approximately 11.1-fold to apparent homogeneity on SDS-PAGE from a cell-free extract of strain DOM grown on methanol (Table 3-2). The specific activity of the purified enzyme was 139 U/mg protein, with a yield of 10.3%. The apparent molecular mass of the purified DAO was estimated to be 41 kDa by SDS-PAGE or 43 kDa by gel filtration (data not shown). The molecular mass calculated from the deduced amino acid sequence of DAO1 was approximately 38 kDa. Only one

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N-terminal amino acid sequence, GDQIVVLGSGIIGLYTTY, was found on
Edman degradation, which is identical to the deduced amino acid sequence of
DAO1. Judging from these results, the *C. boidinii* DAO is a monomeric enzyme,
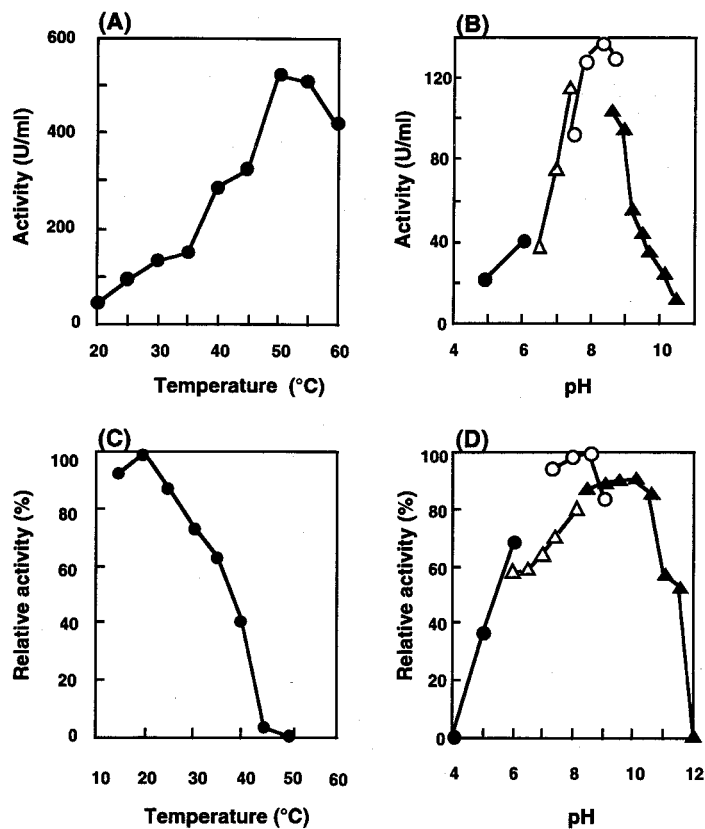while the DAOs reported so far are mostly dimeric enzymes.

Fig. 3-2. Effects of temperature and pH on *C. boidinii* DAO. (A) Effects of
temperature on the activity: enzyme activity was measured under the standard
conditions at various temperatures. (B) Effects of pH on activity: enzyme activity
was measured under the standard conditions at various pHs. (C) Thermostability:
the enzyme was incubated at various temperatures for 30 min in 0.1 M Tris-HCl
buffer, pH 8.0, and then the remaining activity was measured under the standard
conditions. (D) pH stability: the enzyme was incubated at 30°C for 30 min in
various buffers (0.1 M), and then the remaining activity was measured. Buffers
used: ●, acetate buffer; △, phosphate buffer; ○, Tris-HCl buffer; ▲, glycine-
NaOH buffer.

The optimum temperature and pH of the purified DAO were 50°C and 8.4, respectively (Fig. 3-2A and B). On incubation of this enzyme at various temperatures for 30 min, the DAO activity gradually decreased above 30°C and was completely lost at 50°C (Fig. 3-2C). The enzyme was stable within the pH range of 6 to 10.5 (Fig. 3-2D).

The enzyme was active toward several D-amino acids such as D-Ala, D-Met, D-Ser, D-Leu, and D-Val, D-Ala being the best substrate for this enzyme; the K_m for D-Ala was 4.28 mM (Table 3-3). C. boidinii DAO was not active toward other tested D-amino acids (D-Arg, D-Asp, D-Cys, D-Glu, D-His, D-Lys, D-Trp, and D-Tyr), Gly, L-Ala, or CPC.

Table 3-3.   Substrate specificity of DAO from C. boidinii

Substrate	Relative activity (%)[a]	K_m (mM)	V_{max} (U / mg)
D-Ala	100	4.28	176
D -Ser	32.4	33.7	116
D -Met	14.4	27.4	191
D -Val	8.24	3.95	11.1
D -Leu	7.47	10.9	25.2
D -Phe	1.50	n.t.[b]	n.t.
D -Pro	1.41	n.t.	n.t.
D -Thr	1.17	n.t.	n.t.
D -Asn	0.175	n.t.	n.t.

The enzyme did not show activity toward D -Arg, D -Asp, D -Cys, D -Glu, D -His, D -Lys, D -Trp, and D -Tyr, Gly, L -Ala, or CPC.

[a] Activity is given relative to that measured for D -Ala.

[b] n.t., not tested.

The enzyme was completely inhibited by Ag^+ (1 mM) and Hg^{2+} (1 mM), and also by p-chloromercuribenzoate (0.1 mM) and iodoacetamide (1 mM) (data not shown). These results suggest that a sulfhydryl group of the enzyme is important in the activity. Indeed, C. boidinii DAO has seven cysteine residues.

A cysteine residue (Cys298) in *T. variabilis* DAO and Cys208 in *R. gracilis* DAO were reported to be reactive cysteine residues located near the FAD-binding domain (61, 79). However, these cysteine residues are not conserved in DAOs from other organisms including *C. boidinii* (Fig. 1-11). Further site-directed analyses may clarify the amino acid residue which had a direct catalytic role in *C. boidinii* DAO.
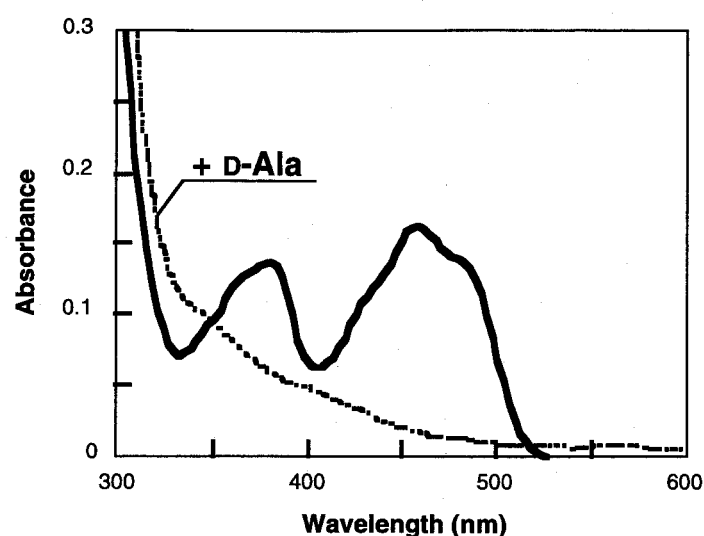

Fig. 3-3. Absorption spectra of *C. boidinii* DAO. Solid line: purified DAO (2.0 mg/ml) in 0.1 M KPB, pH 8.0; broken line: purified DAO (2.0 mg/ml) incubated with 0.5 M D-alanine in 0.1 M KPB, pH 8.0, at 30°C for 30 min.

The absorption spectrum of the purified enzyme had two absorption maxima at 366 and 448 nm, which is typical of a flavoprotein (Fig. 3-3). When the enzyme was incubated with a substrate, D-Ala, the absorption maximum at 448 nm disappeared. Therefore, this flavin may be involved in the enzyme reaction. To identify the AMP moiety of the chromophore, the purified enzyme was digested with Pronase, and then AMP liberated on

phosphodiesterase treatment was measured by the enzymatic method with adenylate kinase. As a result, 37.3 nmoles AMP was obtained for 35.0 nmoles DAO, indicating that DAO has one mole of FAD as a prosthetic group. This FAD was liberated from the enzyme by trichloroacetic acid precipitation and yellow fluorescence was observed in the supernatant, indicating that the FAD is noncovalently bound to the protein.

Localization of DAO in *C. boidinii* strain DOM

The three C-terminal amino acid residues, -AKL, which belong to typical peroxisomal targeting signal 1, were found to be necessary and sufficient for the import of DAO into peroxisomes (Chapter I, Section 2). To investigate the localization of the high-level of DAO produced by strain DOM, the author prepared an organellar fraction from methanol-grown cells, and then this fraction was put through sucrose density-gradient centrifugation.
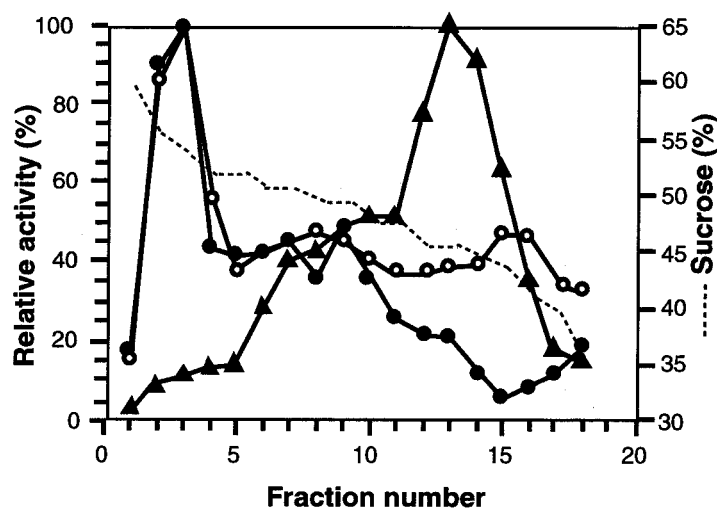

Fig. 3-4. Localization of the produced DAO. An organellar pellet was obtained by subcellular fractionation of methanol-grown cells of strain DOM and then put through density-gradient centrifugation on sucrose. Fraction 1 represents the bottom of the gradient. AOD and cytochrome c oxidase are the peroxisomal and mitochondrial marker enzymes, respectively. ○, DAO; ●, AOD; ▲, cytochrome c oxidase. Broken line, sucrose concentration.

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Figure 3-4 shows the good resolution of mitochondria and peroxisomes, as reflected by the different activity peaks for cytochrome c oxidase (fraction number 13) and AOD, (fraction number 3) as marker enzymes for mitochondria and peroxisomes, respectively. Furthermore, the author observed that DAO shows a similar distribution of the peroxisomal marker AOD, implying that the high-level of DAO produced was correctly targeted to peroxisomes.

DAO overproduction by the *aod1Δ* strain

When the *aod1Δ* strain was used as the host, the author used methylamine as the nitrogen source. Since the *aod1Δ* strain cannot oxidize methanol to formaldehyde, the level of induction of the *AOD1* promoter in the *aod1Δ* strain is lower than that in the wild-type strain on methanol medium (Chapter II, Section 2). When methylamine is used as the nitrogen source, methylamine is oxidized to formaldehyde by amine oxidase, and then formaldehyde and its metabolites can induce the *AOD1* promoter to a comparable level to that in the wild-type strain.

In this study, the author compare the wild-type strain and the *aod1Δ* strain as hosts for *DAO1* expression. The *aod1Δ* strain was transformed with pHAD2, and then a single integrant, strain DOSaΔ, and a multiple integrant, strain DOMaΔ, were obtained. Southern analysis and ODC assaying showed that strain DOMaΔ had eight copies of pHAD2 (data not shown). The levels of DAO produced by these transformants on methanol medium or methanol/glycerol/methylamine medium were compared (Table 3-1). Strains DOSaΔ and DOMaΔ could not grow on methanol as the sole carbon source. Therefore, these strains were grown on glucose and then incubated in methanol medium, or they were grown on glycerol-containing medium.

In the wild-type strain used as the host, the DAO activity of the multiple integrant strain DOM was significantly higher than that of the single integrant strain DOS on both media, as described in the first paragraph of Results and Discussion. The DAO activities of strains DOS and DOM on methanol/glycerol/methylamine medium (9.79 U/mg and 11.6 U/mg, respectively) were slightly lower than those on methanol medium (11.5 U/mg and 13.7 U/mg, respectively). A possible explanation for this phenomenon is that the cells use NH_4Cl better than methylamine as a nitrogen source.

With the $aod1\Delta$ strain as the host, the DAO activities of strains DOSaΔ and DOMaΔ on methanol/glycerol/methylamine medium (14.9 U/mg and 41.6 U/mg, respectively) were 1.5-fold higher than those on methanol medium (9.83 U/mg and 26.5 U/mg, respectively). Also, the DAO activity of strain DOSaΔ on methanol/glycerol/methyl-amine medium was 1.5-fold higher than that of strain DOS (9.79 U/mg). These results suggested that the $aod1\Delta$ strain was a more suitable host for the production of DAO than the wild-type strain. Finally, strain DOMaΔ which had eight copies of pHAD2 on its chromosome, grown on methanol/glycerol/methylamine medium showed the maximum DAO activity (41.6 U/mg), which corresponded to approximately 30% of the total soluble proteins estimated from the specific activity of the purified DAO (139 U/mg).
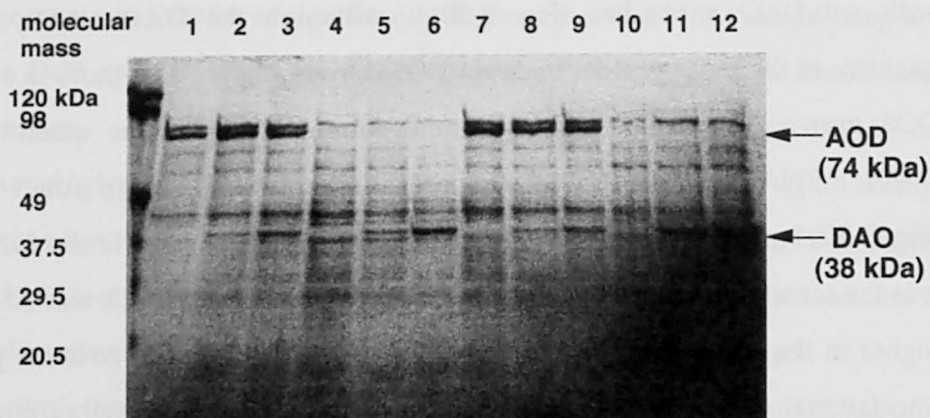
The expression levels of DAO1 in various transformants

The expression levels of the DAO1 gene were also determined by SDS-PAGE, Western analysis and Northern analysis. On SDS-PAGE, a 38-kDa band was detected that was correlated with the DAO activity (Fig. 3-5A, lanes 2, 3, 5, 6, 8, 9, 11, and 12), and a 74-kDa band corresponding to AOD was not observed when the $aod1\Delta$ strain was used as the host (Fig. 3-5A, lanes 4, 5, 6, 10,
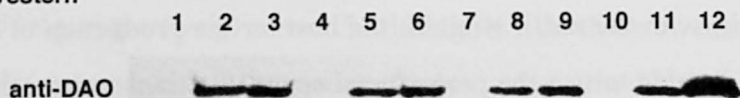
11, and 12). The 38-kDa band was verified to be DAO by Western analysis with anti-DAO antibodies (Fig. 3-5B). Although the DAO activity and quantity of the DAO protein in strain DOSaΔ were higher than those in strain DOS grown on methanol/glycerol/methylamine medium, the quantity of *DAO1* mRNA in strain DOSaΔ was nearly the same as that in strain DOS (Fig. 3-5C, lanes 8 and 11). In other words, while the transcript level of *DAO1* was the same in the two strains, the quantity of the product, DAO, was 1.5-fold higher in the *aod1Δ* strain. This difference could be attributed to some post-translational effects, e.g. transport of peroxisomes and/or FAD supply, as shown in the study on the production of fructosyl amino acid oxidase in next section. That is, with the *aod1Δ* strain as the host for the production of DAO, DAO seems to be able to use the peroxisomal space, the transport machinery into peroxisomes, and FAD as a cofactor, which are used by AOD in the wild-type strain. Based on this study, the *aod1Δ* strain of *C. boidinii* can be considered to be a general host for the production of heterologous proteins within peroxisomes.

DAO produced with *C. boidinii* will be suitable for practical use, such as in the measurement of D-amino acids and the production of α–keto acids. Unfortunately, *C. boidinii* DAO cannot oxidize CPC. However, the *aod1Δ* strain of *C. boidinii* will be a promising host for high-level production of DAOs from other sources that can use CPC as a substrate.

(A) SDS-PAGE

molecular mass

120 kDa
98

49

37.5

29.5

20.5

1 2 3 4 5 6 7 8 9 10 11 12

← AOD (74 kDa)

← DAO (38 kDa)

(B) Western

1 2 3 4 5 6 7 8 9 10 11 12

anti-DAO
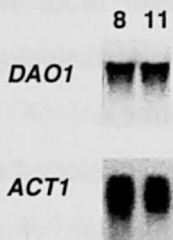
(C) Northern

8 11

DAO1

ACT1

Fig. 3-5. Expression analysis of the *DAO1* gene in the wild-type strain and the *aod1Δ* strain on various media.   (A) SDS-PAGE of cell-free extracts (10 μg protein) of the wild type strain (lanes 1 and 7), strain DOS (lanes 2 and 8), strain DOM (lanes 3 and 9), the *aod1Δ* strain (lanes 4 and 10), strain DOSaΔ (lanes 5 and 11), and strain DOMaΔ (lanes 6 and 12) incubated for 24 h on methanol medium (lanes 1-6) or grown for 48 h on methanol/glycerol/methylamine medium (lanes 7-12). Harvested cells were disrupted by sonication.   After centrifugation at 20,000 x g the supernatants were used as cell-free extracts.   (B) Western analysis with anti-DAO antibodies.   (C) Northern analysis with the ^{32}P labeled, 0.4-kb *DAO1* fragment (top), or ^{32}P-labeled, 0.9-kb *C. boidinii ACT1* fragment (bottom).   Five μg of total RNA was put on each lane.

Summary

D-Amino acid oxidase (DAO, EC 1.4.3.3) from a methylotrophic yeast, *C. boidinii*, was produced at a high level under the control of the alcohol oxidase gene promoter in the original host. The enzyme was a peroxisomal and monomeric enzyme, and contained noncovalently-bound FAD as a cofactor. The enzyme was active toward several D-amino acids such as D-Ala, D-Met, and D-Ser. An alcohol oxidase-depleted strain (*aod1Δ*) was found to be a more suitable host for DAO production than the wild-type strain. Several post-translational effects may be responsible for the improvement of the DAO productivity by the *aod1Δ* strain. Finally, an *aod1Δ* strain transformant having multi-copies of an expression plasmid on its chromosome could produce DAO amounting up to 30% of the total soluble proteins.

Section 2 Production of fungal fructosyl amino acid oxidase useful for diabetic diagnosis in the peroxisome of *Candida boidinii*

Introduction

Many kinds of oxidases are used for quantitative and qualitative colorimetric assay coupled with a peroxidase reaction. It has been reported that fructosyl amino acid oxidase (FAOD) from several fungal species can be used for enzymatic determination of the quantity of glycated proteins (85, 86, 101, 102). Non-enzymatic glycation of proteins has been implicated in the pathogenesis of diabetic complications. Since glycation of blood proteins is not affected by transient increases in blood glucose, the level of glycated proteins is a good index for monitoring patients with diabetes mellitus during therapy. Since the original fungi did not produce a sufficient quantity of FAOD and since the cultivation of fungi is costly, a high-level and economical means of producing FAODs needs to be established to supply the large amount of FAOD needed for clinical use. Although the active form of FAOD can be obtained from *Escherichia coli*, high-level expression was toxic to the host strain probably because of the fortuitous production of H_2O_2 or the retrieval of the coenzyme, flavin adenine dinucleotide (FAD), via covalent bonding to FAOD (101).

To avoid the problems encountered in the *E. coli* expression system, peroxisomes of the alcohol oxidase-depleted strain of the methylotrophic yeast *Candida boidinii* (55) (strain $aod1\Delta$) were chosen as the place for enzyme production. The $aod1\Delta$ strain has been shown to produce peroxisomal oxidases, D-amino acid oxidase and acetylspermidine oxidase (Chapter III, Section 1, (58)). This study was conducted to seek an economical means of producing *Penicillium janthinellum* FAOD (101) with the aim of establishing

a general expression method for toxic proteins or oxidases in *C. boidinii* strain *aod1Δ*.

Materials and Methods

Strains and media

C. boidinii S2 was used as the wild-type strain. *C. boidinii* strain TK62 (*ura3* (66)) and strain *aod1Δ-U* (*ura3, aod1Δ* (55)) were used as hosts for transformation.

The concentrations of the carbon and nitrogen sources were 1.5% (v/v) methanol, 3.0% (v/v) glycerol, 0.5% (w/v) NH_4Cl, and 0.5% (w/v) methylamine.

DNA synthesis

The sequence of the synthesized FAOD gene was submitted to GenBank and was assigned accession number AF181866. The synthetic gene was assembled from three separate fragments of the FAOD gene, that is, the 495-bp *Not* I-*Pst* I fragment, the 415-bp *Pst* I-*Hin*d III fragment, and the 415-bp *Hin*d III-*Not* I fragment. Each fragment was generated by annealing 9 oligos followed by the conventional PCR reaction using VENT DNA polymerase (New England Biolabs, Beverly, MA, USA).

Vectors and molecular methods

pNOTeI was used as the expression vector (64). Linearized DNA was transformed to *C. boidinii* strain TK62 and to strain *aod1Δ-U*. The cells were selected by Ura+ phenotype. The expected chromosomal integration and the copy number of the integrated plasmid were confirmed by Southern analysis

and orotidylate decarboxylase (ODC) activity. The nucleotide sequence of the synthesized FAOD gene was confirmed using the Shimadzu DSQ-1000L DNA sequencer.

Enzyme assay

FAOD activity in the cell-free extract was determined as previously described (101) using fructosyl valine as the substrate. ODC activity was determined by the method of Yoshimoto *et al.* (103). Western analysis was performed using an Amersham ECL Detection Kit (Arlington Heights, IL, USA) with rabbit polyclonal antibody raised against purified FAOD (77).

Results

Expression of synthetic FAOD gene optimized for *C. boidinii* codon usage

While the original FAOD cDNA of *P. janthinellum* contains a G or C nucleotide at the third position of most codons (101), highly expressed genes in *C. boidinii* have an A or T at the third position of most codons. The synthetic FAOD gene was designed based on the deduced amino acid sequence of FAOD and the preferred codon usage in *C. boidinii*. The original FAOD cDNA and the synthetic FAOD DNA were each ligated to pNOTeI under the *AOD1* promoter, yielding pNEP and pNEPS, respectively. After transformation of strain *C. boidinii* TK62 (*ura3*), one-copy transformants of pNEP integrated at the *URA3* locus and one-copy transformants of pNEPS integrated at the *URA3* locus were selected. The level of FAOD expression of the transformants was compared. The copy number of each transformant was estimated from both Southern analysis (data not shown) and the level of ODC activity, which is encoded by the *URA3* gene (Table 3-4). The pNEPS transformant (strain

NEPS1) expressed FAOD at a level approximately 2.3-fold higher than the pNEP transformant (strain NEP1), which reflects a difference in the translation efficiency of the codons. Further studies were performed with pNEPS transformant strains.

Table 3-4. FAOD production in various transformants of *C. boidinii* [a]

DNA/Strain	ODC activity (fold) (U/mg)	Copy number	FAOD activity (fold) (U/mg)
Control strains			
C. boidinii wild type	0.0258 (1)		n.d. [b]
C. boidinii aod1Δ	0.0267 (1)		n.d.
C. boidinii ura3	n.d.		n.d.
C. boidinii ura3 aod1Δ	n.d.		n.d.
FAOD cDNA (pNEP)/*C. boidinii ura3*			
Strain NEP1	0.0262 (1.02)	1	0.520 (1.00)
Synthetic FAOD (pNEPS)/*C. boidinii ura3*			
Strain NEPS1	0.0233 (1.03)	1	1.20 (2.30)
Strain NEPS2	0.0553 (2.43)	2	2.75 (5.29)
Strain NEPS3	0.0781 (3.44)	3	4.33 (8.32)
Synthetic FAOD (pNEPS)/*C. boidinii ura3 aod1Δ*			
Strain NEPSΔa1	0.0261 (0.98)	1	4.95 (9.52)
Strain NEPSΔa2	0.0553 (2.07)	2	11.7 (22.5)
Strain NEPSΔa5	0.133 (4.96)	5	24.5 (47.1)

[a] Each transformant was grown on methanol/methylamine medium to the stationary phase (72 h). The enzyme activity was then determined.   The copy number of the integrated plasmids was also estimated from Southern analysis.
[b] n.d., not detected.

C. boidinii aod1Δ as the expression host

Previously, the *aod1Δ* strain was shown to be a useful host for the expression of D-amino acid oxidase and acetylspermidine oxidase (Chapter III, Section 1, (58)). The author used the *aod1Δ* strain as the expression host for FAOD production. The level of FAOD expressed by the one-copy pNEPS1 transformants of the wild type *C. boidinii* strain (strain NEPS1) and that

(A) pNEPS-transformant (FAOD)

Strain NEPS1 (wild type)   Strain NEPSΔa1 (aod1Δ)

(B) Western
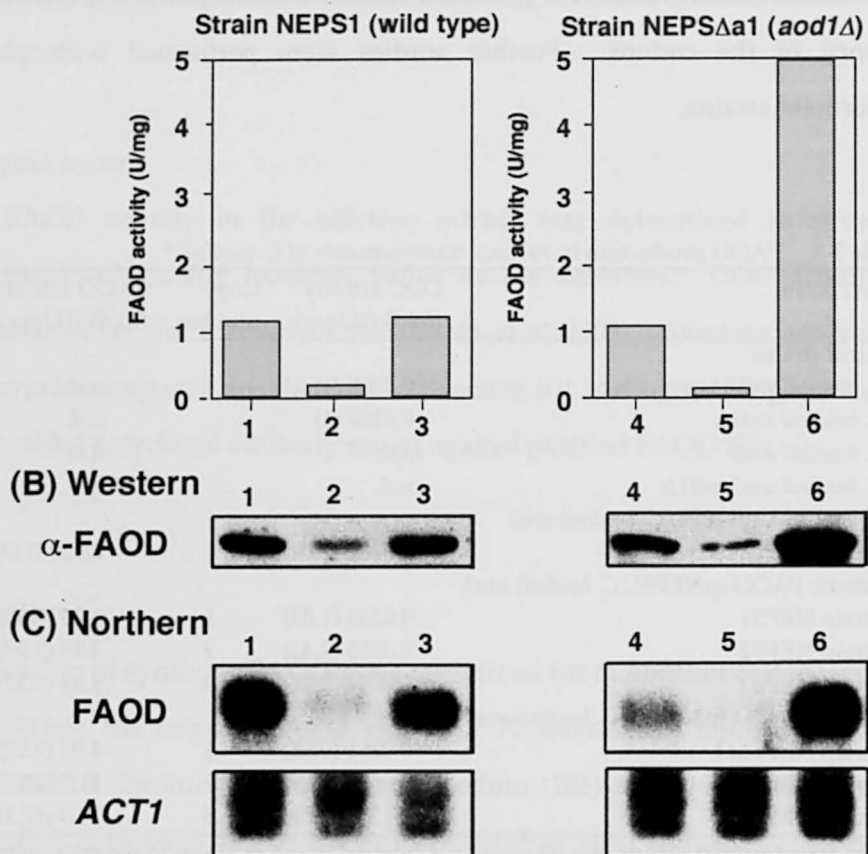
α-FAOD

(C) Northern

FAOD

ACT1

Fig. 3-6. Expression analysis of the synthesized FAOD gene under the *AOD1* promoter in one-copy integrants on various media. A-C: Lanes 1-3, strain NEPS1 (wild type), and lanes 4-6, strain NEPSΔa1 (*aod1Δ*). Cells were harvested at the early logarithmic phase (36 h). A: FAOD activity. B: Western analysis using anti-FAOD anti-body. About 15 μg of protein was loaded on each lane. C: Northern analysis using the ^{32}P-labeled, 1.3-kb synthesized FAOD fragment (upper), or ^{32}P-labeled, 0.9-kb *C. boidinii ACT1* (lower). About 3 μg of total RNA was loaded on each lane. Each lane number indicates the used carbon and nitrogen sources in the medium: lanes 1 and 4, methanol plus glycerol/NH_4Cl; lanes 2 and 5, glycerol/NH_4Cl; lanes 3 and 6, methanol plus glycerol/methylamine.

expressed by one-copy pNEPS1 transformants of *C. boidinii* strain *aod1Δ* (strain NEPSΔa1), were compared. Since strain NEPSΔa1 could not grow on methanol as the sole carbon source, glycerol was added to the media of both transformant strains (methanol+glycerol/NH_4Cl). Although the author expected the enzyme activity in strain NEPSΔa1 to be higher than that in strain NEPS1, the level of FAOD activity and the quantity of FAOD protein detected on immunoblot analysis were the same in both strains (Fig. 3-6A, lanes 1 and 4 (FAOD activity); Fig. 3-6B, lanes 1 and 4 (immunoblot)). Unexpectedly, Northern analysis of these transformant strains revealed a smaller amount of FAOD mRNA in strain NEPSΔa1 than in strain NEPS1 (Fig. 3-6C, lanes 1 and 4), suggesting imperfect activation of the *AOD1* promoter in strain NEPSΔa1.

These results can be explained by the fact that the *AOD1* promoter is induced not only by methanol but also by formaldehyde (Chapter II, Section 2), since methanol is not oxidized to formaldehyde in strain *aod1Δ*. However, addition of formaldehyde is not practical for industrial-scale production due to its extreme toxicity and the technical difficulties in the addition itself. To overcome this problem, we changed the nitrogen source from NH_4Cl to methylamine. In *C. boidinii*, methylamine is metabolized to formaldehyde and then oxidized to formate (69). Therefore, formaldehyde can be supplied continuously *in vivo*. The level of FAOD expression was 4.1-fold higher and acid phosphatase activity was 4.3-fold higher in the *aod1Δ* strains grown in media which contained methylamine as the nitrogen source instead of NH_4Cl (Fig. 3-6A, lanes 4 and 6); the mRNA level and protein level of FAOD was also comparably higher in the *aod1Δ* strains grown in media which contained methylamine (Figs. 3-6B and 3-6C, lanes 4 and 6). In contrast, the level of expression of FAOD did not change in the *C. boidinii* wild type strain upon changing the nitrogen source from NH_4Cl to methylamine (Fig. 3-6A-C, lanes 1

and 3). Therefore, FAOD productivity in the *aod1Δ* strain was 4.1-4.9-fold higher than that in the wild type strain under optimized cultivation conditions (Fig. 3-6A and Table 3-4).

Production of FAOD in multi-copy transformants

Multicopy integrants of pNEPS1 were derived from the wild type and from the *aod1Δ* strain. The level of FAOD expressed by these transformants on a glycerol+methanol/methylamine medium was compared (Table 3-4). The level of FAOD expression was nearly proportional to the copy number of pNEPS1 in both the wild type strain and the *aod1Δ* strain. Transformants with higher copy numbers had larger quantities of FAOD mRNA (data not shown). Therefore, it is suggested that the increase in FAOD expression is due to an increase in the quantity of the mRNA which encodes FAOD. Strain NEPSΔa5, the five-copy integrant of pNEPS in strain *aod1Δ*, was selected as the best producer, which produced FAOD at a level as high as 18% of the total soluble protein. Western analysis did not show any protein aggregates due to improper folding of FAOD (data not shown). FAOD was purified from a 500-ml culture through two-step column chromatography (butyl-Toyopearl and Sepharose Q), yielding 75% recovery (data not shown).

Peroxisomal localization of produced FAOD

Although the NEPSΔa5 strain produced a large amount of FAOD, the author anticipated that all of the produced FAOD would not be transported into the peroxisomes due to overproduction. To study this issue, the author analyzed localization of FAOD in *C. boidinii* strain NEPS1 and strain NEPSΔa5 through subcellular fractionation experiments. After osmotic lysis of the FAOD-producing strain and removal of cell debris and nuclei, the sample was

centrifuged at 20 000 x g for 20 min. The organellar pellet fraction, which contained mainly peroxisomes and mitochondria, was fractionated by sucrose-gradient ultracentrifugation. Since yeast peroxisomes are fragile, only a portion of catalase and FAOD corresponding to approximately 65-75% of catalase and FAOD activity was recovered in the pellet fraction of each strain. Fractionation experiments on each strain indicated that the produced FAOD co-migrated with the catalase fraction but showed a peak distinguishable from that of the mitochondrial marker enzyme, cytochrome c oxidase (Fig. 3-7A, B). The peroxisomal peak fraction was found in lighter fractions of strain NEPSΔa5 than strain NEPS1. This may be due to depletion of the peroxisomal core enzyme, alcohol oxidase. By immuno-electron microscopy, immuno-gold labeling was observed only in peroxisomes (data not shown). Therefore, the author concluded that FAOD produced in C. *boidinii* is transported efficiently to the peroxisome.

(A) Strain NEPS1 (wild type)   **(B) Strain NEPSΔa5 (*aod1Δ*)**
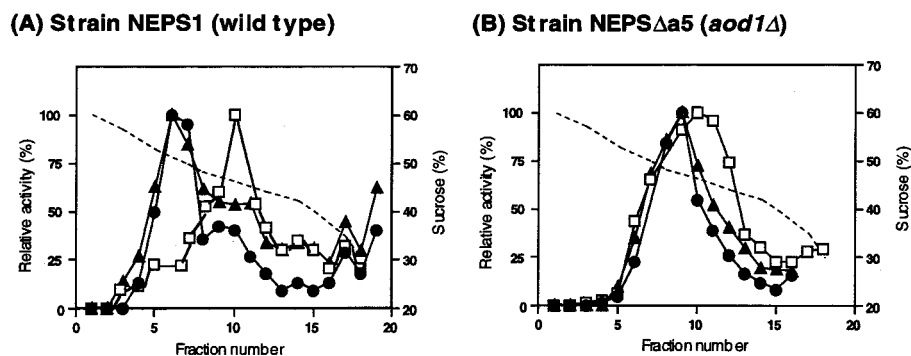

Fig. 3-7. Localization of the produced FAOD. Results of the subcellular fractionation experiments on (A) strain NEPS1 and (B) strain NEPSΔa5. Catalase and cytochrome c oxidase are the peroxisomal and mitochondrial marker enzymes, respectively. ●, FAOD; ▲, catalase; □, cytochrome c oxidase. Dotted line, sucrose concentration.

Discussion

Many successful examples of heterologous protein production in methylotrophic yeast have been reported (9, 64, 72, 76). Other investigators have demonstrated intraperoxisomal production of peroxisomal and non-peroxisomal model proteins in yeast cells to avoid toxicity or to stabilize the produced proteins (18, 32, 57). However, some problems that have been encountered include: i) the expressed protein was not fully targeted to peroxisomes, ii) the protein was not properly folded, and iii) the quantity of expressed protein was low (18, 57). Using *C. boidinii* strain *aod1Δ*, which contains no alcohol oxidase, we have overcome these problems and have shown the first example of high-level intraperoxisomal production of a commercially important protein. The advantages of using *C. boidinii* are: i) an efficient gene expression system was established, coupled with robust peroxisome proliferation. Indeed, intraperoxisomal production caused the increase in FAOD productivity. When three amino acids at the carboxyl-terminus representing peroxisome targeting signal type 1 of FAOD were deleted, the FAOD productivity was decreased to one fourth (data not shown); ii) alcohol oxidase is encoded by a single gene in *C. boidinii* ; iii) glycerol does not repress methanol induction of the *AOD1* promoter and supported the growth of strain *aod1Δ*; iv) the peroxins and FAD originally used for alcohol oxidase would be available for transport and activation of heterologous oxidase to the peroxisomes.

Another outcome of this study besides the establishment of a new expression system was the achievement of an economical means of producing FAOD. Expression of the active form of FAOD in *C. boidinii* reached as high as 18% of the total soluble protein, which exceeds the expression level in *E. coli* (maximum 0.2%) (101). Since the purification yield was also high (75%),

a 3-day 500-ml culture enabled us to produce and purify 2290 U of FAOD, which was sufficient for assaying approximately 14000 patient samples for determination of the content of glycated proteins (1 ml of assay volume). Although many kinds of oxidases and 'assay kits' are currently commercially available, the cost of production has often been the economical limiting factor to large-scale industrial production, as is the case for FAOD. The production system and strategy taken here will not be limited to the production of FAOD from *P. janthinellum*. The same system was also efficient for the production of FAOD from *Aspergillus terreus* (data not shown) (101). The next interest is to produce non-peroxisomal oxidases in the peroxisomes of *C. boidinii*.

Summary

A high-level production of fructosyl amino acid oxidase (FAOD), whose production was toxic in *E. coli*, was investigated through attempts to utilize the peroxisome of *C. boidinii* as the place for protein accumulation. The alcohol oxidase-depleted strain (strain *aod1Δ*) produced FAOD at a four to five times higher level than the wild type strain in terms of protein amount and enzyme activity, although the transcriptional level was similar. As a result of this study, the author could improve FAOD productivity approximately 47-fold from the original transformant, and FAOD accumulated within membrane-bound peroxisomes up to 18% of the total soluble proteins.

Conclusion

This thesis concerns the gene regulation and its applied aspects of peroxisomal enzymes in the methylotrophic yeast *Candida boidinii*.

At first, the regulation of the expression of peroxisomal proteins and peroxisome proliferation by carbon and nitrogen sources were studied (Chapter I). Three peroxisome-inducing carbon sources, *i.e.*, methanol, oleate, and D-alanine, induces respective and specific peroxisomal proteins concomitant with peroxisome proliferation. As for the sensitivity of the peroxisomal induction to glucose repression, the D-alanine-induced activities of D-amino acid oxidase (DAO) and peroxisome proliferation are not repressed by glucose, while the peroxisomal induction by methanol or oleate is completely repressed. Since D-alanine is used not only as a carbon source but also as a nitrogen source, the expression of DAO seems to be distinct from other peroxisomal oxidases. Next, the author cloned and disrupted the gene encoding DAO to elucidate the physiological role of this enzyme. The *dao1*Δ strain did not grow on D-alanine as a carbon source, but did on D-alanine as a sole nitrogen source. These results suggested that DAO is essential for growth on D-alanine as a carbon source and that there should be another enzyme involved in utilizing D-alanine as a nitrogen source.

The author isolated the promoter regions of five methanol-inducible genes from *C. boidinii*, and evaluated their strength and studied their regulation (Chapter II). The promoter of dihydroxyacetone synthase gene (*PDAS1*) is the strongest promoter whose strength is 1.5 times higher than that of alcohol oxidase gene promoter (*PAOD1*). The results of induction kinetics of *PAOD1* and *PDAS1* at the early stage of methanol-induction suggest that the activation of *PDAS1* precedes that of *PAOD1*. In the *aod1*Δ*das1*Δ strain, while both promoters

are activated by methanol and formaldehyde, *PAOD1* is also activated by some anabolites, such as dihydroxyacetone or glyceraldehyde. From these results, the author proposed a new metabolic control system that *PAOD1* is regulated by the unique transcriptional regulation, "anabolite induction", coupled with the flow of C_1-metabolism in order to avoid the accumulation of formaldehyde which is toxic to the cell.

Finally, the author established a novel high-level production system for useful oxidases, which are packaged exclusively in peroxisomes of the *aod1*Δ strain of *C. boidinii* (Chapter III). As an example of endogeneous oxidase, DAO was produced up to 30% of the total soluble proteins, and as an example of heterologous oxidase, fungal fructosyl amino acid oxidase useful for diabetic diagnosis, up to 18% of the total soluble proteins. Through these results, the author showed that peroxisomes of the *aod1*Δ strain are the appropriate place for the high-level production of useful oxidases.

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Acknowledgment

Publications

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