YEAST
Yeast **15**, 1223–1230 (1999)

Alcohol Oxidase Hybrid Oligomers Formed *In Vivo* and *In Vitro*

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Cell-free extract prepared from methanol-grown cells of the methylotrophic yeast *Pichia methanolica* showed nine multiple alcohol oxidase (AOD) bands on active staining in native polyacrylamide gel electrophoresis. Their molecular basis was investigated and two AOD-encoding genes, *MOD1* and *MOD2*, were cloned from *P. methanolica* genome. When the two genes were expressed in a heterologous host, an alcohol oxidase-depleted strain of *Candida boidinii* (*aod1Δ* strain), both *MOD1* and *MOD2* partially complemented growth defect of the host strain on methanol. While expression of either *MOD1* or *MOD2* in *C. boidinii aod1Δ* strain gave a single AOD band corresponding to the band with the largest and smallest mobility among the nine AOD bands, respectively, co-expression of *MOD1* and *MOD2* resulted in multiple band formation. Mixed oligomerization of Mod1p and Mod2p *in vitro* also gave nine multiple bands. From these results, we concluded that the nine multiple forms of AOD observed on native–PAGE represent two homo-octamers and seven hetero-octamers of Mod1p and Mod2p. Using this zymogram analysis, we also found that Mod1p was preferably produced at low methanol concentrations in the media, while Mod2p was produced at higher methanol concentrations. This shows distinct regulatory features of the two AOD-encoding genes in this methylotrophic yeast. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS — methylotrophic yeast; *Pichia methanolica*; isozyme; *Candida boidinii*; alcohol oxidase; methanol metabolism

INTRODUCTION

The methylotrophic yeast has been studied for years because of its unique ability to grow on methanol and to express heterologous genes under strong methanol-inducible promoters (Cregg, 1993). The first reaction in the utilization of methanol is the oxidation of methanol to formaldehyde, catalysed by alcohol oxidase (AOD; EC1.1.3.13). AOD is a homooctameric flavoprotein consisting of eight subunits of ca. 70 kDa, each containing flavin adenine dinucleotide molecule (FAD) as a prosthetic group (Kato *et al.*, 1976).

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Contract/grant sponsor: Ministry of Education, Science, Sports and Culture, Japan.

AOD-encoding genes, together with their promoter regions, have been cloned and characterized from *Hansenula polymorpha* (Ledeboer *et al.*, 1985), *Pichia pastoris* (Koutz *et al.*, 1989), *C. boidinii* (Sakai and Tani, 1992b) and *Pichia methanolica* (Raymond *et al.*, 1998). The primary structures of these AOD amino acid sequences show 73–85% similarity. Among these methylotrophic yeast strains, *P. pastoris* and *P. methanolica* possess two sets of genes for AOD (*AOX1* and *AOX2* for *P. pastoris*, and *AUG1* and *AUG2* for *P. methanolica*). In both *Pichia* strains, the genes *AOX2* and *AUG2* have been reported to be expressed at significantly lower levels than *AOX1* and *AUG1* (Cregg *et al.*, 1989; Raymond *et al.*, 1998). Furthermore, disruption of *AOX1* and *AUG1* caused a severe defect in the growth of the disruptant strains on methanol, while the growth of gene disruptants of *AOX2* and *AUG2* was comparable to that of the wild-type strain.

CCC 0749–503X/99/121223–08$17.50
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Received 8 March 1999
Accepted 15 April 1999

Therefore, the physiological role of *AOX2* and *AUG2* remained unclear.

Nakagawa *et al.* (1996) and Gruzman *et al.* (1996) independently observed nine multiple forms of AOD in *P. methanolica* on a zymogram of native–polyacrylamide gel electrophoresis (native–PAGE). The AODs having the smallest and the largest mobility among the nine ladder bands were named MOD-A and MOD-B, respectively, and each was purified and characterized. MOD-A and MOD-B were clearly different from each other in enzymological characteristics, e.g. the K_m value for methanol (0·56 mM for MOD-A and 5·6 mM for MOD-B), the substrate specificity, and the content of modified form of FAD.

So far, two hypotheses have been framed for the molecular mechanism of the formation of multiple AOD bands (AOD-ladder) on the zymogram. The first hypothesis, by Gruzmann *et al.* (1996), was that nine forms of AODs originated from a single AOD-gene product and that the bands represented each isozyme that was modified after translation. The second hypothesis, postulated by Nakagawa *et al.* (1996) was that the nine AOD-ladder bands represent a mixture of two gene products. The present study was designed to reveal the molecular basis for the formation of the nine AOD-ladder bands of *P. methanolica* AOD and to reveal the physiological importance of the second AOD-encoding gene (*AUG2*) in this organism.

MATERIALS AND METHODS

Yeast and bacterial strains, media and cultivation

P. methanolica IAM 12901 was the source for MOD-A, MOD-B and chromosomal DNA. Transformation of the *C. boidinii aod1*Δ-U strain constructed in this study was performed by the modified lithium acetate method, as described previously (Sakai *et al.*, 1993).

Yeast strains were cultivated in YPD medium or in a basal M1 medium (Sakai *et al.*, 1995) containing 0·12% yeast extract. The concentration of the carbon source was either 1·0% glucose or 2·0% methanol (v/v). The initial pH of the media was adjusted to 6·0. Cultivation was aerobic at 28°C with shaking, and growth was followed by measuring the optical density at 610 nm (OD_{610}).

Escherichia coli JM109 (Sambrook *et al.*, 1989) used for plasmid propagation and for construction of the *P. methanolica* genomic library was grown at 37°C in 2 × YT medium, when necessary supplemented with ampicilin (50 µg/ml).

Purification and determination of N-terminal amino acid sequences of MOD-A and MOD-B

MOD-A and MOD-B were each purified from the cell-free extract of *P. methanolica* IAM 12901 grown on methanol, as previously described (Nakagawa *et al.*, 1996). The N-terminal amino acid sequence analysis of each peptide was performed with a gas-phase sequencer, Shimadzu PSQ-2.

Electrophoresis and assay of alcohol oxidase

A cell pellet of methanol-grown *P. methanolica* was suspended in five-fold volume of 50 mM potassium phosphate buffer (pH 7·5), and then transferred to a 2 ml Eppendorf tube containing an equal volume of 0·5 mm zirconium beads. The tube was shaken vigorously for 30 s on a 3110BX mini-beadbeater (Biospec Products) and then chilled on ice for 30 s. This procedure was repeated six times, and then the cell debris was removed by centrifugation at 16 000 × *g* for 5 min at 4°C. The resultant supernatant cell-free extract was immediately subjected to enzyme activity assay and electrophoresis.

For zymogram analysis, 5–20 µl of the cell-free extract was subjected to non-denatured 5% polyacrylamide gel electrophoresis (native–PAGE) at 4°C by the method of Laemmli (1970). After electrophoresis, the polyacrylamide gel was stained by oxidation of guiyacol (Lee and Komagata, 1980).

Alcohol oxidase activity was determined by the ABTS/POD method, as described by Tani *et al.* (1985).

Dissociation and reassembly of Mod1p and Mod2p in vitro

In vitro dissociation and re-assembly of Mod1p and Mod2p were performed as described by Evers *et al.* (1995), with minor modifications. After concentration of the cell-free extract (0·5 U/ml AOD activity) by precipitation under 55% ammonium sulphate saturation, glycerol was added to a concentration of 80%, and then Mod1p and Mod2p were mixed at appropriate activity ratios, and each mixture was 10-fold diluted with 50 mM potassium phosphate buffer. After incubation on ice overnight, samples were subjected to native–PAGE, and the zymogram of AOD was analysed.

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DNA methods

P. methanolica genomic DNA was purified by the method of Cryer *et al.* (1975) or Davis *et al.* (1980). Southern blot analysis was performed as described previously (Sakai *et al.*, 1991). PCR amplification was performed in a Perkin-Elmer thermal cycler 480 using Ex. Taq polymerase (Takara co. Ltd, Kyoto, Japan).

The primers used for PCR amplification were as follows: MODAFp, 5′-GCGGCCGCAAAATG GCTATTCCAGATGAATT-3′; MODARp, 5′-GCGGCCGCAAAATATCCAAACAGGCAGC C-3′; MODBFp, 5′-GCGGCCGCAAAATGGCT ATTCCTGAAGAATT-3′; MODBRp, 5′-GCGG CCGCTTAGAATCTACCTAATCCAGTAG-3′; AOD1Tp, 5′-CCACTAGTGCCAGTGCCAAGC TTAATTAAT-3′; AOD1Pp, 5′-GGACTAGTAA CAGCTATGACCATGATTACG-3′. These were designed based on DNA sequences obtained in this study or according to information reported by Raymond *et al.* (1998).

DNA sequences of the cloned fragments were determined by using a PRISM Dye Deoxy Terminator Cycle Sequencing Kit and a 373A DNA sequencer (Applied Biosystems), as described previously (Sakai *et al.*, 1997) or a Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Thermo sequenase™) and DSQ-2000 DNA sequencer (Shimadzu Co., Ltd).

Cloning of the two AOD genes, MOD1 *and* MOD2

In a previous study, we obtained two species of 308 bp PCR products, Probe 1 and Probe 2, coding for partial amino acid sequences for *MOD1* and *MOD2*, respectively (Nakagawa *et al.*, 1996). The two probes showed different patterns on Southern analysis using genomic DNAs digested by several restriction enzymes (data not shown). Probe 1 hybridized specifically to a band size of 5·4 kb in *Bgl*II-digested genomic DNA. On the other hand, Probe 2 hybridized specifically to a band size of 6·2 kb when genomic DNA was digested with *Cla*I. The DNAs approximately corresponding to these hybridizing bands were each gel-purified and ligated into the *Bam*HI or the *Cla*I site of pBluescript II SK$^+$, respectively, for Probes 1 and 2.

Transformants for semi-library were transferred onto a Biodyne nylon membrane (Pall Bio Support, New York). After lysis of the bacteria and binding of the liberated DNA to the nylon membrane, the blot was used for colony hybridization under high-stringency conditions using Church buffer (1% bovine serum albumin, 1 mM EDTA, 0·25 M NaCl, 0·25 M $NaPO_4$, pH 7·2, 7% SDS) (Church and Gilbert, 1984). Hybridization was performed overnight at 65°C, and then the membranes were washed three times in 0·3 × SSC (1 × SSC is 0·15 M NaCl plus 0·015 M sodium citrate) at the same temperature. The positive clones that showed strong signals were found to harbour a reactive 5·4 kb *Bgl*II fragment and 6·2 kb *Cla*I fragment, for Probes 1 and 2, respectively. Although the cloned 6·2 kb *Cla*I fragment harboured a complete ORF for *MOD2*, the 5·4 kb *Bgl*II fragment missed some part of 3′-flanking sequence of *MOD1*. A full-size clone for the *MOD1*-coding region was obtained by PCR amplification from genomic DNA using the primers MODAFp and MODARp.

Expression vectors

The expression vector pNoteI (Sakai *et al.*, 1996) was used for the expression of *MOD1* and *MOD2* in the *C. boidinii aod1Δ* strain. *MOD1*- and *MOD2*-coding regions were each PCR amplified, using either primers MODAFp and MODARp or primers MODBFp and MODBRp, respectively. Each fragment was introduced into the *Not*I site of pNoteI under the *AOD1* promoter. The expression vectors for *MOD1* and *MOD2* were named pMOD1 and pMOD2, respectively. pMOD12 was constructed by ligating the two expression cassettes from pMOD1 and pMOD2, in tandem, to co-express *MOD1* and *MOD2* under the *AOD1* promoter.

All three vectors, pMOD1, pMOD2 and pMOD12 were linearized by *Bam*HI, and used for transformation of the *C. boidinii aod1Δ*-U strain.

One-step gene disruption of the C. boidinii AOD1 *gene*

The 3·2 kb *Xba*I–*Hin*dIII fragment derived from pMOX620 (Sakai and Tani, 1992a) (harbouring the entire coding sequence of *AOD1*) was gel-purified and then introduced into *Xba*I–*Hin*dIII digested pBluescript II SK$^+$. The obtained plasmid was digested with *Sty*I to remove the ca. 1·5 kb fragment including most of the coding sequence of *AOD1*. The remaining linearized plasmid and the 4·6 kb *Sac*I–*Xho*I fragment derived from pSPR, which had the *C. boidinii URA3* gene (*CbURA3*)

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with repeated flanking sequences (Sakai and Tani, 1992b), were gel-purified, blunt-ended with T4 polymerase, and then subjected to ligation. The resulting plasmid was digested with *Sac*I–*Xho*I and used to transform *C. boidinii* TK62 to uracil prototrophy (strain *aod1Δ*). Since the integrated plasmid had tandem repeated sequences, the *CbURA3* gene was expected to pop out from the chromosome by site-specific recombination at these repeated sequences at high frequency (Alani *et al.*, 1987). Strain *aod1Δ*-U (*aod1Δ*, *ura3*) was isolated as a resistant colony to 5-fluoro-orotic acid, as described previously (Sakai and Tani, 1992b). The disruption of *AOD1* and popping out of the *CbURA3* were confirmed by genomic Southern analysis of *Eco*RI- and *Hin*dIII-digested DNA from transformants by using the 550 bp PCR-amplified fragment harbouring the *AOD1* terminator region.

Nucleotide sequence Accession No.

The nucleotide sequences of *MOD1* and *MOD2* have been submitted to GenBank and assigned Accession Nos AF141329 and AF141330, respectively.

RESULTS

Cloning of MOD1 *and* MOD2 *coding for alcohol oxidase in* P. methanolica

Nakagawa *et al.* (1996) observed an AOD-ladder formation on native PAGE of the cell-free extract of methanol-grown *P. methanolica* cells. At first, MOD-A and MOD-B, showing the smallest and largest mobility on the zymogram, were each purified to homogeneity showing single bands on SDS–PAGE. The N-terminal amino acid sequences of MOD-A and MOD-B were determined to be AIPDEFDIIVVGGGSTGXALA GRL and AIPEEFDIIVVGGGSAGXPTAGRL, respectively (undetermined amino acid residue X was deduced to be cystein by DNA-sequencing analysis). The two N-terminal amino acid sequences were different at the positions underlined. This showed that two different subunits of AOD were indeed produced in *P. methanolica*.

While our cloning experiments were in progress, Raymond et al. (1998) reported two AOD genes from *P. methanolica*, i.e. a complete nucleotide sequence of *AUG1* and a partial nucleotide sequence of *AUG2*. Independently, we cloned two complete AOD-coding genes, *MOD1* and *MOD2*, from the chromosomal DNA of *P. methanolica* (as described in Materials and Methods), and determined their nucleotide sequences. The deduced amino acid sequences of the cloned genes revealed that the product of *MOD1* (=*AUG1*) and *MOD2* (=*AUG2*) was identical to MOD-A and MOD-B, respectively. The *MOD2* gene coded for a protein of 664 amino acid residues which was one amino acid shorter than Mod1p. The amino acid sequences of Mod1p and Mod2p, because of their homologous origin, share a moderate level of similarity to each other, 85%. In the case of *P. pastoris*, the identity of the amino acid sequences between Aox1p and Aox2p was much higher, 99%.

Disruption of the C. boidinii AOD1 *gene causes a loss of ability to grow on methanol*

To characterize the products of the *MOD1* and *MOD2* genes, we had to establish an expression system for the two AOD genes. Since the transformation system for *P. methanolica* was developed for commercial use and was not available to us, we decided to use *C. boidinii* as the expression host. First, the gene disruptant of *C. boidinii AOD1*, strain *aod1Δ* (*aod1*, *URA3*), was obtained, and the *ura3* marker was regenerated in strain *aod1Δ*-U (*aod1*, *ura3*) (Figure 1A). Southern analysis with genomic DNA from each transformant confirmed the proper gene disruption of the *AOD1* gene and a subsequent loss of the *Cb URA3* gene (Figure 1B).

The wild-type and strain *aod1Δ* of *C. boidinii* were compared for their ability to grow on various carbon sources. The growth of strain *aod1Δ* on glucose, glycerol, oleate or D-alanine as a sole carbon source, was comparable to that of the wild-type strain (data not shown). In contrast, strain *aod1Δ* could not grow on methanol (Figure 2A). Consequently, it was confirmed that *C. boidinii* contains only one gene coding for AOD and that *AOD1* had an indispensable role in methanol utilization.

P. methanolica MOD1 *and* MOD2 *genes could partially complement methylotrophic growth of* C. boidinii *strain* aod1Δ

The coding region of each of *MOD1* and *MOD2* was PCR-amplified and placed into the *Not*I site of pNoteI under the *AOD1*-promoter. Each of the obtained plasmids, pMOD1 (for expression of *MOD1*) and pMOD2 (for expression of *MOD2*),

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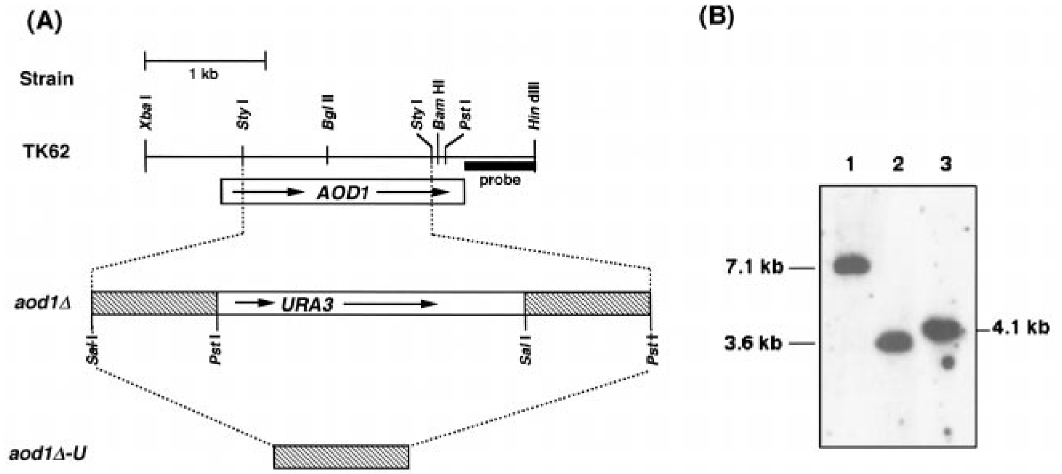

Figure 1. One-step disruption of the *C. boidinii AOD1* gene. (A) Restriction map of the cloned fragment and disruption strategy. The shaded boxes at both ends of *URA3* represent repeated sequences for homologous recombination to remove the *URA3* gene after gene disruption. Arrows indicate coding regions. (B) Genomic Southern analysis of *Eco*RI-digested total DNAs (3 μg of each) from: lane 1, *aod1Δ* strain; lane 2, *aod1Δ*-U strain; and lane 3, the host strain TK62, probed with the ^{32}P-labelled 550-bp PCR-amplified fragment of 3′ flanking region of *AOD1*.

was introduced into *C. boidinii* strain *aod1Δ*-U. Each transformant was then grown on methanol. As shown in Figure 2A, *C. boidinii* strain *aod1Δ* harbouring pMOD1 or pMOD2 grew slowly on methanol, while strain *aod1Δ* did not. The growth rates of the two transformants were essentially similar. These results indicated that both of the cloned genes *MOD1* and *MOD2* were at least functional in a heterologous host, *C. boidinii*. The level of AOD enzyme activity was about eight-fold higher in a homologous *C. boidinii AOD1* transformant (0·92 U/mg protein) than in *P. methanolica MOD1* and *MOD2* transformants (0·12 and 0·11 U/mg protein, respectively). Inefficient expression of *P. methanolica MOD1* or *MOD2* genes in *C. boidinii*, which may originate from the differences in codon usage between these organisms, is considered to cause the partial complementation of *C. boidinii aod1Δ* by these genes.

Next, we asked whether a single gene product from either *MOD1* or *MOD2* could give AOD-ladder bands on a zymogram. Cell-free extract was prepared from the pMOD1 or pMOD2 transformants of *C. boidinii* strain *aod1Δ*-U and subjected to zymogram analysis. As shown in Figure 2B (lane 2), the pMOD1 transformant gave a single band corresponding to the size of MOD-A. Similarly, the pMOD2-transformant gave a single band corresponding to the size of MOD-B (Figure 2B, lane 3).

AOD hybrid octamers observed in vitro *and* in vivo

The results obtained supported the hypothesis that AOD-ladder bands on a zymogram were due to hybrid oligomers of two gene products, Mod1p and Mod2p (Nakagawa *et al.*, 1996). On the other hand, Evers *et al.* (1995) reported that AOD from *H. polymorpha* and AOD from *P. pastoris*, once dissociated into monomers, could be reassembled into active forms of heteromeric mixtures, resulting in nine ladder bands on native–PAGE. Based on these observations, we then asked whether the mixture of Mod1p and Mod2p *in vitro* could give nine ladder bands on a zymogram. Mod1p and Mod2p was each produced in the corresponding *C. boidinii aod1Δ* transformant, and partially purified by ammonium sulphate precipitation. After dissociation of subunits in 80% glycerol solution, Mod1p and Mod2p were mixed in various activity ratios, reassembled and subjected to zymogram analysis (Figure 3).

As a result, a ladder band of nine species appeared in the mixture of Mod1p and Mod2p subunits, whose profile coincided with the profile observed in methanol-grown *P. methanolica* cells. Furthermore, as the relative amount of Mod2p to Mod1p was increased, AODs of larger mobility became dominant. Therefore, the band profile reflected the ratio of subunit species.

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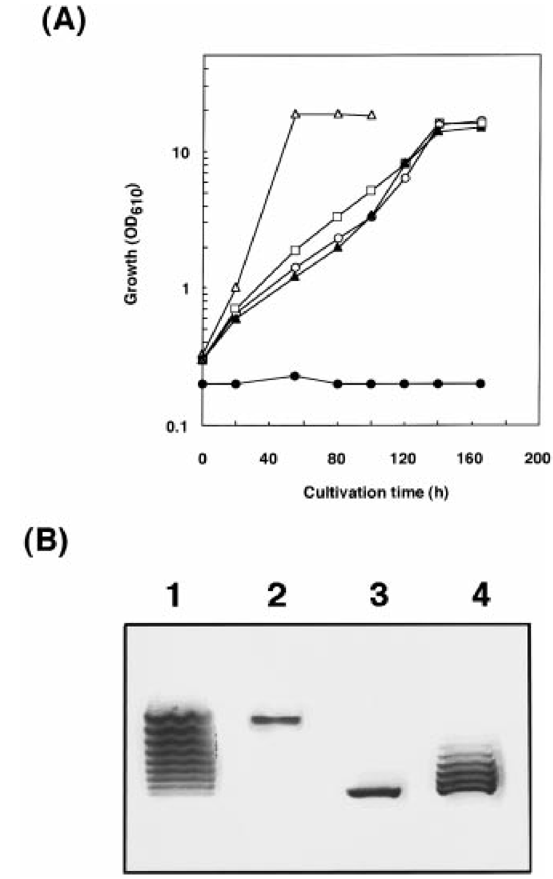

Figure 2. (A) Growth of *C. boidinii* strains on 2% methanol as a sole carbon source. Symbols: △, wild-type strain; ●, *aod1Δ* strain; ○, pMOD1 transformant; □, pMOD2 transformant; ▲, pMOD12 transformant; OD_{610}, optical density at 610 nm. (B) Zymogram of *P. methanolica* MODs. Cell-free extracts from: lane 1, methanol-grown *P. methanolica*; lane 2, pMOD1 transformant; lane 3, pMOD2 transformant; lane 4, pMOD12 transformant. Each sample (20 µl) contained 1·5 mU AOD activity.

Next, we tried to reconstitute the AOD-ladder bands *in vivo*. Both *MOD1* and *MOD2* genes were placed under the *AOD1* promoter, and the resultant plasmid pMOD12 was introduced into *C. boidinii* strain *aod1Δ*-U. The pMOD12-transformant showed AOD-ladder bands (Figure 2B, lane 4), and co-expression of *MOD1* and *MOD2* complemented the growth of *C. boidinii* strain *aod1Δ*, comparable to the growth of pMOD1- or pMOD2-transformant (Figure 2A).

Experiments both *in vitro* and *in vivo* showed AOD-ladder formation only under conditions in which heteromeric assembly of Mod1p and Mod2p was possible. Since AOD is composed of eight subunits, the nine bands most likely represent two homo-oligomers and seven hybrid octamers.

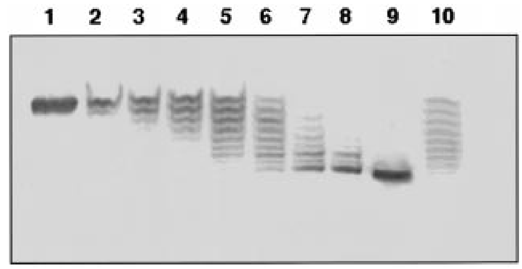

Figure 3. *In vitro* formation of AOD-ladder bands by mixing partially purified Mod1p and Mod2p. Each cell-free extract contained 12 mU AOD in total. The activity ratios between Mod1p and Mod2p (Mod1p:Mod2p) were: lane 1, 1:0; lane 2, 16:1; lane 3, 4:1; lane 4, 2:1; lane 5, 1:1; lane 6, 1:2; lane 7, 1:4; lane 8, 1:16; and lane 9, 0:1. Lane 10. Cell-free extracts were from methanol-grown cells of *P. methanolica*.

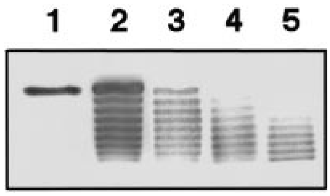

Figure 4. The ratio of Mod2p to Mod1p increased with the methanol concentration in the induction media. Cell-free extract (containing 10 mU AOD) was applied to each lane. Lane 1, no carbon source; lane 2, 1% methanol; lane 3, 2% methanol; lane 4, 3% methanol; lane 5, 4% methanol.

The levels of Mod1p and Mod2p are regulated differently

Based on the data presented in Figure 3, we were easily able to estimate the AOD subunit composition in the cell-free extract from zymogram analysis. From our previous and present results, AOD-ladder formation was only detected in methanol-grown *P. methanolica* cells (Nakagawa *et al.*, 1996). To obtain a regulatory insight of the two species of AOD subunit Mod1p and Mod2p in *P. methanolica*, glucose-grown cells were transferred to media containing different concentrations of methanol (0–4%), induced for 36 h, and the cell-free extract was prepared and subjected to zymogram analysis (Figure 4). When the cells were grown in the basal medium without an additional carbon source, Mod1p was the only AOD subunit produced (Figure 4, lane 1). By contrast, the ratio of Mod2p to Mod1p was found to increase with the increase of initial methanol concentration

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(Figure 4, lanes 2–5). The results obtained show that *MOD1* was induced at lower methanol concentrations, or could be induced even without the addition of methanol, whereas *MOD2* was induced at higher methanol concentrations in the media.

DISCUSSION

In this study, we revealed the molecular basis for the observation of nine AOD-ladder bands in the cell-free extract of methanol-grown *P. methanolica* cells. Although Gruzman *et al.* (1996) suggested that multiple forms of AOD appeared by modification after translation of one gene, we conclude from the present study that AOD-ladder formation is due to an oligomeric mixture of two gene products, Mod1p and Mod2p.

Since AOD is the first enzyme involved in methanol metabolism and the reaction it catalyses produces two toxic compounds, formaldehyde and H_2O_2, the level of AOD must be strictly regulated. Using zymogram analysis, we found that *MOD1* and *MOD2* are regulated differently during growth on methanol. *MOD2* gene expression required methanol as an inducer, while *MOD1* did not. Also, the expression ratio of *MOD1* to *MOD2* increased as methanol concentration in the medium decreased. A homo-octamer of Mod1p, MOD-A, had ca. 10-fold affinity against methanol (K_m value=0·56 mM) than a homo-octamer of Mod2p, MOD-B (K_m value=5·6 mM) (Nakagawa *et al.*, 1996), and heteromeric octamer had an intermediate affinity to methanol (Gruzman *et al.*, 1996). During the first stage of methylotrophic growth, Mod2p with low methanol affinity may be the major subunit for AOD. With a decrease in methanol concentration coupled with cell growth, Mod1p with high affinity would in turn become dominant. A zymogram change observed during methylotrophic growth (Gruzman *et al.*, 1996) fits well with this scheme.

Our results indicated that AOD activity in *P. methanolica* is not regulated simply by the total quantity of two species of AOD subunits, Mod1p and Mod2p. The existence of two AOD subunits of different affinity to methanol enables cells a qualitative regulation of AOD through coordinated and harmonized expression of two different genes, *MOD1* and *MOD2*, making adaptation to the concomitantly changing environmental conditions possible.

ACKNOWLEDGEMENTS

We are grateful to Professor K. Komagata and Dr T. Uchimura, Tokyo University of Agriculture, for permitting us to continue their work on *P. methanolica* AODs. This work was partly supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan, to Y.S.

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