Molecular Basis of Methanol-Inducible Gene Expression and Its Application in the Methylotrophic Yeast *Candida boidinii*

Hiroya YURIMOTO

*Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan*

Online Publication, April 7, 2009

**Key words:** methylotrophic yeast; methanol metabolism; methanol-inducible gene; gene regulation; heterologous gene expression

Methanol is a promising feedstock for biotechnological and chemical processes as well as a primary source of energy to replace coal and petroleum. Methylotrophic yeasts, that can utilize methanol as the sole source of carbon and energy, have been studied intensively in terms of both physiological activities and potential applications. During growth on methanol, the enzymes involved in methanol metabolism are massively produced in these yeasts, indicating that the gene promoters of these enzymes are strong methanol-inducible promoters. Using these promoters, high-level heterologous gene expression systems have been developed in several methylotrophic yeast strains, such as *Pichia pastoris*, *Hansenula polymorpha*, and *Candida boidinii*. To achieve efficient industrial use of methanol and efficient protein production by methylotrophic yeasts, it is important to elucidate the molecular basis of methanol-inducible gene expression in these yeasts. This review describes recent advances in understanding of the regulation of methanol-inducible gene expression and the molecular mechanism of transcriptional activation in the methylotrophic yeast *C. boidinii*. Application of this gained knowledge led to successful production of useful enzymes in this yeast, which is also reviewed.

**This review was written in response to the author’s receipt of The Japan Bioscience, Biotechnology, and Agrochemistry Society Award for the Encouragement of Young Scientists in 2007.**

**Correspondence:** Fax: +81-75-753-6454; E-mail: yury@kais.kyoto-u.ac.jp
ic and industrial fields, due to the following reasons: (i) these yeasts can grow to high cell density on a cheap and simple synthetic medium; (ii) they have strong and tightly regulated gene promoters induced by methanol and repressed by glucose or ethanol; (iii) these yeasts have the ability to perform typical eukaryotic protein processing and other posttranslational modification essentially common to all eukaryotic cells. With these expression systems, a large number of useful proteins, such as enzymes, antibodies, cytokines, plasma proteins, and hormones, have been produced. However, the molecular mechanism of methanol-inducible gene expression has yet to be elucidated.

In order to achieve efficient industrial use of methanol and efficient protein production by methylotrophic yeasts, it is important to thoroughly understand the molecular mechanism of methanol-inducible gene expression in these yeasts. This review covers recent advances in the understanding of the regulation of methanol-inducible gene expression and of the molecular mechanism of transcriptional activation of methanol-inducible genes in the methylotrophic yeast C. boidinii. Successful production of useful enzymes in this yeast is also introduced in this review.

I. Methanol Metabolism in the Methylotrophic Yeast

1. Outline of methanol metabolism

Methanol metabolism in methylotrophic yeasts is summarized in Fig. 1.1 Methanol is first oxidized by alcohol oxidase (AOD) to generate formaldehyde and H₂O₂, which are highly toxic compounds. Formaldehyde is a central intermediate situated at the branch point between assimilatory and dissimilatory pathways.2 A portion of formaldehyde is fixed to xylulose 5-phosphate (Xu5P) by dihydroxyacetone synthase (DAS) forming dihydroxyacetone (DHA) and glyceraldehyde 3-phosphate (GAP), which are used for the synthesis of cell constituents and the regeneration of Xu5P. AOD and DAS are localized to peroxisomes together with catalase (CTA), which decomposes H₂O₂.

Another portion of formaldehyde is further oxidized to CO₂ by the cytosolic dissimilatory pathway. Formaldehyde generated by AOD reacts non-enzymatically with the reduced form of glutathione (GSH) to generate S-hydroxymethyl glutathione (S-HMG). Until recently, the formation of S-HMG was thought to occur in the cytosol. However, GSH was also shown to be present in peroxisomes at a physiologically significant level, indicating that S-HMG is formed not only in the cytosol but also within the peroxisomes. S-HMG is then oxidized to CO₂ through the cytosolic GSH-dependent oxidation pathway, which is ubiquitously present in nature from lower to higher organisms and could be a general formaldehyde detoxification pathway. NAD⁺-linked and GSH-dependent formaldehyde dehydrogenase (FDH) uses S-HMG as a substrate to yield S-formylglutathione (S-FG) and NADH. S-FG is then hydrolyzed to formate and GSH by S-formylglutathione hydrolase (FGH). NAD⁺-linked formate dehydrogenase (FDH) is the last enzyme involved in the methanol dissimilatory pathway and generates CO₂ and NADH by the oxidation of formate.

We have studied the physiological role of the methanol-metabolizing enzymes by cloning and disrupting the corresponding genes in C. boidinii. The cloned genes and their disruptive phenotypes are summarized in Table 1.

2. Physiological function of formaldehyde oxidation pathways

The dissimilatory formaldehyde oxidation pathway has been considered to have two physiological roles: formaldehyde detoxification and energy generation through NADH production. We have studied the physiological role of each enzyme involved in the GSH-dependent formaldehyde oxidation pathway by

Fig. 1. Outline of Methanol Metabolism in the Methylotrophic Yeast C. boidinii.

Enzymes: ADH (MFS), alcohol dehydrogenase (methylformate-synthesizing enzyme); AOD, alcohol oxidase; CTA, catalase; DAK, dihydroxyacetone kinase; DAS, dihydroxyacetone synthase; FDH, formate dehydrogenase; FGH, S-formylglutathione hydrolase; FLD, formaldehyde dehydrogenase; GLR, glutathione reductase; Pmp20, peroxisome membrane protein which has glutathione peroxidase activity.

Abbreviations: DHA, dihydroxyacetone; DHEP, dihydroxyacetone phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; GS-CH₂OH, S-hydroxymethyl glutathione; GS-CHO, S-formylglutathione; GSH, a reduced form of glutathione; GSSG, an oxidized form of glutathione; RCOOH, alkyl hydroperoxide; Xu5P, xylulose 5-phosphate.
disrupting the corresponding genes, FLD1, FGH1, and FDH1, in C. boidinii.\textsuperscript{22-23} For precise evaluation of the growth ability of gene-disrupted strains on methanol, we performed not only batch culture experiments but also methanol-limited chemostat culture experiments, in which formaldehyde toxicity could be minimized. The FLD1-disrupted strain (fld1Δ) was unable to grow on methanol under chemostat conditions even with a low dilution rate, indicating that FLD is essential for growth on methanol. However, the FGH1-disrupted strain (fgh1Δ) and the FDH1-disrupted strain (fdh1Δ) were able to grow on methanol under methanol-limited culture conditions, although the growth yields were only 10% and 25% of that of the wild-type strain, respectively. These results suggest that FGH and FDH are not essential but necessary for optimal growth on methanol.\textsuperscript{24}

In the wild-type formaldehyde oxidation pathway, 2 moles of NADH were generated from 1 mole of dissimilated formaldehyde. While 1 mole of NADH could be generated in the fgh1Δ and fdh1Δ strains, no NADH was formed in the fld1Δ strain. From these results, 1 mole of NADH from 1 mole of dissimilated formaldehyde was considered sufficient to support suboptimal growth on methanol. Our results indicate that the GSH-dependent formaldehyde oxidation pathway has a dual physiological function: detoxification of formaldehyde and energy generation through NADH production. In addition, FGH seems to play an important role in the regeneration of GSH.\textsuperscript{25}

In yeast methanol metabolism, GSH is thought to be involved not only in detoxification of formaldehyde but also in the antioxidant system (see below). GSH is normally the predominant form of glutathione, as its oxidized form, glutathione disulfide (GSSG), is reverted to the reduced form by glutathione reductase (GLR) (Fig. 1). In order to assess the importance of GSH for yeast methanol metabolism, we have analyzed the phenotype of strains that no longer produce enzymes associated with glutathione metabolism. Our recent results suggest that the regulation of the level of the reduced form of glutathione (GSH) mediated by GLR is crucially important in yeast methanol metabolism (unpublished results).

We further showed that methyl formate synthesis catalyzed by cytosolic alcohol dehydrogenase (ADH) contributed to formaldehyde detoxification through GSH-independent formaldehyde oxidation during growth on methanol.\textsuperscript{26}

\section{Peroxiosomal antioxidant enzymes}

Another toxic compound generated in methanol metabolism is H\textsubscript{2}O\textsubscript{2}, which is broken down by a peroxisomal catalase (CTA). In C. boidinii, a CTA-depleted strain (cta1Δ) was able to grow on methanol as the sole carbon source although its growth rate was much lower than that of the wild-type strain.\textsuperscript{26} It is thought that in CTA-deficient strains, hydrogen peroxide is degraded by other systems, e.g., cytochrome c peroxidase.

A 20-kDa peroxisomal peripheral membrane protein of C. boidinii (Pmp20) was identified as peroxiredoxine, an antioxidant enzyme necessary for methylotrophic growth.\textsuperscript{27} Pmp20 showed glutathione peroxidase activity and was reactive against alkyl hydroperoxides and hydrogen peroxide. Interestingly, the pmp20Δ strain had a more severe growth defect than the cta1Δ strain. During incubation of these strains in methanol medium, the cta1Δ strain accumulated H\textsubscript{2}O\textsubscript{2}, while the pmp20Δ strain did not. Therefore, the main function of Pmp20 is thought to be to degrade reactive oxygen species generated at the peroxisomal membrane surface, e.g., lipid hydroperoxides, rather than to degrade hydrogen peroxide. These results have revealed the physiological significance of Pmp20 as an antioxidant enzyme within peroxisomes rich in reactive oxygen species.

\section{II. Regulation of Methanol-Inducible Genes in the Methylotrophic Yeast}

The key enzymes of methanol metabolism mentioned above are induced by methanol. These enzymes are virtually absent in cells growing on glucose, but present at high levels in cells growing on methanol. We have studied the regulatory profile of the expression of genes encoding enzymes involved in methanol metabolism under various carbon and nitrogen sources. Furthermore, we have evaluated the strength and regulation of the methanol-inducible gene promoters in detail using a sensitive promoter-reporter assay system with the acid phosphatase gene (PHOS) from Saccharomyces cerevisiae as the reporter.\textsuperscript{27,28}

\subsection{1. Regulation of the expression of AOD and DAS}

In methanol-induced cells, AOD and DAS can account for up to 20–30\% each of the total soluble protein. This indicates that the promoters of the genes encoding these enzymes are strongly activated under methanol. These strong and tightly-regulated methanol-inducible promoters are used in heterologous gene expression systems of methylotrophic yeasts, but, the molecular mechanism of methanol-inducible gene expression has yet to be elucidated.

Methanol-induced gene expression is exerted through two activation steps, glucose derepression (gene activation without methanol) and methanol-specific induction (gene activation by methanol) (Fig. 2). When grown on glycerol, C. boidinii and H. polymorpha exhibited ~10\% and 80\% of the methanol-induced maximum AOD expression level, respectively. Glucose-limited chemostat culture experiments also showed that the levels of AOD in H. polymorpha gradually increased with decreasing dilution, whereas the derepression of AOD was lower in C. boidinii than in H. polymorpha.\textsuperscript{29}
Therefore, the extent and mode of derepression differs among the methylotrophic yeast species.

We isolated the promoter regions of five methanol-inducible genes from \textit{C. boidinii}, evaluated their strengths, and studied their regulation in detail.\textsuperscript{28} Among the tested promoters, the \textit{DAS1} promoter was the strongest, and attained transcription rates several times higher than that of the \textit{AOD1} gene promoter. The \textit{AOD1} promoter was shown to have a maximum level of expression in cells grown on methanol, a derepressed level of expression in cells grown on glycerol or oleate, and was repressed in cells grown on glucose or ethanol (Fig. 2). Thus, the maximum expression of the \textit{AOD1} gene required not only glucose derepression but also methanol-specific induction. In contrast, the \textit{DAS1} promoter did not show a derepressed level of expression in any of the alternate carbon sources.

Methanol-grown cells contain clusters of large organelles, peroxisomes.\textsuperscript{16} Both \textit{AOD} and \textit{DAS} are major components of the peroxisomal matrix, suggesting that the generation and fixation of formaldehyde is primarily confined to this organelle. Compartmentalization of the formaldehyde conversion reactions in a membrane-bound organelle may be one cellular strategy to avoid formaldehyde toxicity. However, mechanisms for the prevention of a sudden increase in the concentration of formaldehyde in peroxisomes are likely to exist. In this respect, an elegant mechanism for induction of \textit{AOD} and \textit{DAS} seems to prevent an excess accumulation of formaldehyde in the peroxisome during growth on methanol. Induction of \textit{DAS} preceded that of \textit{AOD} during the early stages of methanol induction.\textsuperscript{30} This timely fashioned induction of \textit{AOD} and \textit{DAS} helps to minimize the toxicity of formaldehyde in the peroxisome.

2. Regulation of the expression of GSH-dependent formaldehyde oxidation pathway enzymes

The genes involved in the GSH-dependent formaldehyde oxidation pathway, \textit{FLD1}, \textit{FGH1}, and \textit{FDH1}, were induced by methanol, methylamine, and choline.\textsuperscript{22–24} Since the latter two compounds yield formaldehyde when they are used as nitrogen sources, the formaldehyde-induced expression of \textit{FLD1}, \textit{FGH1}, and \textit{FDH1} was not subject to glucose repression, being distinct from that of \textit{AOD1} and \textit{DAS1}. These features may provide a novel way to produce heterologous proteins in methylotrophic yeasts.\textsuperscript{31} The promoters of \textit{FLD1}, \textit{FGH1}, and \textit{FDH1} can be used for the expression of heterologous genes in glucose-containing medium.

III. Molecular Mechanism of the Activation of Methanol-Inducible Gene Expression

As mentioned above, compared to other methylotrophic yeasts, \textit{C. boidinii} has several distinct features with respect to regulation of methanol-inducible gene expression. Hence, genes responsible for the regulation of methanol-inducible promoters, especially in regard to methanol-specific induction as opposed to glucose derepression, can be characterized by using \textit{C. boidinii}.

We isolated mutants with defects in transcriptional activation of methanol-inducible promoters by a newly developed gene-tagging mutagenesis method in \textit{C. boidinii}, and identified a novel gene, \textit{TRM1}, as a putative regulator of methanol-specific induction.\textsuperscript{32,33} The encoded protein, Trm1p, belongs to a Zn(II)$_2$Cys$_6$-type zinc cluster protein family, members of which are known as transcriptional regulators in many fungi. Deletion of \textit{TRM1} completely inhibited growth on methanol, but caused no growth defect on glucose or other nonfermentative carbon sources, glycerol, ethanol, or oleate. These results suggest that Trm1p is involved more in the regulation of methanol-specific induction than in glucose derepression. In the case of all the methanol-inducible promoters tested, the transcriptional activities in methanol medium drastically decreased in the \textit{trm1A} strain. Thus, the \textit{TRM1} gene was shown to encode a master transcriptional regulator responsible for methanol-specific gene activation in the methylotrophic yeast \textit{C. boidinii}.
Fig. 3. Methanol Induction Regulated by Trm1p and Trm2p in C. boidinii.

During growth on glucose, expression of methanol-inducible genes is repressed. When cells are shifted to methanol, at first, Trm2p-related glucose derepression mechanism occurs, and then, Trm1p-related methanol-specific induction mechanism occurs. Trm1p is likely to form a heterodimer with an unknown protein.

With respect to the AOD1 promoter, the reporter activity in the trml Δ strain retained 6.6% of that in the wild-type strain when induced by methanol. This indicates that the AOD1 promoter is regulated not only by methanol-specific induction but also by glucose derepression. Recently we have isolated and characterized the Trm2p which is homologous to P. pastoris Mxr1p and S. cerevisiae Adr1p (unpublished results). In P. pastoris, Mxr1p was shown to control the transcriptional level of methanol-metabolizing genes, especially AOD1, as well as the PEX genes. Trm2p could be a transcription factor regulating glucose derepression of methanol-inducible gene expression in C. boidinii. The trm2 Δ strain and the trml Δtrm2 Δ double disrupted strain had much lower basal levels of the AOD1 promoter activity than the trml Δ strain when grown on methanol, suggesting that the function of Trm2p is not directly required for methanol-specific induction regulated by Trm1p, but mainly for the preceding step of glucose derepression (Fig. 3).

We showed that among all the methanol-inducible promoters tested, the DAS1 promoter was the most highly inducible. Furthermore, the transcriptional activity of the DAS1 promoter in the trml Δ strain was reduced below 1% compared with the wild-type strain. These features prompted us to determine the DNA sequences that are responsible for methanol specific induction in the DAS1 promoter. Two cis-acting methanol response elements (MREs), MRE1 and MRE2, are present in the promoter of the dihydroxyacetone synthase gene. Reporter analysis using the trml Δ strain showed that Trm1p is required for MRE1-dependent methanol-inducible gene expression.

Chromatin immunoprecipitation assays revealed that Trm1p bound to five methanol-inducible promoters upon methanol induction but did not bind in glucose-grown cells. In many cases, fungal Zn(II)2Cys6 proteins are known to act as homodimers or heterodimers. However, the purified His6-Trm1p did not retard migration in a band shift assay using MRE1 as a probe. This indicates that Trm1p by itself does not bind to the MRE1 sequence in vitro. It is possible that an interaction of Trm1p with another protein(s) is required for binding to MRE1, while other possibilities e.g., activation by protein modification, determining its association with DNA, cannot be ruled out. The heterodimeric binding partners of the Zn(II)2Cys6 type zinc cluster are often other proteins of the same type, although some exceptions are reported. In this context, a homolog of H. polymorpha Mpp1p is one possible partner of Trm1p. In H. polymorpha, Mpp1p is known to be a Zn(II)2Cys6 type zinc cluster protein and was shown to regulate the synthesis of peroxisomal proteins and peroxins. However, since Mpp1p homolog in C. boidinii have not yet been found, it remains to be seen whether Mpp1p can mediate DNA-binding of Trm1p by the formation of a heterodimer.

IV. Production of Useful Enzymes in C. boidinii

We reported efficient heterologous protein production using C. boidinii and set up a novel system for the production of proteins toxic for host cells by utilizing peroxisomes as sites for these proteins to accumulate. An efficient secretion system for the production of active transglutaminase was also developed.

I. Oxidase production within peroxisomes of the aod1 Δ strain

From a biotechnological point of view, peroxisomes of the methylotrophic yeasts are attractive organelles in which to pack the produced heterologous proteins where they are protected from degradation by cytosolic proteases and, in the case of proteins of a toxic nature, have to remain separated from the cytosol. Most peroxisome matrix proteins are destined for peroxisomes by a three amino acid sequence, -SKL and its derivatives, located at the extreme carboxyl end, PTS1 (peroxisome targeting signal 1). An enzyme can be easily targeted to peroxisomes just by adding these three amino acids. Previously, we showed that the aod1 Δ strain of C. boidinii was a useful host for the expression of peroxisomal oxidases that have FAD as a cofactor.

We have studied fungal fructosyl amino acid oxidases (FAODs), which can be used for the determination of glycated proteins. The deduced amino acid sequence of FAOD-encoding cDNAs from several fungi, such as Penicillium janthinellum and Aspergillus terreus, contained the typical PTS1 sequence, -SKL and -AKL, respectively, indicating that the fungal FAODs were peroxisomal enzymes. High-level production of FAOD from P. janthinellum, whose production was toxic in E. coli, was investigated through attempts to utilize the peroxisome of the C. boidinii aod1 Δ strain as
the place for protein accumulation.\(^{39}\) The reason we used the \(aod1\Delta\) strain as a host was that the heterologous FAOD would be able to use the peroxisomal space, the transport machinery into peroxisomes, and FAD as a cofactor, which was used by AOD in the wild-type strain. We expressed the synthetic FAOD gene optimized for \(C.\) boidinii codon usage in the wild type and \(aod1\Delta\) strains under the control of the \(AOD1\) promoter. The \(aod1\Delta\) strain could not grow on methanol as the sole carbon source, therefore glycerol was added to the medium, which further contained ammonium chloride as the source of nitrogen. Unexpectedly, the levels of FAOD activity were the same in both strains (Table 2). Since the \(aod1\Delta\) strain cannot oxidize methanol to formaldehyde, the level of induction of the \(AOD1\) promoter in the \(aod1\Delta\) strain was expected to be lower than that in the wild-type strain on methanol medium. When methylamine was used as the nitrogen source, methylamine was oxidized by amine oxidase to formaldehyde, which then induced the \(AOD1\) promoter to comparable levels in both strains. As a result, the \(aod1\Delta\) strain 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 (Table 2). Using the \(aod1\Delta\) strain, we could also produce \(\delta\)-amino acid oxidase (Table 2) and acetylperoxidimide oxidase from \(C.\) boidinii at higher levels than with the wild-type \(C.\) boidinii strain as a host.\(^{40,41}\) Therefore, it seems important that resources taken up by AOD, like FAD, are freed to enable overproduction of these oxidases. Based on these studies, the peroxisomes of the \(aod1\Delta\) strain of \(C.\) boidinii can be considered to be a suitable storage place for overproduction of heterologous useful oxidases.

2. Efficient secretion of transglutaminase

Transglutaminase (TGase) catalyzes intramolecular or intermolecular cross-link within or between certain proteins through \(\gamma\)-glutamyl-\(\epsilon\)-lysine side chain bridges. This cross-linking reaction is used to control gelatinization of proteins in foods and to provide various food textures. TGase from the actinomycete \(Streptomyces mobaraensis\) is a useful enzyme in the food industry.\(^{48}\) The TGase-encoding gene from \(S.\) mobaraensis consists of three parts: a signal peptide for its secretion, a pro-peptide cleaved off after its secretion into the medium, and the mature form of TGase.\(^{49}\) The pro-peptide inhibits enzyme activity and also increases enzyme thermostability, but it is not necessary for catalytic activity. The pro-peptide appears to be cleaved by a specific metalloprotease in the culture medium of \(S.\) mobaraensis.\(^{50,51}\)

Heterologous gene expression of microbial TGase has also been reported using \(Escherichia coli\) as the host, although difficulties were encountered in getting TGase secreted in an active form.\(^{52}\) Recently, secretion of active TGase was reported by coexpression of a subtilisin-like protease in a \(Streptomyces\) or \(Corynebacterium\) expression system.\(^{49}\) But co-expression of the protease was difficult to control, and protease overproduction may lead to degradation of the produced TGase. We have reported the secretion of TGase in an enzymatically active form by the methylotrophic yeast as an expression host.\(^{52}\)

The coding regions for the pro-peptide and the mature TGase were synthesized according to the preferred codon usage in \(C.\) boidinii. Then we constructed different kinds of expression cassettes under the control of the \(AOD1\) promoter (Fig. 4). The expression cassette \(aTG\) was constructed by fusing DNA encoding \(S.\) cerevisiae \(\alpha\)-factor to the N-terminus of DNA encoding the mature TGase. The expression cassette \(aPKTG\) contained the pro-peptide from TGase between the \(\alpha\)-factor and mature TGase-coding regions. The expression cassette \(aPKTG\) contained an additional DNA sequence encoding a dibasic amino acid sequence (-KR-) cleavable by the Kex2 protease present in the yeast Golgi.

\(C.\) boidinii strain harbouring \(aTG\) and \(aPKTG\) expression cassettes did not give a detectable level of TGase protein (Fig. 4). On the other hand, active TGase was secreted when the \(aPKTG\) expression cassette was used. Thus, secretory production of active TGase required both a pro-peptide from TGase and the release of this pro-peptide.

Next, we tested coexpression of an unlinked pro-peptide and a mature TGase by introducing expression cassettes \(aPro\) and \(aTG\), respectively, into \(C.\) boidinii. Unexpectedly, coexpression of unlinked pro-peptide with mature TGase yielded efficient secretion of the active enzyme. These results indicate that the pro-peptide from TGase functions not only in an intramolecular but also in an intermolecular manner.

Furthermore, site-directed mutagenesis of putative \(N\)-glycosylation sites increased the productivity of the active TGase further (Fig. 4). \(C.\) boidinii strain \(aProN120Q/aTGN388Q\) produced about 10 times more active TGase than strain \(aPKTG\), despite similar expression conditions, i.e., the same promoter driving the expression from a single \(aTG\) expression cassette integrated at the same chromosomal locus (with the separately transcribed \(aPro\) in the case of \(aProN120Q/aTGN388Q\) integrated at another site). Finally, \(C.\) boidinii strain \(aProN120Q/aTGN388Q\) secreted active TGase up to 1.83 U/ml (about 90 mg/l) after 119 h of cultivation in a fermentor.

In the course of optimizing the expression cassette for efficient TGase production, we discovered important characteristics of the pro-peptide of TGase. Our experiments indicated that the expression of this pro-peptide from the original host was essential for the secretion of active TGase from yeast cells. Unexpectedly, unlinked expression of the pro-peptide was found to be very effective for the production of active TGase in terms of both quality and quantity (Fig. 4). Furthermore, when

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Host strains(^a)</th>
<th>N-source</th>
<th>Enzyme activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAOD</td>
<td>wild type</td>
<td>NH(_4)Cl</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>wild type</td>
<td>Methylamine</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>(aod1\Delta)</td>
<td>NH(_4)Cl</td>
<td>1.13</td>
</tr>
<tr>
<td></td>
<td>(aod1\Delta)</td>
<td>Methylamine</td>
<td>4.95</td>
</tr>
<tr>
<td>DAO</td>
<td>wild type</td>
<td>NH(_4)Cl</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>wild type</td>
<td>Methylamine</td>
<td>9.79</td>
</tr>
<tr>
<td></td>
<td>(aod1\Delta)</td>
<td>NH(_4)Cl</td>
<td>9.83</td>
</tr>
<tr>
<td></td>
<td>(aod1\Delta)</td>
<td>Methylamine</td>
<td>14.9</td>
</tr>
</tbody>
</table>

\(^a\) Strains were grown in media containing glycerol and methanol as carbon sources.
the normal precursor TGase was expressed, Kex2-dependent release of the pro-peptide from the precursor was also necessary to yield active TGase. Recent analysis of the 3D-crystal structure of TGase has suggested that the pro-peptide hindered access of the substrate to the catalytic site located at the bottom of the cleft.\(^{53}\) These facts may reflect a regulatory function of the TGase pro-peptide in the original host. Because TGase can be toxic within a bacterial cell, activation of the TGase precursor through processing may be advantageous to avoid negative effects of the synthesized protein within cells, i.e., before secretion. Thus, the pro-peptide of TGase could act as an intermolecular chaperone to control or stimulate protein folding, enzymatic activity, or competence for secretion, via direct interaction with the mature enzyme. These possible roles of the TGase pro-peptide as a potential chaperone call for further investigation.

V. Conclusion and Perspectives

The heterologous gene expression system of methylotrophic yeasts, such as \(P.\) pastoris, \(H.\) polymorpha, and \(C.\) boidinii, is based on their unique methanol metabolism and is characterized by strong and tightly-regulated methanol-inducible gene expression. In this review, I summarize the physiological function and regulatory profile of enzymes involved in methanol-metabolism in \(C.\) boidinii, and describe the molecular mechanism of methanol-inducible gene expression, especially as to the transcriptional factors we have isolated recently. The molecular mechanism of methanol-inducible gene expression has been studied intensively also in other methylotrophic yeasts, and recently, a number of transcriptional factors involved in methanol-inducible gene expression have been identified.\(^{4,34,36}\) Further studies on transcriptional machinery and signal transduction in methanol-inducible gene expression will lead to improvement of the heterologous gene expression system in these yeasts, and thus, to the creation of methylotrophs that would be an ideal host for the biotechnological production of useful compounds from methanol.

Successful examples of heterologous protein production in \(C.\) boidinii are also described in this review. High-level production of useful oxidases in peroxisomes of the \(aodI\alpha\) strain and efficient secretory production of TGase were both achieved by improvements acting at the post-translational stage. In addition to the understanding of molecular mechanisms of gene activation by methanol, the understanding of molecular dynamics involved in post-translational protein folding, sorting, and degradation is required for improvement of the productivity of heterologous protein production in methylotrophic yeasts.

Acknowledgments

I wish to express my sincerest thanks to Professor Emeritus Nobuo Kato (Kyoto University) and Professor Yasuyoshi Sakai (Kyoto University) for their guidance and encouragement throughout the course of our research. I also thank collaborators and colleagues for their kind support and helpful discussion. This research was supported in part by a Grant-in-Aid for Scientific Research and a Grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References
