Molecular Cloning, Sequencing, and Heterologous Expression of the vaoA Gene from *Penicillium simplicissimum* CBS 170.90 Encoding Vanillyl-Alcohol Oxidase*

(Received for publication, December 3, 1997, and in revised form, January 28, 1998)

Jacques A. E. Benen‡, Paloma Sánchez-Torres‡, Matthé J. M. Wagemaker‡, Marco W. Fraaije§, Willem J. H. van Berkel§, and Jaap Visser‡‡

From the ‡Section Molecular Genetics of Industrial Microorganisms, Wageningen Agricultural University, Dreyenlaan 2, 6703 HA Wageningen, The Netherlands and the §Department of Biochemistry, Wageningen Agricultural University, Dreyenlaan 3, 6703 HA Wageningen, The Netherlands

The cDNA encoding vanillyl-alcohol oxidase (EC 1.1.3.7) was selected from a cDNA library constructed from mRNA isolated from *Penicillium simplicissimum* CBS 170.90 grown on veratryl alcohol by immunochromatographic screening. The vaoA-cDNA nucleotide sequence revealed an open reading frame of 1680 base pairs encoding a 560-aminoo acid protein with a deduced mass of 62,915 Da excluding the covalently bound FAD. The deduced primary structure shares 31% sequence identity with the 8α-(O-tyrosyl)-FAD containing subunit of the bacterial flavocytochrome *p*-cresol methyl hydroxylase.

The vaoA gene was isolated from a *P. simplicissimum* genomic library constructed in λEMB3 using the vaoA-cDNA as a probe. Comparison of the nucleotide sequence of the vaoA gene with the cDNA nucleotide sequence demonstrated that the gene is interrupted by five short introns.

*Aspergillus niger* NW156 prf F pyrA leuA cspA transformed with the pyrA containing plasmid and a plasmid harboring the complete vaoA gene including the promoter and terminator was able to produce vaoA mRNA and active vanillyl-alcohol oxidase when grown on veratryl alcohol and anisyl alcohol. A similar induction of the vaoA gene was found for *P. simplicissimum*, indicating that similar regulatory systems are involved in the induction of the vaoA gene in these fungi.

Introduction of a consensus ribosome binding site, AGAAGGAG, in the vaoA-cDNA resulted in elevated expression levels of active vanillyl-alcohol oxidase from the lac promoter in *Escherichia coli* TG2. The catalytic and spectral properties of the purified recombinant enzyme were indistinguishable from the native enzyme.

Vanillyl-alcohol oxidase (EC 1.1.3.7) is a novel type of flavoprotein oxidase that was first isolated from *Penicillium simplicissimum* CBS 170.90 grown on veratryl alcohol (1). The enzyme is a homo-octamer with each 65-kDa subunit harboring an 8α-(N3-histidyl)-FAD (2). Vanillyl-alcohol oxidase has a broad substrate specificity. In addition to the conversion of vanillyl alcohol to vanillin (Equation 1), the enzyme catalyzes the conversion of a wide range of phenolic compounds bearing side chains of variable size at the para-position of the aromatic ring (3, 4). Due to its broad substrate spectrum, vanillyl-alcohol oxidase may be applied in the fine chemical industry (5). Based on induction experiments, 4-(methoxymethyl)phenol has been proposed to represent the physiological substrate (6). Recently, from rapid reaction kinetics conclusive spectral evidence was obtained that the vanillyl-alcohol oxidase-mediated oxidative demethylation of 4-(methoxymethyl)phenol proceeds through the formation of a quinone-methide product intermediate (4).

In the absence of oxygen, this intermediate is stabilized in the active site of the reduced enzyme. Upon flavin reoxidation, the quinone methide of 4-(methoxymethyl)phenol readily reacts with water, yielding 4-hydroxybenzaldehyde, methanol, and hydrogen peroxide as final products.

Recently, the three-dimensional structure of vanillyl-alcohol oxidase was elucidated (7). The crystallographic analysis corroborated earlier observations (2, 8) that the vanillyl-alcohol oxidase octamer can be described as a tetramer of tightly interacting dimers. Each vanillyl-alcohol oxidase monomer consists of two domains. The larger domain creates a binding site for the ADP moiety of the FAD, whereas the smaller cap domain covers the active site that is located between the two domains. Furthermore, from the structures of several vanillyl-alcohol oxidase inhibitor complexes, it could be deduced that the shape of the active site cavity controls substrate specificity.

In this paper we describe the cloning, sequencing, and expression of the gene encoding vanillyl-alcohol oxidase from *P. simplicissimum* CBS 170.90, providing the necessary amino acid sequence information which together with the three-dimensional structure establishes the basis for future protein engineering studies.

**EXPERIMENTAL PROCEDURES**

**Strains and Media—** Growth conditions for *P. simplicissimum* (CBS 170.90: ATCC 90172) have been described before (6). *Aspergillus niger* NW156 (prfF28, leuA6, pyrA1, cspA1) a derivative of A. niger N400 (CBS 120.49) from the laboratory collection was used for transformation and was grown in minimal medium (9), pH 6.0, containing 1% (mass/volume) fructose, 0.1% (mass/volume) yeast extract, 0.02% (mass/volume) leucine, 0.02% (mass/volume) uridine, and a trace element solution according to Ref. 10. *Escherichia coli* DH5α [F endA1 metB7 (trpC metE) supE44 thi-1 recA1 gyrA96 (Nal') relA1 ΔlacZΔM15 argF1U169 deoR (Δ860lacZ Δ1acZI M15) (11) was used for cloning throughout. λ phages were propagated in *E. coli* LE392 [F endA1 (McrA') hisD514 (lacC' m<sub>5</sub>°) supE44 supF58 ΔlacZΔM15 galK2 galT22 metB1 trpR55] (12). *E. coli* BB4 [F lacF1ΔZΔM15 proAB Tn10 (tet') and
cDNA Cloning of the Vanillyl-Alcohol Oxidase Gene

E. coli SOLR (ΔmcrAΔlac::mch2-dsRed::nmtR) Tn5 [kan' lac gyrA96 relA1 thi-1 endA1 ΔF' lacZΔM15 proAB Tn10 (tet') Su'] were used for cDNA cloning according to the suppliers recommendations (Stratagene Cloning Systems, La Jolla, CA). E. coli TG2 (Δlac-pro) thi supE recA [Res' Mod' (kl)] F' [traD36 proA' B' lacZΔM15] (13) was used for expression of vanillyl-alcohol oxidase. E. coli strains were grown in Luria-Bertani medium supplemented with the appropriate antibiotic with the exception of E. coli LE392 which was grown in NZCYM medium. Media were solidified using 1.5% (mass/volume) bacto-agar. Top agar was prepared using NZCYM medium solidified with 0.7% agarose (electrophoresis grade).

Preparation of Cell Extracts—Cell extracts were prepared by sonication as described (6).

Enzyme Activity—Vanillyl-alcohol oxidase activity was determined at 25 °C in 50 mM potassium phosphate buffer, pH 7.5, by measuring the reduction of vanillin at 540 nm (ε280 vanillin = 14 mm−1 cm−1).

Protein Sequence Analysis—Protein sequence analysis of vanillyl-alcohol oxidase was performed by Edman degradation at Eurosequence, Groningen, The Netherlands.

Manipulation of DNA—Isolation of plasmid and chromosomal DNA and other molecular manipulations were carried out essentially as described (12). A. niger and P. simplicissimum chromosomal DNA was isolated according to de Graaff et al. (14). Restriction enzymes were used as recommended by the supplier (Life Technologies, Inc., Gaithersburg, MD).

The nucleotide sequence was determined using either the Cy5™ AutoCycle™ Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden) or the Cy5™-dATP Labeling Mix (Pharmacia Biotech). The reactions were performed as recommended by the supplier (Life Technologies, Inc., Gaithersburg, MD).

Isolation of RNA—Total RNA was isolated by grinding frozen mycelium in liquid nitrogen-cooled shake flasks and grinding balls using a micro dismembrator (Braun Melsungen AG, Melsungen, Germany). Ground mycelium was extracted with TRIzol™ Reagent according to the supplier (Life Technologies, Inc., Gaithersburg, MD). Isolation of poly(A) tail mRNA was performed with oligo(T)-cellulose obtained from Stratagene.

Construction and Screening of the cDNA Library—Using 5 μg of poly(A) tail mRNA, isolated from P. simplicissimum grown for 54 h on 0.1% (mass/volume) veratryl alcohol as the sole source of carbon (see "Results"), a cDNA library was constructed with a ZAP-cDNA Synthesis Kit using Uni-ZAP XR Vector Arms and a ZAP-cDNA Gigapack Gold III packaging extract (Stratagene). All procedures were carried out as described in the manual supplied with the cDNA Kit. The cDNA library was screened with purified antibodies raised against vanillyl alcohol oxidase, detection limit less than 10 pg (15), as described in the picoBlu-e™ Immunoscreening Kit instruction manual (Stratagene) using E. coli BB4 as a host. After a second immuno-screening in vivo excision of the pBlueScript phagemid from the Uni-ZAP XR Vector was performed using the EκAssist Helperphage with the E. coli SOLR strain according to the Single-Clone Excision Protocol (Stratagene).

Construction and Screening of the Genomic Library—P. simplicissimum chromosomal DNA was partially digested with SstI and size-fractionated by agarose gel electrophoresis. 10–15-kilobase pair fragments were recovered from the gel by electroelution and cloned into the BamHI sites of pEMB11 vector arms (Stratagene). Following packaging, the phages were used to infect E. coli LE392 and plated. After overnight growth on selective medium plates, phages were recovered from the top agar by extraction with SM buffer (12) yielding the primary library. The library was amplified by replating an aliquot of the recovered phages with E. coli LE392.

For screening of the amplified library dilutions were prepared yielding 7000–8000 plaques per plate. Four plates of phage were transferred in duplicate to Hybond N+ membranes (Amersham International plc, Little Chalfont, Buckinghamshire, UK) and processed as recommended by the supplier. Prehybridization and hybridization were carried out in 6× SSC, 5× Denhardt’s solution, 0.5% (mass/volume) SDS at 65 °C. A probe a 1215-bp Xbal/BamHI fragment of the vaoA-cDNA was used (Xbal cuts at position 451 and BamHI cuts at position 1923 in the genomic sequence as shown in Fig. 1). The fragment was labeled with [32P]dATP as described previously (16). Washing steps were carried out for 30 min at 65 °C in the following solutions: twice in 2× SSC, 0.5% (mass/volume) SDS, and once in 0.5× SSC, 0.5% (mass/volume) SDS. For secondary screening identical conditions were applied with the exception that phase dilutions were used yielding 200–300 plaques per plate allowing the selection of individual plaques. Phages were propagated in E. coli LE392 and phage DNA isolated as described (12).

Transformation of A. niger NW156 was transformed as described (13), using 1 μg of the selection plasmid pGW35 and 20 μg of cotransforming pM3971. The copy number of the P. simplicissimum vaoA gene in A. niger NW156-T10 was determined by Southern blot analysis. 5 μg of SalI-digested P. simplicissimum chromosomal DNA and undiluted (5 μg) and serially diluted SalI-digested A. niger NW156-T10 chromosomal DNA were separated by agarose gel electrophoresis, blotted, and subsequently hydridized with [32P]dATP-labeled vaoA-cDNA.

Expression Studies in P. simplicissimum and A. niger—Expression studies were carried out via transfer experiments. P. simplicissimum was precultured in medium described above using fructose (1%) as a carbon source. A. niger NW156-T10 was pregrown on complete medium (9) with 1% fructose as a carbon source. After 16 or 30 h of growth for A. niger and P. simplicissimum, respectively, mycelium was harvested and transferred to fresh media supplemented with carbon sources as described under "Results." At regular intervals mycelia were harvested and processed for RNA extraction or preparation of cell extract as described above.

Northern and Southern Analysis—Northern and Southern blots were carried out as described previously (12). [32P]dATP-labeled vaoA-cDNA was used as a probe (see above). The membranes were stripped according to the instructions of the manufacturer and rehybridized with a 900-bp EcoRI fragment encoding part of the 3' end of the 28 S rDNA of Agaricus bisporus (18) to provide for a loading control.

Western Analysis—Western blot analysis was done as described previously (6) using the same antibodies as used for the cDNA library screening. Bound antibodies were detected by an alkaline phosphatase based immunassay.

UV Visualization of Vanillyl-Alcohol Oxidase—Vanillyl-alcohol oxidase was visualized after SDS-PAGE as described (6).

PCR Mutagenesis to Introduce a Shine-Dalgarno Sequence—To enhance the expression of the vaoA-cDNA a consensus Shine-Dalgarno sequence was introduced via PCR mutagenesis. This was done using the pBlueScript harboring plasmid pIM3970, taking advantage of the XbaI site at position 451 (numbering according to Fig. 1) and the KpnI site from the following sequence: 5' GCGGAGCTCGTTTTAAAGAGGATATA CATTATGTCACAGCACAGCAATCTC 3' and primer 2, a 17-mer with the sequence 5' CCGAGAATTTCGCTCCCT 3'. Primer 1 is a mutagenic oligonucleotide. The sequence shown in boldface is identical to the N-terminal coding sequence, and the italicized sequence represents the Shine-Dalgarno sequence. Primer 2 is complementary to the sequence from positions 587 to 603 in Fig. 1 downstream of the XbaI site. PCR was performed in a Biometra thermocycler in a 25-μl reaction volume containing PCR buffer (Pharmacia Biotech), 1.25 mM dNTP (each), 1 ng of vaoA-cDNA, 100 pmol of each oligonucleotide, and 0.5 units of Taq polymerase (Pharmacia) with the following regime: 5 min at 95 °C; 30 cycles: 1 min at 95 °C, 1 min at 43 °C, and 1 min at 72 °C; 10 min at 72 °C.

The PCR fragment generated with primers 1 and 2 was cloned into pGEM-T (Promega Corp., Madison, WI) and sequenced to check the orientation and to check for undesired mutations. Next, the fragment in the right orientation with respect to the polylinker-encoded PstI site was excised from pGEM-T by PstI/XbaI digestion and ligated into PstI/XbaI-digested pEMBL19. The gene was completed by cloning the XbaI/KpnI fragment isolated from pM3970 into the XbaI/KpnI-digested previous pEMBL construct yielding plasmid pM3972. The orientation of the gene is such that transcription takes place from the vector-encoded lac promoter.

Purification of Vanillyl-Alcohol Oxidase from E. coli TG2—E. coli TG2 cells carrying plasmid pM3972 were grown batchwise in 500 ml of

1 The abbreviations used are: bp, base pair(s); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; vaoA, vanillyl-alcohol oxidase encoding gene.
LB medium supplemented with 80 μg/ml ampicillin and 0.1 mM isopropyl-1-thio-β-D-galactopyranoside in 2-liter flasks in a rotary shaker set at 250 rpm at 37 °C. From 5-liter batch cultures cells were harvested by centrifugation and resuspended in 55 ml of 50 mM potassium phosphate buffer, 0.5 mM phenylmethylsulfon fluoride, 0.5 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM MgSO4, 10 mg of DNase I, pH 7.0. Cells were disrupted by passing three times through a precooled French pressure cell press operated at 10,000 p.s.i. Following centrifugation for 15 min at 15,000 × g to remove cellular debris, the supernatant was made 0.5% in protamine sulfate from a 2% mass/volume stock solution. Subsequently, the protamine sulfate aggregates were precipitated by centrifugation for 15 min at 15,000 × g for 15 min at 15,000 × g, the supernatant was adjusted to 65% ammonium sulfate saturation. After centrifugation for 15 min at 15,000 × g, the supernatant was adjusted to 65% ammonium sulfate saturation. The precipitate was collected by centrifugation at identical settings as before. The pellet was dissolved in 50 mM potassium phosphate buffer, 0.5 mM EDTA, 0.5 mM ammonium sulfate, pH 7.0, and loaded onto a phenyl-Sepharose column (30 × 2.6 cm) pre-equilibrated in the same buffer. The enzyme was eluted with a linear gradient of 25–300 mM potassium phosphate, pH 7.0. After washing the column with 3 volumes of starting buffer, the enzyme was eluted with a linear gradient of 25–300 mM potassium phosphate, pH 7.0. Fractions containing vanillyl-alcohol oxidase were pooled and concentrated in an Amicon ultrafiltration unit equipped with a YM-30 membrane.

RESULTS

Construction of the cDNA Library, Selection, and Sequence of the vaoA-cDNA—To ensure high abundance of the vanillyl-alcohol oxidase mRNA, total RNA was isolated from P. simplicissimum after 54 h of growth on minimal medium containing veratral alcohol when vanillyl-alcohol oxidase activity was at 75% of its maximum (not shown). The primary library of 7 × 10⁶ plaque-forming units was amplified to a titer of 1.4 × 10¹⁰ plaque-forming units/ml. The first screening of the amplified library revealed that approximately 4.5% of the phages reacted with the vanillyl-alcohol oxidase-specific antibodies. From the second screening five positive phages were selected, and the phagemid was excised. Restriction enzyme analysis showed that the five phagemids contained identical inserts. From two such phagemids the nucleotide sequence of the insert was determined over both strands by sequencing subclones and by the use of specific oligonucleotides. The vaoA-cDNA sequence and the derived amino acid sequence are presented in Fig. 1 (see below). The vaoA-cDNA nucleotide sequence revealed an open reading frame of 1680 bp encoding a protein of 560 amino acids with a deduced mass of 62,915 Da excluding FAD. Amino acid sequences obtained by automated Edman degradation of the N terminus of purified vanillyl-alcohol oxidase and of a purified internal peptide obtained by tryptic digestion of the enzyme were both identified in the deduced primary amino acid sequence (bold type in Fig. 1). The nucleotide sequence revealed that the first five amino acids were missing from the N-terminal peptide sequence suggesting some limited proteolytic processing of the enzyme in P. simplicissimum.

Cloning and Sequencing of the vaoA Gene—By using methods and conditions described under “Experimental Procedures” a genomic library of P. simplicissimum was constructed and subsequently screened with the [³²P]cATP-labeled vaoA-cDNA as a probe. Two of over 250 subclones containing positive phages were characterized by restriction enzyme analysis and Southern blot analysis (results not shown). It was concluded that the entire gene should be located on an 8-kilobase pair Sall fragment which was subsequently subcloned into Sall-digested pUC18 yielding pLM3971. pLM3971 was used for sequencing either using specific oligomers based on the vaoA-cDNA sequence and on sequences from the gene or by subclones generated. Fig. 1 shows the complete vaoA gene, sequenced over both strands, including 582-bp promoter sequence and 293 bp of downstream sequence.

Comparison of the cDNA sequence with the genomic sequence revealed that the coding region is interrupted by five introns. The intron sequences follow the rules for filamentous fungi as proposed earlier (19): they are short, 61, 60, 70, 52, and 95 bp for introns i, ii, iii, iv and v, respectively, and the introns have consensus 5’ and 3’ splice sites, GTPuNGPy and PyAG, respectively, and lariat sequences, NNCuPuApy (where Pu indicates purine and Py indicates pyrimidine), with only slight deviations.

The promoter region was analyzed for the presence of sequences involved in transcription regulation. The sequences 5’ GATA 3’ and 5’ GCCARG 3’ involved in nitrogen (20) and pH regulation (21), respectively, were not present. The context independent CreA binding site of A. nidulans 5’ GYGGGG 3’ (22) which is probably also recognized in Penicillium (23) was found once (position −557 to −552 in Fig. 1). The 5’ CAAT 3’ sequence, shown to be involved in transcription activation in Saccharomyces and other fungi (24), was found at positions −38, −61, −112, and −373 (lowercase italic letters). No TATA box was found in the sequence immediately upstream of the start codon. However, CT-rich sequences thought to direct transcription initiation (19) were present.

Vanillyl-Alcohol Oxidase Induction in P. simplicissimum and A. niger—Earlier studies have shown that vaoA expression is gratuitously induced in P. simplicissimum when grown on veratral alcohol (1) or anisyl alcohol (6). We have readdressed this issue in a transfer experiment in which veratral alcohol was used as the sole source of carbon. Analysis was carried out by Northern and Western blotting and by vanillyl-alcohol oxidase activity determinations. In Fig. 2 the time course of induction is presented. Panel A shows the Northern blot probed with ³²P-labeled vaoA-cDNA. In panels B and C the Western blot probed with the vanillyl-alcohol oxidase antibodies and the relative vanillyl-alcohol oxidase activity are presented, respectively. The results clearly show that at the start of the transfer no vaoA-mRNA or vanillyl-alcohol oxidase enzyme is present. The vanillyl-alcohol oxidase activity and enzyme concentration follow the vaoA-mRNA concentration with a lag, i.e. while the mRNA is maximal at 36 h the maximal activity is observed between 48 and 72 h. Furthermore, when P. simplicissimum was grown on the combination of 0.1% (mass/volume) veratral or 0.1% (mass/volume) anisyl alcohol and 1% (mass/volume) glucose no vanillyl-alcohol oxidase was produced (results not shown) indicating that the gene is carbon catabolite-repressed. This was also suggested by the presence of the context independent CreA binding site in the promoter region.

A. niger NW156-T10, a pLM3971 multicopy transformant harboring 25–80 vaoA copies, was used to study vanillyl-alcohol oxidase expression. Southern analysis using the vaoA-cDNA as a probe demonstrated that A. niger NW156 does not contain a vaoA gene with sufficient homology with the P. simplicissimum vaoA gene to be detected under the conditions applied. In transfer experiments with A. niger NW156-T10 the inducing properties of veratral alcohol, anisyl alcohol, vanillyl alcohol, veratic acid, vanillic acid, and 4-hydroxybenzoic acid were assessed 3 h after transfer (see Table I and Fig. 3). The highest level of vanillyl-alcohol oxidase activity was obtained with veratral and anisyl alcohol, whereas no activity was seen when A. niger NW156-T10 was grown on fructose and when an untransformed A. niger strain was transferred to veratral or anisyl alcohol (not shown). Both 4-methoxybenzyl alcohols were used alone and in combination with fructose to study the
time course of vanillyl-alcohol oxidase induction (results not shown). With anisyl alcohol already after 3 h strong induction was observed both by Northern and Western analysis, whereas after 6 h the induction decreased. When fructose was included the induction was weaker both at 3 and 6 h. With veratryl alcohol induction was retarded when compared with anisyl alcohol. Fructose alone or a mixture of veratryl alcohol and fructose resulted in no detectable \( \text{vao} \)-A-mRNA, and no vanillyl-alcohol oxidase was found by Western analysis.

Expression of \( \text{vaoA} \)-cDNA in \( \text{E. coli} \) TG2 and Purification of Recombinant Vanillyl-Alcohol Oxidase—Although vanillyl-alcohol oxidase could easily be detected with the vanillyl-alcohol oxidase-specific antibodies during screening of the cDNA library, the expression of the gene was quite low in \( \text{E. coli} \) (less than 0.5% of total protein, based on the specific activity of vanillyl-alcohol oxidase). In addition to the codon usage (see “Discussion”), the apparent lack of a good ribosome binding site may be the cause of this. To enhance the expression level of vanillyl-alcohol oxidase, a consensus Shine-Dalgarno sequence was introduced via PCR mutagenesis as described under “Experimental Procedures.” Cell extracts of \( \text{E. coli} \) TG2 harboring \( \text{pIM3972} \) showed a 7-fold increase of expression of vanillyl-alcohol oxidase when compared with \( \text{E. coli} \) TG2 transformed with \( \text{pIM3970} \).

Recombinant vanillyl-alcohol oxidase was purified from \( \text{E. coli} \) TG2 harboring \( \text{pIM3972} \) in a two-column procedure (Table II). The recombinant enzyme migrated as a single band in SDS-PAGE (Fig. 4) and was identical with vanillyl-alcohol oxidase.
oxidase from *P. simplicissimum* (1) in all aspects tested: spectral properties (250–520 nm), steady state kinetic parameters for vanillyl alcohol, and the association into octamers.

**DISCUSSION**

In this paper we have described the cloning and sequencing of the gene encoding vanillyl-alcohol oxidase from *P. simplicissimum*, the first *8a*-histidyl-FAD containing enzyme of known three-dimensional structure (7). The gene is strongly induced in *P. simplicissimum* when the fungus is grown on 4-methoxybenzyl alcohols (6). This high amount of enzyme is reflected in the abundance of 4-methoxybenzyl alcohols (6). Apart from 4-(methoxymethyl)phenol, which may represent the natural substrate, the flavin is bound to the N-3 atom of His-422 (7), whereas in flavocytochrome *a* the flavin is bound to the phenolic oxygen of Tyr-384 (25). Furthermore, as can be seen from Fig. 5, Tyr-384 of *p*-cresol methylhydroxylase is shifted 8 residues toward the N terminus compared with His-422 of vanillyl-alcohol oxidase.

In vanillyl-alcohol oxidase, these residues facilitate substrate deprotonation upon binding the phenolic moiety of the substrate. In vanillyl-alcohol oxidase, these residues are conserved parts of the sequence (101–147, 178–219, and 245–271) concern residues that are located in the FAD binding cavity. Several active site residues are also conserved. The crystal structure of vanillyl-alcohol oxidase shows that the flavin is bound to the N-3 atom of His-422 (7), whereas in *p*-cresol methylhydroxylase, the 8a-carbon of the flavin is bound to the phenolic oxygen of Tyr-384 (25). Furthermore, as can be seen from Fig. 5, Tyr-384 of *p*-cresol methylhydroxylase is shifted 8 residues toward the N terminus compared with His-422 of vanillyl-alcohol oxidase.

The crystal structure of vanillyl-alcohol oxidase shows that the flavin is bound to the N-3 atom of His-422 (7), whereas in *p*-cresol methylhydroxylase, the 8a-carbon of the flavin is bound to the phenolic oxygen of Tyr-384 (25). Furthermore, as can be seen from Fig. 5, Tyr-384 of *p*-cresol methylhydroxylase is shifted 8 residues toward the N terminus compared with His-422 of vanillyl-alcohol oxidase.

Moreover, it has been suggested that this unusual FAD-binding fold is shared by other flavoprotein oxidoreductases (30, 31). In *P. simplicissimum* the *vao* gene is induced by a limited amount of aromatic compounds (6). Apart from 4-(methoxymethyl)phenol, which may represent the natural substrate, the non-vanillyl-alcohol oxidase substrates anisyl alcohol and veratral alcohol are potent inducers. Also in *A. niger* NW156-T10,
transformed with 25–30 copies of the vao gene, strong expression of the gene was observed with these methoxybenzyl alcohols. With 4-hydroxybenzoic acid, ferulic acid, vanillic acid, and vanillyl alcohol vanillyl-alcohol oxidase was detected as well. This indicates that the vao gene in A. niger NW156-T10 is rendered under the control of at least one regulator involved in regulation of genes involved in the metabolism of aromatic compounds. Furthermore, the vao gene is both in A. niger NW156-T10 and P. simplicissimum under the control of carbon catabolite repression. Since the vao gene is expressed from its own promoter, this means that both in A. niger NW156-T10 and P. simplicissimum similar regulation mechanisms must be operative. In both organisms a surprisingly high expression level of vanillyl-alcohol oxidase is observed in the presence of veratryl and anisyl alcohol. This may be explained by assuming that these compounds, or one of their metabolites, have a high affinity for the common aromatic pathway regulator(s), most likely repressor(s). The affinity must be much higher than the

### Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>0.000</td>
</tr>
<tr>
<td>Anisyl alcohol</td>
<td>0.484</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>0.079</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>0.124</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>0.175</td>
</tr>
<tr>
<td>Vanillyl alcohol</td>
<td>0.136</td>
</tr>
<tr>
<td>Veratric acid</td>
<td>0.209</td>
</tr>
<tr>
<td>Veratryl alcohol</td>
<td>0.346</td>
</tr>
</tbody>
</table>

FIG. 2. Time course of induction of P. simplicissimum vaoA transcription and translation. P. simplicissimum was pregrown on fructose and transferred to medium containing veratryl alcohol (see “Experimental Procedures”). Samples were taken at times indicated. Panel A, Northern blot probed with 32P-labeled vaoA-cDNA (I) and reprobed with 32P-labeled A. bisporus 28S rDNA as a loading control (II). Panel B, Western blot screened with antibodies raised against vanillyl-alcohol oxidase. Panel C, vanillyl-alcohol oxidase relative activity profile.

**TABLE II**

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein Activity</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>2640 46</td>
<td>1.7 x 10^-2</td>
<td>100</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>1648 42</td>
<td>2.5 x 10^-2</td>
<td>91</td>
</tr>
<tr>
<td>Ammonium sulfate treatment</td>
<td>801 37</td>
<td>4.6 x 10^-2</td>
<td>80</td>
</tr>
<tr>
<td>Phenyl-Sepharose chromatography</td>
<td>88 38</td>
<td>0.4</td>
<td>83</td>
</tr>
<tr>
<td>Hydroxyapatite chromatography</td>
<td>24 35</td>
<td>1.5</td>
<td>76</td>
</tr>
</tbody>
</table>

FIG. 3. Effect of various carbon sources on P. simplicissimum vaoA expression in A. niger NW156-T10. A. niger NW156-T10 transformed with 25–30 copies of the P. simplicissimum vaoA gene pregrown on fructose was transferred to the following substrates: fructose (lane 2), anisyl alcohol (lane 3), ferulic acid (lane 4), 4-hydroxybenzoic acid (lane 5), vanillic acid (lane 6), vanillyl alcohol (lane 7), veratric acid (lane 8), and veratryl alcohol (lane 9) and analyzed 3 hours after transfer. Extracts were prepared by sonication. P. simplicissimum grown on veratryl alcohol (lane 10) served as a control. Lane 1, marker. Panel A, 10% SDS-PAGE gel stained with Coomassie Brilliant Blue. Panel B, UV-visualized vanillyl-alcohol oxidase. Panel C, Western blot screened with vanillyl-alcohol oxidase-specific antibodies.

FIG. 4. SDS-PAGE of purification steps of recombinant vanillyl-alcohol oxidase from E. coli TG2 transformed with plM3972 harboring the P. simplicissimum vaoA-cDNA. The Coomassie Brilliant Blue-stained 10% SDS-PAGE gel contains the following: lanes 1 and 7, marker S4; lane 2, cell extract; lane 3, 65% ammonium sulfate precipitate; lane 4, phenyl-Sepharose pool; lane 5, hydroxyapatite pool; lane 6, vanillyl-alcohol oxidase purified from P. simplicissimum.
affinity of 4-hydroxybenzoic acid, ferulic acid, and vanillyl alcohol or their metabolites. These latter compounds induce vanillyl-alcohol oxidase expression in A. niger NW156-T10 but not in P. simplicissimum. The discrepancy in the level of vanillyl-alcohol oxidase expression between A. niger NW156-T10 and P. simplicissimum during growth on 4-hydroxybenzoic acid, ferulic acid, and vanillyl alcohol may be accounted for by the following: (i) in A. niger NW156-T10 25–30 copies of the vaoA gene are present versus 1 copy in P. simplicissimum, and (ii) in the present study A. niger NW156-T10 mycelia were harvested 3 or 6 h following transfer, whereas in the studies with P. simplicissimum mycelia were allowed to grow 2 days after transfer which may have caused degradation of vanillyl-alcohol oxidase due to toxic effects and/or the poor carbon sources these aromatic compounds represent (6).

Although expression of vaoA-cDNA in E. coli TG2 cells was evident, since specific antibodies could be used to select the cDNA, the expression level was low. It was previously observed that E. coli TG2 cells are capable of producing relatively high amounts (>50 mg/liter of culture) of recombinant enzymes from multicopy plasmids like pBlueScript and pUC under the direction of the plasmid-encoded lac promoter (32, 33). Inspection of the vaoA-cDNA sequence revealed two possible explanations for the low expression. The first reason may be the codon usage. Codons that are considered modulator codons in E. coli, suppressing high expression (34), occur with a relatively high frequency in the cDNA. The second reason may be the apparent absence of a good ribosome binding site. The sources these aromatic compounds represent (6).

Although expression of vaoA-cDNA in E. coli TG2 cells was evident, since specific antibodies could be used to select the cDNA, the expression level was low. It was previously observed that E. coli TG2 cells are capable of producing relatively high amounts (>50 mg/liter of culture) of recombinant enzymes from multicopy plasmids like pBlueScript and pUC under the direction of the plasmid-encoded lac promoter (32, 33). Inspection of the vaoA-cDNA sequence revealed two possible explanations for the low expression. The first reason may be the codon usage. Codons that are considered modulator codons in E. coli, suppressing high expression (34), occur with a relatively high frequency in the cDNA. The second reason may be the apparent absence of a good ribosome binding site. The
introduction of a consensus *E. coli* ribosome binding site at the correct distance from the start codon increased the expression level only 7-fold indicating that the low expression is related to the codon usage.

Finally, this study has clearly established that expression of the *vaoA* gene in a prokaryotic or eukaryotic host results in active, fully covalently flavinylated enzyme. This suggests that the flavinylation is an autocatalytic process as shown for 6-hydroxy-p-nicotine oxidase (35, 36). However, for *p*-cresol methyldihydroxylyase it was shown that autocatalytic flavinylation only occurred after binding of the cytochrome subunit (28).

Acknowledgments—We thank Sailaja Koduri, Nanne Kamerbeek, and Robert van den Heuvel for experimental assistance.

REFERENCES