Aryl-alcohol Dehydrogenase from the White-rot Fungus *Phanerochaete chrysosporium*

**GENE CLONING, SEQUENCE ANALYSIS, EXPRESSION, AND PURIFICATION OF THE RECOMBINANT ENZYME**

(Received for publication, February 3, 1994, and in revised form, August 19, 1994)

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A cDNA clone encoding a ligninolytic aryl-alcohol dehydrogenase (AAD; EC 1.1.1.91) from the white-rot basidiomycete fungus *Phanerochaete chrysosporium* was isolated in lignin and biocharacterized. The nucleotide sequence obtained reveals an open reading frame encoding a protein of 385 amino acids. Substantial homology (49.3% identity and 67.3% similarity, respectively) was observed between AAD and an open reading frame sequence present on chromosome III of *Sacharomyces cerevisiae*. A Southern blot analysis showed the presence of multiple AAD gene-related sequences in *P. chrysosporium* and in other white-rot fungi including *Berkendrea adusta* and *Fomes lignosus*. Northern blot analyses are in line with the view that the levels and appearance of AAD mRNA correlate with the level and appearance of AAD activity and that, under conditions of nitrogen limitation, the AAD mRNA levels are higher than in carbon limited cultures. This is consistent with the regulation of the enzyme by carbon or nitrogen limitation being at the level of transcription. Moreover, the appearance of AAD-specific transcripts correlates with the appearance of lignin peroxidase-specific transcripts in the same cultures. This co-appearance is in line with the proposed synergistic interaction of the two enzymes in lignin biodegradation, which suggests a similar regulation. The AAD encoding cDNA was expressed in *Escherichia coli* to yield high levels of active enzyme, and the recombinant enzyme was purified by using metal chelate affinity chromatography.

Lignin depolymerization by white-rot basidiomycetes such as *Phanerochaete chrysosporium* is catalyzed by extracellular enzymes including lignin peroxidases and manganese-dependent peroxidases. When the successful search for lignin peroxidase was reported several years ago (Tien and Kirk, 1983; Glenn et al., 1983), it was considered the key enzyme in lignin degradation. However, although the extensive characterization of these extracellular peroxidases revealed their necessity for lignin degradation, subsequent studies also indicated the need for additional enzymes (reviewed by Kirk and Farrell (1987), Schoemaker et al. (1989), Pease and Tien (1991), Cullen and Kersten (1992), and Fiechter (1993)). The constant oxidation of lignin by peroxidases will end up in a very oxidized state of the polymer and subunit or degradation products thereof, with many aldehyde-, quinone-, and possibly also acidic groups present. As a consequence, peroxidase catalysis will stop. Thus, aside from oxidative reactions, reductive ones are also likely to be needed for lignin biodegradation to occur (Schoemaker et al., 1989). Such activities were found in white-rot fungi some decades ago, but their activities were not linked to lignin biodegradation at that time (Farmer et al., 1959; Zenk and Gross, 1965). In 1988 a model was put forward (Leisola et al., 1988) in which the symphonic action of oxidative peroxidases and reductive, intracellular enzymes is needed for complete degradation of the lignin biopolymer. These reductive activities have recently been identified in *P. chrysosporium* (Muheim et al., 1991; Constam et al., 1991). A reducing activity was detected in agitated as well as non-agitated, nitrogen-limited cultures of *P. chrysosporium* 2 days after inoculation, reaching its maximum after 6 days using veratraldehyde as a substrate, but in carbon-limited, agitated cultures, the veratraldehyde reduction rates reached were considerably lower. The activity of this aryl-alcohol dehydrogenase (AAD) appeared synchronously with the ligninolytic activity, and the production of AAD during secondary metabolism points to its possible involvement in lignin biodegradation. The enzyme showed a major band with an apparent molecular mass of 47 kDa, whereas gel filtration experiments suggested a molecular mass of 280 kDa. Polyclonal antibodies raised against the highly purified 47-kDa protein were able to immunoprecipitate the activity, indicating that it is part of the enzyme. Further biochemical characterization of the purified enzyme showed a broad specificity toward aromatic compounds and the ability to also reduce dimeric compounds, underlining the direct involvement of the enzyme in lignin degradation. Leisola et al. (1988) proposed the possible need of AAD in the biodegradation of veratryl alcohol, which is one of the most simple lignin model compounds, and later on Shoemaker et al. (1989) and Muheim (1991) expanded this view to other model compounds and to lignin in general.

With a view toward investigating the regulatory mechanism(s) controlling AAD production and assessing the importance of AAD in lignin biodegradation, we have cloned and...
determined the nucleotide sequence of an AAD cDNA. In addition, we have expressed the cloned cDNA in *Escherichia coli* to yield high levels of active enzyme.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Radiochemicals, and Enzymes**—All chemicals were commercial preparations, of reagent or sequencing grade whenever possible. ([α-35S]Thio-dATP (1000 Ci mmol⁻¹) and ([α-32P]dCTP (3000 Ci mmol⁻¹) were from Amersham Corp. [1].

**Strains, Media, and Growth Conditions**—Bacterial strains used were *E. coli* DH5α (Life Technologies, Inc.), Y102 (Young and Broda, 1983a), and XL1-Blue (Bullock et al., 1987). They were cultivated in LB or 2 x YT medium (Maniatis et al., 1982). The fungal strains used have been described before (Waldner et al., 1988). They included *P. chrysosporium* BKM-F-1767 (Streptosporangium purpurascens, ATCC 24725), *B. adusta* (Polyporus adustus, CBS 42530), *P. ostreatus* (ATCC 32783), *C. versicolor* (ATCC 42530), and *F. lignosus* (CBS 50050). Cultures of *P. chrysosporium* were inoculated either using conidia or basidiospores. For cultures of *B. adusta*, *P. ostreatus*, *C. versicolor*, and *F. lignosus*, the inoculum consisted of mycelial anasts previously homogenized with a Polytron homogenizer (Kinematik, Luzern, Switzerland). *P. chrysosporium* cultures were start at 37 °C, and *B. adusta*, *C. versicolor*, and *P. ostreatus* were grown at 30 °C. *P. lignosus* and *F. lignosus* were cultivated at 25 °C. Two media, modifications of that used by Kirk et al. (1978), were used. The nitrogen-limited medium contained 0.22 g liter⁻¹ (2.4 mM) ammonium tartrate as a nitrogen source and 10 g liter⁻¹ glucose and 0.66 g liter⁻¹ (7.2 mM) ammonium tartrate contained 2 g liter⁻¹ glucose and 0.66 g liter⁻¹ (7.2 mM) ammonium tartrate as a nitrogen source and 10 g liter⁻¹ glucose. The nitrogen-limited medium was supplemented with 2 g liter⁻¹ glucose and 0.66 g liter⁻¹ (7.2 mM) ammonium tartrate diluted with 3 ml of 100/40 and applied to the column. The column was washed with 15 ml of BC 100/40, followed by elution with 7.5 ml each of BC 100/40 and BC 100/400. For enzymatic assays, the column eluates were used directly, and for SDS-PAGE analyses the eluates were dialyzed against 100 mM sodium phosphate buffer, pH 7.2. 0.9% NaCl and subsequently concentrated using StrataClean™ resin (Strategene Cloning Systems) (Nielsen et al., 1993). The enzyme activity was assessed by analyzing the reaction products by HPLC (Muheim et al., 1991).

**SDS-PAGE and Western Blot Analysis**—Proteins were separated by SDS-PAGE (Neville, 1971) and transferred electrophoretically onto a nitrocellulose membrane (Schleicher & Schuell, 0.45 μm) using 15 mM sodium phosphate buffer, pH 6.8, as described by Reiner and Stark (1983). After the transfer, the filters were blocked in TBS (20 mM Tris-HCl, 150 mM NaCl) containing 5% Bacto skim milk (Difco) for 1 h at room temperature. The primary rabbit anti-AAD antibody (Muheim et al., 1991) was diluted 1:500, essentially as described above for the Western blot analysis. The background could be reduced by preadsorption of the antisera with total *E. coli* cell proteins immobilized on CNBr-activated Sepharose (Sambrook et al., 1989). In a first cycle, several positive phage clones were isolated from a total of 100,000 clones tested. These were then purified in a second cycle. A total of 14 phage clones isolated from library 2 proved to be positive in the second cycle, whereas only 2 of the clones from library 1 reacted positively.

**Antibody Screening of P. chrysosporium agt11-based cDNA Expression Libraries**—The *P. chrysosporium* cDNA libraries used have been described before (Walter et al., 1988; Prinnow et al., 1989). They were originally prepared using RNA from 6-day-old carbon-limited cultures of *P. chrysosporium* BKM-F-1767 (Walter et al., 1988) or from 5- and 6-day-old nitrogen-limited cultures of *P. chrysosporium* OCG101 (Schule, 1983). Positive clones were subsequently subjected to a simple screening procedure (Young et al., 1983b; Sambrook et al., 1989) with anti-AAD antibodies (Muheim et al., 1991) diluted 1:500, essentially as described above for the Western blot analysis. The background could be reduced by preadsorption of the antisera with total *E. coli* cell proteins immobilized on CNBr-activated Sepharose (Sambrook et al., 1989).

**Purification of Recombinant AAD from E. coli**—The *E. coli* expression vector pTrc99A (Pharmacia Biotech Inc.) (Amann et al., 1988) containing the AAD gene sequence data. The proteolytic fragments of the protein were separated by gel electrophoresis on a 0.8% agarose gel. The following primers were used: primer 1, 5'-AGCGGATAACAAT'CACACAGGA, primer 2, 5'-CTCGTGAGATCCTGTGGGCCGAGTACG.

**Subcloning and DNA Sequencing**—Deletion subclones were created using restriction enzymes by following the procedures compiled by Sambrook et al. (1989). Plasmids were transformed into *E. coli* as described by Chung and Miller (1988). Nucleotide sequencing was carried out according to Tabor and Richardson (1987) using Sequenase (U. S. Biochemical Corp.) or T7 DNA polymerase (Pharmacia) following the protocols provided by the supplier. The cloned PCR fragment was sequenced using six different oligonucleotides (18-mers) as primers. For Maxam-Gilbert sequencing (Maxam and Gilbert, 1980), restriction fragments were extracted from low melting temperature agarose gels. The following primers were used: primer 1, 5'-AGCGGATAACAAT'CACACAGGA, primer 2, 5'-CTCGTGAGATCCTGTGGGCCGAGTACG.

**DNA Isolation and Southern Blot Analysis**—Total DNA from *P. chrysosporium*, *B. adusta*, *C. versicolor*, *P. ostreatus*, and *F. lignosus* was isolated essentially as described by Raeder and Broda (1988). For Southern blot analysis (Southern, 1975), the fragments were separated in a 0.8% agarose gel and transferred onto a GeneScreen Plus™ membrane (DuPont NEN) according to the procedure recommended by the manufacturer. Hybridization was carried out overnight in the presence of 50% formamide, 1% SDS, 1x NaCl, and 10% dextran sulfate at 62 °C. The blot was washed twice at 62 °C in 1x SSC, 0.1% SDS followed by a wash step at 55 °C in 0.1 x SSC, 0.1% SDS for 30 min. For the preparation of radioactively labeled probes, the

**Expression of AAD cDNA in E. coli**—The *E. coli* expression vector pTrc99A (Pharmacia Biotech Inc.) (Amann et al., 1988) containing the strong tac (trc) promoter was used for overexpression of the AAD cDNA in *E. coli* XL1-Blue cells (Bullock et al., 1987). For AAD activity assays, cells were harvested by centrifugation, resuspended in 0.04 volume of ice-cold 50 mM Tris-HCl, pH 7.5 and disrupted by sonication (six times, 15 s each). After centrifugation for 10 min at 11,000 x g and 4 °C, the enzyme activity was analyzed by following the oxidation of NADPH to NADP⁺ in the presence of veratraldehyde as described earlier (Muheim et al., 1991).

**Polymerase Chain Reaction**—For PCR amplification, 10 μg of each genomic DNA 10 μl of 2 μM dNTP mixture, 1 μl of 10 μl of 10x PCR polymerase buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl), 15 μM MgCl₂, 1% Triton X-100, 0.1% gelatin; ANAWA, Wangen, Switzerland, 2.5 units of Taq polymerase (ANAwa) were combined and the volume adjusted to 100 μl. Denaturation was at 15 min for 54 °C, annealing for 2 min at 50 °C, and polymerization for 2 min at 72 °C using a Perkins-Elmer GeneAmp 9600 system. The cycle was repeated 30 times. After a 10-min incubation at 72 °C, 10 μg of carrier glycerogen (Boehringer Mannheim) were added and the reaction extracted with 100 μl of 1:1 mixture of phenol and chloroform. The DNA was precipitated with ethanol and washed with 70% ethanol. The DNA was resuspended in 50 μl of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 70% ethanol, 1 μl of resuspended DNA was used for PCR amplification using gene-specific primers described in Table 1.

**Preparation and Sequence Determination of Cytosine Bromide Fragments**—Purified AAD was electrophoresed on a 12% SDS-polyacrylamide gel (Laemmli, 1970) and, after electrophoretic transfer onto a Bio-Rad polyvinylidene difluoride membrane using 10 μs CAPS buffer, pH 11, 20% methanol (LeGendre and Matsudaira, 1989) for 45 min at 500 mA. The blot was stained with 0.02% Coo-
method of Feinberg and Vogelstein (1984) was used.

Isolation of RNA and Northern Blotting—The pellets from a 600-ml *P. chrysosporium* culture were washed with distilled water, filtered, and frozen immediately in liquid nitrogen and subsequently opened using a mortar. The powder obtained was then thawed in a solution of 4 M guanidine isothiocyanate, 0.5% sodium laurylsarcosinate, 0.5 M sodium citrate, pH 7.0, 0.1 M β-mercaptoethanol (Teeri et al., 1987). After removing the cell debris by centrifugation, the RNA was purified by pelleting through a CsCl cushion (5.7 M) for 24 h at 33,000 rpm (15°C) using a Beckman SW41 rotor. The pellet was dissolved in diethylpyrocarbonate-treated water and the RNA precipitated with 2.5 volumes of ethanol. For Northern blot analysis, 30 μg of total RNA were separated on a 6% formaldehyde, 1% agarose gel.

**RESULTS AND DISCUSSION**

Isolation of AAD Encoding cDNAs—Two *P. chrysosporium*Agt11-based cDNA libraries were used. One of them was initially prepared using RNA isolated from 6-day-old carbon-limited cultures (library 1; Walther et al., 1988), while the other had been prepared using RNA from 5- and 6-day-old nitrogen-limited cultures (library 2; Prinnow et al., 1989). Both libraries were screened through two cycles using polyclonal antibodies directed against AAD (Muheim et al., 1991). To compare the immunopositive phage clones at the molecular level, the phage DNAs were isolated, digested with EcoRI, and analyzed in a Southern blot. For this comparison the insert fragment of one of the clones was labeled. While 12 of the clones isolated from library 2 all turned out to be related at the nucleotide sequence level (data not shown), the clones isolated from library 1 did not react positively.

**Nucleotide Sequence Analysis of an AAD Encoding cDNA**—The phage clone containing the largest insert was used for further studies. The 1.3-kilobase pair insert fragment was subcloned into the pUC18 vector (Yanisch-Perron et al., 1985) previously digested with EcoRI and treated with alkaline phosphatase resulting in plasmid pAM1. The complete DNA sequence of the insert was determined on both strands using the dideoxy method (Sanger et al., 1980) and proved to be identical with the experimentally determined open reading frame. The ATG codon closest to the 5'-end of the cDNA is at position 28 (Fig. 1C). It is within the sequence ACAGCAATGAA, which is similar to the ATG context of a range of filamentous fungal genes (Gurr et al., 1987) and includes the important -3 A residue of the Kozak consensus sequence (Kozak, 1987). However, since the NHz-terminal amino acid sequence of the AAD protein is unknown, the methionine residue at position 28 is not necessarily the starting amino acid. The size of the putative AAD monomer has previously been estimated to be roughly 47 kDa, corresponding to a protein chain of about 427 amino acids and thus to a mRNA of at least 1280 nucleotides in length. This is an overestimation, however, since the *E. coli*-produced AAD originating from the cloned cDNA comigrated at around 43 kDa with highly purified *P. chrysosporium* AAD in front of the 45-kDa ovalbumin marker in SDS-polyacrylamide gels (see Fig. 5B) and thus the cDNA analyzed appears to be complete as judged by this fact. This is
also supported by the observation that the sequence can be expressed in an active form in E. coli (see below).

Correlation between the cDNA-encoded AAD and the Fungus-derived Enzyme—In an attempt to analyze the amino terminus of the AAD protein, the highly purified dehydrogenase from \textit{P. chrysosporium} was cleaved with \textit{Cia}I. The fragments were separated in a 0.8% agarose gel and transferred onto a GeneScreen Plus\textsuperscript{TM} membrane and probed with the radiolabeled AAD cDNA fragment. Note that there are no \textit{Cia}I sites in the AAD cDNA. \textit{Lane 1}, \textit{P. chrysosporium} DNA; \textit{lane 2}, \textit{F. lignosus} DNA; \textit{lane 3}, \textit{C. versicolor} DNA; \textit{lane 4}, \textit{B. adusta} DNA; \textit{lane 5}, \textit{P. ostreatus} DNA. The positions of the size markers, HindIII fragments of a DNA, are indicated.

\textbf{AAD Gene Cloning, Expression, and Purification}

**Fig. 2. Comparison of AAD sequence and \textit{S. cerevisiae} chromosome III ORF.** The UWGGC align program (Devereux et al., 1984) was used to compare the derived AAD protein sequence (upper line) with the \textit{S. cerevisiae} YCR107w ORF sequence (Oliver et al., 1992). Asterisk indicates a match across the two sequences, and dot indicates a conservative substitution.

**Fig. 3. Comparison of several white-rot fungi for the presence AAD-related gene sequences by Southern blot analysis.** The DNA (5 pg) was cleaved with \textit{Cia}I. The fragments were separated in a 0.8% agarose gel and transferred onto a Genescreen Plus\textsuperscript{TM} membrane and probed with the radiolabeled AAD cDNA or a 32P-labeled synthetic oligonucleotide corresponding to the CLG4 lignin peroxidase cDNA (De Boer et al., 1987).

\textit{P. chrysosporium} was treated with cyanogen bromide in an acidic medium. This procedure led to the scission of the protein at the various methionine residues. The newly created amino termini were subsequently reacted with phenylisothiocyanate according to the procedure of Frank et al. (1993). The phenylisothiocyanatoamino acids liberated were analyzed, and two more cycles of degradation and analysis were subsequently carried out. The pattern of the amino acids liberated in the second cycle correlates as far as this qualitative amino acid fingerprinting method is concerned.

**Correlation between \textit{S. cerevisiae} chromosome III ORF and \textit{P. chrysosporium} gene expression in \textit{P. chrysosporium}.** RNA was isolated from 2-, 4-, and 6-day carbon- and nitrogen-limited cultures of \textit{P. chrysosporium}. 30 pg of total RNA were analyzed. A, AAD gene probe; B, lignin peroxidase gene probe. \textit{Lanes 1}, 2, and 3, RNA from 2-, 4-, and 6-day-old nitrogen-limited cultures, respectively; \textit{lanes 4}, 5, and 6, RNA from 2-4, and 6-day-old carbon-limited cultures, respectively. Probing was done either using the radiolabeled AAD cDNA or a 32P-labeled synthetic oligonucleotide corresponding to the CLG4 lignin peroxidase cDNA (De Boer et al., 1987).
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A

AGGAAACAGACC ATG GAA TTC GTT GTC GAC TGT CAG TAC AGC ATG AAC ATC
M E F V V D C Q Y S M N I

EcoRI

B

C

Fig. 5. Expression of AAD cDNA in E. coli. A, nucleotide sequence of the beginning of the protein-encoding region of the AAD expression plasmid pTrc99A-AAD. Sequences derived from the AAD cDNA are shown in bold. The Shine-Dalgarno sequence is underlined, and the EcoRI site, which was used for cloning the AAD cDNA into the pTrc99A expression vector, is indicated. B, heterologous production of AAD in E. coli XL1-Blue. Cells containing either plasmid pTrc99A-AAD (lanes 2 and 3) or pTrc99A with a control insert (lanes 4 and 5) were grown in 2x YT medium at 30 °C and processed as described under "Experimental Procedures." A 12% SDS-polyacrylamide gel was prepared and run according to Neville (1971) and stained with Coomassie Brilliant Blue R-250. Lane 1, molecular size markers (Bio-Rad) the sizes of which (in kDa) are indicated on the left; lanes 2 and 4, extracts from IPTG-induced cells; lanes 3 and 5, extracts from uninduced cells; lane 6, purified AAD from P. chrysosporium (peak fraction from a phenyl-Superose column; see Muheim et al. (1991)). The 30-kDa band is due to a contaminating protein. C, Western blot analysis. Extracts were analyzed in a 12.5% SDS-polyacrylamide gel (Neville, 1971). Lane 1, prestained molecular size markers (Bio-Rad); lane 2, AAD from P. chrysosporium (phenyl-Superose peak fraction); lanes 3 and 4, extracts from XL1-Blue cells harboring pTrc99A with a control insert; lanes 5 and 6, extracts from XL1-Blue cells harboring pTrc99A-AAD. Lanes 4 and 6 represent extracts from IPTG induced cells.

Similarity between AAD and an ORF Sequence Encoded by Chromosome III of Saccharomyces cerevisiae—The inferred AAD protein sequence was compared with those of other known proteins using the UWGCG BESTFIT and ALIGN programs (Devereux et al., 1984). The best agreement was obtained with an ORF sequence (YCR107w) present on chromosome III of S. cerevisiae (Oliver et al., 1992). This comparison revealed 49.3% identity and 67.3% similarity between the two sequences (Fig. 2). The S. cerevisiae sequence shows similarity with a Nicotiana tabacum auxin-induced mRNA (Oliver et al., 1992). A comparison between the AAD sequence and the N. tabacum derived sequence revealed 27.7% identity and 51.7% similarity. Since the functions of the S. cerevisiae and N. tabacum proteins are not known (Oliver et al., 1992; Tanaka and Isono, 1993; Van der Zaal et al., 1987), the significance of the observed homologies remains open.

AAD Gene-related Sequences in Different White-rot Fungi—Since the AAD activity is assumed to be important for lignin biodegradation, the presence of AAD within the group of white-rot fungi is expected. Therefore, several lignin degrading basidiomycetes including B. adusta, P. ostreatus, F. lignosus, and C. versicolor were tested for the presence of AAD-related DNA sequences. The fungal DNAs were digested with ClaI and analyzed in a Southern blot using the AAD cDNA as a probe (Fig. 3). This probe fragment does not to contain a restriction site for ClaI. DNA from P. lignosus, B. adusta, and P. chrysosporium revealed strong signals, suggesting the presence of related sequences in these fungi. On the other hand, DNA from...
**Fig. 6. Generation and purification of AAD6His fusion proteins.** A, construction of the pTrc99A-AAD6His expression plasmid. The AAD PCR fragment was generated as described under “Experimental Procedures.” It was ligated together with a BgIII-EcoRI adaptor fragment to pTrc99A vector DNA previously treated with EcoRI and alkaline phosphatase (CIP). B, sequence at the COOH terminus of the AAD6His fusion protein. C, purification of AAD6His fusion protein by NTA-agarose chromatography. Samples: 1-3, extract of uninduced XL1-Blue cells; 4-6, extract of induced cells. Lanes 1 and 4, column input; lanes 2 and 4, column flow-through; lanes 3 and 6, BC 1000 eluates; lane 7, purified AAD from *P. chrysosporium*. The samples were analyzed on a 12.5% SDS-polyacrylamide gel (Neville, 1971).

*C. versicolor* and *P. ostreatus* showed rather faint bands, and it is difficult to predict at this point whether these bands reflect related DNA sequences or not. Interestingly, several bands hybridized in cases where strong hybridization signals were obtained. Some of the bands may have been caused by restriction fragment length polymorphisms. Such a situation has been observed in the case of the lignin peroxidase gene family (Raeder et al., 1989; Gaskell et al., 1991), and this complication was circumvented by the use of basidiospores, which are known to be monokaryotic (Gold and Cheng, 1979; Alic and Gold, 1985). The hybridization pattern seen in Fig. 3 (lane 1) was also obtained using DNA from a basidiospore-derived culture of *P. chrysosporium*. Nevertheless multiple bands hybridizing to the probe are apparent, possibly indicating the presence of multiple AAD genes. In *P. chrysosporium* multiple genes have been observed for lignin peroxidases (Raeder et al., 1989; Huoponen et al., 1990), for Mn(II)-dependent peroxidases (Pribnow et al., 1989; Pease et al., 1989), and for cellulases (Covert et al., 1992),
and such a multicytope family may exist for AAD as well.

Expression of AAD RNA by *P. chrysosporium*—To investigate the regulation of AAD synthesis in *P. chrysosporium*, the corresponding RNA levels were determined in both carbon- and nitrogen-limited cultures. Total RNA was isolated, 2, 4, and 6 days after inoculation and analyzed in Northern blots using the labeled AAD cDNA as a probe. It was evident from this analysis that carbon-limited cultures showed lower levels of AAD transcripts than nitrogen-limited cultures (Fig. 4A). In nitrogen-limited conditions at days 4 and 6, almost equal amounts of AAD RNA were detected, whereas 2 days after inoculation no dehydrogenase RNA could be detected indicating that the gene is expressed during secondary metabolism. The same situation applied to carbon-limited cultures. Such cultures showed no AAD RNA at day 4. However, on day 6 low levels of AAD transcripts could be detected. The levels of AAD RNA in carbon-limited cultures were more than 10 times lower than the corresponding levels found in nitrogen-limited cultures. The patterns of appearance of the lignin peroxidase-specific transcripts (Fig. 4B) and the ones of the AAD-specific transcripts correlate well (see also Reiser et al., 1993). This suggests that the accumulation of the dehydrogenase is controlled similarly to lignin peroxidase. The appearance of AAD activity in *P. chrysosporium* cultures has been investigated previously (Muheim et al., 1991). The pattern seen at the level of the activity parallels that seen at the RNA level. Thus, AAD enzyme production appears to be regulated at the level of transcription by nitrogen or carbon limitation.

Heterologous Expression of the AAD cDNA in *E. coli*—To unequivocally demonstrate that an AAD-specific sequence had been cloned, attempts were made to express the cDNA in *E. coli*. For this purpose, the cDNA was subcloned into the pTrc99A expression vector (Aman et al., 1988) to yield plasmid pTrc99A-AAD. In this plasmid the expression of the AAD cDNA is controlled by the regulatable *E. coli* tac promoter (De Boer et al., 1983). The DNA sequence including the Shine-Dalgarno sequence and the beginning of the protein encoding region is shown in Fig. 5A. Plasmid pTrc99A-AAD was transformed into *E. coli* XL1-Blue (Bullock et al., 1987), and the cells were cultivated at 30°C in 2 × YT medium. IPTG was added to the cells during the early exponential phase to induce the production of AAD, and the cells were collected 3 h after induction and analyzed by SDS-PAGE (Fig. 5B) and by Western blotting (Fig. 5C). It is evident from this analysis that an additional band of around 43 kDa appeared in IPTG-induced cultures of *E. coli* XL1-Blue cells harboring plasmid pTrc99A-AAD (Fig. 5B, lane 2) but not in cells harboring pTrc99A with a control insert (Fig. 5B, lane 4). In addition, this band comigrated with purified AAD (Fig. 5B, lane 6) and it reacted specifically with anti-AAD antibody in Western blots (Fig. 5C, lane 6).

Having demonstrated abundant production of AAD in *E. coli* XL1-Blue cells, AAD activity was determined to see if the protein was functional. The cells were grown at 30°C in 2 × YT medium containing 0.4 mM sucrose to an *A*_{600} of 0.3, and the enzyme production was induced by adding 1 mM IPTG. Aliquots were removed before induction and at various times after induction. After opening the cells using sonification, the NADPH oxidizing activity in the supernatant was measured. Significant levels of such an activity were detected before induction, but 4–5 times more activity was present in IPTG-induced cultures, indicating that the AAD was active and that NADPH oxidizing activities unrelated to AAD are also present in crude extracts of *E. coli* (data not shown). 100 ml of a culture produced up 19 units of active AAD (data not shown).

**Purification of Recombinant AAD by Metal Chelate Affinity Chromatography**—To investigate more directly the activity of the *E. coli*-derived AAD, the recombinant AAD was purified by metal affinity chromatography (Hochuli et al., 1988). This single-step strategy was facilitated by the addition of 6 histidine residues at the carboxyl terminus of the protein according to the strategy depicted in Fig. 6A. In this construct, the last amino acid of the AAD protein was changed from Lys to Arg and 8 additional amino acids (His)\textsubscript{6}-Arg-Ser were spliced on (Fig. 6B). Crude cell extracts of *E. coli* XL1-Blue harboring pTrc99A-AAD6His were prepared and applied onto a Ni\textsuperscript{2+}-NTA-agarose column. In the presence of 40 mM imidazole, most of the soluble bacterial proteins did not bind to the Ni\textsuperscript{2+}-NTA column, whereas a significant portion of the AAD6His fusion protein was retained as judged from the SDS-gel analysis shown in Fig. 6C (lane 5) and from a Western blot analysis (data not shown). A step of 1 M KCl and 100 mM imidazole (BC 1000/1000) released substantial amounts of the fusion protein (Fig. 6C, lane 6) and a final wash of 1 M KCl, 2 mM imidazole (BC 1000/2000) eluted the remaining 10% of the fusion protein (data not shown). Compared to the highly purified enzyme from the fungus (lane 7), the fusion protein had a slightly reduced mobility. This difference is most likely due to the 10 additional amino acids at the amino terminus (see Fig. 5A) and to the 8 additional residues at the carboxyl terminus. Small amounts of other proteins were also eluted (lanes 3 and 5), and trace amounts of a 43-kDa protein were also present in the BC 1000/100 eluate of uninduced cell extract (lanes 3). Up to 75% of the applied AAD activity was recovered in the BC 1000/100 eluate (data not shown), and up to 600 μg of highly purified AAD fusion protein were obtained per 100 ml of culture. The specific activity of the fusion protein was in the order of 15–25 units/mg, which is about half to one-third of that measured for the fungus-derived enzyme (Muheim et al., 1991), indicating that the affinity tail at the carboxyl terminus and/or the extra amino acids at the amino terminus may have affected the enzyme. The protein present in the BC 1000/100 eluate from induced cells was capable of reducing veratraldehyde to veratral alcohol as shown by HPLC analysis (data not shown) indicating that bona fide AAD activity was present in this fraction.

Acknowledgments—We are most grateful to M. H. Gold for providing us with the cDNA library and to J. Nagle for help with the DNA sequence analysis. We thank S. Hochmann for competent technical assistance.

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