Molecular Cloning and Heterologous Expression of the Gene Encoding Dihydrogeodin Oxidase, a Multicopper Blue Enzyme from Aspergillus terreus*

(Received for publication, May 15, 1995, and in revised form, July 6, 1995)

Ke-xue Huang†, Issao Fujii†, Yutaka Ebizuka†, Katsuya Gomi†, and Ushio Sankawa†

From the †Faculty of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, J apan and ‡National Research Institute of Brewing, 2-6-30 Takinogawa, Kita-ku, Tokyo 114, J apan

Aspergillus terreus dihydrogeodin oxidase (DHGO) is an enzyme catalyzing the stereospecific phenol oxidative coupling reaction converting dihydrogeodin to (+)-geodin. We previously reported the purification of DHGO from A. terreus and raised polyclonal antibody against DHGO. From the first cDNA library constructed in λgt11 using mRNA from 3-day-old mycelium of A. terreus, four clones were identified using anti-DHGO antibody, but all contained partial cDNA inserts around 280 base pairs. This cDNA fragment was used as a probe to clone the genomic DNA and cDNA for dihydrogeodin oxidase from A. terreus. The sequence of the cloned DHGO genomic DNA and cDNA predicted that the DHGO polypeptide consists of 605 amino acids showing significant homology with multicopper blue proteins such as laccase and ascorbate oxidase. Four potential copper binding domains exist in DHGO polypeptide. The DHGO gene consists of seven exons separated by six short introns. Expression of the DHGO gene in Aspergillus nidulans under the starch or maltose-inducible Taka-amyrase A promoter as an active enzyme established the functional identity of the gene. Also, introduction of the genomic DNA for DHGO into Penicillium frequentans led to the production of DHGO polypeptide as judged by Western blot analysis.

Phenol oxidative coupling is one of the most important reactions in the biosynthesis of natural products. In 1957, Barton and Cohen (1) proposed that new C–C or C–O bonds of either intra- or intermolecules could be formed by the pairing of radicals that are produced by one-electron oxidation of phenols. Since then, importance of phenol oxidative coupling reaction that is involved in the biosynthesis of a wide range of natural products such as alkaloids (e.g. morphine, lycorine), lignans, and other phenolic compounds (e.g. usnic acid, griseofulvin) of versatile origins has been recognized (2). Therefore, a variety of chemical and biological oxidizing systems has been developed for oxidations of phenolic compounds (3–5). However, model enzyme systems using laccase or peroxidase have been unable to reproduce regio- and stereospecificities, which are often observed in natural product biosynthesis. Hence, elucidation of the true nature of the biocatalysts involved in specific biosynthetic phenol oxidative couplings has been the subject of intensive research for many years.

Fungi produce a group of compounds called grisans. Formation of the unique spiro structures of grisanes such as griseofulvin, geodin, and their derivatives from corresponding benzenophenone precursors had been thought to be catalyzed by the specific phenol oxidative coupling enzymes (6). Enzymatic formation of grisan structure of geodin was first demonstrated by Komatsu (7) using cell-free extracts of Penicillium estingenum. The enzyme, which was suggested to be copper protein, catalyzed regio- and stereospecific formation of (+)-geodin from benzophenone dihydrogeodin. On the other hand, model enzyme systems using laccase or peroxidase gave optically inactive geodin (8). No specific enzyme activity has been detected for dehydrogriseofulvin formation.

We have been working on the biosynthesis of (+)-geodin in Aspergillus terreus (Fig. 1) at the enzyme level (9–13) and previously reported the purification of dihydrogeodin oxidase (DHGO) from A. terreus (9), which catalyzes the regio- and stereospecific formation of (+)-geodin from dihydrogeodin. This was the first example of purification and characterization of the specific phenol oxidative coupling enzyme. The purified DHGO showed an intense blue color and had absorption maximum at 600 nm. The EPR spectrum of DHGO clearly indicated the presence of type-1 and type-2 copper atoms in the enzyme molecule. These facts suggested that DHGO is a multicopper blue protein. Similar properties were also reported for sulochrin oxidases from Penicillium frequentans and Oospora sulfree-ochracea by Nordlöf and Gatenbeck (14). Both enzymes catalyze specific formation of bisdechlorogeodin but in opposite stereospecificity. Recently, we purified sulochrin oxidase from P. frequentans; although the clarified properties were found to be quite different from those reported by Nordlöf and Gatenbeck.

Zenk et al. (15) reported that cytochrome P-450 enzymes catalyze phenol oxidative coupling reactions involved in benzyisoquinoline alkaloid biosynthesis. Recently, they purified a cytochrome P-450 enzyme that mediates specific intermolecular phenol oxidative coupling reaction to form bisbenzylisoquinoline alkaloid berbamunine (16). Presence of peroxidase-like stereoselective phenol coupling enzyme is reported for ligan biosynthesis (17). Therefore, it is very interesting to know how the same type of phenol oxidative coupling reactions are driven by the different kind of oxidation enzymes such as cytochrome P-450s, peroxidase-like enzymes in plants, and

1 The abbreviations used are DHGO, dihydrogeodin oxidase; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; MS, mass spectrum; bp, base pair(s); kb, kilobase(s).

* This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) D49538.

To whom correspondence should be addressed. Tel./Fax: 81-3-5689-2962; E-mail: yebiz@mol.f.u-tokyo.ac.jp.
multicopper blue enzymes in fungi.

To elucidate the precise nature of the biocatalysts involved in the specific phenol oxidative coupling reactions, we decided to clone the gene for DHGO from A. terreus. In this paper, we describe the cloning, sequencing, and heterologous expression of the DHGO gene as the first example of a multicopper blue protein that catalyzes regio- and stereospecific phenol oxidative coupling reaction.

MATERIALS AND METHODS

Strains and Culture Conditions—Escherichia coli strains DH5α (Life Technologies, Inc.), XL1-Blue (Strategene), and Y1090 and Y1090r (18) were used.

A. terreus strain IMI 16043 was used, and its culture condition was previously described (9). Aspergillus nidulans strain FGSC 89 (argB, biA) was maintained on arginine and biotin-supplemented Aspergillus minimal medium described by Pontecorvo et al. (19). A. nidulans strain FGSC 4 (wild type) was obtained from Fungal Genetics Stock Center (The University of Kansas Medical Center, Kansas).

P. frequentans CMI 96659 was obtained from Commonwealth Mycological Institute (United Kingdom), which was maintained and cultured in the same way as that of A. terreus IMI 16043 (9).

Vectors—The expression phage vector pH3.11 was obtained from Promega. λZAP II and pBluescripts were from Stratagene. pT7 Blue from Novagen was used for direct cloning of polymerase chain reaction products. pTAex33 is a shuttle vector carrying the ampicillin resistance gene and the argB gene of A. nidulans for selection in E. coli and arginine prototrophy selection in fungi, respectively. The plasmid also carries the promoter and terminator region of the Taka-amylase Agene (10) for selection in fungi, respectively. The plasmid also carries the promoter and terminator region of the Taka-amylase Agene (10) for selection in fungi, respectively.

Cloning of Dihydrogeodin Oxidase Gene from A. terreus

The N-terminal amino acid sequence of the purified DHGO was determined as described (22) using Applied Biosystems 473A peptide sequencer according to the manufacturer's instructions.

Antibody Production—Polyclonal antibodies recognizing DHGO were obtained by immunizing rabbits with the purified native DHGO first with complete Freund adjuvant and then with incomplete Freund adjuvant as a booster 2 weeks after first immunization. IgG fraction was obtained by passage through a protein A affinity column.

SDS-PAGE and Western Blot Analysis—SDS-PAGE was performed essentially as described by Laemmli (23). Gels of 10.5% acrylamide and 0.5% bisacrylamide were used and electrophoresed in a modified Laemmli buffer system (0.025 M Tris base, 0.192 M glycine, 0.05% SDS). Gels were stained with a silver stain kit of Kanto Chemicals (Japan) or Coomassie Brilliant Blue using Quick CBB kit (Wako Pure Chemicals, Japan). After separation on SDS-PAGE, proteins were blotted onto nitrocellulose membrane (Schleicher and Schuell) by Bio-Rad electro-blotting apparatus according to the manufacturer’s instructions. The filter was detected by enzyme-linked immunodetection with anti-DHGO antibody and anti-rabbit IgG-peroxidase conjugate using Konica immunostain horseradish peroxidase kit (Konica, Japan).

Fungal Genomic DNA Isolation and Southern Blot Analysis—Genomic DNAs of fungi were isolated by the modified procedure of Bien and Crouch (24) previously described (25).

Genomic DNAs were subjected to restriction enzyme digestion and transferred from agarose gel onto nylon membrane (Schleicher and Schuell) as described by Southern (26). DNA probes were labeled with digoxigenin-dUTP using DIG DNA labeling kit (Boehringer Mannheim, Germany) according to the manufacturer’s instructions. Hybridization was carried out in a solution containing 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS, 1% blocking reagent (Boehringer Mannheim), 0.002% sodium N-lauroyl sarcosine at 60°C overnight. Filters were washed twice with 0.1 × SSC, 0.1% SDS at 60°C for 30 min. Enzyme-linked immunodetection was carried out using DIG nucleic acid detection kit (Boehringer Mannheim) as recommended by the manufacturer. Chemiluminescence detection with 3-(2-pyridylazo)-5-ethylbenzene disulfonic acid (ECL) was carried out according to the manufacturer’s instructions. The filter was detected by enzyme-linked immunodetection with anti-DHGO antibody and anti-rabbit IgG-peroxidase conjugate using Konica immunostain horseradish peroxidase kit (Konica, Japan).

Fungal RNA Isolation and Northern Blot Analysis—Total RNAs were isolated by phenol-SDS method (27) with additional LiCl precipitation and CsCl ultracentrifugation. Poly(A)^+ RNA was separated by oligotex™-dT30 (Daichi Chemicals, Japan) according to the manufacturer’s instructions. Total RNAs were resolved on 1% formaldehyde-containing agarose gels and transferred onto GeneScreen Plus membrane (Amersham) by capillary action. Size estimation of mRNAs was determined using rRNAs as internal size standards. Probe DNAs were labeled with [α-32P]dCTP by random primer labeling using Megaprime DNA labeling systems of Amersham. Hybridization (buffer: 5 × SSC, 1 × SSPE (1 × SSPE = 0.15 M NaCl, 0.25 M NaH2PO4, pH 7.4, 1 mM EDTA), 0.1% formaldehyde, 5 × Denhardt’s solution (1 × Denhardt’s = 0.2% (w/v) Ficol1, 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) bovine serum albumin fraction V), 100 μg/ml denatured salmon sperm DNA) was performed at 42°C for 16 h. Filters were washed five times in 1 × SSC, 0.1% SDS for 5 min and twice in 0.1 × SSC, 0.1% SDS at 68°C for 15 min.
Cloning of Dihydrogeodin Oxidase Gene from A. terreus

A rapid amplification of cDNA ends method (28) was used to extend the 5'-end of DHGO cDNA. The template DNA, 100 pmol of each primer, 8 μl of molybdate buffer (100 mM Tris-HCl (pH 9.0), 500 mM molybdate, 1% Triton X-100), and 2.5 units of Taq polymerase were combined in a total volume of 200 μl. The cycling conditions were as follows. EI-MS (m/z 402 (M+ sodium), 370, 368). 1H NMR (CDCl3/TMS) δ: 2.57 (3H, s), 3.68 (3H, s), 3.73 (3H, s), 5.79 (1H, s), 7.18 (1H, s), [α]D = 140° (c = 0.054, CHCl3).

RESULTS

Isolation of cDNA for Dihydrogeodin Oxidase from A. terreus—The first A. terreus cDNA library constructed in λgt11 was screened with polyclonal antibody against DHGO. Four positive phase clones were obtained from 1.4 × 10^6 recombinant phages (λDHGO21, λDHGO22, λDHGO41, λDHGO81). Screening analysis showed that λDHGO21 and λDHGO22 were the same clones containing 280-bp inserts. λDHGO41 and λDHGO81 contained shorter inserts with deletion before the open reading frame. A. terreus genomic DNA and cDNA for dihydrogeodin oxidase were determined from both strands. DNA Sequence Analysis—DNA sequence analysis was carried out by dye-terminator chain termination method using Sequenase version 2.0 (Amerham) and fluorescein isothiocyanate-labeled primer or terminator (Yuki Gosei Kogyo Co. Ltd., Japan) with Hitachi auto DNA sequencer SQ-3000. Fragments for sequencing were generated either by restriction digestion or deletion with exonuclease III and mungbean nuclease. DNA sequence data were organized and analyzed using the DNALAND program (Hitachi Software Engineering Co., Ltd., Japan) and DNA analysis package of Human Genome Center (Institute of Medical Science, The University of Tokyo). The final sequence of the cloned genomic DNA and cDNA for dihydrogeodin oxidase was determined from both strands.

Construction and Screening of cDNA Libraries—The first cDNA library was constructed from A. terreus mRNA in the expression phase vector λgt11 using cDNA synthesis kit (Boehringer Mannheim) according to the manufacturer’s instruction. A library containing 10^6 recombinants was made, and its 1.4 × 10^9 plaques were screened. The second cDNA library was constructed using a TimeSaver cDNA Synthesis kit (Pharmacia) with digoxigenin-labeled primer. Packaging by Lambda II packaging system (Nippon Gene, Japan) resulted in a library of 3 × 10^6 plaque-forming units, of which approximately 98% were recombinants. Screening of the first cDNA expression library was carried out as follows. After plaque lift on nitrocellulose filter, plaques presenting DHGO epitopes by isopropl-1-thio-β-galactopyranoside induction were detected by anti-DHGO primary antibody and anti-rabbit IgG peroxidase conjugate secondary antibody using Konica Immunostain horseradish peroxidase kit.

The second cDNA library was screened by plaque hybridization using a 280-bp cDNA fragment cloned from the first cDNA library as a probe. Labeling of the probe DNA with digoxigenin-dUTP was done by DIG DNA labeling kit (Boehringer Mannheim). Hybridization was carried out in a solution containing 5 × SSC, 0.1% SDS, 1% blocking reagent, 0.02% sodium azide, 15% dextran sulfate. Filters were washed twice with 0.1 × SSC, 0.1% SDS at 60°C for 30 min and then detected by DIG nucleic acid detection kit (Boehringer Mannheim) using nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate.

Selected XAP II recombinant phages were rescued to pBluescript recombinants by helper phage infection as described in the XAP II manufacturer’s manual (Stratagene).

Construction and Screening of A. terreus Partial Genomic DNA Library—A. terreus genomic DNA was digested with restriction endonuclease SacI, and the digestion products were separated by agarose gel electrophoresis. DNA fragments of approximately 5-7 kb recovered by Suprec-01 filtration (Takara Shuzo, Japan) were ligated with SacI-cleaved and dephosphorylated XAP II phage DNA arms. Packaging of the ligation mixture gave a library comprising of 1.4 × 10^9 plaque-forming units.

Screening of the genomic DNA library (5 × 10^10 plaques) was carried out in the same way as that of second cDNA library.

Screening of 5'-End of DHGO DNA—We synthesized the following primers: P1, 5' sense primer 5'-AAGACCATGAGCAGCTTCCGCGTATCTG-3'; P2, 3' antisense primer 5'-TACTACTGGCGATCTCAGC-3'; P3, 3' antisense primer 5'-CTGAGTCGCTCGAAATTCAT-3'; P4, 3' antisense primer 5'-GATGCCTCCTTTGCGTTTACA-3'; P5, 5' antisense primer 5'-GATGCCTCCTTTGCGTTTACA-3'; P6, 5' sense primer 5'-AAGACCATGAGCAGCTTCCGCGTATCTG-3'. The cycle of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min was repeated 30 times.

A rapid amplification of cDNA ends method (28) was used to extend the 5'-end of DHGO cDNA. One microgram of poly(A)+ RNA was reverse transcribed using P3 primer. The reverse-transcribed first strand cDNA was polyadenylated by terminal deoxynucleotidyltransferase (Takara, Japan). P2 primer and XbaI-dT16 primer adaptor were used to perform amplification at 94°C for 1 min, at 50°C for 1 min, and 72°C for 1 min for 30 cycles. The cDNA fragment was then directly subcloned into pT7 Blue. The result was confirmed by DNA sequencing.

Enzyme Assay and HPLC Conditions—DHGO activity was assayed as previously described (9). HPLC analysis of DHGO reaction product was performed on ODS-80 column (Tosoh) using CH3CN/H2O (1:1), 1% acetic acid at a flow rate of 0.6 ml/min. Elution was monitored at 254 nm. The recombinant DHGO enzymatic product was identified to be (+)-geodin by MS and NMR spectrometric analyses and measuring optical rotation.

Product Identification—Incubation mixture containing 20 mg of dihydrogeodin in 10 ml of ethylene glycol monomethyl ether, 200 ml of 0.5 M potassium phosphate buffer (pH 6.0), and recombinant DHGO enzyme solution (60 ml) was incubated at 30°C for 18 h. The reaction product was exhaustively extracted with ethyl acetate and recrystallized from ethyl acetate to give 10 mg of (+)-geodin. Physico-chemical data were as follows. El-MS (m/z: 402 (M+) ), 400 (M+, [35Cl, 37Cl]), 398 (M+, [35Cl, 35Cl]), 370, 368). 1H NMR (CDCl3/TMS): δ: 2.57 (3H, s), 3.68 (3H, s), 3.73 (3H, s), 5.79 (1H, s), 7.18 (1H, s), [α]D = 140° (c = 0.054, CHCl3).
Fig. 3. Nucleotide and deduced amino acid sequences of A. terreus dihydrogeodin oxidase. Putative TATA and CAAT boxes are underlined in the 5'-flanking region. Copper binding sites are boxed. Introns are underlined. Sequences of oligonucleotides used in the polymerase...
A DNA fragment comprising the 5'-end of the cDNA amplified using P2 primer and XbaI-dT15 primer adaptor was cloned into pT7 Blue and sequenced (cDNA 5 in Fig. 2).

Isolation of the Genomic DNA Encoding Dihydrogeodin Oxidase—To isolate the genomic DNA clones encoding DHGO, genomic Southern analysis was performed using the 280-bp cDNA fragment obtained from the first cDNA library as a probe. Southern blot analysis of BamHI-digested A. terreus genomic DNA gave two hybridizing bands of about 15 kb and 800 bp since there is a single BamHI site in the 3'-non-coding region of the cloned 280-bp cDNA. cDNA coding region probe indicated that the 800-bp band is the 5'-upstream region of DHGO gene. To know the suitable restriction enzyme fragment for genomic DNA cloning, single digestion of A. terreus genomic DNA with variable restriction enzymes or double digestion with the cDNA probe. This result indicated that 1) the 5.5-kb BamHI fragment contained a 5'-coding region including the BamHI 800-bp fragment and 2) the distance of 3'-downstream SacI site from the BamHI site in the 3'-non-coding region is very short because no detectable band other than 800 bp was observed in double digestion analysis, suggesting that DHGO is most likely encoded by a single copy gene, and the whole coding region of DHGO gene exists in the 5.5-kb SacI fragment.

A partial genomic DNA library of size-fractionated SacI-digested fragments around 5 kb was constructed using AscI-ZAP II phage vector and screened with the DIG-labeled 280-bp cDNA probe. Five positive clones selected were in vivo rescued to pBluescript recombinants, and all contained the 5.5-kb SacI fragments (pBDSHGO). The restriction endonuclease map of this 5.5-kb SacI fragment and the DHGO coding region located in the 2.8-kb BamHI fragment are illustrated in Fig. 2.

Structural Organization of the Gene for Dihydrogeodin Oxidase—The nucleotide sequence of the region corresponding to the DHGO genomic DNA was determined as shown in Fig. 3. Comparing the nucleotide sequences of the genomic DNA and cDNA, introns and the polyadenylation site of the gene were determined. The DHGO gene consisted of 6 introns and 7 exons. All 6 introns possess consensus 5'-splicing signals (GT(A/G/T)(A/G)(A/T/G)) and 3'-signals (C(T)AG). The length of introns (48, 59, 61, 49, 59, and 66 bases) are typical for fungal introns (49–85 bases) (31).

Sequence analysis of the 5'-flanking region revealed the presence of structural features considered to be important for gene transcription. A TATA box-like sequence (TATAAA) was found 214 bp upstream from the presumptive translation start (ATGCCC), and a CAAT element (C(AA)T) was located at 437 bp upstream from the ATG.

A canonical consensus sequence for the polyadenylation signal of higher eukaryotes, AATAAA (32), is not present in the C-terminal of the DHGO gene. The indicated methionine starts an open reading frame comprised of 605 amino acids, and the N-terminal amino acid of the purified enzyme is found in the same open reading frame after the 21-amino acid leader sequence, as shown in Fig. 3. The molecular mass of the mature DHGO deduced from the amino acid sequence was 68 kDa, whereas that estimated by SDS-PAGE was 76 kDa (9). Since the predicted DHGO peptide contained six potential N-glycosylation sites (Asn-X-Ser or Thr) (33) at amino acids 27, 106, 111, 282, 467, and 483, post-translational modification by glycosylation might have occurred. The fact that digestion of the purified native enzyme with N-glycanase (34) increased the mobility on SDS-PAGE (Fig. 4) revealed the existence of N-glycosylation in the mature DHGO protein.

Sequence Comparison of Dihydrogeodin Oxidase with Other Blue Copper Proteins—The deduced amino acid sequence of the DHGO from A. terreus was compared with other multicopper blue proteins to analyze the structural relationships among them.

Significant homology was observed with multicopper oxidases, including fungal laccase genes from Neurospora crassa (35), Coriolus hirsutus (36), Cryphonectria parasitica (37), cucumber (Cucumis sativus) ascorbate oxidase (38), and human ceruloplasmin (39). The degrees of amino acid sequence identity between A. terreus DHGO and laccase of N. crassa (35) and ascorbate oxidase of C. sativus (38) are 36 and 29%, respectively.

Closer inspection of the sequences revealed four conserved regions (A–D) shown in Fig. 5. These highly homologous regions contain clusters of histidine residues, which constitute the proposed 12 copper binding ligands to type-1, type-2, and type-3 coppers, on the basis of the results of x-ray crystallographic analysis for zucchini ascorbate oxidase (40). The homologous region D (amino acids 546–557 of DHGO) contains a cysteine, histidine, and methionine in positions homologous to the type-1 copper binding domain of small blue copper proteins, such as algal plastocyanin (41), azurin from Alcaligenes denitrificans (42), pseudoozurin from Alcaligenes faecalis (43), and plantacyanin from C. sativus (44).

Expression of Dihydrogeodin Oxidase Genomic DNA in P. frequentans—The cloned 5.5-kb SacI fragment, which contained whole DHGO genomic DNA, was first introduced into A. nidulans wild type strain FGSC 4. Although the integration of the introduced DNA fragment was confirmed by Southern blot analysis, no expression of DHGO poly-peptide was detected by Western analysis (data not shown).

P. frequentans CM196659 possesses similar secondary metabolite biosynthetic pathway to that of A. terreus and produces asterric acid as an end product. The fungus has its own phenol oxidative coupling enzyme, sulochrin oxidase,2 though whose nature such as molecular weight and immunoreactivity to anti-DHGO antibody is significantly different from DHGO. We introduced the cloned 5.5-kb SacI fragment (pBDSHGO) into P. frequentans by cotransformation with pDH25, hoping that the promoter of DHGO gene could function under similar secondary metabolism regulation in P. frequentans as that in A. terreus. All cotransformants obtained gave immunoreactive bands corresponding to the native DHGO protein by Western blot

chain reaction amplification of the dihydrogeodin oxidase cDNA are overlined (P1, P2, P3, P4). N-terminal amino acid sequence obtained from the purified A. terreus dihydrogeodin oxidase is double-underlined in the amino acid sequence. Potential N-glycosylation sites are shown in boldface type. The polyadenylation site is indicated (*)

Fig. 4. SDS-PAGE analysis of the purified dihydrogeodin oxidase from A. terreus. Lane 1, dihydrogeodin oxidase digested with N-glycanase; lane 2, untreated dihydrogeodin oxidase; M, molecular weight marker.
**Cloning of Dihydrogeodin Oxidase Gene from A. terreus**

**FIG. 5. Amino acid sequence comparison of copper binding motifs of the multicopper blue proteins.** The boxed regions A, B, C, and D, aligned according to the study by Ohkawa et al. (38), contain 12 potential ligands of type-1, type-2, and type-3 copper on the basis of the results of x-ray crystallographic analysis for zucchini ascorbate oxidase (40). Sequences of dihydrogeodin oxidase (DHGO) from A. terreus, lac-case (LAC) from N. crassa, ascorbate oxidase (ASO) from cucumber, and human ceruloplasmin (CP) are shown. The number on the left of each sequence represents the position of the amino acid residues of the protein. Identical amino acids are boxed. The numbers 1, 2, and 3 indicate potential coordination sites for three different types of copper ions.

**FIG. 6. Immunological detection of dihydrogeodin oxidase expressed in P. frequentans by immunoblotting.** The mycelial protein fractions were separated by SDS-polyacrylamide gel electrophoresis. After blotting to the nitrocellulose filter, immunological detection with the polyclonal antibodies against dihydrogeodin oxidase was carried out. The samples are as follows: lane A, crude proteins from P. frequentans wild type; lanes T1–T5, crude proteins from P. frequentans transformants; lane B, 0.1 μg of purified dihydrogeodin oxidase.

**FIG. 7. Expression plasmid pTAexDHGO.** The dihydrogeodin oxidase cDNA is shown as an open box. The EcoRI fragment of dihydrogeodin oxidase cDNA cloned in pT7 Blue vector was ligated into pTAex3 at the EcoRI site to construct pTAexDHGO. The promoter of A. oryzae Taka-amylase A (amyB) directs the starch or maltase-inducible expression of dihydrogeodin oxidase (20).

**FIG. 8. Analysis of dihydrogeodin oxidase derived from recombinant A. nidulans by immunoblotting.** The concentrated supernatant and the mycelial protein fractions of A. nidulans transformants were separated by SDS-polyacrylamide gel electrophoresis. After blotting to the nitrocellulose filter, immunological detection with the polyclonal antibodies against dihydrogeodin oxidase was carried out. The samples are as follows: lane B, 0.05 μg of dihydrogeodin oxidase; lanes 1–3, 20 μl of the mycelial fractions from the pTAexDHGO transformants; lanes 1–3, respectively; lane 4, 20 μl of mycelial fractions derived from the pTAexDHGO-R transformants; lane 5, 20 μl of the concentrated culture liquid from pTAexDHGO transformant 1; lane A, 0.1 μg of dihydrogeodin oxidase.

**DISCUSSION**

Dihydrogeodin oxidase catalyzes highly regio- and stereospecific phenol oxidative coupling of dihydrogeodin to form (+)-geodin. This is the first report of cloning of the cDNA and
Cloning of Dihydrogeodin Oxidase Gene from A. terreus

We have sequenced total 3041 bp of A. terreus genomic DNA, including 702 bp of the 5'-untranslated and 189 bp of the 3'-untranslated sequences. In the 702-bp region upstream from the translation initiation site, there are one TATA box-like sequence TATAAA and one CAAT box-like sequence CAAAT. This is consistent with the finding of similar putative eukaryotic regulatory sequences by Cordes et al. (45). The cloned 5.5-kb genomic DNA of A. terreus possibly contained upstream regulatory sequence(s) for the expression of DHGO gene. Since DHGO expression was observed in P. frequentans transformants but not in A. nidulans transformants, P. frequentans might possess regulatory mechanism for the expression of secondary metabolism, asterric acid biosynthesis, analogous to that for (+)-geodin biosynthesis in A. terreus, although A. nidulans might lack such a system.

In our previous study, DHGO was purified to be multicopper blue protein (9). In the deduced amino acid sequence of DHGO were found four regions homologous to regions strongly conserved among multicopper oxidases as shown in Fig. 5. The copper atoms bound in these domains are classified into three types corresponding to their distinct spectroscopic properties, type-1 (or blue), type-2 (or normal), and type-3 (or coupled binuclear) copper. Twelve potential ligands to these copperers were assigned on the basis of the results of x-ray crystallographic analysis for zucchini ascorbate oxidase (40). Similarities between ascorbate oxidase and DHGO, the size (about 600 amino acids), solution structure (homodimer), and EPR spectra suggested that DHGO might contain four copper ions, one type-1, one type-2, and two type-3 coppers per subunit as is in ascorbate oxidase. Sequence alignment of these homologous regions of DHGO and other multicopper blue proteins indicated that the coordination sites for the three types of copper ions in DHGO areas follows. Type-1 copper ion coordinates at His-115 and His-490. Type-2 copper ion pair coordinates at His-117, His-162, His-164, His-494, His-546, and His-548 as shown in Fig. 5.

A catalytic mechanism for ascorbate oxidase has been proposed based on the available kinetic data, the three-dimensional structure, and the associated electron-transfer processes (46). Presence of the same type copper binding domains in DHGO suggests the involvement of a similar electron-transfer
mechanism in the phenol oxidative coupling reaction catalyzed by DHGO. Type-1 copper is first reduced by one-electron transfer from the substrate dihydrogeodin, which is oxidized to a free radical. The electron is then transferred from type-1 copper to the type-3 copper pair. After reduction with four equivalents of reductant, the fully reduced enzyme is formed, which is able to bind dioxygen into the trinuclear copper center bridging the type-3 copper pair and the type-2 copper. This species must accept protons to release H₂O, while intramolecular C–O coupling of dihydrogeodin diradicals gives (+)-geodin under regio- and stereospecific control of DHGO enzyme (Fig. 10). It is very interesting to make further study by site-directed mutagenesis of the DHGO gene and heterologous production of mutated DHGO proteins to conduct mechanistic investigation on electron transfer in multicopper blue protein and regio- and stereochemical control by the enzyme. The success of DHGO expression in A. nidulans makes this study possible.

DHGO catalyzes the final step of (+)-geodin biosynthesis in A. terreus. Emodinanthrone, the initial cyclized biosynthetic precursor, is assumed to be elaborated by the specific polypeptide synthase we call emodinanthrone synthase. However, no such anthrone-synthesizing enzyme activity has been detected ever in any of fungal cell-free systems. Recent studies suggested the clustered structure of genes for aflatoxin (47) and melanin (48) biosynthesis. By Northern blot analysis, two other message signals were detected by the cloned 5.5-kb SacI probe, indicating that two other enzymes are encoded along with DHGO in this 5.5-kb region. We are now analyzing whether these genes are involved in the biosynthesis of (+)-geodin or not.

Acknowledgments—We thank Dr. Y. Nakano and Prof. M. Tomita (Showa University, Tokyo) for amino acid sequence analysis. We also thank Human Genome Center (Institute of Medical Science, The University of Tokyo) for the use of nucleotide and protein sequence analysis program.

REFERENCES

1. Barton, D. H. R., and Cohen, T. (1957) in Festschrift Prof. Dr. Arthur Stoll, pp. 117–144, Birkhäuser, Basel
2. Brown, B. R. (1967) in Oxidative Coupling of Phenols (Taylor, W. I., and Battersby, A. R., eds) pp. 1–94, Marcel Dekker, New York
3. Mayer, A. M. (1987) in Angewandte Chemie 26, 11–20
4. Thurston, C. F. (1994) in Microbiology 140, 19–26
5. Muko, H. (1963) in Angew. Chem. 75, 945–977
6. Brown, B. R. (1967) in Oxidative Coupling of Phenols (Taylor, W. I., and Battersby, A. R., eds) pp. 109–119, Marcel Dekker, New York
7. Konatsu, E. (1958) in Nippon Kogaku Kaishi 31, 564–567
8. Scott, A. I. (1965) in Q. Rev. Chem. Soc. 19, 1–35
9. Fujii, I., Iijima, H., Tsukita, S., Ebizuka, Y., and Sankawa, U. (1987) in J. Biochem. (Tokyo) 101, 11–18
10. Fujii, I., Chen, Z. G., Ebizuka, Y., and Sankawa, U. (1991) in Biochem. Int. 25, 1043–1049
11. Fujii, I., Ebizuka, Y., and Sankawa, U. (1988) in J. Biochem. (Tokyo) 103, 878–883
12. Chen, Z. G., Fujii, I., Ebizuka, Y., and Sankawa, U. (1995) in Phytochemistry 38, 373–380
13. Chen, Z. G., Fujii, I., Ebizuka, Y., and Sankawa, U. (1992) in Arch. Microbiol. 158, 29–34
14. Nordlöv, H., and Gatenbeck, S. (1982) Arch. Microbiol. 131, 208–211
15. Zenk, M. H., Gerardy, R., and Studier, F. (1989) in J. Chem. Soc. Chem. Commun. 1725–1727
16. Studier, R., and Zenk, M. H. (1993) in Bioc. Chem. 268, 823–831
17. Davin, L. B., Bedgar, D. L., Katayama, T., and Lewis, N. G. (1991) in Phytochemistry 31, 399–3974
18. Young, R. A., and Davis, R. W. (1983) in Science 222, 778–782
19. Pontecorvo, G., Roper, J. A., Hemmone, L. M., MacDonald, K. D., and Button, A. W. J. (1953) in Adv. Genet. 5, 141–238
20. Tada, S., Goni, K., Kitamoto, K., Kumagai, C., Tamura, G., and Hara, S. (1991) in Agric. Bioc. Chem. 55, 1939–1941
21. Cullen, D., Leong, S. A., Wilson, L. J., and Henner, D. J. (1987) in Gene (Amst.) 51, 21–28
22. Matsudaïra, P. T. (1989) in A Practical Guide to Protein and Peptide Purification, pp. 3–88, Academic Press Inc., San Diego
23. Laemmli, U. K. (1970) in Nature 227, 680–685
24. Biel, S. W., and Parrish, F. W. (1986) in Anal. Biochem. 154, 21–25
25. Huang, K., Iwakami, N., Fujii, I., Ebizuka, Y., and Sankawa, U. (1995) inCurr. Genet., in press
26. Southern, E. M. (1975) in Mol. Biol. 98, 503–517
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Frohman, M. A., Dush, M. K., and Martin, G. R. (1988) in Proc. Natl. Acad. Sci. U. S. A. 85, 8998–9002
29. Tilburn, J., Sczczuchcia, E., Taylor, G. G., Zabicky-Zisman, J. H., Lockington, R. A., and Davies, R. W. (1983) in Gene (Amst.) 26, 205–221
30. Goni, K., Iimura, Y., and Hara, S. (1987) in Agric. Bioc. Chem. 51, 2549–2555
31. Gurr, S., Unkles, S. E., and Kinong, J. R. (1987) in Gene Structure in Eukaryotic Microbes (Kinghorn, J. R., ed.) pp. 93–139, IRL Press, Oxford
32. Proudfoot, N. (1984) in Nature 307, 412–413
33. Kornfeld, R., and Kornfeld, S. (1985) in Annu. Rev. Biochem. 54, 651–664
34. Plummer, T. H. J., Elder, J. H., Alexander, S., Phédan, A. W., and Tarentino, A. L. (1984) in J. Biol. Chem. 259, 10700–10704
35. Germann, U. A., Muller, G., Hunziker, P. E., and Lerch, K. (1988) in J. Biol. Chem. 263, 885–896
36. Kojima, Y., Tsukuba, Y., Kawai, Y., Tsukamoto, A., Sugii, J., Sakaino, M., and Kitai, Y. (1990) in J. Biol. Chem. 265, 15224–15230
37. Chd, G. H., Larson, T. G., and Nuss, D. L. (1992) in Mol. Plant-Microbe Interact. 5, 21–28
38. Ohkawa, J., Okada, N., Shinmyo, A., and Takanu, M. (1989) in Proc. Natl. Acad. Sci. U. S. A. 86, 1239–1243
39. Takashashi, N., Ortí, T., and Putman, F. W. (1984) in Proc. Natl. Acad. Sci. U. S. A. 81, 390–394
40. Messerschmidt, A., Rossi, A., Ladenstein, R., and Huber, R. (1989) in Mol. Biol. 206, 513–529
41. Guss, J. M., and Freeman, H. C. (1983) in J. Mol. Biol. 169, 521–563
42. Norris, G. E., Anderson, B. F., and Baker, E. N. (1983) in J. Mol. Biol. 165, 501–521
43. Petratos, K., Dauter, Z., and Wilson, K. S. (1988) in Acta Crystallogr. Sect. Struct. Chem. B 44, 628–636
44. Guss, J. M., Merritt, E. A., Philzackerly, R. P., Hedman, B., Murata, M., Hodgson, K. O., and Freeman, H. C. (1988) in Science 241, 806–811
45. Corden, J., Wasylyk, B., Buchwald, A., Sassone-Corsi, P., Kedinge, C., and Chambon, P. (1980) in Science 209, 1406–1414
46. Messerschmidt, A., Ladenstein, R., Huber, R., Bolognesi, M., Awi Lipan, L., Petruzzelli, P., Rossi, A., and Finazzi-Agrò, A. (1992) in J. Mol. Biol. 224, 179–205
47. Chang, P. K., Skory, C. D., and Lin, J. E. (1992) in Curr. Genet. 21, 231–233
48. Kimura, N., and Tsuge, T. (1993) in J. Bacteriol. 175, 4427–4435