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Site-directed mutagenesis of gentisate 1,2-dioxygenases from *Klebsiella pneumoniae* M5a1 and *Ralstonia* sp. strain U2

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Summary

Gentisate 1,2-dioxygenase (GDO, EC 1.13.11.4) is the first enzyme in gentisate pathway that catalyses the ring fission of gentisate to form maleylpyruvate. Phylogenetic tree of amino acid sequences from 11 GDOs demonstrates that the GDOs from different genus share identities between 12.1% and 64.8%. According to the alignment result, four highly conserved histidine residues in GDO from *Klebsiella pneumoniae* M5a1 and *Ralstonia* sp. strain U2 were chosen to be substituted with aspartate residues. Enzyme analysis indicated that substitution of any of these four histidine residues had resulted in the complete loss of its catalytic activity.

Introduction

Degradation of aromatic compounds by microorganisms is not only a basic step of carbon cycle in the nature, but also plays a key role in the detoxification of these compounds in environment. Large amounts of polycyclic aromatic compounds are aerobically transformed by monooxygenases or dioxygenases to several central dihydroxylated intermediates, such as catechol, protocatechuate, and gentisate. Aromatic rings of these dihydroxylated intermediates could be consequently cleaved by dioxygenases, and the ring cleavage has been classified into three pathways, *ortho*-, *meta*-, and gentisate pathway according to the site of ring fission (Werwath et al., 1998).

In the gentisate pathway, gentisate is cleaved by gentisate 1,2-dioxygenase (GDO) to form maleylpyruvate (Fig. 1). Maleylpyruvate can be converted to fumarylpyruvate by isomerase and subsequently hydrolysed to fumarate and pyruvate, both of which are intermediates for the TCA cycle (Gao and Zhou,

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2003). The first report on an integrated gentisate pathway at molecular level is from *Ralstonia* sp. strain U2, which metabolises naphthalene via gentisate (Zhou et al., 2001). In another example, *Klebsiella pneumoniae* M5a1 has been demonstrated to metabolise 3-hydroxybenzoate via gentisate, and the catabolic genes have been cloned (Jones and Cooper, 1990) and sequenced (Gao, 2003) (GenBank accession number AY648560).

Up to now, the purification of GDO has been reported in several microorganisms (Crawford et al., 1975; Harpel and Lipscomb, 1990a; Suarez et al., 1996; Fu and Oriel, 1998; Werwath et al., 1998; Feng et al., 1999), and the catalytic mechanisms of GDO are studied only in one report (Harpel and Lipscomb, 1990b). GDO from *Pseudomonas alcaligenes* NCIB 9867(P25X) was mutated through random and site-directed mutagenesis, and nearly 300 mutants were obtained (Chua et al., 2001). The results of enzyme assay of these mutants indicated that the substitution of any of four highly conserved His residues to Asp residues lead to the complete loss of enzyme activity. However, we cannot yet make a conclusion that the four His residues in other GDOs are as essential as that in P25X since the crystal structure of GDO has not yet been determined and the identity values among the GDOs' amino acid sequences vary dramatically (12.1–64.8%). In this study, through site-directed mutagenesis, we demonstrate that these four His residues in GDO from *K. pneumoniae* M5a1 and *Ralstonia* sp. strain U2 are also critical to the catalytic activity of GDO.

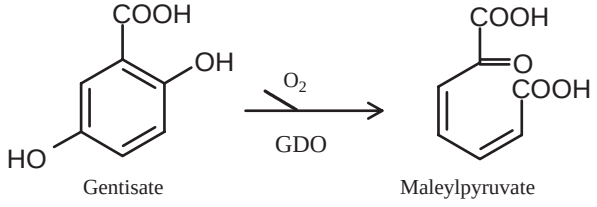

Figure 1. Gentisate 1,2-dioxygenase converts gentisate to form maleylpyruvate.

Materials and methods

Strains and plasmids

Escherichia coli DH5α[ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169*] (Life Technologies, UK), *E. coli* Rosetta(DE3) pLysS[F *ompT hsdS*$_B$(r_B m_B *gal dcm lac*Y1(DE3) pLysSRARE^2(Cm^r)] (Novagen, USA), the plasmids used and constructed in this study are listed in Table 1.

Enzymes and reagents

Restriction endonuclease and T4 ligase were purchased from Takara (Dalian, China), *Pfu* DNA polymerase and dNTPs were purchased from Shanghai Shenergy Biocolor Bioscience & Technology Company (Shanghai, China), gentisate from Sigma (Sigma, USA), PCR purification kit from V-gene Biotechnology Limited Company (Hangzhou, China). Nucleotide sequences were determined by United Gene Holdings, Ltd. (Shanghai, China). PCR primers were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

Media and culture condition

Strains used in this study were grown overnight in LB at 37 °C. When necessary 100 mg/L ampicillin and 34 mg/L chloramphenicol were added.

Table 1. Plasmids used and constructed in this study

Plasmid	Description	Source or reference
pET5a	Expression vector	Studier and Moffatt, 1986
pZWGD5	1076 bp *Eco*RI-*Nde*I-cut PCR fragment containing *mhbD* inserted into pET5a	Gao, 2003
pBSI	8216 bp *Sph*I-cut fragment from *Klebsiella pneumoniae* M5a1 inserted into pUC18	Gao, 2003
pWWF19-25	1286 bp *Eco*RI-*Nde*I-cut PCR fragment containing *nagI* inserted into pET5a	Zhou et al., 2001
pWWF60	8.9 kb *Eco*RI-cut fragment from *Ralstonia* sp. U2 inserted into pUC18	Zhou et al., 2001
pZWLSD108	pZWGD5, in which CAT code for His^{108} was changed to GAT	This study
pZWLSD110	pZWGD5, in which CAC code for His^{110} was changed to GAC	This study
pZWLSD149	pZWGD5, in which CAC code for His^{149} was changed to GAC	This study
pZWLSD151	pZWGD5, in which CAC code for His^{151} was changed to GAC	This study
pZWLSI118	pWWF19-25, in which CAC code for His^{118} was changed to GAC	This study
pZWLSI120	pWWF19-25, in which CAC code for His^{120} was changed to GAC	This study
pZWLSI159	pWWF19-25, in which CAC code for His^{159} was changed to GAC	This study
pZWLSI161	pWWF19-25, in which CAC code for His^{161} was changed to GAC	This study

Site-directed mutagenesis of GDO

Site-directed mutagenesis of *mhbD* and *nagI* was carried out by overlap-extension PCR (Pogulis et al., 1996), and pBSI and pWWF60 were used as templates for PCR, respectively. Primers used in this study are listed in Table 2.

PCR fragments of GDO were digested with *Eco*RI and *Nde*I, and then inserted into expression vector pET5a. The recombinant plasmids were transformed into *E. coli* DH5α and positive clones were screened according to restriction enzyme analysis. The resulting mutant plasmids (pZWLSD108, pZWLSD110, pZWLSD149, pZWLSD151, pZWLSI118, pZWLSI120, pZWLSI159, and pZWLSI161) were sequenced to ensure the mutations occurred as desired and no unintended mutation had been incorporated during the PCR before they were transformed into *E. coli* Rosetta for expression.

Expression of wild-type and mutant enzymes in *E. coli* Rosetta

The GDO genes, cloned in vector pET5a, were expressed in *E. coli* Rosetta cells which were grown at 37 °C on LB media supplemented with ampicillin and chloramphenicol. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the final concentration of 0.4 mM before the culture density, measured by the absorbance at 600 nm, reached 0.6. Cultures were then grown for another 4 h at 30 °C before the cells were harvested by centrifugation.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and enzyme assay

The expression of GDO was analysed by SDS–PAGE (Schaggfr and Jagow, 1987) with 12% acrylamide separating gel and 5% stacking gel.

Cell extracts were prepared by resuspending the bacterial pellets in ice-cold 50 mM phosphate buffer (pH 7.4) and lysed by sonication in an ice-water bath, MhbD and its mutants for 15 min, and NagI and its mutants for 5 min (disrupting for 6 s with 9 s intervals). Cell debris was removed by centrifugation at 15,000*g* for 30 min at 4 °C.

GDO activity was assayed spectrophotometrically at 30 °C by measuring the formation of maleylpyruvate at 330 nm (Zhou et al., 2001). Activity assay was conducted in 500 μL of reaction mixture containing 0.2 mM gentisate in 50 mM phosphate buffer (pH 7.4). The molar extinction coefficient of 13,000/M cm was used. One unit of enzyme activity is defined as the amount of enzyme required for production of 1 μmol maleylpyruvate per minute at 30 °C. Specific activities are expressed as units per milligram of protein. Protein concentrations were determined by Bradford kit according to the manufacture's instructions (Beyotime Institute of Biotechnology, Haimen, China).

Sequence and phylogenetic analysis

The sequences were aligned by using CLUSTALX, and phylogenetic analyses were performed using

Table 2. Oligonucleotides used for site-directed mutagenesis

mhbD forward	5′ GAACG**CATATG**TCCCAGTCCACCACGG 3′
mhbD reverse	5′ TCGGCA**GAATTC**CAGAAGAAGAACGGC 3′
mhbD H108D forward	5′ GAGCGATCGGCACAACCAGTCGG 3′
mhbD H108D reverse	5′ TGCCGATCGCTCGGCGCCA 3′
mhbD H110D forward	5′ GAGCCATCGGGACAACCAGTCGG 3′
mhbD H110D reverse	5′ TGGTTGTCCCGATGGCTCGGCG 3′
mhbD H149D forward	5′ GCGCTGGGACGATCACGGCAAC 3′
mhbD H149D reverse	5′ CGTGATCGTCCCAGCGCCACTG 3′
mhbD H151D forward	5′ GCACGATGACGGCAACCCCGG 3′
mhbD H151D reverse	5′ TGCCGTCATCGTGCCAGCGCC 3′
nagI forward	5′ TCCCCTATC**CATATG**CTTGATGAAGAG 3′
nagI reverse	5′ CTCACTT**GAATTC**CTGTTGTGTGGTCG 3′
nagI H118D forward	5′ CAGCGACCGCCACACCCC 3′
nagI H118D reverse	5′ GGTCGCTGGGCGCCCAC 3′
nagI H120D forward	5′ CACCGCGACACCCCCAACG 3′
nagI H120D reverse	5′ GGTGTCGCGGTGGCTGGG 3′
nagI H159D forward	5′ TGGGACGAGCACGGCCAC 3′
nagI H159D reverse	5′ GCTCGTCCCACAGGCCGGT 3′
nagI H161D forward	5′ CACGAGGACGGCCACGACG 3′
nagI H161D reverse	5′ GGCCGTCCTCGTGCCACAG 3′

The bases representing the restriction sites are in boldface. The bases changed are underlined.

the neighbour-joining algorithm of PHYLIP (version 3.572c). BLASTP was used for the amino acid identity search.

Results

Sequence and phylogenetic analysis

The GDO sequences, which share different identities with MhbD and NagI in GenBank, were selected to perform multiple sequences alignment and phylogenetic tree. The phylogenetic tree is shown in Fig. 2. The result of alignment indicates that the amino acid sequences of GDO from different organisms share different degrees of identities, ranging from 12.1% to 64.8% (data not shown). NagI demonstrates poor identities with MhbD and XlnE, which are 32.9% and 25.6%, respectively. The low identities among GDOs indicate that the origins of gentisate pathway are apparently diverse, which may be attributed to adapt themselves to gentisate derivatives (Gao, 2003).

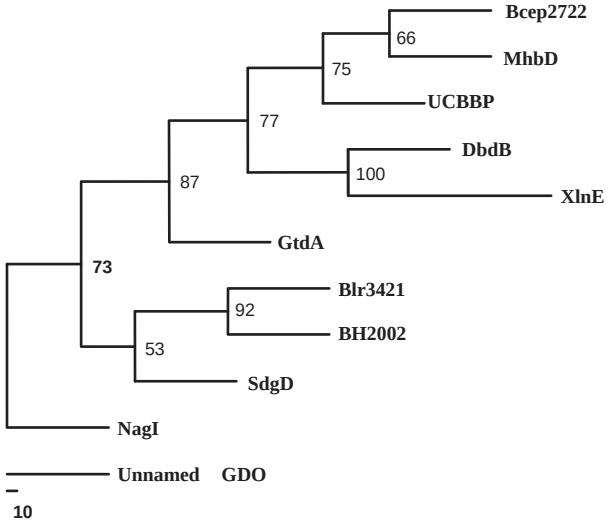

Figure 2. Phylogram of consensus tree obtained from neighbour-joining bootstrap analysis of 11 GDO amino acid sequences with GenBank accession numbers. Bcep2722 is from *Burkholderia fungorum* Bcep_225 (ZP_00029914.1), MhbD from *Klebsiella pneumoniae* M5a1 (AAW63413.1), UCBBP from *Pseudomonas aeruginosa* UCBPP-PA14 Paer_1 (ZP_00135722.1), DbdB from *Xanthobacter polyaromaticivorans* (BAC98955.1), XlnE from *Pseudomonas alcaligenes* NCIB 9867(P25X) (AAD49427.1), GtdA from *Sphingomonas* sp. RW5 (CAA12267.1), Blr3412 from *Bradyrhizobium japonicum* USDA 110 DNA (BAC45375.1), BH2002 from *Bacillus halodurans* (NP_242868.1), SdgD from *Streptomyces* sp. WA46 (BAC78375.1), NagI from *Ralstonia* sp. U2 (AAD12619.1). An unnamed GDO from *Haloferax* sp. D1227 (AAC25761.1) was used as an outgroup. Bootstrap values refer to which they are closest. The scale bar represents a Jukes–Cantor distance.

Site-directed mutagenesis of conserved His residues

According to the result of sequences alignment of MhbD, NagI and XlnE, motif scanning (http://hits.isb-sib.ch/cgi-bin/PFSCAN), and PHI-Blast in GenBank (http://www.ncbi.nlm.nih.gov), it is found that the residues from position 101 to 152 in MhbD (from 110 to 163 in NagI and 102 to 153 in XlnE) are highly conserved (shown in Fig. 3). It has been predicted that GDO belongs to the cupin superfamily, which refers to a β-barrel structural domain, on the basis of primary sequence (Dunwell et al., 2000). Figure 3 shows that these conserved sequences seem to contain the characteristic cupin domain, which comprises two histidine-containing motifs, the conserved sequences of these motifs are G-X(5)-H-X-H-X(3,4)-E-X(6)-G and G-X(5)-P-X-G-X(2)-H-X(3)-N, respectively (Khuri et al., 2001). The four highly conserved His residues in GDO are located in these motifs and three of them are also conserved in the cupin domain.

Expression of wild-type and mutant enzymes and activity assay

Through overlap-extension PCR, eight mutant plasmids were obtained (they are pZWLSD108, pZWLSD110, pZWLSD149, and pZWLSD151 of MhbD, pZWLSI118, pZWLSI120, pZWLSI159, and pZWLSI161 of NagI). Sequence determination confirmed that each His residue at different positions had been substituted by Asp residues in these mutants, respectively. Expressions of wild-type and mutants MhbD and NagI were performed in *E. coli* Rosetta strain, and highly soluble form proteins were obtained. Figure 4 shows the expressions of wild-type and mutants MhbD and similar results were obtained with the expressions of wild-type and mutants NagI (data not shown). There is no difference in the expression of GDO genes between wild type and mutants, and it can be concluded that the substitutions of these four conserved His residues individually had no impact on the expressions of GDO from the two different bacterial strains.

Wild-type MhbD and NagI exhibited evident GDO activity (the specific activities are 0.54 and 0.37 U/mg, respectively), but no GDO activity was detected in any of the eight mutants of MhbD and NagI. The comparison of GDO activities between

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                  *** ** ♦**♦   * *  * * ** * **** *  **  **  **♦ ♦
MhbD  100  PGEVAPSHRHNQSALRFVVEGEGAFTAVDGERTAMRAGDFILTPQWRWHDHGN
NagI  110  PGEWAPSHRHTPNAVRMIVEGEGAYTTVDGEKCPMSRGDLILTPTGLWHEHGH
XlnE  101  PGEVAPTHRHSQSALRFVVDGGACTSVDGERTTMQVGDFVITPPWAWHDHVN
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Figure 3. Partial multiple sequence alignment of the amino acid sequences from three GDOs. Conserved His residues are marked with ♦ and amino acid residues conserved in all three GDO species are marked with *, the positions of the two conserved cupin motifs are boxed.

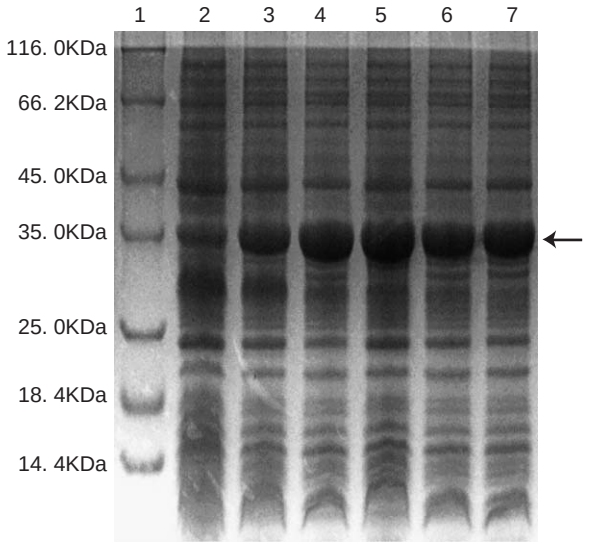

Figure 4. SDS–PAGE of overexpressed MhbD and its mutants in *E. coli* Rosetta on a 12% gel. Lane 1, molecular weight markers; lane 2, blank control (pET5a was transformed into *E. coli* Rosetta); lanes 3–7, cell extracts containing H151D, H149D, H110D, H108D, and MhbD obtained after induction with IPTG, respectively. The molecular mass of the overexpressed polypeptide (indicated by an arrow on the right) is ~35 kDa.

wild-type and mutant MhbD is shown in Fig. 5 (the corresponding comparison of NagI is similar to that of MhbD, but the results were not shown).

Discussion

The substitutions of any of the four highly conserved histidine to aspartate residues in GDO from strain M5a1 and strain U2 had resulted in the complete loss of their catalytic activities. This is consistent with the results of mutagenesis studies of GDO in *P. alcaligenes* NCIB 9867(P25X) (Chua et al., 2001). Furthermore, it should be noted that the GDO genes of the above studies originated from different genus and the identities of their amino acid sequences is less than 33%. The result clearly suggests that these four His residues are vital for GDO catalytic activity.

It has been reported that iron in GDO is the primary site for substrate interaction. Gentisate binds directly to the iron through the carbon 1 carboxylate and carbon 2 hydroxyl substituents, and it results in the active site iron coming close to the site of ring cleavage (Harpel and Lipscomb, 1990b). Imidazole group in His residue is very active, and it could rapidly offer or receive electron at almost the same rate. It is proposed that during the biological evolution imidazole group might exist as a catalytic structure in an enzyme rather than an ordinary part of the protein structure (Yu, 1990). His residues are often found to be coordinated with iron in active sites in conserved motif in many dioxygenases whose crystallographic data have been obtained. For example, His335 and His371 in homogentisate dioxygenase (Fernandez-Canon and Penaval, 1995), His145 and His209 in 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) (Han et al., 1995), His153 and His214 in catechol 2,3-dioxygenase (2,3-CTD) (Kita et al., 1999), His12, His61 in protocatechuate 4,5-dioxygenase (Sugimoto et al., 1999), His 208, His 213, and His 362 in naphthalene dioxygenase (Carredano et al., 2000). Moreover, some dioxygenases that have not been crystallographically characterised also exhibit similar motif with His residues function as iron ligands. His 222 and His 228 in toluene dioxygenase is a good case in point (Jiang et al., 1996).

On the other hand, it is found that within some cupin superfamily members, the metal binding motif is highly conserved, according to structure-based sequence alignment (Pang et al., 2004). A cupin protein, quercetin 2,3-dioxygenase from *Bacillus subtilis*, whose crystal structure had been recently determined, has irons in active sites and six conserved His residues, His 62, His 64, His 103, His 234, His 236, and His 275 in two cupin motifs, respectively. These six His residues coordinate the Fe^{2+} at the two active sites (Gopal et al., 2005). A similar situation is also found in another newly identified cupin nuclear protein pirin, in which iron is in active site and three His residues bind to the iron (Pang et al., 2004).

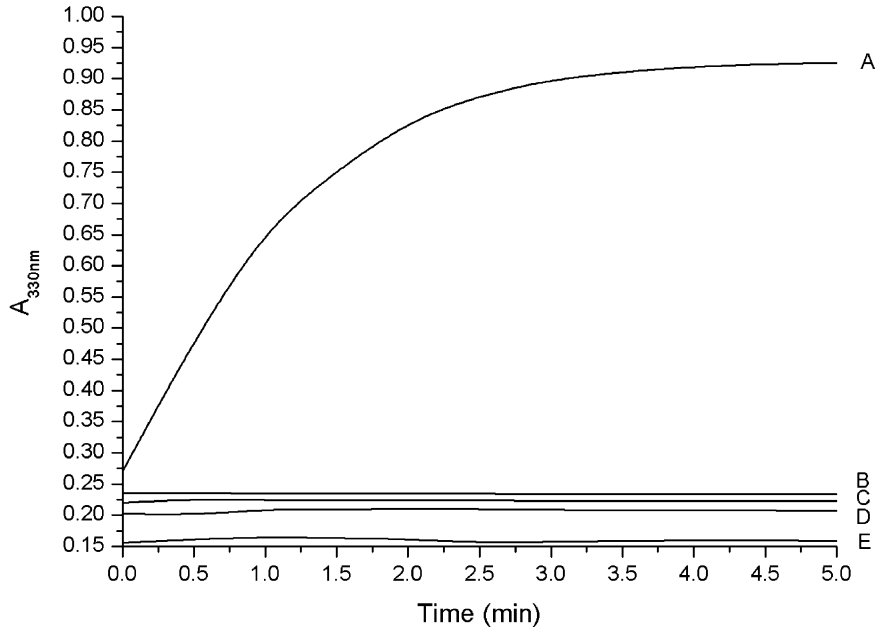

Figure 5. Time course of production of maleylpyruvate from gentisate by MhbD and its mutants. Cell extracts of *E. coli* Rosetta containing pZWGD5 (MhbD), pZWLSD108 (H108D), pZWLSD110 (H110D), pZWLSD149 (H149D), and pZWLSD151 (H151D) obtained after induction with IPTG. (A) MhbD; (B) H151D; (C) H108D; (D) H149D; (E) H110D.

In addition, His residues not only act as the iron binder, but also was found to play different roles in extradiol-cleaving dioxygenases. A prominent example is the 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) in *Pseudomonas* sp. KKS102. The His residues in the enzyme seem to deprotonate the hydroxyl group of the substrate, to stabilise a negative charge on the O_2 molecular, and to function as a proton donor (Sato et al., 2002).

Nevertheless, only based on the above data, it would be plausible to draw a conclusion that these His residues in this study play the key role of iron coordination as the same in other dioxygenases or cupin proteins. The illustration of particular catalytic mechanisms of GDO would not be clarified until the resolution of GDO crystal structure was performed.

Acknowledgements

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