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NOTE

Replacement of Tyrosine 181 by Phenylalanine in Gentisate 1,2-Dioxygenase I from *Pseudomonas alcaligenes* NCIMB 9867 Enhances Catalytic Activities

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***xlnE*, encoding gentisate 1,2-dioxygenase (EC 1.13.11.4), from *Pseudomonas alcaligenes* (P25X) was mutagenized by site-directed mutagenesis. The mutant enzyme, Y181F, demonstrated 4-, 3-, 6-, and 16-fold increases in relative activity towards gentisate and 3-fluoro-, 4-methyl-, and 3-methylgentisate, respectively. The specific mutation conferred a 13-fold higher catalytic efficiency (k_{cat}/K_m) on Y181F towards 3-methylgentisate than that of the wild-type enzyme.**

Pseudomonas alcaligenes NCIMB 9867 (strain P25X) degrades *m*-cresol, 2,5-xylenol, 3,5-xylenol, and their catabolites via the gentisate pathway (7, 9). A critical step in the gentisate pathway is the fission of the gentisate aromatic ring catalyzed by gentisate 1,2-dioxygenase (GDO I; EC 1.13.11.4) that initiates this reaction by destabilizing the aromatic ring, employing Fe^{2+} as a cofactor (5) and yielding maleylpyruvate, which is then channeled to the tricarboxylic acid cycle (7). P25X harbored isofunctional GDOs, with one set being constitutively expressed yet further inducible (14) and the other set being strictly inducible (9). Both GDOs were reported to possess broad substrate specificities towards unsubstituted, alkylated, and halogenated gentisate analogs. The constitutive GDO I enzyme was shown to have marked differences in substrate specificities compared to the inducible GDO II enzyme (9).

Enhancing the catalytic properties of biodegradative enzymes by site-directed mutagenesis (SDM) represents a potential strategy for improving the efficacy of biodegradation processes (10, 11). Mutations of specific amino acids have been known to alter either the substrate specificity or the kinetic properties of an enzyme (1, 8). In this study, site-specific mutations were targeted in the *xlnE* gene to assess the effects these have on the substrate specificities and catalytic properties of the variant enzymes.

A Clustal W alignment of GDOs from P25X (GenBank accession no. AF173167), *Pseudomonas aeruginosa* (AE004674), *Escherichia coli* O157:H7 (AE005174), *Bacillus halodurans* I, II (AP001514), *Ralstonia* sp. strain U2 (AP001514), *Haloferax* sp. strain D1227 (AF069949), and *Sphingomonas* sp. strain RW5 (AJ224977) showed the presence of a highly conserved double-stranded β-helix domain (data not shown). To evaluate the influence of specific amino acid residues on the catalytic properties of the enzyme, amino acid residues located outside and within the β-helix domain were randomly selected and subjected to SDM (Table 1). Random substitutions with different amino acid residues were constructed, for instance, a polar amino acid was replaced with a nonpolar amino acid and an acidic amino acid was replaced with a basic amino acid. The mutagenized genes were fully sequenced and transformed into *E. coli* BL21(DE3) for protein expression and purification. The recombinant glutathione *S*-transferase (GST)-tagged GDO proteins were overexpressed in the respective mutants of *E. coli* BL21 when induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). A one-step purification of GST-tagged GDO and its mutants was performed using GSTrap Fast Flow columns (Amersham Biosciences, NJ) via affinity purification. The formation of substituted maleylpyruvates from various substrates was monitored with purified enzymes in the presence of 0.1 mM ferrous ammonium sulfate, 2 mM L-cysteine, and glycerol at 10% (vol/vol) at the following wavelengths: for gentisate, 330 nm; for 3-methylgentisate, 327 nm; for 4-methylgentisate, 316 nm; for 3-bromogentisate, 335 nm; for 3-fluorogentisate, 331 nm; for 3-isopropylgentisate, 325 nm; and for 4-chlorogentisate, 335 nm. The reaction mixture reported by Feng et al. (4) was employed, and GDO-specific activities were calculated using various molar extinction coefficients based on the reaction products derived from unsubstituted and substituted gentisates. A value of 12,500 M^{-1} cm^{-1} was used for gentisate, 11,900 M^{-1} cm^{-1} was used for 3-methylgentisate, 11,400 M^{-1} cm^{-1} was used for 4-methylgentisate, 11,200 M^{-1} cm^{-1} was used for 3-isopropylgentisate, 13,000 M^{-1} cm^{-1} was used for 3-bromogentisate, 11,700 M^{-1} cm^{-1} was used for 3-fluorogentisate,

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TABLE 1. Amino acid substitutions in mutant enzymes generated by SDM and their resultant activities

Mutant enzyme	Amino acid position	Nucleotide change	Amino acid change	Relative activity[a] (%)
Y17C	17	TAT→TGT	Tyr→Cys	161
V36A	36	GTT→GCT	Val→Ala	ND
N43T	43	AAT→ACT	Asn→Thr	317
S113P	113	TCT→CCT	Ser→Pro	ND
D120K	120	GAT→AAA	Asp→Lys	ND
G123N	123	GGC→AAC	Gly→Asn	75
M146T	146	TGG→ACG	Met→Thr	ND
N153H	153	AAT→CAC	Asn→His	183
G164T	164	GGC→AAC	Gly→Thr	ND
M169H	169	ATG→CAT	Met→His	ND
Y181F	181	TAT→TTT	Tyr→Phe	464
Y181D	181	TAT→GAT	Tyr→Asp	68
Y181H	181	TAT→CAT	Tyr→His	98
E223A	223	GAG→GAC	Glu→Ala	102
T260C	260	GAG→GCG	Thr→Cys	ND
V2841	284	GTT→ATC	Val→Iso	260
V326Q	326	GTT→AAG	Val→Gln	ND
K338Y	338	AAG→TAC	Lys→Tyr	156

[a] The relative activities of the mutant enzymes were calculated in comparison with that of the GST-tagged GDO enzyme, which exhibited a specific activity of 0.5 μmol of product formed min^{-1} mg^{-1}, with this value taken as 100%. ND, mutant enzymes with nondetectable GDO activities. Each value was derived from the mean of at least three separate experiments.

and 12,400 M^{-1} cm^{-1} was used for 4-chlorogentisate (9). For the determination of kinetic parameters, K_m values were calibrated from Hanes plots with substrate concentrations ranging from 50 μM to 600 μM. Protein concentrations were determined by the Bradford assay (2), employing bovine serum albumin as the standard. Relative activities of GDO were calculated with respect to the activity of recombinant GST-GDO expressed in *E. coli*. One enzyme unit is the amount of enzyme required to produce 1 μmol of maleylpyruvate per min at 23°C.

The GST-GDO fusion protein expressed in *E. coli* exhibited a similar specific activity and substrate affinity towards gentisate to those of the previously reported purified GDO enzyme from P25X (wild type [WT]) (4). WT GDO I exhibited specific activity towards gentisate at 0.63 U/mg, whereas recombinant GST-GDO I displayed a specific activity at 0.58 U/mg. The apparent K_m values for WT GDO I and GST-GDO I differed slightly, at 92.0 μM and 86.02 μM, respectively. This showed that the purified GST-GDO I enzyme did not differ markedly in catalytic properties from purified WT GDO I.

Eight of the 18 mutants, namely, V36A, S113P, D120K, M146T, G164T, M169T, T260C, and V326Q, exhibited a total loss of enzyme activity, whereas the mutant enzyme G123N showed a 25% reduced GDO activity (Table 1). These amino acid substitutions were found to be mainly located within the periphery of the highly conserved region and could have resulted in an alteration of the quaternary structure of the enzyme that led to a nonfunctional enzyme. However, several mutant enzymes exhibited higher relative activities, ranging from 103% to 464% of the WT activity. The mutant enzyme Y181F was selected for further characterization of its substrate affinities (K_m) and catalytic efficiencies (k_{cat}/K_m) towards gentisate and substituted gentisates.

The mutant enzyme Y181F showed a 464% increase in relative activity towards gentisate compared to that of the recombinant wild-type GST-GDO protein. Significantly higher relative activities towards 3-methyl- and 4-methylgentisates, at 1,638% and 667%, respectively, of the WT activities, were observed. Relative activities towards 3-bromo- and 3-fluorogentisates were also found to be higher than the WT GDO activities, at 254% and 373%, respectively. Replacement of the tyrosine at position 181 of GDO by phenylalanine had altered the catalytic properties significantly.

To further characterize the importance of tyrosine (Tyr) at position 181 in the *xlnE* gene product, tyrosine was replaced with either an acidic or a basic amino acid by SDM. When an acidic (aspartic acid) or a basic (histidine) amino acid was introduced to generate two new variant enzymes, Y181D and Y181H, respectively, there was no improvement in specific activity towards gentisate compared to that of the WT. These observations demonstrated that a single amino acid exchange in the mutant enzyme Y181F was responsible for the significantly altered catalytic properties observed. The substitution at position 181 favors catalysis, and the absence of a hydroxyl group might have contributed to favorable hydrophobic interactions between the polypeptide chains and aromatic hydrocarbons such as gentisate and substituted gentisates.

All enzymes displayed Michaelis-Menten kinetics, and Hanes plots of the enzyme activities yielded apparent K_m values towards gentisate of 86.02 μM for the WT and 68.43 μM for the mutant enzyme (Table 2). Remarkably, the catalytic

TABLE 2. Specific activities and catalytic efficiencies of recombinant P25X GST-GDO and the mutant enzyme Y181F

Substrate	Value (mean ± SD) for WTGST-GDO				Value (mean ± SD) for Y181F mutant			
	Sp act (U/mg)	Relative activity (%)	K_m (μM)[a]	k_{cat}/K_m (10^4 M^{-1} s^{-1})[a]	Sp act (U/mg)	Relative activity (%)	K_m (μM)[a]	k_{cat}/K_m (10^4 M^{-1} s^{-1})[a]
Gentisate	0.58 ± 0.12	100	86.02 ± 5.73	54.2 ± 2.8	2.69 ± 0.68	464	68.43 ± 3.60	260.3 ± 5.1
3-Bromogentisate	0.28 ± 0.04	100	45.91 ± 3.05	18.1 ± 1.2	0.71 ± 0.06	254	78.26 ± 4.25	25.4 ± 1.6
3-Fluorogentisate	0.11 ± 0.08	100	76.69 ± 2.08	12.4 ± 0.2	0.41 ± 0.03	373	89.62 ± 5.19	13.3 ± 2.5
3-Isopropylgentisate	0.06 ± 0.05	100	79.37 ± 1.50	7.9 ± 1.3	0.09 ± 0.01	150	67.69 ± 1.81	4.9 ± 1.3
3-Methylgentisate	0.16 ± 0.03	100	62.34 ± 3.23	15.6 ± 1.6	2.62 ± 0.31	1,638	87.34 ± 3.46	211.9 ± 3.7
4-Chlorogentisate	0.13 ± 0.05	100	23.26 ± 2.45	56.3 ± 3.3	0.13 ± 0.05	100	60.69 ± 3.10	9.2 ± 1.4
4-Methylgentisate	0.03 ± 0.01	100	74.26 ± 4.39	3.5 ± 2.5	0.20 ± 0.02	667	72.58 ± 2.68	8.1 ± 0.9

[a] K_m and k_{cat} were determined in 0.1 M KH_2PO_4 buffer (pH 7.4) at 23°C. Their values were calibrated from Hanes plots in the presence of the respective substrates at concentrations ranging from 50 μM to 600 μM. Each value was derived from the mean of at least three separate experiments.

efficiency of the mutant enzyme towards 3-methylgentisate was 13-fold higher than that of the WT. The mutant enzyme Y181F also exhibited a 4.8-fold increase in the turnover rate of gentisate compared to that of the WT.

The K_m value of the WT towards gentisate was close to the K_m values reported for *Pseudomonas testosteroni* (85.0 μM) and *Pseudomonas acidovorans* (74.0 μM), but this value was considerably higher than those reported for *Moraxella osloensis* (7.1 μM) and *Sphingomonas* sp. strain RW5 (15.0 μM) (3, 6, 13). The K_m values towards gentisates for the mutant enzyme Y181F showed that the alkylated and halogenated gentisates were well tolerated. The mutant enzyme Y181F displayed a 17-fold higher catalytic efficiency towards 3-methylgentisate than the enzyme from *Pseudomonas testosteroni* (6). The higher catalytic efficiencies observed for the mutant enzyme towards alkylated and halogenated gentisates could be explained either by the favorable steric effects encountered in the active sites of the mutant enzyme or by the interactions with the electron attracting/donating alkyl/halogen groups present in the substituted gentisates.

Several extradiol dioxygenases have been studied extensively, while the GDOs are relatively undercharacterized (12, 15). Enzymes with broader substrate specificities or enhanced levels of production are important in industrial applications and could be employed in the bioremediation of toxic aromatic hydrocarbons. Since the three-dimensional crystal structure of the enzyme remains to be elucidated, our results indicated that GDOs with improved catalytic properties could be generated by SDM of randomly selected amino acid residues in the absence of a three-dimensional structure of GDO.

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