Cloning, purification and characterization of two components of phenol hydroxylase from Rhodococcus erythropolis UPV-1

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Abstract

Phenol hydroxylase that catalyzes the conversion of phenol to catechol in *Rhodococcus erythropolis* UPV-1 was identified as a two-component flavin-dependent monooxygenase. The two proteins are encoded by the genes *pheA1* and *pheA2*, located very closely in the genome. The sequenced *pheA1* gene was composed of 1,629 bp encoding a protein of 542 amino acids, whereas the *pheA2* gene consisted of 570 bp encoding a protein of 189 amino acids. The deduced amino acid sequences of both genes showed high homology with several two-component aromatic hydroxylases. The genes were cloned separately in cells of *Escherichia coli* M15 as hexahistidine-tagged proteins, and the recombinant proteins His_6PheA1 and His_6PheA2 were purified and its catalytic activity characterized. His_6PheA1 exists as a homotetramer of four identical subunits of 62 kDa that has no phenol hydroxylase activity on its own. His_6PheA2 is a homodimeric flavin reductase, consisting of two identical subunits of 22 kDa, that uses NAD(P)H in order to reduce flavin adenine dinucleotide (FAD), according to a random sequential kinetic mechanism. The reductase activity was strongly inhibited by thiol-blocking reagents. The hydroxylation of phenol in vitro requires the presence of both His_6PheA1 and His_6PheA2 components, in addition to NADH and FAD, but the physical interaction between the proteins is not necessary for the reaction.

Introduction

Phenol and similar aromatic compounds are environmental pollutants present in effluents from many industrial processes. For the removal of phenol from wastewaters, a number of physical, chemical, and biological methods have been developed (Rajkumar and Palanivelu 2004; Neves et al. 2006). Biological treatments provide an effective and practical strategy to depollute these wastewaters leading to a complete mineralization of phenol (Annadurai et al. 2002). A great number of microorganisms capable of degrading natural or anthropogenic aromatic compounds have been found, and many degradation pathways have been elucidated (Smith 1990; Dunaway-Mariano and Babbitt 1994; Harwood and Parales 1996; Kulkarni and Chaudhari 2007).

Phenol-degrading aerobic bacteria are able to convert phenol into nontoxic intermediates of the tricarboxylic acid cycle via an ortho or meta pathway (Harwood and Parales 1996). The monooxygenation of the aromatic ring constitutes the first step in the biodegradation of many phenolic compounds. This process is carried out by flavoprotein monooxygenases, which use electrons of NAD(P)H to activate and cleave a molecule of oxygen through the formation of an intermediate flavin hydroperoxide and enable the incorporation of an oxygen atom into the substrate (Moonen et al. 2002). These reactions can be catalyzed by a single polypeptide chain or by multicomponent enzymes (van Berkel et al. 2006). It has been reported as a class of monooxygenases, consisting of a small reductase component that uses NAD(P)H to reduce a flavin that diffuses to a large oxygenase component that catalyzes the hydroxylation of aromatic substrate (van Berkel et al. 2006). Some of the proteins that belong to this family are *p*-hydroxyphenylacetate 3-hydroxylase of *Escherichia coli* (Galán et al. 2000) and *Acinetobacter baumannii* (Chaiyen et al. 2001), 4-nitrophenol monooxygenase (Kadiyala and Spain 1998), and chlorophenol-4-monooxygenase of *Burkholderia cepacia* (Gisi and Xun 2003). Among the

reported phenol hydroxylases, only those of *Bacillus thermoglucosidasius* and *Bacillus thermoleovorans* have been described as two-component flavin-dependent monooxygenases (Duffner and Muller 1998; Kirchner et al. 2003).

In previous papers, we reported that *Rhodococcus erythropolis* UPV-1 was able to grow on phenol as the sole carbon and energy source, removing, concomitantly, the formaldehyde present in phenolic industrial wastewaters (Hidalgo et al. 2002a, b). Moreover, the efficiency of this strain in a bench-scale depolluting system was proven (Prieto et al. 2002a, b). After the identification and characterization of the enzyme responsible for the elimination of formaldehyde from wastewaters (Jaureguibeitia et al. 2007), our interest has focused on the enzymes involved in the hydroxylation of phenol into catechol in the initial catabolic step for the degradation of phenol in *R. erythropolis* UPV-1.

Although the sequences of phenol hydroxylase genes of a *R. erythropolis* strain are available (GenBank accession no. AJ973228.1), no functional or biochemical information about phenol hydroxylase in representatives of the genus *Rhodococcus* has been reported so far. In this paper, we have identified the genes encoding the phenol hydroxylase from *R. erythropolis* UPV-1. We propose that this enzyme is a new member of the two-component flavin-dependent monooxygenases that are composed of a flavin reductase and an oxygenase component that uses the reduced flavin to carry out the hydroxylation of the aromatic ring of phenol. We have cloned and overexpressed separately *pheA1* and *pheA2* genes, encoding the oxygenase and flavin reductase components, respectively. Finally, the recombinant components of phenol hydroxylase (i.e., His_6PheA1 and His_6PheA2 proteins) have been purified, and their main structural and catalytic properties have been characterized.

Materials and methods

Chemicals

NADH, NADPH, and restriction endonucleases were from Roche Diagnostics (Mannheim, Germany). Flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), riboflavin, thiol-blocking reagents, ampicillin, and kanamycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ni^{2+} Chelating Sepharose™ Fast-Flow and isopropyl-thio-β-d-galactopyranoside (IPTG) were obtained from GE Healthcare (Uppsala, Sweden). Phenol was a product from Merck (Darmstadt, Germany).

Microorganisms and culture conditions

A strain of *R. erythropolis* UPV-1 (deposited in the Spanish Type Culture Collection as strain CECT 3054) was isolated in our laboratory from a phenol-formaldehyde-polluted site in Bizkaia, Spain. Cells were grown as described previously (Prieto et al. 2002b). The recombinant proteins were overexpressed in *E. coli* strain M15 [pREP4] grown at 37°C with shaking in Luria–Bertani (LB) medium which contained 1% (*w*/*v*) tryptone, 0.5% (*w*/*v*) yeast extract, and 1% (*w*/*v*) NaCl, supplemented with 100 mg l^{-1} ampicillin and 25 mg l^{-1} kanamycin.

Cloning and construction of expression plasmids

The genomic DNA of *R. erythropolis* UPV-1 was extracted and purified using the NucleoSpin® Tissue Kit (Macherey Nagel, Düren, Germany) and stored at 4°C. Polymerase chain reaction (PCR) was performed using primers designed based on conserved sequences of phenol hydroxylase

components from other bacteria. DNA fragments amplified in each PCR were purified from the agarose bands. For cloning of *pheA1*, PCR was performed using the primers 5'-GAAGGATCCATGACGACCACCGAAATCCCT-3' and 5'-ATAAAGCTTTTACCGGTTCTTGAAGAACGA-3', containing *Bam*HI and *Hin*dIII sites, respectively. A DNA fragment of 1.5 kb was amplified, digested with *Bam*HI and *Hin*dIII and cloned into the *Bam*HI and *Hin*dIII sites of vector pQE-9 (Qiagen, Hilden, Germany) to create the expression plasmid pQE9A1. For cloning *pheA2*, PCR was performed using the primers 5'-ATGGAACAAAATCAGCTTCGAGGA-3' and 5'-TCAGATCGTTTGGGGTGCGGGCTG-3'. A DNA fragment of 0.6 kb was amplified and directly cloned into the vector pQE-30UA (Qiagen, Hilden, Germany) to create the expression plasmid pQE30A2. The final expression plasmids were verified by DNA sequencing and used to transform *E. coli* strain M15 [pREP4]. The plasmids pQE9A1 and pQE30A2 permit the expression of His_6PheA1 and His_6PheA2 proteins, respectively, that contain a 6× His-tag extension in their N-terminal segment. The *pheA1* and *pheA2* nucleotide sequences identified in this study have been submitted to GenBank (accession no. EU004078 and EU004079, respectively).

Expression and purification of His_6PheA1 and His_6PheA2

Transformed cells carrying the pQE9A1 or pQE30A2 plasmids were grown overnight at 37°C in LB medium (2× 100 ml) supplemented with 100 mg l^{-1} ampicillin and 25 mg l^{-1} kanamycin. This culture was used to inoculate 1 l of LB medium supplemented with 100 mg l^{-1} ampicillin and 25 mg l^{-1} kanamycin. When the OD_{600} was 0.60, 0.1 mM IPTG was added. After induction for 1 h at 30°C, cells were harvested by centrifugation (10 min, 8,000×*g*, 4°C), and the pellet was resuspended in 25 ml of buffer A (50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole). Cell suspension was disrupted by sonication for 7 min in an ice bath, and the cell lysate was centrifuged (25 min, 13,000×*g*, 4°C). The clear supernatant was loaded onto a Ni^{2+} Chelating Sepharose Fast-Flow column equilibrated in buffer A. All purification steps were carried out at room temperature. The column was washed with buffer A, and the retained proteins were eluted with an increasing gradient from 20 to 300 mM of imidazole in buffer A. In both cases, the absorbance of eluate was monitored continuously at 280 nm, and fractions of 1 ml were collected. The purity of fractions was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot. Finally, those fractions containing the pure protein were pooled and desalted in a PD-10 column (GE Healthcare, Uppsala, Sweden) equilibrated and eluted with 100 mM phosphate buffer, pH 6.8. The purified proteins were maintained at 4°C and were used immediately after purification.

Enzyme assays

Flavin reductase activity was assayed at 25°C monitoring the decrease in absorbance of NADH at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) in 100 mM phosphate buffer, pH 6.8, containing 400 µM NADH and 150 µM FAD. Assays were initiated by the addition of the enzyme. One unit (U) of activity was the amount of enzyme required to catalyze the consumption of 1 µmol of NADH per minute. To determine the specificity of the enzyme, FMN, riboflavin, and NADPH were used as substrates. The kinetic reaction mechanism of the enzyme was determined by assaying (in triplicate) the activity at different flavin concentrations at a fixed NADH concentration and vice versa. Kinetic data were fitted by iterative nonlinear regression to the Michaelis–Menten equation using the Enzyme Kinetics v.1.10 module of SigmaPlot 8.0 (SPSS Inc., Chicago, IL, USA).

Phenol hydroxylase activity was determined by measuring the disappearance of phenol in the reaction mixture (2.5 ml), which contained 100 mM phosphate buffer, pH 6.8, 0.1 mM phenol, 1 mM NADH, 10 µM FAD, and a variable amount of purified His_6PheA2. After the addition of the

purified His_6PheA1, aliquots of 200 µl were withdrawn at different times and mixed with 20 µl of trichloroacetic acid to stop the reaction. After centrifugation (10 min, 10,000×*g*), the supernatants were analyzed by high-performance liquid chromatography (HPLC) as indicated below. One unit (U) of activity was the amount of enzyme required to catalyze the consumption of 1 µmol of phenol per minute. The presence of H_2O_2 was determined after the reaction of phenol hydroxylation. Samples (100 µl) of the reaction mixture were added to 100 µl of 1 mM 3,3′,5,5′-tetramethylbenzidine and 0.3 U ml^{-1} of horseradish peroxidase in 100 mM citrate buffer, pH 4.0, and the increase of the absorbance at 655 nm was recorded.

The physical interactions between His_6PheA1 and His_6PheA2 were investigated by gel filtration chromatography (see below) or by the two-compartment reaction assay of phenol hydroxylase (Galán et al. 2000). A solution (1 ml) containing His_6PheA2 was placed inside a dialysis bag (Visking tubing, 12–14-kDa molecular mass cutoff, Medicell Int. Ltd, London, UK). The bag was immersed into a solution (4 ml) of 100 mM phosphate buffer, pH 6.8, 1 mM NADH, 10 µM FAD, 100 µM phenol, and His_6PheA1. The reaction was initiated by the addition of NADH to the reaction mixture. Samples were withdrawn at different times and analyzed as described above. A parallel control experiment was performed under identical conditions but with both proteins located in the same compartment.

Analytical methods

The measurement of phenol and other analogs was performed in an HPLC system (Waters Corporation, Milford, MA, USA) composed by a photodiode Waters™ 996, two pumps Waters™ 510, and a Tracer Lichrosorb RP18 column (250 × 4 mm). The mobile phase was ultrapure MilliQ water/acetonitrile 70:30 (*v*/*v*) containing 1% (*v*/*v*) acetic acid. The detection was carried out spectrophotometrically at 270 nm, and the compounds were identified by comparison of their retention times with those of pure substances. Protein concentration was quantified by the method of Bradford (1976) using bovine serum albumin as a standard.

Electrophoretical analysis

The purification progress of the recombinant proteins and the molecular mass of the native enzymes and its subunits were assessed by PAGE under denaturing (SDS-PAGE) and nondenaturing conditions (ND-PAGE). SDS-PAGE was performed in the buffer system of Laemmli (1970) with a homogeneous 12% (*w*/*v*) acrylamide resolving gel and a 4% (*w*/*v*) acrylamide stacking gel. For ND-PAGE, commercially available gels (Bio-Rad, Hercules, CA, USA) with a linear gradient of 4–20% (*w*/*v*) acrylamide were used. In both cases, proteins were electrophoresed in a Mini Protean III cell (Bio-Rad, Hercules, CA, USA) at 200 V. Appropriate molecular mass standards were run in all cases. Protein bands were visualized after staining with Coomassie Brilliant Blue (PhastGel® Blue R, GE Healthcare, Uppsala, Sweden) or with silver reagent (Shevchenko et al. 1996).

Western blots

Protein bands of polyacrylamide gels were electrotransferred at 150 mA for 1 h into nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA, USA) using a semidry system with transfer buffer: Tris (0.30% *w*/*v*), glycine (1.44% *w*/*v*), and methanol (20% *v*/*v*). The membranes were then incubated for 1 h at 25°C in phosphate-buffered saline (PBS), pH 7.0, containing 1% (*w*/*v*) bovine serum albumin, 0.05% (*w*/*v*) Tween 20, and 10% (*w*/*v*) defatted milk. After incubation with blocking buffer, the membranes were washed with PBS and incubated for 1 h with the same buffer containing 1/3,000 (*v*/*v*) diluted anti-His_6-tag mouse monoclonal

antibodies. Then, the membranes were washed with PBS and further incubated for 1 h with 1/5,000 (*v*/*v*) diluted secondary rabbit antibodies antimouse IgG conjugated to alkaline phosphatase. Protein bands were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution.

Molecular mass determination

The molecular mass of the native enzymes and the interactions between His_6PheA1 and His_6PheA2 were determined by gel filtration chromatography using a Superdex 200 10/300 GL column controlled by an ÄKTA UPC Basic equipment (GE Healthcare, Uppsala, Sweden). The column was equilibrated and developed with 50 mM Tris–HCl, pH 8.0, containing 150 mM NaCl at 0.5 ml min^{-1} flow rate. The standard proteins used to calibrate the column were ferritin (440 kDa), catalase (213 kDa), aldolase (182 kDa), bovine serum albumin (67 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa).

Results

Identification of pheA1 and pheA2 genes

We have determined the sequence of *pheA1* and *pheA2* genes present in the genome of *R. erythropolis* UPV-1. Several known sequences of two-component phenol hydroxylases from other bacteria were used to design primers to amplify the sequence of DNA that encodes both genes. The identified genes are closely located in the genome, only separated by 11 bp.

The sequenced *pheA1* gene was composed of 1,629 bp encoding a protein of 542 amino acids with a theoretical molecular mass of 60.72 kDa and a p*I* of 5.75, whereas the *pheA2* gene consisted of 570 bp encoding a protein of 189 amino acids with a predicted molecular mass of 20.35 kDa and a p*I* of 5.16.

The deduced amino acid sequence of *pheA1* gene was compared with that of several phenol hydroxylases, using Vector NTI™ software package (InforMax Inc., Bethesda, MD, USA). When PheA1 was aligned with the protein of *R. erythropolis* CCM2595 (GenBank accession no. CAJ01325), values of 99.1% homology and identity were obtained. In addition, high values of identity 87.8%, 87.3%, 59.7%, and 47.5% were deduced when the amino acid sequence was compared, respectively, with the putative large component of phenol hydroxylase of *Nocardia farcinica* IFM 10152 (GenBank accession no. YP120578.1), *Rhodococcus jostii* RHA1 (GenBank accession no. YP_702343), *Thermus thermophilus* (GenBank accession no. YP_145488), and *B. thermoglucosidasius* (GenBank accession no. AAF66546). When PheA1 from *R. erythropolis* UPV-1 was compared with two-component flavin-dependent monooxygenases, identity values of 50.2% for HpaB from *E. coli* (GenBank accession no. CAA82321), 49.5% for HpaA from *Klebsiella pneumoniae* (GenBank accession no. AAC37120), 19.4% for TftD from *B. cepacia* (GenBank accession no. AAC23548), and 9.5% for nitrilotriacetate monooxygenase component A from *Chelatobacter heintzii* (GenBank accession no. P54989) were calculated.

The deduced amino acid sequence of *pheA2* gene was compared with the corresponding protein of several bacteria. The predicted PheA2 protein was very similar to the putative phenol hydroxylase small subunit from *R. erythropolis* CCM2595 (GenBank accession no. CAJ01324), sharing 99.5% of homology and 98.4% of identity. Values of identity of 78.4%, 73.0%, and 29.0% were obtained, respectively, when PheA2 was aligned with phenol hydroxylase small subunits from *R*. *jostii* RHA1 (GenBank accession no. YP_702342), *N*. *farcinica* IFM10152 (GenBank accession no. BAD59215), and *B. thermoglucosidasius* (GenBank accession no. AAF66547).

When deduced PheA2 was compared with the reductase component of two-component monooxygenases, identity values of 21.6% for 4-nitrophenol hydroxylase from *Rhodococcus* sp. PN1 (GenBank accession no. BAB86379), 15.5% for TftC from *B. cepacia* (GenBank accession no. AAC23547), 16.8% for HpaC from *E. coli* (GenBank accession no. CAA82322), 15.3% for HpaH from *K. pneumoniae* (GenBank accession no. AAC37121), and 16.2% for DszD from *R. erythropolis* (GenBank accession no. BAB18470) were calculated.

Expression and purification of His_6PheA1 and His_6PheA2 proteins

The *pheA1* and *pheA2* genes were cloned separately into pQE vectors (Qiagen, Hilden, Germany) that allow the overexpression of hexahistidine-tagged proteins to facilitate further their purification. The constructed plasmids were used to transform *E. coli* M15 cells. The recombinant proteins His_6PheA1 and His_6PheA2 were purified to electrophoretic homogeneity (Fig. 1) from the soluble fraction of transformed *E. coli* cells by immobilized metal affinity chromatography (IMAC). SDS-PAGE analysis of pure His_6PheA1 and His_6PheA2 revealed, respectively, the presence of a single protein band of 62 kDa (Fig. 1A) and 22 kDa (Fig. 1B). These results are in agreement with the predicted molecular mass of both recombinant enzymes. The proteins were also detected by Western blot using antibodies against His_6-tagged polypeptides.

Molecular properties of His_6PheA1

When the purified His_6PheA1 was analyzed by ND-PAGE followed by staining with Coomassie Brilliant Blue, a single band of about 238 kDa was detected (Fig. 2A). Also, His_6PheA1 eluted from the Superdex 200 10/300 GL column as a single peak with an elution volume corresponding to an apparent molecular mass of 236 kDa. When the protein was analyzed by SDS-PAGE, a subunit mass of 62 kDa was calculated (Fig. 2B). These results indicate that His_6PheA1 is a homotetrameric enzyme consisting of four identical subunits of about 62 kDa. Further analysis of the purified enzyme by liquid chromatography–mass spectrometry (LC–MS) revealed an exact subunit molecular mass of 62,078 Da.

Molecular properties of His_6PheA2

Concentrated samples of purified His_6PheA2 were colorless, indicating that the enzyme did not contain any chromophore group absorbing in the visible. The enzyme eluted from the Superdex 200 10/300 GL column as a single peak of an apparent molecular mass of 45 kDa. This result suggests that the enzyme is a homodimer consisting of two identical subunits of about 22 kDa. Further analysis of the purified enzyme by LC–MS revealed an exact subunit molecular mass of 22,550 Da.

Catalytic activity of His_6PheA2

The oxidoreductase activity of His_6PheA2 in vitro depended only on the presence of NADH and an oxidized flavin as electron acceptor in the reaction mixture. No additional cofactors were required apparently. The maximum NADH:FAD oxidoreductase activity was measured at pH 6.8 and at 40°C. However, the enzyme was routinely assayed at 30°C to ensure the stability of the components of the reaction mixture. The pure enzyme showed a specific activity of 411.7 U mg^{-1} of protein when assayed in the conditions described in "Materials and methods."

The affinity of the pure enzyme for its substrates NADH and FAD was determined. A K_m value for NADH of 53.3 μM and for FAD of 13.4 μM was calculated after fitting data by nonlinear regression to a hyperbola. The kinetic mechanism of His_6PheA2 was determined by assaying the activity with different concentrations of NADH (from 0 to 100 μM) and FAD (from 0 to 100 μM)

and analyzing the kinetic data in Lineweaver–Burk plots. Thus, both the double reciprocal plots 1/*v* vs 1/[NADH] and 1/*v* vs 1/[FAD] provided a family of straight lines which, in both cases, intersected in a common point of the negative abscise axis (Fig.3). These results suggest that His_6PheA2 catalyzes the reaction according to a random sequential two-substrate mechanism with independent binding sites.

The substrate specificity of pure His_6PheA2 was studied using other reduced nicotinamide coenzyme (NADPH) as electron donor and oxidized flavins (FMN and riboflavin) as electron acceptors. Both FMN and riboflavin can accept the electrons of NADH but with lower affinity and catalytic efficiency than FAD (Table 1). Also, NADPH can be used instead of NADH as electron donor (using either FAD or FMN as electron acceptor) but with an affinity fivefold or tenfold lower than NADH, respectively.

The possible effect of different ions and reagents on the activity of His_6PheA2 was studied (Table 2). Iodoacetamide, phenylmethylsulfonyl fluoride, and ethylenediaminetetraacetic acid (EDTA), at 1 mM, did not affect the activity of the pure enzyme, whereas Mg^{2+} and Mn^{2+} activated it slightly. However, 20 µM of the thiol-blocking reagent *p*-hydroxymercuribenzoate inhibited completely the enzymic activity, suggesting that a sulfhydryl group would be involved in the catalytic process or somehow related to the conformation or stability of His_6PheA2. Also *N*-ethylmaleimide inhibited the activity significantly. The presence of Ag^{+} or Cu^{2+} (at 20 µM) and of Fe^{3+} or Ni^{2+} (at 1 mM) also inhibited totally the activity, whereas other cations (Zn^{2+}, Fe^{2+}, or Co^{2+}) inhibited the activity more moderately (Table 2).

Catalytic activity of His_6PheA1

We firstly investigated the His_6PheA1 activity expressed in cultures of *E. coli* and in vitro using enzyme extracts. Thus, when *E. coli* M15 cells (transformed with the plasmid pQE9A1) growing in LB medium were supplemented with 1 mM phenol, the color of cultures turned brown after 4 h of addition. However, when control cells (*E. coli* M15 carrying pQE9 plasmid without *pheA1* gene) were grown under the same conditions, no coloration change was observed. To ascertain if this effect was due to the accumulation of some compounds in the culture medium, the supernatant of cultures was analyzed by HPLC (Fig. 4). The peak corresponding to catechol was only detected in samples of cells carrying *pheA1* gene. These results indicated that the expressed His_6PheA1 was able in vivo to hydroxylate phenol yielding the corresponding dihydroxylated compound without any apparent additional requirement. Moreover, the product of its activity (i.e., catechol) was accumulated in the culture medium.

When the activity of phenol hydroxylase was assayed in extracts of *E. coli* transformed with pQE9A1 plasmid in the absence of His_6PheA2 protein, phenol decreased at a rate of 0.123 µmol min^{-1}, whereas the addition of His_6PheA2 to the reaction mixture doubled the rate of phenol disappearance (up to 0.260 µmol min^{-1}). Phenol hydroxylase activity was also determined using the purified enzyme His_6PheA1 in the presence or absence of pure His_6PheA2. Thus, phenol disappearance and concomitant catechol formation only occurred when His_6PheA2 was present in the reaction mixture. This result suggested that His_6PheA2 could be replaced by another flavin reductase present in the crude extract of *E. coli*. However, the enzymic activity catalyzed in vitro by His_6PheA2 is essential to carry out the hydroxylation of phenol by His_6PheA1.

Furthermore, the hydroxylation of phenol in vitro depended on the molar ratio of His_6PheA2 and His_6PheA1 present in the reaction mixture. Thus, an increase of the amount of His_6PheA1 in the assay resulted in a higher phenol hydroxylase activity (data not shown), suggesting that the

reaction catalyzed by His_6PheA1 would be the limiting step in the hydroxylation of phenol. For this reason, in the experiments to characterize the phenol hydroxylase activity, a reductase/oxygenase molar ratio of 1:10 was used.

Although the reductase His_6PheA2 could use several flavins (FAD, FMN, and riboflavin) with different efficiency, the activity of the oxygenase component (His_6PheA1) of phenol hydroxylase was strictly dependent on FAD. Thus, FMN and riboflavin could not substitute FAD in this coupled reaction, and phenol consumption was negligible when any of these flavins were used instead of FAD (Fig. 5A). The effect of FAD concentration on the total phenol hydroxylase activity was investigated. An excess of FAD in the reaction mixture resulted in a decrease of the phenol disappearance, and FAD concentrations higher than 10 μM inhibited the catalyzed reaction (Fig. 5B). The formation of H_2O_2 was checked after the reaction of hydroxylation. We detected H_2O_2 when His_6PheA2 was present in the reaction mixture, but not in control experiments carried out in the absence of either His_6PheA2 or FAD.

Several aromatic compounds (at a final concentration of 100 μM) were tested as an alternative substrate of the purified components of phenol hydroxylase in vitro. In addition to phenol, 4-nitrophenol, 3-nitrophenol, and resorcinol could be also hydroxylated, indicating that the enzyme is not absolutely specific for phenol. However, the two-component phenol hydroxylase of *R. erythropolis* UPV-1 was completely unable to hydroxylate benzoate, 4-hydroxybenzoate, and orcinol.

In order to investigate the physical interaction of the two purified components, phenol hydroxylase activity was determined by a two-compartment reaction assay (Galán et al. 2000). The two components His_6PheA1 and His_6PheA2 were placed in two different compartments separated by a selective membrane with pores that allowed the passage of molecules smaller than 14 kDa. In this assay, a phenol hydroxylation rate of 24.13 μmol min^{-1} mg^{-1} protein was calculated. In control experiments, with both proteins placed in the same compartment, similar values of phenol hydroxylase activity were measured. These results indicated that the physical interaction of the reductase and hydroxylase components of enzyme was not compulsory for the hydroxylation of phenol. Moreover, they showed that His_6PheA2 would reduce FAD to $FADH_2$ which then can diffuse rapidly to the oxygenase component, providing the reducing power required to hydroxylate the phenol. Furthermore, we investigated the protein–protein interactions by gel filtration studies. For this purpose, purified His_6PheA1 and His_6PheA2 were mixed and analyzed. Only two peaks corresponding with the elution time of the tetrameric His_6PheA1 and the dimeric His_6PheA2 were detected, indicating that His_6PheA1 and His_6PheA2 did not comigrate in the gel filtration column.

Discussion

In this study, we have identified and characterized the phenol hydroxylase activity of *R. erythropolis* UPV-1. This enzymic activity is linked to two separate protein components encoded by genes *pheA1* and *pheA2*, which are located very closely in the genome of *R. erythropolis* UPV-1. This proximity between the two genes has been also described in the phenol hydroxylase of *B. thermoglucosidasius* (Duffner et al. 2000), 4-nitrophenol hydroxylase of *Rhodococcus* sp. PN1 (Takeo et al. 2003), and 4-hydroxyphenylacetate-3-hydroxylase of *E. coli* (Prieto et al. 1996) whose genes are separated by 20, 28, and 18 bp, respectively. A similar genetic arrangement has been reported among the members of several two-component monooxygenases (Prieto et al. 1996; Xu et al. 1997; Hubner et al. 1998; Duffner et al. 2000) although there are also some examples in which the location of the reductase component is far away from the oxygenase one

(Xi et al. 1997). The deduced amino acid sequences of *pheA1* and *pheA2* are practically identical to that of sequenced putative phenol hydroxylase components of *R. erythropolis* strain CCM2595 (Vesely et al. 2007). In addition, we found high identity and similarity values with the corresponding sequences of *N. farcinica* IFM 10152 and *R. jostii* RHA1. Moreover, we performed a conserved domain search of two deduced amino acid sequences. The deduced amino acid sequence for *pheA1* showed high similarity with the 4-hydroxyphenylacetate 3-hydroxylase family (HpaB; pfam 03241), and the deduced amino acid sequence for *pheA2* contained a flavin reductase-like domain (pfam 01613; Bateman et al. 2002). These results suggested that the *pheA1* and *pheA2* genes identified in this work corresponded to the two components of phenol hydroxylase from *R. erythropolis* UPV-1 and indicated that *pheA2* and *pheA1* encoded a flavin reductase and a flavin-dependent monooxygenase, respectively. In order to confirm this hypothesis, we cloned both genes separately in expression vectors that allowed the overexpression of the recombinant proteins containing a hexahistidine extension to facilitate their purification by IMAC and yielding sufficient amount of proteins to characterize their structure and main catalytic properties.

Thus, the predicted molecular mass of His_6PheA1 and His_6PheA2 (62 and 22 kDa, respectively) are in good agreement with the values calculated by SDS-PAGE analysis. The molecular mass of subunits was similar to that found for the subunits of two-component monooxygenases characterized from other sources.

His_6PheA2 apparently exists as a dimer composed of two identical subunits. The characterized flavin reductase subunits from two-component monooxygenases exist in the form of dimers (Galán et al. 2000; Chaiyen et al. 2001; Kirchner et al. 2003) or monomers (Xun 1996; Ohshiro et al. 2004). On the other hand, both electrophoretic and chromatographic analyses show that His_6PheA1 is a tetramer composed of four identical subunits. Similar homotetrameric structure has been reported for EDTA monooxygenase in the bacterial strain DSM 9103 (Witschel et al. 1997), the oxygenase component (SsuD) of alkanesulfonate monooxygenase from *E. coli* (Eichhorn et al. 1999), and the large component of *p*-hydroxyphenylacetate hydroxylase from *A. baumannii* (Chaiyen et al. 2001). However, there is reported in the literature a variety in the structure of the large component of two-component monooxygenases. Thus, TcpA from *R. eutropha* is a monomer (Louie et al. 2002), HpaB from *E. coli* is a dimer (Galán et al. 2000), and the large subunit of pyrrol-2-carboxylate monooxygenase from *Rhodococcus* sp. exists as a trimer (Becker et al. 1997).

The incubation of His_6PheA2 with a flavin and NAD(P)H resulted in the oxidation of the nicotinamide nucleotide used as electron donor, indicating that the protein possesses NAD(P)H:flavin oxidoreductase activity. Analysis of the catalytic properties of His_6PheA2 showed that the enzyme can use FAD, FMN, and riboflavin as an electron acceptor but with remarkably different efficiency. Moreover, NADPH can replace NADH with lower affinity. This behavior has been described in flavin reductases that do not contain a flavin as prosthetic group and in which the flavin acts as a real free substrate of the reaction (Galán et al. 2000).

The phenol hydroxylase activity required the presence of both the His_6PheA1 and His_6PheA2 components and the redox coenzymes NADH and FAD. The molar ratio of His_6PheA1 to His_6PheA2 appears to affect the rate of phenol hydroxylation, and higher phenol hydroxylase activity was measured by increasing the molar amounts of His_6PheA1. A similar effect was found in pyrrol-2-carboxylate monooxygenase of *Rhodococcus* sp. (Becker et al. 1997), *p*-hydroxyphenylacetate hydroxylase of *A. baumannii* (Chaiyen et al. 2001), and phenol

hydroxylase of *B. thermoglucosidasius* (Kirchner et al. 2003), where the maximum activity was found with a 200:1 molar ratio of oxygenase to reductase.

A divergence has been proposed in the classification of two-protein component aromatic hydroxylases based on the selectivity of the oxygenase components for the flavin and sequence analysis (Thotsaporn et al. 2004). Thus, one type of enzymes uses reduced FMN, FAD, or riboflavin for hydroxylation, whereas the other type of oxygenase uses specifically $FADH_2$. Certainly, His_6PheA1 belongs to the second class of enzymes because its hydroxylase activity was undetected when either FMN or riboflavin was used as a substitute of FAD. Furthermore, the FAD concentration used appears to play an important role on the phenol hydroxylase activity. Thus, according to our results, FAD concentrations higher than 10 μM diminished the measured reaction rate. The same result was reported for 4-hydroxyphenylacetate 3-hydroxylase of *E. coli* W (Xun and Sandvik 2000). Free reduced flavin is unstable and reacts easily with molecular oxygen to yield hydrogen peroxide (Massey 1994; Ballou et al. 2005). It has been proposed that the autoxidation of the accumulated $FADH_2$ leads to an excess of H_2O_2 in the assay, which may be responsible for the observed inhibitory effect. The detection of H_2O_2 in our experiments supports this observation. An alternative explanation was proposed for the two-component styrene monooxygenase of *Pseudomonas* sp. VLB12 (Otto et al. 2004). The authors suggested that the autoxidation of reduced flavin yielded a highly reactive flavin radical, and its formation rate depended on the FAD concentration. The use of a tenfold excess of oxygenase component in the reaction would decrease the amount of auto-oxidated $FADH_2$ to H_2O_2.

Our studies indicate that both His_6PheA1 and His_6PheA2 are essential to catalyze the hydroxylation of phenol in the presence of NADH and FAD. According to sequence analysis and activity characterization, we propose that His_6PheA2 is a flavin reductase that uses NADH to reduce FAD to $FADH_2$ and that the resulting reduced flavin is used by His_6PheA1 to hydroxylate the phenolic substrate (Fig. 6). Nevertheless, the fact that the *E. coli* cells transformed with the plasmid encoding only the His_6PheA1 protein also could hydroxylate phenol to catechol, indicated that other flavin reductases present in *E. coli* would supply the necessary reduced flavin to the oxygenase component. It has been observed a similar activity in vivo in *E. coli* cells expressing the oxygenases HpaB (Prieto and Garcia 1994) and TcpA (Louie et al. 2002), and several *E. coli* flavin reductases that can supply the reduced flavin have been reported (Coves et al. 1993; Ingelman et al. 1999).

In spite of the fact that two components are essential for hydroxylase activity, our results in the two-compartment and gel filtration experiments clearly indicate that no physical interactions between His_6PheA1 and His_6PheA2 are necessary to catalyze the hydroxylation of phenol, suggesting that the reduced flavin diffuses rapidly to the oxygenase component, without being oxidized by molecular O_2, to complete the reaction. These results are in agreement with recent studies in *p*-hydroxyphenylacetate hydroxylase of *A. baumannii* (Sucharitakul et al. 2007) and in FMN-dependent monooxygenase ActVA–ActVB from *Streptomyces coelicolor* (Valton et al. 2008) that indicate that the transfer of the reduced flavin between the two components of the enzyme can occur by a rapid-diffusion process and no protein–protein interactions between the two components are required. However, there are also reports indicating that protein–protein interaction between the reductase and oxygenase components was essential for the efficient transfer of reduced FAD in the alkanesulfonate monooxygenase of *E. coli* (Abdurachim and Ellis 2006) and arylamine oxygenase of *Pseudomonas fluorescens* Pf-5 T (Lee and Zhao 2007).

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Fig. 1

Analysis of the expression and purification of (**A**) His_6PheA1 and (**B**) His_6PheA2 from cells of *E. coli* M15. (*a*) SDS-PAGE in a 12% (*w*/*v*) acrylamide gel stained with Coomassie Brilliant Blue. (*b*) Western blot using specific antibodies against His_6-tagged polypeptides. *Lanes 1* and *6*: molecular mass standards. *Lane 2*: crude extracts of *E. coli* M15 harboring pQE9A1 (**A**) or pQE30A2 (**B**). *Lane 3*: soluble fraction after cellular lysis. *Lane 4*: IMAC column flow-through. *Lane 5*: eluate of IMAC with 300 mM imidazole. *Lane 7*: Western blot of purified protein

Fig. 2

Analysis by PAGE of pure His_6PheA1 under nondenaturing (**a**) or denaturing (**b**) conditions. *Lane 1*: high weight molecular standards (GE Healthcare, Uppsala, Sweden). *Lane 2*: purified His_6PheA1 after staining with Coomassie Brilliant Blue. *Lane 3*: molecular mass standards (Bio-Rad, Hercules, CA, USA). *Lane 4*: purified His_6PheA1 after silver staining

Fig. 3

Kinetics of NADH:FAD oxidoreductase activity of His_6PheA2. (**A**) Double reciprocal plots of rates as a function of NADH concentration at different FAD concentrations: 5 µM (*filled circles*), 10 µM (*filled squares*), 25 µM (*filled upright triangles*), 50 µM (*filled inverse triangles*), and 100 µM (*empty circles*). (**B**) Double reciprocal plots of rates as a function of FAD concentration at different NADH concentrations: 5 µM (*filled circles*), 10 µM (*filled squares*), 25 µM (*filled upright triangles*), 50 µM (*filled inverse triangles*), and 100 µM (*empty circles*)

Fig. 4

Phenol hydroxylase activity in *E. coli* M15 cells. HPLC chromatogram showing the consumption of phenol and concomitant formation of catechol in cells of *E. coli* M15 transformed with pQE9A1 plasmid grown in LB medium supplemented with 1 mM phenol (*solid line*). Catechol formation was undetectable in control cells transformed with pQE9 plasmid (*dashed line*)

Fig. 5

Flavin specificity of phenol hydroxylase activity of His_6PheA1. **a** Phenol hydroxylase activity in the presence of FAD (*filled circles*), FMN (*filled inverse triangles*), or riboflavin (*filled squares*). The reaction mixture contained 1 mM NADH, 80 µM phenol, 104 µg of His_6PheA1, 3.7 µg of His_6PheA2, and 10 mM flavin in 100 mM phosphate buffer, pH 6.8. **b** Effect of FAD concentration on phenol hydroxylase activity. The reaction mixture contained 1 mM NADH, 100 µM phenol, 104 µg of His_6PheA1, 3.7 µg of His_6PheA2, and the indicated concentration of FAD in 100 mM phosphate buffer, pH 6.8

Fig. 6

Scheme of the proposed reaction catalyzed by the two-component phenol hydroxylase of *R. erythropolis* UPV-1

Fig 1

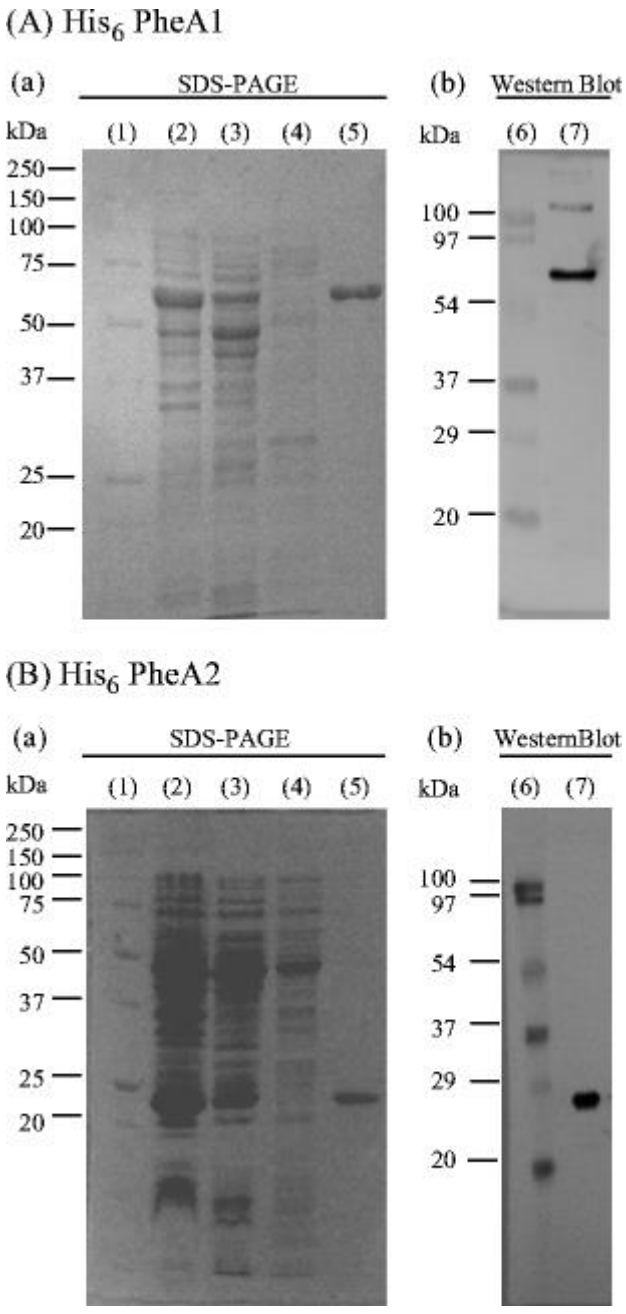
(A) His6 PheA1
(a)
SDS-PAGE
kDa
(1) (2) (3) (4) (5)
250
150
100
75
50
37
25
20
(b)
Western Blot
kDa
(6) (7)
100
97
54
37
29
20
(B) His6 PheA2
(a)
SDS-PAGE
kDa
(1) (2) (3) (4) (5)
250
150
100
75
50
37
25
20
(b)
WesternBlot
kDa
(6) (7)
100
97
54
37
29
20

Fig 2

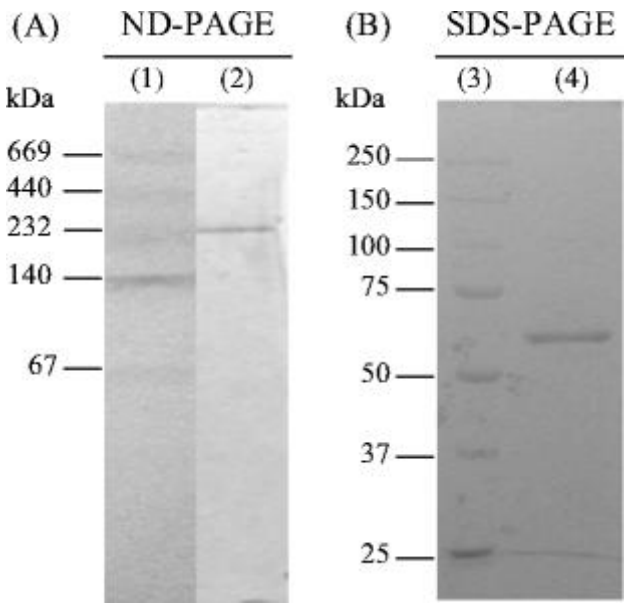
(A)
ND-PAGE
(1)
(2)
kDa
669
440
232
140
67
(B)
SDS-PAGE
(3)
(4)
kDa
250
150
100
75
50
37
25

Fig3

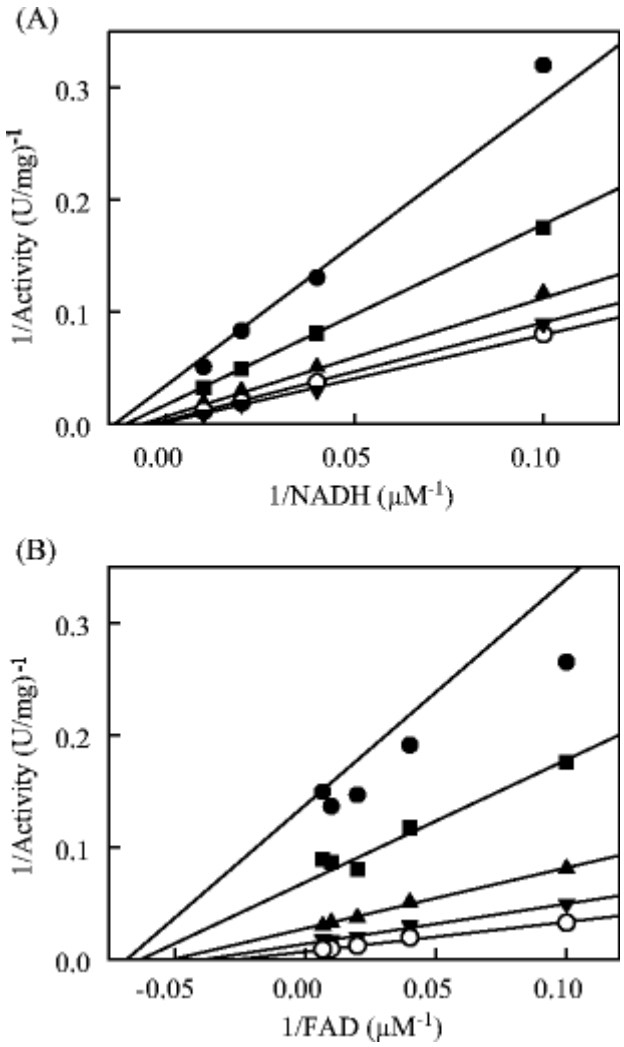
(A)
0.3
0.2
0.1
0.0
1/Activity (U/mg)-1
0.00
0.05
0.10
1/NADH (μM-1)
(B)
0.3
0.2
0.1
0.0
1/Activity (U/mg)-1
-0.05
0.00
0.05
0.10
1/FAD (μM-1)

Fig 4

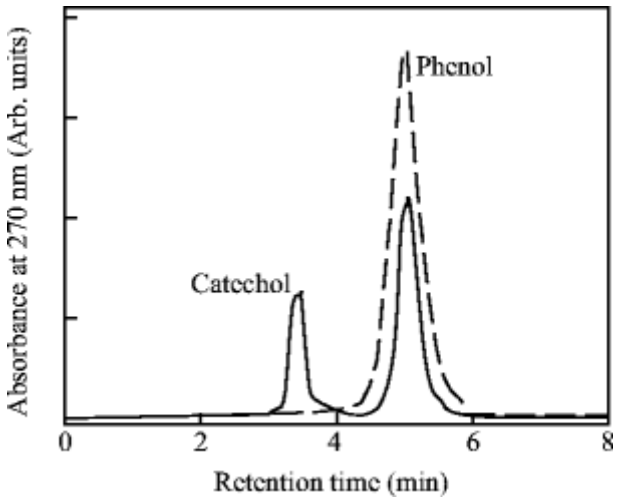
Absorbance at 270 nm (Arb. units)
Phenol
Catechol
0
2
4
6
8
Retention time (min)

Fig 5

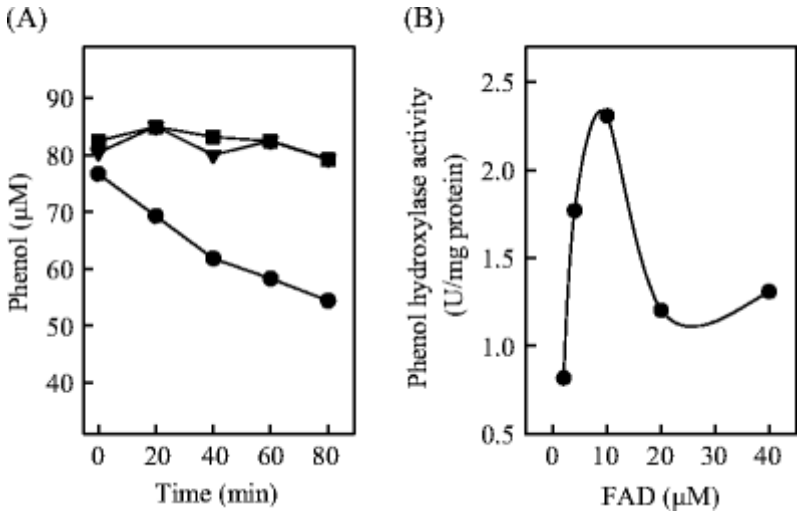
(A)
Phenol (μM)
90
80
70
60
50
40
0
20
40
60
80
Time (min)
(B)
Phenol hydroxylase activity
(U/mg protein)
2.5
2.0
1.5
1.0
0.5
0
10
20
30
40
FAD (μM)

Fig 6

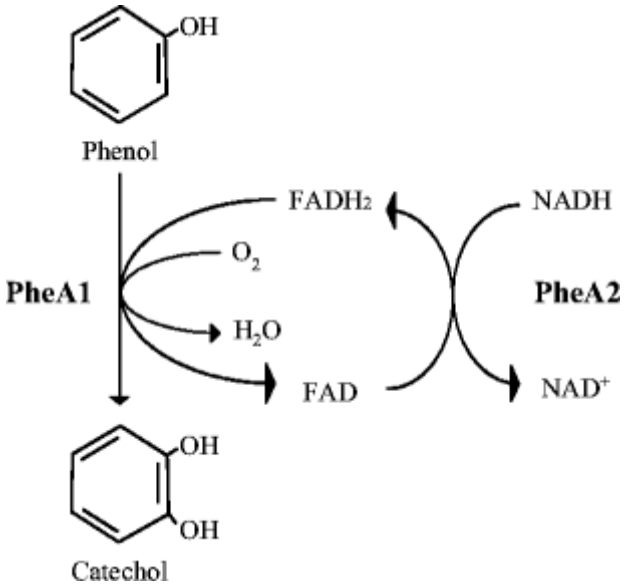
OH
Phenol
FADH2
NADH
O2
PheA1
PheA2
H2O
FAD
NAD+
OH
OH
Catechol

Table 1 Kinetic parameters of His_6PheA2 activity

Substrate	K_m (μM)	V_{max} (U mg^{-1} protein)	V_{max}/K_m (U mg^{-1} protein μM^{-1})
FAD	13.4	411.7	30.72
FMN	69.1	181.1	2.62
Riboflavin	67.7	83.6	1.23
$NADPH_{FAD}$	271	184.2	–
$NADPH_{FMN}$	606	79.0	–

Activity was determined as described in "Materials and methods." Kinetic parameters were calculated fitting data to a hyperbola by nonlinear regression. One unit of enzyme (U) catalyzed the consumption of 1 μmol of NAD(P)H per minute. K_m and V_{max} of flavins were determined using 400 μM NADH and variable concentrations of flavins. K_m and V_{max} of NADPH were determined using 150 μM of the indicated flavin nucleotide and variable concentrations of NADPH

Table 2 Effect of some cations and reagents on His_6PheA2 activity

Effector	Concentration (mM)	Activity (%)
None (control)	–	100
Iodoacetamide	1	100
Phenylmethylsulfonyl fluoride	1	100
EDTA	1	100
N-Ethylmaleimide	0.1	62
	1	0
p-Hydroxymercuribenzoate	0.0005	47
	0.02	0
Ag^{+}	0.02	0
Cu^{2+}	0.02	0
Ni^{2+}	0.1	21
	1	0
Fe^{3+}	0.1	77
	1	0
Zn^{2+}	1	23
Fe^{2+}	1	51
Co^{2+}	1	80
Mg^{2+}	1	127
Mn^{2+}	1	113

Activity was assayed in reaction mixtures containing 400 µM NADH, 150 µM FAD, and 6.2 µg of His_6PheA2 in 100 mM phosphate buffer, pH 6.8. Results are expressed as percentages of the values of their respective control assayed in parallel. The specific activity in the control assay corresponded to 189 U mg^{-1} protein. Cations were added as chlorides, except in the case of Zn^{2+} and Fe^{2+} which were added as sulfates