A novel phenol hydroxylase (PheA) that catalyzes the first step in the degradation of phenol in *Bacillus thermoglucosidasius* A7 is described. The two-protein system, encoded by the *pheA1* and *pheA2* genes, consists of an oxygenase (PheA1) and a flavin reductase (PheA2) and is optimally active at 55 °C. PheA1 and PheA2 were separately expressed in recombinant *Escherichia coli* BL21(DE3) pLysS cells and purified to apparent homogeneity. The *pheA1* gene codes for a protein of 504 amino acids with a predicted mass of 57.2 kDa. PheA1 exists as a homodimer in solution and has no enzyme activity on its own. PheA1 catalyzes the efficient ortho-hydroxylation of phenol to catechol when supplemented with PheA2 and FAD/NADH. The hydroxylase activity is strictly FAD-dependent, and neither FMN nor riboflavin can replace FAD in this reaction. The *pheA2* gene codes for a protein of 161 amino acids with a predicted mass of 17.7 kDa. PheA2 is also a homodimer, with each subunit containing a highly fluorescent FAD prosthetic group. PheA2 catalyzes the NADH-dependent reduction of free flavins according to a Ping Pong Bi Bi mechanism. PheA2 is structurally related to ferric reductase, an NAD(P)H-dependent reductase from the hyperthermophilic Archaea *Archaeoglobus fulgidus* that catalyzes the flavin-mediated reduction of iron complexes. However, PheA2 displays no ferric reductase activity and is the first member of a newly recognized family of short-chain flavin reductases that use FAD both as a substrate and as a prosthetic group.

Phenolic compounds constitute one of the largest groups of natural products. They are predominantly found in plants, where they occur in a great variety of structures and functions. During the last century, the natural pool of phenolic compounds has been increased with products of industrial origin. Many of these synthetic compounds cause environmental pollution and human health problems as a result of their persistence, toxicity, and transformation into hazardous intermediates (1, 2).

The aerobic mineralization of natural and xenobiotic phenolic compounds by mesophilic microorganisms has been intensively investigated, and numerous pathways are known (3). Almost invariably, phenols are first converted into more reactive dihydroxylated intermediates and then subjected to intracellular degradation by molecular oxygen. The initial hydroxylation of the phenolic ring usually is catalyzed by single-component NAD(P)H-dependent flavoprotein monooxygenases (4). These enzymes share a common dinucleotide-binding fold for complexation of the FAD cofactor while lacking a common NAD(P)-binding fold (5, 6). Because the regioselective hydroxylation of phenols is notoriously difficult to achieve by chemical methods, the mechanistic and structural features of single-component flavoprotein monooxygenases have received much attention (7–11). The reduced forms of these enzymes react with molecular oxygen to yield a transiently stable flavin C4a-hydroperoxide species that is involved in substrate oxygenation. For *Pseudomonas* *p*-hydroxybenzoate hydroxylase (12–14) and phenol hydroxylase from yeast (15), it was shown that the oxygenation reaction takes place in the inner part of the protein and that this aprotic environment is crucial for preventing uncoupling of flavin reduction from substrate hydroxylation. With 4-hydroxyphenylacetate 3-hydroxylase from *Pseudomonas putida*, the binding of a second protein component prevents the uncoupling of substrate hydroxylation (16–18). This enzyme is an unusual example of a two-component flavoprotein hydroxylase in which flavin reduction and substrate oxygenation take place in the same protein.

Relatively little is known about the mineralization of phenolic compounds by thermophilic microorganisms (19). Several *Bacillus* species isolated from geographically distinct thermal sources degrade phenol at 65 °C via the *meta*-cleavage pathway (Fig. 1) (20–22). The initial conversion of phenol to catechol in these microorganisms requires two protein components that are encoded by the *pheA1* and *pheA2* genes (23). Characterization of the *Bacillus* phenol hydroxylase system appeared to be severely hampered by the low yield and instability of the purified enzymes. This prompted us to clone the *Bacillus pheA* genes in an *Escherichia coli* expression system (24).

In this study, we describe the overexpression, purification, and characterization of the recombinant phenol hydroxylase of *Bacillus thermoglucosidasius* A7. We show that this two-protein enzyme belongs to a newly recognized family of flavin-dependent monooxygenases that carry out the reductive and oxidative half-reactions on separate polypeptide chains. Members of this family are involved in various biological processes, including the biosynthesis of antibiotics (25–27), the desulfurization of fossil fuels (28), the degradation of chelating agents (29), and the oxidation of aromatic compounds (30). In contrast to most other family members, the reductase component of phenol hydroxylase from *B. thermoglucosidasius* A7 harbors a tightly bound FAD. Evidence is provided that the FAD cofactor is directly involved in the reduction of free flavins.
EXPERIMENTAL PROCEDURES

Chemicals, Bacterial Strains, and Plasmids—E. coli strain BL21(DE3) pLyS8 and plasmid pET3a were used for expression of the pheA genes (31). Isopropyl-β-D-thiogalactopyranoside was purchased from Invitrogen. Phenyl-Sepharose, Q-Sepharose Fast Flow, HiLoad Q-Sepharose, Superdex 200 pg, Superdex 200 HR10/30, Superdex 75 HR10/30, and PhastGel precast isoelectric focusing gels were from Amersham Biosciences. Macro-prep ceramic hydroxylapatite (type I, particle size of 20 μm) was obtained from Bio-Rad. FAD, FMN, riboflavin, and 3-acyethylpyridine-adениne dinucleotide (AcPyAD) were purchased from Sigma. NADH, NADPH, NAD+, and glucose oxidase (grade II) were from Roche Applied Science. Phenolic compounds were obtained from Aldrich. All other chemicals were from Merck and were of the purest grade available.

Enzyme Purification—All purification steps were carried out at room temperature. PheA1 and PheA2 were purified from transformed E. coli cells harboring the pheA1 and pheA2 genes (24), respectively. Recombinant cells were grown for 16 h in medium containing 10 g/liter Tryptone, 5 g/liter yeast, and 5 g/liter NaCl (pH 7.4) at 30 °C, followed by induction with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h.

Purification of PheA1 (Oxidase Component)—Washed recombinant E. coli cells (15 g) were suspended in 15 ml of 50 mM sodium phosphate (pH 7.0) containing 0.5 mM EDTA, 0.5 mM phenylmethylsulfon-aryl fluoride, and 0.5 mg of DNase and passed two times through a precooled French press operating at 10,000 p.s.i. Following centrifugation for 15 min at 15,000 × g to remove cellular debris, the supernatant was made 0.5% in protamine sulfate from a 2% stock solution. The protamine sulfate aggregates were removed by centrifugation for 15 min at 15,000 × g, and the resulting supernatant was applied to a Q-Sepharose Fast Flow column (1.6 × 10 cm) equilibrated with 50 mM sodium phosphate (pH 7.0) containing 0.5 mM EDTA (starting buffer). After washing with starting buffer, the bound protein was eluted with a linear gradient of 0–0.6 M NaCl in 100 ml of starting buffer. The PheA1 protein eluting between 0.3 and 0.5 M NaCl was concentrated by ultrafiltration, and loaded onto a Superdex 200 prep column (2.6 × 60 cm) equilibrated with 50 mM potassium phosphate (pH 7.0) containing 150 mM NaCl. Pure reductase fractions were pooled, concentrated, and stored in 50 mM potassium phosphate (pH 7.0) at −70 °C.

Purification of PheA2 (Reductase Component)—The preparation of cell extract from recombinant E. coli cells, the proteamine sulfate precipitation, and the ion exchange chromatography step were carried out under the same conditions as described above. HiLoad Q-Sepharose fractions containing FAD reductase activity (eluting between 0.1 and 0.5 M NaCl) were pooled and concentrated by ultrafiltration (Amicon YM-30 membrane). After the addition of pulverized ammonium sulfate to a final concentration of 1.4 M, the protein solution was loaded onto a phenyl-Sepharose column (1.6 × 10 cm) pre-equilibrated with 5 mM sodium phosphate (pH 7.0). The PheA1 protein was then loaded onto a hydroxylapatite column (1.6 × 10 cm) pre-equilibrated with 5 mM sodium phosphate (pH 7.0). The PheA1 protein was then loaded onto a hydroxylapatite column (1.6 × 10 cm) pre-equilibrated with 5 mM sodium phosphate (pH 7.0). The PheA1 protein was then loaded onto a hydroxylapatite column (1.6 × 10 cm) pre-equilibrated with 5 mM sodium phosphate (pH 7.0). The PheA1 protein was then loaded onto a hydroxylapatite column (1.6 × 10 cm) pre-equilibrated with 5 mM sodium phosphate (pH 7.0). The PheA1 protein was then loaded onto a hydroxylapatite column (1.6 × 10 cm) pre-equilibrated with 5 mM sodium phosphate (pH 7.0).

Activity Determinations—NADH:flavin reductase activity was determined at 25 or 53 °C in 25 mM potassium phosphate and 150 mM KCl (pH 7.0). To determine the specificity of the reaction, FMN, riboflavin, and NADPH were tested as substrates. NADFeichrome c reductase activity was determined spectrophotometrically at 25 °C by recording the NADH-dependent reduction of cytochrome c at 550 nm (ε550 = 21.1 mM−1 cm−1). The assay mixture contained 0.2 mM cytochrome c and 0.2 mM NADH in 50 mM potassium phosphate (pH 7.5). NADH oxidase activity was determined spectrophotometrically at 25 °C by monitoring the decrease in absorption of NADH at 340 nm (ε340 = 6.22 mM−1 cm−1). The assay mixture contained 0.2 mM NADH in 50 mM potassium phosphate (pH 7.0). The NADH-dependent reduction of 2,6-dichloropheno- lindophenol was measured at 25 °C by following the decrease in absorption of 2,6-dichlorophenolindophenol at 600 nm (ε600 = 21.0 mM−1 cm−1), pH 7.0. The assay mixture contained 25 mM potassium phosphate (pH 7.0), 150 mM KCl, 200 μM NADH, and varying concentrations of 2,6-dichlorophenolindophenol. The NADH-dependent reduction of AcPyAD (transhydrogenase activity) was measured at 25 °C by following the increase in absorption of reduced AcPyAD at 365 nm (ε365 = 5.6 mM−1 cm−1). The assay mixture contained 25 mM potassium phosphate (pH 7.0), 150 mM KCl, 200 μM NADH, and varying concentrations of AcPyAD.

Kinetic Analysis—NADH:flavin reductase activity was determined at 25, 40, and 53 °C in 25 mM potassium phosphate and 150 mM KCl (pH 7.0). For estimation of kinetic parameters, the NADH concentration was varied at a fixed flavin concentration and vice versa. Kinetic parameters were determined from saturation curves, fitted with the Michaelis-Menten equation, using a Levensberg-Marquardt algorithm. For estimation of the type of mechanism, the NADH:flavin reductase activity was determined as a function of FAD concentration at several constant levels of NADH and as a function of NADH at several constant levels of FAD. Reciprocals of initial velocities were plotted against reciprocal substrate concentrations and fitted with a straight line determined by a linear regression program. Inhibition constants for NAD+ inhibition were determined according to Fromm (32).

Other Assays—Phenol monooxygenase activity was determined by measuring phenol consumption colorimetrically (33). The reaction mixture (6 ml) contained 0.1 mM phenol, 0.5 mM NADH, 10 μM FAD, and 1.0 mM PheA2 in 50 mM potassium phosphate (pH 7.0). After equilibration at 50 °C and the addition of the desired amount of PheA1 (10–200 nm), samples of 1 ml were taken for 10 min at 1-min intervals. The reaction was stopped by the addition of 12 μl of 2% 4-aminoantipyrine followed by 40 μl of 2% ammonium hydroxide and 40 μl of 2% potassium ferricyanide, and the final volume was adjusted to 2 ml with water. After 15 min of incubation at room temperature, the absorbance at 510 nm was read and compared with those of phenol standards. 1 unit is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate/min under the assay conditions. Oxygen consumption was determined polarographically at 50 °C in a closed reaction vessel fitted with a Clark-type oxygen electrode. Reaction mixtures contained 50 mM sodium phosphate (pH 7.0), 0.1 mM phenol, 5–10 μM FAD, and 0.5 mM PheA2 in the absence or presence of 100 nM PheA1. NADH was added to a final concentration of 0.1 mM to initiate the reaction. When oxygen consumption ceased, 90 units of catalase were added to determine the degree of uncoupling of hydroxylation.

Thermostability—Studies on the thermostability of PheA1 and PheA2 were performed by incubating the enzymes (46 μM PheA1 or 48 μM PheA2) in closed vials at different temperatures (50, 60, 70, and 85 °C) in 25 mM phosphate (pH 7.0) for 2 h. At timed intervals, aliquots were withdrawn from the incubation mixtures and assayed for residual phenol hydroxylase (PheA1) and NADH:flavin reductase (PheA2) activities.

Analytical Methods—HPLC experiments were performed with an Applied Biosystems pump equipped with a Waters 996 photodiode array detector. Reaction products were separated with a 3.9 × 100-mm Lichrospher RP18 column running in methanol/water (50:50, v/v) containing 0.7% acetic acid. The flow rate was 1 ml/min. Protein was
determined by the method of Bradford (34) with bovine serum albumin as a standard. SDS-PAGE was carried out with 15% slab gels (35) or with 16.5% Tricine gels (36). The gels were scanned and analyzed with a computing densitometer system (Scanjet ADF, Hewlett-Packard Inc.) with 16.5% Tricine gels (36). The gels were scanned and analyzed with a Bio-Rad Densitometer system (Scanjet ADF, Hewlett-Packard Inc.).

**Results**

**Overexpression of pheA Genes**—Both protein components PheA1 and PheA2 from *B. thermoglucosidasius* A7 are required for phenol hydroxylase activity (24). However, *E. coli* cells containing a plasmid with the genes for PheA1 and PheA2 in tandem did not express PheA2, as only a clear protein band for PheA1 (but not for PheA2) could be detected by SDS-PAGE analysis of cell extracts (24). A likely explanation is that, in this construct, PheA2 expression must use its own *B. thermoglucosidasius* A7 ribosome-binding site, which might not be effective in *E. coli*. When PheA2 was expressed separately, expression was under the control of the highly efficient phage T7 promoter, and a clear protein band of PheA2 was visible upon SDS-PAGE analysis of cell extracts (24). Therefore, PheA1 and PheA2 were separately purified from *E. coli* cells containing the appropriate plasmid with the gene for PheA1 or PheA2.

**Purification of PheA1**—Extracts of *E. coli* cells containing the plasmid with only the pheA1 gene exhibited phenol hydroxylase activity. Because these cells do not contain PheA2, this indicates that a flavin reductase activity present in the *E. coli* host substitutes for PheA2 to give substrate conversion. A similar observation was made for 4-hydroxyphenylacetate 3-monoxygenase from *E. coli* W (30). During purification of PheA1, phenol hydroxylase activity was determined with and without complementation with PheA2. The phenol hydroxylase activity determined in the absence of PheA2 decreased after every purification step (Table I). The specific activity of purified PheA1 determined in the presence of PheA2 was 0.32 units/mg. SDS-PAGE analysis of the purified PheA1 protein revealed the presence of a single polypeptide chain corresponding to a molecular mass of ~57 kDa (Fig. 2). This value is in good agreement with the molecular mass predicted from gene sequence analysis (24).

Purified PheA1 did not contain any chromophore as judged by absorption spectral analysis and eluted from an analytical Superdex 200 HR10/30 column in a single symmetrical peak with an apparent molecular mass of 120 ± 5 kDa. This suggests that the PheA1 protein is a dimer composed of identical subunits.

Isoelectric focusing of PheA1 revealed a main protein band with an isoelectric point of 5.2 ± 0.1. This value is considerably lower than the theoretical value of 6.29 calculated from the amino acid sequence. Purified PheA1 was stable for 2 h at 60 °C, as no significant decrease in phenol hydroxylase activity was observed in complementation experiments with PheA2. In contrast, at 70 °C, inactivation of PheA1 was complete after 10 min of incubation. The purified PheA1 protein was not very stable when stored at −70 °C because it formed aggregates after thawing. Therefore, PheA1 was stored as a protein precipitate in 80% ammonium sulfate at 4 °C.

**Purification of PheA2**—The PheA2 protein expressed in *E. coli* cells containing the plasmid with the pheA2 gene was purified to apparent homogeneity using three chromatographic steps (Table II). The specific NADH:FAD reductase activity of the purified PheA2 protein at 53 °C was 800 units/mg. Analysis by SDS-PAGE revealed the presence of a single band corresponding to a polypeptide chain molecular mass of ~18 kDa (Fig. 3). Again, this value is in good agreement with the mo-
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lecular mass predicted from gene sequence analysis (17,660 Da) (24). The purified PheA2 protein was bright yellow and eluted from an analytical Superdex 200 HR10/30 column in a single symmetrical peak with an apparent molecular mass of 35 ± 3 kDa, indicative of a homodimeric structure. When PheA2 was mixed with PheA1 and analyzed by gel filtration, no interaction between both proteins was observed.

The isoelectric point of PheA2 was determined by isoelectric focusing to be 5.2 ± 0.1. This value agrees with the theoretical value (5.36) estimated from the amino acid sequence. PheA2 was stable in the frozen state because no significant loss of activity was observed during 4 months of storage at −70 °C. When purified PheA2 was incubated for 2 h at 65 °C, >65% of the NADH:FAD reductase activity was maintained. Time-dependent analysis showed that the partial loss of activity at 65 °C occurred within the first 10 min of incubation. When the enzyme was incubated at 75 or 85 °C, the activity decreased faster, but a similar behavior was observed. After 2 h of incubation at 85 °C, still 15–20% of the NADH:FAD reductase activity remained.

PheA2 contains a single cysteine residue (Cys112) per polypeptide chain (24). Absorption measurements at 412 nm revealed that this cysteine reacted rapidly with 5,5'-dithiobis(2-nitrobenzoate) when the protein was unfolded with 0.5% SDS. In the native enzyme, the side chain of Cys112 is not accessible for solvent. This is concluded from the lack of reactivity of native PheA2 with Ellman’s reagent.

Spectral Properties of PheA2—The optical spectrum of PheA2 was typical of a flavoprotein with maxima at 376 and 455 nm and characteristic shoulders around 355 and 485 nm (Fig. 4A). The $A_{376}/A_{455}$ ratio for the purified enzyme was 4.1. This value is in good agreement with the low amount of aromatic amino acid residues (24) and suggests that each PheA2 subunit binds one molecule of flavin. Extraction of the cofactor revealed that the flavin was noncovalently bound. Moreover, the HPLC retention time and fluorescence properties of the extracted flavin were identical to those of authentic FAD. From the absorption spectra of PheA2 in the absence and presence of 0.5% SDS (Fig. 4A), a molar absorption coefficient of $ε_{455} = 11.8 \text{ m}^{-1} \text{cm}^{-1}$ for protein-bound FAD was estimated.

The fluorescence quantum yield of the protein-bound FAD of PheA2 was rather high. Upon excitation at 450 nm, a maximum fluorescence emission was observed around 525 nm (data not shown). Upon unfolding of PheA2 in 0.5% SDS, the fluorescence intensity of the flavin decreased by almost an order of magnitude. This indicates that the flavin fluorescence quantum yield of PheA2 is comparable to that of free FMN (38) and in the same range as that of lipoamide dehydrogenase, one of the most fluorescent flavoproteins (49).

Fig. 4B shows the flavin circular dichroism spectrum of PheA2 in the visible region. This spectrum resembles those of flavin reductase from Streptomyces viridifaciens (27), ferredoxin:NADP$^+$ oxidoreductase (50), and the enzyme-substrate complex of p-hydroxybenzoate hydroxylase (51) by displaying negative circular dichroism in the 450 nm region and positive optical activity in the near-UV band.

When PheA2 was (photo)chemically reduced under anaerobic conditions, the flavin hydroquinone was formed, and no thermodynamic stabilization of semiquinone species was observed. Upon titration with NAD$^+$, the two-electron reduced enzyme formed a complex with NAD$^+$, as evidenced by the formation of a long wavelength absorption band. Anaerobic reduction of PheA2 with NADH also led to the reduced enzyme-NAD$^+$ complex (Fig. 5). At pH 8.0, the enzyme could not be fully reduced with NADH, in contrast to reduction at pH 6.0 (Fig. 5).

Catalytic Properties of PheA2—In addition to NADH:FAD reductase activity, PheA2 exhibited also NADH:cytochrome c reductase and NADH: dichlorophenolindophenol and NADH:cytochrome $c$ oxidoreductase (50), and the enzyme-substrate complex ($p$-hydroxybenzoate hydroxylase (51) by displaying negative circular dichroism in the 450 nm region and positive optical activity in the near-UV band.

The pH and temperature optima of the NADH:FAD reductase activity of PheA2 were pH 6.7 and 55 °C (Figs. 6 and 7). Determination of kinetic parameters revealed that NADH is a much better coenzyme for PheA2 than NADPH (Table IV). The FAD reductase activity exhibited a linear dependence on NADPH concentration over a range 10–500 $\mu$M, and no indication of saturation was observed. Thus, $K_m$ and $k_{cat}$ values for NADPH could not be determined under these conditions. However, from the slope of the kinetic plot, a second-order rate constant of $k_{cat}/K_m = 0.066 \text{s}^{-1}$ could be estimated. This value...
is at least two orders of magnitude lower than the corresponding value for NADH (Table IV).

When the NADH:FAD reductase activity of PheA2 was studied under anaerobic conditions, all free FAD was rapidly reduced, confirming that the free FAD acts as a true substrate. In addition to FAD, PheA2 was also active with FMN and riboflavin. The turnover rate of PheA2 with free flavins was strongly dependent on temperature. At $25^\circ C$, the activity with FMN and riboflavin was much higher than with FAD (Table III). However, when the temperature was raised to $53^\circ C$, the turnover rates with the different flavins became nearly identical (Table IV). On the other hand, the Michaelis constants for NADH, FAD, riboflavin, and FMN did not vary with temperature (Tables III and IV).

Initial velocity measurements in dependence of both FAD and NADH were performed to provide insight into the kinetic mechanism of PheA2. When the NADH concentration was varied at a fixed FAD concentration, a series of parallel lines was observed in a double-reciprocal plot (Fig. 8A). A similar pattern was observed when the FAD concentration was varied at a fixed NADH concentration (Fig. 8B). This suggests that PheA2 acts according to a Ping Pong Bi Bi reaction mechanism in which NADH reduces the FAD cofactor, which in turn transfers electrons to the FAD substrate. A similar kinetic behavior was observed when the NADH:AcPyAD$^+$ transhydrogenase activity of PheA2 was determined as a function of AcPyAD$^+$ concentration at several constant levels of NADH and as a function of NADH at several constant levels of AcPyAD$^+$ (data not shown). The NADH:FAD reductase activity of PheA2 was inhibited by NAD$^+$. In line with the above-described mechanism, the inhibition was noncompetitive to NADH and competitive to FAD (data not shown).

**Phenol Hydroxylase Activity**—Purified PheA1 showed almost no phenol hydroxylase activity when assayed at $50^\circ C$ and pH 7.0 in the absence of PheA2. The PheA1-mediated conversion of phenol to catechol was strongly stimulated in the presence of catalytic amounts of PheA2. At relatively low concentrations of PheA1, phenol hydroxylase activity was not linear with time. The addition of catalase to the reaction mixture to avoid the
accumulation of hydrogen peroxide had no effect. At a constant concentration of PheA2 and increasing concentrations of PheA1 in the presence of phenol, the oxygen consumption increased, and the relative amount of hydrogen peroxide formed decreased. When the dependence of the hydroxylation reaction on the concentration of free FAD at constant concentrations of PheA1 and PheA2 (200:1 molar ratio) was studied, the oxygen consumption became faster with increasing concentrations of free FAD, but the amount of uncoupling increased (Table V). In contrast to the NADH:flavin reductase activity of PheA2, the hydroxylase activity of PheA was strictly FAD-dependent, and neither FMN nor riboflavin could replace FAD in this reaction.

Under optimal assay conditions with PheA1 in large excess over PheA2, maximum phenol hydroxylase activity was reached at 55°C and pH 7.0. HPLC analysis revealed that PheA from B. thermoglucosidasius A7 also catalyzed the conversion of 4-methylphenol, 4-chlorophenol, and 4-fluorophenol to the corresponding catechols. No monooxygenase activity was observed with 4-hydroxybenzoate, 4-hydroxyacetophenone, and 4-hydroxyphenylacetate.

To test whether a nonspecific FAD reductase might substitute for PheA2 to give substrate conversion, the phenol hydroxylase activity of purified PheA1 was measured in the presence of E. coli NADH oxidoreductase, which is physiologically associated with the so-called hybrid cluster protein (53). At 50°C, this iron-sulfur and FAD-containing reductase initiated the formation of catechol. However, the E. coli reductase was not

![Fig. 5](image.png)  
**Fig. 5.** Spectral changes upon anaerobic reduction of PheA2 from B. thermoglucosidasius A7. Trace 1 is the absorption spectrum of 16 μM oxidized PheA2 in 25 mM potassium phosphate (pH 8.0) and 150 mM KCl. The spectrum of oxidized PheA2 is identical at pH 6.0. Spectrum after anaerobic reduction by 25 μM NADH at pH 8.0 (trace 2), after reduction by 25 μM NADH at pH 6.0 (trace 3), and after reduction by sodium dithionite at pH 8.0 (trace 4).

![Fig. 6](image.png)  
**Fig. 6.** Dependence of the NADH:FAD reductase activity of flavin reductase (PheA2) from B. thermoglucosidasius A7 on pH. The assay mixture contained 25 mM potassium phosphate buffer at different pH values, 150 mM KCl, 200 μM NADH, and 20 μM FAD. The assay temperature was 53°C.

![Fig. 7](image.png)  
**Fig. 7.** Dependence of the NADH:FAD reductase activity of flavin reductase (PheA2) from B. thermoglucosidasius A7 on temperature. The assay mixture contained 25 mM potassium phosphate buffer (pH 7.0), 150 mM KCl, 200 μM NADH, and 20 μM FAD. The assay temperature was varied from 18 to 64°C.
very stable under these assay conditions, and several aliquots of enzyme were needed to consume all of the phenol.

Sequence Homology—N-terminal amino acid sequence analysis of the purified recombinant PheA1 and PheA2 proteins revealed sequences Met-Lys-Asp-Met-Met and Met-Asp-Asp-Arg-Leu, respectively. These sequences are identical to those deduced from the nucleotide sequences of the pheA1 and pheA2 genes (24).

Multiple sequence alignment revealed that PheA1 shares the most sequence identity (up to 54%) with the oxygenase component of (i) phenol hydroxylase from Bacillus thermoleovorans A2 (23), (ii) chlorophenol 4-monoxygenase from Ralstonia picketii (54) and Burkholderia cepacia (55), and (iii) several (putative) bacterial 4-hydroxyphenylacetate 3-hydroxylases (30, 56, 57).

PheA2 resembles a larger number of proteins than PheA1. Except for the reductase components of the above-mentioned two-component enzymes, PheA2 shares considerable sequence identity (up to 57%) with the reductase component of (i) styrene monooxygenase (58); (ii) dibenzothiophene monooxygenase (59); (iii) actinorhodin-polyketide dimerase (25); (iv) nitrolotri-acetate monooxygenase (60); (v) VlmR, an NADPH:FAD oxidoreductase from S. viridifaciens (27); (vi) SnaC, the FMN reductase component of pristinamycin IIA synthase from Streptomyces pristinaespiralis (26); and (vii) a number of hypothetical flavin-binding proteins, including the putative reductase component of phenol hydroxylase from Oceanobacillus iheyensis (61) and the NimA protein (GenBankTM/EBI Data Bank accession number Q8VQV3).

Structure Homology Modeling of PheA2—So far, no crystal structures of members of the family of two-component flavoprotein monooxygenases have been reported. However, the PheA2-related flavin reductases are homologous to the NAD(P)H-dependent FeR from the hyperthermophilic Archaea A. fulgidus (62), for which the three-dimensional structure was recently solved (46). FeR reduces FAD and FMN, but not riboflavin, whereas PheA2 shows no ferric reductase activity. Another remarkable difference between PheA2 and FeR is that the latter enzyme was purified as an apoprotein (62). Furthermore, in the FeR crystal structure, only one subunit contains FMN, and NADP+ was found to bind only to the subunit that harbors FMN (46). PheA2 shares 46% sequence homology (24% sequence identity) with FeR (Fig. 9), which prompted us to build a model of the three-dimensional structure of PheA2 (Fig. 10). As found for FeR, each PheA2 subunit consists of a single domain that is organized around a six-stranded antiparallel β-barrel that is homologous to the FMN-binding protein from Desulfoviridium vulgaris (63). The FMN part of FAD was fit in the model structure at the same position and orientation as the FMN in FeR. The AMP part of FAD was docked into the PheA2 model in a groove elongating the FMN cofactor-binding site. However, because of steric constraints and the poor alignment with a flexible loop in the FeR structure comprising residues 85–100, the location of this part of the FAD molecule remained elusive.

DISCUSSION

PheA is a novel type of phenol hydroxylase that is induced in the thermophilic bacterium B. thermoglucosidasius A7 when the strain is grown on phenol as a source of carbon and energy. The phenol hydroxylase system consists of two homodimeric proteins with subunit molecular masses of 57 and 18 kDa, respectively. The large PheA1 component contains no cofactors and has no enzyme activity on its own. The small PheA2 component is an NADH-dependent FAD-containing reductase that supplies PheA1 with reduced FAD to catalyze the ortho-hydroxylation of simple phenols to the corresponding catechols.

During the past few years, several two-protein component aromatic hydroxylases relying on reduced flavin as a substrate...
have been identified. These enzymes show a remarkable variety in protein size and flavin specificity (52). So far, no structural data of these enzymes are available, and their mechanisms of reduced flavin transfer and substrate hydroxylation remain to be elucidated. For 4-hydroxyphenylacetate 3-hydroxylase from *E. coli* (64) and *A. baumannii* (52), it was reported that the oxygenase component is able to use chemically reduced flavin for 4-hydroxyphenylacetate monooxygenation and that a physical interaction between the oxygenase and reductase protein components is not required for substrate hydroxylation (30, 65). Here we have shown that the purified dimeric PheA1 and PheA2 proteins do not strongly interact, but that for the *in vitro* conversion of phenol to catechol, a large excess of PheA1 over PheA2 is needed to prevent the unproductive formation of hydrogen peroxide.

Another important finding described in this study is the fact that PheA2 harbors a tightly bound FAD. The interaction between PheA2 and FAD is much stronger than found for other short-chain flavin reductases (30, 64, 66). Here we have shown that the purified dimeric PheA1 and PheA2 proteins do not strongly interact, but that for the *in vitro* conversion of phenol to catechol, a large excess of PheA1 over PheA2 is needed to prevent the unproductive formation of hydrogen peroxide.

The kinetic mechanism and flavin-binding properties of PheA2 are clearly different from those of the related ActVB NADH:flavin reductase participating in the last step of actinorhodin synthesis in *Streptomyces coelicolor* (66). This homodimeric FMN-binding enzyme acts according to an ordered sequential mechanism and is not classified as a flavoprotein. The mechanism depicted in Scheme 1 also differs from the ternary complex mechanism proposed for Fre, the prototype of the class I flavin reductases (69–71). In this monomeric enzyme, which does not contain any prosthetic group, flavins are recognized mainly through hydrophobic interactions with the isoalloxazine ring, providing an explanation of why Fre uses riboflavin as a substrate rather than as a cofactor (72, 73). The mechanism depicted in Scheme 1 is more consistent with the mechanism proposed for the homodimeric FMN-containing flavin reductase P from *Vibrio harveyi*, which is involved in bioluminescence by providing reduced FMN to luciferase (74, 75). With the latter enzyme, a shift in the kinetic mechanism in the luciferase-coupled assay was taken as evidence that the reduced FMN cofactor is transferred to luciferase via protein-protein complex formation (76). Subsequent studies with the bioluminescence enzymes from *V. fisheri* (77) and *V. harveyi* (78, 79) have indicated that the mechanism of reduced flavin transfer may depend on the constituent protein partners in the reductase-luciferase couple and that, under *in vitro* reconstitution conditions, direct flavin product transfer is feasible as well.

PheA2 belongs to a newly recognized family of short-chain flavin reductases (30). These enzymes are considerably smaller in size than members of the Fre family (71, 72) and the flavin...
reductases from luminous bacteria (68, 74, 75). Our studies show that PheA2 is structurally related to FeR, a ferric reductase from the hyperthermophilic Archaea *A. fulgidus* that uses its single domain to provide both the flavin- and NAD(P)/H-binding sites (46). However, PheA2 displays no ferric reductase activity, binds FAD instead of FMN, and is specific for NADH.

For FeR, it was proposed that the substrate binds in a pocket near the flavin ring and that Thr31, Leu35, Cys45, and His128 might serve as protein ligands for the metal ion. Except for the conserved histidine (His128), these residues are replaced in PheA2, disfavoring iron ligandation. In FeR, the FMN ribityl phosphate interacts with Asp51, Thr52, Arg59, Ser84, and Lys89. These residues are not conserved or lacking in the PheA2 sequence. This suggests that the specificity for FAD binding is related to the shorter loop (region 84–92, PheA2 numbering) (Fig. 9).

For FeR, it was found that the 2'-phosphate of NADP+ does not make strong interactions with the polypeptide chain. This might explain why FeR shows no preference for NADH or NADPH. From the three-dimensional model of PheA2, no indication was obtained as to why this enzyme is very specific for NADPH. Therefore and especially because both PheA1 and PheA2 have the specific interaction of PheA1 with the AMP moiety of FAD.

**Acknowledgments**—We thank Dr. Fiona Duffner for valuable discussions, Dr. Walter van Dongen for the gift of E. coli NADH oxidoreductase, and Bert Janssen for help in some of the experiments.

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