On the Role of DmpK, an Auxiliary Protein Associated with Multicomponent Phenol Hydroxylase from *Pseudomonas* sp. Strain CF600*

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DmpK from *Pseudomonas* sp. strain CF600 represents a group of proteins required by phenol-degrading bacteria that utilize a multicomponent iron-containing phenol hydroxylase. DmpK has been overexpressed in *Escherichia coli* and purified to homogeneity; it lacks redox cofactors and was found to strongly inhibit phenol hydroxylase in *vitro*. Chemical cross-linking experiments established that DmpK binds to the two largest subunits of the oxygenase component of the hydroxylase; this may interfere with binding of the hydroxylase activator protein, DmpM, causing inhibition. Since expression of DmpK normally appears to be much lower than that of the components of the oxygenase, inhibition may not occur in *vivo*. Hence, the interaction between DmpK and the oxygenase manifested in the inhibition and cross-linking results prompted construction of *E. coli* strains in which the oxygenase component was expressed in the presence and absence of a low molar ratio of DmpK. Active oxygenase was detected only when expressed in the presence of DmpK. Furthermore, inactive oxygenase could be activated in *vitro* by adding ferrous iron, in a process that was dependent on the presence of DmpK. These results indicate that DmpK plays a role in assembly of the active form of the oxygenase component of phenol hydroxylase.

There is currently much interest in the biochemistry of oxygenases, which control the entry of toxic compounds into catabolic pathways that allow microorganisms to break them down to carbon dioxide and water. A recently discovered class of aromatic oxygenases includes phenol (1–3) and toluene (4–6), monoxygenases that are in many ways similar to the relatively well characterized binuclear iron-containing enzyme, methane monooxygenase (reviewed in Ref. 7). Various types of experimental evidence indicate that these systems all utilize an FAD/[2Fe-2S] center containing reductase, a low molecular weight activator protein (similar to MmoB), and a heteromultimeric (αβγ) oxygenase component.

The degradation of phenol by *Pseudomonas* sp. strain CF600 requires the participation of polypeptides encoded by the 15 genes of the *dmp* operon (3), and is initiated by the hydroxylation of phenol to catechol. The gene products of *dmpLMNOP* were found to be necessary for phenol hydroxylase activity in an *in vitro* assay containing phenol, NADH, and Fe^2+ (2). The reductase component of this enzyme is encoded by *dmpP*, the oxygenase component by *dmpLNO*, and the activator protein by *dmpM* (2, 3).

Polypeptide requirements for enzyme activity are summarized in Scheme 1.

An additional gene, *dmpK*, is located immediately upstream of the phenol hydroxylase genes in the operon. The presence of *dmpK* was found to be necessary to allow growth on phenol of a strain of *Pseudomonas* harboring *dmpLMNOP* together with enzymes necessary to catalyze catechol, the product of phenol hydroxylase (1). However, the function of the 10.5-kDa gene product remained obscure.

Nucleotide sequencing has since revealed the presence of genes encoding similar proteins clustered together with phenol hydroxylase genes in other strains of *Pseudomonas* (96% identity) (8, 9) and in *Acinetobacter* (52% identity over 68 amino acids) (10). Southern hybridization experiments also established the presence of DNA homologous to *dmpK* in a number of phenol-degrading marine bacteria, and in the archetypal phenol degrader, *Pseudomonas* U (11). Furthermore, a gene (*tbmA*) associated with a multicomponent toluene monoxygenase system exhibits 38% identity, over 55 amino acids, with DmpK (12). The functions of these DmpK homologues have not been defined, nor have any homologous proteins of known function been identified in sequence data base searches.

In this report we have overexpressed and purified DmpK, and characterized its interaction with the oxygenase component of phenol hydroxylase. The data are consistent with the involvement of DmpK in iron-dependent assembly of an active oxygenase.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were reagent grade or higher. The purification from phenol-grown *Pseudomonas* sp. strain CF600 of DmpM and the oxygenase component (DmpLNO) of phenol hydroxylase will be described elsewhere.† The reductase component, DmpP, was purified using a previously published method (2). Catechol 2,3-dioxygenase was purified essentially as described previously (13) from *Escherichia coli* harboring pMMB26 (14).

**Plasmid Constructs and Strains**—Strains and plasmids used in this study are listed in Table I. DNA manipulations were performed according to standard techniques (20). In order to express DmpK alone and as a fusion protein with TrpE, the *dmpK* gene was first amplified by polymerase chain reaction as an *NdeI* to BamHI fragment (base pairs 745–1052; Ref. 1) and cloned into a pBluescript (Stratagene) derivative, in which an *NdeI* site had previously been introduced into the polylinker, to generate pV1202. Both strands of the resulting fragment were sequenced to ensure that no mutations had been introduced.

The *dmpK* gene was introduced into a PET expression vector (16), and the resulting plasmid was used for overexpression of DmpK. The

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harvest were as described above.

Tricine, ampicillin (100 µg/ml) and casamino acids (0.5%) was inoculated with BL21 (DE3). Fresh transformants were grown on LB containing ampicillin (100 µg/ml) to induce expression from the P\text{trp} promoter. Cross-linkers were dissolved just before use in ice-cold 50 mM MOPS buffer, pH 7.4, and then stored as a paste at \(-80\) °C until further use.

**Immunological Methods**—To obtain high titer antisera to the low molecular weight DmpK polypeptide (92 amino acids), a strategy employing both the TrpE-DmpK fusion and DmpK proteins was employed. Cross-links of BL21(DE3)pV1203 (expressing DmpK) and BL21(DE3)pV1205 (expressing TrpE-DmpK) were prepared by sonication of cells resuspended in 50 mM Tris-HCl, pH 7.5. After removal of cell debris by ultracentrifugation, soluble proteins were separated using 10–20% SDS-PAGE, and the polypeptide bands corresponding to the proteins of interest were excised and homogenized in phosphate-buffered saline. These preparations were used for rabbit antisera production by sequential immunization with TrpE-DmpK (2 injections) and DmpK (3 injections), using methods described previously (21).

For Western blot analysis, proteins were transferred from 10–20% SDS-PAGE gradient gels to polyvinylidene difluoride membranes using standard electrod blotting methods (22). The blots were developed using an immunoblot assay kit (Bio-Rad) with goat anti-rabbit IgG conjugated to alkaline phosphatase. The serum containing primary antibody was diluted 1:1000 for use.

**Purification of DmpK**—The buffer used during the purification procedure was 50 mM Tris-HCl, pH 8.0, containing 10% glycerol and 1 mM diethytheriol (“Buffer A”). All purification procedures were performed at 4 °C. The presence of DmpK in column fractions was monitored using SDS-PAGE.

**Crude Extract Preparation**—Crude extract of BL21(DE3)pV1203 (expressing DmpK) was prepared by sonication of a suspension of cells (51 g, wet weight) in buffer A (100 ml). A few milligrams of DNase I were then added, and the cell suspension was incubated at 4 °C for 15 min with occasional stirring. The cell suspension was then sonicated in 15-s bursts (five times), followed by centrifugation at 70,500 \(\times g\) for 1 h. The supernatant was decanted carefully and used for further purification.

**Ion Exchange Chromatography**—The crude extract was loaded (6 ml/min) onto a Fast-Flow DEAE-Sepharose column (36 × 2.6 cm) equilibrated with Buffer A. The column was then washed with approximately 200 ml of this buffer, followed by a linear gradient of 0–2 M NaCl in Buffer A (1500 ml). Fractions (12 ml) containing DmpK were combined and brought to 80% saturation with ammonium sulfate, and, after 15 min on ice, the precipitate was collected by centrifugation. Precipitated protein was dissolved in Buffer A (50 ml) containing ammonium sulfate (1 M) in preparation for the next step.

**Hydroporphic Interaction Chromatography**—One-third of the sample from the previous step was loaded (2 ml/min) on a Phenyl-Sepharose (Pharmacia) High Performance column (36 × 2.6 cm) equilibrated with Buffer A containing ammonium sulfate (1 M). The column was washed with 50 ml of this buffer and then developed with a linear gradient of ammonium sulfate (1–0 M in 1200 ml). Two peaks containing DmpK eluted, and fractions from each peak were pooled and subjected separately to the next, and final, purification step.

**Gel Filtration Chromatography**—In preparation for this step, proteins from the previous step were concentrated by precipitation with ammonium sulfate, as described above. Precipitated proteins were redisolved in Buffer A and loaded (1 ml/min) on a Sephacryl S-300HR column (95 × 2.5 cm) equilibrated with Buffer A. Proteins were eluted using the same buffer, and fractions (12 ml) were collected. Those fractions found to contain DmpK were combined, concentrated by ultracentrifugation using an Amicon PM-10 membrane, and then stored as small aliquots at –80 °C.

On occasion, DmpK collected from the DEAE-Sepharose column was concentrated by precipitation with ammonium sulfate and applied directly to the gel filtration column. In these cases, a major peak eluted from the gel filtration column early on, and a minor one eluted much later; both contained DmpK, as judged by SDS-PAGE. However, preparations were less pure than those obtained when the Phenyl-Sepharose step was included.

**Chemical Cross-linking Experiments**—Cross-linking reactions were carried out at room temperature in 50 mM MOPS buffer, pH 7.4. Cross-links were dissolved just before use in ice-cold 50 mM MOPS buffer, pH 7.4. The cross-linking reactions with bi(sulfosuccinimidyl)suberate (BS\textsuperscript{2}+) were done in a volume of 200 µl, containing oxygenase component (4.5 µM DmpK\textsubscript{NO}), DmpK (4.8 or 24 µM), and BS\textsuperscript{2}+ (230 µM). Control reactions were also run in which cross-linker or proteins were omitted. Cross-links were added to protein solutions at room temperature, and the mixtures were incubated for 30–60 min, after which reactions were quenched by the addition of SDS-PAGE sample buffer. Cross-linked samples were then analyzed by SDS-PAGE and/or Western blotting. Although both prestained (Bio-Rad) and unstained (Pharmacia) molecular weight markers were used, the reported molecular
weights were estimated by comparison of $R_f$ values with those of the non-pretained standards.

**Analytical Methods**—Protein concentrations were generally estimated using the bicinchoninic acid (BCA) method (Pierce) following the 60 °C protocol supplied by the manufacturer and modified when necessary to remove interfering substances (23). Bovine serum albumin was used as the standard.

Polyacrylamide gel electrophoresis was carried out using standard techniques with Tris-glycine or Tricine buffer systems (24, 25). Tris-glycine gels were run as 10–20% linear gradient gels under denaturing conditions, or as 5–30% native (reducing or nonreducing) gels. Occasionally precast gels supplied by ICN were used (Tris-SDS minigels or Bio-Rad, 0.78 cm) equilibrated with 50 mM MOPS buffer, pH 7.4, containing 0.15 M NaCl, and running at a flow rate of 1 ml/min. The column was calibrated using proteins obtained from Boehringer-Mannheim: bovine serum albumin, ovalbumin, chymotrypsinogen, cytochrome $c$, and aprotinin ($M_w = 66,000, 45,000, 25,000, 12,500,$ and 6500, respectively).

Amino-terminal sequencing of purified DmpK was done by Dr. Per Ingvar Ohlson, Department of Medical Chemistry, Umeå University, using an Applied Biosystems model 477A peptide sequencer.

Circular dichroism spectra were collected at room temperature using a Jasco J-710 CD spectrometer. UV-visible measurements were performed using a Philips PU8710 spectrophotometer.

Iron concentrations were estimated colorimetrically using o-phenanthroline after precipitation of protein with trichloroacetic acid (26).

**Enzyme Assays**—Phenol hydroxylase assays were carried out at 25 °C in 0.05 M Tris acetate buffer, pH 7.5, (1 ml) containing: NADH (0.12 mM), DmpM (0.38 mM), and catechol 2,3-dioxygenase (4–5 units, where 1 unit catalyzes the appearance of 1 μmol of 2-hydroxyxymuconic semialdehyde, the catabolite ring-fission product, was monitored at 400 nm (under these conditions, $ε_{400}$ was estimated to be 18,800 M$^{-1}$ cm$^{-1}$). Background rates in the absence of phenol were generally undetectable. After 30–60 s, the reaction was initiated by the addition of phenol (1.25 mM). Where indicated, ferrous ammonium sulfate was added to assays, from a 10 mM stock solution in 50 mM HCl, to a final concentration of 5 mM. The addition was made immediately after the addition of the oxygenase preparation, and assay mixtures were then incubated for 2 min before the addition of phenol to initiate the reaction. Addition of DmpK was made after the addition of oxygenase preparation, and prior to the addition of ferrous ammonium sulfate.

**RESULTS**

**Expression of DmpK by Pseudomonas spp. Strain CF600**—Although the phenol hydroxylase polypeptides, DmpLMNO, are expressed at high enough levels in *Pseudomonas* spp. strain CF600 to be readily distinguished in crude extracts run on SDS-polyacrylamide gels (in comparison with extracts from uninduced cells), it was impossible to detect DmpK on SDS-PAGE gels by inspection. However, Western blotting revealed the presence of DmpK in crude extracts from phenol-grown cells but not in extracts from acetate-grown cells (data not shown). The low level of expression of DmpK is consistent with the suboptimal position of its poor ribosome binding site (1) and has physiological implications, discussed below.

In order to express sufficient DmpK to allow purification, *dmpK* was cloned and expressed in *E. coli* from the strong viral T7 promoter in pVI203, as described under “Experimental Procedures.”

**Purification and Properties of DmpK**—Recombinant DmpK was purified from *E. coli* harboring pVI203 using Fast-Flow DEAE chromatography, followed by chromatography on gel filtration and Phenyl-Sepharose columns. Samples from each stage of a representative purification are shown in Fig. 1. Approximately 125 mg were obtained from the equivalent of 17 g (wet weight) of cell paste.

The protein exhibited some heterogeneity on the gel filtration and Phenyl-Sepharose columns. When the Phenyl-Sepharose step was omitted and the eluate from the DEAE column was applied directly to the gel filtration column, high and low molecular weight forms of DmpK were observed. The high molecular weight form predominated, with more than 90% of the total DmpK eluted from the column existing in this form. Two broad peaks of similar size were observed when the eluate from the DEAE column was instead chromatographed on the Phenyl-Sepharose column; one eluted early in the salt gradient, and the other eluted immediately after. Protein from each peak was further purified by gel filtration chromatography; the two fractions behaved identically on this column, each eluting at the position of the high molecular weight form referred to above (data not shown).

All fractions of DmpK were essentially pure, as judged by SDS-PAGE (e.g., Fig. 1) and electrospray mass spectrometry (not shown), and were indistinguishable using either of these techniques or native gel electrophoresis; circular dichroism spectroscopy of the different fractions showed only minor differences. The molecular weight obtained by electrospray ionization-mass spectroscopy was 10,451 ± 1 mass unit, which corresponds well with the molecular weight deduced from the nucleotide sequence (10,586), assuming that the amino-terminal methionine had been removed. This was confirmed by amino-terminal sequencing of the purified protein. The native mo-

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TABLE I

| Strain or Plasmid | Properties/genes* | Reference/source |
|-------------------|-------------------|-----------------|
| *E. coli DH5*     | $r$ m$^3$ recAI   | (15)            |
| *E. coli BL21 (DE3)* | Protease $^-$ strain expressing T7 RNA | (16) |
| *Pseudomonas sp. CF600* | Polymerase under control of the tac promoter | (17) |
| Plasmid (vector)  |                  |                 |
| pBluescript SK (+) |                  |                 |
| pM9MB66HE        | $Ap^R$ cloning and sequencing vector | Stratagene       |
| pATH11           | $Ap^R$ Inc Q broad host range lacF $^+$/tac promoter expression vector | (18) |
| pET3a            | $trpE$ fusion vector | (19)            |
| pVI202 (pBluescript SK (+)) | intermediate construct | This study |
| pVI203 (pET3a)   | DmpK expression | This study |
| pVI204 (pBluescript SK (+)) | intermediate construct | This study |
| pVI205 (pATH11)  | TrpE-DmpK fusion expression | This study |
| pVI293 (pMMB66HE) | dmpLNOP           | (1,2)           |
| pFOW100 (pMMB66HE) | dmpLNOK           | This study |
| pFOW101 (pMMB66HE) | dmpLNO             | This study |

* $r$ and m refer to host restriction and modification systems respectively. $Ap^R$, ampicillin-resistant.
lecular weight of the protein was estimated to be approximately 37,000 using a calibrated high pressure liquid chromatography gel filtration column: thus, DmpK appears to exist mainly as a trimer or a tetramer.

Purified DmpK fractions were colorless, with a peak observed only in the UV region of the spectrum (data not shown). Therefore, the purified protein does not contain redox-active prosthetic groups such as flavin, heme, or iron-sulfur centers. Additionally, preparations of DmpK were found to contain only traces of iron, so the purified protein does not contain a spectroscopically invisible iron center either.

Inhibition of Phenol Hydroxylase Activity by DmpK—The dose-dependent effects of adding a purified preparation of DmpK to phenol hydroxylase assays, in the presence and absence of DmpM, are summarized in (Fig. 2). DmpK-dependent inhibition of the hydroxylase in the presence of DmpM was dramatic, with inhibition occurring at low levels of DmpK. The phenol hydroxylase-catalyzed reaction was much slower in the absence of DmpM, but was still readily detectable, and appeared to be insensitive to the presence of DmpK (Fig. 2). These results suggest that DmpK can interfere with the interaction between the oxygenase component (DmpLNO) and DmpM, the activator protein. However, since the in vivo ratio of DmpK:DmpNO appears to be very low (see above), inhibition of the hydroxylase is of questionable physiological significance.

Direct Evidence for Interaction between DmpK and Phenol Hydroxylase—Since the inhibition experiments suggested that DmpK interacts with one or more of the phenol hydroxylase polypeptides (excluding DmpP), chemical cross-linking was used to probe potential interactions. Direct evidence for the interaction of DmpK with the two larger subunits of the hydroxylase was obtained using the homobifunctional N-hydroxysuccinimide-ester cross-linker, BS₃.

DmpK (10.5 kDa) appeared to be cross-linked to itself by this reagent, as indicated by three prominent bands of cross-linked products with apparent molecular masses ranging from 19.5 to 24 kDa observed upon Coomassie Blue staining and Western blotting using a DmpK-specific antibody (Fig. 3). These bands are likely to represent DmpK dimers; the range of products observed may be ascribed to additional modifications and/or internal cross-linking of the dimeric protein, either of which could alter the mobility of cross-linked products. An additional band visible on the Western blot (Fig. 3b, lane D) just below the 35.1-kDa marker is consistent with the gel filtration results, which suggested that the protein is tri- or tetrameric. However, the yield of this cross-linked product is low, and it is not visible on the Coomassie-stained gel.

Upon exposure to BS₃, the oxygenase component of phenol hydroxylase, which is composed of subunits running at 54, 36, and 13.5 kDa on SDS-PAGE (molecular masses predicted from the gene sequences are 60.5 (DmpN), 38.5 (DmpL), and 13.2 kDa (DmpO); see Ref. 1), gave rise to products running at 92 and 120 kDa (Fig. 3a). The 92-kDa band is likely to be the cross-linked product of DmpL (36 kDa, observed) and Dmp N (54 kDa, observed). It is difficult to identify the 120-kDa product without additional information.

In the presence of BS₃ a mixture of DmpK and oxygenase component gave rise to new cross-linked products (Fig. 3a), each of which reacted with the DmpK-specific antibody (Fig. 3b). The major products had molecular masses of 46 kDa and 69 kDa, which most likely represent DmpK linked to DmpL (12.5 plus 36 kDa, observed) and DmpK and DmpN (12.5 plus 54 kDa, observed), respectively. Two larger complexes containing DmpK were also observed (Fig. 3b): that running at 96 kDa most likely represents a complex of DmpKLN. It should be noted that although DmpK can clearly bind to polypeptides of the oxygenase complex, it does not co-purify with it (Fig. 3b, lane E).

These data provided clear evidence that DmpK binding sites exist on the two largest subunits of the hydroxylase. In light of the cross-linking and inhibition results, the effects of co-expressing it at a low level (i.e. at far less than a molar ratio) with the oxygenase (DmpLNO) were examined in order to provide more information about a potential physiological role.

Expression of the Oxygenase Component of Phenol Hydroxylase in the Presence and Absence of DmpK—In a previous study, recombinant phenol hydroxylase was expressed in the presence and absence of DmpK, and phenol hydroxylase activity was measured in the presence of Fe⁺⁺ (2). Since then, we have developed a sensitive assay for phenol hydroxylase activity in the absence of added iron and here re-examine the activity of the recombinant oxygenase component when expressed with or without DmpK.

Preliminary experiments (data not shown) indicated that expression levels of the oxygenase component polypeptides, DmpLNO, differed substantially in two earlier plasmid constructs, pVT290 and pVI293, which express DmpKLNOP and DmpLNOP, respectively (1, 2). In an attempt to eliminate oxygenase component expression levels as a variable, two new plasmids, pPOW100 and pPOW101, were constructed such that transcription started at dmpL in both cases. This was done by replacing dmpP in pVI293 by dmpK to generate pPOW100.

![Fig. 1. SDS-PAGE of fractions obtained during purification of DmpK. Lane 1, crude extract; lane 2, after Fast-Flow DEAE chromatography; lane 3, after Phenyl-Sepharose chromatography (peak 1); lane 4, after Sephacryl S-300 chromatography of Phenyl-Sepharose chromatography peak 1 fractions.](image1)

![Fig. 2. Effects of DmpK on phenol hydroxylase activity in the presence (●) and absence (○) of DmpM. The concentration of the purified oxygenase component (DmpLNO) in each assay was 0.16 μM (●) and 0.82 μM (○), respectively, and activity is expressed per milligram of DmpLNO. The assay procedure was as outlined under “Experimental Procedures,” except that DmpK was added to DmpLNO and this mixture was incubated on ice for several minutes before addition to the assay; however, premixing was not essential for observing inhibition. This data was obtained using DmpK purified from the first peak of the Phenyl-Sepharose column eluate, but similar results were obtained for other purified fractions of DmpK.](image2)
and by deleting dmpP in pVI293 to generate pPOW101 (see “Experimental Procedures”). Since DmpP is expressed at a much lower level in E. coli than the DmpLNO polypeptides (see Figs. 4 and 5 in Ref. 1), DmpK should be produced from the dmpP ribosome binding site in pPOW100 at a low level, which interferes minimally with in vitro hydroxylase activity measurements. Neither of the other two polypeptides required for optimal hydroxylase activity (DmpM and DmpP) are expressed in these constructs, but are provided in activity assays.

As can be seen in Fig. 4, expression of the polypeptides of the oxygenase component (cf. lane 7) from pPOW100 and pPOW101 is low in the absence of IPTG induction (lanes 1 and 2) and increases at 1.25 (lanes 3 and 4) and 3 h (lanes 5 and 6) post-induction, with essentially the same levels observed in both crude extracts. Note also the position of the DmpK band (lane 8), which is not distinguishable in the samples of crude extract (lanes 1–6), although it was readily detectable by Western blotting in extracts from cells harboring pPOW100 (data not shown). Based on these results, one would expect both crude extracts to exhibit the same levels of phenol hydroxylase activity in vitro, with some inhibition by DmpP possible in the extract from cells harboring pPOW100.

As is shown in Table II, this was not what was observed. In crude extracts obtained from the construct expressing DmpK (pPOW100), levels of oxygenase activity were high compared to extracts prepared from cells in which DmpK was absent (pPOW101). The possibility that extracts from DmpK-lacking cells contain some inhibitor was excluded by the observation that the oxygenase activity was additive in assays where both extracts were present (data not shown).

The addition of ferrous iron to the enzyme assay resulted in increased oxygenase activity for each extract (Table II), indicating that even when produced in the presence of DmpK not all of the oxygenase component was active. Since the levels of expression of the oxygenase component polypeptides appear to be essentially identical in extracts prepared from cells harboring the two plasmid constructs, it is interesting that the oxygenase activity was not activated to the same level in each case. In order to determine if the observed difference in ferrous iron stimulation was due to the presence or absence of DmpK, small amounts of purified DmpK were added to the in vitro assays. Dramatically, in the presence of added ferrous iron, inactive DmpLNO oxygenase expressed by cells harboring pPOW101 was activated by a small amount of purified DmpK. The level of activity achieved was almost the same as that of the oxygenase when co-expressed with DmpK in cells harboring pPOW100 (Table II).

**DISCUSSION**

While functional roles have been assigned to most of the 15 dmp operon-encoded proteins required for the mineralization of phenols by Pseudomonas sp. strain CF600, that of DmpK has remained obscure. Although the gene encoding this protein is clustered together with five phenol hydroxylase genes, its product is not essential for in vitro phenol hydroxylase activity. However, when the gene encoding this protein is deleted, strains that might normally be expected to be able to grow using multicomponent phenol hydroxylase fail to do so. These results led to the initial suggestion that DmpK might be involved in phenol binding/transport or phenol hydroxylase regulation (3).

In this paper, we have examined the interactions of DmpK with phenol hydroxylase in some detail. Since DmpK appears to be present at very low levels in crude extracts of phenol-grown cells, it was necessary to overexpress the protein in order to purify it. Very high levels of expression of this protein were attained from the T7 promoter in pVI203, and this made it possible to obtain large quantities of pure DmpK. Spectral characteristics of the purified protein indicated that it did not contain any common redox cofactors (e.g., FAD, heme, Fe-S centers), and iron assays indicated no significant quantities of

**FIG. 4.** Expression of phenol hydroxylase components in E. coli DH5 strains. Lanes 1, 3, and 5, extracts from cells harboring pPOW100 (dmpLNOK) at 0, 1.25, and 3 h after IPTG induction, respectively. Lanes 2, 4, and 6, extracts from cells harboring pPOW101 (dmpLNO) at 0, 1.25, and 3 h after induction, respectively. Approximately 14 μg of crude extract protein was loaded onto each lane. Lane 7, purified DmpLNO. Lane 8, purified DmpK.
DmpK and Multicomponent Phenol Hydroxylase

### Results

Purified DmpK was found to have a marked inhibitory effect on phenol hydroxylase activity when assays were performed in the presence of DmpM, an accessory protein required for optimal turnover of the hydroxylase. In the absence of DmpM, DmpK had little effect on activity. The simplest interpretation of this observation is that DmpK in some way interferes with the interaction between DmpM and the oxygenase component of the hydroxylase. This could be mediated by binding of DmpK either to the oxygenase component or to DmpM, such that it would interfere with the normal DmpM-oxygenase interaction.

In relatively dilute solutions of oxygenase component containing a severalfold excess of DmpK, it was found that DmpK could be cross-linked to both DmpL and DmpN, the two largest subunits of the oxygenase component. In contrast, when mixtures of DmpM and DmpK were exposed to various cross-linking reagents, no evidence of cross-linked products was found. Thus, while interactions between DmpM and DmpK cannot be ruled out, it is clear that DmpK interacts with oxygenase component on at least two of its subunits. It is interesting to note that we have also found DmpM to interact with at least one of the same subunits (DmpN). These observations at least partially explain the inhibition of phenol hydroxylase activity in vitro. However, since DmpK is expressed at much lower levels than the polypeptides of the oxygenase component, inhibition of phenol hydroxylase is unlikely to be the main physiological function of this protein.

Results obtained using the two plasmid constructs, pPOW100 and pPOW101, are consistent with a role for DmpK in the formation of active oxygenase component. When the oxygenase component polypeptides were expressed in the absence of DmpK from pPOW101, no oxygenase activity was detected in crude extracts; expression in the presence of DmpK from pPOW100 led to the formation of active oxygenase. Although DmpK is inhibitory to phenol hydroxylase even when it is present at a less than molar ratio, the level of expression of DmpK from pPOW100 is apparently low enough relative to the oxygenase polypeptides so that inhibition is minimal. The low level of DmpK expression from pPOW100 is likely to be similar to the level at which DmpK is expressed in *Pseudomonas* sp. strain CF600. Therefore, activation of the oxygenase by DmpK in the recombinant strain probably reflects the physiological role of this protein.

### Table II

| Crude extract from *E. coli* harboring: | Additions to assay | Induction time h | Phenol Hydroxylase Activity (nmol/min/mg extract protein) |
|----------------------------------------|-------------------|-----------------|--------------------------------------------------------|
| pPOW100 (DmpLNO + DmpK)                | None              | 0               | ND<sup>a</sup>                                         |
|                                        | DmpK              | 1.25            | 62                                                     |
|                                        | Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> | 3               | 52                                                     |
|                                        | Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> + DmpK | 3               | 43                                                     |
| pPOW101 (DmpLNO - DmpK)               | None              | 0               | ND<sup>a</sup>                                         |
|                                        | Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> | 3               | 130                                                    |
|                                        | Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> + DmpK | 3               | 100                                                    |
|                                        | None              | 0               | ND<sup>a</sup>                                         |
|                                        | Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> | 3               | ND<sup>a</sup>                                         |
|                                        | Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> + DmpK | 3               | ND<sup>a</sup>                                         |

<sup>a</sup> Additions were Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> (5 μM) and DmpK (5.8 nM). All assays were carried out in the presence of DmpM and DmpP, as described under "Experimental Procedures."

<sup>b</sup> ND, not detectable.

Iron. This latter observation was of interest, as some types of redox-active iron centers are spectroscopically inviable. Purified DmpK was found to have a marked inhibitory effect on phenol hydroxylase activity when assays were performed in the presence of DmpM, an accessory protein required for optimal turnover of the hydroxylase. In the absence of DmpM, DmpK had little effect on activity. The simplest interpretation of this observation is that DmpK in some way interferes with the interaction between DmpM and the oxygenase component of the hydroxylase. This could be mediated by binding of DmpK either to the oxygenase component or to DmpM, such that it would interfere with the normal DmpM-oxygenase interaction.

In relatively dilute solutions of oxygenase component containing a severalfold excess of DmpK, it was found that DmpK could be cross-linked to both DmpL and DmpN, the two largest subunits of the oxygenase component. In contrast, when mixtures of DmpM and DmpK were exposed to various cross-linking reagents, no evidence of cross-linked products was found. Thus, while interactions between DmpM and DmpK cannot be ruled out, it is clear that DmpK interacts with oxygenase component on at least two of its subunits. It is interesting to note that we have also found DmpM to interact with at least one of the same subunits (DmpN). These observations at least partially explain the inhibition of phenol hydroxylase activity in vitro. However, since DmpK is expressed at much lower levels than the polypeptides of the oxygenase component, inhibition of phenol hydroxylase is unlikely to be the main physiological function of this protein.

Results obtained using the two plasmid constructs, pPOW100 and pPOW101, are consistent with a role for DmpK in the formation of active oxygenase component. When the oxygenase component polypeptides were expressed in the absence of DmpK from pPOW101, no oxygenase activity was detected in crude extracts; expression in the presence of DmpK from pPOW100 led to the formation of active oxygenase. Although DmpK is inhibitory to phenol hydroxylase even when it is present at a less than molar ratio, the level of expression of DmpK from pPOW100 is apparently low enough relative to the oxygenase polypeptides so that inhibition is minimal. The low level of DmpK expression from pPOW100 is likely to be similar to the level at which DmpK is expressed in *Pseudomonas* sp. strain CF600. Therefore, activation of the oxygenase by DmpK in the recombinant strain probably reflects the physiological role of this protein.

### Clues about the mechanism of oxygenase component activation

Clues about the mechanism of oxygenase component activation are provided by the results obtained when ferrous iron and purified DmpK were added to the hydroxylase assays (Table II). The 2-fold increase in activity caused by adding iron suggests that crude extract from cells co-expressing DmpK and oxygenase contained some inactive oxygenase, probably apo-enzyme. Possible explanations are that the level of DmpK co-expression was not optimal, or that some apoenzyme was produced during cell breakage. By contrast, in the absence of DmpK co-expression, all of the oxygenase was present in an inactive form, and addition of ferrous iron resulted in very little activation. However, when a slightly inhibitory concentration of DmpK was also present in the assay, activation in the presence of iron was dramatic for the inactive DmpLNO oxygenase generated in the absence of DmpK. These results strongly suggest a role for DmpK in post-translational insertion of iron into apo-oxygenase. In this context, it is interesting to note that cross-linking experiments revealed a direct interaction between DmpK and the oxygenase component subunit, DmpN, which encompasses the putative ligands for the binuclear iron center (3, 5).

Experiments using bacterial strains in which *dmpK* was deleted showed that DmpK is essential for allowing growth on phenol using the multicomponent phenol hydroxylase (1). The results reported here indicate a role for DmpK in post-translational incorporation of iron into the oxygenase component of the phenol hydroxylase. Since the oxygenase component was expressed in the absence of phenol, reductase (DmpP) and the activator protein (DmpM), it was incapable of turning over at any significant rate. This means that DmpK does not simply play a repair role for oxygenase, which might inadvertently lose iron via a turnover event, as oxygenases are sometimes prone to do; instead, it must be essential for proper de novo assembly of the active oxygenase. However, an ancillary role for DmpK in oxygenase repair is certainly possible.

Requirements for accessory proteins in the assembly of metalloproteins have previously been well documented for some proteins, including the nickel-containing enzyme, urease, and the FeMo protein of nitrogenase (reviewed in Refs. 27 and 28, respectively). Although the functions of some of these accessory proteins are not known, others appear to function as scaffold proteins for the assembly of a metal cofactor (NiFe (see Ref. 28, and references therein), potential metal donors (of iron for NiFe U (29)), and of nickel for UreE (30)), and chaperone or "molecular prop" proteins thought to be important in metalloprotein assembly or metal center incorporation (UreD (31) and NiFY (32)). Like some of these proteins, DmpK appears to be able to function post-translationally. However, unlike NiFe or

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<sup>3</sup> E. Cadieux and J. Powlowski, unpublished observation.
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NiF, purified DmpK does not contain any iron, and unlike the scaffold protein, NiFEN, DmpK exhibits no sequence similarity to the putative iron center-containing polypeptide (DmpN). Thus, although it is possible that DmpK loses an essential cofactor during isolation, the available evidence is consistent with a function as some sort of molecular prop required to allow correct or efficient incorporation of iron into the oxygenase component of phenol hydroxylase.

The outstanding questions about how DmpK functions are important ones, both in furthering our understanding of phenol hydroxylase, and in determining how best to express recombinant phenol hydroxylase for future studies of structure-function relationships. It is interesting to note that activator proteins like DmpK have not been reported previously for binuclear iron center oxygenases other than phenol hydroxylases, although a gene encoding a protein with some sequence similarities is associated with toluene monooxygenase genes. Considering the relatively recent discovery of enzymes like the multicomponent phenol hydroxylase, it may well be that proteins that resemble DmpK are more widespread than is currently known.

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