Characterization of Active and Inactive Forms of the Phenol Hydroxylase Stimulatory Protein DmpM†

Elisabeth Cadieux and Justin Powlowski*

Department of Chemistry and Biochemistry, Concordia University, 1455 de Maisonneuve Boulevard, West, Montreal, Quebec, H3G 1M8 CANADA

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ABSTRACT: The stimulatory protein DmpM of phenol hydroxylase from methylphenol-degrading *Pseudomonas* sp. strain CF600 has been found to exist in two forms. DmpM purified from the native strain was mostly active in stimulating phenol hydroxylase activity, whereas an inactive form accumulated in a recombinant strain. Both forms exhibited a molecular mass of 10 361.3 ± 1.3 Da by electrospray mass spectrometry, but nondenaturing gel filtration showed molecular masses of 31 600 Da for the inactive form and 11 500 Da for the active form. Cross-linking and sedimentation velocity results were consistent with the inactive form being a dimer. Partial thermal or chemical denaturation, or treatment with trifluoroethanol, readily activated dimeric DmpM. A combination of circular dichroism and fluorescence spectroscopies, activity assays, and native and urea gel electrophoresis were used to further characterize reactivation with urea. These results showed that dissociation of the dimeric form of DmpM precedes denaturation at low protein concentrations and results in activation. The same concentration of urea that effects dissociation also converts the monomeric form to a different conformation.

The *meta*-cleavage pathway encoded by the *dmp* operon of *Pseudomonas* sp. strain CF600 efficiently degrades phenol and methyl-substituted phenols. Phenol hydroxylase is the first enzyme in the pathway and hydroxylates phenol to form catechol, which is then further degraded. Five polypeptides, encoded by *dmpLMNOP*, are required for phenol hydroxylase activity (*1*, *2*). These five polypeptides are arranged as three components: *dmpP* encodes a reductase containing a ferredoxin type [2Fe-2S] center and an FAD cofactor; *dmpLNO* encodes an oxygenase component with a binuclear iron center; and *dmpM* encodes a protein lacking any cofactors or metal (*3*). An additional polypeptide, DmpK, is required for the production of active oxygenase (*4*).

The reductase (DmpP) apparently accepts electrons from NADH and transfers them to the oxygenase component (DmpLNO), where they are required for insertion of oxygen into phenol (*2*). *DmpM* encodes a polypeptide with a molecular mass of 10.4 kDa that is essential for efficient catalysis of phenol hydroxylation (*4*), but its mechanism of activation has not previously been studied in any detail.

The components of phenol hydroxylase from *Pseudomonas* sp. strain CF600 are similar to the components of methane and toluene monooxygenases (*5*, *6*). Amino acid sequence analyses show strong similarity between DmpM, MmoB, and the small component of toluene monooxygenase (*7*, *8*). These proteins appear to have similar roles in catalysis, with by far the best-studied example being MmoB. Its effects include stimulation of methane monooxygenase activity by up to 150-fold (*9*), alteration of the regioselectivity of the hydroxylase component (*10*), changes in the environment of the binuclear iron center (*11*), and increases in the O_2 reactivity of the oxygenase (*12*).

Reports in the literature suggest that the methane monooxygenase regulatory protein, MmoB, can exist in multiple forms. Although MmoB from *Methylosinus trichosporium* OB3b has been shown by sedimentation velocity experiments to have a molecular mass of 15 kDa, other evidence suggests that MmoB can also exist as a dimer (*5*, *9*). The existence of a dimer form has also been suggested for MmoB from *Methylococcus capsulatus* (Bath) (*13*). These authors also reported that MmoB is subject to proteolysis and requires protease inhibitors to prevent its degradation to two inactive forms, B′ and B″. The physiological significance of this observation is not clear.

We have found that DmpM, the activator protein of phenol hydroxylase from *Pseudomonas* sp. strain CF600, can also exist in more than one form. While DmpM purified from the native strain is mostly in the active form, recombinant DmpM tends to accumulate in an inactive form. In this report, we have compared the properties of the inactive and active forms. Reconstitution of active DmpM from inactive preparations is also described.

MATERIALS AND METHODS

Materials. EDC[1] was purchased from Sigma Chemical Co. DTT and NADH were obtained from Roche Molecular

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* To whom correspondence should be addressed: Phone (514) 848-8727; Fax (514) 848-2868; E-mail Powlow@vax2.concordia.ca.

[1] Abbreviations: BCA, bicinchoninic acid; CD, circular dichroism; DTT, dithiothreitol; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; IPTG, isopropyl 1-thio-β-D-galactopyranoside; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NHS, *N*-hydroxysuccinimide; PCR, polymerase chain reaction; Tris, tris(hydroxymethyl)-aminomethane; TFE, trifluoroethanol.

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Biochemicals; phenol, urea, guandine hydrochloride and glycerol (ultrapure grade) were from ICN Biomedicals. Catechol 2,3-dioxygenase was purified as described previously (*4*). Purification of the oxygenase component (DmpLNO) of phenol hydroxylase will be described elsewhere, and the reductase (DmpP) was purified by a previously published protocol (*2*). All restriction enzymes were purchased from Promega.

Analytical Methods. Protein concentrations were estimated by the bicinchoninic acid (BCA) method (Pierce) following the 60 °C protocol supplied by the manufacturer. Interfering substances such as DTT were removed by a modification of this method (*14*). Fluorescence measurements were carried out on an Aminco Bowman series 2 spectrofluorometer. Circular dichroism spectra were collected on a Jasco J-710 CD spectrometer. Electrospray mass spectrometry was done on a Finnigan SSQ 7000 single-quadrupole mass spectrometer. All samples were passed through a 1 cm C-18 small molecule cartridge (Michrom BioResources) interfaced to the mass spectrometer. Initially the cartridge was equilibrated with 95% water−5% acetonitrile−0.05% trifluoroacetic acid, and elution was with 35% water−65% acetonitrile−0.05% trifluoroactic acid. Sedimentation velocity experiments were performed on a Beckman XL-A analytical ultracentrifuge. SDS−polyacrylamide gel electrophoresis was carried out using a Tris−glycine buffer (*15*) or Tricine buffer system (*16*). Native gels were purchased precast from ICN (CAP-Gels, 10−20% Tris/borate/EDTA, pH 8.3, native format, 8 × 10 cm), or from Bio-Rad Laboratories (Ready Gels, 10−20% Tris-HCl, native format, 8 × 10 cm).

DNA Manipulations. DNA manipulations were done according to standard techniques (*17*). DNA was purified with Wizard Miniprep kits, and DNA sequencing was performed with the Silver Stain sequencing kit (Promega).

The strategy for cloning *dmpM* into pET3a, a T7-polymerase based expression vector (*18*), was essentially as described previously (*7*). First, *dmpM* was amplified from pVI258 (*1*) by PCR. The 5′-end primer, 5′-GCCGCGAGGAATAACATATGTCATCAC-3′, introduced an *Nde*I restriction enzyme site at the ATG start codon of *dmpM*, while the 3′-end primer 5′-CTTGTTGTGGGGATCCATGAGCTTG-3′ introduced a *Bam*HI restriction enzyme site after the stop codon. The amplified PCR fragment was incorporated directly into the pCRII vector (Invitrogen). DmpM was then subcloned into pET3a on an *Nde*I−*Bam*HI restriction enzyme fragment. Thus, pCRII-*dmpM* and pET3a were digested with *Nde*I and *Bam*HI, and the fragments were purified from agarose gels with a Sephaglas kit (Pharmacia). After ligation, the resulting plasmids were transformed into *Escherichia coli* DH5. Successful subcloning was verified by restriction digest followed by agarose gel electrophoresis, and the sequence was confirmed.

Bacterial Growth and DmpM Expression. The pET3a−*dmpM* construct was transformed into *E. coli* BL21(DE3) (*18*). Cells were grown either in LB or M9 minimal medium (*17*), using ampicillin or carbenecillin selection (100 μg/mL). IPTG (0.5 mM) was added when the OD_{600} of the culture reached 0.6−0.9, and growth was continued for an additional 1−4 h. Cells were collected by centrifugation, and the cell paste was stored at −80 °C until used.

Purification of Native DmpM from Phenol-Grown Pseudomonas sp. Strain CF600. The buffer used was 0.05 M Tris-HCl, pH 8.0, containing glycerol (10%) and DTT (1 mM) (referred to as TGD buffer). *Pseudomonas* sp. strain CF600 was grown in a fermenter in M9 minimal medium (16 L) supplemented with metals (*2*). Phenol was used as the carbon source and was added (3−5 mM) whenever culture oxygen demand decreased abruptly. The pH of the growth medium was maintained at 7.0−7.5 by addition of NH_4OH. When an OD_{650} of approximately 5 was reached, cells were concentrated with a tangential-flow filtration unit (Millipore) and then collected by centrifugation at 3800*g* for 20 min. Cell pastes were stored at −80 °C until used.

Cell paste was suspended in TGD (2 volumes), and then a spatula tip of DNase was added to the thawed cells prior to sonication. Aliquots (30 mL) were sonicated in 10 bursts of 15 s each, maintaining a temperature of less than 10 °C by immersion in a salt−ice water bath. The sonicated cell suspension was centrifuged at 70400*g* for 60 min. The supernatant ("crude extract") was collected and centrifuged again for 5 min at 3800*g* before being loaded onto the first column.

Crude extract was loaded (6 mL/min) onto a fast-flow DEAE-Sepharose column (30 × 2.6 cm), which was then washed with TGD buffer containing 75 mM NaCl for 40 min. The oxygenase component and DmpM eluted in the middle of a 75−275 mM NaCl gradient. Fractions (12 mL) containing DmpM and the oxygenase component were combined and loaded onto a phenyl-Sepharose high-performance column (36 × 2.6 cm) equilibrated with TGD buffer containing 0.15 M NaCl. DmpM eluted in the first few fractions following a wash with 0.15 M NaCl, whereas the oxygenase component eluted when the column was washed with 5 mM TGD. Fractions containing DmpM were combined and stored at −80 °C until purification of the more labile oxygenase component was completed.

DmpM-containing fractions were thawed, centrifuged for 5 min at 3800*g*, and brought to 1 M ammonium sulfate. This solution was centrifuged for 5 min at 3800*g* before being loaded (1.5 mL/min) onto a phenyl-Sepharose high-performance column (36 × 2.6 cm) equilibrated with TGD buffer containing 1 M ammonium sulfate. Then the column was washed with 1 M ammonium sulfate in TGD for approximately 10 min, prior to eluting the column with a gradient of 1−0 M ammonium sulfate in TGD (600 mL).

Fractions (6 mL) containing DmpM were identified by SDS−PAGE, combined, and brought to 80% saturation with ammonium sulfate. After 30 min on ice the mixture was centrifuged at 7000*g* for 30 min. The precipitate was collected, redissolved in TGD (10 mL), and then centrifuged for 5 min at 3800*g*. This sample was then loaded (1 mL/min) onto a Sephacryl S-300HR column (78 × 2.6 cm), which was subsequently eluted with TGD buffer, collecting 6 mL/fraction.

Fractions that contained DmpM were combined and loaded (6 mL/min) onto a fast-flow DEAE-Sepharose column (18 × 1.6 cm) previously equilibrated with TGD buffer. The column was eluted (1 mL/min) with a gradient of 0−0.3 M NaCl (600 mL). Fractions (6 mL) containing DmpM were combined and concentrated with an Amicon PM10 ultrafiltration membrane. The concentrated protein was passed through a desalting column equilibrated with TGD before it was stored it at −80 °C.

Purification of Recombinant DmpM. Purification of recombinant DmpM was carried out essentially as described previously (*7*), except that a phenyl-Sepharose chromatography step (see below) was usually necessary in order to obtain good yields of homogeneous protein. In addition, ammonium sulfate precipitation at 80% saturation was used to concentrate the protein between ion-exchange and gel-filtration chromatography steps.

After gel-filtration chromatography (*7*), ammonium sulfate was added to the DmpM preparation to a final concentration of approximately 1 M. After centrifugation for 5 min at 3800*g*, the supernatant was loaded (3 mL/min) onto a phenyl-Sepharose high-performance column (36 × 2.6 cm) previously equilibrated with TGD buffer containing 1 M ammonium sulfate. The column was then eluted (2 mL/min) with a gradient of 1−0.075 M ammonium sulfate in TGD (600 mL), collecting 6 mL fractions. Fractions containing DmpM, which eluted at the end of the gradient, were combined and concentrated by ultrafiltration with an Amicon PM-10 membrane. The concentrated protein was then aliquoted and stored at -80 °C. Before use, samples were dialyzed against an appropriate buffer.

Phenol Hydroxylase Activity Assays. Phenol hydroxylase activity was assayed essentially as described earlier (*4*) by coupling phenol hydroxylase with catechol 2,3-oxygenase: catechol 2,3-oxygenase catalyzes the *meta*-cleavage of catechol, the product of phenol hydroxylase, to form 2-hydroxymuconic semialdehyde. Formation of 2-hydroxymuconic semialdehyde was monitored by observing the increase in absorbance at 400 nm. Unless noted otherwise, assay mixtures (1 mL) contained 50 mM MOPS buffer (pH 7.4), DmpM (0.12 μM), NADH (300 μM), DmpP (0.38 μM), catechol 2,3-oxygenase (4−5 units), and varying amounts of oxygenase component. The background rate was recorded for 30 seconds and the reaction was initiated by adding phenol to 1.25 mM. An extinction coefficient for 2-hydroxymuconic semialdehyde of 20 500 M^{-1} cm^{-1} at 400 nm was used in all calculations (*4*).

Chemical Cross-Linking of DmpM by EDC. Cross-linking reactions with EDC were carried out according to the protocol described by Grabarek and Gergley (*19*). Prior to cross-linking, samples of DmpM were dialyzed to exchange Tris buffer for 50 mM phosphate buffer, pH 7.5, containing 10% glycerol. DmpM (77 μM; 25 μg) was incubated for 60 min at 25 °C in 50 mM phosphate buffer, pH 7.5, containing glycerol (10%), EDC (2 mM), and NHS (5 mM), and then the reaction was terminated by the addition of β-mercaptoethanol (20 mM). More DmpM was then added at a 1:1 molar ratio with DmpM already in the reaction mixture, and the solution was further incubated for 1 h at 25 °C. Progress of the cross-linking reaction was monitored by SDS−polyacrylamide gel electrophoresis.

Denaturation of DmpM with Urea. In early experiments, inactive recombinant DmpM (0.4 mL) was added to 10 M urea (1.6 mL) to a final DmpM concentration of 160 μM, and the solution was incubated on ice for 30 min. The sample was then dialyzed for 3 h at 4 °C against 20 mM MOPS buffer, pH 7.4 (1 L). The dialysis buffer was replaced and dialysis was continued overnight, after which the buffer was replaced again and dialysis continued for an additional 3 h.

When the concentrations of urea were varied, DmpM was first dialyzed as above, but with 50 mM phosphate buffer, pH 7.5, containing 10% glycerol, prior to preparation of denatured samples. Nondenaturing gel electrophoresis of dialyzed samples was used to confirm that the DmpM sample remained in its original form (monomer or oligomer) after dialysis treatment. Protein concentrations of the dialyzed samples were estimated by the BCA method, prior to addition of urea. A stock solution of urea (10 M) was freshly prepared in 50 mM phosphate buffer, pH 7.5, with 10% glycerol, and the pH was adjusted to 7.5 after the urea dissolved. Stock solutions of DmpM were then added to diluted samples of the stock urea solution to generate concentrations of 0−7 M urea. Proteins in these samples were allowed to denature over 14 h at room temperature prior to CD and fluorescence measurements.

CD spectra of all samples were scanned on a Jasco J-710 CD spectrometer. Spectra were acquired in the region 195−250 nm with a 0.1-cm path length cell (∼200 μL) and a scan speed of 100 nm/min with a response time of 0.25 s. The UV/CD spectra reported are the average of 5 scans at 0.5 nm resolution and a bandwidth of 1.0 nm.

Fluorescence emission spectra of protein samples were obtained on an Aminco Bowman series 2 luminescence spectrometer. Spectra were collected from 300 to 400 nm following excitation at 290 nm with a bandwidth of 4 nm; samples were contained in a 400 μL quartz cuvette.

Analysis of the UV/CD and Fluorescence Transition Curves. The unfolding of DmpM by urea was analyzed using the method of Pace et al. (*20, 21*) assuming that unfolding of DmpM follows a two-state transition. A nonlinear least-squares fitting procedure was used to obtain the unfolding parameters (*22*). These parameters were then used to calculate the fraction folded as a function of urea concentration (*20*). Transitions were analyzed from the signals at 222 nm for circular dichroism data and 315 nm for fluorescence data.

Urea Gradient Gels. Inactive recombinant and native DmpM samples were electrophoresed on discontinuous transverse urea gradient gels prepared by the method of Gentile et al. (*23*) with the buffers of Laemmli (*15*), including 10% glycerol (final concentration). Urea concentrations ranged from 0 to 7 M with an acrylamide countergradient of 16−10.4%. Gels were run for 16 h at 50 V. Recombinant DmpM was also electrophoresed on urea gels with a gel buffer of pH 7.4. These gels were prepared following the protocol of McLellan (*24*), and included 10% glycerol.

Treatment of DmpM with Trifluoroethanol. Inactive recombinant and native DmpM samples were dialyzed against 50 mM phosphate buffer, pH 7.5, containing 10% glycerol, which ensured that neither form of the protein was interconverted during dialysis (see Results). Retention of the different forms of the protein was monitored by nondenaturing polyacrylamide gel electrophoresis. Protein−TFE samples were prepared by adding DmpM (27 μg) to 50 mM phosphate buffer, pH 7.5, with 10% glycerol and different TFE concentrations to a total volume of 200 μL (13 μM DmpM). CD spectra were acquired with the same instrument parameters as described above.

Temperature Denaturation of DmpM. Thermal denaturation between 25 and 90 °C was monitored at 222 nm, with a heating rate of 1 °C/min in a 0.1 cm jacketed cell in the circular dichroism instrument described above. Temperature was controlled by a Neslab RTE-111 water bath. The

concentrations of native and inactive recombinant DmpM during temperature denaturation were 13 and 15 μM, respectively, and the buffer used was 50 mM sodium phosphate, pH 7.5, containing 10% glycerol.

Gel-Filtration Chromatography. A HiPrep S-100 (Pharmacia) gel-filtration column (60 $\times$ 1.6 cm) was calibrated with proteins of known molecular weight. Each standard protein (4 mg), cytochrome *c* (M_r 12 500), chymotrypsin A (M_r 25 000), and albumin (M_r 45 000), was dissolved in 50 mM MOPS, pH 7.4, containing 0.15 M NaCl (0.5 mL) and eluted from the column with this buffer. A flow rate of 0.4 mL/min was used, and 1.5 mL fractions were collected for analysis. Molecular masses of unknowns were estimated from the standard curve of log M_r vs elution time of the standard proteins.

Resolution of Two Forms of DmpM by Gel-Filtration Chromatography. Inactive recombinant DmpM (124 μM) was treated with 6 M guanidiumine hydrochloride and then loaded onto the S-100 column equilibrated with 50 mM MOPS, pH 7.4, containing 0.15 M NaCl. The column was then developed (0.3 mL/min) with this buffer, and 1.4 mL fractions were collected. Samples of fractions were analyzed by electrophoresis on a native polyacrylamide gel. Fractions that contained the faster-migrating form were combined and concentrated with a Centricon 10 concentrator. Fractions containing the slower-migrating form were concentrated separately.

Analytical Ultracentrifugation. Sedimentation velocity experiments were performed on a Beckman Coulter XL-A analytical ultracentrifuge at 20 °C with a rotor speed of 56 000 rpm. Data processing was done with the computer program Origin, provided by Beckman Coulter. The molecular mass of recombinant DmpM was calculated from the time derivative of the sedimentation velocity concentration profile according to the method of Stafford (*25*). The calculated molecular weight from this algorithm was corrected by using values for the partial specific volume of the protein and solution density of 0.7291 mL/g (calculated with the program Sedinterp) and 1.029 g/mL (*26*), respectively. Protein solutions were dialyzed against 50 mM sodium phosphate buffer, pH 7.5, + 10% glycerol, and the protein concentration for the sedimentation velocity experiment was approximately 0.2 mg/ml.

RESULTS

Purification of DmpM from Pseudomonas sp. Strain CF600. DmpM from phenol-grown *Pseudomonas* CF600 was purified according to the steps outlined in Materials and Methods. Purified protein migrated as a single band on SDS-polyacrylamide gels (data not shown). Identification as the *dmpM* gene product was confirmed by amino-terminal sequencing in the case of native DmpM. Electrospray mass spectrometry revealed a single species in both recombinant and native samples, with identical masses of 10 361.3 $\pm$ 1.3 Da. This agrees well with the molecular mass of 10 359.55 Da calculated from the amino acid composition (*1*) minus the amino-terminal methionine residue.

Identification of Two Forms of DmpM. Under some conditions fast- and slow-migrating forms of DmpM were distinguished by nondenaturing polyacrylamide gel electrophoresis (Figure 1). Although DmpM purified from phenol-grown *Pseudomonas* sp. strain CF600 was always isolated mainly as the faster-migrating form (Figure 1, lane A), a slower-migrating form was isolated from *E. coli* overexpressing DmpM (Figure 1, lane B).

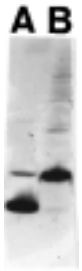

FIGURE 1: Nondenaturing polyacrylamide gel of native and recombinant DmpM (2.2 μg). Samples were purified from *Pseudomonas* sp. CF600 (lane A) or purified inactive recombinant DmpM (lane B).

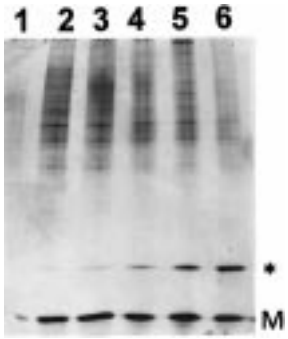

FIGURE 2: Nondenaturing gel electrophoresis monitoring expression of recombinant DmpM after induction with 0.5 mM IPTG. Crude extracts (5 μg of protein) of cells from various time points after induction were loaded in each well. Lane 1, crude extract from uninduced cells; lanes 2−6, crude extracts from cells at 15, 30, 60, 120, and 180 min after induction, respectively. Migration positions for fast-(M) and slow-(*) migrating DmpM are indicated.

Both forms were present in crude extracts from *E. coli* overexpressing DmpM (Figure 2). The first step for purification of recombinant DmpM involved a DEAE anion-exchange column where these two forms separated (data not shown). Fractions containing each of the two forms were combined separately and then carried on through the purification procedure separately. The two forms did not appear to interchange substantially during further purification (data not shown). While the fast-migrating form was active in stimulating phenol hydroxylase activity, the slow-migrating form was not (Table 1). In subsequent studies the properties of slow-migrating inactive recombinant DmpM are compared to the properties of active DmpM isolated from *Pseudomonas* sp. strain CF600.

In order to test whether inactive, slow-migrating DmpM was formed in the cell or only during purification, recombinant DmpM production was monitored in aliquots of culture taken at different time points after induction. Cells were collected by centrifugation and sonicated to obtain crude extract. Crude extracts from each time point were subjected to nondenaturing polyacrylamide gel electrophoresis (Figure 2), which reveals that slow-migrating DmpM is apparent at 60 min after induction but not before. It has not been determined whether slow-migrating DmpM is newly synthesized or derived from the faster-migrating form. However, it is clear that the inactive form of recombinant DmpM accumulates over time *in vivo*.

Table 1: Phenol Hydroxylase Activity with Native and Recombinant DmpM before and after Urea Denaturation[a]

DmpM added to assay (μg)	specific activity (nmol/min)		
	native DmpM	recombinant (+ urea)	recombinant (− urea)
2.5	506	527	11.7
0.5	244	123	
0.25	130	71.5	
0.125	63.5	31.5	

[a] Inactive recombinant DmpM was treated with 6 M urea followed by dialysis to remove the denaturant, as described in Materials and Methods. Nondenaturing gels indicated that equal amounts of fast and slow migrating forms were present after this treatment, while the native DmpM preparation contained only the faster-migrating species. A control recombinant sample was dialyzed but not treated with urea.

Activation of Inactive DmpM. Initial attempts at activating inactive recombinant DmpM involved unfolding with 6 M urea, followed by dialysis to remove the denaturant. After this treatment, native gels showed 50% conversion to the faster-migrating band when the DmpM concentration was 124 μM (1.3 mg/mL) (data not shown). Phenol hydroxylase activity assays on the urea-treated recombinant DmpM sample showed 100% activity relative to the active (native) form when the amount of DmpM added (2.5 μg) was above that required to saturate the hydroxylase (Table 1). Under nonsaturating conditions, the urea-treated recombinant sample, containing approximately 50% slower-migrating protein, exhibited exactly half the activity of native DmpM, which contained only fast-migrating protein (Table 1). Thus, incubation with urea activates DmpM by promoting conversion of the slow-migrating form to the fast-migrating form.

Size-Exclusion Chromatography. The nondenaturing gel electrophoresis results are consistent with the inactive form of DmpM being either an oligomer or a different conformation of the native form that migrates more slowly. Since DmpM contains no cysteine residues, dithiol and disulfide forms are ruled out as a possible explanation for the two bands observed on nondenaturing, nonreducing polyacrylamide gels. In order to estimate molecular masses of the two forms, size-exclusion chromatography was performed.

The two forms in a denaturant-treated recombinant sample were successfully separated on a gel-filtration column in which the elution buffer contained 0.15 M NaCl but lacked glycerol (Figure 3). Resolution of the peaks was dependent on the addition of 0.15 M NaCl to the elution buffer: omission of the salt resulted in elution patterns where the two peaks merged. Samples from the first peak were inactive, consisted of the slow-migrating band on nondenaturing polyacrylamide gels (Figure 3A, inset), and eluted at a position corresponding to a molecular mass of 32 000 Da. The second peak to elute from the gel-filtration column eluted at a position corresponding to a molecular mass of 12 500 Da, contained active protein, and when analyzed on nondenaturing polyacrylamide gels it consisted only of the faster-migrating band (Figure 3A, inset). Although the higher molecular weight species was the major component when the gel-filtration column was run in the absence of glycerol (Figure 3A), the lower molecular weight form was predominant when glycerol was included in the dialysis and elution buffers (Figure 3B and inset). These data suggest that the active form of DmpM is monomeric, that the inactive form

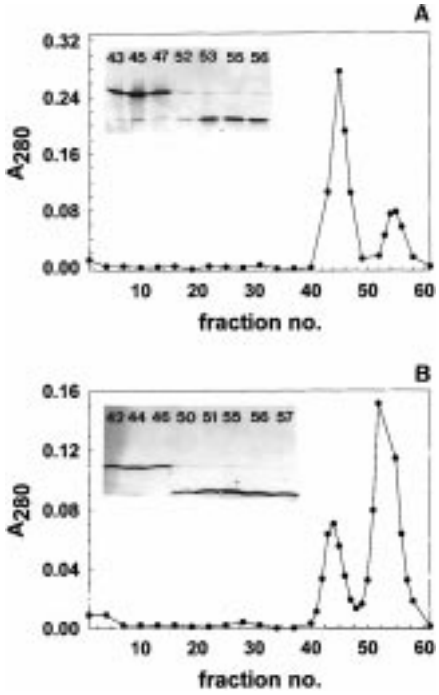

FIGURE 3: Gel filtration of recombinant DmpM in the absence (A) and presence (B) of glycerol. Nondenaturing gel electrophoresis of peak fractions is also shown (insets). (A) Elution profile of inactive recombinant DmpM (1.3 mg/mL) treated with GdHCl (6 M), dialyzed against 50 mM MOPS, pH 7.4, lacking glycerol, and eluted through the S-100 column with the same buffer containing 0.15 M NaCl. (B) Elution profile of inactive recombinant DmpM (1.3 mg/mL) treated with GdHCl (6 M), dialyzed against 50 mM MOPS, pH 7.4, + 10% glycerol, and eluted with the same buffer containing 0.15 M NaCl.

is an oligomer, and that glycerol affects the equilibrium between the two forms.

Interconversion between the Two Forms of DmpM Is Prevented by Glycerol. The differing gel-filtration profiles in the presence and absence of glycerol suggested that glycerol influences the distribution of DmpM between monomeric and oligomeric forms. Inactive recombinant DmpM was initially purified, like the native protein, in buffer containing 10% glycerol. Removal of glycerol by dialysis resulted in formation of active DmpM (Table 2); therefore glycerol appears to stabilize the inactive, slow-migrating form of DmpM. Conversely, dialysis of native DmpM against buffer lacking glycerol resulted in formation of the inactive, slow-migrating form (Table 2). Therefore, both forms appear to be stabilized by the presence of glycerol.

Evidence That Oligomeric DmpM Is a Dimer. Inactive recombinant and native DmpM preparations were exposed to various chemical cross-linkers in order to further characterize the oligomeric form. Figure 4 shows that inactive recombinant DmpM gave rise to bands corresponding to the molecular weight of dimeric DmpM when exposed to the cross-linker EDC. These cross-linked products were not observed when native (fast-migrating) DmpM was incubated with the same cross-linkers (Figure 4). These results suggest that the inactive form is a dimer, rather than the trimer suggested by gel-filtration experiments. This was confirmed in sedimentation velocity experiments, which gave a molecular weight of 20 913 Da for the inactive recombinant form of DmpM: a single boundary was observed, as would be expected for a homogeneous preparation.

Table 2: Relative Phenol Hydroxylase Activity with Native and Recombinant DmpM after Various Treatments

preparation	treatment	relative activity (%)
native DmpM (1.3 mg/mL)	none	100
	dialyzed against MOPS, pH 7.5	26
	dialyzed against MOPS pH 7.5, + 10% glycerol	108
	heated at 90 °C[b]	96
inactive recombinant DmpM (1.3 mg/mL)	none	ND[c]
	dialyzed against TGD	ND
	dialyzed against MOPS, pH 7.5	29
	dialyzed against MOPS, pH 7.5, + 10% glycerol	ND
	heated at 90 °C[b]	96

[a] The phenol hydroxylase activity assays were carried out as described in Materials and Methods with 0.25 μg of DmpM. [b] The protein concentrations of native and recombinant DmpM in the heated samples were 0.13 and 0.15 mg/mL, respectively. [c] ND, not detectable.

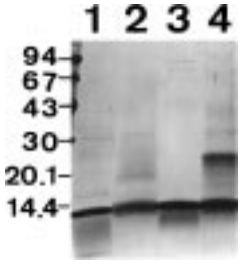

FIGURE 4: Cross-linking of native (lanes 1 and 2) and inactive recombinant (lanes 3 and 4) DmpM with EDC at 25 °C. Lane 1, native DmpM without EDC; lane 2, native DmpM + EDC; lane 3, recombinant DmpM without EDC; lane 4, recombinant DmpM + EDC.

Activation of DmpM by Heating. Inactive recombinant DmpM was also activated by heating to 90 °C at a rate of 1 °C/min. Table 2 shows that recombinant DmpM gained 96% activity relative to native DmpM activity after this treatment.

The state of DmpM resulting from heating was examined by both nondenaturing gel electrophoresis and CD spectroscopy. The CD spectrum of dimeric DmpM at 90 °C indicated that the protein was not completely denatured at 90 °C (data not shown), suggesting that refolding from a completely unfolded form is not necessary for activation. Furthermore, nondenaturing polyacrylamide gel electrophoresis indicated that heat-activated recombinant DmpM migrated in the fast-migrating band characteristic of active protein (data not shown).

Activation with Trifluoroethanol. The action of denaturants on the activation process was further probed by examining the effects of trifluoroethanol (TFE), which is known to affect secondary, tertiary, and quaternary structures of proteins (*27*). As is shown in Figure 5, native DmpM remained active at all TFE concentrations tested. Inactive recombinant DmpM underwent a transition at 10−17.5% TFE, becoming fully active at the higher concentration of TFE (Figure 5). Nondenaturing polyacrylamide gel electrophoresis analysis of the samples at each TFE concentration showed a progressive increase in the proportion of the faster-migrating form over the same range of TFE concentrations, with essentially 100% in this form at 17.5% TFE (data not shown).

Although native DmpM maintained activity at all TFE concentrations, the far-UV CD spectrum of native DmpM indicated increasing α-helical content as TFE was added (data not shown). These structural changes may be reversed when DmpM is diluted into assay mixtures lacking TFE, as activity is maintained (Figure 5). Increasing α-helical content was also observed when TFE was titrated into the inactive recombinant form (data not shown). Thus, addition of 17.5% TFE induces structural changes that result in activation of recombinant DmpM.

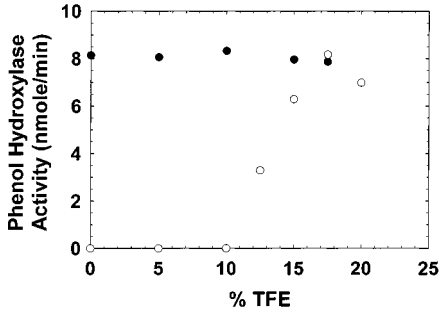

FIGURE 5: Activation of dimeric DmpM by treatment with TFE. Phenol hydroxylase activity assays with DmpM (2.2 nmol) were as described in Materials and Methods. Dimeric recombinant (○) and native DmpM (●) were incubated at the indicated TFE concentrations prior to activity assay.

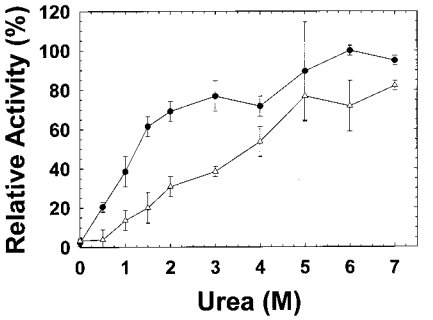

FIGURE 6: Protein concentration dependence of recombinant DmpM activation with urea. DmpM (2.2 nmol) was used in phenol hydroxylase activity assays as described in Materials and Methods. Different concentrations of dimeric DmpM [(●) 0.08 mg/mL and (△) 1.2 mg/mL] Were incubated with various concentrations of urea prior to activity assays.

Dependence of Activation on Urea and Protein Concentrations. The effects of different concentrations of urea on inactive recombinant DmpM were examined both by activity assays and by nondenaturing gel electrophoresis. DmpM samples were allowed to equilibrate overnight in buffer containing different concentrations of urea. Activation of DmpM by treatment with urea was dependent on protein concentration (Figure 6). Samples were incubated in the presence of urea at two different protein concentrations: 0.08 and 1.2 mg/mL (7.6 and 114 μM). The higher protein concentration required a greater concentration of urea for

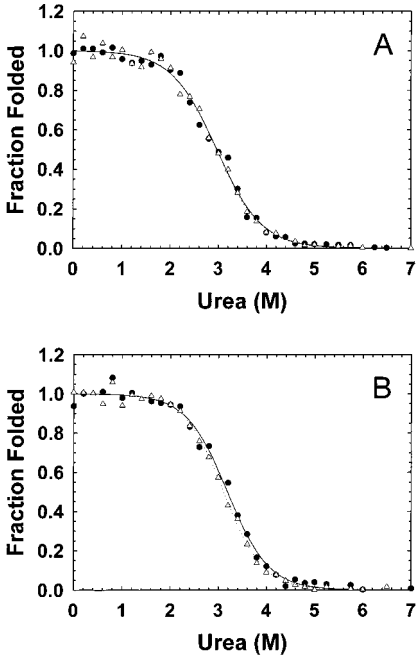

FIGURE 7: Urea denaturation curves of native and recombinant dimeric DmpM monitored by circular dichroism and fluorescence spectroscopies at 25 °C. (**A**) Native DmpM (8.7 μM) in 50 mM sodium phosphate buffer, pH 7.5, + 10% glycerol. (**B**) Recombinant DmpM (9.1 μM) in 50 mM sodium phosphate buffer, pH 7.5, + 10% glycerol. (●) CD data; (—) fitted CD data; (△) fluorescence data; (•••) fitted fluorescence data). Fitted ΔG_{H_2O} obtained from CD data: 3.67 ± 0.45 kcal/mol for native DmpM and 4.33 ± 0.54 kcal/mol for recombinant DmpM. ΔG_{H_2O} obtained from fluorescence data: 3.74 ± 0.40 kcal/mol for native DmpM and 4.44 ± 0.37 kcal/mol for recombinant DmpM.

activation (Figure 6) as well as for the parallel conversion to the faster-migrating electrophoretic form (data not shown). The observed dependence on protein concentration is consistent with the existence of an inactive dimeric form of DmpM that is progressively dissociated as the urea concentration increases.

Urea-Induced Unfolding of DmpM Monitored by CD Spectroscopy. Denaturation of native (100% fast-migrating) and recombinant (100% slow-migrating) DmpM at different concentrations of urea was monitored by CD spectroscopy. The observed transitions were essentially identical when protein concentrations were kept at or below 29 μM (Figure 7). Similar results were obtained with fluorescence spectroscopy (see below). At higher concentrations of recombinant DmpM, denaturation curves were protein concentration-dependent (data not shown). These results suggest that at low protein concentrations the recombinant dimer dissociates before denaturation occurs and that it is the monomeric form that denatures.

Urea Gradient Gel Electrophoresis To Monitor DmpM Dissociation and Unfolding. Urea gradient gels were used in order to obtain evidence for dissociation of oligomeric DmpM prior to unfolding. In the absence of urea, most of the sample is in the dimeric (slow-migrating) form, with a small amount of the monomer (faster-migrating form) also present (Figure 8). Between 2 and 2.5 M urea, the slow-migrating form of DmpM is converted to the faster-migrating form characteristic of native DmpM monomer (Figure 8). The smear is indicative of slow equilibration between the two forms on the time scale of the experiment (*28*).

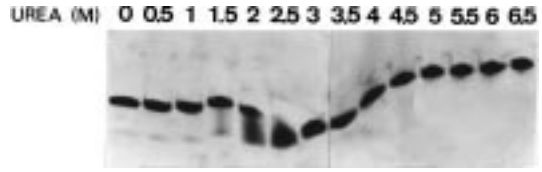

FIGURE 8: Discontinuous urea gradient gel electrophoresis of recombinant (dimeric) DmpM. Inactive recombinant DmpM (0.1 mg/mL) in 50 mM phosphate buffer, pH 7.5, + 10% glycerol was applied to urea gradient polyacrylamide gels ranging from 0 to 6.5 M urea.

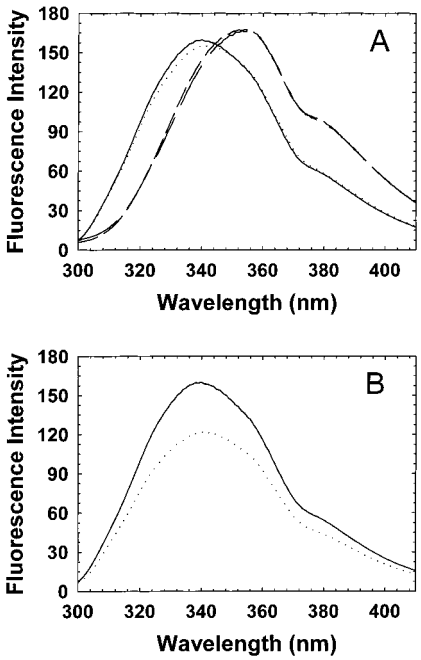

FIGURE 9: Fluorescence spectra of recombinant and native DmpM. (**A**) Native (•••) and recombinant (—) DmpM at 2 M urea and native (— — —) and recombinant (---) DmpM at 7 M urea. (**B**) Native (•••) and recombinant (—) DmpM in 0 M urea. A small correction factor was applied to account for different protein concentrations in recombinant and native samples by assuming that the spectra of native and recombinant DmpM should be the same in 7 M urea.

Denaturation then occurs between 3 and 4.5 M urea, producing the slowest-migrating, unfolded, form.

Figure 8 indicates a small shift, in both dissociation and denaturation, to a higher urea concentration compared with the CD and fluorescence denaturation data (Figure 7 and see below). This shift may be due to the difference in pH during electrophoresis, which is about 2 pH units higher than the pH of the solutions prepared for CD and fluorescence denaturation experiments. A urea gradient gel run in a buffer system at pH 7.5 (*23*) showed that, at this pH, denaturation occurs between 2 and 4.5 M urea (data not shown), while dissociation occurs between 1 and 2 M urea. This behavior agrees more closely with the CD and fluorescence denaturation data, suggesting pH is indeed important.

Fluorescence-Monitored Unfolding of DmpM. Denaturation of both native and recombinant DmpM above 2 M urea was also monitored by using the red shift of the fluorescence emission maximum at 338 nm (Figure 9A). Fitting this data to a two-state unfolding model gave results almost identical to those obtained by using CD spectroscopy to monitor unfolding (Figure 7).

Native DmpM, already in the monomer form, has a fluorescence spectrum that is less intense than that of the

recombinant dimer when urea is not present (Figure 9B). Native DmpM undergoes a transition between 0 and 2 M urea resulting in an increase in fluorescence intensity at 338 nm (Figure 9), so that the fluorescence spectra of the two forms are identical in 2 M urea (Figure 9A). At 2−2.5 M urea, dissociation of the dimer has been shown to occur by urea gel electrophoresis (Figure 8). Interestingly, no significant fluorescence intensity changes were observed between 0 and 2 M urea for the recombinant, dimeric form of DmpM (Figure 9). These data are consistent with formation of an altered conformation of DmpM monomer, with the spectral characteristics of the dimeric form, produced from either native or recombinant DmpM in the presence of 2 M urea. It is this form of the monomer that is capable of undergoing dimerization.

DISCUSSION

Stimulatory proteins similar to DmpM are associated with multicomponent binuclear iron center-containing oxygenases such as toluene and methane monooxygenases. The best-studied example is MmoB, the stimulatory protein for methane monooxygenase. Studies of this protein have focused on how interactions of the oxygenase with MmoB affect the reactivity of the oxygenase (*5*, *9*−*12*). In the course of studying phenol hydroxylase, we found that recombinant DmpM could readily be isolated in an inactive form. In order to carry out site-directed mutagenesis studies of this protein, we needed to be able to obtain fully active preparations. In addition, we wanted to determine why these preparations were inactive and whether this might be related to regulation of the oxygenase by this protein. Some of the impetus for this work came from recent findings that MmoB is converted to inactive forms by proteolytic events (*13*), although the physiological significance of this finding is uncertain.

No evidence was found for proteolysis of recombinant DmpM, which instead appeared to undergo dimerization to produce an inactive form. This form was readily distinguished from the active form by polyacrylamide gel electrophoresis under nondenaturing conditions. While some dimerization may occur during purification, it was also observed that the inactive form accumulated as induction progressed during cell growth. Thus, one way of minimizing the amount of the inactive form is to use relatively short induction times during expression.

The observation of a slower-migating form on nondenaturing polyacrylamide gels is consistent with dimer formation but was not the sole piece of evidence for it. It was possible to separate the two forms of DmpM by gel filtration: the higher molecular weight form corresponded to the slower-migrating form and was inactive. The estimated molecular mass was consistent with a trimeric structure, although cross-linking studies provided evidence only for a dimer. Sedimentation velocity experiments confirmed that the inactive slow-migrating form of DmpM is a dimer. The low molecular weight peak from the gel-filtration column corresponded to the faster-migrating electrophoretic form and was active: the estimated molecular mass is consistent with a monomer. Finally, urea denaturation studies indicated a two-state transition for native DmpM, consistent with an equilibrium between folded and unfolded forms of the monomer. By contrast, the unfolding of recombinant DmpM was more complex and dependent on protein concentration, as would be expected for a dimeric form of the protein.

The observation that DmpM is active as a monomer, but inactive as a dimer, is interesting since similar proteins have been reported to exist in both forms (*5*, *6*). The stimulatory activities of these different forms have not been reported. Since, like methane monooxygenase (*5*), phenol hydroxylase appears to be an $(\alpha\beta\gamma)_2$ complex (this laboratory, unpublished results), it is likely that it is fully activated by 2 molecules of DmpM acting independently rather than by a DmpM dimer.

A number of methods for recovering the active form of DmpM were investigated. Heat denaturation successfully reactivated the protein and, as was evident from CD spectroscopy data, reactivation by this method did not involve full refolding. Incubation of inactive preparations with trifluoroethanol (10−20%) also activated inactive preparations, and reactivation correlated with the conversion of dimer to monomer. However, the effects of this denaturant on DmpM were complex. Nondenaturing concentrations of urea also activated recombinant DmpM, in a protein concentration-dependent manner. It was established that at a concentration of dimeric DmpM of 7.6 μM, incubation with 3 M urea resulted in approximately 80% activity upon dilution in the assay cuvette (Figure 6). Furthermore, dissociation of the dimer to the monomer was observed to occur at 2−2.5 M urea by urea gradient gel electrophoresis, indicating that conversion to the monomer form can account for activation of the recombinant form.

Although inactive dimeric DmpM has not been observed in significant quantities after purification of native DmpM from *Pseudomonas* sp. strain CF600, simply removing glycerol allowed its partial conversion to the inactive form. Conversely, conversion of dimeric DmpM to monomer also occurred after removal of glycerol. Since glycerol stabilizes both the active monomer and inactive dimer forms of DmpM, the two species cannot be in direct equilibrium. Possibly, a conformational change allows DmpM to achieve a form that can dimerize. Evidence for this was obtained from urea-induced changes monitored by fluorescence spectroscopy. These data were consistent with the following model:

$$\begin{array}{ccccc} M & \rightleftharpoons & D & \rightleftharpoons & U \\ & & \Vert & & \\ & & DD & & \end{array}$$

where M is the native form, DD is the recombinant dimer, D is the monomer form present at 2−2.5 M urea, and U is the unfolded form of DmpM. In this model, glycerol binding to both M and DD may prevent interconversion of the two forms. In any event, it is only the D form of the monomer that can readily dimerize.

A number of possibilities can be entertained for how active DmpM is maintained in *Pseudomonas* sp. strain CF600 growing at the expense of phenol. The most obvious explanation is that DmpM readily associates with the other components of phenol hydroxylase, thus preventing the transition. Another factor may be that the concentration of DmpM in the cell remains low enough to favor folding into the monomeric, active form.

No evidence for significant inactive dimer formation in the native strain was obtained, yet it is possible that it does exist and could play a physiological role in the regulation

of phenol hydroxylase activity. This might be required, for example, to prevent unnecessary oxidation of NADH by phenol hydroxylase in the absence of phenol. Interestingly, an inactivation event also occurs with the DmpM homologue, MmoB of soluble methane monooxygenase from *M. capsulatus* (Bath) (*13*). In this case, the inactive form of MmoB resulted from proteolysis between Met12 and Gly13. However, amino acid residues 2−34 of MmoB of methane monooxygenases are lacking in DmpM, and we have never observed any proteolysis in samples of DmpM that have been subjected to mass spectrometry analysis.

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