Ligand Binding Induces an Ammonia Channel in 2-Amino-2-desoxyisochorismate (Dic) Synthase PhzE*

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PhzE utilizes chorismate and glutamine to synthesize 2-amino-2-desoxyisochorismate (ADIC) in the first step of phenazine biosynthesis. The PhzE monomer contains both a chorismate-converting menaquinone, siderophore, tryptophan, and secondary metabolites such as e.g. the aromatic amino acids, ubiquinone, folate, vitamin K, and the siderophores enterobactin and pyochelin. Because the production and utilization of chorismic acid are limited to prokaryotic microorganisms and plants, enzymes involved in chorismate metabolism are attractive targets for the development of biologically active compounds as exemplified by the herbicide glyphosate, which inhibits an enzyme of the shikimate pathway required for chorismate biosynthesis.

Four chorismate-utilizing enzyme families are known (1). First, two unrelated types of chorismate mutases, AroH and AroQ, generate prephenate as a precursor of phenylalanine and tyrosine. A third family consists of the chorismate lyases, which cleave chorismic acid to p-hydroxybenzoate and pyruvate in the biosynthesis of ubiquinone. Finally, the fourth group comprises members of the menaquinone, siderophore, tryptophan (MST)3 biosynthesis family, which utilize water or ammonia to perform nucleophilic substitution at the six-membered ring of chorismate with or without concomitant rearrangement of the double bond system in an Mg2+-dependent reaction (Fig. 1). Of these enzymes, isochorismate, 2-amino-2-desoxyisochorismate (ADIC), and 4-amino-4-desoxycumarate synthases release the isomerized product, whereas anthranilate (AS) and salicylate synthase initiate a subsequent sigmatropic elimination of pyruvate within the same active center, leading to the synthesis of anthranilate or salicylate, respectively (2, 3). The aminating members of the MST family generate ammonia from glutamine in a type 1 glutamine amidotransferase (GA1ase1) domain, which either forms a heterodimer with or is fused to the N or C terminus of the MST domain. It is believed that glutamine hydrolysis by GA1ase1 is only initiated once chorismate has bound to the MST domain and that NH3 is delivered through a tunnel to the chorismate binding site to avoid its loss to the solvent (4, 5).

Crystallographic and biochemical studies have led to considerable structural and mechanistic insight into members of the MST family in recent years, yet structures of a MST/GA1ase1 fusion protein and of an ADIC synthase are lacking. In addition, the existence of an intramolecular channel has never been demonstrated in these enzymes. We have aimed at filling these gaps...
by determining the crystal structure of ADIC synthase PhzE from *Burkholderia lata* 383, an enzyme that consists of an MST/GATase1 fusion in a single chain, in an uncomplexed open and a ligand-bound closed conformation. In addition, we have measured small angle x-ray scattering (SAXS) and biochemical data. PhzE catalyzes the first step in the biosynthesis of phenazines, a class of redox-active secondary metabolites that a number of bacterial strains produce as virulence factors, antibiotics, and probably also as respiratory pigments (6, 7). Because PhzE commits chorismate to phenazine biosynthesis, we also investigated whether the enzyme is subject to feedback inhibition similar to some of the related anthranilate synthases. In addition, we confirmed the functional assignment of PhzE as an ADIC synthase by mass spectrometry and by visualizing PhzE-produced ADIC in the active center of an inactive mutant of PhzD from *Pseudomonas fluorescens* 2-79. PhzD is an isocho- 

rismatase that catalyzes the step following PhzE in phenazine biosynthesis. Together, our new data extend the spectrum of structurally characterized MST proteins to ADIC synthases and also provide new insights into several mechanistic aspects of this enzyme family.

**EXPERIMENTAL PROCEDURES**

For full experimental details, refer to the supplemental material.

Overexpression and Purification of *B. lata* 383 PhzE and of *P. fluorescens* 2-79 PhzD—*B. lata* phzE was amplified from genomic DNA and cloned into a modified pET vector extending the protein for an N-terminal protease-cleavable His6 tag. After overexpression in *Escherichia coli* and purification on immobilized Ni2+ resin, the tag was cleaved with tobacco etch virus protease, and the protein was further purified by size exclusion chromatography. Preparation of PhzD from *P. fluorescens* 2-79 was achieved with a similar protocol. Seleno-L-methionine labeling of PhzE was performed by the methionine biosynthesis suppression method (8), and mutations were introduced into PhzE and PhzD with the QuikChange method (Stratagene).

Crystallization, Data Collection, and Structure Determination—Crystals of ligand-free *B. lata* PhzE (native and seleno-L-methionine-labeled) were obtained at room temperature by the hanging-drop vapor diffusion method with a 500-µl reservoir consisting of 0.1M BisTris propane, pH 7.0, 0.2M KSCN, and 22% (w/v) PEG 3350, of which 1 µl was mixed with 2 µl of protein solution containing 1 mM MgCl2 and 20 mM glutamine. For the ligand-bound form, the protein was incubated with 50 mM MgCl2, 20 mM chorismate, and 25 mM L-glutamine for 30 min before crystallization against a reservoir containing 0.1M HEPES buffer, pH 7.1, 0.2M MgCl2, and 15% isopropyl alcohol at 4 °C. Streak seeding was required to obtain suitable crystals. To remove Zn2+ bound to the GATase1 active site in the complex structure (see below), PhzE was incubated with 10 mM EDTA followed by buffer exchange prior to setting up crystallization experiments.

Crystals of the D38A mutant of PhzD from *P. fluorescens* 2-79 were obtained with a precipitant consisting of 0.1 M sodium cacodylate, pH 6.5, 0.2M sodium acetate, and 25% (w/v) PEG 4000. They were soaked for 1 h in a solution containing 0.1 M BisTris, pH 6.5, 20% (w/v) PEG 4000, 0.1 M NaCl, and 1 mM ADIC. ADIC was synthesized enzymatically and purified by HPLC as described in the supplemental materials.

Diffraction data of cryoprotected PhzE crystals were collected on a MX-225 CCD detector (marresearch) at beamline X10SA of the Swiss Light Source (Paul Scherrer Institute, Villigen, Switzerland) and integrated with XDS (9). The structure of apo-PhzE was determined from selenium MAD data, using SHELXD (10) and SHARP (11) for initial phasing. The model was traced in O (12) and refined with REFMAC5 (13). Final corrections were applied in COOT (14). The refined structure was used for molecular replacement of the ligand-bound form in Phaser (15). Ligand restraints dictionaries were generated.
with PRODRG (16). Diffraction data of cryptoprotected *P. fluorescens* 2-79 PhzD crystals were collected at 100 K on a mar345 image plate (marresearch) using a Micro-MAX-007 HF rotating copper anode (Rigaku) as x-ray source. The structure was solved by molecular replacement using coordinates of *Pseudomonas aeruginosa* PhzD (Protein Data Bank (PDB) ID code 1NF8) (17). Full data collection and refinement statistics are shown in Table 1 and supplemental Table S2.

SAXS—SAXS experiments were performed at beamline X33 of the DORIS III storage ring (DESY and EMBL Hamburg, Germany) (18), using concentrations of 3 and 6 mg/ml PhzE to assess protein interactions. Bovine serum albumin (BSA) was taken as a reference for calculating the molecular mass of PhzE particles in solution. Data were processed with PRIMUS (19), using GNOM (20) to compute forward scattering, radius of gyration and distance distribution.

Low resolution models of PhzE were built with DAMMIN (21), applying a 2-fold symmetry constraint. Finally, 10 independent reconstructions were superimposed with SUBCOMP and DAMAVER (21) to derive the most probable PhzE model after filtering to the molecular mass of the dimer.

 Isothermal Titration Calorimetry—Isothermal titration calorimetry measurements were performed at 25 °C with a VP-ITC system from MicroCal LLC. PhzE was diluted to 100 μM in buffer containing 50 mM Tris, pH 7.5, and 150 mM NaCl with or without 1 mM MgCl₂. Chorismate was dissolved in the same buffer as used for the protein to a final concentration of 1 mM. Data were analyzed with Origin version 7 (OriginLab Corporation).

Enzymatic Activity Assays—Activity assays were performed by modification of a previously described method (22). Reaction mixtures contained 0.1 M HEPES-Na, pH 7.0, 1 mM MgCl₂, 20 mM glutamine, and 1–50 μM chorismic acid at 25 °C. The reaction was initiated by addition of PhzE to a final concentration of 100 nM, and initial velocities were determined by following the increase in absorption at 280 nm. *Kₘ* and *kᵥ* values were determined by fitting to a Michaelis–Menten equation in GraFit (Erithacus Software). Experiments were repeated at least in triplicate. Turnover of ADIC by PhzD was confirmed by HPLC.

**RESULTS**

**PhzE is an ADIC Synthase**—ADIC is an intermediate of anthranilate biosynthesis (23), but rather than being released from the PhzE-related AS, it is further converted to anthranilate within the active site of the MST domain of this enzyme. ADIC was first postulated as a phenazine precursor in 1982 (24), and full incorporation of ADIC into phenazines has been demonstrated (25), but direct proof of its generation and release from PhzE has not been brought forward. In fact, the role of PhzE as an ADIC synthase has recently been questioned due to the finding that PhzE could complement an AS mutant of *E. coli* (26) and because it possesses only 12% sequence identity to and a different domain arrangement than SgcD, an ADIC synthase involved in the biosynthesis of the enediyne antitumor antibiotic C-1027 (27).

We therefore investigated the product of PhzE by several methods, which are detailed in the supplemental materials. First, incubation of chorismic acid with glutamine, MgCl₂, and PhzE produced a species with increased absorption at 280 nm (supplemental Fig. S1A), as has been described for ADIC previously (22). The compound possessed the expected molecular mass (supplemental Fig. S1B) and could further be converted to *trans*-2,3-dihydro-3-hydroxyanthranilic acid with PhzD (supplemental Fig. S1C), an isochorismatase that catalyzes the step following PhzE in phenazine biosynthesis (17). In addition, it was possible to visualize ADIC at 1.9 Å resolution in crystals of the inactive D38A mutant of PhzD from *P. fluorescens* 2-79 by soaking with HPLC-purified product of PhzE (*R* <sub>work</sub> = 16.4%, *R* <sub>free</sub> = 21.0%; supplemental Fig. S1D). Together, this confirms the assignment of PhzE as an ADIC synthase.

After treatment with EDTA to remove bound Zn²⁺ (see below), enzyme parameters for chorismic acid were *Kₘ* = 2.4 ± 0.5 μM and *kᵥ* = 0.3 ± 0.01 s⁻¹ for chorismic acid, which is similar to other MST enzymes (supplemental Table S1). Unlike

**Crystal Structure of ADIC Synthase PhzE**

**Table 1** Data collection and refinement statistics.

| Parameters                  | Open PhzE | Closed PhzE + Zn²⁺ | Closed PhzE | PhzD-D38A/ADIC |
|-----------------------------|-----------|--------------------|-------------|----------------|
| Space group                 | P₆₁₂₂     | P₂₁₂₂              | P₂₁₂₂       | P₂₁₂₂         |
| Cell dimensions             | 1274, 1274, 2164 | 2597, 945, 536     | 2582, 978, 540 | 68.5, 72.1, 79.0 |
| α, β, γ (°)                 | 90, 90, 120 | 90, 90, 90         | 90, 90, 90   | 90, 90, 90     |
| Resolution (Å)²             | 20.2-2.9 (3.0-2.9) | 20.2-2.1 (2.2-2.1) | 20.2-2.6 (2.7-2.6) | 20.2-2.6 (2.7-2.6) |
| R<sub>work</sub> (I)        | 7.0 (52.5) | 6.6 (30.5)         | 6.9 (41.2)   | 5.0 (28.0)     |
| Completeness (%)            | 99.6 (99.9) | 98.3 (98.3)        | 99.8 (100)   | 97.7 (98.2)    |
| Redundancy                  | 4.8 (4.9) | 2.2 (2.2)          | 4.0 (4.1)    | 2.3 (2.4)      |
| No. atoms/B-factors (Å²)    | 20.2-2.9 (3.0-2.9) | 20.2-2.1 (2.15-2.1) | 20.2-2.6 (2.65-2.6) | 20.1-1.9 (1.95-1.9) |
| No. reflections             | 204850 (2476) | 324405 (146170) | 173817 (43071) | 29748 (2271) |
| Root mean square deviations | 0.017/1.693 | 0.023/1.901 | 0.016/1.576 | 0.025/1.904 |
| Bond lengths/angles (Å/°)   | 0.017/1.693 | 0.023/1.901 | 0.016/1.576 | 0.025/1.904 |

**Table 1—Continued.**

| Closed PhzE | PhzD-D38A/ADIC |
|-------------|---------------|
| 0.5 s⁻¹     | 0.3 s⁻¹       |
| 0.01 s⁻¹    | 0.01 s⁻¹      |
| 0.5 s⁻¹     | 0.3 s⁻¹       |
some AS, PhzE was not inhibited by tryptophan or by phenazine biosynthesis intermediates.

Crystralization and Structure Determination of PhzE—It was not possible to obtain crystals with PhzE from several Pseudomonas spp., which we initially investigated because pseudomonads are the best studied phenazine producers. Crystallization experiments were therefore extended to the protein of B. lata 383, which possesses the same domain arrangement as the Pseudomonas orthologs at an average sequence identity of 56% (supplemental Fig. S2). Crystals of ligand-free B. lata 383 PhzE diffracted to 2.9 Å, and the structure was determined by anomalous diffraction data from crystals of seleno-L-methionine-labeled protein. The model was refined to $R_{\text{work}}/R_{\text{free}} = 18.7/23.1\%$, and could, with exception of the termini and two flexible regions, be traced completely missing residues in chain A: Met1–Asn6, Thr28–Glu33, Glu433–Leu439, Lys637–Ala643; chain B: Met1–Pro5, Gly429–Leu439, Arg634–Ala643). The structure of PhzE crystallized in the presence of glutamine, chorismic acid and $\text{Mg}^{2+}$ was solved by molecular replacement. These crystals diffracted to 2.1 Å, and the refinement converged at $R_{\text{work}} = 14.5\%$ and $R_{\text{free}} = 20.0\%$, and the final model showed a similar amount of disorder as the apo structure. Data collection and refinement statistics are shown in Table 1 and supplemental Table S2.

Overall Structure of PhzE—Size exclusion chromatography indicated that PhzE forms homodimers in solution, and indeed one 140-kDa homodimer was found in the asymmetric unit of both crystal forms investigated here. We initially determined the structure of PhzE crystallized in the absence of ligands, which will be referred to as the open form. In this crystal form, the dimer adopts a butterfly-shaped structure in which the N-terminal chorismate-binding MST domains (residues 1–395) lie near the 2-fold symmetry axis of the dimer and the GATase1 domains (residues 442–643) occupy the far ends of the wings (Fig. 2A). The linker (residues 396–441) is partially disordered (Gly429–Leu439 and Glu433–Leu439 missing in chain A and chain B, respectively) but crystal packing and tracing considerations only allow for an intertwined dimer in which the GATase1 domain of one chain forms intimate contact with the MST domain of the other chain B. This indicates that the functional unit is established by the MST domain of one chain and the GATase1 domain of the other. The quaternary structure of the dimer is unprecedented within the MST family and to exclude that the dimer is a crystallization artifact, we performed SAXS experiments that confirmed the functional unit of the dimer.

FIGURE 2. Overall structure of PhzE. A, ligand-free open form. One chain has been colored in magenta, the other monomer is colored according to domains. B, same as in A, but seen from the top. C, ligand bound closed form, fitted to the SAXS envelope (gray) with SITUS (37). Circles indicate the positions of active sites in the MST and GATase1 domain. Ligands are shown in ball-and-stick representation. The NH3 channel (black mesh) was located with CAVER (30).

This figure was prepared with PyMOL.
the unusual arrangement (Fig. 2C and supplemental Fig. S3). The intertwined structure is also retained in the closed form, but here the dimer is more outstretched. In fact, this closed conformation fits the SAXS envelope even better than the open form, which is also reflected in a better fit of the calculated scattering curve ($\chi^2 = 1.5$ for the closed conformation versus $3.2$ for the apo structure; supplemental Fig. S3). This indicates that the quaternary structure observed in the crystal form obtained in the absence of ligands is to a certain extent a crystallization artifact, resulting from the relatively weak interaction between the two MST domains of the dimer. Apparently, this interface can be distorted by crystal packing forces, and the distortion of the open structure manifests itself in a positional change of approximately 60 Å at the outer edge of the GATase1 domain when one of the MST domains from each of the two crystal forms are superimposed (Fig. 2D).

Interestingly, in both crystal forms the relative arrangement of the MST and GATase1 domains of the functional unit in PhzE is similar to that found in the heterotetrameric TrpE/GAS, even if the quaternary structures are different. However, because the positions of the two MST domains also differ among the structurally characterized AS (supplemental Fig. S4), this finding alone does not explain the catalytic differences between ADIC synthases and AS.

**Chorismate Binding MST Domain**—A hallmark of the MST domain is a large $\beta$-sandwich structure covered with $\alpha$-helices from both sides. The active center lies in the core of the domain and is built from residues of both sides of the sandwich together with amino acids from flanking $\alpha$-helices (Figs. 2C and 3A). It was found to contain benzoate and pyruvic acid (Fig. 3A and supplemental Fig. S5), even if the closed conformation was obtained by crystallizing PhzE in the presence of chorismic acid. This breakdown of either the substrate chorismic acid or of the product ADIC was also observed in the ligand-bound crystal structure of *Serratia marcescens* AS (PDB ID code 1I7Q) (2). The position and interactions of benzoate and pyruvate are very similar to those of the ADIC isoster isochorismate in the recently published isochorismate synthase EntC structure (28), showing that they serve as a good template for the enzyme/substrate complex (supplemental Fig. S5).

Pyruvic acid is bidentately coordinated to the guanidino group of Arg$^{352}$ and also makes a hydrogen bond with Tyr$^{328}$. With respect to the unliganded form, the side chain of Arg$^{352}$ adopts a different rotamer conformation to bind pyruvate, whose carbonyl oxygen atom is 3 Å away from C3 of benzoate, clearly indicating that the C-C bond is broken (Fig. 3A and supplemental Fig. S5). The carboxylate group of benzoate occupies two positions within the octahedral coordination sphere of an Mg$^{2+}$ cation (Figs. 3A and 4A). It also involves hydrogen bonds with the amide bonds between Ser$^{217}$ and Gly$^{218}$ and between Ser$^{368}$ and Thr$^{369}$ as well as with the side chains of Ser$^{217}$, Thr$^{319}$, and Lys$^{386}$. Other residues within the first coordination sphere of the ligands include a handful of nonpolar residues together with Glu$^{201}$ and His$^{279}$. This histidine moves by nearly 4 Å with respect to the unliganded structure to interact with the new rotamer of Arg$^{352}$ (Fig. 3A).

**GATase1 Domain**—The two crystal structures of PhzE allow comparison of the structure of the ligand-free GATase1 domain with that of an intermediate of the catalytic cycle in which Cys$^{526}$ in the active site is covalently modified by a glutamyl moiety (supplemental Fig. S6). Superimposition reveals that only small structural changes accompany ligand binding. With the exception of hydrogen bonds to the side chains of Gln$^{530}$ and Glu$^{548}$, other polar contacts of the glutamyl group involve backbone atoms of Gly$^{493}$, Gly$^{495}$, Thr$^{468}$, and Tyr$^{569}$ (Fig. 3B). The active site itself is located close to the surface and is solvent-accessible in the ligand-free structure. Following a ligand-induced rigid body movement, it becomes secluded through interactions with the MST domain (see below).

Interestingly, in the highest resolution data set of the ligand-bound crystal form collected for this study, we observed additional electron density, including anomalous diffraction in the vicinity of Cys$^{526}$ of the GATase1 active site (see Fig. 6B and supplemental Fig. S7A). This electron density could be removed by treating the enzyme with EDTA prior to crystallization. Because of the tetrahedral coordination, we assumed that this density results from Zn$^{2+}$ cations, and the presence of zinc was indeed revealed by x-ray fluorescence of crystals (supplemental Fig. S7C). Ni$^{2+}$, which was used to capture His$_{6}$-tagged...
PhzE in the first step of the purification procedure, was not discovered in these experiments (data not shown). It was, however, found that Ni\(^{2+}/\)H\(_{11001}\), Mn\(^{2+}/\)H\(_{11001}\), and Zn\(^{2+}/\)H\(_{11001}\) inhibit the enzyme (supplemental Fig. S7B and supplemental Table S1). Because the zinc-free and zinc-bound structures of the closed form of PhzE are otherwise identical, the discussion is based on the higher resolution Zn\(^{2+}/\)H\(_{11001}\)-contaminated diffraction data.

**Linker**—The linker comprises amino acids 396–441 and connects the MST and GATase1 domains at a distance of over 60 Å. It is in part proline- and glycine-rich but also contains \(\alpha\)-and \(\eta\)-helical secondary structure. Due to flexibility, it was not possible to trace the linker fully (Gly\(^{429}\)–Leu\(^{439}\) and Glu\(^{433}\)–Leu\(^{439}\) missing in chain A and chain B of the apo structure, Gln\(^{432}\)–Leu\(^{439}\) missing in both chains of the closed structure), but it is clear that

![Diagram of PhzE](https://example.com/diagram)

**FIGURE 4.** Ligand binding induces structural changes in PhzE. A, stereo plot of the clamp-like closing of the MST active site during ligand binding. Moving parts in the closed form are shown in yellow, and their position in the open structure is shown as a black ribbon. Residues from the linker of the other monomer are in magenta. Black arrows indicate the repositioning of single residues. B, stereo plot showing the formation of new hydrogen bonds in the MST/GATase1 interface of the ligand-bound structure. Note the reorientation of Asp\(^{243}\) with respect to the open form. C, cluster of acidic residues and electrostatic surface around the entry to the active site of the MST domain in the open form, calculated with APBS (38). D, formation of the ammonia channel between the active centers of MST domain and GATase1 of the second chain. Glu\(^{151}\) likely acts as a gatekeeper. The arrow indicates the trajectory of NH\(_{3}\) to Cys\(^{2}\) of chorismate.
the central α-helix of the linker (residues 403–416) moves in concert with residues Pro^215–Leu^249 when ligands bind the MST domain. Comparison of the open and closed forms reveals that the backbone conformation of amino acids 395–414 differs most strongly between the two structures. These local changes neutralize the large changes in the MST/MST monomer/monomer interface between the two crystal forms of PhzE and keep the relative orientation of the MST and GATase1 domains within the two functional units of the dimer intact (Fig. 2).

**DISCUSSION**

The data presented here confirm the assignment of PhzE as an ADIC synthase, and the two crystal structures provide insight into structural changes that accompany its catalytic cycle. They also give an opportunity to discuss differences that may lead to the evolution of pyruvate-eliminating and non-eliminating MST enzymes.

First, it is interesting to note that the ligand-bound structure of PhzE fits the SAXS envelope much better than the crystal structure obtained in the absence of substrates, even if the SAXS measurements were performed without ligands (Fig. 2C and supplemental Fig. S3). Therefore, the relative arrangement of the two MST/GATase1 functional units found in the apo crystal structure seems to be influenced by crystal packing forces. Only 219 Å² of the solvent-accessible surface area are buried in the MST/MST interface of the ligand-bound structure per monomer. Given this very weak interaction, the occurrence of crystal-packing-induced distortions is not very surprising. More contacts between the two MST domains exist in the apo crystal form (471 Å² buried per monomer; Fig. 2, A and B), but these additional interactions involve residues Arg^221–Pro^227, which undergo ligand-induced movement such that the butterfly shape of the open structure cannot be retained in the crystal structure of the complex (see below).

Structural changes are triggered by the binding of Mg^{2+} and chorismic acid, which apparently bind simultaneously. This is indicated by the observation that no Mg^{2+} was found in the crystal structure of the open form despite being present in the crystallization buffer, and chorismate alone did not show affinity toward PhzE in isothermal titration calorimetry experiments without Mg^{2+} (supplemental Fig. S8). Interestingly, the binding of Mg^{2+}/chorismate is endothermic, i.e. entropy-driven. This may be a further indication of structural changes that accompany the process. Obviously, glutamine can only bind second, as shown by the fact that glutamine was not observable in the open form despite being included in the crystallization experiment. Together, this argues for sequentially ordered substrate binding in which Mg^{2+}/chorismate binds first, enabling the GATase1 domain to capture glutamine for hydrolysis.

The octahedral coordination sphere of Mg^{2+} involves a bidentate interaction with the carboxylate group of benzoate, the side chains of Glu^244 and Glu^382, and two water molecules that are held in place by Glu^241 and Glu^779 (Fig. 4A). These highly conserved acidic residues approach each other with respect to the open structure, leading to a closing of the active center in a clamp-like movement of the α-helix containing Glu^779 and Glu^382 on one side and the large structural element from Pro^215 to Leu^249 on the other (Fig. 4A). It thus is conceivable that neutralization of the negative charges in the formation of the active site in part explains the requirement for Mg^{2+} in the MST enzymes. This is also corroborated by the observation that repositioning of the glutamic acids is accompanied by reorientation of the conserved Lys^241, Glu^244, and Glu^379, thus adding to the anchoring of both jaws of the clamp (Figs. 3A and 4A).

Vice versa, the open conformation of PhzE seems to be stabilized by repulsion of negative charges between the helix containing Glu^399 and Glu^802 on one side and the highly acidic stretch from Asp^238 to Glu^244 on the other (Fig. 4C). Of these amino acids, Asp^238–Glu^241 also change their backbone conformation from coil to α-helical structure, which is the most significant local structural rearrangement within the MST domain on ligand binding (Fig. 4A). This is likely to add further to the stabilization of the closed form and creates a hinge that triggers the concerted motion of residues Pro^215–Leu^249. Similar refolding, but to a lesser extent, is also observed in AS (PDB ID codes 1I7Q and 1I7S) (2) and isochorismate synthase (PDB ID codes 3BZM and 3HWO) (28, 29).

Because the structural element Pro^215–Leu^249 contains part of a central strand of one of the β-sheets (Cy^210–Tyr^222), it pulls the two strands in Val^266–Ile^284 with it, together with residues Ala^393–Gly^419 of the linker, which lines the moving structures in this region. Anchoring of Pro^215–Leu^249 also involves interactions between the carboxylate group of benzoate and the backbone amide between Ser^217 and Gly^218, which leads to a peptide flip with respect to the open conformation (Fig. 3A).

The position of Pro^215–Leu^249/Val^266–Ile^284 of the closed structure is not compatible with the more compressed MST/MST interface observed in the apo crystal structure because it would lead to steric conflicts with Arg^221–Pro^227 of the MST domain in the second chain. Differences in the MST/MST interfaces between the two crystal structures accumulate to a rotation of approximately 50° between the two MST/GATase1 pairs when one MST domain of the open and closed structure are superimposed (Fig. 2D). Within the functional units, however, these differences are nearly completely compensated through backbone changes of linker residues Asp^399–Arg^414.

The MST/GATase1 interface is also subject to changes, yet these are much less pronounced and proceed around a different rotation axis than the crystal-packing-induced MST/MST reorientation. It seems that the refolding within Asp^238–Glu^241 mentioned above also triggers motion of the GATase1 domain because it reorients Asp^248 outward to form hydrogen bonds with Arg^372 and His^373. As a consequence, the side chain of Arg^372 repositions to interact tightly with the carboxyl groups of Gln^548 and Gly^549 (Fig. 4B), which may be sufficient to pull the GATase1 into an 8° rotation with a concomitant 8 Å movement at positions most distant to the rotation axis (Fig. 2D).

The clamping motion in the MST fold and the rotation of the GATase1 domain shield both active centers toward the solvent. Because the modification of chorismic acid requires ammonia as a nucleophile, both active centers must coordinate their activities and establish a means for transporting the toxic NH₃ without loss to the environment. Indeed, analysis with CAVER (30) reveals two halves of an ammonia channel that connect the
active centers of the MST and GATase1 domain. The channel is blocked by the side chain of Glu$^{251}$, which forms two hydrogen bonds with Asn$^{149}$. Mutation of these residues leads to an inactive enzyme. Glu$^{251}$ adopts a different rotamer in the open structure to interact with Lys$^{354}$, and the required space for this rotamer is still available in the closed conformation crystallized here (Fig. 4D). This implies that Glu$^{251}$ acts as a gatekeeper between the MST and GATase1 active centers, similar to Trp$^{74}$ in glucosamine-6-phosphate synthase from *E. coli* (PDB ID code 1JXA) (31). The closed conformation therefore likely represents a late state of the catalytic cycle in which NH$_3$ has already been passed to the chorismic acid binding site. The ammonia channel is approximately 25 Å long and ends at C2 of the si-face of chorismic acid, thus establishing the stereochemical configuration of ADIC. The nucleophilic attack at C2 is probably assisted by the carbonyl groups of Ile$^{216}$ and Thr$^{304}$ together with the side chains of Ser$^{217}$ and Thr$^{369}$, which are all positioned to act as hydrogen bond acceptors aiding the deprotonation of NH$_3$. Protonation of the leaving hydroxyl group at C4 of chorismic acid is achieved by Glu$^{201}$, whose mutation to glutamine rendered the enzyme inactive (Figs. 3A and 5).

It is unclear why benzoate and pyruvate and not chorismate or ADIC were found in the MST active site of the closed crystal form. Although the cleavage of pyruvate seems to be rooted in the tendency of chorismic acid and possibly also of ADIC to undergo sigmatropic reactions including the elimination of pyruvate, the generation of benzoate requires reduction, and the nature of the reducing agent in crystallization of the closed form is unknown at present. However, the breakdown seems associated with an intrinsic enzymatic activity of PhzE because it was not possible to crystallize any of the inactive mutants in the closed conformation. ADIC is a relatively unstable molecule with a half-life of 34 h (22), which is similar to the time required for crystallization. It therefore is likely that the breakdown products originate from ADIC captured in the active center of PhzE before the enzyme has completed its catalytic cycle with product release. This is also corroborated by the observation that Cys$^{526}$ is still covalently modified by glutamine and the fact that it was not possible to crystallize PhzE in the presence of exogenously added benzoate and pyruvate. Further, the breakdown of ADIC seems to be linked to the crystallization conditions because no breakdown products were observed in HPLC analysis of enzyme activity assays.

Further in this context, questions remain concerning the differences between ADIC synthase PhzE and AS, which lyse ADIC to anthranilate and pyruvate (Fig. 1). We tried to employ structural comparisons to identify residues that could convert PhzE into an AS and hence mutated Ser$^{217}$, Ser$^{368}$, and Thr$^{369}$ to alanine, alanine, and glycine, respectively, which are the three differences in the first coordination sphere of chorismic acid in which PhzE and AS differ (supplemental Fig. S9). Whereas S368A was unstable, S217A and T369G retained lower levels of ADIC synthase activity and the S368A/T369G double as well as the S217A/S368A/T369G triple mutant were inactive. Increased anthranilate production was not observed, indicating that more subtle differences determine the fate of ADIC in these enzymes. In this respect, it is also interesting to note that the mutation of His$^{398}$ to methionine in AS from *Salmonella enterica* abolishes pyruvate lyase activity nearly completely to create an ADIC synthase (22). However, the corresponding His$^{279}$ is also conserved in PhzE (Fig. 3A and supplemental Fig. S9), showing that this residue alone cannot be responsible for the different activities. Convincing theoretical and experimental evidence for a pericyclic pyruvate elimination mechanism in AS and salicylate synthase has been brought forward (32, 33). In this reaction, the enzyme would be expected to act mainly in properly orienting the methylene group of the enolpyruvyl moiety toward the hydrogen atom at C2 of ADIC. In PhzE, binding of the enolpyruvyl group is achieved by the conserved Arg$^{352}$, which in the closed conformation interacts with His$^{279}$ (Fig. 3A). It is therefore conceivable that the H398M mutation in *S. enterica* AS interferes with the proper conformation of ADIC for pyruvate elimination. Because Arg$^{352}$ and His$^{279}$ are conserved in ADIC synthases, however, it seems

**Figure 5. Mechanistic proposal for the generation of ADIC in the active site of the MST domain.** The identity of the base B required for deprotonation of the NH$_3$ nucleophile is unclear at present.

**Figure 6. Inhibition of PhzE.** A, the allosteric tryptophan binding site of *S. marcescens* anthranilate synthase is blocked by Arg$^{26}$ and Trp$^{184}$ in PhzE. Surface and Trp coordinates (black) are from PDB ID code 1175 (2). B, Zn$^{2+}$ (gray sphere) binds to the GATase1 active center. Anomalous difference electron density of data collected at 12.4 keV at 5σ are displayed as a gray mesh.
likely that the difference between these enzymes lies in the sub-
strate release mechanism, which avoids pyruvate elimination in
the ADIC synthases by providing a different release path or by
shortening the ADIC residence time in the active center. The
latter will be strongly prolonged in protein crystals, explaining
why pyruvate elimination was observed in PhzE crystals but not
in solution. Although it is tempting to speculate that different
release mechanisms are a consequence of different quaternary
structures, it has to be mentioned that at least one example of an
ADIC synthase in which the MST and GATase1 domains are
provided from separate polypeptides is known (27) and that an
anthranilate synthase encoded in a single polypeptide with a
linker similar to PhzE has been described in the literature (34).

An interesting property of some related AS is their feedback
inhibition by L-tryptophan, which is the major downstream
product of anthranilate in primary metabolism. We therefore
investigated whether PhzE is also feedback inhibited by L-trypt-
ophan or by phenazine-1-carboxylic acid, the end product of
enzymes in the phz operon. No inhibition was observed with
PhzE from B. lata or from Pseudomonas strains. This presum-
ably is a consequence of a structural variation with respect to
AS in that the potential allosteric regulatory site is blocked by
the side chains of Arg26 and Trp184 in PhzE due to differences in
the N terminus and an insertion near β-strand 9 (Fig. 6A and
supplemental Fig. S8). This insertion is also present in the
unregulated isochorismate synthases (35).

It is intriguing that we observed Zn2+ binding to the GATase1
active center (Fig. 6B) and that we could confirm inhibition of
PhzE by divalent cations including Zn2+, Mn2+, and Ni2+. The
origin of the bound Zn2+ is unclear, but it likely stems from the
culture media because the only other conceivable source would be
NiSO4 used in the first purification step. This chemical contains
many). E. coli strain KA12 for the overproduction of chorismic acid was a
Source. SAXS experiments were performed at beamline X33 of the EMBL
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in quorum sensing in the overall control of phenazine biosynthesis.

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