Crystal Structures of the HypCD Complex and the HypCDE Ternary Complex: Transient Intermediate Complexes during [NiFe] Hydrogenase Maturation

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SUMMARY

[NiFe] hydrogenase maturation represents one of the most dynamic and sophisticated processes in metallocenter assembly. The Fe(CN)2CO moiety of [NiFe] hydrogenases is assembled via unknown transient interactions among specific maturation proteins HypC (metallochaperone), HypD (redox protein), and HypE (cyanide synthesis/donor). Here, we report the structures of the HypC-HypD and HypC-HypD-HypE complexes, providing a view of the transient interactions that take place during the maturation process. HypC binds to the conserved region of HypD through extensive hydrophobic interactions. The ternary complex formation between HypE and the HypCD complex involves both HypC and HypD, rendering the HypE conformation favorable for cyanide transfer. In the complex, the conserved cysteines of HypC and HypD form an Fe binding site. The conserved C-terminal cysteine of HypE can access the thiol redox cascade of HypD. These results provide structural insights into the Fe atom cyanation in the HypCDE complex.

INTRODUCTION

Hydrogenases catalyze hydrogen production/consumption in microorganisms. The enzymes are classified into the [NiFe], [FeFe], and [Fe] hydrogenases based on the complex metal cofactor in their active site (Vignais and Billoud, 2007). The biosynthesis and maturation of their metal centers are complicated processes requiring the function and transient interaction of specific auxiliary proteins (Böck et al., 2006). Understanding these maturation mechanisms will not only provide valuable insight into the mechanisms of biologic metallocenter assembly, but also assist in future biotechnologic application of hydrogen-producing microorganisms and the development of biomimetic models of hydrogenases.

[NiFe] hydrogenases contain a Ni-Fe center in which the Fe atom carries two CN and one CO (Fontecilla-Camps et al., 2007; Volbeda and Fontecilla-Camps, 2003). The maturation of [NiFe] hydrogenases proceeds through a multistep pathway, in which the six Hyp proteins (HypA, -B, -C, -D, -E, and -F) function as the conserved core machinery in the [NiFe] hydrogenase maturation (Böck et al., 2006). Most of the current model for the [NiFe] hydrogenase maturation, based on the studies on hydrogenase 3 from Escherichia coli, is thought to be conserved in all organisms expressing the enzyme (Böck et al., 2006). First, the Fe(CN)2CO moiety is synthesized and inserted into the precursor of the large subunit (LS) of the [NiFe] hydrogenase by four Hyp proteins (HypCDEF) (Paschos et al., 2002). At present, the biologic source of CO is still unclear (Bürstel et al., 2011; Roseboom et al., 2005). After the insertion of the Fe ligand, a nickel chaperone HypA and a GTPase HypB perform the Ni insertion into the LS precursor (Kaluarachchi et al., 2010). Finally, proteolytic modification by a specific endopeptidase completes the maturation process (Fritsche et al., 1999). In addition, some organisms require other auxiliary proteins such as E. coli SlyD to obtain fully active [NiFe] hydrogenases (Zhang et al., 2005).

In the maturation process, HypC functions as a small metallochaperone that transfers the Fe ligand to the precursor LS and maintains a conformation of LS that is capable of accepting Ni (Drapal and Böck, 1998; Magalon and Böck, 2000a). HypD is a 4Fe-4S protein and is assumed to be a scaffold for the Fe cyanation (Blokesch and Böck, 2006). HypE together with HypF synthesizes the CN ligand using carbamoylphosphate and ATP. First, HypF carbamylates the C-terminal cysteine residue of HypE to produce HypE-thiocarboxamide. HypE catalyzes an ATP-dependent dehydration of the carbamoyl group to yield the HypE-thiocyanate (Reissmann et al., 2003).

Among the Hyp proteins, HypC, HypD, and HypE are involved in the cyanation of the Fe atom. This requires a tightly controlled mechanism to ensure the fidelity of the incorporation of the two CN ligands onto the Fe atom, because free cyanide is toxic to cells. It was demonstrated that a transient complex formation between HypC, HypD, and HypE occurs in vivo, but with unknown stoichiometry (Blokesch et al., 2004; Blokesch and Böck, 2002; Jones et al., 2004). An in vitro experiment under anaerobic conditions showed that a complex between HypC and HypD receives the cyano group from the HypE-thiocyanate (Blokesch et al., 2004). Previously, we reported the crystal structures of HypC, HypD and HypE from Thermococcus kodakarensis (Watanabe et al., 2007a). HypC and HypE proteins as well as other Hyp proteins from several species also have recently...
been reported (Gasper et al., 2006; Petkun et al., 2011; Rangarajan et al., 2008; Shimura et al., 2007; Wang et al., 2007; Watanabe et al., 2009; Xia et al., 2009). The structure of HypD reveals that HypD contains a putative ferrodoxin/thioredoxin reductase-like redox cascade (Dai et al., 2007), which consists of a [4Fe-4S] cluster and two pairs of disulfide bonds, suggesting that the cyanation reaction is catalyzed by the thiol redox cascade in the HypCDE complex (Watanabe et al., 2007a). However, it is unclear how HypC, HypD, and HypE interact with one another and form the transient ternary complex corresponding to an intermediate for the Fe atom cyanation.

To obtain fundamental understanding of the cyanation reaction in the maturation process, we have determined the crystal structures of transient intermediate complexes in the maturation process: the HypC-HypD binary complex (the HypCD complex) and the HypC-HypD-HypE ternary complex (the HypCDE ternary complex) from T. kodakarenensis. The present structures, in conjunction with biochemical and mutational analysis, reveal the detailed interactions among these proteins, and provide a structural basis for the cyanation reaction mechanism.

RESULTS

Transient Interactions between HypC, HypD, and HypE

To determine the stoichiometry and affinity among these proteins, size exclusion chromatography (SEC) and isothermal titration calorimetry (ITC) experiments were performed (Figure 1). At first, we investigated the binary complex formation of HypC, HypD, and HypE. SEC and ITC analyses showed that HypC and HypD form a complex at a molar ratio of 1:1 with a nanomolar affinity ($K_d$ value of 140 ± 20 nM) (Figures 1A and 1B), suggesting that the interaction between HypC and HypD is a strong transient interaction (Nooren and Thornton, 2003a, 2003b; Perkins et al., 2010). On the other hand, neither HypC nor HypD forms complexes with HypE individually (data not shown). We then examined the interaction between the HypCD complex and HypE. SEC showed that the HypCD complex weakly interacts with the HypE dimer (Figure 1C). The ITC experiments between the HypCD complex and HypE showed that two HypCD complexes interact with the HypE dimer with a $K_d$ value of 1.9 ± 0.2 μM (Figure 1D). The affinity in the micromolar range suggests that the HypC-HypD-HypE ternary complex is a weak transient complex that exists in a dynamic equilibrium between the complex and free states in solution (Nooren and Thornton, 2003a, 2003b; Perkins et al., 2010).

Overall Structure of the HypCD Complex

We have determined the crystal structure of the HypCD complex at 2.55 Å resolution by the molecular replacement (MR) method using the structures of HypD (PDB ID 2Z1D) and HypC (2Z1C) (Watanabe et al., 2007a) (Figure 2A; Table 1). The initial electron density map calculated using only the MR solution of the HypD structure clearly showed the existence of the β-barrel domain of HypC (Watanabe et al., 2007a) at the center of HypD and large conformational changes of the C-terminal long α helix of HypC. Therefore, the site of HypC was searched by using only the β-barrel domain of HypC, and the C-terminal α helix of HypC was manually fitted in the electron density.

The structure of HypD consists of two α/β domains (I and II), and an FeS cluster binding domain (FeSBD) carrying the [4Fe-4S] cluster (Watanabe et al., 2007a). In the structure of the HypCD complex, the β-barrel domain of HypC is bound to the central cleft between HypD α/β domains I and II (Figure 2B). The contact area (~1080 Å² of the accessible surface area (ASA)) corresponds to a highly conserved region of HypD (Figure S1 available online). The β4 and β5 strands of HypC fit into the cleft formed by the $x_1$, $x_6$, $x_7$, $x_9$, and $x_{12}$ helices of HypD (Figure 2B). On the other hand, the C-terminal α-helix of HypC, which is a highly variable region (Watanabe et al., 2007a), unexpectedly undergoes a large conformational change and does not interact with any parts of HypD. Compared to the isolated structures, the HypC C-terminal α-helix in the complex bends by about 130° at HypC Asp54 (Asp54>HypC) (Figure 2C). Instead of interacting with HypD, the HypC C-terminal α-helix interacts with that of the symmetric molecule in the crystal packing.

Compared to the isolated structure, the HypD α/β domain I in the complex rotates by ~4° relative to the HypD α/β domain II (Figure 2D). Furthermore, the N-terminal region of the $x_1$ helix of HypD bends sharply at HypD Ser21 (Ser21>HypD) toward HypC. As a consequence, these closing movements of the two domains of HypD make the central cleft deeper, allowing it to better recognize and trap the molecular shape of the β-barrel domain of HypC.

HypC-HypD Interface

The complex interface between HypC and HypD is formed mainly by hydrophobic interactions (Figures 3A and 3B). Leu32 hypC-Thr46 hypC, and Phe48 hypC interact with a hydrophobic patch in the $x_6$, $x_7$, and $x_{12}$ helices in the HypD α/β domain II (Figure 3A). Trp41 hypC, Ile43 hypC, Ile50 hypC, and Leu53 hypC make van der Waals contacts with hydrophobic residues in HypD α/β domain I (Figure 3B). The extensive hydrophobic interaction is consistent with the endothermic interactions observed between HypC and HypD (Figure 1B). These hydrophobic residues are well conserved among both proteins (Watanabe et al., 2007a). Mutation of these residues to alanine or polar residues impaired the binding of HypC to HypD in the pull-down assays (Figure S2). Therefore, these conserved hydrophobic residues play a major role in establishing the complex interface.

Hydrogen bonds also stabilize the complex interface between HypC and HypD. A hydrogen bond occurs between the Nε of His45 hypC and the Oε of Thr152 hypD (Figure 3A). The Nε of Asn264 hypD makes a hydrogen bond and van der Waals contact with the carbonyl oxygen of Leu32 hypC and Leu50 hypC, respectively (Figures 3A and 3C). The high conservation of these three residues suggests that they play important roles in the molecular recognition between the two proteins. Asn264 hypD also interacts with the HypD $x_9$ and $x_{12}$ helices through several hydrogen bonds (Figure 3C). Upon complex formation with HypC, Asn264 hypD moves toward HypC by ~0.4 Å, leading to substantial displacement of the HypD $x_9$ and $x_{12}$ helices. Therefore, Asn264 hypD appears to function as a key residue for the induced conformational changes of HypD required for the complex formation with HypC.

Structure Determination of the HypCDE Ternary Complex

The three purified proteins were mixed in equal molar amounts and were successfully crystallized in two different crystal forms.
Figure 1. SEC and ITC Analyses of Interactions between Hyp Proteins

(A) Elution profiles of HypD and the mixture of HypD with excess HypC. The SEC analysis was performed with a Superdex 75 10/300 GL column equilibrated with 20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 1 mM DTT.

(B) ITC raw data (upper panel) and binding isotherm data (lower panel) for titration of HypC to HypD at 20°C. After a 4 μL initial injection, 8 μL of 100 μM HypC was injected at 4-min intervals into the calorimetric cell containing 10 μM HypD with stirring at 310 rpm. Data were fit to a one-site binding model. The best fit parameters were n = 1.13, K_d = 0.14 ± 0.02 μM, ΔH = 13.8 ± 0.2 kcal/mol and TΔS = 23.0 kcal/mol.
Both crystal forms belong to the space group C2, but with different crystal packing. The crystal structures of the HypCD ternary complex have been determined at 2.25–2.75 Å resolution with MR using the sequential search of HypE (PDB ID: 2Z1E), HypD, and HypC (Table 1). The structure of HypE consists of N-terminal and C-terminal α/β domains (domains A and B). The HypE dimer is formed by interactions between the domains A from each monomer, which form a pseudo β-barrel (Watanabe et al., 2007a). The N-terminal region (residues 3–40) of HypE, which was missing in the previously determined structures (Watanabe et al., 2007a), is well ordered in the present structures. On the other hand, the C-terminal α-helix (residues 55–75) of HypC is disordered in the ternary complex structure. The overall structures in crystal forms I and II are nearly identical, but local conformational differences are observed (described below).

See also Figure S1.

(C) Elution profiles from HypE, a mixture of HypC and HypD, and a mixture of all three proteins. (D) ITC raw data (upper panel) and binding isotherm data (lower panel) for titration of HypE to the HypCD complex at 20 °C. After a 4 μl initial injection, 8 μl of 200 μM HypE was injected at 4-min intervals into the sample cell containing 20 μM HypCD complex with stirring at 307 rpm. Data were fit to a one-site binding model. The best fit parameters were n = 1.01, K_d = 1.9 ± 0.2 μM, ΔH = 5.64 ± 0.13 kcal/mol, and TΔS = 13.3 kcal/mol.
Overall Structure of the HypCDE Ternary Complex

The overall structure of the HypCDE ternary complex resembles a crab with big pincers (Figure 4A). The HypE dimer constitutes the body and two HypCD complexes attach to the individual sides of the HypE dimer, forming the pincers. The crystallographic asymmetric unit in both crystal forms contains one protomer of the HypCDE complex with a 1:1:1 stoichiometry, and the dimer of the HypCDE complex is located on a crystallographic 2-fold axis (Figure 4A).

The ternary complex interface (1248 Å² of ASA) involves both HypC and HypD (Figures 4A and 4B). HypD α/β domain I and FeSBD are associated with the α6 helix, β11, and β12 strands and C-terminal tail in the HypE C-terminal domain (domain B) (Figures 4B and S3). In addition, the HypC β2-β3 loop interacts with the HypE α6 helix and its adjacent loop between the α3 and α4 helices, acting as a “thumb” to trap HypE (Figures 4B and S3B). The position of the HypC β2-β3 loop is fixed by complex formation with HypD. These observations confirm that complex formation between HypC and HypD precedes formation of the HypCDE ternary complex.

HypC-HypE Interface

In the HypC-HypE contact interface, Val24HypC in the HypC β2-β3 loop is stuck in a hydrophobic pocket formed by HypE Ile187 (Ile187HypE), Phe189HypE, Ala258HypE, and Met261HypE (Figure 5A). Mutation of Val24HypC to aspartate did not affect the complex formation with HypD, but reduced the binding of HypE to the HypCD complex in pull-down assays (Figures S2 and S4). These hydrophobic residues are well conserved in HypC and HypE proteins, respectively (Watanabe et al., 2007a). In addition, several hydrogen bonds and van der Waals contacts are formed between the amides and carbonyl oxygen atoms surrounding Val24HypC. These interactions indicate that the HypC-binding site of HypE is sophisticatedly constructed to recognize the HypC β2-β3 loop.

Interaction between HypE and HypD through a Hydrophobic Anchor

The contact interface of HypD with HypE is a flat surface structure formed by the HypD α1, α2, α3, α11, and α14 helices and loops between them (Figure S3A). The HypE β11 and β12 strands lie...
along the flat interface (Figure 5B). A HypD four-helix bundle (the $\alpha_1$, $\alpha_2$, $\alpha_3$, and $\alpha_{11}$ helices) traps the HypE $b_{11}$-$b_{12}$ loop, where Ile320HypE is stuck in a hydrophobic pocket formed by hydrophobic residues of HypD (Figure 5C). Mutation of Ile320HypE to alanine/glutamate severely impaired the ternary complex formation in the pull-down assays (Figure S4). Other HypE proteins also have hydrophobic residues (Ile, Phe, Tyr, Val, and Ala) at position 320 (Watanabe et al., 2007a). Furthermore, comparison of the structures in the two different crystal forms reveals that the HypCD complex in crystal form II pivots by $\sim3^\circ$ on Ile320$_{\text{HypE}}$ compared to that in form I (Figure S5). These results indicate that the hydrophobic residue at position 320 plays a critical role in the ternary complex formation and that the HypCD complex binds loosely to HypE, using Ile320$_{\text{HypE}}$ as a hydrophobic anchor. The HypE-HypD interface is also stabilized by several hydrogen bonds and van der Waals contacts (Figure 5B).
of R324_HypE to glutamate also prevented complex formation (Figure S4). A salt bridge is formed between Arg47_HypD and Glu260_HypE, which are also conserved (Figure 5C). Mutation of Glu260_HypE to arginine did not have a significant effect on the complex formation (Figure S4), suggesting that the salt bridge plays an ancillary role in the ternary complex formation.

Conformational Changes of HypE upon the Ternary Complex Formation

The ternary complex formation induces domain movements of HypE (Figure 6A). Compared to the isolated structure, HypE domain A is rotated by ~4° (form I), or by ~8° (form II) with respect to domain B, resulting in the opening movements of the two domains of HypE. The opening movement of HypE causes the displacement of the HypE C terminus (Figure 6B). Electron density maps showed that the C-terminal tail of HypE in crystal form I adopts both the outward and inward conformations (Figure 6C). When HypE was incubated with ATP prior to the ternary complex preparation, the C-terminal tail mainly assumed the inward conformation (Watanabe et al., 2007a), suggesting that the salt bridge plays an ancillary role in the ternary complex formation.

Electron density maps showed that the C-terminal tail of HypE in crystal form I adopts both the outward and inward conformations (Figure 6C). When HypE was incubated with ATP prior to the ternary complex preparation, the C-terminal tail mainly assumed the inward conformation (Watanabe et al., 2007a), suggesting that the salt bridge plays an ancillary role in the ternary complex formation.

The Conserved Motifs of HypC, HypD, and HypE Form the Fe Binding and Cyanation Sites in the Ternary Complex

In the HypCDE ternary complex, the conserved motifs of HypC and HypE are located in close proximity to the conserved motifs of HypD (Figures 7A–7C). The four conserved motifs of HypD (CGXH, GPGCPVC, GFETT, and PXHVS motifs) are assembled at the central cleft of HypD (Watanabe et al., 2007a). In particular, three cysteine residues (Cys38_HypD, Cys66_HypD, and Cys69_HypD) are essential for the [NiFe] hydrogenase maturation (Blokesch and Böck, 2006). On the other hand, the conserved N-terminal cysteine residue (Cys2) of HypC (Cys2_HypC) is essential for maturation, and is assumed to transfer the Fe ligand to the precursor of the [NiFe] hydrogenase LS (Drapal and Böck, 1998; Magalon and Böck, 2000a, 2000b). In the complex structure, Cys2_HypC is located close to Cys38_HypD in the HypD CGXH motif, although the N-terminal residues of HypC have relatively high B-factors (Figures 7A and B). The position of Cys2_HypC in the HypCD complex strongly suggests that Cys2_HypC and Cys38_HypD form the binding site for Fe, which is eventually inserted into the active site of [NiFe] hydrogenases (Blokesch and Böck, 2006; Watanabe et al., 2007a).

Attempts to obtain the Fe-bound structure of the HypCDE complex by soaking or co-crystallization have not been successful. Instead, we performed ITC analyses to investigate whether the HypCD complex can bind Fe ions. HypC or HypD alone did not show Fe (II) binding heats (data not shown). Fe (II) binding heats were only observed for the HypCD complex (Figure S6A). Furthermore, mutation of Cys38_HypD to Ala disrupted the Fe binding by the HypCD complex (Figure S6B). These results confirm that Cys38_HypD together with Cys2_HypC is involved in the Fe binding in the HypCD complex. Depending on the sample concentration, dissociation and stoichiometry values for Fe binding determined by ITC varied from ~0.5 to ~0.3, respectively. This was because small precipitates occurred after the ITC experiments and the recollected sample after the experiments gradually aggregated. These observations suggest that the HypCD complex was destabilized in the
presence of Fe ions under these conditions. Therefore these values from ITC can be considered as minimum estimates.

In the HypCDE ternary complex, the HypE C-terminal residues in the outward conformation are located near Tyr125HypD and Tyr358HypD (Figure 7A). It was difficult to build the HypE C terminus (residues 336–338) because the electron density around the HypE C terminus was not continuous (Figure 7C). However, the positive peaks in the maps indicate the existence of the HypE C terminus near the HypD GPGCPCV motif (Figure 7C, green stick model). These results suggest that the HypE C-terminal cysteine residue (Cys338HypE), which is eventually converted to thiocyanate, can gain access to Cys66HypD in the GPGCPCV motif.

**DISCUSSION**

In this study, we have determined the crystal structures of the HypCD complex and the HypCDE ternary complex, revealing the structural details of transient intermediate complexes in the maturation process. The \( \beta \)-barrel domain of HypC tightly interacts with the conserved central cleft of HypD, with unexpected conformational changes of the C-terminal \( \alpha \)-helix (Figure 2A). The nanomolar affinity of HypC for HypD (\( K_d = 140 \, \text{nM} \)) and the large conformational change of HypC suggest that the HypC-HypD complex is a strong transient complex that requires a trigger for dissociation (Nooren and Thornton, 2000b, 2003a; Perkins et al., 2010). On the other hand, HypC...
has been shown to more weakly interact with the [NiFe] hydrogenase LS HyhL than HypD (Sasaki et al., 2012). These observations indicate that HypC prefers binding to HypD rather than HyhL in the initial step of the maturation process. Completion of the biosynthesis of the Fe(CN)2CO moiety in the HypCD complex may be a molecular trigger that promotes the dissociation of HypC from HypD, followed by transfer of the Fe ligand from HypC to HyhL.

The ternary complex structures reveal that the HypCD complex interacts weakly with HypE through specific binding sites for HypC and HypD, respectively (Figure 4B). The conformational changes of HypE induced by the ternary complex formation (Figure 6) indicate that the transient interaction between HypE and the HypCD complex renders the conformation of the HypE C terminus favorable for the cyanide transfer (Figure 8). First, the HypE C terminus assumes the outward conformation and receives carboxamide from carboxamidophosphate with the assistance of HypF. Second, the conformational changes of HypE accompanying ATP binding lead to formation of the inward conformation (Watanabe et al., 2007a), allowing HypE to dehydrate the thiocarboxamide group to the thiocyanate group. Next, the ternary complex formation with the HypCD complex causes the opening movements of HypE, resulting in disruption of the interactions stabilizing the inward conformation. Consequently, the HypE C-terminal tail again assumes the outward conformation and is able to transfer the cyano group of HypE-thiocyanate to the HypCD complex.

The HypCD complex has been shown to bind an Fe(II) ion with micromolar affinity. A similar metal affinity is observed in the iron chaperon frataxin (Cook et al., 2006; Yoon and Cowan, 2003) and the copper chaperons (Abajian et al., 2004; Wernimont et al., 2004), suggesting that the observed affinity of the HypCD complex for Fe (II) is reasonable for the metal delivery. However, higher affinity for Fe (II) will be required to retain the Fe ion during the cyanation reaction. The present structures with the mutant analysis show that Cys38HypD and Cys2HypC are involved in Fe binding (Figures 7 and S6). On the other hand, the high B-factors of the N-terminus of HypC and the low Fe affinity imply that the Fe binding site in the HypCD complex is incomplete. In fact, the typical coordination number of Fe is more than 4 (Holm et al., 1996). Another conserved residue (Glu359HypD) near Cys38HypD might be involved in the Fe binding, but the Fe coordination by Glu359HypD requires main chain conformational changes. Therefore, a cofactor seems to be required to provide additional O/N ligands for coordination of the Fe ion (Figure 9). The structural similarity of the HypD α/β domains to the sugar-binding proteins (Watanabe et al., 2007a) raises the possibility that sugar or carbonate is involved in the Fe binding in the HypCD complex.

The proximity of the conserved motifs of HypC, HypD and HypE in the HypCDE ternary complex (Figures 6A–6C) indicates that the conserved motifs of HypD form the scaffold for the cyanation of the Fe atom (Figure 9). In the HypCDE ternary complex, the C-terminal residues of HypE can be located close to Cys66HypD. The relative positions of these conserved motifs of HypD and HypE in the ternary complex strongly support the hypothesis that the free thiol of Cys66HypD attacks the Cys338HypE-thiocyanate and the cyano group is transferred from HypE to the Fe atom coordinated by Cys2HypC and Cys38HypD (Figure 9, step 1). The HypD GPGPCV motif leads
to the thiol redox cascade composed of two pairs of cysteine residues (Cys66\textsubscript{HypD} and Cys69\textsubscript{HypD}, Cys325\textsubscript{HypD} and Cys354\textsubscript{HypD}) and the \[4Fe4S\] cluster (Watanabe et al., 2007a). Therefore, the resulting heterodisulfide bond between Cys66\textsubscript{HypD} and Cys338\textsubscript{HypE} can be continuously reduced by the thiol redox cascade (Figure 9, steps 2–5), allowing Cys66\textsubscript{HypD} and Cys69\textsubscript{HypD} to catalyze the transfer of the second CN ligand (Figure 8, step 6).

In the ternary complex, the path from the complex surface to the HypD GPGCPVC motif is a short narrow channel (Figures 7A and 7C). The N-terminal region of HypC (yellow) also constitutes a part of the channel. Therefore, the disorder of the HypE C-terminal residues probably reflects the relatively high B-factors of the N-terminal region of HypC. These observations suggest that the flexibility of the N-terminal region of HypC occludes the insertion of the HypE C terminus into the HypD GPGCPVC motif. The Fe-bound state of the HypCD complex with the HypC N-terminus fixed would allow the HypE C terminus to enter into the active site of HypD.

**EXPERIMENTAL PROCEDURES**

**Crystallization**

The overexpression and purification of HypC, HypD and HypE from *T. kodakarenensis* were performed as described previously (Arai et al., 2007; Watanabe et al., 2007a, 2007b). For HypD, incubation of cells at 18°C–20°C for 24 hr after addition of isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) allowed the high-level production of soluble proteins. The sample of the HypCD complex for crystallization was prepared by mixing the two proteins at an equal molar ratio and incubating overnight at 4°C. For data collection, cryoprotectant solution

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**Figure 7. Scaffold for the Cyanation in the HypCDE Ternary Complex**

(A) Stereo view of the scaffold for cyanation in the HypCDE complex in a stick representation. The four conserved motifs of HypD are shown in pink (the CGXH motif), magenta (the GPGCPVC motif), orange (the GFETT motif), and blue (PXHVS motif). Cys66\textsubscript{HypD} and Cys69\textsubscript{HypD} in the GPGCPVC motif are partially reduced. The N-terminal region of HypC (yellow) and the C-terminal region of HypE (cyan) are also shown.

(B) A close-up view of the HypC N-terminus. The electron density of the simulated annealing omit map around the N-terminal residues of HypC is shown at 2.5σ.

(C) Stereo view of a close-up of the HypE C terminus. The electron densities are shown for a 2Fo-Fc map at 1σ in gray and for a Fo-Fc map at 3.5σ in red. One of the possible models for the HypE C terminus (residues 336–338) is shown as a green stick model.

See also Figure S6.
(1.6 M (NH₄)₂ citrate/citric acid pH 4.5–4.7, 0.7% MPD, and 25% glycerol) was added in the drops several times and the crystals were flash-cooled in a nitrogen stream.

The crystallization sample of the HypC-HypD-HypE complex was prepared by mixing purified HypC, HypD, and HypE at a ratio of 1:1:1 at a concentration of 0.5–0.6 mM in 20 mM Tris-HCl pH 7.5, 150 mM NaCl and 1 mM TCEP, and incubating overnight at 4°C. The crystals of the HypCDE complex were obtained in two different crystal forms by the sitting drop vapor diffusion method at 20°C. Crystal form I was grown in 2–4 weeks by mixing 0.7 ml of protein solution with 0.7 ml of reservoir solution (50 mM MES pH 6.4, 12%–16% PEG400 and 10 mM MgCl₂). Prior to data collection, cryoprotectant solution (50 mM MES pH 6.4, 14%–18% PEG400, 10 mM MgCl₂, and 20% ethylene glycol) was added into the drops several times. Crystal form II was grown in a week using reservoir solution containing 0.1 M Tris-HCl pH 8.7, 12%–16% (w/v) PEG8000, 2% ethylene glycol. Drops of 1.4 ml were made by mixing protein solution, reservoir solution, and additive solution (0.1 M spermidine) at a ratio of 1:0.8:0.2, and equilibrated against 100 μl of reservoir solution. Cryoprotectant solution (0.1 M Tris-HCl pH 8.7, 20% w/v PEG8000 and 15% ethylene glycol) was added to the drops in a stepwise manner.

**Structure Determination and Refinement**

The X-ray diffraction data were collected on the BL41XU beamline at SPring-8 and the BL17A and AR-NW12 beamlines at the Photon factory, and were processed with the HKL suites (Otwinowski and Minor, 1997). The present structures were determined by the molecular replacement method using the program MOLREP (Vagin and Teplyakov, 1997). The previously determined monomer structures of HypC (PDB ID: 2Z1C), HypD (2Z1D), and HypE (2Z1E) were used as search models. For the HypCD complex, the site of HypD was first searched and then the final site of HypC was searched by using only the β-barrel domain of HypC. For the HypCDE complex, the site of HypE was first searched, followed by HypD and HypC. Although the search of HypC did not produce a clear solution, the electron density map calculated using only the HypD and HypE structures clearly showed that the β-barrel domain of HypC is located at the same position as that of the HypCD complex. Therefore, the refined HypCD complex structure without the HypC C-terminal α-helix was finally used as a search model. The initial electron density maps were of good quality. Manual model rebuilding was performed with COOT (Emsley and Cowtan, 2004) and validated with Coot validation tools and MolProbity (Chen et al., 2010). The final model of the HypCD complex includes one HypC (residues 4–75) and one HypD (3–372) with 97.7% of residues in the favored region of the Ramachandran plot and 2.3% of residues in the disallowed region. HypCDE complex I (inward) includes one HypC (3–54), one HypD (4–371) and one
HypE (3–338) with 95.6% of residues in the favored region, 4.3% in the allowed region, and 0.1% in the disallowed region. The HypCDE form II includes one HypC (3–56), one HypD (5–371), and one HypE (20–336) with 94.8% of residues in the favored region and 5.2% in the allowed region. Structural figures were prepared with PyMOL (DeLano, 2008). Structural superposition was performed with CCP4/LSQKAB (CCP4, 1994).

Construction, Overexpression, and Purification of HypDStrep
A Strep-tag II sequence was fused at the C terminus of HypD by an inverse PCR method using the primers in Table S1 with the pET21a(+)hypD(Watanabe et al., 2007b) as a template. After introduction of the plasmid into E. coli strain Rosetta 2 (DE3) plysS cells (Merck/Novagen), gene expression was induced with 0.1 mM IPTG at the mid-exponential growth phase with further incubation for 24 hr at 18°C. After sonication and heat treatment (80°C, 10 min), the supernatant was applied to a Strep-Tactin superflow column (IBA). After washing with five column volumes (CV) of wash buffer (0.1 M Tris-HCl pH 8.0, 150 mM NaCl, 1 mM dithiothreitol [DTT]), the proteins were eluted with elution buffer (wash buffer containing 2.5 mM desthiobiotin). The fractions containing HypDStrep were concentrated by Amicon ultrafiltration with buffer exchange, and were applied to a gel filtration column (Superdex200 10/300 GL [GE Healthcare]) equilibrated with buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT).

Pull-Down Assay
Mutants of HypC and HypE were constructed by PCR-based site-directed mutagenesis using the primers listed in Table S1, and were purified by heat treatment, anion exchange and size exclusion chromatography. The assay solution (60 μl) was made by mixing 1 nmol of HypDStrep and 4 nmol of each mutant of HypC (for the HypCD complex), or 1 nmol of HypDStrep, 2 nmol of HypC, and 1 nmol of each HypE mutant (for the ternary complex), in 20 μl Tris-HCl pH 8.0, 150 mM NaCl, 1 mM dithiothreitol (DTT), the proteins were eluted with elution buffer (wash buffer containing 2.5 mM desthiobiotin). The fractions containing HypDStrep were concentrated by Amicon ultrafiltration with buffer exchange, and were applied to a gel filtration column (Superdex200 10/300 GL [GE Healthcare]) equilibrated with buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT).

Isothermal Titration Calorimetry Measurements
Isothermal titration calorimetry (ITC) experiments were carried out with a VP-ITC Microcalorimeter (MicroCal, Northampton, MA). All protein samples were purified with a Superdex 75 10/300 GL column equilibrated with 20 mM HEPES pH 7.0, 150 mM NaCl, and 1 mM TCEP. The C38A HypD mutant could be purified and formed a stable complex with HypC, much like wild-type HypD. For the Fe (II) binding assay, all solutions were prepared under anaerobic conditions. Fe(NH4)2(SO4)26H2O was dissolved in the same buffer. To prevent oxidation of ferrous ions, 8 mM Na2S2O4 was added to both the protein and Fe solutions. The samples were thoroughly degassed and quickly loaded into the cell or syringe. The data acquisition and analysis were performed using Microcal Origin Software (version 7.0). The heat of dilution was measured by the average heat of injections after saturation and was subtracted before curve fitting. All experiments were repeated at least twice.

ACCESSION NUMBERS
The Protein Data Bank accession numbers for the coordinates and structure factors are 3VYR (the HypCD complex), 3VYS (the HypCDE complex in crystal form I), 3VYT (the HypCDE complex in crystal form I, inward form) and 3VYU (the HypCDE complex in crystal form II).

SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.str.2012.09.018.

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