HypF, a Carbamoyl Phosphate-converting Enzyme Involved in [NiFe] Hydrogenase Maturation*

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HypF has been characterized as an auxiliary protein whose function is required for the synthesis of active [NiFe] hydrogenases in Escherichia coli and other bacteria. To approach the functional analysis, in particular the involvement in CO/CN ligand synthesis, HypF was purified from an overproducing strain to apparent homogeneity. The purified protein behaves as a monomer on size exclusion chromatography, and it is devoid of nickel or other cofactors. As indicated by the existence of a sequence motif also present in several O-carbamoyltransferases, HypF interacts with carbamoyl phosphate as a substrate and releases inorganic phosphate. In addition, HypF also possesses ATP cleavage activity that gives rise to AMP and pyrophosphate as products and that is dependent on the presence of carbamoyl phosphate. This and the fact that HypF catalyzes a carbamoyl phosphate-dependent pyrophosphate ATP exchange reaction suggest that the protein catalyzes activation of carbamoyl phosphate. Extensive mutagenesis of the putative functional motifs deduced from the derived amino acid sequence showed a full correlation of the resulting variants between their activity in hydrogenase maturation and the in vitro reactivity with carbamoyl phosphate. The results are discussed in terms of the involvement of HypF in the conversion of carbamoyl phosphate to the CN ligand.

Hydrogen metabolism in enterobacteria involves the activity of the products of three functional classes of genes that code for structural proteins, regulatory proteins, or for proteins involved in metal center biosynthesis and enzyme maturation. In Escherichia coli, the structural genes are organized in four operons, responsible for the formation of hydrogenase 1 (hyd operon), hydrogenase 2 (hyb operon), hydrogenase 3 (hyc operon), and the putative hydrogenase 4 (hyf operon). Each operon contains the genes for the large and small hydrogenase subunit, for redox carriers, membrane anchor proteins plus components required for the maturation of the hydrogenase encoded by that specific operon, like the endopeptidase involved in proteolytic processing of the large subunit (for review see Refs. 1 and 2). Because the hydrogenases in E. coli serve different physiological functions, the expression of these operons is differentially regulated. Hydrogenase 3, for example, which is a component of the formate hydrogen lyase system, is synthesized under fermentative conditions. Its formation requires the activity of the transcriptional activator FhIA and formate as inducer (3).

Considerable efforts have been directed to understand the incorporation of the [NiFe] metal center into the large hydrogenase subunit. A scenario is emerging indicating that iron and nickel insertion proceeds separately, whereby the incorporation of iron together with its CO and CN ligands precedes that of nickel (1, 4, 5). The function of the HypA and HypB proteins has been related to nickel insertion because hypA and hypB mutations can be phenotypically complemented by inclusion of high nickel concentrations in the medium (6, 7) and because the HypB protein binds nickel (8). HypC, which is a small acidic protein, forms a complex with the precursor of the large hydrogenase subunit and, accordingly, has been postulated to play the function of a specific chaperone, maintaining a folding state to render the large subunit amenable for metal acquisition (9, 10). The most detailed information for all maturation proteins is available for the endopeptidase which cleaves the C-terminal extension from the precursor of the large subunit once nickel has been inserted (5, 11, 12). It is thought that the endopeptidase controls the fidelity of nickel insertion (13) and also, by cleavage, induces the conformational shift required to thread the finished metal center into the interior of the large subunit (1, 5, 14).

An intriguing issue of [NiFe] center synthesis concerns the source and the pathway of biosynthesis of the CO and CN ligands. Circumstantial evidence indicates that the HypD, HypF, and HypE proteins are involved in this process. HypD is an Fe/S protein with unusual spectroscopy properties,2 and HypF and HypE display sequence signatures that implicate functions in organic synthesis. HypF, for example, has been reported to carry a sequence motif characteristic of acyl phosphatases (15), and HypE shares motifs that are also found in aminomimidazole ribonucleotide synthetase and thiamin-phosphate kinase (16, 17).

An important discovery in this connection was the finding that maturation of [NiFe] hydrogenases requires the availability of carbamoyl phosphate (CP).2 A mutant of E. coli lacking CP synthetase (carAB genes) activity was unable to synthesize active hydrogenases 1–3. The deficiency was shown to be caused by a blockade of the maturation process (18). Citrulline in the medium was able to complement the lesion of the carAB mutant indicating that the carbamoyl moiety was crucial. It was postulated that CP is the precursor of either CO or CN or

1 N. Drapal, S. P. P. Albracht, and A. Böck, unpublished results.
2 The abbreviations used are: CP, carbamoyl phosphate; DTT, dithiothreitol.
of both of them (18). In the present communication we show that the HypF protein recognizes CP as a substrate. A detailed mutational analysis of conserved sequence traits was performed, and it is shown that abolition of HypF function in the maturation process is fully correlated with its ability to interact with CP as a substrate. It is further shown that HypF cleaves ATP into AMP and pyrophosphate in the presence of CP.

EXPERIMENTAL PROCEDURES

E. coli Strains, Plasmids, and Growth Conditions—Strain MC4100 (19) from E. coli was used as wild type, and strains JM109 (20) and DH5α (21) served as hosts for transformations. DHP-F (22) and DHP-F2 are derivatives of MC4100 with different deletions in the hypF gene; DHP-F lacks the segment of the gene reaching from amino acids 59 to 629. DHP-F thus forms a shortened version of HypF that is detectable by anti-HypF antibodies in immunoblotting experiments of crude extracts, whereas no immunologically reacting material can be found in extracts from DHP-F2.

The plasmids employed in this work are listed in Table I, together with their genotype and source or derivation. Maintenance of plasmids from DHP-F2.

Purification of the HypF Protein

Aerobic cultures were grown in Erlenmeyer flasks under rigorous rotatory in LB medium (23) or anaerobically in TGYEP medium (24). Cultures of strain JM109 transformed with plasmid pUCF18 were grown in 300 ml of LB medium in 2-liter Erlenmeyer flasks. After reaching an A600 of 1.0, expression of hypF was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to 1 mM final concentration. The cells were harvested after 3 h by centrifugation, resuspended in 10 mM Tris/Cl, pH 7.4, and sedimented again. The washed cells were suspended in 10 mM Tris/Cl, pH 7.4, 1 mM DTT (1% of the cultivation volume), and the suspension was brought to 20 μg/ml phenylmethylsulfonyl fluoride and DNase I each.

The cells were broken by passage through a French press cell at 118 MPa, and the homogenate was clarified first by centrifugation at 10,000 × g for 30 min (S10 supernatant) and subsequently for 2 h at 100,000 × g (S100). The S100 fraction was adjusted to a protein concentration of 15 mg/ml and brought to an ammonium sulfate saturation of 35% by the addition of solid (NH4)2SO4, followed by stirring at 0 °C for 30 min. The precipitate formed was collected by centrifugation (30 min at 15,000 × g), dissolved in a minimum of buffer (10 mM Tris/Cl, pH 7.4, 1 mM DTT), and dialyzed against the same buffer.

The dialysate was fractionated by anion exchange chromatography on a Mono-Q HR 5/5 (Amersham Biosciences) column (1 ml) equilibrated with 10 mM Tris/Cl, pH 7.4, 1 mM DTT. Elution was with a gradient from 0 to 1 M sodium chloride at a flow rate of 60 ml/h. Five fractions (1.5 ml) were monitored for presence of HypF by SDS-PAGE (27) and staining with Coomassie Blue. HypF-containing fractions were combined and subjected to ammonium sulfate precipitation at 50% saturation, and the precipitate formed was collected by centrifugation. The sediment was dissolved in a small volume of Tris/Cl (10 mM, pH 7.4), 1 mM DTT containing ammonium sulfate at 20% saturation and applied to a 1-ml phenyl-Superose HR 5/5 column (Amersham Biosciences) that had been equilibrated with the same buffer. Bound proteins were eluted with a decreasing linear gradient from 20 to 0% ammonium sulfate at a flow rate of 30 ml per min. The elution of the HypF protein was monitored by SDS-PAGE. Fractions containing HypF were combined, and the protein was concentrated by ultrafiltration with the aid of the MICROSEP™-Microconcentration system (Pall GmbH, Laboratory, Dreieich, Germany).

The final purification step consisted of a gel filtration over a 300-ml Superdex XK 26/60 (Amersham Biosciences) column. It was developed with 25 mM Tris/Cl, pH 7.4, 100 mM sodium chloride, 1 mM DTT at a flow rate of 0.5 ml per min. 1-ml fractions were collected and assayed for HypF content by SDS-PAGE. Fractions containing HypF of apparent homogeneity were dialyzed against 25 mM Tris/Cl, pH 7.4, 1 mM DTT, 100 mM NaCl, 50% glycerol (v/v) and stored at −20 °C.

Electrophoretic Techniques—SDS-PAGE was performed according to Laemmli (27) and non-denaturing PAGE as described by Drapal and Böck (9). Immunological detection of HypF in polyacrylamide gels after electrophoretic transfer onto nitrocellulose membranes was achieved with anti-HypF antibodies in a 1:1000 dilution and the use of the Lumi-Light Western blotting Substrate (Roche Diagnostics). Antibodies directed against HypF were generated by Eurogentec (Seraing, Belgium).

Determination of Carbamoyl-phosphate Phosphatase Activity—CP phosphatase activity was followed via the liberation of inorganic phos-
Hydrogenase Maturation Protein HypF

Fig. 1. Schematic representation of the HyfP sequence indicating the position of the acylphosphatase (AP), the two zinc fingers (ZF1 and ZF2), and the carbamoyltransferase (O-CT) motifs. The residues replaced by targeted mutagenesis are denoted. On the top of the figure the sequence motifs shared between HypF and acylphosphatases and O-carbamoyltransferases are shown. Sequences were taken from the NCBI data base (www.ncbi.nlm.nih.gov) for Streptomyces (S.) lavendulae, S. spheroides, Bradyrhizobium sp. WM9, and Mesorhizobium (M.) loti.

Acylphosphate phosphatases

| C. acetoxyfusis | V. cholerae |
|----------------|-------------|
| AP | VhGKVQVYSPF |
| O-CT | VhGKVQVYSPF |

Sequence Characteristics of the HypF Protein—A schematic representation of sequence motifs strongly conserved in HypF homologs from bacteria and archaea is given in Fig. 1. The most intriguing characteristic is the existence of two perfect apparent “zinc finger” motifs from amino acid positions 109–184 (30). They have been implicated in the binding of some bivalent cation, but experimental studies on such a function are lacking thus far. On the N-terminal side of these motifs a putative acylphosphatase motif ranging from position 13–23 was discovered (15), but biochemical results demonstrating such an activity and its role in hydrogenase maturation are not available yet. Finally, in the C-terminal one-third of the protein there is a conserved sequence segment containing three histidine residues in a characteristic pattern. A data base search delivered best hits for enzymes with an O-carbamoylation activity in the synthesis of antibiotics or nodulation factors (see Fig. 1, top). Proteins possessing a similar motif, however, are also present in organisms like Pyrococcus horikoshii (31) or Sulfolobus tokodaii (32), which are not known to synthesize antibiotics or nodulation factors. The existence of this motif had prompted us to investigate whether CP is required for maturation of hydrogenases. Indeed, a mutant of E. coli devoid of CP synthetase activity was unable to develop active hydrogenases (18).

Purification and Properties of the HypF Protein from E. coli—The hypF gene on plasmid pUC18 was overexpressed in strain JM109, and purified HypF was isolated from crude extracts by ammonium sulfate precipitation, Mono-Q HR anion exchange chromatography, hydrophobic interaction chromatography on a phenyl-Superose HR 5/5 residue, and gel filtration over a Superose XK 26/60 column, as detailed under “Experimental Procedures.” The path of purification is displayed in Fig. 2A which presents a Coomassie-stained SDS-polyacrylamide gel in which the pooled fractions of each purification step were separated. Characteristically, from 142 mg of protein of a crude 30,000 × g supernatant 9 mg of apparently purified HypF protein were obtained.

The UV-visible absorption spectrum of the purified protein, taken between 200 and 500 nm, did not indicate the presence of any cofactor absorbing in this range (data not shown). Size exclusion chromatography on a calibrated Superdex 200 column showed that HypF eluted at a position characteristic of a
molecular mass of about 80 kDa that corresponds with the size 
retrieved from the migration in SDS gels and that delineated 
from the gene sequence, namely 81.9 kDa. Therefore, HypF as 
purified appears to be a monomeric protein. Analysis of purified 
HypF by atomic absorption spectroscopy and inductively coupled 
plasma atomic emission spectroscopy revealed that the protein 
(dialyzed against buffer lacking chelators) does not contain 
nickel, cobalt, copper, manganese, or molybdenum. However, the 
protein contained 1 iron atom per 7.8 HypF and 1 zinc atom per 
2.5 HypF molecules. The substoichiometric ratio of the two 
metals may indicate that they are bound non-specifically. However, 
this needs further experimental analysis.

HypF Displays Carbamoyl-phosphate Phosphatase Activity—

HypF shares a sequence signature motif with O-carbamoyl-

transferases, and it was tested to determine whether the pro-
tein purified can interact with CP. The liberation of inorganic 
phosphate was taken as a measure. Table II shows that HypF 
cleaves carbamoyl phosphate rather specifically; other 
acylphosphates or phosphoesters are only marginally hydro-
lyzed. The kinetics of CP cleavage by HypF were determined by 
assaying the hydrolysis rate at different substrate concentra-
tions (Fig. 3). The liberation of phosphate followed Michaelis-

Menten-type saturation kinetics. In several experiments 
Kₘ values ranging from 260 to 330 μM were obtained.

Next it was tested whether the CP phosphatase activity 
exhibited by purified HypF was also present in a freshly pre-
pared crude extract. Cells of a mutant of E. coli (DHF-F) 
carrying a deletion in the chromosomal hypF gene were trans-
formed either with plasmid pAF1 carrying hypF or the vector. Extracts were prepared, and samples from S30 extracts 
were loaded on non-denaturing polyacrylamide gels, and the 
gels were analyzed for CP phosphatase activity (Fig. 2B).

Enzyme activity developed specifically in the extract of the trans-
formant carrying hypF on a plasmid, and it migrated in a 
position where the major band of the purified HypF was 
detected. Therefore, CP hydrolysis in the crude extract is cata-
lized by the hypF gene product, and the purified HypF protein 
exhibits the same activity.

Carbamoyl Phosphate-dependent Liberation of AMP from 

ATP—The primary structure of HypF also contains a sequence 
motif (GXXGXXGALA) that resembles the “glycine-rich loop” motif 
of a family of ATP-binding proteins (GXXGXXG(R/K)) (33). Its 
presence prompted the investigation whether HypF possesses 
ATP cleavage activity. [α-³²P]ATP was therefore incubated with 
HypF protein, and the samples were applied to polyeth-

yleneimine thin layer plates and separated with a solvent of 0.5 
M KH₂PO₄ (pH 3.4). Hydrolysis of ATP with the liberation of 
AMP could be observed but only when CP was included in the 
reaction mixture. In its absence, no significant ATP hydrolysis 
took place (data not shown).

To characterize the CP-dependent ATP hydrolysis activity of 
the HypF protein, initial reaction velocities were followed at 
different ATP concentrations keeping CP constant at 100 μM 
and also at varying CP concentrations in the presence of 100 μM 
ATP. Fig. 4 shows that the reaction follows Michaelis-Menten 
kinetics with both substrates. The kinetic constants of HypF in 
the CP phosphatase and the ATP hydrolysis reaction are sum-
marized in Table III.

The apparent affinity of HypF for CP in the phosphatase 
reaction is far below that measured for it as substrate in the 
ATP hydrolysis reaction. By taking into account the rates of the 
two reactions, ATP cleavage in the presence of CP is kinetically 
favored.

The CP dependence of the ATP cleavage reaction indicates 
that CP may form a covalent intermediate either with AMP or 
with pyrophosphate. If this is the case, it should be visualized 
by the CP dependence of an exchange of radioactive pyrophos-
phate with ATP (34, 35) catalyzed by the HypF protein. For an

FIG. 2. Purification of HypF protein from an overexpressing strain as followed by SDS-PAGE (10%) of the pooled fractions of each 
step. A: lanes 1 and 8, molecular mass standards (β-galactosidase, bovine serum albumin, ovalbumin, lactate dehydrogenase, and restriction 
endonuclease Bsp981I); lane 2, S10; lane 3, S100; lane 4, 0–35% ammonium sulfate fraction; lane 5, MonoQ pool; lane 6, phenyl-Sepharose pool; lane 
7, Superdex 200 pool. B shows a non-denaturing gel in which purified HypF (lanes 5–7) and a crude extract of a ΔhypF strain overexpressing hypF 
from a plasmid (ΔhypF/pHypF (lanes 3 and 4) were separated. In the control lanes 1 and 2, the identically treated extract of the ΔhypF strain 
carrying the empty vector was applied (ΔhypF/pACYC184). The gel of in B was stained for CP phosphatase activity. Lanes 1 and 3 contain 60 μg 
of protein, and lanes 2 and 4 contain 120 μg of protein. Lanes 5–7 contain 2, 4, and 6 μg of protein, respectively.

| Substrate                                                                 | Phosphatase activity | pmol Pi × pmol HypF⁻¹ × s⁻¹ |
|---------------------------------------------------------------------------|----------------------|-----------------------------|
| Phosphoenolpyruvate                                                       | <0.01                |
| Creatine phosphate                                                        | <0.01                |
| Acetyl phosphate                                                          | <0.01                |
| Glycerol 2-phosphate                                                      | <0.01                |
| Carbamoyl phosphate                                                       | 6.7                  |

* 2.5 mM final concentration each.

FIG. 3. Lineweaver-Burk plot of the substrate concentration dependence of HypF activity in the phosphatase reaction.
assessment, radioactively labeled pyrophosphate was incubated in the presence or absence of CP with unlabeled ATP, and samples were taken and assayed for the generation of radioactive ATP. The results obtained showed that such a PPi-ATP exchange indeed takes place when CP is present in the reaction mixture (Fig. 4B). The results also prove that HypF cleaves ATP into AMP and pyrophosphate and not by the sequential removal of two phosphate moieties, like in the selenophosphate synthetase reaction (36). The entry into a plateau might either be because of the attainment of the equilibrium in the PP-ATP exchange reaction or be caused by CP substrate limitation. The retardation of the reaction in the presence of 100 μM CP is unspecific because CP at this concentration and higher inhibits HypF activity. 3

Mutational Analysis of HypF

To gain further insight into the function of HypF in the process of hydrogenase maturation and, in particular, to correlate the in vitro activities of the protein with the formation of the CO and CN ligands of the metal center, an extensive mutagenesis of the hypF gene has been carried out. Residues from the acylphosphatase, the zinc finger, and the O-carbamoyltransferase motifs were replaced either singly or in combination (see Fig. 1). The mutant gene

Table III

| Reaction                  | 1. Substrate | 2. Substrate | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (μM$^{-1}$ s$^{-1}$) |
|--------------------------|--------------|--------------| -----------|----------------------|-------------------------------|
| CP phosphatase           | CP           | H$_2$O       | 330        | 9.6                  | 0.03                          |
| ATP cleavage             | ATP          | CP           | 15         | 1.5                  | 0.1                           |
|                          | CP           | ATP          | 5.8        | 17                   | 2.93                          |

3 At 100 μM each.

Table IV

| CP phosphatase activity of wild type and mutant HypF variants | pmol P$_{i}$ liberated × s$^{-1}$ |
|--------------------------------------------------------------|----------------------------------|
| DHP-F2 carrying plasmid expressing HypF variants             |                                  |
| R23E, R23K, R23H                                             |                                  |
| R23EV1720A                                                  |                                  |
| C109A, C109A/C112A                                          |                                  |
| C159A, C159A/C162A                                          |                                  |
| H755A/H476A                                                | <0.01                           |
| H475Y/H476Y/H477Y/A479Y                                     | 2.2                             |
| H475A                                                      | 16.2                            |
| H476A                                                      | 21.2                            |
| H479A                                                      | 18.2                            |
| Wild type HypF                                              | 25                              |

FIG. 4. Kinetics of carbamoyl phosphate-dependent ATP cleavage reaction. A, Lineweaver-Burk plot of ATP cleavage at different CP or ATP concentrations. When the CP concentration was varied, ATP was present at 100 μM, and when ATP concentration was varied the CP concentration was held at 100 μM. B, [32P]PPi-ATP exchange catalyzed by HypF at different carbamoyl phosphate concentrations.

FIG. 5. Analysis of mutant HypF proteins. A, in vivo stability of the HypF variants produced by the transformants indicated. An immunoblot is shown from a 10% SDS gel in which lysates of 0.1 A$_{600}$ units of cells were separated. The gel was developed with antibodies directed against HypF protein. B, processing of the precursor of the large subunit of hydrogenase 3 from E. coli in the ΔhypF strain DHP-F2 transformed with plasmids carrying the wild type and the mutant hypF alleles indicated. An immunoblot is shown from a 10% SDS gel in which lysates of 0.1 A$_{600}$ units of cells were separated. The gel was developed with antibodies directed against synthetic peptides of HycE. Lanes 1 and 18, wild type hypF; lanes 2 and 17, ΔhypF; lanes 3–16, HypF variants; lane 3, R23E; lane 4, R23H; lane 5, R23K; lane 6, R23Q; lane 7, R23EV17A/V20A; lane 8, C112A; lane 9, C109A/C112A; lane 10, C162A; lane 11, C159A/C162A; lane 12, H475A; lane 13, H476A; lane 14, H479A; lane 15, H475A/H476A; lane 16, H475Y/H476Y/H477Y/H479Y.

HypF cleaves ATP into AMP and pyrophosphate and not by the sequential removal of two phosphate moieties, like in the selenophosphate synthetase reaction (36). The entry into a plateau might either be because of the attainment of the equilibrium in the PP-ATP exchange reaction or be caused by CP substrate limitation. The retardation of the reaction in the presence of 100 μM CP is unspecific because CP at this concentration and higher inhibits HypF activity.

Mutational Analysis of HypF—To gain further insight into the function of HypF in the process of hydrogenase maturation and, in particular, to correlate the in vitro activities of the protein with the formation of the CO and CN ligands of the metal center, an extensive mutagenesis of the hypF gene has been carried out. Residues from the acylphosphatase, the zinc finger, and the O-carbamoyltransferase motifs were replaced either singly or in combination (see Fig. 1). The mutant gene

3 A. Paschos, A. Bauer, and A. Böck, unpublished data.
products were analyzed for their in vivo stability when the mutated genes were expressed from a plasmid in strain DHP-F2 (ΔhypF) (Fig. 5A). With some remarkable exceptions, most of the mutant alleles yielded products at approximately the same level. The exceptions were HypF-R23K, HypF-C162A, and HypF-H475A/H476A. Intriguingly, exchange of a single cysteine from the C-terminal “zinc finger motif” (see Fig. 1, ZP2) destabilized the protein completely, whereas the replacement of the distal (neighboring) one in addition yielded a stable product (Fig. 5A, lanes 10 and 11). Surprisingly, the identical replacements at the N-terminal zinc finger motif delivered a stable product when one of the cysteines was exchanged but not in case of the double replacements (Fig. 5A, lanes 8 and 9).

The consequences of the mutations carried by the different HypF variants on their role in hydrogenase maturation was assessed by following the proteolytic processing of the precursor of HycE, which is the large subunit of hydrogenase 3 (Fig. 5B). The immunoblot indicates that exchange of the histidine residues in positions 475, 476, and 479 against alanine delivers gene products that are still functional in hydrogenase maturation. All the other variants were inactive. This was corroborated by measuring hydrogenase activity in non-denaturing polyacrylamide gels after separation of 100 μg of protein of crude extracts from the mutants. Upon substrate staining, bands reflecting hydrogenase 2 and hydrogenase 1 activity were detected in case of the mutant variants HypF-H475A, HypF-H476A, and HypF-H479A.

Finally, it was important to correlate the proficiency of the HypF variants in the hydrogenase maturation process with their in vitro capacity to interact with CP. The transformants harboring the mutant alleles on a plasmid were grown anaerobically to an A600 of 1.0. The cells were harvested, and crude extracts were prepared that were partially purified by centrifugation at 100,000 × g and by ammonium sulfate precipitation. 200 μg of protein of the 30% sediment fraction was analyzed for CP phosphatase activity (Table IV). Variants carrying the H475A, H476A, or H479A exchanges displayed full activity, whereas variant R23Q had detectable CP phosphatase activity but at a very low level. All the other variants that produced stable HypF protein were devoid of activity.

DISCUSSION

The synthesis of the CO and CN ligands of [NiFe] hydrogenases and their attachment to the iron poses a number of intriguingly novel features of bioinorganic chemistry. As some of the most toxic compounds in biology, it is predictable that they are not synthesized in the free state but rather bound to some adaptor, thus preventing their interaction with susceptible and essential cellular components. It is still unclear whether they are attached to the iron when it has been inserted into the large hydrogenase subunit or whether they are preformed, e.g. at some scaffold protein and transferred as the complete entity into the apoprotein. Moreover, it is still unclear how the precise stoichiometry of 2 CN and 1 CO per iron atom is achieved.

We have reported recently (18) that CP is required for the synthesis of active [NiFe] hydrogenases. The experimental evidence is as follows: (i) a mutant of E. coli devoid of CP synthetase activity was unable to synthesize all three hydrogenases based on the inability to mature and process the large subunits, and (ii) supplementation with citrulline as a source of CP restored this capacity. It has also been pointed out that reactions in metallo-organic chemistry have been described that convert a carbamoyl into a carbonyl or cyanyl moiety (18).

The results reported here convincingly show that the hydrogenase maturation protein HypF is interacting with CP as a substrate and that this interaction is involved in hydrogenase maturation. Purified HypF dephosphorylates CP specifically, and this activity can be discovered in electrophoretically separated extracts from an E. coli strain overexpressing hypF. Because the HypF protein has been purified under aerobic conditions, this also means that the CP phosphatase activity is oxygen-stable. A causal connection between CP and hydrogenase maturation by HypF is then provided by the mutational analysis. There is a clear and quantitative parallelism between maturation activity of HypF and the in vitro CP phosphatase activity. Somewhat surprising is the discovery that mutations in all three major signature motifs, the acylphosphatase, the zinc fingers, and the O-carbamoyltransferase motifs, can lead to the blockade of CP phosphatase activity. It indicates an integrated cooperativity between these domains in the cleavage reaction.

The delineation of the route how HypF could convert the carbamoyl moiety into one or both of the CO/CN ligands is not possible yet. It needs detailed analysis of the product formed in the CP-dependent ATP cleavage reaction and, especially, also the analysis of the activity of the HypE protein, since it has been shown recently that HypF and HypE form a complex (37).

We strongly emphasize in this context that the reactions assayed are those of the sole HypF protein in the absence of HypE or putative additional substrates. Although they present information on the existence of substrate-binding sites and types of reactions catalyzed, they do not preclude the possibility that the products measured are those of side reactions when reaction partners are missing, and the flux is blocked at the state of the same intermediate.

Bearing all this caveats in mind, speculations can be attempted on the fate of carbamoyl phosphate when acted upon by HypF and HypE. First, from the two reactions catalyzed by HypF, the CP phosphatase activity is kinetically somewhat less favored in comparison to the CP-dependent ATP cleavage. There are several explanations for this inefficiency. First, CP hydrolysis may represent a side reaction that is followed in the absence of other substrates, reflecting the transfer of the carbamoyl residue to a water molecule. Second, and alternatively, it could represent the carbamoyl transfer to some acceptor group of the HypF protein that is paralleled by the liberation of inorganic phosphate. The carbamoylated amino acid residue could then lose its acyl group because the conversion into the adduct is blocked, possibly due to the absence of HypE.

On the other hand, the incorporation of labeled pyrophosphate into ATP that is catalyzed by HypF in the presence of CP supports the view that CP is adenylated. Although exchange of the phosphoryl group of CP by an adenyl residue cannot be excluded (energetically this would not make sense), our favorite hypothesis is that adenylation takes place at the hydroxyl of the tautomeric form of the carbamoyl moiety yielding the iminoform of the carbamoyl adenylate. Removal of a hydrogen from the imino group would directly lead to the cyano moiety. It resembles the well known dehydration reaction with the formation of a phosphorylated intermediate. Interestingly, HypE shares structural similarity with PurM which catalyzes such a reaction in the purine biosynthetic pathway (16). Further work is aimed at this possibility.

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